# The Metabolism of Benz[*a*]anthracene and Dibenz[*a*,*h*]anthracene and their 5,6-Epoxy-5,6-Dihydro Derivatives by Rat-Liver Homogenates

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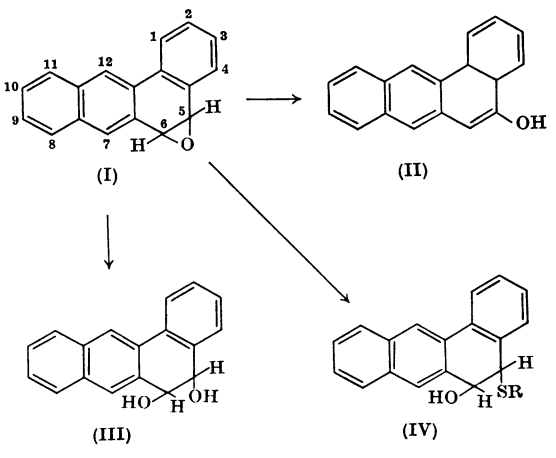
1. Benz[*a*]anthracene was hydroxylated by rat-liver homogenates on the 3,4-, 5,6- or 8,9-bond to yield phenols and dihydrodihydroxy compounds. Metabolic action at the 7- and 12-positions was also detected. 5,6-Epoxy-5,6-dihydrobenzanthracene was converted into a phenol that is probably 5-hydroxybenzanthracene and 5,6-dihydro-5,6-dihydroxybenzanthracene. Both substrates yielded a product that is probably *S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)glutathione. 2. Dibenz[*a*,*h*]anthracene was hydroxylated by rat-liver homogenates to yield products that are probably 3- and 4-hydroxydibenzanthracene, 1,2-dihydro-1,2-dihydroxydibenzanthracene, 3,4-dihydro-3,4-dihydroxydibenzanthracene and 5,6-dihydro-5,6-dihydroxydibenzanthracene. There was no evidence for metabolic action at the 7- and 14-positions. 5,6-Epoxy-5,6-dihydrodibenzanthracene was converted into a phenol that is probably 5-hydroxydibenzanthracene and 5,6-dihydro-5,6-dihydroxydibenzanthracene. Both substrates yielded a glutathione conjugate that is probably *S*-(5,6-dihydro-6-hydroxy-5-dibenzanthracenyl)glutathione. 3. The synthesis of 5,6-epoxy-5,6-dihydrodibenzanthracene is described and the reactions of this epoxide and 5,6-epoxy-5,6-dihydrobenzanthracene with water and thiols have been investigated. 4. The oxidation of dibenzanthracene in the ascorbic acid–$Fe^{2+}$ ion–oxygen model system is described.

5,6-Epoxy-5,6-dihydrobenz[*a*]anthracene (I; Scheme 1) has been postulated as an intermediate in benz[*a*]anthracene metabolism (Boyland & Sims, 1964*b*) and experiments described below suggest that 5,6-epoxy-5,6-dihydrodibenz[*a*,*h*]anthracene (VIII; Scheme 2) is an intermediate in the metabolism of dibenz[*a*,*h*]anthracene (V). The metabolism of these epoxides by rat-liver homogenates is described and is compared with the metabolism of the parent hydrocarbons in the same systems.

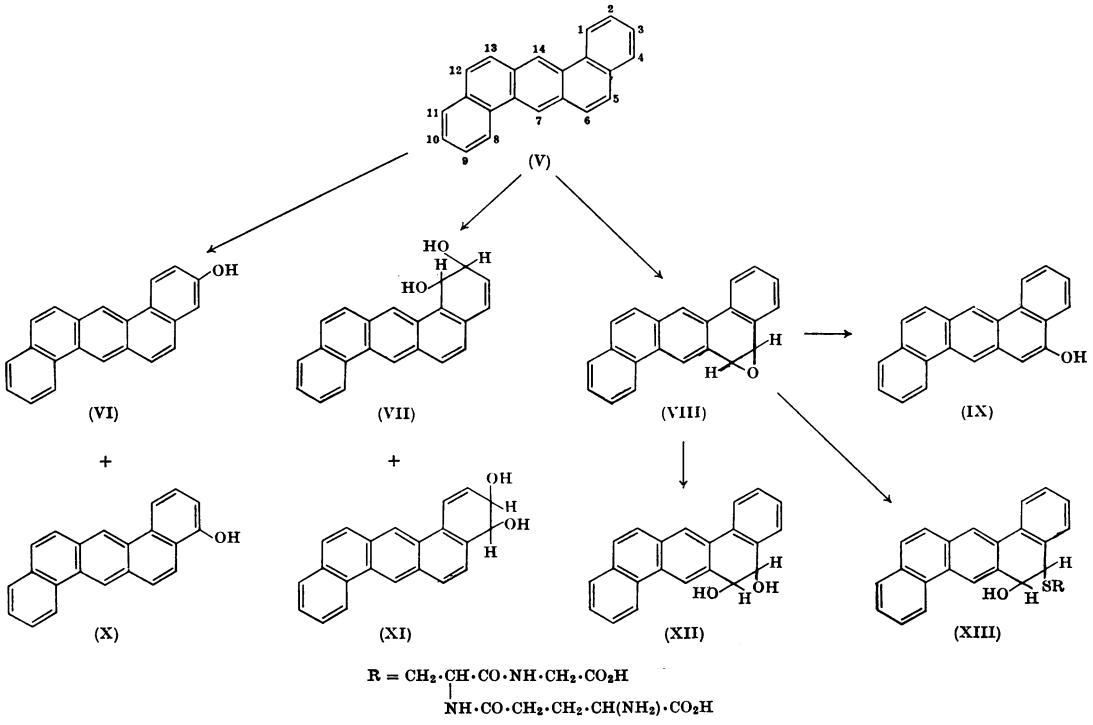
## EXPERIMENTAL

Melting points are uncorrected.

*Absorption spectra.* These were measured either on a Unicam SP.500 spectrophotometer or on a Perkin–Elmer model 137 ultraviolet spectrophotometer. The absorption spectra of compounds eluted from paper or thin-layer chromatograms were measured on the latter instrument: solutions of these compounds were obtained by eluting with methanol containing 1% (v/v) of aq. $NH_3$ (sp.gr. 0·88) the appropriate bands cut from paper chromatograms, or with ethanol the bands removed from thin-layer chromatograms. The positions of the bands on the chromatograms were determined by the fluorescence in u.v. light.




Scheme 1. Probable routes in the metabolism of 5,6-epoxy-5,6-dihydrobenz[*a*]anthracene by rat-liver homogenate. For the metabolic routes of benz[*a*]anthracene in rats see Boyland & Sims (1964*b*).




Scheme 2. Metabolism of dibenz[*a*,*h*]anthracene and 5,6-epoxy-5,6-dihydrodibenz[*a*,*h*]anthracene by rat-liver homogenate. Compound (IX) was formed from (VIII) but not from (V).

*Chromatography.* Paper chromatography was carried out on Whatman no. 1 chromatography paper by downward development for 18 hr. Chromatograms were developed with either (1) butan-1-ol–propan-1-ol–aq. 2N-$NH_3$ (2:1:1, by vol.) or (2) butan-1-ol–acetic acid–water (2:1:1, by vol.). The dried chromatograms were examined in u.v. light and dipped in either ninhydrin (0·2%) in acetone or in the platinic iodide reagent of Toennies & Kolb (1951).

Thin-layer chromatograms, prepared from silica gel G (E. Merck A.-G., Darmstadt, West Germany) of 0·25 mm. thickness, were developed for 10 cm. with (a) light petroleum (b.p. 60–80°)–benzene (19:1, v/v) or (b) benzene or (c) benzene–ethanol (19:1, v/v). The chromatograms were examined in u.v. light while still wet both before and after exposure to $NH_3$ and were then sprayed with a 0·5% solution of 2,6-dichloroquinonechloroimide in ethanol, followed by aq. 10% (w/v) $Na_2CO_3$, either immediately, or after spraying with conc. HCl and heating in an oven to 80°. Dihydro-dihydroxy compounds were detected on two-dimensional thin-layer chromatograms, which were developed in the first direction with (c), sprayed with conc. HCl and heated to 80° for 10 min. and developed in the second direction with (b). The chromatograms were examined as before and the formation of any phenols arising from the acid decompositon of the dihydrodihydroxy compounds was noted. The properties on thin-layer chromatograms of the compounds described below are listed in Table 1.

*Materials.* (*a*) Benz[*a*]anthracene derivatives. With the exception of 5,6-epoxy-5,6-dihydrobenz[*a*]anthracene (I), these were obtained as described by Boyland & Sims (1964*b*). The epoxide (I) was prepared from 2-phenylnaphthalene-2′,3-dicarboxaldehyde by the method of Newman & Blum (1964): when recrystallized from cyclohexane it had m.p. 118–119° (decomp.). Newman & Blum (1964) found m.p. 119–120°.

(*b*) Dibenz[*a*,*h*]anthracene derivatives. Dibenz[*a*,*h*]-anthracene (V), obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.) and from Roche Products Ltd. (Welwyn Garden City, Herts.), was converted into dibenz-[*a*,*h*]anthracene-7,14-quinone by oxidation with $Na_2Cr_2O_7$ in acetic acid as described by Cason & Fieser (1940). 7,14-Dihydrodibenz[*a*,*h*]anthracen-7-one was prepared from the reduction of the quinone with aluminium powder by the method of Cook (1931). 4,11- and 7,14-Dimethoxydibenz-[*a*,*h*]anthracene were samples that had been prepared in this Institute. The 4,11-dimethoxy compound was converted into the dihydroxy compound by heating under reflux with an excess of HBr (sp.gr. 1·7) for 24 hr. 2,9-Dihydroxydibenz[*a*,*h*]anthracene was obtained from the urine of rabbits dosed with dibenzanthracene (Boyland, Levi, Mawson & Roe, 1941).

*cis*-5,6-Dihydro-5,6-dihydroxydibenz[*a*,*h*]anthracene was prepared from the hydrocarbon by the action of $OsO_4$: a portion of the product heated with conc. HCl in acetic acid yielded 5-hydroxydibenz[*a*,*h*]anthracene (IX) (Cook & Schoental, 1948). The *cis*-dihydrodihydroxy compound

Table 1. *Properties on thin-layer chromatograms of compounds related to benz*[a]*anthracene and dibenz*[a,h]*anthracene*

Details are given in the text. For the properties of compounds related to benzanthracene that are not listed below see Boyland & Sims (1964*b*).

| Compound | $R_F$ Benzene | $R_F$ Benzene–ethanol (19:1, v/v) | Fluorescence Immediate | Fluorescence After exposure to $NH_3$ | Colour with 2,6-dichloroquinone-chloroimide–$Na_2CO_3$ |
|---|---|---|---|---|---|
| 5,6-Epoxy-5,6-dihydrobenz[*a*]anthracene (I)* | 0·42 | 0·03 | Dark violet turning blue | Yellow | Blue–green |
| 5,6-Epoxy-5,6-dihydrodibenz[*a,h*]-anthracene (VIII)* | 0·38 | 0·92 | Dark violet turning violet | Green | Blue–green |
| *cis*- and *trans*-5,6-Dihydro-5,6-dihydroxydibenz[*a,h*]anthracene (XII) | 0·00 | 0·25 | Dark violet | Dark violet | None (blue–green)† |
| 7,14-Dihydro-7,14-dihydroxydibenz-[*a,h*]anthracene | 0·00 | 0·41 | Dark violet | Dark violet | None (red)† |
| 5-Hydroxydibenz[*a,h*]anthracene (IX) | 0·35 | 0·52 | Violet | Green | Blue–green |
| 7,14-Dihydrodibenz[*a,h*]anthracen-7-one | 0·65 | — | Violet | Violet | None |
| Dibenz[*a,h*]anthracene-5,6-quinone | 0·05 | 0·64 | Dark-absorption | Dark-absorption | (Orange)‡ |
| Dibenz[*a,h*]anthracene-7,14-quinone | 0·78 | 0·92 | Orange | Orange | (Yellow)‡ |
| Dimeric compound | 0·16 | — | Dark orange | Dark orange | (Pale yellow)‡ |
| 4,11-Dihydroxydibenz[*a,h*]anthracene | 0·28 | 0·47 | Violet | Orange | Blue |
| 2,9-Dihydroxydibenz[*a,h*]anthracene | 0·00 | 0·12 | Violet | Yellow | Grey |
| Dibenz[*a,h*]anthracene (V) | (0·51)§ | | Violet | Violet | None |

* These compounds are decomposed on the chromatograms, particularly on those developed with benzene, to give the related phenols which formed fluorescent 'tails' to the epoxide spots.

† These colours appeared on chromatograms that were treated with acid as described in the text.

‡ These colours appeared on untreated chromatograms.

§ Measured in solvent (a) (see the text).

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was oxidized with $Na_2Cr_2O_7$ in acetic acid as described by Bhargava, Hadler & Heidelberger (1955) to yield dibenz[*a,h*]anthracene-5,6-quinone, and this was reduced with lithium tetrahydroaluminate in ether by using the method of Booth, Boyland & Turner (1950) to yield trans-5,6-*dihydro*-5,6-*dihydroxydibenz*[a,h]*anthracene* (XII), separating from benzene as a pale-pink powder, m.p. 227° (Found: C, 84·95; H, 5·4. $C_{22}H_{16}O_2$ requires C, 84·6; H, 5·2%). On a two-dimensional thin-layer chromatogram heated with acid between runs, the dihydrodihydroxy compound yielded a product indistinguishable from 5-hydroxydibenzanthracene (IX).

*cis*-5,6-Dihydro-5,6-dihydroxydibenzanthracene (3g.) was oxidized with sodium periodate (8g.) in aq. methanol under the conditions described by Hadler & Kryger (1960) for the oxidation of *cis*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene. The product obtained on evaporation of the methanol was recrystallized from light petroleum (b.p. 80–100°) to yield 2-*phenylphenanthrene*-2′,3-*dicarboxaldehyde* (1·8g.) as yellow crystals, m.p. 157–158° (Found: 84·9; H, 4·8. $C_{22}H_{14}O_2$ requires C, 85·1; H, 4·55%). The dialdehyde (500mg.), in dry benzene (5ml.), was heated under reflux with tri(dimethylamino)phosphine (0·5ml.). Crystals separated from the solution and, after 15min., the mixture was cooled in ice. The crystals were filtered off and recrystallized from benzene to yield 5,6-*epoxy*-5,6-*dihydrodibenz*[a,h]*anthracene* (250mg.) (VIII) in flat needles (Found: C, 89·65; H, 4·85. $C_{22}H_{14}O$ requires C, 89·8; H, 4·8%). A second crop (100mg.) of the epoxide was obtained when the benzene mother liquors were evaporated to dryness and the residue was treated with dry, ice-cold ether (5ml.). The epoxide had an indefinite melting point, turning yellow at 150° and brown with softening at 190° and finally decomposing at 200°: this is presumably due to the rearrangement of the epoxide to the related phenol (IX). Newman & Blum (1964) have found that other related epoxides undergo this rearrangement at elevated temperatures. The epoxide (100mg.), in acetone (1ml.), was treated with a few drops of conc. HCl and the solution heated under reflux for 30min. and diluted with water. The product that separated was identical with 5-hydroxydibenzanthracene in its u.v. spectrum and its mobility on thin-layer chromatograms in solvents (b) and (c). Newman & Blum (1964) have shown that 5,6-epoxy-5,6-dihydrodibenzanthracene (I) yields 5-hydroxybenzanthracene (II) with acid.

Dibenzanthracene-7,14-quinone (590mg.) and lithium tetrahydroaluminate (500mg.) were heated under reflux in dry ether (100ml.) for 30min. The unchanged hydride was decomposed by the cautious addition of water, and 2N-$H_2SO_4$ (30ml.) was added and the mixture shaken. The solid (450mg.) that separated mainly in the aqueous layer was collected and the aqueous and ethereal layers were separated. The ethereal layer was washed with aq. 2N-NaOH (100ml.) and dried and the solvent evaporated. The residue contained a mixture of products, which could not be separated from each other by crystallization. The material

was therefore applied to the base lines of thin-layer chromatograms, which were developed in (c) for 10 cm. and examined in u.v. light. Dark-violet fluorescent bands at $R_F$ 0·42 were removed and the absorbed material was eluted from the silica gel with ether. Removal of the solvent yielded 7,14-*dihydro*-7,14-*dihydroxydibenz*[a,h]*anthracene* (15 mg.), separating from benzene in needles, m.p. 238–240° (decomp.) (darkening at 170° and softening at 220°) (Found: C, 84·5; H, 5·0. $C_{22}H_{16}O_2$ requires C, 84·6; H, 5·2%). Its u.v. spectrum is in agreement with the proposed structure and differs from that of 7,14-dimethoxydibenzanthracene (see Table 3).

The solid that first separated from the reaction mixture was dissolved in a minimum amount of boiling ethanol, and the product that separated from the cooled solution was filtered off and examined on thin-layer chromatograms developed with (b) and found to contain compounds identical in their mobilities and properties with dibenzanthracene-7,14-quinone and 7,14-dihydrodibenzanthracen-7-one. An unidentified product, $R_F$ 0·92, that gave a red colour with the 2,6-dichloroquinonechloroimide–$Na_2CO_3$ reagent was also detected. The filtrate was diluted with water and the product (30 mg.) that separated recrystallized from aq. ethanol in pale-yellow needles, m.p. 250–252°. It is probably a dimeric dibenz[*a,h*]anthracenone, related to the product obtained as a by-product in the reduction of benz[*a*]anthracene-7,12-quinone with lithium tetrahydroaluminate (Boyland & Sims, 1964*b*) (Found: C, 90·1; H, 4·5. Calc. for $C_{44}H_{24}O_2$: C, 90·4; H, 4·1%).

When the dihydrodihydroxy compound was heated to 100° with conc. HCl and the ether-soluble products were examined on thin-layer chromatograms, compounds identical in their mobilities and properties with dibenzanthracene-7,14-quinone, 7,14-dihydrodibenzanthracene-7-one, the dimeric compound and the unidentified product described above were detected.

*Reactions of the epoxides with water.* The epoxides (I and VIII) (50 mg.) were each heated under reflux with aq. 50% (v/v) acetone (50 ml.) for 24 hr. The acetone was distilled off and the solids that separated were each dissolved in ether (20 ml.). The solutions were washed twice with 2N-NaOH (10 ml.) and evaporated under reduced pressure. The residues were dissolved in the minimum of boiling benzene and the solutions were allowed to crystallize. The crystals that separated from the product of the reaction of (I) were recrystallized from benzene to yield *trans*-5,6-dihydro-5,6-dihydroxybenzanthracene (III) (10 mg.), m.p. 210° (decomp.), undepressed in admixture with an authentic specimen. The dihydrodihydroxy compounds were identical with each other in their mobilities and properties on thin-layer chromatograms developed with solvent (c). The solution of the product of the reaction of (VIII) failed to crystallize but on thin-layer chromatograms it was seen to contain a product that was indistinguishable from *cis*- or *trans*-5,6-dihydro-5,6-dihydroxydibenzanthracene (XII). The dihydrodihydroxy compound probably has the *trans*-configuration since both the epoxide (I) and 9,10-epoxy-9,10-dihydrophenanthrene (Boyland & Sims, 1965*b*) yield *trans*-dihydrodihydroxy compounds with water.

The aqueous layers were acidified with conc. HCl and extracted with ether (20 ml.). The ether extracts were examined on thin-layer chromatograms, when that from the

Table 2. *Properties on paper chromatograms of amino acid conjugates related to benz[a]anthracene and dibenz*[a,h]*anthracene*

Details are given in the text. ++, Strong, and +, weak, yellow colour on pink background.

| Probable identity of conjugate | $R_F$: Butan-1-ol–propan-1-ol–2N-$NH_3$ (2:1:1, by vol.) | $R_F$: Butan-1-ol–acetic acid–water (2:1:1, by vol.) | Fluorescence | Colour with ninhydrin | Reaction with platinic iodide |
|---|---|---|---|---|---|
| *N*-Acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benz[*a*]-anthracenyl)-L-cysteine | 0·35 | 0·82 | Dark violet | None | ++ |
| 5-Benz[*a*]anthracenylmercapturic acid | 0·51 | 0·86 | Violet | None | + |
| *S*-(5,6-Dihydro-6-hydroxy-5-benz[*a*]anthracenyl)-L-cysteine | 0·30 | 0·62 | Dark violet | Purple | ++ |
| *S*-(5-Benzanthracenyl)-L-cysteine | 0·45 | 0·75 | Violet | Purple | + |
| *S*-(5,6-Dihydro-6-hydroxy-5-benz[*a*]anthracenyl)-glutathione (IV) | 0·11 | 0·47 | Dark violet | Purple | ++ |
| *S*-(5-Benzanthracenyl)glutathione | 0·19 | 0·62 | Violet | Purple | + |
| *N*-Acetyl-*S*-(5,6-dihydro-6-hydroxy-5-dibenz[*a,h*]-anthracenyl)-L-cysteine | 0·33 | 0·81 | Dark violet | None | ++ |
| 5-Dibenz[*a,h*]anthracenylmercapturic acid | 0·50 | 0·85 | Violet | None | + |
| *S*-(5,6-Dihydro-6-hydroxy-5-dibenz[*a,h*]anthracenyl)-L-cysteine | 0·28 | 0·60 | Dark violet | Purple | ++ |
| *S*-(5-Dibenz[*a,h*]anthracenyl)-L-cysteine | 0·41 | 0·74 | Violet | Purple | + |
| *S*-(5,6-Dihydro-6-hydroxy-5-dibenz[*a,h*]anthracenyl)-glutathione (XIII) | 0·10 | 0·46 | Dark violet | Purple | ++ |
| *S*-(5-Dibenz[*a,h*]anthracenyl)glutathione | 0·17 | 0·61 | Violet | Purple | + |

reaction of (I) was found to contain a compound identical in its mobility and properties with 5-hydroxybenzanthracene (II) and that from (VIII) a compound similarly identical with 5-hydroxydibenzanthracene (IX).

*Reactions of the epoxide with N-acetylcysteine, cysteine and glutathione.* The epoxide (I or VIII) (10mg.), $NaHCO_3$ (20mg.) and the thiol compound (10mg.) in aq. 50% (v/v) acetone (20ml.) were heated under reflux for 4hr. In each experiment the acetone was distilled off under reduced pressure and the aqueous mixture extracted with ether (10ml.). The residue obtained on evaporation of the ether was examined. 5,6-Epoxy-5,6-dihydrobenzanthracene (I) yielded compounds with the properties on thin-layer chromatograms of 5-hydroxybenzanthracene (II) and 5,6-dihydro-5,6-dihydroxybenzanthracene (III), and 5,6-epoxy-5,6-dihydrodibenzanthracene (VIII) similarly yielded 5-hydroxydibenzanthracene (IX) and 5,6-dihydro-5,6-dihydroxydibenzanthracene (XII). The aqueous layer was acidified to pH4 with acetic acid and activated charcoal (British Drug Houses Ltd.) (2g.) was added. The charcoal was filtered off, washed with water (50ml.) and the absorbed material eluted with methanol (250ml.) containing 5% (v/v) of aq. $NH_3$ (sp.gr. 0·88). The solution was evaporated and the residue applied to the base line of a paper chromatogram developed with (1). The conjugate was located as a violet fluorescent band, seen when the chromatogram was examined in u.v. light. The band was cut out and the conjugate eluted with methanol (25ml.) containing 5% (v/v) of aq. $NH_3$ (sp.gr. 0·88). The solution was evaporated to yield a gum that was probably a mixture of the diastereoisomers of the conjugate. The reactions with cysteine proceeded less readily than the reactions with the other thiol compounds. The properties of the conjugates are recorded in Table 2 and their u.v.-absorption spectra in Table 3.

Portions of the gums were each dissolved in water (0·5ml.) and acidified with conc. HCl (0·1ml.). The mixtures were applied to the base lines of paper chromatograms which were developed as before. Each chromatogram showed two fluorescent bands when examined in u.v. light and these were cut out and the absorbed material was eluted as before. The materials from the faster-moving bands were examined on thin-layer chromatograms developed with either (a) or (b). The conjugates derived from 5,6-epoxy-5,6-dihydrobenzanthracene (I) all yielded compounds identical in their u.v. spectra and mobilities with benzanthracene, and those derived from 5,6-epoxy-5,6-dihydrodibenzanthracene (VIII) yielded compounds similarly identical with dibenzanthracene. Hydroxybenzanthracenes and hydroxydibenzanthracenes were not detected. The materials from the slower-moving bands are presumed to contain the related benzanthracenyl and dibenzanthracenyl conjugates. The properties of these conjugates on paper chromatograms are recorded in Table 2.

It has been shown (e.g. Boyland, Ramsay & Sims, 1961) that, when conjugates of the type described above are decomposed with conc. HCl, the oxidized forms of the sulphur-containing amino acid side chains are formed. The glutathione conjugates described above both yielded a compound with acid identical with oxidized glutathione in its mobility and properties on paper chromatograms developed with solvent (2): reduced glutathione was not detected.

Small portions of the conjugates were heated under reflux with 5% (w/v) KOH in methanol (1ml.) for 2hr. The mixtures were diluted with water, acidified with conc. HCl and extracted with ether. Examination of the ether extracts on thin-layer chromatograms showed the presence of small amounts of phenols that had similar properties to those of

Table 3. *Ultraviolet-absorption spectra of compounds related to benz*[a]*anthracene and dibenz*[a,h]*anthracene*

Measurements were made as described in the text. When measured the molecular extinction coefficient is shown in parentheses (as log $\epsilon_{max.}$). For the spectra of benz[*a*]anthracene derivatives not listed below see Boyland & Sims (1964*b*).

| Compound | $\lambda_{max.}$ (m$\mu$) |
|---|---|
| 5,6-Epoxy-5,6-dihydrobenz[*a*]anthracene (I) | 217(4·66), 250(4·51), 260(4·67), 270(4·75), 293·5(4·10), 304·5(4·20), 317·5(4·18) and 348(3·76) |
| 5,6-Epoxy-5,6-dihydrodibenz[*a,h*]anthracene (VIII) | 223(4·56), 274*(4·76), 283(4·91), 299(4·56), 321(4·53), 351(3·01) and 369(2·80) |
| *cis*-5,6-Dihydro-5,6-dihydroxydibenz[*a,h*]anthracene† | 226*(4·32), 270(4·67), 280(4·79), 296(4·50), 315·5(4·47), 347(2·95) and 365(2·75) |
| 5-Hydroxydibenz[*a,h*]anthracene (IX) | 224, 273, 293, 302, 321, 337, 353 and 386 |
| 7,14-Dihydro-7,14-dihydroxydibenz[*a,h*]anthracene | 234(5·03) and 278(4·19) |
| 7,14-Dihydrodibenz[*a,h*]anthracen-7-one | 222, 235, 281, 291, 303, 321, 345, 362 and 382 |
| 4,11-Dimethoxydibenz[*a,h*]anthracene | 237, 279, 295, 306·5, 324, 337, 354 and 381 |
| 7,14-Dimethoxydibenz[*a,h*]anthracene | 222, 282, 291, 301, 318, 334, 350, 367 and 381 |
| Conjugate, probably *S*-(5,6-dihydro-6-hydroxy-5-benz[*a*]-anthracenyl)glutathione (IV) | 259, 268, 300 and 311 |
| Conjugate, probably *S*-(5,6-dihydro-6-hydroxy-5-dibenz-anthracenyl)glutathione (XIII)‡ | 272*, 282, 298, 317, 348 and 367 |
| Dibenz[*a,h*]anthracene (V) | 221, 230, 274, 278, 288, 296, 320, 332, 347 and 372 |

* Inflexion.

† The *trans*-isomer gave a virtually identical spectrum.

‡ The related cysteine and *N*-acetylcysteine conjugates gave identical spectra.

5-hydroxybenzanthracene (II) with the conjugates related to benzanthracene and to those of 5-hydroxydibenzanthracene (IX) with the conjugates related to dibenzanthracene. It has been shown, however, that the mercapturic acid obtained when the products of the oxidation of benzanthracene with perbenzoic acid are allowed to react with *N*-acetylcysteine yielded 6-hydroxybenzanthracene with alkali (Boyland & Sims, 1964*b*). This phenol could not be distinguished from the 5-hydroxy derivative on thin-layer chromatograms so that it is probable that the phenols derived from the mercapturic acids are 6-hydroxybenzanthracene and 6-hydroxydibenzanthracene respectively and that the conjugates are 5,6-dihydro-6-hydroxy-5-benzanthracenyl and 5,6-dihydro-6-hydroxy-5-dibenzanthracenyl derivatives, the glutathione conjugates being (IV) and (XIII) respectively.

*Oxidation of dibenzanthracene in the ascorbic acid–*$Fe^{2+}$ *ion–oxygen model systems.* A brisk current of $O_2$ was passed for 24hr. through a solution of dibenzanthracene (1g.), ascorbic acid (20g.) and $FeSO_4,7H_2O$ (3g.) in acetone (750ml.) and water (750ml.). The acetone was distilled off and dibenzanthracene (800mg.) was separated. The filtrate was extracted with ether (100ml.) and the residue obtained on evaporation of the ether was applied to the base lines of thin-layer chromatograms, which were developed for 15cm. in (a). About 30 bands were detected on the chromatograms, by their colour on the untreated chromatograms, or by their fluorescence in u.v. light or their colour with the 2,6-dichloroquinonechloroimide–$Na_2CO_3$ reagent. Of these bands, five were examined in greater detail. The silica gel forming the bands was removed from the chromatograms and the absorbed material was eluted with ether and the residues were examined on thin-layer chromatograms. The fastest-moving of these bands contained a product that was identical in its mobility and properties with dibenzanthracene-7,14-quinone. The second band contained a phenol (A), $R_F$ 0·65 in (b), that had a violet fluorescence in u.v. light, turning pink–violet in the presence of $NH_3$, and that gave a blue–purple colour with the 2,6-dichloroquinonechloroimide–$Na_2CO_3$ reagent. The third band contained a phenol (B), $R_F$ 0·37 in (b), that had a violet fluorescence in u.v. light, turning orange with $NH_3$, and that gave a blue colour with the 2,6-dichloroquinonechloroimide–$Na_2CO_3$ reagent. Its light-absorption curve showed $\lambda_{max.}$ at 282, 292, 330, 336, 352 and 379mμ and an inflexion at 300mμ. The fourth band contained a phenol (C), $R_F$ 0·29 in (b), that had a violet fluorescence in u.v. light, turning green with $NH_3$, and that gave a pale-pink colour with the 2,6-dichloroquinonechloroimide–$Na_2CO_3$ reagent. The light-absorption curves of A and C did not show sharp maxima, presumably because of the presence of absorbing impurities.

The fifth band, consisting of material left at the base lines of the chromatograms, was examined on two-dimensional thin-layer chromatograms. Two dihydrodihydroxy compounds were detected, one, $R_F$ 0·29 in (c), yielding compounds identical in their properties with phenols B and C after treatment with acid and one, $R_F$ 0·25 in (c), yielding phenol A and a second phenol (D), $R_F$ 0·29 in (b), that had a violet fluorescence in u.v. light, turning green with $NH_3$, and that gave a pale-grey colour with 2,6-dichloroquinonechloroimide–$Na_2CO_3$. The probable structures of the phenols and the dihydrodihydroxy compounds are discussed below.

*Experiments with rat-liver homogenates.* The incubations were carried out with rat-liver homogenates prepared as described by Boyland & Sims (1965*a*), nicotinamide, glucose 6-phosphate and $NADP^+$ being added as before. Usually the homogenate from four rat livers was used in each experiment, the substrates (10mg.) being added as solutions in ethanol (5ml.). At the end of the incubations the reaction mixtures were extracted twice with ethyl acetate (100ml.), with centrifuging if necessary, and the extracts were dried ($Na_2SO_4$) and evaporated and the residues examined on thin-layer chromatograms as described above. The aqueous layers were heated to 100° for a few minutes to coagulate protein and filtered and the filtrates acidified to pH4 with acetic acid. Charcoal (5g.) was added, the mixtures were filtered and the charcoal was washed with water (100ml.). Absorbed material was eluted from the charcoal with methanol containing 5% (v/v) of aq. $NH_3$ (sp.gr. 0·88). The residues obtained on evaporation of the solvents were examined on paper chromatograms.

Control experiments were carried out in which the homogenates were heated at 100° for a few minutes before the additions of the cofactors and the substrates. In an incubation of homogenate carried out in the absence of a substrate none of the products described below was detected.

## RESULTS

*Metabolism of benzanthracene.* The products present in the ethyl acetate-soluble fraction were similar to those detected in the hydroxylation of the hydrocarbon in the microsomal hydroxylating system (Boyland, Kimura & Sims, 1964) and included 3- and 4-hydroxybenzanthracene, 5,6-dihydro-5,6-dihydroxybenzanthracene, 8,9-dihydro-8,9-dihydroxybenzanthracene and benzanthracene-7,12-quinone, together with small amounts of a compound that yielded 1-hydroxybenzanthracene after heating with acid and is probably 1,2-dihydro-1,2-dihydroxybenzanthracene. A product with the mobility and properties on thin-layer chromatograms of 12-hydroxybenzanthracen-7-one was also present, but 7,12-dihydro-7,12-dihydroxybenzanthracene was not detected. It is possible that the quinone and the hydroxyketone were formed from the dihydrodihydroxy compound by oxidation.

The water-soluble fraction contained a compound that was identical in its u.v. spectrum and in its mobility and properties on paper chromatograms, developed with solvents (1) and (2), with the glutathione conjugate (IV) derived from 5,6-epoxy-5,6-dihydrobenzanthracene (I) that is described above. When the fraction was acidified with a few drops of conc. hydrochloric acid, the conjugate was no longer detected but products indistinguishable from benzanthracene and the product believed to be *S*-(5-benzanthracenyl)glutathione were present.

None of the above products was detected when heated homogenates were used.

*Metabolism of* 5,6-*epoxy*-5,6-*dihydrobenzanthracene* (I). The ethyl acetate-soluble fraction was examined on thin-layer chromatograms. Compounds identical in their mobilities and properties with 5-hydroxybenzanthracene (II) and 5,6-dihydro-5,6-dihydroxybenzanthracene (III) were detected. The water-soluble fraction contained a compound that was identical with the synthetic glutathione conjugate (IV) in its u.v. spectrum and mobility on paper chromatograms developed with solvents (1) and (2) and that yielded *S*-(5-benzanthracenyl)glutathione and benzanthracene with conc. hydrochloric acid as before.

In experiments with heated homogenate, 5,6-epoxy-5,6-dihydrobenzanthracene (I), 5-hydroxybenzanthracene (II) or, less likely, the 6-isomer and 5,6-dihydro-5,6-dihydroxybenzanthracene (III) were detected in the ethyl acetate-soluble fraction but glutathione conjugates were not found in the water-soluble fraction.

*Metabolism of dibenzanthracene* (V). The ethyl acetate-soluble fraction was applied to the base lines of two thin-layer chromatograms, which were developed with (b). The chromatograms were examined in u.v. light and three bands were removed from each chromatogram and the absorbed material was eluted from the silica gel with ether. The fastest-moving bands contained a phenol, $R_F$ 0·39 in (b), that had a violet fluorescence in u.v. light, turning orange with ammonia, and that gave a blue colour with the 2,6-dichloroquinonechloroimide–sodium carbonate reagent. Its light-absorption curve had $\lambda_{max.}$ at 282, 292, 330, 336, 352 and 379m$\mu$ and an inflexion at 300m$\mu$. In all these respects the phenol was identical with phenol B described above. The second pair of bands contained a phenol, $R_F$ 0·30 in (b), that had a violet fluorescence in u.v. light, turning green with ammonia, and that gave a pale-pink colour with the 2,6-dichloroquinonechloroimide–sodium carbonate reagent. In these respects the phenol was indistinguishable from phenol C described above.

The eluate of the third pair of bands, which contained material left at the base lines of the chromatograms, was examined on two-dimensional thin-layer chromatograms. Three dihydrodihydroxy compounds were detected. The first, $R_F$ 0·31 in (c), was identical in its mobility and properties with *cis*- and *trans*-5,6-dihydro-5,6-dihydroxybenzanthracene (XII). After being heated with acid it yielded a phenol identical with 5-hydroxydibenzanthracene (IX) in its mobility and properties on thin-layer chromatograms. The second compound, $R_F$ 0·29 in (c), yielded phenols indistinguishable from B and C described above, and the third compound, $R_F$ 0·25 in (c), yielded phenols indistinguishable from phenols A and D after being heated with acid. 7,14-Dihydro-7,14-dihydroxydibenzanthracene, which might be expected in this fraction, was not detected.

Dibenzanthracene-5,6- and -7,14-quinone, 2,9- and 4,11-dihydroxydibenzanthracene and 7,14-dihydrodibenzanthracen-7-one were not detected in the ethyl acetate-soluble fraction.

The water-soluble fraction contained a small amount of a product that was identical in its u.v. spectrum and its mobility and properties on paper chromatograms, developed with solvents (1) and (2), with the glutathione conjugate (XIII). When the product was treated with conc. hydrochloric acid, products indistinguishable from dibenzanthracene and the conjugate believed to be *S*-(5-dibenzanthracenyl)glutathione were detected.

None of the above products was formed when heated homogenate was used.

*Metabolism of* 5,6-*epoxy*-5,6-*dihydrodibenzanthracene* (VIII). The ethyl acetate-soluble fraction was examined on thin-layer chromatograms: it contained small amounts of 5,6-dihydro-5,6-dihydroxydibenzanthracene (XII) and of 5-hydroxydibenzanthracene (IX) or, less likely, the 6-isomer. The water-soluble fraction was shown on paper chromatograms developed with solvent (1) and (2) to contain a product identical in its u.v. spectrum and properties with the glutathione conjugate (XIII). *S*-(5-Dibenzanthracenyl)glutathione and dibenzanthracene were detected when the conjugate was acidified with conc. hydrochloric acid as before.

Experiments with heated homogenate showed that the epoxide was largely unchanged, but that small amounts of the dihydrodihydroxy compound (XII) and the phenol (IX) were formed. No glutathione conjugate was detected.

*Metabolism of* 7,12-*dihydro*-7,12-*dihydroxybenzanthracene and* 7,14-*dihydro*-7,14-*dihydroxydibenzanthracene*. These incubations were carried out with 2mg. samples of the dihydrodihydroxy compounds and only the ethyl acetate-soluble fractions were examined.

The benzanthracene derivative yielded small amounts of compounds indistinguishable from benzanthracene-7,12-quinone, 12-hydroxybenzanthracen-7-one and 7,12-dihydrobenzanthracen-7-one (or 7-hydroxybenzanthracene), but most of the dihydrodihydroxy compound appeared to be unchanged. The dibenzanthracene derivative yielded small amounts of 7,14-dihydrodibenzanthracen-7-one and an unidentified product that had $R_F$ 0·53 and 0·92 in (a) and (b), a violet fluorescence in u.v. light and appeared identical with the product described above that was formed in the acid hydrolysis of the dihydrodihydroxy compound. Benzanthracene-7,14-quinone was not detected and a large proportion of the dihydrodihydroxy compound was unchanged.

## DISCUSSION

The results show that, in the metabolism of benzanthracene in rat-liver homogenate, the hydrocarbon is hydroxylated on the 3,4-, 5,6-, 8,9-, 10,11- and, to a smaller extent, on the 1,2-bond, to yield either phenols or dihydrodihydroxy compounds. These results are in agreement with those previously obtained in the metabolism of the hydrocarbon in whole animals (Boyland & Sims, 1964*b*) and with rat-liver microsomes (Boyland *et al.* 1964).

With dibenzanthracene (V), most of the hydroxylated products were not compared directly with authentic compounds, but it is possible to deduce their probable structures by a comparison of their properties with those of known compounds. It seems probable that phenols A, B, C and D are monohydroxy compounds: their properties on thin-layer chromatograms differed from those of 2,9- and 4,11-dihydroxydibenzanthracene. Phenols B and C are related in that both are derived from the same dihydrodihydroxy compound. The u.v. spectrum of B is similar to, but not identical with, that reported for 1-hydroxydibenzanthracene (Cook & Schoental, 1952), and in its $R_F$ on thin-layer chromatograms, its fluorescence in u.v. light and its colour with the 2,6-dichloroquinonechloroimide–sodium carbonate reagent it resembles 4-hydroxybenzanthracene (Boyland & Sims, 1964*b*). It also resembles 4,11-dihydroxydibenzanthracene in its fluorescence in u.v. light and in its colour reactions, but the two phenols differ in their chromatographic properties. The light-absorption curve of B differs from that of 4,11-dimethoxydibenzanthracene (see Table 3) and from that of 4,11-dihydroxydibenzanthracene reported by Cason & Fieser (1940). All these properties suggest that B is 4-hydroxydibenzanthracene (X) and that C is therefore 3-hydroxydibenzanthracene (VI). The chromatographic properties of C are in fact similar to those reported for 3-hydroxybenzanthracene (Boyland & Sims, 1964*b*). The dihydrodihydroxy compound from which these phenols are derived is therefore presumed to be 3,4-dihydro-3,4-dihydroxydibenzanthracene (XI). Similar arguments suggest that A and D are 1- and 2-hydroxydibenzanthracene respectively, which are derived from 1,2-dihydro-1,2-dihydroxydibenzanthracene (VII). Dobriner, Rhoads & Lavin (1939) isolated 4,11-dihydroxydibenzanthracene from the urine and faeces of rats and mice dosed with dibenzanthracene, and Boyland *et al.* (1941) obtained a dihydroxydibenzanthracene and Cook & Schoental (1952) a monohydroxydibenzanthracene from the urines of rabbits dosed with the hydrocarbon, which were shown by La Budde & Heidelberger (1958) to be 2,9-dihydroxy- and 2-hydroxy-dibenzanthracene respectively. The significance of this species difference is not clear since both rats and rabbits hydroxylate benzanthracene on the 3,4-bond, with little or no hydroxylation on the 1,2-bond (Boyland & Sims, 1964*b*). In the present work it is clear from a comparison of the sizes of spots on thin-layer chromatograms that with rat-liver homogenate larger amounts of hydroxylated products are formed on the 3,4- than on the 1,2-bond of dibenzanthracene. 2,9- and 4,11-Dihydroxydibenzanthracene were not detected in the work now described: presumably they arise in the body by the further hydroxylation of the monohydroxy derivatives.

The formation of the phenols and dihydrodihydroxy compounds in the ascorbic acid–$Fe^{2+}$ ion–oxygen system is analogous to the formation of the related benzanthracene derivatives in this system (Boyland *et al.* 1964) and provides further evidence for the proposed structures of A, B, C and D and of the dihydrodihydroxy compounds from which the phenols were derived. The two systems differed in that the biological hydroxylation of the hydrocarbon yielded 3- and 4-hydroxydibenzanthracene (VI and X) and 1,2-dihydro-1,2-dihydroxydibenzanthracene (VII) and 3,4-dihydro-3,4-dihydroxydibenzanthracene (XI), whereas the chemical oxidation yielded these products together with 1- and possibly 2-hydroxydibenzanthracene.

The 7- and 12-positions of benzanthracene and the 7- and 14-positions of dibenzanthracene are *meso* positions (or 'L regions') of the hydrocarbons. With anthracene, it has been shown (Sims, 1964) that one of the metabolic routes of the hydrocarbon in rats involves the *meso* position with the formation of 9,10-dihydro-9,10-dihydroxyanthracene, which could have arisen through a 9,10-epoxy-9,10-dihydro intermediate. With benzanthracene, previous work has shown that some metabolic action occurs at the *meso* position (Boyland & Sims, 1964*b*; Boyland *et al.* 1964) and this has been confirmed by the work now described since benzanthracene-7,12-quinone and 7,12-dihydro-12-hydroxybenzanthracen-7-one were detected in the enzymic oxidation products. Similar products were not detected in the enzymic hydroxylation of dibenzanthracene although it has been shown by Heidelberger, Hadler & Wolf (1953) and by Van Duuren (1963) that the hydrocarbon is converted into dibenzanthracene-7,14-quinone either by whole animals or by rat lung. 7,12-Dihydro-7,12-dihydroxybenzanthracene and 7,14-dihydro-7,14-dihydroxydibenzanthracene, which might be expected as products of the metabolism of the parent hydrocarbon, were recovered largely unchanged after incubation in rat-liver homogenate although small amounts of oxidation products and dehydration products were formed. Dibenzanthracene-7,14-quinone did not appear to be a product of the action of rat-liver homogenate on the related dihydrodihydroxy compound. 4,11-

Dihydroxydibenzanthracene-7,14-quinone (Heidelberger *et al.* 1953) and its oxidation product, 5-hydroxy-1,2-naphthoic acid (Heidelberger & Wiest, 1951), have also been detected as metabolites of the hydrocarbon in mice. Products with the expected properties of these compounds have not been detected in the incubations with rat-liver homogenate.

Both benzanthracene (Boyland & Sims, 1964*b*) and dibenzanthracene (Van Duuren, Bekersky & Lefar, 1964) appear to form epoxides across the *meso* positions when oxidized with perbenzoic acid but there is yet no direct evidence that these intermediates are involved in the metabolism of the hydrocarbons. In other chemical oxidations of dibenzanthracene the hydrocarbon is attacked at the 5,6-position by ozone (Moriconi, O'Connor, Schmitt, Cogswell & Fürer, 1960) and at the 7,14-position to yield the quinone by perbenzoic acid (Roitt & Waters, 1949). Benzoyl peroxide (Roitt & Waters, 1952) and hydrogen peroxide in *tert.*-butyl alcohol catalysed by osmium tetroxide (Cook & Schoental, 1950) yield mixtures of the 5,6- and 7,14-quinone.

With rat-liver homogenate benzanthracene and dibenzanthracene yielded the dihydrodihydroxy compounds (III) and (XII) and the glutathione conjugates (IV) and (XIII) respectively, all formed by reaction on the 5,6-bonds (the 'K regions') of the hydrocarbons, but not the corresponding quinones. In contrast with dibenzanthracene, the potent carcinogen 7,12-dimethylbenz[*a*]anthracene does not appear to be metabolized on the 'K region' (Boyland & Sims, 1965*a*). Heidelberger *et al.* (1953) showed that dibenzanthracene is converted into dibenzanthracene-5,6-quinone in whole animals, and Van Duuren (1963) that the hydrocarbon is converted into the quinone, 5,6-dihydro-5,6-dihydroxydibenzanthracene (XII) and 5-hydroxydibenzanthracene (IX) by rat lung. In the present work, 5-hydroxydibenzanthracene (IX) has not been detected as a metabolite of the hydrocarbon. This is in agreement with earlier observations that phenols formed on the 'K regions' of hydrocarbons such as phenanthrene (Boyland & Sims, 1962), pyrene (Boyland & Sims, 1964*a*) and benzanthracene (Boyland & Sims, 1964*b*) are not metabolic products.

The metabolic pathways of 5,6-epoxy-5,6-dihydrobenzanthracene (I) and 5,6-epoxy-5,6-dihydrodibenzanthracene (VIII) in rat-liver homogenate resemble those of the parent hydrocarbons in that corresponding dihydrodihydroxy compounds (III and XII) and the glutathione conjugates (IV and XIII) are formed, but differ in that the epoxides (I and VIII) also yield phenols that are probably 5-hydroxybenzanthracene (II) and 5-hydroxydibenzanthracene (IX) respectively, since the epoxides are known to rearrange to these isomers. A possible explanation for this is that, if epoxides are formed in the metabolism of the hydrocarbons, their concentration at any one moment would be low, whereas in the experiments with the epoxides there are high concentrations of epoxides at the beginning of the experiments. With heated homogenates the epoxides similarly rearrange to the corresponding phenols. The amounts of the dihydrodihydroxy compounds formed with fresh and with heated homogenates are of the same orders of magnitude (as judged by the sizes of their spots on thin-layer chromatograms) so that the reactions of the epoxides with water are probably non-enzymic. The configurations of the dihydrodihydroxy compounds formed from the hydrocarbons and the epoxides by rat-liver homogenates and from the epoxide (VIII) with water were not established because the *cis*- and *trans*-isomers could not be separated on thin-layer chromatograms. It has been shown, however, that 9,10-epoxy-9,10-dihydrophenanthrene yields *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene with water and that both the epoxide and the hydrocarbon are converted into the *trans*-dihydrodihydroxy compound in whole animals (Boyland & Sims, 1965*b*). Benzanthracene yields *trans*-5,6-dihydro-5,6-dihydroxybenzanthracene (III) in rats and this isomer is also formed during the oxidation of the hydrocarbon with perbenzoic acid, a reaction that is believed to yield the epoxide as an intermediate (Boyland & Sims, 1964*b*). 5,6-Epoxy-5,6-dihydrodibenzanthracene (VIII) is apparently formed in the perbenzoic acid oxidation of dibenzanthracene (Van Duuren *et al.* 1964).

The conjugations of the epoxides (I) and (VIII) are probably catalysed by the enzyme present in rat liver that catalyses the conjugation of a number of epoxides with glutathione (Boyland & Williams, 1965). The conjugation of the epoxides with glutathione in the presence of this enzyme has been demonstrated (Boyland, Sims & Williams, 1965).

The amino acid conjugates are all immediately decomposed with acid to yield mainly the parent hydrocarbons together with small amounts of the corresponding aryl conjugates. The acid-labile conjugates thus resemble those related to phenanthrene (Boyland & Sims, 1962, 1965*b*) and to pyrene (Boyland & Sims, 1964*a*), but differ from those related to naphthalene (Boyland & Sims, 1958) and anthracene (Sims, 1964) where phenols are also formed.

The u.v.-absorption spectra of 5,6-epoxy-5,6-dihydrobenzanthracene (I) and 5,6-epoxy-5,6-dihydrodibenzanthracene (VIII) are similar to those of the related dihydrodihydroxy compounds (III) and (XII) except that the peaks are displaced about 4 m$\mu$ towards longer wavelengths (see Table 3). The

epoxides are crystalline compounds that are stable at least for a few days at room temperature and that keep indefinitely at 0°. They readily rearrange to the related phenols (with which they are isomeric) in the presence of mineral acid and they react rather slowly with water and with thiol compounds to yield the products described above. It now seems likely that the reason why a mercapturic acid was not always obtained when the products of the oxidation of benzanthracene with perbenzoic acid were allowed to react with *N*-acetylcysteine (Boyland & Sims, 1964*b*) was not that the epoxide intermediate had decomposed, but that reactions were not allowed to proceed for long enough periods of time.

Bhargava & Heidelberger (1956) have shown that, when mice are painted with solutions of dibenzanthracene, the hydrocarbon is bound to protein, the hydrolysis of which yielded 2-phenylphenanthrene-2′,3-dicarboxylic acid. It is possible that 5,6-epoxy-5,6-dihydrodibenzanthracene (VIII) is involved in this protein binding. Although the above results indicate that the epoxides are probably intermediates in the metabolism of the hydrocarbons it is not known whether they are involved in the carcinogenic process. 5,6-Epoxy-5,6-dihydrobenzanthracene (I) is derived from an inactive (or very feebly active) hydrocarbon and 5,6-epoxy-5,6-dihydrodibenzanthracene (VIII) from an active carcinogen, but there appears to be little difference between the chemical and the metabolic reactivities of the two epoxides. Both are being tested for carcinogenic activity.

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