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Metabolism of Polycyclic Compounds

18. THE SECRETION OF METABOLITES OF NAPHTHALENE, 1:2-DIHYDRONAPHTHALENE AND 1:2-EPOXY-1:2:3:4-TETRAHYDRONAPHTHALENE IN RAT BILE*

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A metabolite of sulphobromophthalein has been detected in the bile of rats (Combes, 1959) and of human subjects (Grodsky, Carbone & Fanska, 1959), which gives rise to glycine and glutamic acid on acid hydrolysis. Grodsky *et al.* (1959) suggested that the metabolite is a glutathione conjugate. In a study of the metabolism of naphthalene, 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene by rat-liver slices (Booth, Boyland & Sims, 1960*b*; Booth, Boyland, Sato & Sims, 1960*a*), it was found that these compounds are converted into glutathione conjugates, which yield the corresponding cysteine conjugates on incubation with rat-kidney homogenates. The present work shows that the bile of rats treated with either naphthalene, 1:2-dihydronaphthalene or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene contain conjugates of glutathione, cysteinyl glycine, cysteine and *N*-acetylcysteine in addition to other metabolites previously reported as urinary excretion products.

EXPERIMENTAL

Materials. 1:2-Dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene were prepared as described by Boyland & Sims (1960). *S*-(1-Naphthyl)glutathione, *S*-(1-naphthyl)-L-cysteine and 1-naphthylmercapturic acid were prepared by the methods of Booth *et al.* (1960*b*). A product which appeared to be mainly *S*-(1-naphthyl)-L-cysteinylglycine was obtained by boiling *S*-(1-naphthyl)-glutathione (100 mg.) with water (150 ml.) under reflux for 48 hr. The solid obtained on evaporation of the water was extracted with saturated aq. $NaHCO_3$ and the extract acidified with acetic acid to yield a light-brown powder (25 mg.). On paper chromatograms developed with solvent 1 described below, the solid yielded one large and two small spots, the latter being indistinguishable from *S*-(1-naphthyl)glutathione and *S*-(1-naphthyl)-L-cysteine. The large spot was eluted from chromatograms and treated by the methods described below to yield compounds indistinguishable from *S*-(1-naphthyl)-L-cysteine and glycine after hydrolysis with HBr and from alanine and glycine after hydrogenolysis with Raney nickel followed by acid hydrolysis. No other amino acids were detected. A similar result was obtained when *S*-(1-naphthyl)glutathione was heated with N-H_2SO_4 at 100° for 1 hr.

S-(1:2:3:4-Tetrahydro-2-hydroxy-1-naphthyl)glutathione was prepared by the method of Booth *et al.* (1960*a*) and *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine and *N*-acetyl-*S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine by the methods of Boyland & Sims (1960). A preparation which appeared to be a mixture of the stereoisomeric forms of *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine was obtained as a gum when L-cysteinylglycine was treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene under the conditions used by Booth *et al.* (1960*a*) for the reaction of the epoxide with glutathione. The gum formed one spot on paper chromatograms, and

* Part 17: Booth, Boyland, Sato & Sims (1960*a*).

this gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ and ninhydrin reagents described below, and yielded glycine, cysteine (and cystine) and 2-tetralone [detected by the test of Straus & Rohrbacher (1921)] after acid-hydrolysis, and glycine, alanine and 2-naphthol after hydrogenolysis with Raney nickel (followed by acid-hydrolysis of the dipeptide thus obtained). When the epoxide was treated with the products obtained by boiling glutathione with water for 24 hr., compounds were detected on paper chromatograms indistinguishable from the glutathione and cysteinylglycine derivatives described above and a third product, which gave a positive reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent but no ninhydrin reaction and is probably a derivative of the diketopiperazine which Hopkins (1929) had shown to be a reaction product of glutathione with boiling water.

Other naphthalene metabolites were isolated from the urine of rabbits dosed with naphthalene as described by Boyland & Sims (1957, 1958) and Sims (1959) and other metabolites of 1:2-dihydronaphthalene were isolated from the urine of rabbits dosed with 1:2-dihydronaphthalene as described by Boyland & Sims (1960).

Paper chromatography. Except where stated, paper chromatography was carried out by downward development for 18 hr. on Whatman no. 1 paper in: 1, butanol–propan-1-ol–aq. 2N-NH_3 soln. (2:1:1, by vol.); 2, butanol–acetic acid–water (2:1:1, by vol.); or 3, 0·1N-NH_3 (upward development for 8 hr.) [this solvent was used in the identification of 1- and 2-naphthol (Sims, 1959)]. The dried chromatograms were examined under u.v. light and sprayed with (i), freshly diazotized *p*-nitroaniline (0·02% in 0·1N-HCl) followed by aq. 10% Na_2CO_3; (ii), 0·1M-$K_2Cr_2O_7$–acetic acid (1:1, v/v) followed by 0·1M-$AgNO_3$ (Knight & Young, 1958); or (iii), aq. 2% (w/v) $NaIO_4$ followed after 30 min. by Schiff's reagent (Brookes & Young, 1956). Other chromatograms were dipped in a solution of ninhydrin in acetone (0·2%) and heated to 70° in an oven for 10 min. In the detection of naphthalene metabolites, chromatograms were also sprayed with 2N-HCl, heated to 70° for 10 min. and sprayed with reagent (i) above.

The presence or absence of the various metabolites in the bile of treated animals was determined by a direct comparison with authentic materials on paper chromatograms wherever possible and by their reactions to the reagents described above. Many of these reactions have been described (Boyland & Sims, 1958, 1960; Sims, 1959).

The amino acids obtained after the acid-hydrolysis of some of the metabolites as described below were characterized on paper chromatograms both by direct comparison with authentic materials with solvent 2 and, after conversion into 2:4-dinitrophenyl derivatives (Sanger, 1945), by comparison with authentic derivatives with the solvents and conditions of Phillips (1958). Glycine and taurine were also detected on chromatograms by the *o*-phthalaldehyde reagent of Curzon & Giltrow (1954).

Establishment of biliary fistulae in rats. Tributaries from the main lobes of the liver join to form the common bile duct which opens into the anterior wall of the duodenum about 3 cm. from the pylorus. The pancreatic ducts drain into the lower one-third of the common bile duct and therefore the upper part of the duct must be cannulated in order to obtain uncontaminated bile.

Under ether anaesthesia an upper right paramedian incision was made and the bile duct displayed by traction on the duodenum. The duct was ligated at its midpoint and then opened just above the ligature, and polythene tubing (0·35 mm. diameter) was introduced by using binocular lenses. The end of the tube was bevelled to facilitate its introduction. (Nylon tubing of equal bore was unsatisfactory because it kinked.) The tube was pushed up the duct to the junction of the lowest tributaries when the flow of bile stopped: it was then withdrawn 0·5 cm. and an atraumatic suture placed around the duct just above the site of cannulation. The suture was tied but not cut. A stab wound was made through the abdominal muscles 1·5 cm. from the right margin of the wound with a needle, which was pushed into the end of the tube and withdrawn, carrying the tube into the subcutaneous tissues. The atraumatic suture then picked up a fold of peritoneum just above the emerging tube and thus anchored it flush with the abdominal wall. A second suture fixed the tube to the muscles in the subcutaneous space and the tube was passed along a hollow needle of suitable calibre to emerge in the midline at the nape of the neck, so that the rat cannot bite the tube. Bile was collected in a glass saddle-shaped container (Van Zyl, 1958) held in position by encircling strips of Elastoplast.

The rats with biliary fistulae were given solutions of naphthalene (75 mg.), 1:2-dihydronaphthalene (50 mg.) or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (25 mg.) in arachis oil (0·5 ml.) by intraperitoneal injection on the day after the operations and on each successive day until the animals were ill. Bile was collected daily and stored at 0°. The rats each produced about 25 ml. of bile every day and usually lived for about 5 days after the operations.

Treatment of bile. The biles collected from each animal were pooled and treated separately. A direct examination of the bile on paper chromatograms showed that the normal biliary constituents had little effect on the R_F values of the metabolites. Usually, however, the bile was diluted with 3 vol. of water and the solution acidified to pH 4 with acetic acid and activated charcoal (1 g. for every 25 ml. of undiluted bile) added with stirring. The charcoal was filtered off and washed with water (250 ml.) and the absorbed material eluted by passing first 500 ml. of methanol containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88) and then methanol containing 5% of benzene through the filter. The combined eluates were evaporated to dryness under reduced pressure, and the residue (which contained bile salts) was examined on paper chromatograms.

The examination of the bile for amino acid conjugates was carried out separately. The materials obtained with the charcoal procedure described above were applied along the base-lines of chromatograms [one sheet of Whatman no. 1 paper (46 cm. × 57 cm.) was used for the material obtained from each 20 ml. of bile] which were developed with solvent 1. With naphthalene, the zones on the dried chromatograms containing the conjugates were located as dark absorbent regions in u.v. light, and on test strips cut from the main chromatograms as regions giving blue colours with diazotized *p*-nitroaniline and Na_2CO_3 and positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent. Except for the *N*-acetylcysteine conjugate, positive ninhydrin reactions were also obtained. With 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, the zones were located with the $K_2Cr_2O_7$–$AgNO_3$ reagent, applied to test strips cut from the main chromatograms.

The zones were cut from the chromatograms, and the conjugates were eluted with either aq. 2N-NH_3 or with methanol containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88). The eluates were evaporated to small volumes under reduced pressure to give solutions of the conjugates under investigation, which in most cases also probably contained naturally occurring taurine and glycine conjugates. Grodski *et al.* (1959) found that Whatman 3MM chromatography paper contained substances which gave rise to glycine and glutamic acid on acid hydrolysis. In the present work it was found that the amounts of amino acids arising in this way were negligible as compared with those arising from the hydrolysis of the metabolites. On the other hand, experiments in which bile from untreated animals was examined on chromatograms under the above conditions showed that contamination from biliary glycine and taurine conjugates could easily arise. With the conjugates arising from naphthalene, a third source of contamination was present, namely the conjugates themselves which break down spontaneously during the working-up procedures to give small amounts of peptides which yield glycine and sometimes glutamic acid on acid hydrolysis (a similar but faster breakdown of the metabolites in the presence of mineral acid is described later). This possibility of contamination was also present, but to a much lesser extent, with the amino acid conjugates of 1:2-dihydronaphthalene, since this hydrocarbon is converted into naphthalene amino acid conjugates in the body to some extent.

The solutions containing the amino acid conjugates of naphthalene were each divided into two portions, one of which was applied to the base-lines of chromatograms which were developed with solvent 2. The other portions were acidified with HCl and the solutions applied to the base-lines of chromatograms which were developed with solvent 1. The conjugates were all related to *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, which yields 1-naphthylmercapturic acid on acidification (Boyland & Sims, 1958) and all gave similar acid decomposition products which had R_F values different from those of the original conjugates. Normal biliary constituents would not be expected to be affected by this mild treatment, and their R_F values would remain the same. The new conjugates were located on the chromatograms by their characteristic orange fluorescence under u.v. light. The elution of the appropriate zones of the chromatograms with aq. 2N-NH_3 soln. afforded solutions of the conjugates, or of their acid decomposition products, which were evaporated under reduced pressure to yield gums which were treated as described below.

The bile from animals treated with 1:2-dihydronaphthalene contained, besides the amino acid conjugates of this hydrocarbon, detectable amounts of amino acid conjugates which were indistinguishable from those formed from naphthalene as described above. The corresponding amino acid conjugates of the two hydrocarbons were difficult to separate from each other on chromatograms, so that the solutions of the conjugates of 1:2-dihydronaphthalene obtained as described above contained small amounts of those of naphthalene. If the solutions were acidified with HCl and chromatographed in solvent 1, however, the amino acid conjugates of naphthalene were decomposed, in the manner indicated above, to give products of different R_F values from the corresponding conjugates of 1:2-dihydronaphthalene, which are unaffected by cold dilute acid. The amino acid conjugates of 1:2-dihydronaphthalene, now free from the corresponding conjugates of naphthalene, were eluted from the appropriate zones on the chromatograms with aq. 2N-NH_3 soln., and the solutions applied to the base-lines of chromatograms which were developed in solvent 2. The conjugates were located and eluted as before and the solutions evaporated to yield gums which were treated as described below.

The solution obtained from the initial fractionation of the bile of animals treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, which did not contain amino acid conjugates of naphthalene, were applied to the base-lines of chromatograms which were developed with solvent 2. The conjugates were located and eluted, and the solutions evaporated as before to yield gums which were treated as described below.

With the above techniques, a number of gummy products (in mg. quantities) were obtained, which gave single spots on paper chromatograms with the $K_2Cr_2O_7$–$AgNO_3$ reagent and (except for the mercapturic acids) with ninhydrin. From the bile of animals treated with naphthalene six pairs of products (from normal and acidified fractions) were obtained, whereas bile from animals treated with 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene each yielded five products.

Examination of the amino acid conjugates obtained from naphthalene. Small portions of the conjugates were each dissolved in a few drops of water and the solutions acidified with HCl. The products were examined on paper chromatograms with solvents 1 and 3.

Portions of the conjugates were separately dissolved in methanol (5 ml.) and heated under reflux with Raney nickel (W2 grade, 100 mg.) for 2 hr. The mixtures were filtered and the filtrates diluted with water (25 ml.) and extracted with ether (2 × 25 ml.). The ethereal extracts were evaporated and the residues examined for naphthols with solvent 3. The aqueous phases were evaporated and the residues heated with HBr (sp.gr. 1·7) at 100° for 4 hr. The solutions were evaporated and the residues examined for amino acids, both directly with solvent 2 and after treatment with 2:4-dinitrofluorobenzene as indicated above.

Other portions of the conjugates were heated with HBr (sp.gr. 1·7) for 4 hr. at 100°, and the residues obtained by evaporation of the solutions were examined for amino acids. Portions of the products obtained after the acidification of the conjugates were similarly hydrolysed, and the products obtained examined in the same way. The results are shown in Table 1.

Examination of the amino acid conjugates obtained from 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene. Small portions of the conjugates were heated to 100° with 2N-HCl (0·5 ml.) for 5 min. The solutions were cooled and 10N-NaOH (1 ml.), ethanol (0·5 ml.) and ether (0·5 ml.) were added. Blue colours developing in the ether layer on shaking the mixture were specific for the presence of 2-tetralone (Straus & Rohrbacher, 1921).

Other portions of the conjugates were treated with Raney nickel and the products examined for naphthols and amino acids as described above. The products were also hydrolysed with HBr as before and the products examined for amino acids. The results are summarized in Table 2.

Other animal experiments. A rat with a biliary fistula was given *S*-(1-naphthyl)glutathione (100 mg.), suspended

in water (1 ml.), by intraperitoneal injection. The bile was collected for 48 hr. after the injection and examined for amino acid conjugates with the methods described above.

Two rats were each given a solution of *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)glutathione (100 mg.) in water (1 ml.) daily for three successive days by intraperitoneal injection. The urines were collected, pooled and treated with charcoal as described by Boyland & Sims (1960). The material subsequently eluted from the charcoal with methanol containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88) was examined on paper chromatograms.

RESULTS

Biliary metabolites of naphthalene. The paper chromatograms of the bile of rats dosed with naphthalene showed a pattern of metabolites differing in a number of features from that seen on

Table 1. *Degradation products of the amino acid conjugates obtained from naphthalene*

Probable identity of the conjugate	Products obtained: With cold HCl*	By hydrolysis with HBr at 100°	By hydrogenolysis with Raney nickel and hydrolysis of the products with HBr†
N-Acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine	1-Naphthylmercapturic acid‡ *NN*-Diacetylcystine	*S*-(1-Naphthyl)-L-cysteine	Alanine
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine	*S*-(1-Naphthyl)-L-cysteine‡ Cystine	*S*-(1-Naphthyl)-L-cysteine	Alanine
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine	*S*-(1-Naphthyl)-L-cysteinylglycine‡ Substance, possibly oxidized cysteinylglycine	*S*-(1-Naphthyl)-L-cysteine Glycine	Alanine Glycine
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)glutathione	*S*-(1-Naphthyl)glutathione Oxidized glutathione	*S*-(1-Naphthyl)-L-cysteine Glycine Glutamic acid	Alanine Glycine Glutamic acid
Two unidentified conjugates	Unidentified ninhydrin positive substances‡	*S*-(1-Naphthyl)-L-cysteine Glycine Glutamic acid Taurine	Alanine Glycine Glutamic acid Taurine

* All the conjugates gave rise to 1- and 2-naphthol.
† All the conjugates gave rise to 2- (but not 1-) naphthol.
‡ These products gave rise to the same amino acids on hydrolysis with HBr as the parent conjugates.

Table 2. *Chromatographic properties and degradation products of the amino acid conjugates derived from* 1:2-*dihydronaphthalene and* 1:2-*epoxy*-1:2:3:4-*tetrahydronaphthalene*

All the conjugates gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Probable identity of the conjugate	R_F: Solvent 1	R_F: Solvent 2	Colour with ninhydrin	Products obtained: By hydrolysis with HBr at 100°*	By hydrogenolysis with Raney nickel and hydrolysis of the products with HBr at 100°
N-Acetyl-*S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine	0·56	0·88	—	Cysteine†	Alanine
Unidentified metabolite	0·39	0·79	—	Cysteine† Glycine	Alanine Glycine
S-(1:2:3:4-Tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine	0·57	0·72	Purple	Cysteine†	Alanine
S-(1:2:3:4-Tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine	0·37	0·72	Brown, turning purple	Cysteine† Glycine	Alanine Glycine
S-(1:2:3:4-Tetrahydro-2-hydroxy-1-naphthyl)glutathione	0·25	0·45	Purple	Cysteine† Glycine Glutamic acid	Alanine Glycine Glutamic acid

* All metabolites gave a positive test (Straus & Rohrbacher, 1921) for 2-tetralone on heating with HBr or HCl for 5 min. at 100°.
† Cystine was often detected in the hydrolysates. It is presumed to be an artifact.

chromatograms of the urine of similarly treated animals. The bile did not contain either 1-naphthyl sulphate or the isomeric sulphuric esters of 1:2-dihydroxynaphthalene, although rats dosed with naphthalene excrete large amounts of these esters in the urine (Sims, 1959). 1-Naphthylglucosiduronic acid, the 1- and 2-glucosiduronic acids of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and substances believed to be the 1- and 2-glucosiduronic acids of 1:2-dihydroxynaphthalene were

Table 3. *Properties of the biliary naphthalene metabolites (other than the amino acid conjugates) on paper chromatograms*

Metabolite	R_F Solvent 1	R_F Solvent 3	Fluorescence	Colour with diazotized *p*-nitroaniline and Na_2CO_3: Immediate	Colour with diazotized *p*-nitroaniline and Na_2CO_3: After heating the chromatogram with 2N-HCl at 100° for 10 min.
1-Naphthol	0·97	0·62	Blue	Blue	Blue
2-Naphthol	0·97	0·55	Violet	Orange	Orange
trans-1:2-Dihydro-1:2-dihydroxy-naphthalene*	0·89	—	Dark-absorption	—	Blue
1-Naphthylglucosiduronic acid	0·28	—	Dark violet	—	Blue
trans-1:2-Dihydro-2-hydroxy-1-naphthylglucosiduronic acid*	0·18	—	Dark-absorption	Pale orange†	Blue
trans-1:2-Dihydro-1-hydroxy-2-naphthylglucosiduronic acid*	0·18	—	Dark-absorption	Blue†	Blue
2-Hydroxy-1-naphthylglucosiduronic acid	0·15	—	Bright blue	Yellow	Purple
1-Hydroxy-2-naphthylglucosiduronic acid	0·14	—	Bright blue	Blue	Purple

* The optical isomers of the compounds could not be separated on paper chromatograms.

† These colours are evidently due to decomposition of the metabolites by the reagent to give 1- or 2-naphthol. On two-dimensional chromatograms which were sprayed with 2N-HCl and allowed to dry between the first and second development (with solvent 1), compounds indistinguishable from 1- or 2-naphthol were detected.

Table 4. *Properties on paper chromatograms of the amino acid conjugates derived from naphthalene and their related acid-decomposition products*

All the compounds gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Compounds	R_F Solvent 1	R_F Solvent 2	Fluorescence	Colour: With diazotized *p*-nitroaniline and Na_2CO_3	Colour: With ninhydrin
N-Acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0·37	0·84	Dark-absorption	Blue	—
1-Naphthylmercapturic acid	0·51	0·86	Pink*	—	—
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0·31	0·63	Dark-absorption	Blue	Purple
S-(1-Naphthyl)-L-cysteine	0·46	0·75	Pink*	—	Purple
S-(1:2-Dihydro-2-hydroxy-1-naphthyl-L-cysteinylglycine	0·25	0·62	Dark-absorption	Blue	Brown, then purple
S-(1-Naphthyl)-L-cysteinylglycine	0·40	0·74	Pink*	—	Brown, then purple
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-glutathione	0·10	0·47	Dark-absorption	Blue	Purple
S-(1-Naphthyl)glutathione	0·21	0·67	Pink*	—	Purple
Unidentified metabolites, possibly taurine conjugates 1	0·05	0·40	Dark-absorption	Blue	Purple
Unidentified metabolites, possibly taurine conjugates 2	0·01	0·35	Dark-absorption	Blue	Purple
Acid decomposition products of unidentified metabolites 1	0·10	0·49	Pink*	—	Purple
Acid decomposition products of unidentified metabolites 2	0·05	0·42	Pink*	—	Purple

* After exposure to NH_3 vapour.

readily identified by methods previously described (Boyland & Sims, 1957, 1958; Sims, 1959) (see Table 3). Small amounts of free 1- and 2-naphthol were always detected, but the amounts of free *trans*-1:2-dihydro-1:2-dihydroxynaphthalene found in the bile varied. Often none could be detected. A similar variation in the amounts of this diol in the urines of animals treated with naphthalene has been noted (Sims, 1959).

The main difference between the bile and urine of rats treated with naphthalene, however, was in the number of amino acid conjugates present. Only one such conjugate, the mercapturic acid *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, has been found in urine (Boyland & Sims, 1958), whereas six were found in bile. The properties and degradation products of these conjugates are listed in Tables 4 and 1 and they are clearly related to each other, differing only in the nature of the amino acid side chain. Of the six, one was indistinguishable from the above mercapturic acid, one was indistinguishable from *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione, which is formed by the action of rat-liver slices on naphthalene (Booth *et al* 1960*b*), and a third was indistinguishable from *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, which is formed from the above glutathione conjugate by rat-kidney homogenate (Booth *et al.* 1960 *b*). A fourth conjugate, which ran on chromatograms in a position intermediate to the glutathione and cysteine derivatives and which gave on acidification a product indistinguishable from *S*-(1-naphthyl)-L-cysteinylglycine, is believed to be *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine. The hydrogenolysis and acid-hydrolysis products listed in Table 1 provide supporting evidence for these structures, whereas the acid-decomposition products were similar in every respect on paper chromatograms to the synthetic materials.

On one occasion, the fraction of bile (from a rat which had received a total of 750 mg. of naphthalene) containing the glutathione derivative was dissolved in water (3 ml.), and 1 ml. of the resulting solution acidified with HCl. After 24 hr. the solid was collected to yield *S*-(1-naphthyl)glutathione (15 mg.), m.p. and mixed m.p. 222–225° (decomp.), indistinguishable from the synthetic material on paper chromatograms. The infrared spectra of the two samples were identical, and both yielded compounds indistinguishable from glycine, glutamic acid and *S*-(1-naphthyl)cysteine on paper chromatograms after being heated to 100° with HBr (sp.gr. 1·7) for 4 hr.

The relative amounts of the four conjugates described above as determined by the sizes and intensities of their spots on paper chromatograms were somewhat variable, but usually the glutathione derivative was the predominant metabolite, whereas the mercapturic acid was often difficult to detect, particularly in the bile of animals which had received only one dose of naphthalene. A similar pattern of amino acid conjugates was observed in the bile of the animal treated with *S*-(1-naphthyl)-glutathione: much unchanged glutathione conjugate was found, together with smaller amounts of *S*-(1-naphthyl)-L-cysteinylglycine and *S*-(1-naphthyl)-L-cysteine and traces of 1-naphthylmercapturic acid.

The two remaining amino acid conjugates from the bile of animals treated with naphthalene were slower running than the glutathione conjugate on paper chromatograms, so presumably they have longer amino acid side chains than this metabolite. Apart from *S*-(1-naphthyl)cysteine, glutamic acid and glycine, the only amino acid found to be present in the hydrolysates of both of these conjugates was taurine, so that they may be taurine conjugates of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione. Since only small amounts of these conjugates were present in bile, as compared with the other conjugates their structures must remain uncertain.

Biliary metabolites of 1:2-*dihydronaphthalene.* The bile from rats treated with this hydrocarbon contained small amounts of all the metabolites of naphthalene described above. This is not surprising since naphthalene metabolites have been detected in the urine of rabbits dosed with 1:2-dihydronaphthalene by Boyland & Sims (1960). Like this urine, the bile contained relatively large amounts of 2-naphthol, which may not have arisen by the same route as the naphthalene metabolites, since it is also a product of the action of rat-liver slices on 1:2-dihydronaphthalene (Booth *et al.* 1960*a*). The bile resembled that of animals treated with naphthalene in that no sulphuric esters were detected. *trans*-1:2:3:4-Tetrahydro-1:2-dihydroxynaphthalene and the corresponding glucosiduronic acid, which was isolated from the urine of treated rabbits by Boyland & Sims (1960), were detected in the bile.

As in the bile of rats treated with naphthalene, the most prominent feature of the biles of rats treated with 1:2-dihydronaphthalene was the number of compounds giving positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent. The properties and degradation products of these compounds are listed in Table 2. Of the five compounds detected, four were identified as the mercapturic acid, *N*-acetyl-*S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine, and the corresponding cysteine, cysteinylglycine and glutathione derivatives. Of these, the mercapturic acid and the cysteine derivative have been found in the urine of animals treated with 1:2-dihydronaphthalene (Boyland & Sims, 1960), and the glutathione derivative is a

product of the action of rat-liver slices on 1:2-dihydronaphthalene (Booth *et al.* 1960*a*). On paper chromatograms the conjugates were indistinguishable from the corresponding synthetic compounds.

The fifth biliary metabolite was indistinguishable on paper chromatograms from a product previously detected in the urine of animals treated with 1:2-dihydronaphthalene (Boyland & Sims, 1960), which did not react with ninhydrin, and which yielded 2-tetralone, cysteine (and cystine) and glycine on acid-hydrolysis. A possible structure for this compound would be *N*-acetyl-*S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteinylglycine, but the product from the acetylation of the corresponding cysteinylglycine conjugate with acetic anhydride in aqueous sodium hydroxide (which also yielded 2-tetralone, cysteine and glycine on acid-hydrolysis) was not identical with the metabolite on paper chromatograms. The metabolite was not identical on paper chromatograms with the corresponding diketopiperazine derivative described above. Further evidence that the metabolite was closely related to the above amino acid derivatives was obtained by an examination on paper chromatograms of the urine of rats treated with *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)-glutathione. The urine contained compounds indistinguishable from the above mercapturic acid, the cysteine and cysteinylglycine derivatives and the unidentified metabolite. A third possible structure of this metabolite would be the *N*-acetylglycylcysteine derivative, which would also yield 2-tetralone, cysteine and glycine on acid-hydrolysis. This could arise by ring-opening of the diketopiperazine derivative, followed by acetylation of the amino group.

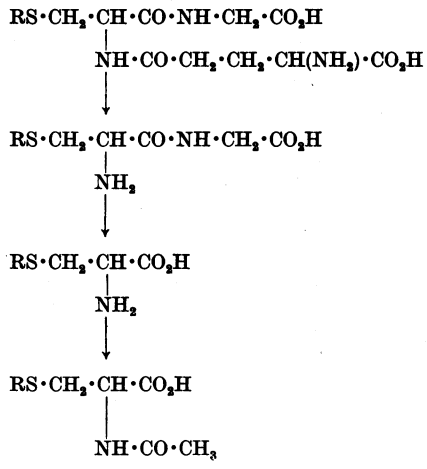
Biliary metabolites of 1:2-*epoxy*-1:2:3:4-*tetrahydronaphthalene.* These closely resembled those of 1:2-dihydronaphthalene except that the naphthalene metabolites mentioned above and 2-naphthol were not detected. The bile contained *trans*-1:2:3:4-tetrahydro-1:2-dihydroxynaphthalene and its glucosiduronic acid and compounds indistinguishable on paper chromatograms from the amino acid conjugates obtained from 1:2-dihydronaphthalene described above. The amounts of the various amino acid conjugates from both 1:2-dihydronaphthalene and the corresponding epoxide were variable, but usually the glutathione derivative was most prominent, whereas the mercapturic acid and the unidentified metabolite sometimes occurred only in traces, particularly in animals which had received few doses of the compounds. Substances corresponding to the supposed taurine conjugates derived from naphthalene were not detected in the bile of animals receiving 1:2-dihydronaphthalene or its epoxide.

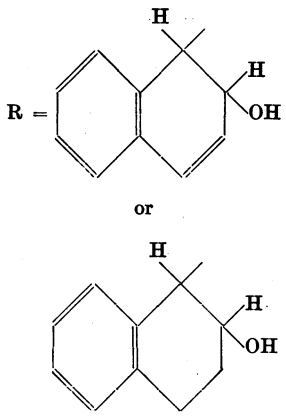
DISCUSSION

The presence of metabolites other than amino acid conjugates in the biles of rats treated with naphthalene, 1:2-dihydronaphthalene or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene calls for little comment, since these metabolites are presumably formed in the liver and are known to be excreted in the urine. The absence of sulphuric esters in the biles of animals treated with the two hydrocarbons is remarkable since large amounts of these esters are excreted in the urines. Moreover, taurocholic acid, a sulphonic acid, is a normal constituent of bile and sulphuric esters of bile alcohols are present in the biles of lower vertebrates (cf. Haslewood, 1955).

The proposed structures for the amino acid conjugates found in bile after the administration of naphthalene, 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene are confirmed by the nature of their degradation products and they are clearly related to the mercapturic acids detected in the urine of animals after similar treatment. Moreover, the present work provides a connecting link between these metabolites and the glutathione conjugates formed from the same compounds in experiments *in vitro* with rat-liver slices (Booth *et al.* 1960*a*, *b*). In these experiments, the breakdown of the glutathione derivative to the cysteine derivative by rat-liver slices was not observed, but it could readily be brought about by rat-kidney homogenates. On the other hand, Bray, Franklin & James (1959) found that *S*-(*p*-chlorobenzyl)glutathione was converted into the corresponding *S*-cysteine derivative by guinea-pig-liver slices and by homogenates of guinea-pig, rat and rabbit liver. The intermediate formation of cysteinylglycine derivatives has not been observed in experiments *in vitro*. The absence of γ-glutamylcysteine derivatives in the biles of treated animals suggests that the breakdown of the glutathione conjugates proceeds in the same way as the breakdown of the glutathione itself, the first step being the loss of the γ-glutamyl groups, either by hydrolysis, by transfer to acceptors, or by cyclization to pyrrolidonecarboxylic acid (cf. Jocelyn, 1959). This is followed by the loss of glycine and the acetylation of the amino groups to give the mercapturic acids. The isolation of *S*-(1-naphthyl)-glutathione (liberated from its precursor by acid) provides direct evidence for the formation of a conjugate of this tripeptide in the body.

There is an interesting difference between the metabolism of naphthalene and that of 1:2-dihydronaphthalene and its related epoxide in that with naphthalene only the mercapturic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, has been detected in the urine of treated





animals, whereas with the latter compounds both the corresponding cysteine conjugate and the mercapturic acid are always present. Moreover, the corresponding cysteinylglycine conjugate was detected in the urine of animals treated with *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)glutathione. The unidentified metabolite described above was present in these urines as well as in the urines of animals treated with 1:2-dihydronaphthalene and its epoxide, but no analogous compound has been detected in the urines (or in the bile) of animals treated with naphthalene.

The amino acid conjugates obtained from naphthalene are all related to *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, which breaks down with cold acid to give mainly 1-naphthylmercapturic acid (Boyland & Sims, 1958) together with small amounts of 1- and 2-naphthol and *NN*-diacetylcystine. Table 1 shows that in the breakdown of the related amino acid conjugates, side reactions yielded small amounts of the naphthols and oxidized forms of the sulphur-containing amino acids or peptides: the reduced forms (which would be expected) were not detected. Solutions of the conjugates did not give positive reactions for SH groups with sodium nitroprusside on acidification but always produced strong odours of naphthalene (although the hydrocarbon has not been detected chemically).

The formation of mercapturic acids from both naphthalene and 1:2-dihydronaphthalene appears to involve the reaction of intermediates, possibly epoxides (Booth *et al.* 1960*a*; Boyland & Sims, 1960) with glutathione. The glutathione conjugates are broken down to yield the cysteinylglycine and the cysteine derivatives and finally, after the acetylation of the amino groups, the mercapturic acids.

SUMMARY

1. The bile of rats treated with naphthalene contains 1- and 2-naphthol, 1-naphthylglucosiduronic acid, the 1- and 2-glucosiduronic acids of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and 1:2-dihydroxynaphthalene, and sometimes the free dihydrodihydroxy compound. Sulphuric esters were not detected.

2. The bile also contained *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione, together with the corresponding *S*-cysteinylglycine, *S*-cysteine and *N*-acetyl-*S*-cysteine conjugates. Two other conjugates present in small amounts appeared to be taurine conjugates of the glutathione derivative.

3. The bile of rats treated with 1:2-dihydronaphthalene contained all the above naphthalene metabolites, together with *S*-(1:2:3:4-tetrahydro-2-hydroxy-1-naphthyl)glutathione and the corresponding *S*-cysteinylglycine, *S*-cysteine and *N*-acetyl-*S*-cysteine conjugates. An unidentified metabolite, which yielded cysteine, glycine and 2-tetralone on acid-hydrolysis, appeared to be related to the above conjugates and it was also present in the urine of animals treated with the above glutathione conjugate. The bile also contained *trans*-1:2:3:4-tetrahydro-1:2-dihydroxynaphthalene and its glucosiduronic acid and relatively large amounts of 2-naphthol.

4. The bile of rats treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene contained five amino acid conjugates indistinguishable from those present in the bile of rats treated with 1:2-dihydronaphthalene. *trans*-1:2:3:4-Tetrahydro-1:2-dihydroxynaphthalene and its glucosiduronic acid were also present.

5. A method for the establishment of bile fistulae in rats is described.

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Spectrophotometric Measurements on Ascorbic Acid and Their Use for the Estimation of Ascorbic Acid and Dehydroascorbic Acid in Plant Tissues

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The ultraviolet-light-absorption of ascorbic acid in neutral solutions was described by Herbert, Hirst, Percival, Reynolds & Smith (1933), Baird *et al.* (1934) and Morton (1942). Racker (1952) suggested application of spectrophotometry to enzymic microestimation of ascorbic acid or assay of ascorbic acid oxidases but did not investigate the conditions for such methods or the effects of pH on the absorption spectrum. Effects of pH were studied in detail by Daglish (1951), who described a spectrophotometric assay based on effects of pH on extinction. We have compared enzymic and chemical methods for estimating ascorbic acid and dehydroascorbic acid by spectrophotometric and visual-titration methods. Molar extinction coefficients of L-ascorbic acid found in this work were higher than those recorded by all previous workers except Lawendel (1956, 1957).

The spectrophotometric method we now describe was designed to estimate ascorbic acid in as little as 10 mg. of plant tissue. Between 1 and 75μg. of ascorbic acid or dehydroascorbic acid can be determined in 3 ml. of extract, 2μg. within about 5% and 10–20μg. within about 2% of the amount present. Non-enzymic methods of comparable sensitivity, with 2:6-dichlorophenolindophenol (Bessey, 1938; Glick, Alpert & Stecklein, 1953), are liable to interference, especially by reducing agents other than ascorbic acid. Chromatographic separation from reductones and thiols has been used by Chen, Isherwood & Mapson (1953) and by Strohecker, Heimann & Matt (1955). These methods also separate L-ascorbic acid from D-arabo- and D-gluco-ascorbic acid, but are lengthy and result in substantial losses with some plant tissues.

The estimation of dehydroascorbic acid with an indophenol dye by the difference in ascorbic acid found before and after reduction with hydrogen sulphide (Bessey, 1938) or with homocysteine (Hughes, 1954, 1956) is dependent on no other reducing compounds being produced by these treatments. We have compared the effects of reduction by hydrogen sulphide or homocysteine on the determination of dehydroascorbic acid by independent enzymic or chemical means.

EXPERIMENTAL

Materials

L-Ascorbic acid (British Drug Houses Ltd.) was found to be 99·4% pure by titration with KIO_3 in 4N-HCl in the presence of $CHCl_3$ (1 drop) to detect free I_2 at the end point