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Metabolism of Polycyclic Compounds

THE METABOLISM OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE BY RAT-LIVER HOMOGENATES

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1. The main products of the metabolism of 7,12-dimethylbenz[*a*]anthracene by rat-liver homogenates are the isomeric monohydroxymethyl derivatives. The syntheses of these compounds are described. 2. Two phenolic products and two dihydrodihydroxy compounds were formed, but none of these appeared to have been formed by hydroxylation at the 'K region'. There was little evidence for the formation of a glutathione conjugate of the hydrocarbon. 3. The monohydroxymethyl derivatives are products of the hydroxylation of the hydrocarbon in the ascorbic acid–Fe^{2+}–oxygen model hydroxylating system.

Earlier work on the metabolism of 7,12-dimethylbenz[*a*]anthracene (I) showed that the hydrocarbon is converted into the 4-hydroxy derivative by rats (Dickens, 1945), and this was detected in the faeces by the fluorescence spectrum of the methoxy derivative. The present work has shown that the hydrocarbon (I) is converted by rat-liver homogenates mainly into the isomeric monohydroxymethyl derivatives (II and III). Small amounts of phenols and dihydrodihydroxy compounds are also formed. The hydroxymethyl derivatives (II and III) are also formed in the oxidation of the hydrocarbon (I) in the ascorbic acid–Fe^{2+}–oxygen model hydroxylating system of Udenfriend, Clark, Axelrod & Brodie (1954).

EXPERIMENTAL

Melting points. These are uncorrected.

Ultraviolet-absorption spectra. These were measured in ethanol in a Unicam SP.500 spectrophotometer.

Paper chromatography. Paper chromatograms on Whatman no. 1 paper were developed downwards for 18hr. with butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.). The chromatograms were dipped in either the platinic iodide reagent of Toennies & Kolb (1951) or in 0·2% ninhydrin in acetone.

Thin-layer chromatography. This was carried out on glass plates coated with a film of silica gel G (E. Merck A.-G., Darmstadt, Germany) of 0·25mm. thickness. The chromatograms were developed for 10cm. with (*a*) light petroleum (b.p. 60–80°) containing 5% (v/v) of benzene, (*b*) benzene or (*c*) benzene containing 5% (v/v) of ethanol. Two-dimensional chromatograms were run, which were developed in the first direction with solvent (*c*), sprayed with conc. HCl and heated at 80° for 15min. and developed in the second direction with solvent (*b*). All the chromatograms were examined in u.v. light while still wet, both before and after exposure to NH_3. Chromatograms developed in one direction only were finally sprayed with either conc.HCl and heated at 80° for 15min. or with conc. H_2SO_4. The properties of compounds described below are listed in Table 1.

Materials. cis-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene (IV). This was prepared from the parent hydrocarbon by the action of osmium tetroxide, as described by Cook & Schoental (1948), when it formed needles from benzene, m.p. 171° (Found: C, 82·9; H, 6·2. Calc. for $C_{20}H_{18}O_2$: C, 82·7; H, 6·25%). Attempts to oxidize the dihydrodihydroxy compound to the corresponding quinone were unsuccessful (cf. Hadler & Kryger, 1960). Because of this, the *trans* isomer of the dihydrodihydroxy compound could not be prepared.

7,12-Dihydroxy-7,12-dimethylbenz[*a*]anthracene (V). This was prepared from benz[*a*]anthracene-7,12-quinone by the action of methylmagnesium iodide (Bachmann & Bradbury, 1938): when recrystallized from aq. ethanol it had m.p. 180–181°.

7,12-Epidioxy-7,12-dimethylbenz[*a*]anthracene (VI). This was prepared by the method of Cook & Martin (1940). It was recrystallized from carbon disulphide in needles, m.p. 193°.

7,12-Dihydroxymethylbenz[*a*]anthracene. The hydrocarbon was oxidized with lead tetra-acetate in acetic acid, as described by Badger & Cook (1939), to yield 7,12-diacetoxymethylbenz[*a*]anthracene, separating from ethanol in pale-yellow needles, m.p. 166–167°. The diacetoxymethyl compound was hydrolysed with KOH in aq. ethanol to yield the dihydroxymethyl compound, separating from ethanol as a yellow powder, m.p. 221–223°.

Monohydroxymethyl derivatives of 7,12-dimethylbenz[*a*]anthracene. The hydrocarbon (4g.) in acetic acid (150ml.) was heated at 100° and the solution stirred while a suspension of lead tetra-acetate (7·3g.) in acetic acid (20ml.) was added during 15min. The mixture was heated for another 15min. and poured into water (500ml.). The gummy

Table 1. *Properties of compounds related to* 7,12-*dimethylbenz*[a]*anthracene on thin-layer chromatograms*

Details are given in the text.

Compound	R_F		Colour		
	Benzene (*b*)	Benzene–ethanol (19:1, v/v) (*c*)	Fluorescence	After spraying with conc. HCl and heating to 100°	With conc. H_2SO_4
7-Hydroxymethyl-12-methylbenz[*a*]anthracene (II)	0·22	0·41	Violet	Yellow*†	Purple
7-Acetoxymethyl-12-methylbenz[*a*]anthracene	0·62	0·87	Violet	Yellow†	Purple
12-Hydroxymethyl-7-methylbenz[*a*]anthracene (III)	0·40	0·55	Violet	Yellow†	Brown
12-Acetoxymethyl-7-methylbenz[*a*]anthracene	0·62	0·87	Violet	Yellow†	Brown
7,12-Dihydroxymethylbenz[*a*]anthracene	0·00	0·20	Violet	Yellow*†	Purple
7,12-Dihydroxy-7,12-dimethylbenz[*a*]anthracene (V)	0·05	0·30	None	Yellow–green†	Purple
7,12-Epidioxy-7,12-dimethylbenz[*a*]anthracene (VI)	0·58	0·85	Dark blue	Yellow, turning brown†	Brown
cis-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz-[*a*]anthracene (IV)	0·00	0·20	Dark violet	Orange	Orange
7,12-Dimethylbenz[*a*]anthracene (I)	0·95‡	0·98	Violet	Yellow†	Brown

* These compounds had yellow-fluorescent 'tails', seen when the treated chromatograms were examined in u.v. light.
† These colours also appeared when untreated chromatograms were kept exposed to air and daylight for some days.
‡ R_F 0·54 in light petroleum (b.p. 60–80°) containing 5% (v/v) of benzene.

precipitate, which was found on examination on thin-layer chromatograms to contain the hydrocarbon, and its mono- and di-acetoxymethyl derivatives, was chromatographed on a column prepared from 100g. of silica gel (200–300 mesh; L. Light and Co. Ltd., Colnbrook, Bucks.) in benzene–light petroleum (b.p. 80–100°) (1:1, v/v). Fractions (200ml.) were collected and examined on thin-layer chromatograms. Fractions 5–7, which contained mainly the monoacetoxymethyl derivatives, were combined and evaporated and the oil was treated with ether (10ml.). The crystals were filtered and recrystallized from ethanol to yield a product (1·55g.) that, for reasons described below, is believed to be 7-*acetoxymethyl*-12-*methylbenz*[a]*anthracene*. It formed pale-yellow plates, m.p. 145–146° (Found: C, 83·9; H, 6·1. $C_{22}H_{18}O_2$ requires C, 84·05; H, 5·8%). The ether solution was kept at room temperature for 30min. and the crystals that separated were recrystallized from ethanol to yield 12-*acetoxymethyl*-7-*methylbenz*[a]*anthracene* (650mg.) in pale-yellow needles, m.p. 146° (Found: C, 84·1; H, 5·7%). A mixture of the two acetoxymethyl derivatives had m.p. 118–123°. When the ether mother liquors were evaporated, a mixture of needles and plates (m.p. 117–122°) was obtained that could not be separated.

The acetoxymethyl derivatives (100mg.) were separately heated under reflux with KOH (100mg.) in methanol (5ml.). The solutions were poured into water (20ml.) and the products recrystallized from ethanol to yield 7-*hydroxymethyl*-12-*methylbenz*[a]*anthracene* (II) (20mg.) in flat needles, m.p. 162° (Found: C, 88·3; H, 5·7. $C_{20}H_{16}O$ requires C, 88·2; H, 5·9%), and 12-*hydroxymethyl*-7-*methylbenz*[a]*anthracene* (III) (65mg.) in pale-yellow plates, m.p. 164° (Found: C, 88·1; H, 6·2%).

7-Acetoxy-12-methylbenz[*a*]anthracene was also prepared in poor yield from 12-methylbenz[*a*]anthracene. The hydrocarbon (250mg.) was converted into 7-iodomethyl-12-methylbenz[*a*]anthracene by the method of Sandin & Fieser (1940). The crude iodo compound was heated under reflux with potassium acetate (0·5g.) in acetic acid (20ml.) for 2hr., and the solution was poured into water (250ml.) and the gummy solid collected and chromatographed on a silica-gel column as described above. Evaporation of the appropriate fraction yielded the acetoxy derivative (21mg.), which, after three crystallizations from ethanol, formed pale-yellow plates, m.p. and mixed m.p. 144–145°. A portion hydrolysed by brief treatment with boiling 5% (w/v) KOH in methanol yielded a compound that was indistinguishable from 7-hydroxymethyl-12-methylbenz-[*a*]anthracene on thin-layer chromatograms. The structure of this compound is thus established and it follows that the second product described above must be the 12-hydroxymethyl derivative.

Rat-liver homogenates. The livers from four rats of the Chester Beatty strain (body wt. approx. 200g.) were homogenized in 200ml. of ice-cold 1·15% (w/v) KCl in a Potter & Elvehjem (1936) type of homogenizer with a Teflon pestle. The homogenate was centrifuged at 0° for 20min. at $1480g_{av.}$ in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd., Birmingham) and the supernatant diluted with an equal amount of 0·1M-phosphate buffer, pH7·4, prepared from NaH_2PO_4 and Na_2HPO_4. Nicotinamide (1·76g.), obtained from Roche Products Ltd. (Welwyn Garden City, Herts.), $NADP^+$ (30mg.) and glucose 6-phosphate (250mg.), both as the sodium salts obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), were added and the mixture was heated in a water bath to 37°. Air was slowly bubbled through the mixture and, when the temperature of the mixture had reached 37°, a solution of 10mg. of either 7,12-methylbenz[*a*]anthracene (I), 7-hydroxymethyl-12-methylbenz[*a*]anthracene (II) or 12-hydroxymethyl-7-methylbenz[*a*]anthracene (III), each in ethanol (5ml.),

was added. The incubation was continued for 1 hr. and the contents of the flask were cooled and extracted twice with ethyl acetate (100 ml.). It was usually necessary to separate the organic and the aqueous phases by centrifugation: they were examined separately.

The ethyl acetate extracts were combined and dried over Na_2SO_4 and the solvent was evaporated. The residue, which consisted mainly of nicotinamide, was washed with ether and the ether washings were applied to the base lines of two thin-layer chromatograms, which were developed for 15 cm. with benzene. The wet chromatograms were examined in u.v. light and divided into a number of regions, the positions of which were determined by their fluorescence. The silica gel forming these regions was removed from the chromatograms and the absorbed materials were eluted with ether. The ether was evaporated and the residues were examined as described below.

The aqueous phase was heated to 100° for a few minutes to coagulate protein and filtered. The filtrate was acidified to pH4 with acetic acid and activated charcoal (5 g.) (British Drug Houses Ltd., Poole, Dorset) was added. The charcoal was filtered off and washed with water (100 ml.), and the absorbed material eluted with methanol (200 ml.) containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). The solvent was removed under reduced pressure and the residue applied to the base line of a paper chromatogram, which was developed downward for 18 hr. with butan-1-ol–propan-1-ol–aq. 2 N-NH_3 (2:1:1, by vol.). The bottom third of the chromatogram was cut out and the absorbed material eluted from the paper with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). The solvent was removed and the residue examined as described below.

Control experiments were carried out in which the rat-liver homogenates were boiled for 5 min. and cooled to room temperature before the addition of the factors described above. The mixtures were then incubated at 37° in the presence of the substrates, and the mixtures extracted and the products examined as before. One incubation with fresh homogenate was carried out in the absence of substrate.

As far as possible, all operations were carried out either in the dark or in the absence of direct sunlight. This was particularly necessary during the separation of the metabolites on thin-layer chromatograms: Sandin & Fieser (1940) have shown that 7,12-dimethylbenz[*a*]anthracene is readily converted into the epidioxide (VI) by chromatography on an alumina column.

Oxidation of 7,12-*dimethylbenz*[a]*anthracene in the ascorbic acid*-Fe^{2+}*–oxygen system.* 7,12-Dimethylbenz[*a*]anthracene (1 g.), ascorbic acid (20 g.) and $FeSO_4$ (3 g.) were dissolved in a mixture of water (800 ml.) and acetone (800 ml.). A brisk current of oxygen was passed through the solution, which was kept at room temperature. After 24 hr. the acetone was distilled off under reduced pressure, unchanged hydrocarbon (0·7 g.) was filtered off and the filtrate was extracted twice with ether (200 ml.). The combined extracts were dried over Na_2SO_4 and evaporated and the residue was chromatographed on a column prepared from silica gel (200–300 mesh) (25 g.). The column was developed with benzene–light petroleum (b.p. 80–100°) (1:1, v/v) and 20 ml. fractions were collected and examined on thin-layer chromatograms. Fractions containing similar products were combined and the solvent was evaporated to yield six main fractions: most of these contained several oxidation products, only some of which could be identified.

Fraction 1 contained compounds indistinguishable from unchanged hydrocarbon, 7,12-epidioxy-7,12-benz[*a*]anthracene (VI) and benz[*a*]anthracene-7,12-quinone.

Fraction 2 contained a compound, R_F 0·62 in solvent (*b*), that had a green fluorescence in u.v. light. Its absorption spectrum, measured on a solution of the compound eluted with ethanol from the silica gel removed from a thin-layer chromatogram developed with solvent (*b*), had $\lambda_{max.}$ at 265 mμ. The compound was not identified but it appeared to be an oxidation product of 7-hydroxymethyl-12-methylbenz[*a*]anthracene, since solutions of this compound that had been kept in the air for some days contained an identical product. The oxidation product was not formed when 12-hydroxymethyl-7-methylbenz[*a*]anthracene was kept under the same conditions.

Fraction 3 contained a product indistinguishable from 12-hydroxymethyl-7-methylbenz[*a*]anthracene (III). The light-absorption of a solution of this compound obtained from a thin-layer chromatogram as before had $\lambda_{max.}$ at 237, 265, 273, 284, 294, 344, 360 and 374 mμ, and the spectrum was identical with that of the synthetic hydroxy-

Table 2. *Light-absorption spectra of compounds related to* 7,12-*dimethylbenz*[a]*anthracene*

Details are given in the text. The molecular extinction coefficients are shown in parentheses (as $\log \epsilon_{max.}$).

Compound	$\lambda_{max.}$ (mμ)
7-Hydroxymethyl-12-methylbenz[*a*]anthracene	235 (4·28), 265 (4·57), 273·5 (4·63), 284 (4·85), 294 (4·90), 344 (3·84), 359 (3·95), 375 (3·84)
7-Acetoxymethyl-12-methylbenz[*a*]anthracene	236 (4·30), 264 (4·55), 274 (4·62), 284 (4·86), 293·5 (4·91), 344 (3·86), 359 (3·96), 376 (3·86)
12-Hydroxymethyl-7-methylbenz[*a*]anthracene	237 (4·30), 264 (4·54), 274 (4·61), 284 (4·85), 294 (4·90), 344 (3·86), 359 (3·95), 375 (3·86)
12-Acetoxymethyl-7-methylbenz[*a*]anthracene	237 (4·31), 264 (4·54), 274 (4·60), 284 (4·84), 295 (4·88), 344 (3·86), 360 (3·95), 375 (3·85)
7,12-Dihydroxymethylbenz[*a*]anthracene	240 (4·16), 265 (4·50), 273·5 (4·61), 283 (4·85), 294 (4·88), 343 (3·84), 358 (3·94), 374 (3·84)
7,12-Epidioxy-7,12-dimethylbenz[*a*]anthracene	275 (3·64), 288 (3·67), 297 (3·79)
7,12-Dihydroxy-7,12-dimethylbenz[*a*]anthracene	230 (4·56), 270 (2·55), 278 (2·57), 289 (2·39)
cis-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]-anthracene	260 (4·63), 268 (4·68), 303 (4·01)

methyl compound (III) and of other compounds with this type of structure (see Table 2).

Fraction 4 contained a product, R_F 0·35, that appeared on thin-layer chromatograms as a pale-yellow spot in daylight and a green-fluorescent spot in u.v. light. The light-absorption curve, measured in ethanol as before, showed $\lambda_{max.}$ at 250, 251, 262 and 361 mμ. Although the compound appeared to be a major product of the oxidation, it was not identified.

Fraction 5 contained a compound indistinguishable from 7-hydroxymethyl-12-methylbenz[a]anthracene (II). The light-absorption curve in ethanol showed $\lambda_{max.}$ at 235, 264, 273, 284, 294, 344, 360 and 374 mμ, and the curve was identical with that of the synthetic compound (II).

Fraction 6, which was expected to contain dihydrodihydroxy compounds, was examined on acid-treated two-dimensional chromatograms. The fraction contained a large number of products, including one, R_F 0·23 in solvent (*c*), that gave rise to two products with acid that appeared to be phenols arising from the decomposition of a dihydrodihydroxy compound and to be related to two products detected in the experiments with rat-liver preparations. The products had R_F 0·35 and 0·26 respectively in solvent (*b*) and had a pink and a green fluorescence respectively when the chromatograms were exposed to NH_3 and examined in u.v. light. Two other compounds, R_F 0·32 and 0·38 in solvent (*c*) respectively, were detected, that gave rise to compounds indistinguishable from benz[a]anthracene-7,12-quinone after treatment with acid.

RESULTS

Metabolism of 7,12-*dimethylbenz*[a]*anthracene* (*I*) *by rat-liver homogenates.* Eight fractions were obtained when the ethyl acetate fraction from the metabolism of the hydrocarbon was chromatographed in solvent (*b*) on thin-layer chromatograms as described above. Fraction 1, R_F 0·95, appeared to contain only unchanged hydrocarbon. The fraction was rechromatographed on a thin-layer chromatogram in solvent (*a*) and the violet-fluorescent band, R_F 0·54, seen when the chromatograms was examined in u.v. light, was removed and the adsorbed material eluted with ethanol. The absorption spectrum of the solution was identical with that of 7,12-dimethylbenz[a]anthracene.

Fraction 2, R_F 0·82, did not appear to contain metabolites. Because benz[a]anthracene-7,12-quinone was a product of the ascorbic acid oxidation of the hydrocarbon, a careful search was made for the quinone, which would be expected in this fraction: none was found.

Fraction 3, R_F 0·58, contained a compound that had a blue–violet fluorescence in u.v. light and that was indistinguishable from 7,12-epidioxy-7,12-benz[a]anthracene (VI) on thin-layer chromatograms developed with solvent (*b*) or (*c*). The epidioxide was probably formed during the working-up procedures, in spite of the precaution taken, since it was also detected when the products from the control experiment with boiled liver preparation were examined.

Fraction 4, R_F 0·42, contained two compounds, which separated when the fraction was rechromatographed in solvent (*b*) on a thin-layer chromatogram. The first product then had R_F 0·44, and was indistinguishable from 12-hydroxymethyl-7-methylbenz[a]anthracene (III) on thin-layer chromatograms developed with solvent (*b*) or (*c*). A solution of the product in ethanol, obtained as before, had absorption maxima at 235, 263, 275, 285, 290, 345, 360 and 375 mμ and the curve was indistinguishable from that of the 12-hydroxymethyl derivative (III). In one experiment, the fraction was treated with a few drops of acetic anhydride in pyridine, and the product extracted with ether from the solution obtained when the reaction mixture was diluted with water and was chromatographed on a thin-layer chromatogram in solvent (*b*). The original compound was no longer present; instead, a compound indistinguishable from 12-acetoxymethyl-7-methylbenz[a]anthracene was detected. The light-absorption spectrum of a solution in ethanol, obtained as before, showed the same peaks as that of the parent hydroxymethyl compound and was identical with that of the synthetic acetoxymethyl derivative.

The second product in this fraction had R_F 0·34 on thin-layer chromatograms developed with solvent (*b*). It had a violet fluorescence in u.v. light that changed to pink when the chromatograms were exposed to ammonia, and resembled one of the unidentified products obtained in the ascorbic acid oxidation of 7,12-dimethylbenz[a]anthracene that possibly arose from the acid-decomposition of a dihydrodihydroxy compound.

Fraction 5, R_F 0·28, had a violet fluorescence on thin-layer chromatograms examined in u.v. light, changing to yellow–green when the chromatograms were exposed to ammonia. It resembles the second of the unidentified products detected in the ascorbic acid oxidation of the hydrocarbon that appeared to arise from the decomposition of a dihydrodihydroxy compound.

Fraction 6, R_F 0·21, had a violet fluorescence in u.v. light and was indistinguishable on thin-layer chromatograms developed with solvent (*b*) or (*c*) from 7-hydroxymethyl-12-methylbenz[a]anthracene (II). The light-absorption spectrum, measured in ethanol as before, had $\lambda_{max.}$ 237, 265, 273, 284, 294, 344, 360 and 374 mμ and the curve was identical with that of the synthetic hydroxymethyl compound (II). The product was acetylated as before to yield a compound indistinguishable on thin-layer chromatograms from 7-acetoxymethyl-12-methylbenz[a]anthracene and the light-absorption curve was identical with that of the synthetic acetoxy compound. When solutions of the product

in fraction 6 were kept at room temperature for some days and examined on thin-layer chromatograms, a product was detected that had a green fluorescence in u.v. light and was indistinguishable from the product detected in the ascorbic acid oxidation of 7,12-dimethylbenz[*a*]anthracene (I) that had R_F 0·62 in solvent (*b*).

Fraction 7, R_F 0·05, was examined on thin-layer chromatograms developed with solvent (*b*) or (*c*) for the presence of 7,12-dihydroxy-7,12-dimethylbenz[*a*]anthracene (V). The dihydroxy compound was not detected but a substance was present that, on chromatograms sprayed with sulphuric acid, gave an orange colour that changed to purple and then to brown, whereas the dihydro compound (V) itself gives an immediate purple colour with this reagent. When the ethyl acetate extract of the control rat-liver preparation that was incubated in the absence of the hydrocarbon was similarly examined on thin-layer chromatograms, a compound with R_F 0·05 in solvent (*b*) was detected that gave the same series of colours with sulphuric acid.

Fraction 8, which contained material that did not move on thin-layer chromatograms developed in solvent (*b*) and which was expected to contain dihydrodihydroxy compounds, was examined on two-dimensional thin-layer chromatograms as described above. Two such compounds were detected. One, R_F 0·17 in solvent (*c*), gave rise to a product, R_F 0·30 in solvent (*b*) after the treatment with acid, that had a violet fluorescence in u.v. light, changing to bright green when the chromatogram was exposed to ammonia. There appeared to be a second product associated with this compound, which was detected as a violet-fluorescent streak extending up to the top half of the chromatograms when they were examined in u.v. light.

A second compound, which was not always detected as a product of the oxidation, had R_F 0·23 in solvent (*c*) and gave rise with acid to two compounds that were indistinguishable in their properties in u.v. light from the products in fractions 4 and 5 above and from the similar products of the ascorbic acid oxidation of the hydrocarbon detected on two-dimensional chromatograms.

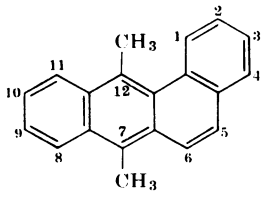
Although *trans*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene was not available, experience has shown that, with other pairs of *cis*- and *trans*-isomers of dihydrodihydroxy compounds related to aromatic hydrocarbons, both isomers have similar properties on thin-layer chromatograms. No metabolite with the expected properties of a 5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene (IV) was detected in fraction 8. Because products were detected in the ascorbic acid oxidation that gave rise to benz[*a*]-anthracene-7,12-quinone on acid-treated two-dimensional thin-layer chromatograms, a search was made for similar products in fraction 8: none was found. 7,12-Dihydroxymethylbenz[*a*]anthracene was not detected in this fraction.

Apart from 7,12-epidioxy-7,12-dimethylbenz-[*a*]anthracene (VI), none of the metabolites described above was detected after 7,12-dimethylbenz[*a*]anthracene (I) was incubated with boiled rat-liver preparation.

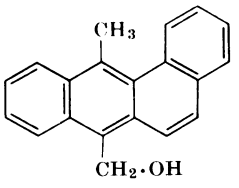
The residue from the treatment of the aqueous phase of the 7,12-dimethylbenz[*a*]anthracene (I) incubation mixture was applied to the base lines of two sheets of chromatography paper, which was developed as described below. Strips were cut from the papers from the base line to a line 10cm. above and the absorbed material was eluted from the paper with methanol containing 5% (v/v) of aq. ammonia (sp.gr. 0·88). The solvent was removed under reduced pressure and the residue was divided into two portions, one of which was acidified with a few drops of concentrated hydrochloric acid. Two paper chromatograms were prepared, to each of which was applied half of the material forming the unacidified and half of that forming the acidified portions. The chromatograms were developed as before and one was treated with the platinic iodide reagent and one with ninhydrin. Any glutathione derivative present should, by analogy with the properties of other related glutathione conjugates, give a positive reaction with platinic iodide reagent and a purple colour with ninhydrin, and have R_F 0·1–0·2. It should also be acid-labile and so be present in the non-acidified but not in the acidified portion. The acidified fraction should contain 7,12-dimethylbenz[*a*]anthracene, arising from the decomposition of the glutathione conjugates. Of seven incubations carried out, a small amount of a compound, R_F 0·15, that could be a glutathione conjugate was detected as a product of one: the results of the other six were negative. In no case was 7,12-dimethylbenz[*a*]anthracene detected as a product after treatment with acid. It is therefore concluded that little conjugation of glutathione with 7,12-dimethylbenz[*a*]anthracene occurs in the system used. In a system of rat-liver microsomes and supernatant, naphthalene and phenanthrene are easily converted into glutathione conjugates (Booth, Boyland & Sims, 1961).

Metabolism of the monohydroxymethyl derivatives of 7,12-*dimethylbenz*[a]*anthracene.* The investigation of the metabolism of compounds (II) and (III) was undertaken to see if any of the metabolites described above were formed by the further metabolism of the hydroxymethyl compounds.

In both cases one fluorescent band was seen when thin-layer chromatograms, developed with solvent (*b*), of the ethyl acetate extracts were examined in u.v. light. They were identified as the unchanged hydroxymethyl derivatives (II and III) by the



(I)



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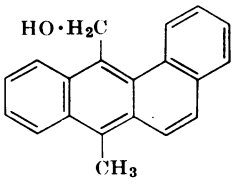

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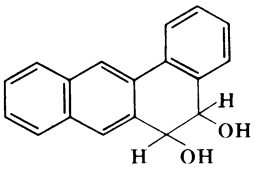
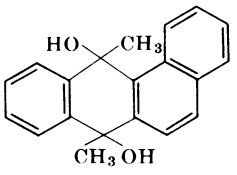
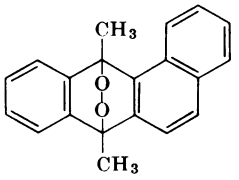


(IV)



(V)



(VI)

methods described above. The material at the base lines of the chromatograms were examined on two-dimensional thin-layer chromatograms for dihydrodihydroxy compounds. Each appeared to contain a small amount of one such compound, neither of which has been recognized as a metabolite of the hydrocarbon itself. 7,12-Dihydroxymethylbenz[a]anthracene was not detected as a metabolite of either monohydroxymethyl derivative.

Examination of the aqueous layer by the methods outlined above did not show the formation of glutathione conjugates.

DISCUSSION

The metabolism of 7,12-dimethylbenz[a]anthracene (I) in rat-liver homogenates differs in many respects from that reported for benz[a]anthracene in whole animals (Boyland & Sims, 1964) and by rat-liver microsomes (Boyland, Kimura & Sims, 1964). The major metabolic route for the former hydrocarbon by rat-liver homogenates involves the hydroxylation of one or other, but not both, of the methyl groups. The other metabolites detected are minor products, and because of this they could not be identified with certainty. 4-Hydroxy-7,12-dimethylbenz[a]anthracene was identified as a metabolic product of the hydrocarbon in rats (Dickens, 1945) and it seems likely that the slower-moving product in fraction 4 is this phenol: in its fluorescence in u.v. light and in its properties on thin-layer chromatograms it resembles 4-hydroxybenz[a]anthracene (Boyland & Sims, 1964). If this is so, then the product in fraction 5 could be 3-hydroxy-7,12-dimethylbenz[a]anthracene: its fluorescence and chromatographic properties resemble those of a metabolite believed to be 3-hydroxybenz[a]anthracene (Boyland & Sims, 1964). Similarly, the dihydrodihydroxy compound detected both as a product of the ascorbic acid–Fe^{2+}–oxygen oxidation and of the metabolic hydroxylation of the hydrocarbon, which appears to give rise to the above phenols with acid, could be 3,4-dihydro-3,4-dihydroxy-7,12-dimethylbenz[a]anthracene. The second dihydrodihydroxy compound detected in the metabolic products of the hydrocarbon could, by analogy with benz[a]anthracene metabolism, be 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[a]-anthracene or more likely a mixture of this dihydrodihydroxy compound with the 10,11-isomer. The phenol formed with acid resembles the phenols believed to be 9- and 10-hydroxybenz[a]anthracene: the other products had apparently decomposed during the treatment with acid. In the benz[a]-anthracene series, 8- and 11-hydroxybenz[a]anthracene are less stable than the 9- and 10-isomers. The absence of detectable amounts of 1- and 2-hydroxylated products is not unexpected, since little or none of these products is formed with benz[a]anthracene and rat-liver microsomes. On the other hand, these products might be expected in the oxidation of the hydrocarbon in the ascorbic acid system, since they are products of the hydroxylation of benz[a]anthracene in this system (Boyland *et al.* 1964).

The apparent absence of metabolites formed by oxidation of the 5,6-bond ('K region') of 7,12-dimethylbenz[a]anthracene is unexpected, since the bond is oxidized chemically by osmium tetroxide (Cook & Schoental, 1948) and by ozone (Moriconi & Taranko, 1963). Although with benz[a]anthracene only small amounts of the dihydrodihydroxy compound are formed by metabolic action on this

bond, the corresponding mercapturic acid is a major product (Boyland & Sims, 1964). With 7,12-dimethylbenz[*a*]anthracene it is not clear whether the absence of metabolites is because there is no initial epoxidation on this bond or whether the epoxide, once it is formed, reacts with other tissue constituents.

It is apparent that in the rat-liver preparations used in these experiments, the monohydroxymethyl (II and III) derivatives are not metabolized further to any appreciable extent, either by conversion into the corresponding aldehydes or acids, by further hydroxylation of the intact methyl group, or by hydroxylation at other positions on the molecules. It is not known whether, in animals treated with 7,12-dimethylbenz[*a*]anthracene (I), the hydroxymethyl compounds (II and III) are excreted, either free or conjugated, in the urine or whether they are first oxidized to the corresponding carboxylic acids. Gillette (1959) has shown that, with *p*-nitrotoluene, hydroxymethylation is the first stage in the oxidation of the methyl group to the acid. The oxidation of methyl groups to carboxylic acids in whole animals is well known (Williams, 1959).

The mode of formation of the monohydroxymethyl derivatives (II and III) is uncertain. They could arise by direct hydroxylation of the methyl groups or alternatively by an initial epoxidation across the 7- and 12- (or meso) positions of the hydrocarbon, followed by a rearrangement to yield the hydroxymethyl derivatives. A possibly analogous chemical reaction is known, for, when 7,12-dihydroxy-7,12-dimethylbenz[*a*]anthracene (V) is treated with hydrochloric acid or picric acid in methanol, 7-methoxy-12-methylbenz[*a*]anthracene is formed (Badger, Goulden, & Warren, 1941; Badger & Pearce, 1950): the 12-methoxy compound is apparently not formed. If the mechanism in the metabolism of the hydrocarbon is one of expoxidation it might explain why only the monohydroxymethyl derivatives are formed. The biological oxidation of the hydrocarbon resembles the oxidation in the ascorbic acid–Fe^{2+}–oxygen system in that only monohydroxymethylation products are formed, and in this respect both differ from the oxidation with lead tetra-acetate, where the diacetoxymethyl derivative is readily formed (Badger & Cook, 1939). 7,12-Dihydroxy-7,12-dimethylbenz[*a*]anthracene (V), which might be expected as a product of the action of water on the epoxide, was not detected as a metabolite. In the metabolism of anthracene (Sims, 1964) and of benz[*a*]anthracene (Boyland & Sims, 1964), products were detected that were formed by reactions at the meso positions of these hydrocarbons.

The oxidation of 7,12-dimethylbenz[*a*]anthracene (I) with the ascorbic acid–Fe^{2+}–oxygen system led to the formation of benz[*a*]anthracene-7,12-quinone, some of which was detected free and some of which was formed during the acid-decomposition of unidentifiable precursors. It is possible that the hydroxymethyl derivatives (II and III) were intermediates in these oxidations, the methyl group being eliminated as formaldehyde. Moriconi & Taranko (1963) have shown that the hydrocarbon (I) is oxidized to benz[*a*]anthracene-7,12-quinone by ozone, but the quinone was not detected as a product of the enzymic oxidation of the hydrocarbon (I).

The relationship between the formation of the hydroxymethyl compounds (II and III) and the carcinogenic action of the parent hydrocarbon, 7,12-dimethylbenz[*a*]anthracene (I), is not clear. In the 7-methylbenz[*a*]anthracene series, however, both the hydrocarbon and the hydroxymethyl compound, together with some related compounds, including the related acetoxymethyl derivative and the aldehyde, have carcinogenic properties in mice (Badger *et al.* 1940; Shear & Leiter, 1940). The methyl ether of 7-hydroxy-12-methylbenz[*a*]anthracene (tested under the name 9,10-dimethyl-1,2-benzanthracene-9,10-oxide; cf. Badger & Pearce, 1950) is also carcinogenic. It is therefore possible that the formation of hydroxymethyl derivatives (or their oxidation products) are necessary steps in the carcinogenic action of methylbenz[*a*]anthracene derivatives. Alternatively, it might be that the carcinogenic properties of these hydrocarbons are due to reactions at other sites in the molecules and that the oxidations of the methyl groups are unimportant.

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