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Metabolism of Polycyclic Compounds

22. THE METABOLISM OF (±)-*trans*-9,10-DIHYDRO-9,10-DIHYDROXYPHENANTHRENE IN RATS

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Dihydrodihydroxy compounds are important intermediates in the metabolic pathways of aromatic hydrocarbons, giving rise to many of the metabolites observed in the urine when the parent hydrocarbons are given to animals. Thus, for example, when *trans*-1,2-dihydro-1,2-dihydroxynaphthalene is given to rats it is excreted partly unchanged in conjugation with glucuronic acid, partly as 1-naphthol in conjugation with sulphuric acid and glucuronic acid, partly as 2-naphthol and partly as 1,2-dihydroxynaphthalene in conjugation with sulphuric acid (Corner & Young, 1955; Sims, 1959), all of which are also products of naphthalene metabolism. Boyland & Wolf (1950) showed that phenanthrene is converted into *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene by rats and rabbits and into *trans*-1,2-dihydro-1,2-dihydroxyphenanthrene by rabbits. The present work has shown that *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene is converted by rats into conjugates of the dihydrodihydroxy compound with sulphuric acid and glucuronic acid and into the sulphuric acid conjugate of 9,10-dihydroxyphenanthrene. Conjugates of 9-hydroxyphenanthrene were not detected, although a small amount of the free phenol was present.

MATERIALS AND METHODS

Melting points. These are uncorrected.

Chromatography. Paper and thin-layer chromatograms, solvents and detection reagents were as described by Sims (1962). The properties of the compounds on paper chromatograms are given in Table 1 and by Sims (1962) and Boyland & Sims (1962).

Materials. (±)-*trans*-9,10-Dihydro-9,10-dihydroxyphenanthrene was prepared from 9,10-phenanthraquinone by the method of Booth, Boyland & Turner (1950). 9-Hydroxy- and 9,10-dihydroxy-phenanthrene, potassium 9-phenanthryl sulphate and ammonium 9-hydroxy-10-phenanthryl sulphate were prepared as described by Sims (1962).

(±)-*trans*-9,10-Dihydro-9,10-dihydroxyphenanthrene (800 mg.) and pyridine–sulphur trioxide (1 g.) (prepared by the method of Fernelius, 1946) were heated under reflux with pyridine (100 ml.) for 18 hr. The solution was cooled and ether (500 ml.) was added. The mixture was kept overnight; the gum which separated was dissolved in methanol (25 ml.), and dry NH_3 gas passed through the solution until no more solid separated. The solid [$(NH_4)_2SO_4$] was filtered off and to the mother liquors was added a solution of potassium acetate (0·5 g.) in methanol followed by ether (5 ml.). The solution was kept at 0° overnight and the solid (120 mg.) which separated was recrystallized from methanol–ether to yield *potassium* (±)-trans-9,10-*dihydro*-9-*hydroxy*-10-*phenanthryl sulphate* in plates (Found: C, 50·5; H, 3·7; S, 9·5. $C_{14}H_{11}KO_5S$ requires C, 50·9; H, 3·4; S, 9·7%).

(±)-*trans*-9,10-Dihydro-9,10-dihydroxyphenanthrene (2 g.) and sulphamic acid (5 g.), in pyridine (150 ml.), were heated under reflux for 18 hr. and the mixture was kept overnight. The solid was filtered off and extracted twice with boiling aq. ethanol (90%, w/v) (150 ml.). A solution of KOH (1 g.) in methanol (50 ml.) was added and the solid which separated was filtered off. The filtrate was evaporated under reduced pressure and the residue recrystallized twice from aq. ethanol to yield *dipotassium* (±)-trans-9,10-

Table 1. *Paper chromatography of compounds related to* trans-9,10-*dihydro*-9,10-*dihydroxyphenanthrene*

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	Colour with diazotized *p*-nitroaniline in 4N-HCl, heating and Na_2CO_3
trans-9,10-Dihydro-9-hydroxy-10-phenanthryl sulphate	0·51	0·31	Dark-violet	Orange
trans-9,10-Dihydro-9,10-phenanthrylene disulphate	0·23	—	Dark-violet	Orange
trans-9,10-Dihydro-9,10-dihydroxyphenanthrene*	0·85	0·92	Dark-violet	Orange

* This compound was also detected on thin-layer chromatograms with the methods previously described (Sims, 1962; Boyland & Sims, 1962).

dihydro-9,10-phenanthrylene disulphate (1·2 g.), in rods (Found: C, 36·7; H, 2·3; S, 14·4. $C_{14}H_{10}K_2O_8S_2$ requires C, 37·5; H, 2·25; S, 14·3%).

Hydrolyses. Hydrolyses with acid, barium methoxide, sulphatase and β-glucuronidase were carried out as described by Boyland & Sims (1962).

Animal experiments. Six rats were given (±)-*trans*-9,10-dihydro-9,10-hydroxyphenanthrene (100 mg.) in arachis oil (1 ml.) by intraperitoneal injection on 3 successive days. The urines were collected for a further 2 days after the last injections, pooled and stored at 0°. The combined urines were filtered, acidified to pH 4 with acetic acid, and charcoal (15 g.) was added. The charcoal was filtered off and washed with water (1 l.), and the adsorbed material was eluted from the charcoal with methanol (2 l.) containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). The solvent was evaporated under reduced pressure to leave a gum (2·5 g.) which was chromatographed on a cellulose-powder column (prepared from 200 g. of Whatman standard-grade cellulose powder) which was developed with butan-1-ol–cyclohexane–aq. 2N-NH_3 (9:2:1, by vol.). Fractions of 100 ml. were collected and evaporated under reduced pressure and the residues were examined on chromatograms and treated as described below.

RESULTS

Examination of fractions from the column

Fraction 1 was examined on thin-layer chromatograms and contained a small amount of a substance indistinguishable from 9-hydroxyphenanthrene.

Fractions 2 and 3 were dissolved in hot benzene and allowed to crystallize, when (±)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene (15 mg.) separated in needles, m.p. and mixed m.p. 187°. The mother liquors were evaporated and the residue acetylated with acetic anhydride in pyridine to yield a few mg. of a substance which separated from aq. ethanol in needles, m.p. 125–127°. A mixture with (+)-*trans*-9,10-diacetoxy-9,10-dihydrophenanthrene had m.p. 122–124°. When hydrolysed with acid the substance yielded a produce indistinguishable from 9-hydroxyphenanthrene on thin-layer chromatograms.

Fractions 4–9 contained substances indistinguishable from 9-hydroxy-10-phenanthryl sulphate and 9,10-dihydro-9-hydroxy-10-phenanthryl sulphate on paper chromatograms. The combined residues were chromatographed on four sheets of Whatman no. 3MM chromatography paper for 18 hr. with butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.). They were examined in u.v. light to determine the positions of the metabolites, and these regions were cut out and the absorbed materials eluted from the paper with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). The fraction containing 9-hydroxy-10-phenanthryl sulphate was evaporated to a light-brown gum (250 mg.) which was dissolved in hot 2N-NH_3 (1·5 ml.) and allowed to crystallize. The ammonium salt of the sulphuric ester (50 mg.) slowly separated in light-brown plates (Found: S, 10·5. Calc. for $C_{14}H_{13}NO_5S$: S, 10·4%). The ester was heated for 15 min. with 5N-HCl and the product which separated on cooling was filtered off and acetylated with acetic anhydride in 2N-NaOH to yield 9,10-diacetoxyphenanthrene, separating from ethanol in prisms, m.p. and mixed m.p. 201°. The mother liquors contained inorganic sulphate. The second product formed a gum (30 mg.) which could not be crystallized. The product was indistinguishable on paper chromatograms from 9,10-dihydro-9-hydroxy-10-phenanthryl sulphate. It yielded 9-hydroxyphenanthrene, identified on thin-layer chromatograms, together with inorganic sulphate, on hydrolysis with 5N-HCl at 100° or on heating with water to 100° for 15 min. No ether-soluble products could be identified after incubation with sulphatase. The light-absorption data confirm the proposed structure (see Table 2).

Fractions 10–15 contained no recognizable metabolites: mercapturic acids, if present, would be expected in these fractions. Fractions 17–32, which contained glucosiduronic acids, were combined and the gum was dissolved in methanol and treated with an excess of diazomethane in ether. The solvent was removed, and the residue was dissolved in pyridine and acetylated with acetic anhydride to yield a

Table 2. *Ultraviolet absorption of compounds related to* trans-9,10-*dihydro*-9,10-*dihydroxyphenanthrene*

Spectra were measured on a Perkin–Elmer model 137 ultraviolet spectrophotometer.

Compound	Solvent	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$
Potassium *trans*-9,10-dihydro-9-hydroxy-10-phenanthryl sulphate	Methanol	268*	17 200
Dipotassium *trans*-9,10-dihydro-9,10-phenanthrylene disulphate	Water	267*	18 000
Potassium 9-phenanthryl sulphate	Water	251	64 000
		274	23 000
		284	20 000
Ammonium 9-hydroxy-10-phenanthryl sulphate	Water	251	68 000
		274	14 000
		284	11 700
		295	13 500
(±)-*trans*-9,10-Dihydro-9,10-dihydroxyphenanthrene	Ethanol	268*	17 000
(+)-Methyl (9-acetoxy-9,10-dihydro-10-phenanthryltri-*O*-acetyl-D-glucosid)uronate	Ethanol	268*	17 800
(−)-Methyl (9-acetoxy-9,10-dihydro-10-phenanthryltri-*O*-acetyl-D-glucosid)uronate	Ethanol	268*	13 000
Metabolite, probably *trans*-9,10-dihydro-9-hydroxy-10-phenanthryl sulphate	Methanol	268*	—

* Compounds showed pronounced shoulders in the absorption curves at 224, 230 and 300 mμ.

mixture of derivatives which was separated into two products by fractional crystallization from ethanol. The less-soluble fraction was recrystallized from ethanol to yield (+)-methyl (9-acetoxy-9,10-dihydro-10-phenanthryltri-*O*-acetyl-D-glucosid)uronate (800 mg.) in needles, m.p. and mixed m.p. 247°, $[\alpha]_D^{25}+68\pm4°$ (*c* 0·5 in $CHCl_3$) (Found: C, 60·7; H, 5·2. Calc. for $C_{29}H_{30}O_{12}$: C, 61·05; H, 5·3%). The more-soluble fraction yielded (−)-*methyl* (9-*acetoxy*-9,10-*dihydro*-10-*phenanthryltri*-O-*acetyl*-D-*glucosid*)*uronate* (350 mg.) in plates (from ethanol), m.p. 167°, $[\alpha]_D^{26}-298\pm4°$ (*c* 0·5 in $CHCl_3$) (Found: C, 61·3; H, 5·3%). Mixtures with the products obtained from rabbits and rats dosed with phenanthrene (Boyland & Sims, 1962) had m.p. 163–164° and 166–167° respectively. Both derivatives yielded compounds indistinguishable from 9-hydroxyphenanthrene on thin-layer chromatograms after hydrolysis with acid, whereas hydrolysis with barium methoxide in methanol yielded compounds indistinguishable on paper chromatograms from the isomeric 9,10-dihydro-9-hydroxy-10-phenanthrylglucosiduronic acids (Boyland & Sims, 1962). The glucosiduronic acids were hydrolysed with glucuronidase to yield the corresponding dihydrodihydroxy compounds. The acid derived from the (+)-methyl ester gave the (−)-isomer, m.p. and mixed m.p. 160–161°, and that from the (−)-methyl ester gave the (+)-isomer, m.p. and mixed m.p. 161–162°. A glucosiduronic acid derivative of 9,10-dihydroxyphenanthrene could not be detected.

Decomposition of sulphuric esters

The decomposition of the sulphuric esters and of 9-phenanthryl sulphate in aqueous media under a variety of conditions was investigated. The products were identified by paper or thin-layer chromatography and the results are shown in Table 3. It was not possible to convert the monosulphuric ester of the dihydrodihydroxy compound into 9-phenanthryl sulphate.

DISCUSSION

In the metabolism of *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene, no conjugates of 9-hydroxyphenanthrene with sulphuric acid or glucuronic acid were observed, although there is good evidence for the presence of a conjugate of the dihydrodihydroxy compound with sulphuric acid as well as with glucuronic acid. It has been suggested (Corner & Young, 1955; Sims, 1959) that, in the metabolism of *trans*-1,2-dihydro-1,2-dihydroxynaphthalene, the 1-naphthol conjugates and the free 2-naphthol observed in the urine arise, not from the dihydrodihydroxy compound itself, but by the breakdown of the corresponding sulphuric acid or, less likely, the glucuronic acid conjugates. An attempt to prepare a mixture of the sulphuric esters of *trans*-1,2-dihydro-1,2-dihydroxynaphthalene was unsuccessful (Sims, 1959), presumably because of the instability of the ester mixture. Nevertheless, the experiments indicated that the 1-isomer breaks down under neutral conditions to yield 1-naphthyl sulphate (by loss of water) and 2-naphthol (by loss of sulphuric acid), whereas the 2-isomer breaks

Table 3. *Hydrolysis of the sulphuric esters related to* trans-9,10-*dihydro*-9,10-*dihydroxyphenanthrene*

Sulphuric ester	Treatment				
	Heated at 100°			Kept at room temp. for 7 days with: (*a*) water; (*b*) 2N-HCl; (*c*) acetate buffer, pH 5·7; (*d*) phosphate buffer, pH 7*	Incubated with sulphatase in acetate buffer, pH 5·7, for 18 hr. at 37°
	With 5N-HCl for 15 min.	With 5N-HCl for 1 min.	With water for 15 min.		
Potassium *trans*-9,10-dihydro-9-hydroxy-10-phenanthryl sulphate	9-Hydroxyphenanthrene	9-Hydroxyphenanthrene; unchanged ester	9-Hydroxyphenanthrene	9-Hydroxyphenanthrene	Unchanged ester
Dipotassium *trans*-9,10-dihydro-9,10-phenanthryl-ene sulphate	9-Hydroxyphenanthrene	9-Hydroxyphenanthrene; 9-phenanthryl sulphate; monosulphuric ester; unchanged ester	9-Hydroxyphenanthrene; 9-phenanthryl sulphate	9-Hydroxyphenanthrene; 9-phenanthryl sulphate; monosulphuric ester; unchanged ester	Unchanged ester
Potassium 9-phenanthryl sulphate	9-Hydroxyphenanthrene	9-Hydroxyphenanthrene; unchanged ester	9-Hydroxyphenanthrene; unchanged ester	9-Hydroxyphenanthrene; unchanged ester	9-Hydroxy-phenanthrene
Ammonium 9-hydroxy-10-phenanthryl sulphate	9,10-Dihydroxyphenanthrene	—	—	—	9,10-Phenanthraquinone

* Each of these treatments yielded the same products.

down only by loss of sulphuric acid to yield 1-naphthol. *trans*-9,10-Dihydro-9-hydroxy-10-phenanthryl sulphate is more stable than the esters of *trans*-1,2-dihydro-1,2-dihydroxynaphthalene, but, when it decomposes under conditions in which 9-phenanthryl sulphate is stable, it yields only 9-hydroxyphenanthrene and inorganic sulphate. The decomposition of the sulphuric ester in this way explains the presence of small amounts of 9-hydroxyphenanthrene and the absence of conjugates of this phenol in the urine of animals given either the dihydrodihydroxy compound or phenanthrene itself. Ukita & Takeshita (1961) have described the preparation of the cyclic phosphoric ester of *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene which decomposes on incubation at 37° in solution with pH values of less than 2·5 or greater than 9·5 to yield the monophosphoric ester of the dihydrodihydroxy compound. Although these results indicate that the phosphoric ester is more stable than the sulphuric ester described above, the mixed phosphoric esters obtained by these workers from the treatment of the cyclic ester with ethanol were unstable in acid, breaking down to give monoalkylphosphoric esters and presumably 9-hydroxyphenanthrene. The glucosiduronic acids of the dihydrodihydroxy compounds are more stable than the sulphuric ester, but are slowly broken down by warm dilute mineral acid to yield 9-hydroxyphenanthrene (by loss of glucuronic acid) and 9-phenanthrylglucosiduronic acid (by loss of water) (Boyland & Sims, 1962). The formation of sulphuric esters of aliphatic alcohols in the body has been shown by Vestermark & Boström (1959).

The disulphuric ester of the dihydrodihydroxy compound is readily synthesized, although 9,10-dihydroxyphenanthrene yields only its monosulphuric ester when treated with sulphamic acid under the same conditions. This difference is probably because there is no steric hindrance in the dihydrodihydroxy compound owing to the *trans* configuration of the hydroxyl groups. In spite of this there was no evidence that the diester is formed in the body. Both the mono- and the di-ester are resistant to the action of the sulphatases of Taka-diastase.

The sulphuric ester of 9,10-dihydroxyphenanthrene arises in the body by the dehydrogenation of the dihydrodihydroxy compound or its conjugates, presumably brought about by enzymes similar to those described by Mitoma, Posner & Leonard (1958) and by Ayengar, Hayaishi, Nakajima & Tomida (1959). Although the (±)-dihydrodihydroxy compound was administered, the amount of the glucosiduronic acid derivative of the (−)-dihydrodihydroxy compound isolated was more than twice that of the derivative of the (+)-isomer. This could be explained in part by the fact that the glucosiduronic acid derivative of the (−)-isomer is less soluble, and therefore easier to isolate, than that of the (+)-isomer. It might be, however, either that the enzymic dehydrogenation proceeds more readily with the (+)- than with the (−)-isomer, or, less likely, that the (+)-isomer is more readily converted into the sulphuric ester. If in the metabolism of phenanthrene the dihydrodihydroxy compound arises by the non-enzymic opening of the oxiran ring of 9,10-epoxy-9,10-dihydrophenanthrene (Boyland & Sims, 1962) to give a racemic mixture, then this difference in the ease of dehydrogenation of the two isomers could explain why more of the (−)- than of the (+)-isomer (and their derivatives) is isolated from the urine of both rabbits and rats treated with the hydrocarbon.

SUMMARY

1. A study of the metabolism of (±)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene in rats has shown that the compound is excreted free and in conjunction with sulphuric acid and glucuronic acid. More of the tetra-acetyl methyl ester derivative of the glucosiduronic acid of the (−)-dihydrodihydroxy compound was isolated than that of the (+)-isomer.

2. 9,10-Dihydroxyphenanthrene is formed and is excreted in conjugation with sulphuric acid.

3. The synthesis of potassium 9,10-dihydro-9-hydroxy-10-phenanthryl sulphate and of dipotassium 9,10-dihydro-9,10-phenanthrylene disulphate are described and the decomposition of the esters in aqueous solutions has been investigated.

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