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Metabolism of Polycyclic Compounds

20. THE METABOLISM OF PHENANTHRENE IN RABBITS AND RATS: MERCAPTURIC ACIDS AND RELATED COMPOUNDS

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Investigations on rats dosed with phenanthrene (Elson, Goulden & Warren, 1945; Crabtree, 1946) showed that there was an increase in the neutral-sulphur output in the urines, and Gutmann & Wood (1950) found an increase in the radioactivity in the urines when animals were fed with phenanthrene and L-[^{35}S]cysteine. Stekol (1935, 1939) had noticed that the growth of dogs and rats was retarded when the animals, maintained on a casein-free diet, were given phenanthrene. The growth was resumed when cysteine or methionine, but not taurine, was given. In the present work, a number of mercapturic acids and related compounds have been isolated or detected in the urines of both rabbits and rats dosed with phenanthrene.

MATERIALS AND METHODS

Melting points. These are uncorrected.

Materials. 9-Nitrophenanthrene, m.p. 115–116°, was prepared by the nitration of phenanthrene (Schmidt & Heinle, 1911). It was reduced with hydrazine hydrate in ethanol in the presence of 5% (w/w) palladium on charcoal to yield 9-aminophenanthrene in needles, m.p. 137°. 1- and 4-Aminophenanthrene, m.p. 144° and 55°, were prepared from 1,2,3,4-tetrahydro-1-oxo- and 1,2,3,4-tetrahydro-4-oxo-phenanthrene respectively by the method of Langenbeck & Weissenborn (1939), and 2- and 3-aminophenanthrene, both of m.p. 85°, by the action of aq. NH_3 (sp.gr. 0·88) on the corresponding hydrochlorides obtained by the hydrolyses of the acetamido compounds with boiling 5N-HCl.

The *S*-cysteine derivatives were obtained from the corresponding amino compounds by the method described by Booth, Boyland & Sims (1960) for the preparation of *S*-(1-naphthyl)cysteine except that the diazotizations of the amines were carried out by adding a solution of the amine in pyridine to nitrososulphuric acid as described by Bachmann & Boatner (1936). The products, which were obtained in poor yield, were difficult to purify because they often separated as jellies both from aqueous solutions and from solutions in 2N-NaOH after the addition of acetic acid. After repeated precipitations from solutions in 2N-NaOH with acetic acid, S-(9-*phenanthryl*)*cysteine* was obtained as a pink powder, m.p. 180° (decomp.) (Found: N, 4·5; S, 10·5. $C_{17}H_{15}NO_2S$ requires N, 4·7; S, 10·8%) and S-(1-*phenanthryl*)*cysteine* as a buff powder, m.p. 180–182° (decomp.) (Found: N, 4·1; S, 10·4%). The products from 2-, 3- and 4-aminophenanthrene, presumed to be mainly the corresponding *S*-phenanthrylcysteine derivatives, formed dark powders with indefinite melting points.

When the cysteine derivatives, in 2N-NaOH, were treated with an excess of acetic anhydride, the corresponding mercapturic acids separated. 9-*Phenanthrylmercapturic* acid formed a pink powder (from water), m.p. 194° (decomp.) (Found: N, 4·0; S, 9·1. $C_{19}H_{17}NO_3S$ requires N, 4·1; S, 9·45%), and 1-*phenanthrylmercapturic acid* a buff powder (from water), m.p. 165–168° (decomp.) (Found: S, 9·0%). 2-, 3- and 4-Phenanthrylmercapturic acid were obtained in the form of dark-brown amorphous solids which formed characteristic pink-fluorescing spots in u.v. light on paper chromatograms.

N-Acetylcysteine was prepared by the method of Pirie & Hele (1933) and *NN'*-diacetylcystine by the method of Hollander & du Vigneaud (1931). The compounds had R_F 0·80 and 0·71 respectively in solvent 2 (see below). The preparations of other compounds used were as described by Sims (1962).

Chromatography. The solvents (1 and 2) and the reagents used for paper and thin-layer chromatography were as described by Sims (1962). Two-dimensional chromatograms developed with solvent 1 were run in which the papers were sprayed with 5N-HCl and allowed to dry between the first and second developments. In this way acid-labile compounds were detected. The properties of the mercapturic acids and related compounds are listed in Table 1.

Acid hydrolysis of the amino acid conjugates. Samples of about 5 mg. of the materials obtained as described below were heated to 100° with HBr (sp.gr. 1·7) for 4 hr., after which the acid was evaporated. The residues obtained were examined for amino acids both by direct comparison with authentic materials on paper chromatograms and, after conversion into the 2,4-dinitrophenyl derivatives (Sanger, 1945), by comparison with authentic derivatives with the solvents and conditions of Phillips (1958).

Hydrogenolysis with Raney nickel catalyst. These were carried out on 5–10 mg. samples of materials obtained as indicated below. The samples, in ethanol (5 ml.), were heated under reflux with Raney nickel catalyst (W-2 grade) (250 mg.) for 6 hr. The mixtures were filtered, and the filtrates were diluted with water (50 ml.) and extracted twice with ether (20 ml.). The ether extracts were combined and evaporated; the residues were examined for hydroxyphenanthrenes on thin-layer chromatograms, and the aqueous layers were evaporated and the residues hydrolysed and examined for amino acids as described above.

Examination of metabolites in urine. The groups of fractions containing mercapturic acids and related compounds obtained by Sims (1962) from the urines of rabbits

Table 1. *Paper chromatography of the amino acid conjugates related to phenanthrene*

Solvent 1, butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.); solvent 2, butan-1-ol–acetic acid–water (2:1:1, by vol.) [for details see Sims (1962)].

Compound	R_F Solvent 1	R_F Solvent 2	Fluorescence	Reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent	Reaction with the platinic iodide reagent	Colour with ninhydrin
N-Acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)-cysteine	0·39	0·83	Dark-violet	+ +	+ +	None
S-(9,10-Dihydro-9-hydroxy-10-phenanthryl)cysteine	0·29	0·73	Dark-violet	+ +	+ +	Purple
Metabolite, probably a glycine conjugate	0·32	0·85	Dark-violet	+ +	+ +	None
Biliary metabolite, probably *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteinylglycine	0·24	0·65	Dark-violet	+ +	+ +	Purple–brown
Biliary metabolite, probably *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)glutathione	0·11	0·52	Dark-violet	+ +	+ +	Purple
9-Phenanthrylmercapturic acid	0·52	0·86	Orange	+	+	None
S-(9-Phenanthryl)cysteine	0·34	0·76	Orange	+	+	Purple
Substance formed from the above glycine conjugate with HCl	0·48	0·90	Orange	+	+	None
Substance, probably *S*-(9-phenanthryl)cysteinylglycine	0·37	0·70	Orange	+	+	Purple–brown
Substance, probably *S*-(9-phenanthryl)glutathione	0·16	0·61	Orange	+	+	Purple
1-Phenanthrylmercapturic acid	0·52	0·86	Pink–violet	+	+	None
S-(1-Phenanthryl)cysteine	0·34	0·74	Pink	+	+	Purple
2-Phenanthrylmercapturic acid	0·52	0·86	Pink	+	+	None
S-(2-Phenanthryl)cysteine	0·34	0·72	Pink	+	+	Purple
3-Phenanthrylmercapturic acid	0·52	0·86	Pink–violet	+	+	None
S-(3-Phenanthryl)cysteine	0·34	0·74	Pink–violet	+	+	Purple
4-Phenanthrylmercapturic acid	0·52	0·86	Pink–violet	+	+	None
S-(4-Phenanthryl)cysteine	0·34	0·75	Pink	+	+	Purple

and rats treated with phenanthrene were examined on paper chromatograms to give the results described below and then chromatographed on cellulose-powder columns (prepared from 250 g. of Whatman standard-grade cellulose powder) with butan-1-ol–cyclohexane–2N-NH_3 (9:2:1, by vol.) as the developing solvent. Fractions of 25 ml. were collected, aq. NH_3 (sp.gr. 0·88) (2 ml.) was added to each fraction and the mixtures were evaporated under reduced pressure. The residues were examined on paper chromatograms and then treated as described below.

Examination of metabolites in bile. Three rats, in which biliary fistulae had been established by the method of Boyland, Ramsay & Sims (1961), were each given phenanthrene (250 mg.) as a 2% (w/v) solution in arachis oil by intraperitoneal injection on the day after the operations. The biles were collected for 3 days, pooled and diluted with 3 vol. of water. The pH of the solution was adjusted to 4 with acetic acid, and charcoal (10 g.) was added with stirring. The charcoal was filtered off and washed with water (500 ml.), and the adsorbed material eluted with methanol (750 ml.) containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). Removal of the solvent gave a brown gum which was treated as described below.

RESULTS

The mercapturic acid fractions from the urines of both rabbits and rats treated with phenanthrene formed clear light-brown gums each weighing about 1·5 g. An examination of the gums on paper chromatograms showed that they consisted mainly of three substances, all of which gave positive reactions with the platinic iodide and $K_2Cr_2O_7$–$AgNO_3$ reagents, although only one reacted with ninhydrin. On the two-dimensional chromatograms described above, these substances gave three pink-fluorescent spots, one of which was indistinguishable from 9-phenanthrylmercapturic acid and one from *S*-(9-phenanthryl)cysteine. The third new substance, which did not react with ninhydrin, was not identified. A fourth, but minor, component of the gum derived from the rabbit urine formed a violet–pink-fluorescing spot in u.v. light on paper chromatograms which was indistinguishable from that of 1-phenanthrylmercapturic acid. Small amounts of 1-, 2-, 3- and 4-hydroxyphenanthrene, which were present in the gums from animals of both species, were detected on thin-layer chromatograms.

Mercapturic acid fractions from rabbit urine

The gum was chromatographed on a cellulose-powder column as indicated above. Fractions 1–4 were evaporated to yield gummy solids which were combined and recrystallized from aq. ethanol to yield phenanthrene (25 mg.), m.p. and mixed m.p. 94°. The mother liquors contained compounds indistinguishable from 1-, 2-, 3- and 4-hydroxyphenanthrene. Fractions 5 and 6 were combined and evaporated and the residue was dissolved in saturated aq. $NaHCO_3$ (1 ml.). The solution was acidified with HCl and kept at 0° for several days when a dark powder separated. This was purified by several precipitations with acetic acid from its solutions in 2N-NaOH to yield 1-phenanthrylmercapturic acid (5 mg.), m.p. and mixed m.p. 163–165° (decomp.). The acid was indistinguishable from the authentic material on paper chromatograms. The combined mother liquors were treated with charcoal (1 g.) which was filtered off and washed with water (100 ml.). The absorbed material was eluted with methanol (100 ml.) containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88) and evaporation of the solvent yielded a brown gum which appeared, on examination on paper chromatograms, to be a mixture of phenanthrylmercapturic acids.

On evaporation, fraction 8 yielded benzoic acid (150 mg.), m.p. and mixed m.p. 121°. Fractions 12 and 13 contained the first of the acid-labile substances mentioned above, together with indoxyl sulphate. The fractions were combined and evaporated and the residual gum was applied to the base lines of six sheets of Whatman no. 1 chromatography paper. The chromatograms were developed with solvent 1 for 18 hr. and the position of the metabolite on the chromatograms was found by means of its dark-violet fluorescence in u.v. light. The appropriate areas were cut from the chromatograms and the metabolite was eluted from the paper with methanol (250 ml.) containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). Removal of the solvent gave a gum which was applied to fresh sheets of chromatography paper and the chromatograms were developed with solvent 2. The metabolite was located and eluted as before, and evaporation of the solvent yielded a clear light-brown gum (900 mg.) which could not be crystallized, although it formed only one detectable spot on paper chromatograms. The gum was soluble in aq. 2N-NaOH and in hot water: from the latter solvent it separated unchanged on cooling. When the gum (250 mg.) was suspended in water (5 ml.) and HCl (0·5 ml.) added, a solid separated which was recrystallized from aq. ethanol to yield phenanthrene (105 mg.) in plates, m.p. and mixed m.p. 94°. The mother liquors from the acidification were examined on chromatograms and contained compounds indistinguishable from 9-phenanthrylmercapturic acid and *NN'*-diacetylcystine. 9-Hydroxyphenanthrene and *N*-acetylcysteine were not detected. The mother liquors were treated with charcoal (0·5 g.), and the charcoal was filtered off and washed with water (100 ml.). The adsorbed material was eluted with methanol (100 ml.) containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88) and the methanol was evaporated to leave a gum which was applied to the base line of a chromatogram which was developed with solvent 1 for 18 hr. The pink-fluorescent band seen when the chromatogram was inspected in u.v. light was cut out and the adsorbed material eluted with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). The solid (12 mg.) obtained on evaporation of the solvent was recrystallized from water to yield 9-phenanthrylmercapturic acid as a pink powder, m.p. and mixed m.p. 193–194° (decomp.).

The remainder of the metabolite was dissolved in methanol and esterified with diazomethane in ether. Removal of the solvent under reduced pressure gave a red gum which crystallized on rubbing with a little methanol. The product was recrystallized from aq. methanol to yield the (−)-*methyl ester* of N-*acetyl*-S-(9,10-*dihydro*-9-*hydroxy*-10-*phenanthryl*)*cysteine* in flat needles, m.p. 94°, $[\alpha]_D^{27}$ −406±4° (*c* 0·5 in methanol) (Found, after drying for 2 hr. at 61° and 10 mm.: C, 59·6, 59·7; H, 6·05, 6·1; N, 3·4, 3·4; S, 8·0, 8·1. $C_{20}H_{21}NO_4S,1{\cdot}5H_2O$ requires C, 60·3; H, 6·1; N, 3·5; S, 8·0%). A sample dried at 61° and 10 mm. for 16 hr. had m.p. 93° (Found: C, 64·0; H, 5·75; N, 3·5; S, 8·4. $C_{20}H_{21}NO_4S$ requires C, 64·6; H, 5·7; N, 3·8; S, 8·6%). At higher temperatures the product began to decompose. Thus, when the ester was heated in a micro-

sublimation apparatus at 120° and 10 mm. for 2 hr., phenanthrene (25 mg.), m.p. and mixed m.p. 94°, sublimed and the residue, after hydrolysis with HBr as described above, yielded compounds indistinguishable on paper chromatograms from *S*-(9-phenanthryl)cysteine and cystine. The ester (100 mg.) in methanol (0·5 ml.) was acidified with HCl (0·25 ml.) and water (2 ml.) added. The product which separated was recrystallized from aq. ethanol to yield phenanthrene (20 mg.), m.p. and mixed m.p. 92–93°. The mother liquors were hydrolysed with HBr as described above to yield products indistinguishable from cystine and *S*-(9-phenanthryl)cysteine on paper chromatograms. The ester (50 mg.) in methanol (10 ml.) was treated with Raney nickel catalyst (500 mg.) as described above and the ether-soluble products were crystallized from aq. methanol: unchanged ester (28 mg.) separated and the mother liquors contained a substance indistinguishable from 9-hydroxyphenanthrene on thin-layer chromatograms. The mother liquors and the aqueous phase were combined and evaporated and the residue was hydrolysed with HBr to yield a small amount of a substance indistinguishable from alanine on paper chromatograms.

Fractions 14 and 15 from the column contained small amounts of the mercapturic acid described above together with a second acid-labile product. The fractions were combined and the product was purified by chromatography on Whatman no. 1 paper as described for the first metabolite. The metabolite formed a gum (15 mg.) which could not be crystallized, although only one spot was detected on paper chromatograms. It was soluble in aq. $NaHCO_3$ and in 2N-NH_3 and reacted with diazomethane in ether to give a substance whose R_F (0·85) in solvent 1 differed from that (0·32) of the original compound. When the product was acidified with HCl, phenanthrene, identified on thin-layer chromatograms, separated; a second compound with a pink fluorescence in u.v. light whose R_F value differed from those of 9-phenanthrylmercapturic acid, *S*-(9-phenanthryl)cysteine and *S*-(9-phenanthryl)cysteinylglycine (see Table 2) was detected on paper chromatograms developed with solvent 1. When the metabolite was hydrolysed with HBr as described above, compounds indistinguishable from *S*-(9-phenanthryl)cysteine, cystine and glycine were detected on paper chromatograms, and hydrogenolysis with Raney nickel catalyst as described above yielded small amounts of compounds indistinguishable from 9-hydroxyphenanthrene, alanine and glycine. This metabolite was, however, different in properties from *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteinylglycine which was present in rat bile. Its structure is not known.

Fractions 17 and 18 contained a substance which appeared to be *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)-cysteine. The product was purified on sheets of chromatography paper as described above to yield a light-brown gum (125 mg.). The gum (100 mg.) was dissolved in water (0·5 ml.) and acidified with HCl when phenanthrene (20 mg.), m.p. and mixed m.p. 93–94°, separated immediately. The pH of the filtrate was adjusted to 5 with $NaHCO_3$ and the solution was kept overnight at 0° when cystine (15 mg.), decomposing at 250°, separated. The amino acid and its 2,4-dinitrophenyl derivative were indistinguishable from the authentic materials on paper chromatograms. The mother liquors were kept in an open dish for several days, when a pink solid (15 mg.) separated, which was purified by several precipitations with acetic acid from its solution in 2N-NaOH to yield *S*-(9-phenan-

Table 2. *Ultraviolet absorption of* N-*acetyl*-S-(9,10-*dihydro*-9-*hydroxy*-10-*phenanthryl*)*cysteine and related compounds*

Measurements were made, in ethanol solution, on a Perkin–Elmer model 137 ultraviolet spectrophotometer. Values in parentheses are those of pronounced shoulders in the curves.

Compound	Source	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$
Methyl ester of *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine	Rabbit urine	269 (230) (300)	17 000 (13 000) (2 800)
	Rat urine	269 (230) (300)	17 000 (13 000) (2 800)
N-Acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine	Rabbit urine	268 (300)	—
	Rat urine	269 (300)	—
S-(9,10-Dihydro-9-hydroxy-10-phenanthryl)cysteine	Rabbit urine	269 (230) (300)	—
	Rat urine	268 (230) (300)	—
	Synthetic (see text)	269 (230) (300)	—
Metabolite, probably a glycine conjugate	Rabbit urine	269 (300)	—
trans-9,10-Dihydro-9,10-dihydroxyphenanthrene	Synthetic	268 (300)	16 000* (2 000)

* Beale & Roe (1951).

thryl)cysteine, m.p. and mixed m.p. 180° (decomp.) (Found: N, 4·4; S, 10·4. Calc. for $C_{17}H_{15}NO_2S$: N, 4·7; S, 10·8%). Hydrogenolysis of the metabolite with Raney nickel as indicated above yielded an ether-soluble compound indistinguishable on thin-layer chromatograms from 9-hydroxyphenanthrene whereas the aqueous layer contained compounds indistinguishable from the unchanged metabolite and alanine on paper chromatograms.

An examination of the urines of rabbits treated with phenanthrene for the presence of acid-labile mercapturic acids other than that described above was carried out separately. About 250 mg. of the gum obtained from the urine of these animals by the charcoal procedure described by Sims (1962) was chromatographed on four sheets of Whatman no. 3MM chromatography paper for 18 hr. with solvent 1. The regions of the papers containing the mercapturic acids were located by means of the platinic iodide reagent applied to test strips and these regions were cut and the material eluted from the paper with methanol containing 5% (v/v) of NH_3 (sp.gr. 0·88). The solvent was removed and a little of the residual gum was dissolved in water (0·5 ml.) and HCl (0·25 ml.) added. The mixture was extracted with ether and it was shown by means of thin-layer chromatography that the ether layer contained compounds indistinguishable from phenanthrene and 1-, 2-, 3- and 4-hydroxyphenanthrene. The aqueous layer contained compounds indistinguishable from *NN'*-diacetylcystine and 9-phenanthrylmercapturic acid on paper chromatograms.

Although the presence of other phenanthrylmercapturic acids was suspected it was not possible to demonstrate their presence in a satisfactory manner. Hydrogenolysis of the gum with Raney nickel catalyst as indicated above also yielded compounds indistinguishable from 1-, 2-, 3- and 4-hydroxyphenanthrene. After acidification, the sizes of the spots formed by the hydroxyphenanthrenes on the thin-layer chromatograms indicated the presence of more 1- and 2- than 3- and 4-isomers, but after hydrogenolysis 2-hydroxyphenanthrene was the predominant isomer. These findings indicate that *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-phenanthryl)cysteine, *N*-acetyl-*S*-(3,4-dihydro-4-hydroxy-3-phenanthryl)cysteine and *N*-acetyl-*S*-(3,4-dihydro-3-hydroxy-4-phenanthryl)cysteine were present.

Mercapturic acid fractions from rat urine

The examination and separation of the metabolites was carried out as above with first a cellulose-powder column and then sheets of Whatman no. 1 chromatography paper to effect the separations. A mixture of phenanthrylmercapturic acids, which formed a brown gum, was present in fractions 4 and 5 from the column.

The compound identical in properties with *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine was present in fractions 9–11. After purification on paper chromatograms it formed a light-brown gum (700 mg.) similar to the product isolated from rabbit urine. The gum was dissolved in methanol and esterified with diazomethane in ether to yield the (−)-methyl ester of *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine (350 mg.) separating from aq. methanol in flat needles, m.p. 94° undepressed in admixture with the (−)-methyl ester obtained from the rabbit, $[\alpha]_D^{27} - 399 \pm 2°$ (*c* 0·5 in methanol) (Found, on a sample dried at 61° and 10 mm. for 2 hr.: C, 60·3, 60·0; H, 6·0, 6·0; N, 3·4; S, 8·0. Calc. for $C_{20}H_{21}NO_4S$, $1{\cdot}5H_2O$; C, 60·3; H, 6·1; N, 3·5; S, 8·0%). The ester had the same chemical properties as those described for the product obtained from the rabbit.

The second, unidentified, metabolite (30 mg.) was present in fractions 13 and 14. It was indistinguishable from the rabbit metabolite on paper chromatograms and, like this metabolite, it yielded *S*-(9-phenanthryl)cysteine and glycine on acid hydrolysis.

Only small amounts of the compound believed to be *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine were

Table 3. *Degradation products of the amino acid conjugates present in the bile of rats dosed with phenanthrene*

Experimental details are given in the text.

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*-(9,10-dihydro-9-hydroxy-10-phenanthryl)-cysteine	9-Phenanthrylmercapturic acid; *NN'*-diacetylcystine	*S*-(9-Phenanthryl)cysteine; cystine	Alanine
S-(9,10-Dihydro-9-hydroxy-10-phenanthryl)cysteine	*S*-(9-Phenanthryl)cysteine; cystine	*S*-(9-Phenanthryl)cysteine; cystine	Alanine
S-(9,10-Dihydro-9-hydroxy-10-phenanthryl)cysteinyl-glycine	*S*-(9-Phenanthryl)cysteinyl-glycine; unidentified peptide, probably oxidized cysteinylglycine	*S*-(9-Phenanthryl)cysteine; cystine; glycine	Alanine; glycine
S-(9,10-Dihydro-9-hydroxy-10-phenanthryl)glutathione	*S*-(9-Phenanthryl)glut-athione; oxidized glut-athione	*S*-(9-Phenanthryl)cysteine; cystine; glycine; glutamic acid	Alanine; glycine; glutamic acid
Metabolite, probably a glycine conjugate	Unidentified amino acid conjugate	*S*-(9-Phenanthryl)cysteine; cystine; glycine	Alanine; glycine

* Phenanthrene was liberated in these reactions.
† 9-Hydroxyphenanthrene was also found in these reactions.

found (in fraction 15) in rat urine, but the compound was indistinguishable on paper chromatograms from the corresponding rabbit metabolite.

A separate examination for other acid-labile mercapturic acids was carried out as described above. Compounds were detected which on acidification gave rise to phenanthrene and 1-, 2-, 3- and 4-hydroxyphenanthrene and, after hydrogenolysis with Raney nickel catalyst, to 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene, indicating that the same mercapturic acids present in rabbit urine were excreted by rats.

Examination of the bile of rats

The gum was examined on paper chromatograms with solvent 1. Five compounds were detected which gave positive reactions with the platinic iodide and $K_2Cr_2O_7$–$AgNO_3$ reagents and, of these, three gave colours with ninhydrin. The gum was applied to the base lines of six sheets of Whatman no. 3MM paper and the chromatograms were developed for 18 hr. with solvent 1. The positions of the five metabolites on the chromatograms were determined by means of test strips dipped in the platinic iodide reagent and the appropriate areas of the chromatograms were cut out and the materials eluted from the paper with methanol containing 5% (w/v) of aq. NH_3 (sp.gr. 0·88). The solutions were evaporated to yield gums, portions of which were hydrolysed with HBr and portions treated with Raney nickel catalyst and the products hydrolysed as described above. The hydrolysis products were examined for amino acids on paper chromatograms to give results which are shown in Table 3. The presence of other amino acid conjugates or other metabolites of phenanthrene such as glucuronic acid conjugates was not investigated in this experiment, but the sulphuric esters found in the urine of animals treated with phenanthrene (Sims, 1962) were not present.

DISCUSSION

The principal mercapturic acid present in the urines of both rabbits and rats dosed with phenanthrene appears to be *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine and the proposed structure is supported by the light-absorption characteristics of the compound (see Table 2). The methyl esters of the mercapturic acids from both rabbits and rats were identical in melting point and optical rotation, indicating that animals of both species excrete the same diastereoisomeric form of the mercapturic acid. If the mercapturic acid is derived from the product obtained by the enzymic reaction between glutathione and 9,10-epoxy-9,10-dihydrophenanthrene (Booth, Boyland & Sims, 1961) then the enzymes catalysing the reaction in animals of the two species appear to be the same or closely related.

Of the two related metabolites that are present in urine in much smaller amounts than the mercapturic acid, the metabolite which is believed to be *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine is indistinguishable on paper chromatograms, in its chemical properties and in its u.v. spectrum, from a compound obtained when the product from the oxidation of phenanthrene with perbenzoic acid was allowed to react with cysteine (E. Boyland & P. Sims, unpublished work). The oxidation product is believed to be 9,10-epoxy-9,10-dihydrophenanthrene, which should, on reaction with cysteine, give rise to a mixture of the diastereoisomers of the cysteine conjugate. The product obtained when the cysteine conjugate was acetylated with acetic anhydride in 2N-sodium hydroxide was indistinguishable on paper chromatograms from the above mercapturic acid.

The second metabolite related to the mercapturic acid, which appears to be a glycine conjugate, could not be identified. It resembles conjugates detected in the urine and bile of rats treated with 1,2-dihydronaphthalene and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene (Boyland & Sims, 1960; Boyland *et al.* 1961) and also in the urine of rabbits and rats treated with certain bromoalkanes (James, 1961). The additional conjugates, *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteinylglycine and the corresponding glutathione conjugate, detected in the bile of rats dosed with phenanthrene, resemble those of the corresponding metabolites detected in the bile of rats dosed with naphthalene (Boyland *et al.* 1961). The products present in bile are thus probably intermediate compounds in the conversion of 9,10-epoxy-9,10-dihydrophenanthrene into the mercapturic acid, which might proceed as follows: 9,10-epoxy-9,10-dihydrophenanthrene → *S*-(9,10-dihydro - 9 - hydroxy - 10 - phenanthryl) - glutathione → *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteinylglycine → *S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine → *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine.

The presence of the early intermediates in this process in the bile but not in the urine suggests that the kidney can reabsorb these derivatives.

The breakdown of the amino acid conjugates with mineral acid is evidently a complex process which differs from the breakdown under similar conditions of the conjugates related to *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine. These yield mainly the corresponding *S*-(1-naphthyl)-cysteine conjugates together with small amounts of 1- and 2-naphthol, naphthalene and the oxidized forms of the sulphur-containing amino acid side chains (Boyland & Sims, 1958; Boyland *et al.* 1961). With the phenanthrene metabolites described above, however, the main products of the acid decomposition are phenanthrene and the oxidized form of the amino acid side chains. Small amounts of the corresponding *S*-(9-phenanthryl)cysteine conjugates are formed but no 9-hydroxyphenanthrene could be detected. Thus, for example, *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine yields 1-naphthylmercapturic acid and small amounts of 1- and 2-naphthol, naphthalene and

NN'-diacetylcystine, whereas *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine yields phenanthrene and *NN'*-diacetylcystine and small amounts of 9-phenanthrylmercapturic acid. The liberation of phenanthrene from the mercapturic acid on acidification could account for some of the phenanthrene observed by Chang & Young (1943) when the urine of animals dosed with the hydrocarbon is acidified.

Other acid-labile mercapturic acids are present in the urines of animals treated with phenanthrene. Although 1-phenanthrylmercapturic acid was isolated, the acid was not detected in the untreated urine and this, together with the facts that 1- and 2-hydroxyphenanthrene are liberated from the mercapturic acid fraction on acidification and that 2-hydroxyphenanthrene was detected after the treatment of the fractions with Raney nickel catalyst, suggests that *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-phenanthryl)cysteine is present in these fractions. The decomposition of this conjugate with mineral acid thus resembles that of the corresponding naphthalene metabolite in that phenols are produced. Since 3- and 4-hydroxyphenanthrene are also liberated from the mercapturic acid fractions on acidification and 1-, 3- and 4-hydroxyphenanthrene are detected after treatment of the fraction with Raney nickel catalyst, it is probable that isomeric mercapturic acids, *N*-acetyl-*S*-(1,2-dihydro-1-hydroxy-2-phenanthryl)-, *N*-acetyl-*S*-(3,4-dihydro-3-hydroxy-4-phenanthryl)- and *N*-acetyl-*S*-(3,4-dihydro-4-hydroxy-3-phenanthryl)-cysteine are also present in the urine, although in small amount.

SUMMARY

1. Both rabbits and rats dosed with phenanthrene excreted in urine the same stereoisomeric form of *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine.

2. The non-acetylated cysteine derivative and a related conjugate which contained glycine but was not a derivative of 9-phenanthrylcysteinylglycine were also excreted.

3. The bile of these rats also contains the related cysteinylglycine and glutathione conjugates.

4. Treatment of the mercapturic acid with mineral acid yielded phenanthrene and *NN'*-diacetylcystine together with small amounts of 9-phenanthrylmercapturic acid, and hydrogenolysis with Raney nickel gave 9-hydroxyphenanthrene. The related amino acid conjugates were decomposed in an analogous manner.

5. *N*-Acetyl-*S*-(1,2-dihydro-2-hydroxy-1-phenanthryl)cysteine, *N*-acetyl-*S*-(1,2-dihydro-1-hydroxy-2-phenanthryl)cysteine, *N*-acetyl-*S*-(3,4-dihydro-3-hydroxy-4-phenanthryl)cysteine and *N*-acetyl-*S*-(3,4-dihydro-4-hydroxy-3-phenanthryl)cysteine were probably also formed. These were decomposed by mineral acid to give phenanthrylmercapturic acids and hydroxyphenanthrenes.

6. The syntheses of 1- and 9-phenanthrylcysteine and of their related mercapturic acids are described.

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