phoretic mobility with A-chain made from either ox or pig insulin. No A-chain was derived from zone X or other areas of the paper.

Biological identification of insulin. Insulin was further identified after elution from paper by its effects in enhancing uptake of glucose and deposition of glycogen in rat hemidiaphragm *in vitro*, by methods already described (Taylor *et al.* 1961). Appropriate areas of paper were cut out and allowed to stand overnight with 1–2 ml. of 0·01N-HCl and tested for biological activity. Effects on isolated rat hemidiaphragm were examined essentially according to Randle (1956); glucose uptake was determined by the use of glucose oxidase (Huggett & Nixon, 1957) and glycogen deposition was determined by the anthrone method (Seifter, Dayton, Novic & Muntwyler, 1950). In some instances the presence of insulin was confirmed by inhibition of effects of eluates on glucose uptake by addition of antiserum, made by injecting guinea pigs with ox insulin (Wright, 1959; Taylor & Randle, 1959). Effects from the two zones on paper corresponding with areas giving A-chain are shown in Table 1.

Since glucose uptake and synthesis of glycogen were markedly enhanced by both extracts, and effects on glucose uptake were abolished by antiserum, it is concluded that both these zones contained insulin. These two insulins are probably identical with those already demonstrated by Smith (1964) to be present in rat pancreas. The B-chains derived from these insulins are known to differ in their lysine content, though the two A-chains made from them are identical. On account of this difference in charge due to an extra lysine residue in one of them, the two insulins are separable by electrophoresis in 20 % formic acid.

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Metabolism of Polycyclic Compounds

24. THE METABOLISM OF BENZ[*a*]ANTHRACENE*

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Berenblum & Schoental (1943) investigated the metabolism of benzanthracene in rats and mice and detected a compound in the faeces which was identified by methylation and spectroscopic examination as 4-hydroxybenzanthracene. Harper (1959*a*) confirmed the presence of this phenol as a metabolite in the faeces of mice and he found evidence for the presence of a second phenol, which was also a metabolite in rabbits (Harper, 1959*b*) and which was claimed to be 2-hydroxybenzanthracene.

In the present work it has been shown that benzanthracene is converted by rats, rabbits and mice into *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)-L-cysteine, 5,6-dihydro-5,6-dihydroxybenzanthracene, 8,9-dihydro-8,9-dihydroxybenzanthracene and 10,11-dihydro-10,11-dihydroxybenzanthracene, together with smaller amounts of 3,4-dihydro-3,4-dihydroxybenzanthracene and possibly 1,2-dihydro-1,2-dihydroxybenzanthracene. 4-, 8- and 9-Hydroxybenzanthracene and a phenol which is probably 3-hydroxybenzanthracene are also formed. 2-Hydroxybenzanthra-

* Part 23: Boyland & Sims (1964).

cene could not be detected as a metabolite in animals of any of the species examined. There is also evidence of hydroxylation in the 7- or 12-position of the benzanthracene nucleus. The chemical oxidation of benzanthracene with perbenzoic acid shows that attack occurs at the 5,6-bond and the 7- and 12-positions on the nucleus.

EXPERIMENTAL

Spectra. Absorption spectra were measured on a Perkin–Elmer model 137 ultraviolet spectrophotometer and fluorescence spectra on an Aminco–Bowman spectrophotofluorimeter. Measurements were made in ethanol.

Melting points. These are uncorrected.

Chromatography. Paper chromatography was carried out on Whatman no. 1 chromatography paper by downward development with butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.) for 18 hr. Chromatograms were examined in u.v. light and dipped in either ninhydrin in acetone (0·2%, w/v) or in the platinic iodide reagent of Toennies & Kolb (1951). Thin-layer chromatograms, prepared from silica gel G, were of 0·25 mm. thickness and were developed with one of the following (1): hexane–benzene (19:1, v/v); (2) benzene; (3) benzene–ethanol (19:1, v/v); (4) benzene–ethanol (9:1, v/v). The chromatograms were examined, whilst still wet, in u.v. light, both before and after exposure to NH_3. They were sprayed with 0·5% of 2,6-dichloroquinonechloroimide in ethanol followed by aq. 10% (w/v) Na_2CO_3 either immediately or after being sprayed with HCl and heated to 80° in an oven for 10 min. The last method was used in the detection of dihydrodihydroxy compounds and these were also detected on two-dimensional chromatograms which were developed in the first direction with solvent (3), sprayed with HCl and heated to 80° for 10 min. and developed in the second direction with solvent (2). In this way the phenols arising from the acid decomposition of the dihydrodihydroxy compounds could be identified and the structures of the latter compounds determined. The phenols were also characterized in both one- and two-dimensional chromatograms by locating the spots containing the phenols in u.v. light and removing the areas of silica gel from the plate, eluting the absorbed materials with ethanol and comparing the fluorescence spectra of the solutions with those of solutions of the authentic phenols. It was sometimes possible also to measure the u.v. spectra of the solutions, but usually the spectra did not show sharp peaks, probably because of absorption by the relatively large amounts of naturally occurring compounds in the solutions being measured. In the metabolic experiments described below, many of the naturally occurring compounds that were present gave colours with the 2,6-dichloroquinonechloroimide–Na_2CO_3 reagent. Because of this, the fluorescence of the metabolites, particularly after the chromatograms were exposed to NH_3, was often a more reliable test of their presence than the colour reaction. The properties on thin-layer chromatograms of the compounds described in this paper are listed in Table 1 and their spectra in Table 2.

Materials. Benz[*a*]anthracene and benz[*a*]anthracene-7,12-quinone were obtained from L. Light and Co. Ltd. *cis*-5,6-Dihydro-5,6-dihydroxybenz[*a*]anthracene was prepared by the oxidation of benzanthracene with osmium tetroxide as described by Cook & Schoental (1948). Benz[*a*]anthracene-5,6-quinone was obtained from the dihydrodihydroxy compound by oxidation with chromium trioxide in acetic acid. The quinone was reduced with lithium tetrahydroaluminate in ether by the method of Booth, Boyland & Turner (1950) to trans-5,6-*dihydro*-5,6-*dihydroxybenz*[a]-*anthracene*, which was recrystallized from benzene in needles, m.p. 210° (decomp.) (Found: C, 82·2; H, 5·4. $C_{18}H_{14}O_2$ requires C, 82·4; H, 5·4%). The compound was acetylated with acetic anhydride in pyridine to yield a *diacetate* in needles from aq. ethanol, m.p. 209° (Found: C, 75·9; H, 5·6. $C_{22}H_{18}O_4$ requires C, 76·3; H, 5·2%). The dihydrodihydroxy compound was oxidized by chromium trioxide in acetic acid to a compound indistinguishable from benzanthracene-5,6-quinone on thin-layer chromatograms and both the dihydroxy compound and its diacetate yielded a compound indistinguishable from 5-hydroxybenzanthracene after being heated for 15 min. at 100° with 5N-HCl.

Benzanthracene-7,12-quinone (5 g.) was reduced in ether (250 ml.) with lithium tetrahydroaluminate (1·7 g.) for 48 hr., by the general method described by Booth *et al.* (1950). At the end of this time 3·8 g. of the quinone had been used up and the reaction was stopped. Water (5 ml.) followed by 2N-H_2SO_4 (30 ml.) were added slowly to decompose the complex and the excess of the hydride. The ether layer was separated, washed with 2N-NaOH (50 ml.), dried (Na_2SO_4) and the ether removed under reduced pressure. The residue was dissolved in the minimum of boiling benzene (about 300 ml.) and the yellow solution was allowed to crystallize overnight at 0°. The clusters of needles, m.p. 149–150°, which separated, were recrystallized twice from aq. ethanol and then from benzene to yield 7,12-*dihydro*-7,12-*dihydroxybenz*[a]*anthracene* (940 mg.) in needles, m.p. 156–158° (decomp.) (Found: C, 82·2; H, 5·5. $C_{18}H_{14}O_2$ requires C, 82·4; H, 5·4%). It was acetylated with acetic anhydride in pyridine to yield a *diacetate* in rods from ethanol, m.p. 146° (decomp.) (Found: C, 76·2; H, 5·5. $C_{22}H_{18}O_4$ requires C, 76·3; H, 5·2%). The dihydrodihydroxy compound (100 mg.) in ethanol (1 ml.) and conc. HCl (1 ml.) was heated to 100° for 5 min. and the solution cooled and diluted with an excess of water. 7-Hydroxybenzanthracene separated and was recrystallized from benzene in yellow prisms (65 mg.), m.p. 152–153°, undepressed in admixture with the authentic phenol described below. The stereochemical form of the dihydrodihydroxy compound could not be determined although it could be the *trans*-isomer since the reduction of quinones with lithium tetrahydroaluminate usually gives *trans*-dihydrodihydroxy compounds. The reduction of 9,10-anthraquinone with lithium tetrahydroaluminate and with aluminium isopropoxide, however, yields mixtures of the *cis*- and *trans*-isomers (Boyland & Manson, 1951; Coffey & Boyd, 1954; Dufraisse, Rio & Lepage, 1958). This seems to be a special case since *trans*-9,10-dihydro-9,10-dihydroxyanthracene is readily converted into the *cis*-isomer, even on incubation in aqueous solution at pH 5·0 overnight at 37° (E. Boyland and P. Sims, unpublished observations). In the present work, however, a second dihydrodihydroxy compound could not be found in the reduction products, either on fractional crystallization or on examination of the products on thin-layer chromatograms.

A second product, which was obtained when the above benzene mother-liquors were evaporated to small volume

Table 1. *Properties of compounds related to benzanthracene on thin-layer chromatograms*

For details see text.

Compound	R_F*		Fluorescence		Colour with 2,6-dichloroquinonechloroimide–Na_2CO_3
	Benzene	Benzene–ethanol (19:1, v/v)	Immediate	After exposure to NH_3	
1-Hydroxybenzanthracene	0·43	0·60	Violet	Violet	Violet
2-Hydroxybenzanthracene	0·23	0·49	Violet	Yellow	Grey, becoming red–brown
Phenol, probably 3-hydroxybenzanthracene	0·22	0·50	Violet	Light green	Pink, becoming purple
4-Hydroxybenzanthracene	0·35	0·53	Violet	Yellow	Violet, becoming blue
5-Hydroxybenzanthracene†	0·39	0·53	Blue	Yellow	Blue–green, becoming brown
Phenol, probably 6-hydroxybenzanthracene†	0·39	—	Blue	Yellow	Blue–green, becoming brown
7-Hydroxybenzanthracene	0·41	0·54	Green	Green	Red–purple after 5 min.
8-Hydroxybenzanthracene	0·35	0·53	Violet	Orange	Purple
Phenol, probably 9-hydroxybenzanthracene	0·22	0·49	Violet	Yellow–green	Green, becoming grey
Phenol, probably 10-hydroxybenzanthracene	0·22	0·49	Violet	Yellow	Green, becoming grey
11-Hydroxybenzanthracene	0·35	0·53	Violet	Orange	Purple
7-Acetoxybenzanthracene	0·50	0·94	Violet	—	Pink, becoming purple
7,12-Diacetoxybenzanthracene	0·13	0·72	Violet	—	Pink, becoming purple
Metabolite, possibly 1,2-dihydro-1,2-dihydroxybenzanthracene‡	—	0·26	—	—	None
Metabolite, probably 3,4-dihydro-3,4-dihydroxybenzanthracene‡	—	0·28	—	—	Blue§
cis-5,6-Dihydro-5,6-dihydroxybenzanthracene‡	0·02	0·29	Dark violet	—	Green, becoming brown§
trans-5,6-Dihydro-5,6-dihydroxybenzanthracene‡	0·02	0·29	Dark violet	—	Green, becoming brown§
Metabolite, probably 8,9-dihydro-8,9-dihydroxybenzanthracene‡	—	0·29	—	—	Purple–brown
Metabolite, probably 10,11-dihydro-10,11-dihydroxybenzanthracene‡	—	0·29	—	—	Purple–brown
7,12-Dihydro-7,12-dihydroxybenzanthracene‡	0·03	0·31	Dark violet	—	Yellow on standing
Benzanthracene-5,6-quinone	0·08	0·65	Dark-absorption	—	(Orange)‖
Benzanthracene-7,12-quinone	0·62	0·98	Orange	—	(Yellow)‖
12-Hydroxybenzanthracen-7-one	0·10	0·50	Green	—	Yellow, then green, then yellow
Dimeric compound	—	0·62	None	None	(Pale yellow in large‖ concentration)
Metabolite giving benzanthracene with acid¶	—	0·28	—	—	—
Methyl ester of *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine	—	0·41**	Dark violet	—	None
Substance, probably a glycine conjugate of the above ester	—	0·45**	Dark violet	—	None

* The absolute R_F values varied between chromatograms, although the relative order of the compounds on the chromatograms was the same. Figures quoted are those from typical chromatograms.

† These compounds usually formed small amounts of benzanthracene-5,6-quinone, presumably because of oxidation during the loading of the chromatograms.

‡ The presence of these compounds was detected on two-dimensional acid-treated chromatograms by the formation of phenols on the chromatograms after the second development as described in the text.

§ These colours were obtained only if the chromatograms were first heated with acid as described in the text.

‖ These colours were present on untreated chromatograms.

¶ This compound was detected by its decomposition to benzanthracene on two-dimensional acid-treated chromatograms developed in the second direction with hexane–benzene (19:1, v/v). In this solvent system benzanthracene had R_F 0·53 and was detected by its violet fluorescence in u.v. light.

** R_F measured in benzene–ethanol (9:1, v/v).

and allowed to crystallize, was apparently identical with one obtained by Fieser & Hershberg (1937, 1938) as a product of the action of pentylmagnesium bromide or *tert.*-butyl hypochlorite on benz[*a*]anthracen-7-one or by the attempted recrystallization of a chlorobenzanthracenone. Fieser's product had an empirical formula $C_{36}H_{22}O_2$ and was apparently a dimeric benz[*a*]anthracen-7-one, which formed needles from toluene, m.p. 265–267° (corr.) (decomp.). The product obtained in the present work formed very pale-yellow needles (540 mg.) from toluene, m.p. 256–257° (decomp.) (Found: C, 88·8; H, 4·7. Calc. for $C_{36}H_{22}O_2$: C, 88·9; H, 4·6%).

1- and 4-Hydroxybenz[*a*]anthracene were given by Dr R. Schoental and 4-hydroxybenzanthracene was also prepared by the method of Sempronj (1939). 2-Hydroxybenz[*a*]anthracene was a sample that had been prepared in this Institute by the method of Ioffe & Fedorova (1941). 5-Hydroxybenz[*a*]anthracene was prepared from *cis*-5,6-dihydro-5,6-dihydroxybenzanthracene by the action of HCl in acetic acid at 100° (Cook & Schoental, 1948).

Table 2. *Absorption and fluorescence spectra of compounds related to benzanthracene*

Details are given in the text. When measured, the molecular extinction coefficients are shown in parentheses (as $\log E_{max.}$). Measurements made on the Aminco–Bowman spectrophotofluorimeter have a possible error of ± 10 mμ.

Compound	$\lambda_{max.}$ (mμ)	Fluorescence: Excitation max. (mμ)	Fluorescence: Fluorescence max. (mμ)
1-Hydroxybenzanthracene	227, 255, 272, 281, 297, 308 326, 341, 352 and 371	280 and 305	400 and 425
2-Hydroxybenzanthracene	227, 270, 279, 287, 297, 326, 341, 355 and 373	300 and 355	410 and 430
Metabolite, probably 3-hydroxybenzanthracene*	—	285 and 335	410 and 430
4-Hydroxybenzanthracene	231, 246, 253, 277, 286, 314, 342, 358 and 374	285, 310, 335, 350, 370 and 395	410 and 430
5-Hydroxybenzanthracene	268, 278, 289, 300, 320, 334, 350 and 374	290 and 355	410 and 430
Phenol, probably 6-hydroxybenzanthracene	260, 269, 285, 340 and 355	285, 335 and 355	410 and 425
7-Hydroxybenzanthracene	260, 270, 279, 289, 335, 350 and 365	295, 370 and 395	415 and 440
8-Hydroxybenzanthracene	235, 272, 282, 295, 340 and 358	290 and 365	430
Metabolite, probably 9-hydroxybenzanthracene*	245, 279, 287, 307 and 340	290, 335 and 390	405 and 420
11-Hydroxybenzanthracene	234, 265, 285, 297, 323, 340, 355 and 384	295, 360 and 380	425
7-Acetoxybenzanthracene	220 (4·60), 231 (4·60), 257 (4·60), 268 (4·68), 278 (4·97), 289 (5·05), 301 (3·83), 320 (3·60), 334 (3·92), 349 (3·99) and 367 (3·83)	—	—
7,12-Diacetoxybenzanthracene	219, 259, 268, 277, 288, 301, 338, 365 and 373	—	—
cis-5,6-Dihydro-5,6-dihydroxybenzanthracene	216, 247, 257, 266, 298 and 308	—	—
trans-5,6-Dihydro-5,6-dihydroxybenzanthracene	216 (4·47), 247 (4·51), 257 (4·58), 266 (4·60), 298 (4·20) and 309 (4·05)	—	—
trans-5,6-Diacetoxy-5,6-dihydroxybenzanthracene	216 (4·47), 248 (4·49), 257 (4·60), 266 (4·62), 297 (4·21) and 309 (4·04)	—	—
7,12-Dihydro-7,12-dihydroxybenzanthracene	231 (4·89) and 283 (3·80)	—	—
7,12-Diacetoxy-7,12-dihydroxybenzanthracene	230 (4·90), 279 (3·94) and 289 (3·94)	—	—
Benzanthracene-7,12-quinone	247, 283 and 336	—	—
12-Hydroxybenzanthracen-7-one	218 (4·59), 260 (4·46), 269 (4·49) and 300 (3·97)	—	—
Methyl ester of *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine (from rat urine)	259 (4·59), 268 (4·53), 300 (4·15) and 311 (4·08)	—	—
N-Acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine (synthetic)	259, 268, 300 and 311†	—	—

* Measurements were made only on the materials eluted from spots on thin-layer chromatograms as described in the text.

† The glycine conjugate of the methyl ester, the mercapturic acid obtained from the urine of benzanthracene-treated rats, rabbits and mice, the mercapturic acid and the corresponding cysteine, cysteinylglycine and glutathione conjugates eluted from spots after the paper chromatography of the bile of benzanthracene-treated rats, and the methyl ester of the synthetic mercapturic acid had similar u.v.-absorption spectra.

7-Hydroxybenz[a]anthracene was prepared by the hydrolysis of 7-acetoxybenz[a]anthracene with methanol and HCl (Fieser & Hershberg, 1937), the acetoxy derivative being obtained by the oxidation of benzanthracene with lead tetra-acetate (Fieser & Hershberg, 1938). The mother-liquors from the hydrolysis yielded a small amount of the dimeric material, described above, which crystallized in pale-yellow needles from toluene, m.p. and mixed m.p. 256–257°. 8-Hydroxybenz[a]anthracene was prepared by the dehydrogenation with palladium black of 8,9,10,11-tetrahydro-8-oxobenz[a]anthracene as described by Cook & Schoental (1952) and 10-hydroxybenz[a]anthracene from the dehydrogenation with sulphur of 8,9,10,11-tetrahydro-10-oxobenz[a]anthracene as described by Fieser & Johnson (1939).

Oxidation of benzanthracene with bromine. The method used was essentially that of Meyer (1911) in the preparation of 9-hydroxy-10-anthrone (oxanthrone). Benzanthracene (5 g.), in acetone (200 ml.) and ice–water (100 ml.), was stirred whilst bromine (11 g.) was added over 30 min., the mixture being kept cold by the addition of crushed ice. The acetone was removed under reduced pressure and the mixture kept at 0° overnight, when the gum which had separated solidified. The solid was chromatographed in benzene on a column prepared from 100 g. of silica gel (200–300 mesh; L. Light and Co. Ltd.), fractions of 100 ml. being collected. Fractions 1–5 contained benzanthracene and were discarded but fractions 6–9, which contained the material forming a yellow band on the column, were combined and evaporated to yield benzanthracene-7,12-quinone in orange–yellow needles, m.p. and mixed m.p. 163–165°. Later fractions, containing the material which formed a band with greenish-yellow fluorescence in u.v. light, were combined and evaporated and the solid was crystallized from ethanol to yield 12-*hydroxybenz*[a]*anthracen*-7-*one* (or possibly the isomeric 7-hydroxybenz[a]anthracen-12-one) (980 mg.) in yellow prisms, m.p. 186° (Found: C, 82·8; H, 4·7. $C_{18}H_{12}O_2$ requires C, 83·1; H, 4·65%). The hydroxybenzanthracenone was acetylated with acetic anhydride in pyridine to yield 7,12-diacetoxybenz[a]anthracene in needles from aq. ethanol, m.p. 214–215° (Found: C, 76·9; H, 4·6. Calc. for $C_{22}H_{16}O_4$: C, 76·7; H, 4·7%). A mixture with 7,12-diacetoxybenzanthracene, of m.p. 215–216°, prepared by the reductive acetylation of benzanthracene-7,12-quinone with zinc in acetic anhydride as described by Badger & Lynn (1950), had m.p. 215–216°.

Later fractions from the column yielded a small amount of the dimeric compound described above, which separated as needles from toluene, m.p. and mixed m.p. 256–257°.

Oxidation of benzanthracene with perbenzoic acid. In a typical oxidation benzanthracene (5 g.) and perbenzoic acid (6 g.) in chloroform (100 ml.) were kept at room temperature in the dark for 7 days with occasional shaking. At the end of this time the solution was washed three times with ice-cold aq. 2N-KOH (250 ml.) and once with water. The aqueous washings were combined and acidified with HCl and the mixture was extracted with ether (250 ml.). The ether solution was washed three times with saturated $NaHCO_3$ soln. (250 ml.) to remove benzoic acid, dried (Na_2SO_4) and evaporated and the residue examined on thin-layer chromatograms for hydroxybenzanthracenes: none was detected.

The chloroform solution was evaporated under reduced pressure in the presence of a solution of *N*-acetyl-L-cysteine (1 g.) and $NaHCO_3$ (1 g.) in water (50 ml.). Acetone (150 ml.) was added and the mixture was heated under reflux for 30 min. The acetone was distilled off under reduced pressure and the mixture extracted three times with ether (100 ml.). The aqueous layer was acidified to pH 4 with acetic acid, and charcoal (10 g.; British Drug Houses Ltd., activated) added. The charcoal was filtered off and washed with water (500 ml.) and the adsorbed material eluted with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88) (1 l.). The methanol was evaporated under reduced pressure to yield a gum, which was chromatographed on Whatman no. 3MM paper for 18 hr. in butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.). A compound, R_F 0·43, which was located by its dark-violet fluorescence in u.v. light and by its reaction with the platinic iodide reagent, was eluted with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88) from the appropriate strips cut from the chromatograms. Removal of the solvent yielded a gum, which was rechromatographed on no. 3MM paper for 18 hr. with butan-1-ol–acetic acid–water (2:1:1, by vol.) as developing solvent. The product was located as before and eluted from the paper with methanol to yield a product which, on the evidence presented below, is presumed to be a mixture of the diastereoisomers of *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benz[a]anthracenyl)-L-cysteine (150 mg.), separating from methanol–ether as a light-brown powder, decomp. 182–195° (Found: N, 3·5; S, 8·1. Calc. for $C_{23}H_{21}NO_4S$: N, 3·4; S, 7·9%). A little of the mercapturic acid in methanol was esterified with diazomethane to yield a methyl ester that formed a gum which could not be crystallized. Solutions of the acid in water or the methyl ester in methanol were acidified with HCl and the products examined on thin-layer chromatograms. In both cases a compound indistinguishable from benzanthracene was detected but no hydroxybenzanthracenes were formed. When the products from the mercapturic acid decomposition were examined on paper chromatograms, a compound indistinguishable from *NN'*-diacetylcystine was detected, together with a small amount of a substance which is presumed to be 5-benz[a]anthracenylmercapturic acid. A little of the original mercapturic acid was heated with a few drops of aq. 2N-NaOH at 100° for 2 hr. and the solution was acidified with HCl and extracted with ether. Examination of the contents of the ether extract on thin-layer chromatograms showed the presence of a phenolic substance with properties on the chromatogram closely resembling those of 5-hydroxybenzanthracene. Its absorption and fluorescence spectra differed, however, from those of the 5-hydroxy compound (see Table 2), and since it was slowly oxidized in air to a compound indistinguishable on thin-layer chromatograms from benzanthracene-5,6-quinone it is presumed to be 6-hydroxybenz[a]anthracene. Both the mercapturic acid and its methyl ester were slowly oxidized when their solutions in water and methanol respectively were exposed to the atmosphere, to yield products on thin-layer chromatograms indistinguishable from benzanthracene-5,6-quinone.

The combined ether extracts described above were dried (Na_2SO_4) and evaporated and the residue was chromatographed in benzene on silica gel (200–300 mesh) (75 g.), fractions of 100 ml. being collected. Fractions 1 and 2 were combined and evaporated to yield benzanthracene (1·8 g.), m.p. and mixed m.p. 156–157°, whilst fractions 3–5 yielded benzanthracene-7,12-quinone (0·95 g.), m.p. and

mixed m.p. 163–165°. Fractions 7–9 were combined and evaporated and the residue was fractionally crystallized from aq. ethanol to yield two unidentified substances, the less soluble as needles (8 mg.), m.p. 194–196°, and the more soluble as needles (5 mg.), m.p. 155–160°. On thin-layer chromatograms, the first substance formed a blue-fluorescent spot in u.v. light (R_F 0·38 and 0·90 in solvents 2 and 3 respectively) and the second a violet-fluorescent spot (R_F 0·32 and 0·85). Both substances had similar u.v. spectra ($\lambda_{max.}$ at 257, 266, 297 and 310 mμ), which resembled that of 5,6-dihydro-5,6-dihydroxybenzanthracene, but no phenolic products could be detected on thin-layer chromatograms after the substances had been heated to 100° with HCl for 30 min. Fractions 10 and 11 yielded the dimeric compound (25 mg.) described above as pale-yellow needles from toluene, m.p. and mixed m.p. 256–257° (decomp.). Fractions 12–15 were combined and evaporated to yield the hydroxybenzanthracenone (155 mg.) described above, which separated from ethanol in yellow prisms, m.p. and mixed m.p. 184–186° (Found: C, 83·5; H, 4·8%). With acetic anhydride in pyridine, the hydroxy ketone yielded 7,12-diacetoxybenzanthracene in needles, m.p. and mixed m.p. 214–215°.

At this point the eluting solvent was changed to benzene containing 1% (v/v) of ethanol and the fractions containing the material moving on the column as a dark-coloured band were combined and evaporated to yield a brown gum (110 mg.). When the gum was examined on acid-treated two-dimensional thin-layer chromatograms, four compounds giving rise to 5-hydroxybenzanthracene and four compounds giving rise to 7-hydroxybenzanthracene and benzanthracene-7,12-quinone with acid were detected together with a number of coloured products. On a typical chromatogram developed with solvent (3) the first group of compounds had R_F 0·65, 0·50, 0·42 and 0·20 and the second group R_F 0·72, 0·57, 0·45 and 0·25 respectively. In an attempt to identify the compounds, the gum was applied to the base lines of six thin-layer chromatograms, which were developed with solvent (3). The appropriate bands on the chromatograms were removed and the adsorbed materials eluted from the silica gel with ethanol and the u.v.-absorption spectra of the solutions measured. The u.v. spectra of the solutions of the first four compounds ($\lambda_{max.}$ at 157, 266, 297 and 309 mμ) were similar to each other and to that of 5,6-dihydro-5,6-dihydroxybenzanthracene. Removal of the solvent from the first three solutions yielded small amounts of gums which could not be crystallized, but the solution containing the product with R_F 0·20 yielded a solid that crystallized from benzene in needles (4 mg.), m.p. 208° (decomp.), undepressed in admixture with *trans*-5,6-dihydro-5,6-dihydroxybenzanthracene.

The u.v. spectra of the solutions of the second group of compounds were more difficult to interpret, possibly because the solutions contained impurities. Those of the solutions of compounds with R_F 0·72 and 0·57 showed no prominent maxima, and those of the solutions of the compounds with R_F 0·45 and 0·20 each showed one maximum at 232 mμ. The properties of the slowest-running compound on thin-layer chromatograms were similar to those of 7,12-dihydro-7,12-dihydroxybenzanthracene described above, but no solid material was obtained.

Enzymic and acid hydrolyses. Hydrolyses with the sulphatases of Taka-diastase (Parke Davis Ltd.) were carried out in 0·1 M-sodium acetate–acetic acid buffer, pH 5·7, and hydrolyses with β-glucuronidase (Ketodase; Warner-Chilcott Laboratories) in 0·1 M-sodium acetate–acetic acid buffer, pH 5·0, the solutions being incubated overnight at 37°. Acid hydrolyses were carried out by heating the material with 5 N-HCl for 15 min. at 100°. The products of the hydrolyses were extracted with ether and examined on thin-layer chromatograms as described above.

Metabolism of benzanthracene in rats

In a typical experiment 12 male rats of the Chester Beatty strain (body wt. 250 g.) were given 2 ml. of a suspension of 5% (w/v) benzanthracene in arachis oil by intraperitoneal injection every other day until a total of 12 g. of the hydrocarbon had been administered. The urines and the faeces were both collected and examined separately.

Metabolites in urine. The urines were combined and filtered and 5 ml. portions of the filtrates hydrolysed with sulphatase, with β-glucuronidase or with acid. The phenols and dihydrodihydroxy compounds detected on thin-layer chromatograms were the same as those found in the large-scale experiment described below and no additional products were detected.

The urine was diluted with an equal volume of water and the solution acidified to pH 4 with acetic acid. Charcoal (50 g.) was added and the mixture was filtered and the charcoal washed with water (2 l.). The adsorbed material was eluted from the charcoal with methanol (10 l.) containing 5% (w/v) of NH_3 (sp.gr. 0·88) followed by methanol–benzene (9:1, v/v) (5 l.). The combined methanol solutions were evaporated under reduced pressure to yield a brown gum, which was chromatographed on a cellulose-powder column prepared from Whatman standard-grade cellulose powder (500 g.). The column was developed with butan-1-ol–cyclohexane–aq. 2 N-NH_3 (9:2:1, by vol.) and fractions of 150 ml. were collected and the solvent was removed under reduced pressure. The residues were examined on paper chromatograms and fractions containing the same products were combined. In this way three main fractions were obtained containing sulphuric acid esters, mercapturic acids and glucosiduronic acids respectively. Free phenols and dihydrodihydroxy compounds were not normally detected in these experiments.

(*a*) Sulphuric ester fraction. This was obtained as a dark gum, which formed only a small proportion of metabolites in the urine. Portions of the gum were hydrolysed with sulphatase and with acid and the products examined on thin-layer chromatograms. The presence of sulphuric acid in the acid hydrolysates was demonstrated with aq. $BaCl_2$ and two major phenolic products were detected on the chromatograms, one of which was indistinguishable from 4-hydroxybenzanthracene and the second appeared to be 3-hydroxybenzanthracene. Two other phenolic products present in small amounts appeared to be 8- and 9-hydroxybenzanthracene. Two dihydrodihydroxy compounds, which were detected in the products of the sulphatase hydrolysis, were presumed to be 5,6-dihydro-5,6-dihydroxy- and 8,9-dihydro-8,9-dihydroxybenzanthracene. The reasons for assigning these structures to the metabolites are discussed in more detail below. Benzanthracene-7,12-quinone was also detected in the hydrolysates and appeared to have arisen from the oxidation by air of one or more hydroxy compounds released from conjugation with sulphuric acid in the hydrolyses.

(*b*) Mercapturic acid fraction. This formed a brown gum (320 mg.; 1·25% of the administered hydrocarbon) which was purified by chromatography on Whatman no. 3MM paper as described for the synthetic acid. The product, which was indistinguishable from the synthetic acid on paper chromatograms, formed a light-brown gum that could not be crystallized. When a solution of the gum in water was acidified, benzanthracene, m.p. and mixed m.p. 155–157°, separated. The mother-liquors were shown by means of paper chromatography to contain a compound indistinguishable from *NN'*-diacetylcystine together with a small amount of a compound which appeared to be 5-benzanthracenylmercapturic acid. When a little of the gum was heated with 2N-NaOH at 100° for 2 hr. and the solution was acidified with HCl and extracted with ether, a phenol was obtained which was indistinguishable in its properties on thin-layer chromatograms and in its fluorescence spectrum from the phenol believed to be 6-hydroxybenzanthracene which had been obtained by the hydrolysis with alkali of the synthetic mercapturic acid.

The gum (100 mg.), in methanol, was esterified with diazomethane in ether to yield a gummy product which was shown on a thin-layer chromatogram to consist of two esters. The mixture was applied to the base lines of six thin-layer chromatograms, which were developed with solvent (4). The two esters were located on the chromatograms as opaque bands, seen when the wet chromatograms were examined in daylight. These bands were removed from the plates and the adsorbed material was eluted from the silica gel with methanol. The solution of the slower-running ester was evaporated and the solid recrystallized from a small amount of methanol to yield the *methyl ester* of *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benz[*a*]anthracenyl)-L-cysteine (58 mg.) as a buff powder, m.p. 104–107°, $[\alpha]_D^{27°} + 108 \pm 4°$ (*c* 0·5 in methanol) (Found: C, 66·3; H, 6·5; N, 3·2; S, 6·9. $C_{24}H_{23}NO_4S,CH_3OH$ requires C, 66·2; H, 6·0; N, 3·1; S, 7·1%). Attempts to remove the solvent of crystallization by drying the ester under reduced pressure at room temperature yielded a gum; drying at 60° caused decomposition. A 5 mg. sample of the ester, in methanol (0·1 ml.), was acidified with 1 drop of conc. HCl and the mixture applied to the base line of a thin-layer chromatogram, which was developed with solvent (1). The fluorescent band of benzanthracene, seen when the chromatogram was examined in u.v. light, was removed from the plate and the hydrocarbon eluted from the silica gel with ethanol. Estimation of the hydrocarbon by measurement of the extinction at 287 mμ in a Unicam SP. 500 spectrophotometer showed that the ester was converted into the hydrocarbon in about 90% yield. A little of the ester was heated with 5% (w/v) KOH in methanol for 1 hr. and the products were examined on thin-layer chromatograms, when a phenol indistinguishable from that believed to be 6-hydroxybenzanthracene was detected. The u.v. and i.r. spectra of the methyl esters of both the synthetic mercapturic acid and the metabolite were identical.

The faster-running ester formed a gum (15 mg.) that could not be crystallized. A little of the ester was hydrolysed with KOH in methanol as before to yield a phenol indistinguishable from the phenol believed to be 6-hydroxybenzanthracene. When the ester was treated with HCl in methanol, a product indistinguishable on thin-layer chromatograms from benzanthracene was obtained. A little of the ester was heated for 4 hr. at 100° with HBr (sp.gr. 1·7) and the products were examined on paper chromatograms developed with butan-1-ol–acetic acid–water (2:1:1, by vol.) to reveal a ninhydrin-positive spot indistinguishable from glycine. The structure of the acid from which the ester was derived could not be determined, although it appears to be closely related to the mercapturic acid and to have a structure analogous to conjugates formed in the metabolism of 1,2-dihydronaphthalene (Boyland & Sims, 1960) and of phenanthrene (Boyland & Sims, 1962*a*). The acid could not be separated from the mercapturic acid on paper chromatograms but the compound spot did not react with ninhydrin, unlike the metabolite in the bile, described below, which appears to be *S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteinylglycine.

There was some evidence of the presence of a second acid-labile mercapturic acid in the mercapturic acid fraction, as, when the fraction was acidified with HCl and the products were extracted with ether, small amounts of 8-hydroxybenzanthracene and a second phenol believed to be 9-hydroxybenzanthracene were detected in the ether layer on thin-layer chromatograms. This could indicate the presence of compounds such as *N*-acetyl-*S*-(8,9-dihydro-9-hydroxy-8-benzanthracenyl)cysteine, which should yield phenols with acid in the same way as the mercapturic acid derived from naphthalene yields naphthols (Boyland & Sims, 1958).

(*c*) Glucosiduronic acid fraction. This was obtained as a dark-brown gum, which gave a positive naphtharesorcinol test for glucuronic acid. The acid-hydrolysis products were seen on thin-layer chromatograms to contain benzanthracene, benzanthracene-7,12-quinone and a number of phenolic products, of which 8-hydroxybenzanthracene and a phenol presumed to be 9-hydroxybenzanthracene were the major products. The products of the hydrolysis of the gum with β-glucuronidase contained benzanthracene-7,12-quinone, a substance yielding benzanthracene after treatment with acid and a complex mixture of dihydrodihydroxy compounds and phenols. A complete identification of these latter products was not possible at this stage since many of the hydroxybenzanthracenes could not be separated from each other on thin-layer chromatograms. A little of the glucosiduronic acid gum was therefore applied along the base line of a sheet of Whatman no. 3MM chromatography paper and the chromatogram was developed with butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.) for 18 hr. The fluorescent band containing the glucuronic acid conjugates was located in u.v. light and cut into six horizontal strips of about equal width. The adsorbed material was eluted from the strips with methanol containing 5% (w/v) of NH_3 (sp.gr. 0·88) to give six fractions, numbered from 1 to 6 in decreasing order of R_F values. The solutions were evaporated, and the residues hydrolysed with β-glucuronidase and the products examined on two-dimensional, acid-treated thin-layer chromatograms. The hydrolysates of fractions 1 and 2 yielded mainly phenolic products, one of which was indistinguishable from 4-hydroxybenzanthracene in R_F, fluorescence and colour reaction on the chromatogram, and in its absorption and fluorescence spectra. A second product was, for reasons described below, believed to be 3-hydroxybenzanthracene. There was also evidence for the presence of 8- (or 11-)hydroxybenzanthracene, since the 4-hydroxybenzanthracene spot on untreated chromatograms became brown on standing in the air, a property of 8- and 11-hydroxybenzanthracene but not of 4-hydroxybenzanthracene. There also

appeared to be a small spot whose chromatographic properties resembled those of the phenol believed to be 9-hydroxybenzanthracene, but identification was not certain because of the close proximity of the phenol believed to be 3-hydroxybenzanthracene. 1- and 2-Hydroxybenzanthracene were not detected in these hydrolysates. Fractions 3 and 4 yielded, besides the above-mentioned phenols, a product which with acid gave a phenol indistinguishable in its chromatographic properties and spectra from 5-hydroxybenzanthracene. The product itself had the same R_F on thin-layer chromatograms as the synthetic 5,6-dihydro-5,6-dihydroxybenzanthracenes. The configuration of the metabolite is presumed to be *trans* by analogy with the corresponding metabolites of other aromatic hydrocarbons. A second dihydrodihydroxy compound, present in relatively small amount, yielded 4-hydroxybenzanthracene with acid, together with a second phenol which was indistinguishable from the product described above, which is believed to be 3-hydroxybenzanthracene. Since the dihydrodihydroxy compound yields 4-hydroxybenzanthracene with acid, it is most probably 3,4-dihydro-3,4-dihydroxybenzanthracene, so that the second phenol produced in the acid decomposition should be 3-hydroxybenzanthracene. A substance yielding benzanthracene with acid was also present in this hydrolysate together with benzanthracene-7,12-quinone. 2-Hydroxybenzanthracene or compounds yielding this phenol with acid were not detected. The hydrolysate of fraction 4 also contained a relatively large amount of a third dihydrodihydroxy compound, which with acid yielded two phenols, one of which was indistinguishable on thin-layer chromatograms and in its absorption and fluorescence spectra from 8-hydroxybenzanthracene. If the parent compound is 8,9-dihydro-8,9-dihydroxybenzanthracene, then the second phenol should be 9-hydroxybenzanthracene. The hydrolysates of fractions 5 and 6 also contained the 8,9-dihydro-8,9-dihydroxy compound and there was evidence of yet another dihydrodihydroxy compound in these hydrolysates, both compounds having the same R_F on thin-layer chromatograms. The u.v.-absorption spectrum of the acid-decomposition product which had the chromatographic properties of 8-hydroxybenzanthracene showed, in addition to the expected maxima, a small maximum at 265 mμ, which is characteristic of 11-hydroxybenzanthracene. In another separation of the glucosiduronic acids on a paper chromatogram the hydrolysate of the slowest-moving fraction contained a compound which yielded a phenol indistinguishable from 11-hydroxybenzanthracene both on thin-layer chromatograms and in its absorption and fluorescence spectra. The parent compound is presumably 10,11-dihydro-10,11-dihydroxybenzanthracene and a second phenol which was detected after its acid-decomposition is probably 10-hydroxybenzanthracene. In a third separation of the glucosiduronic acids, the slowest-running fraction contained a small proportion of a compound which with acid yielded a phenol which in its R_F and colour reactions resembled 1-hydroxybenzanthracene. No 2-hydroxybenzanthracene was detected although this phenol would be expected as a product of the acid-decomposition of 1,2-dihydro-1,2-dihydroxybenzanthracene. Attempts to confirm the presence of the dihydrodihydroxy compound in other samples of urine were unsuccessful.

Metabolites in faeces. The faeces were covered with a layer of methanol and kept for some days at room temperature, the mixture being stirred from time to time. The solid material was filtered off and discarded, and the filtrate was evaporated to dryness under reduced pressure and the gummy residue heated under reflux with ether (250 ml.) for 30 min. The ether layer was separated and evaporated and the residue examined on thin-layer chromatograms. Compounds indistinguishable from 3- and 4-hydroxybenzanthracene and 8,9-dihydro-8,9-dihydroxybenzanthracene were detected. The ether-insoluble residue was hydrolysed with acid and the products were examined on thin-layer chromatograms. 4-, 8- and 9-Hydroxybenzanthracene and benzanthracene-7,12-quinone were formed. In neither of these experiments could 2-hydroxybenzanthracene be detected.

Metabolites in bile. Two rats in which biliary fistulae had been established (Boyland, Ramsay & Sims, 1961) were given 2 ml. of a suspension of benzanthracene (5%, w/v) in arachis oil on 2 successive days and the bile was collected to the end of the third day. The bile was examined by the methods described by Boyland *et al.* (1961) and four acid-labile compounds were detected. From the results of acid hydrolyses, these were identified as *N*-acetyl-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine and the corresponding cysteine, cysteinylglycine and glutathione conjugates. The second glycine conjugate described above was not detected as a metabolite in bile. All the conjugates yielded benzanthracene and the oxidized forms of the peptide or amino acid side chains with HCl and all had u.v.-absorption spectra similar to the mercapturic acid found in the urine of treated animals (see Table 2). The properties of the above conjugates on paper chromatograms are recorded in Table 3. When a little of the bile was hydrolysed with β-glucuronidase and the products were examined on thin-layer chromatograms, 3- and 4-hydroxybenzanthracene and 8,9-dihydro-8,9-dihydroxybenzanthracene were found but 2-hydroxybenzanthracene was not detected.

Metabolism of benzanthracene in rabbits and mice

Two rabbits were each given 10 ml. of a suspension of benzanthracene (5%, w/v) in arachis oil by intraperitoneal injection every other day for 8 days and the urines collected up to the end of the tenth day. Eighteen mice were each given one injection of 0·5 ml. of a suspension of 10% (w/v) of benzanthracene, in arachis oil. The urine and faeces were collected for 3 days after the injections. The metabolites from the urine were adsorbed on to charcoal and eluted with methanol–aq. NH_3 as described for the rat metabolites. The rabbit metabolites were separated into sulphuric acid, mercapturic acid and glucosiduronic acid fractions on a cellulose-powder column as described above and the mouse metabolites into similar fractions by chromatography on sheets of Whatman no. 3MM chromatography paper with butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.). The chromatograms were developed for 18 hr. and the metabolites eluted with methanol from the strips of paper cut from the appropriate parts of the chromatograms.

The fractions were examined in the manner described above to give results essentially the same as those previously obtained. Both sulphuric ester fractions yielded, after hydrolysis with sulphatase, compounds indistinguishable from 3- and 4-hydroxybenzanthracene, 5,6-dihydro-5,6-dihydroxybenzanthracene and 8,9-dihydro-8,9-dihydroxybenzanthracene. Benzanthracene-7,12-quinone was

also present in these hydrolysates. Both mercapturic acid fractions contained compounds indistinguishable on paper chromatograms, in their u.v.-absorption spectra and in their chemical properties from *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine. The glucosiduronic acid fractions both contained conjugates which, after hydrolysis with β-glucuronidase, yielded compounds indistinguishable from 3- and 4- (and probably 8- and 9-) hydroxybenzanthracene, 3,4-dihydro-3,4-dihydroxybenzanthracene, 5,6-dihydro-5,6-dihydroxybenzanthracene, 8,9-dihydro-8,9-dihydroxybenzanthracene and 10,11-dihydro-10,11-dihydroxybenzanthracene. Benzanthracene-7,12-quinone and a compound yielding benzanthracene with acid were also present in the hydrolysates.

The faeces of mice were examined in the same way as those from rats. The results showed the presence of all the phenolic and dihydrodihydroxy compounds found in the urine. 2-Hydroxybenzanthracene was not detected.

Metabolism of 7,12-dihydro-7,12-dihydroxybenzanthracene and benzanthracene-7,12-quinone in rats

Two rats were each given 2 ml. of a solution (5%, w/v) of 7,12-dihydro-7,12-dihydroxybenzanthracene in arachis oil by intraperitoneal injection. The urine was collected for 3 days, filtered, acidified to pH 4 with acetic acid and treated with charcoal (20 g.). The charcoal was filtered off, washed with water (500 ml.) and the adsorbed material eluted with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). The solvent was evaporated under reduced pressure and the gum applied to the base lines of six sheets of Whatman no. 3MM chromatography paper. The chromatograms were developed with butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.) for 18 hr. The areas of the chromatograms in which the sulphuric esters, the mercapturic acids and the glucosiduronic acids respectively might be expected to lie were cut out and any adsorbed material was eluted from the paper with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88) and the solvent evaporated.

The sulphuric ester fraction was treated with sulphatase: the products contained a small amount of a compound indistinguishable on thin-layer chromatograms from benzanthracene-7,12-quinone. The second fraction was examined on paper chromatograms, but no mercapturic acids could be detected. The fraction expected to contain glucosiduronic acids was treated with β-glucuronidase and the products were examined on thin-layer chromatograms. Three phenols were detected, one being indistinguishable from 7-hydroxybenzanthracene. The others were indistinguishable in their properties on the chromatograms and in their fluorescence spectra from 4-hydroxybenzanthracene and the phenol which is probably 3-hydroxybenzanthracene. Two dihydrodihydroxy compounds were detected, one of which was indistinguishable from 7,12-dihydro-7,12-dihydroxybenzanthracene and the second from the product formed in benzanthracene metabolism which is probably 3,4-dihydro-3,4-dihydroxybenzanthracene. The latter compound yielded phenols with acid indistinguishable in their chromatographic properties and fluorescence spectra from the 3- and 4-hydroxybenzanthracene described above. Benzanthracene-7,12-quinone was also present in the hydrolysate.

Two rats were each given 2 ml. of a suspension (5%, w/v) of benzanthracene-7,12-quinone in arachis oil by intraperitoneal injection and the urine was collected for 3 days and treated in the manner described above. Apart from small amounts of compounds in the sulphuric ester and glucosiduronic acid fractions which yielded benzanthracene-7,12-quinone, after treatment with sulphatase and β-glucuronidase respectively, no metabolites were identified.

Table 3. *Paper chromatography of the mercapturic acids and related compounds derived from benzanthracene*

Chromatograms were developed with butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.) as described in the text.

Compound	R_F	Fluorescence	Reaction with the platinic iodide reagent	Colour with ninhydrin
N-Acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine*	0·41	Dark violet	+ +	None
Metabolite, probably a glycine conjugate of the mercapturic acid†	0·41	—	-	None
Metabolite in bile, probably *S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine	0·32	Dark violet	+ +	Purple
Metabolite in bile, probably *S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteinylglycine	0·26	Dark violet	+ +	Purple–brown
Metabolite in bile, probably *S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)glutathione	0·12	Dark violet	+ +	Purple
Acid-decomposition product of the mercapturic acid, probably 5-benzanthracenyl-mercapturic acid	0·53	Bright violet	+	None

* The mercapturic acids derived from the urine of benzanthracene-treated rats, rabbits and mice and from the bile of treated rats and the synthetic acid all had the same properties on paper chromatograms.

† This product was only detected as a benzanthracene metabolite in rat urine and was separated from the mercapturic acid by methylation and thin-layer chromatography as described in the text.

DISCUSSION

In the oxidation of benzanthracene with perbenzoic acid there appear to be two main sites of attack, at the 7,12- and the 5,6-bonds (see Fig. 1). The major product of the oxidation is benzanthracene-7,12-quinone, an oxidation product previously isolated by Roitt & Waters (1949). A second product also involving oxidation at the 7,12-position is 12-hydroxybenzanthracen-7-one (or its isomer), which is presumably an intermediate stage in the formation of the quinone. Of the four compounds present in the oxidation mixture which yield 7-hydroxybenzanthracene with acid, one is undoubtedly 7,12-dihydro-7,12-dihydroxybenzanthracene and could have arisen through the intermediate formation of 7,12-epoxy-7,12-dihydrobenzanthracene. The other products were not identified, but one is clearly related in structure to the dihydrodihydroxy compound and could be the monobenzoate of the dihydrodihydroxy compound arising from the reaction of the epoxy compound with benzoic acid. It is not certain if the quinone and the hydroxybenzanthracenone arise by further oxidation of the epoxide (or of compounds derived from it) since, in the oxidations involving the 5,6-bond of benzanthracene, no benzanthracene-5,6-quinone could be detected. It seems likely that 5,6-epoxy-5,6-dihydrobenzanthracene is the intermediate formed by oxidation at the 5,6-bond since *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine and *trans*-5,6-dihydro-5,6-dihydroxybenzanthracene have been obtained, which could have arisen from the reaction of the epoxy compound with *N*-acetylcysteine and water respectively. In its reaction with perbenzoic acid on the so-called 'K region' to form reactive intermediates, benzanthracene resembles pyrene (Boyland & Sims, 1964) and phenanthrene (Boyland & Sims, 1961). In the oxidation of benzanthracene the intermediate formed on the 5,6-bond appears to be relatively more stable than that across the 7,12-positions since mercapturic acids derived from the latter epoxide were not detected. The epoxide formed about the 5,6-bond is itself quite labile, and a mercapturic acid was isolated in only about half of the oxidations carried out, although the dihydrodihydroxy compound was always detected. The three unidentified products of the oxidation yielding 5-hydroxybenzanthracene with acid are clearly related to the dihydrodihydroxy compound and one could be the monobenzoate of 5,6-dihydro-5,6-dihydroxybenzanthracene, arising from the reaction of the epoxide with benzoic acid. No phenolic products were detected in the oxidation: in this respect the benzanthracene oxidation

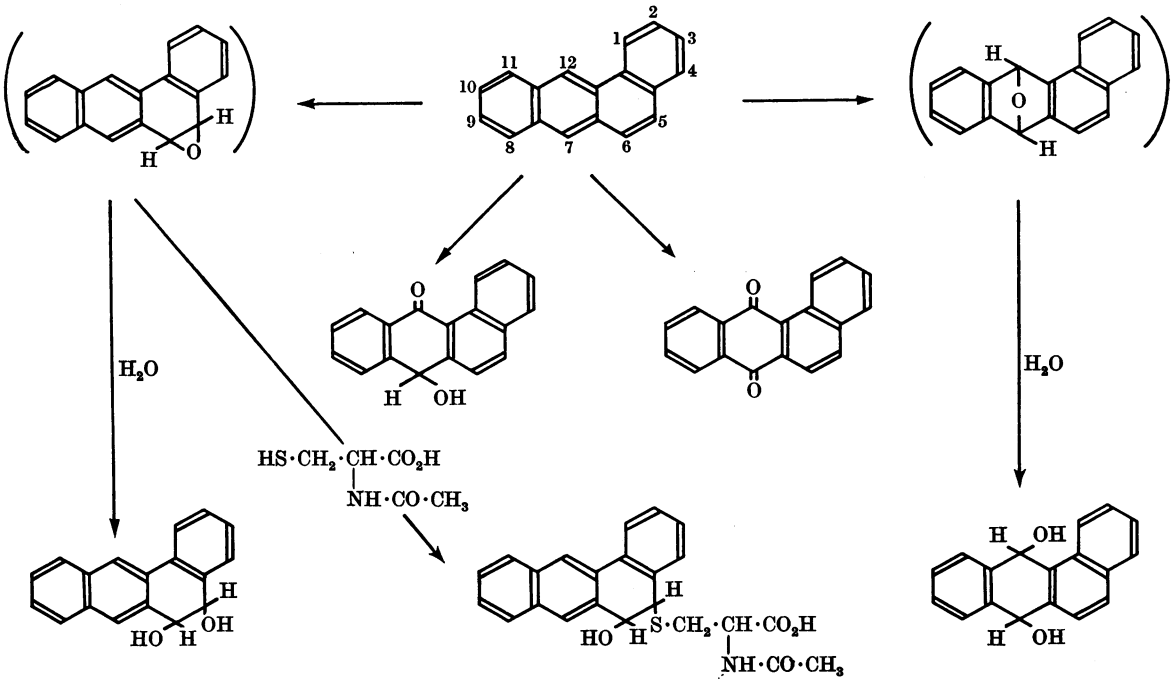

Fig. 1. Oxidation of benz[*a*]anthracene with perbenzoic acid. Formulae in parentheses are those of possible intermediates in the oxidation.

differs from those of pyrene, where 1-hydroxypyrene is formed (Boyland & Sims, 1964), and phenanthrene, where all the possible hydroxyphenanthrenes are formed with 9-hydroxyphenanthrene as the major phenolic product (E. Boyland & P. Sims, unpublished observations).

Perbenzoic acid, which is an electrophilic reagent, should react with molecules at the regions of highest electron density; in benzanthracene, theoretical considerations show these to be the 7- and 12-positions and the 5,6-bond (see e.g., Pullman & Pullman, 1952; Badger, 1954). In other oxidations not previously mentioned, benzanthracene forms benzanthracene-7,12-quinone with ozone (Moriconi, O'Connor & Wallenberger, 1959), hydrogen peroxide (Arnold & Larsen, 1940) and chromic acid (Graebe, 1905). Oxidation with the Milas reagent (Milas & Sussman, 1936) gives a mixture of benzanthracene-7,12- and benzanthracene-5,6-quinone (Cook & Schoental, 1950) and benzoyl peroxide yields 7-benzoyloxybenzanthracene (Roitt & Waters, 1952). Copeland, Dean & McNeil (1961) found 3-*o*-carboxyphenyl-2-naphthoic acid as a product of the ozonolysis as well as the 7,12-quinone.

The comparative metabolism of benzanthracene in rats, rabbits and mice showed no qualitative differences (see Fig. 2). The major metabolite of benzanthracene in the urine of animals of the three species examined is the mercapturic acid, *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine, forming about 1·25% of the dose of the hydrocarbon administered to rats. The related 5,6-dihydro-5,6-dihydroxybenzanthracene appears as a minor metabolite (in rats the amount present in the urine was too small to be isolated) and in this respect the metabolism of the hydrocarbon differs from that of phenanthrene, where the major metabolite involving the 'K region' is 9,10-dihydro-9,10-dihydroxyphenanthrene (Boyland & Sims, 1962*b*). Neither benzanthracene nor phenanthrene nor pyrene (Boyland & Sims, 1964) yielded phenols formed by hydroxylation on the 'K region' as metabolites in the urine. Of the metabolic reactions about the other bonds of benzanthracene, phenols and dihydrodihydroxy compounds formed about the 3,4-, the 8,9- and the 10,11-bonds were found which were excreted in conjugation with sulphuric acid or glucuronic acid but little or no metabolic reaction about the 1,2-bond was detected. In some of the experiments described above, where 8,9-dihydro-8,9-dihydroxybenzanthracene and 8- and 9-hydroxybenzanthracene were found, the presence of the corresponding 10,11-isomers is suspected although they were not specifically sought for because of separation difficulties.

The compound yielding benzanthracene-7,12-quinone, after being released from conjugation

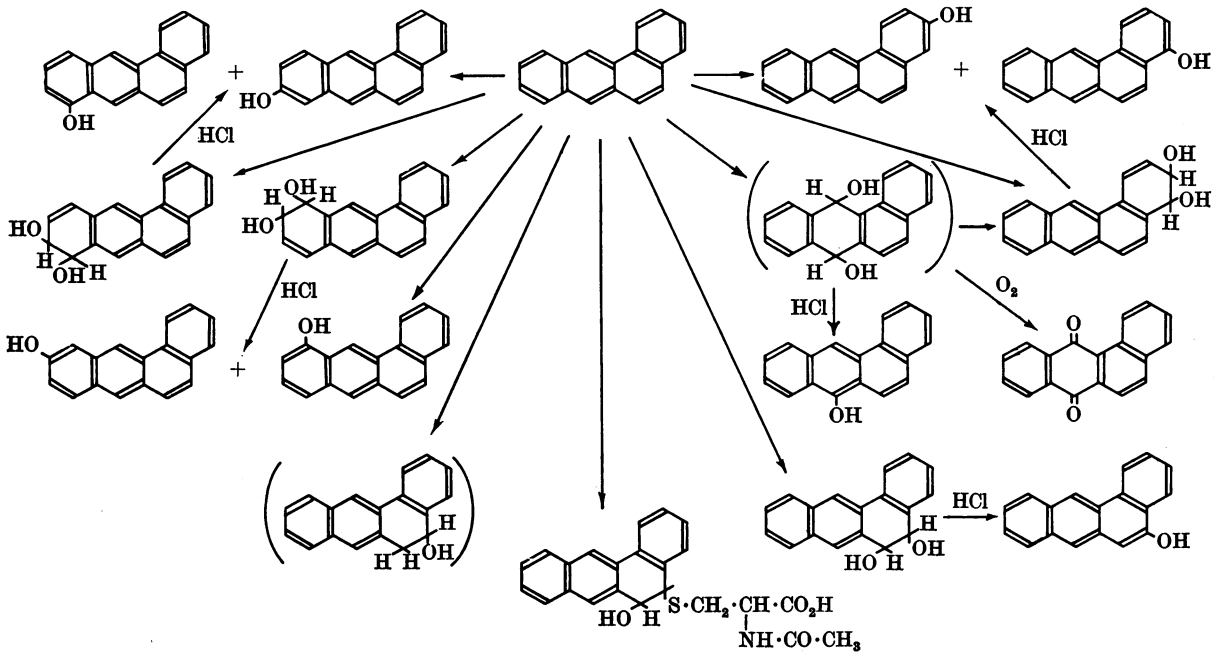

Fig. 2. Probable pathways in benz[*a*]anthracene metabolism. Formulae in parentheses are those of compounds whose presence as metabolites is uncertain.

either enzymically or with acid, could not be identified. The chemical oxidation of benzanthracene suggests that epoxidation across the 7,12-positions is possible, and the corresponding epoxide of 7,12-dimethylbenz[*a*]anthracene has been described (Badger, Goulden & Warren, 1941). It is possible therefore that the metabolite is either 7,12-dihydro-7,12-dihydroxybenzanthracene or 7,12-dihydroxybenzanthracene, which could have arisen in the body by the dehydrogenation of the dihydrodihydroxy compound. 7-Hydroxybenzanthracene, which could have arisen in the body either by direct hydroxylation or from the dihydrodihydroxy compound by loss of water, could also be a metabolite. In control experiments, however, in which 7-hydroxybenzanthracene and 7,12-dihydro-7,12-dihydroxybenzanthracene were incubated with sulphatase and with β-glucuronidase, the phenol was recovered mainly unchanged whereas the dihydrodihydroxy compound was largely oxidized to the corresponding quinone. The dihydroxy compound was even more unstable and attempts to prepare it yielded only the quinone. It is also possible that, during the hydrolyses of the conjugates of the unknown metabolite, the metabolite is converted in part into the dimeric compound described above. This compound is difficult to detect on thin-layer chromatograms.

In the metabolism of 7,12-dihydro-7,12-dihydroxybenzanthracene in rats, only small amounts of the dihydrodihydroxy compound and 7-hydroxybenzanthracene were detected after the enzymic hydrolyses of the sulphuric ester and glucosiduronic acid fractions, the main product of the hydrolyses being benzanthracene-7,12-quinone. The formation of 3- and 4-hydroxybenzanthracene and 3,4-dihydro-3,4-dihydroxybenzanthracene in these experiments is difficult to explain and a further investigation of the reaction is required. Presumably hydroxylation of the 3,4-bond of the 7,12-dihydro-7,12-dihydroxy compound occurs before dehydroxylation at the 7- and 12-positions, otherwise other benzanthracene metabolites would be expected. In related compounds such as benzanthracene-7,12-quinone, the 1-, 2-, 3- and 4-positions are the most reactive chemically, since, for example, sulphonation of the quinone yields the 2- and 4-sulphonic acids (Ioffe & Fedorova, 1941) and ozone yields anthraquinone-1,2-dicarboxylic acid (Moriconi *et al.* 1959). The quinone is not, however, involved in the metabolism of the dihydrodihydroxy compound, since rats treated with the quinone do not excrete any of the above metabolites. Dehydroxylation reactions *in vivo* are known, but it is likely that, normally, intestinal micro-organisms are involved (see, e.g., Booth & Williams, 1963). In the present work, however, 7,12-dihydro-7,12-dihydroxybenzanthracene, on incubation with the rat-liver microsomal hydroxylating system, was converted mainly by a non-enzymic oxidation into benzanthracene-7,12-quinone, the hydroxybenzanthracenone and 7-hydroxybenzanthracene. A small amount of a fourth product was also formed, probably by an enzymic oxidation, which had the R_F on thin-layer chromatograms of 3,4-dihydro-3,4-dihydroxybenzanthracene and which, with acid, yielded phenols closely resembling 3- and 4-hydroxybenzanthracene (E. Boyland, M. Kimura & P. Sims, unpublished work). If 7,12-dihydro-7,12-dihydroxybenzanthracene is formed during benzanthracene metabolism, then it is possible that metabolites hydroxylated in the 3- and 4-positions arise from this dihydrodihydroxy compound and not by direct oxidation of the benzanthracene nucleus. It must be assumed, of course, that in all experiments with the synthetic dihydrodihydroxy compound, the compound has the same configuration about the 7- and 12-positions as the possible metabolite, or that both the *cis*- and the *trans*-compounds undergo the same reactions. The dihydrodihydroxy compound formed in the oxidations which had the same R_F as 7,12-dihydro-7,12-dihydroxybenzanthracene, might be expected to have the *trans* configuration if it arose via an epoxide.

The mercapturic acid appears to arise in the body by the same mechanism as that postulated for the formation of the corresponding mercapturic acid derived from phenanthrene (Boyland & Sims, 1962*a*), that is, by the enzyme-catalysed reaction between an epoxide and glutathione and the subsequent conversion of the glutathione conjugate into the mercapturic acid. The isolation of an optically active methyl ester of the mercapturic acid provides further evidence for the enzymic nature of the original conjugating reaction. The breakdown of the urinary mercapturic acid with acid to yield benzanthracene accounts for most of the benzanthracene liberated when the urine of treated animals is acidified.

The unidentified compound which also yields benzanthracene with acid is apparently a hydroxy compound since it appears in the urine as a glucuronic acid conjugate. It is presumably related to the type of compound previously described in naphthalene metabolism (Boyland & Solomon, 1955) and could be a 5,6-dihydrohydroxybenzanthracene. It appears to be only a minor product in the metabolic pathway of benzanthracene in animals of all the species studied.

It appears therefore that the proportions of the metabolic products of benzanthracene, as judged by the sizes and intensities of the spots on paper and thin-layer chromatograms and by the amount of mercapturic acid isolated, are related to the bond orders of the various bonds in the molecule. The

5,6-bond is the most active biologically and has the highest bond order (see, e.g., Pullman & Pullman, 1952; Badger, 1954). The 8,9- and 10,11-bonds appear to be next in order of activity and in bond order. The bond orders of the 1,2- and 3,4-bond are nearly similar and are less than those of the 8,9- and 10,11-bonds, but the 3,4-bond appears to be active and the 1,2-bond almost inactive biologically. The alternative mechanism of formation of the 3,4-hydroxylated benzanthracene derivatives which is postulated above could account for this. It should be pointed out that only small amounts of the phenols and dihydrodihydroxy compounds as compared with the amount of mercapturic acid are formed in the metabolism of benzanthracene, in contrast with the relatively large amounts of the former compounds in the metabolism of phenanthrene (Boyland & Sims, 1962*a*, *b*). It is difficult to form an estimate of the metabolic reaction about the 7,12-position in benzanthracene, but the amount of benzanthracene-7,12-quinone, presumably arising from the oxidation of 7- and 12-hydroxylated derivatives, was small as compared with the mercapturic acid formed on the 5,6-bond. The 7- and 12-positions in benzanthracene are the most reactive chemically, so that it might be expected that in the body 7- and 12-hydroxylated derivatives would be the most abundant metabolic products if free hydroxyl radicals or cationoid hydroxyl groups are involved in the metabolic hydroxylation. Since this is not so, the enzymic formation of an intermediate epoxy derivative across these positions seems more likely. The enzymic formation of epoxy derivatives about the other bonds in the benzanthracene molecule could explain the observed pattern of metabolites.

Although the major product of the oxidation of benzanthracene with perbenzoic acid is benzanthracene-7,12-quinone, in many other respects the oxidation provides a chemical model of the reactions undergone by the hydrocarbon in the body.

SUMMARY

1. The oxidation of benz[*a*]anthracene with perbenzoic acid yielded benz[*a*]anthracene-7,12-quinone, 12-hydroxybenz[*a*]anthracen-7-one, a dimeric benz[*a*]anthracen-7-one, four compounds which yielded 7-hydroxybenzanthracene and four compounds which yielded 5-hydroxybenzanthracene with acid. One of the latter group of compounds was 5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene, which was also synthesized from benz[*a*]anthracene-5,6-quinone. When the products from the oxidation were caused to react with *N*-acetyl-L-cysteine, *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benz[*a*]anthracenyl)-L-cysteine was obtained.

2. In the metabolism of benzanthracene in rats, rabbits and mice, the above mercapturic acid was the major product. 3-, 4-, 8- and 9-Hydroxybenz[*a*]anthracene and 3,4-dihydro-3,4-dihydroxy-, 5,6-dihydro-5,6-dihydroxy-, 8,9-dihydro-8,9-dihydroxy- and 10,11-dihydro-10,11-dihydroxy-benz[*a*]anthracene, all as sulphuric acid and glucuronic acid conjugates, were also identified as metabolites in the urine and, in some cases, in bile and faeces. 1- and 2-Hydroxybenz[*a*]anthracene were not detected.

3. An unidentified hydroxy compound which yielded benzanthracene-7,12-quinone after enzymic hydrolyses of its conjugates was also a metabolite of benzanthracene. It is possibly 7,12-dihydro-7,12-dihydroxybenz[*a*]anthracene, which was synthesized from benzanthracene-7,12-quinone.

4. In the metabolism of 7,12-dihydro-7,12-dihydroxybenzanthracene, the compound was partly excreted as sulphuric acid and glucuronic acid conjugates, partly converted into conjugates of 7-hydroxybenz[*a*]anthracene and partly converted into conjugates of 3- and 4-hydroxybenz[*a*]anthracene and 3,4-dihydro-3,4-dihydroxybenzanthracene. Benzanthracene-7,12-quinone was not converted into these compounds.

5. The bile of rats treated with benzanthracene contained *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)cysteine and the corresponding cysteine, cysteinylglycine and glutathione conjugates. The urine of rats also contained a second glycine conjugate related to the mercapturic acid. All these conjugates yielded benzanthracene with mineral acid.

6. Another metabolite, present in the urine of treated animals as a glucuronic acid conjugate, which also yielded benzanthracene with acid was not identified.

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A Radiometric Study of Cholinesterase and its Inhibition

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Lipophilic inhibitors of acetylcholinesterase are widely used for insect control. They include organophosphorus compounds, which phosphorylate the esteratic site of the enzyme in a relatively irreversible manner (O'Brien, 1960), and the carbamates, which may form a reversible complex with the enzyme or a compound as a result of carbamoylation (O'Brien & Matthysse, 1961). The estimation of cholinesterase inhibition by these compounds *in vivo* is fundamental to studies of their mode of action (Casida, 1963; Fukuto, 1961). Its measurement in human blood as an index of accidental or occupational exposure is also of considerable importance (Aldridge & Davies, 1952; World Health Organization, 1962; Witter, 1963). The determination of cholinesterase activity usually involves manifold dilution of the tissue sample in buffer followed by the addition of acetylcholine or equivalent substrate to give a suitable initial concentration of about 0·01 M (Augustinsson, 1957). Organophosphorus compounds are usually classified as 'irreversible' inhibitors as a result of phosphorylation of the enzyme itself and there is little or no reversal of inhibition on dialysis against inhibitor-free solution. Carbamates such as eserine are usually classified as 'reversible' (Hearon, Bernhard, Friess, Botts & Morales, 1959). Measurement of cholinesterase inhibition by the former would not be expected to be affected by sample dilution or substrate addition. Dilution of the 'reversibly' inhibited enzyme and addition of substrate might, however, considerably reduce the inhibition it was sought to determine. Thus Aldridge (1950) demonstrated significant reversibility on dilution of erythrocyte cholinesterase inhibited with eserine but not of cholinesterase inhibited by DFP.* These situations have a well-known kinetic basis (e.g. Hearon *et al.* 1959). Wilson and his colleagues (Wilson, Hatch & Ginsburg, 1960; Wilson, Harrison & Ginsburg, 1961) have demonstrated the carbamoylation of acetylcholinesterase prepared from the electric eel by certain *N*-methyl- and *NN*-dimethyl-carbamic esters so that carbamate inhibition might resemble that by organophosphorus compounds. On the other hand, Casida, Augustinsson & Jonsson (1960) concluded that 'if the carbamates do indeed form a carbamoyl-

* Abbreviations: DFP, di-isopropyl phosphorofluoridate; IPMC, *o*-isopropoxyphenyl-*N*-methyl carbamate.