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The Effect of Pretreatment with Adrenal-Protecting Compounds on the Metabolism of 7,12-Dimethylbenz[*a*]anthracene and Related Compounds by Rat-Liver Homogenates

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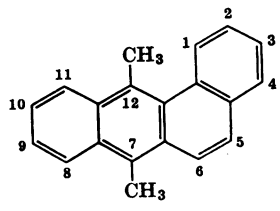
1. 7,12-Dimethylbenz[*a*]anthracene is converted by rat-liver homogenates into products with the properties of the 7- and 12-hydroxymethyl derivatives, the 7,12-dihydroxymethyl derivative, the related carboxylic acids and ring-hydroxylated products such as the 8,9-dihydro-8,9-dihydroxy derivative and phenols. Ring-hydroxylated products and products arising from the further oxidation of the hydroxymethyl groups were formed when the hydroxymethyl derivatives were themselves incubated with rat-liver homogenates. 2. Pretreatment of the animal with 3-methylcholanthrene or with Sudan III, which can protect rat adrenal glands from damage by 7,12-dimethylbenz[*a*]anthracene or by its 7-hydroxymethyl derivative, led to an increased rate of metabolism of 7,12-dimethylbenz[*a*]anthracene and its hydroxymethyl derivatives. The metabolic routes mainly affected were those involving the formation of ring-hydroxylated products. 3. Pretreatment with phenobarbitone led to a small increase in the rate of metabolism of the hydrocarbon and of its hydroxymethyl derivatives, but the increase appeared mainly to involve increased metabolism of the methyl and hydroxymethyl groups. 4. Pretreatment with metyrapone increased the rate of metabolism of the hydrocarbon mainly by increasing the amounts of products resulting from hydroxylation of the methyl groups: small increases in the amounts of ring-hydroxylated products were also produced. 8. Of a number of hydrocarbons and of derivatives of 3-methylcholanthrene tested as enzyme inducers, 3-methylcholanthrene itself was the most effective.

It was shown by Huggins & Morii (1961) that the treatment of rats with 7,12-dimethylbenz[*a*]anthracene (I) led to the destruction of two zones of the adrenal cortex and the induction of adrenal apoplexy. This effect could be inhibited by the administration before the hydrocarbon of any one of a number of compounds that are known to stimulate rat-liver microsomal enzymes (Huggins, Deuel & Fukunishi, 1963; Dao & Tanaka, 1963). Four such compounds are 3-methylcholanthrene, Sudan III [1-(*p*-phenylazophenylazo)-2-naphthol], phenobarbitone (Huggins & Fukunishi, 1964; Huggins & Pataki, 1965) and metyrapone [2-methyl-1,2-bis-(3-pyridyl)propan-1-one] (Currie, Helfenstein & Young, 1962). It was shown by Boyland, Sims & Huggins (1965) that 7-hydroxymethyl-12-methylbenz[*a*]anthracene, which is formed together with the isomeric 12-hydroxymethyl-7-methylbenz[*a*]anthracene from the hydrocarbon in rat-liver homogenates (Boyland & Sims, 1965*a*), is more effective than the hydrocarbon in inducing adrenal apoplexy in rats. The effect of the 7-hydroxymethyl derivative is also inhibited if the animals are pretreated with 3-methylcholanthrene (Professor C. Huggins, personal communication). Rats with impaired liver function are protected against adrenal necrosis caused by 7,12-dimethylbenz[*a*]anthracene (Wheatley, Kernohan & Currie, 1966*a*), whereas no protection is afforded against the action of the 7-hydroxymethyl derivative (Wheatley, Hamilton, Currie, Boyland & Sims, 1966*b*). The action of the 7-hydroxymethyl derivative is, however, inhibited by pretreatment with metyrapone.



(I)

These results suggested that the 7-hydroxymethyl

derivative rather than the parent hydrocarbon was responsible for adrenal necrosis and that the effect of the pretreatments is to alter the metabolism of the hydrocarbon in the body so that the hydroxymethyl derivative either is not formed or is formed in decreased amounts or that it is further metabolized to inactive products. The metabolism of 7,12-dimethylbenz[*a*]anthracene and its hydroxymethyl derivatives in liver homogenates from normal rats and from rats that were pretreated with compounds that inhibit the formation of adrenal necrosis has therefore been examined. Conney & Levin (1966) found that the pretreatment of animals with any one of a number of compounds that are known to induce microsomal enzymes caused increases in the amounts of unidentified metabolites of 7,12-dimethylbenz[*a*]anthracene formed from the hydrocarbon by rat-liver homogenates, and Jellinck & Goudy (1966) found in experiments with 7,12-dimethyl[12-^{14}C]benz[*a*]anthracene that the pretreatment of animals with 3-methylcholanthrene or with dibenz[*a*,*h*]anthracene caused a decrease in the amounts of the hydroxymethyl derivatives and an increase in the amounts of more polar products as compared with homogenates from untreated animals when the labelled hydrocarbon was incubated with rat-liver supernatant.

EXPERIMENTAL

Melting points. These are uncorrected.

Thin-layer chromatography. Thin-layer chromatograms were prepared by coating glass plates (20cm. × 20cm.) with layers of silica gel G (E. Merck A.-G., Darmstadt, Germany) of 0·25mm. thickness. The chromatograms were developed for either 10cm. or 15cm. with either (*a*) light petroleum (b.p. 80–100°)–benzene (19:1, v/v), (*b*) benzene, (*c*) benzene–ethanol (19:1, v/v) or (*d*) benzene–ethanol (9:1, v/v) as indicated below. Products were detected by examining the plates in u.v. light while they were still wet, both before and after exposure to NH_3.

The two-dimensional chromatograms referred to below were developed with solvent (*c*) for 10cm., sprayed with conc. HCl and heated in an oven to 100° for 10min. and developed in the second direction with solvent (*b*). In this way phenols arising from the decomposition of dihydrodihydroxy compounds were detected.

Spectra. The u.v. spectra were measured on a Unicam SP.800 recording spectrophotometer and fluorescence spectra on an Aminco–Bowman spectrophotofluorimeter. The activation and fluorescence maxima recorded below were instrument readings and reproducible to ±5mμ.

Measurements were made in ethanol: solutions of metabolites and other material separated on thin-layer chromatograms were obtained by removing bands or spots of silica gel from the chromatograms, suspending the silica gel in ethanol (3ml.) to elute the absorbed material and centrifuging the suspensions to remove silica gel.

Materials. Sudan III [1-(*p*-phenylazophenylazo)-2-naphthol] was obtained from British Drug Houses Ltd. (Poole, Dorset), metyrapone (Metopirone pure substance) from Ciba Laboratories (Horsham, Surrey) and 7,12-dimethylbenz[*a*]anthracene from Eastman Kodak Ltd. (Kirkby, Liverpool). 7-Hydroxymethyl-12-methylbenz[*a*]-anthracene and 12-hydroxymethyl-7-methylbenz[*a*]anthracene and their acetates were prepared as described by Boyland & Sims (1965*a*). 7,12-Dihydroxymethylbenz[*a*]-anthracene was prepared by the method of Badger & Cook (1940). For the u.v. spectra of the hydroxymethyl derivatives see Boyland & Sims (1965*a*): the fluorescence spectra of the hydrocarbon and its derivatives showed activation maxima at 295 and 360mμ and fluorescence maxima at 410 and 430mμ.

12-Methylbenz[*a*]anthracene-7-carboxaldehyde ($\lambda_{max.}$ at 221, 236, 257 293, 302 and 390mμ) was prepared by the method of Badger & Cook (1940). Reduction of the aldehyde with $LiAlH_4$ in ether yielded 7-hydroxymethyl-12-methylbenz[*a*]anthracene, m.p. and mixed m.p. 164°. The aldehyde was oxidized by $KMnO_4$ in acetone, by using the method of Badger & Cook (1940) for the oxidation of benz[*a*]anthracene-7-carboxaldehyde, to yield 12-*methylbenz*[a]*anthracene-7-carboxylic acid*, which separated from acetic acid in flat pale-yellow needles, m.p. 233° (decomp.) (Found: C, 83·5, H, 5·1. $C_{20}H_{14}O_2$ requires C, 83·9; H, 4·9%), $\lambda_{max.}$ at 221, 232, 262, 272, 281·5, 290·5, 340, 355, 360 and 394mμ (log ε 4·47, 4·30, 4·51, 4·53, 4·79, 4·87, 3·80, 3·88, 3·80 and 3·19 respectively). The *methyl ester*, prepared by the action of diazomethane in ether on the acid in methanol, was recrystallized from ethanol in pale-yellow needles, m.p. 152° (Found: C, 84·5, H, 5·7. $C_{21}H_{16}O_2$ requires C, 84·0; H, 5·4%), $\lambda_{max.}$ at 221, 232, 262, 272, 281, 292, 340, 355, 360 and 394mμ (log ε 4·45, 4·30, 4·51, 4·60, 4·83, 4·88, 3·83, 3·94, 3·83 and 3·18 respectively).

cis-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene was prepared from the hydrocarbon with OsO_4 as described by Cook & Schoental (1948). For the u.v. spectrum see Boyland & Sims (1965*a*). 7,12-Dimethylbenz[*a*]anthracene-5,6-quinone was a gift from Professor M. S. Newman. The quinone (100mg.) was reduced by heating under reflux for 4hr. with $LiAlH_4$ (100mg.) in ether (50ml.). The product, which was isolated in the usual manner (Booth, Boyland & Turner, 1950), formed a colourless gum. It was chromatographed on two thin-layer chromatograms for 15cm. in solvent (*d*), when two bands, both of which showed a violet fluorescence in u.v. light, were obtained. The bands were removed and the absorbed material was eluted with ether. The material from the faster-moving bands was recrystallized from benzene in needles (25mg.), m.p. 171°, undepressed in admixture with *cis*-5,6-dihydro-5, 6-dihydroxy-7,12-dimethylbenz[*a*]anthracene. The product and the authentic dihydrodihydroxy compound were indistinguishable on thin-layer chromatograms developed with solvent (*c*) or (*d*) and the u.v. spectra were identical. The material from the slower-moving band formed a colourless gum (80mg.) that could not be crystallized. It is presumed to consist mainly of *trans*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene. It yielded only one spot on thin-layer chromatograms developed with solvent (*c*) or (*d*), and on two-dimensional thin-layer chromatograms yielded a phenol (presumably 5-hydroxy-7,12-dimethylbenz[*a*]-anthracene) indistinguishable from that formed when the *cis*-dihydrodihydroxy compound was similarly chromatographed. The u.v. spectrum of the reduction product ($\lambda_{max.}$ at 220, 261, 269, 300 and 344mμ and inflexions at 251, 293 and 310mμ) was identical with that of the *cis*-isomer.

The product yielded a *diacetate* with acetic anhydride in pyridine that separated from ethanol in leaflets, m.p. 208–209° (Found: C, 76·8; H, 6·1. $C_{24}H_{22}O_4$ requires C, 77·0; H, 5·9%), $\lambda_{max.}$ at 220, 261, 268, 300 and 344 mμ (log ε 4·46, 4·63, 4·60, 4·10 and 3·25 respectively) and inflexions at 251, 293 and 310 mμ. The diacetate of the *cis*-isomer had m.p. 153–153·5° (Cook & Schoental, 1948).

When 7-acetoxymethyl-12-methylbenz[*a*]anthracene was oxidized with OsO_4, a product was obtained that appeared to be cis-7-*acetoxymethyl*-5,6-*dihydro*-5,6-*dihydroxy*-12-*methylbenz*[a]*anthracene*, which separated from ethanol in needles, m.p. 213° (Found: C, 75·8; H, 6·0. $C_{22}H_{20}O_4$ requires C, 75·8; H, 5·8%). It yielded an *acetate* with acetic anhydride in pyridine, separating from ethanol in prisms, m.p. 168° (Found: C, 72·4; H, 5·7. $C_{26}H_{24}O_6$ requires C, 72·2; H, 5·6%). Hydrolyses of the acetoxymethyl derivative by brief boiling with KOH in methanol yielded cis-5,6-*dihydro*-5,6-*dihydroxy*-7-*hydroxymethyl*-12-*methylbenz*[a]*anthracene*, separating from ethanol in needles, m.p. 217° (Found: C, 77·9; H, 5·7. $C_{20}H_{18}O_3$ requires C, 78·4; H, 5·9%), $\lambda_{max.}$ at 220, 260, 267, 300 and 344 mμ (log ε 4·47, 4·64, 4·67, 4·10 and 3·25 respectively) and inflexions at 250 and 310 mμ.

The 3-methylcholanthrene derivatives were prepared as described by Sims (1966).

Experiments with rat-liver homogenates. (*a*) Qualitative experiments. Young male rats of the Chester Beatty strain (body wt. approx. 180 g.), maintained on rat cubes (diet 8b; Plowco Feeds Ltd., South Godstone, Surrey), were used in these experiments. At 40 hr. before they were killed the rats were treated by intraperitoneal injection with either arachis oil (0·5 ml.), 3-methylcholanthrene (5 mg.) in arachis oil (0·5 ml.) or Sudan III (10 mg.) in arachis oil (0·5 ml.). Phenobarbitone (12 mg.) in arachis oil (0·5 ml.) was administered in two doses at 40 hr. and 20 hr. and metyrapone (10 mg.) in arachis oil (6·5 ml.) at 24 hr., 20 hr., 16 hr. and 4 hr. before the animals were killed.

Each experiment was carried out with the pooled livers from eight rats: by using this number of animals it was possible to purify adequately most of the metabolites before their spectra were measured. The livers were homogenized, the homogenates centrifuged, cofactors were added to the supernatants and the incubations were carried out as described by Sims (1966), except that nicotinamide was omitted from the cofactors: it appeared to have little effect on the nature of the metabolic products and its presence interfered with their separation on thin-layer chromatograms.

The substrates (10 mg.) in ethanol (5 ml.) were added to the homogenates and, after 30 min., the reactions were stopped by the addition of 2 vol. of acetone. The mixtures were each extracted twice with ethyl acetate (500 ml.) and the combined extracts were washed with water (500 ml.), dried over Na_2SO_4 and evaporated under reduced pressure, and the residue from each incubation was applied to the base lines of four thin-layer chromatograms. The chromatograms were developed for 15 cm. in solvent (*b*) and examined in u.v. light. Each chromatogram was divided into a number of zones according to the presence of fluorescent materials. The divisions of the chromatograms depended on the substrate under investigation, but usually five zones were marked off, one towards the solvent front containing fast-moving non-polar material, three intermediate zones containing hydroxymethyl derivatives and phenols, and a zone consisting of material left at the base line that contained polyhydroxy compounds and carboxylic acids. The zones were removed from the chromatograms, the corresponding zones from each set of chromatograms being combined, and the absorbed material was eluted from the silica gel with ether (100 ml.). The solutions were evaporated and the residues rechromatographed for 15 cm. on single chromatograms. Usually the materials from the fastest-moving bands were chromatographed in solvent (*a*), the intermediate bands in solvent (*b*) and the material from the base lines in solvent (*d*). The chromatograms were examined in u.v. light, and the u.v. spectra and in some cases the fluorescence spectra of the metabolites thus detected were measured as described above. The solutions were then evaporated and the residues either compared directly on thin-layer chromatograms with the appropriate synthetic compound or further examined as described below. No attempt was made at an exact determination of the amounts of the various metabolites formed in each incubation in these experiments. However, since the incubations were all carried out under standard conditions it was usually possible to compare, by a comparison of the heights of some of the peaks of the respective u.v. spectra, the amount of one particular metabolite formed by liver homogenate from normal rats with the amount of the same metabolite formed by the liver homogenate from treated animals.

Control experiments were carried out in which the homogenates were boiled before the addition of the cofactors and the substrates. Most of the metabolites described below were not detected under these conditions, but 7,12-dimethylbenz[*a*]anthracene sometimes yielded small amounts of the isomeric hydroxymethyl derivatives and the hydroxymethyl derivatives yielded small amounts of 7,12-dihydroxymethylbenz[*a*]anthracene. The amounts of these products formed were, however, much less than those formed when fresh homogenates were used.

All incubations yielded fast-moving fluorescent material which was also present in homogenates that were incubated without substrate. With 7,12-dimethylbenz[*a*]anthracene itself, the unmetabolized hydrocarbon was also present in this fluorescent material and both the monohydroxymethyl derivatives sometimes yielded unidentified products that moved to this region of the chromatograms.

(*b*) Quantitative experiments. In each experiment the livers of two rats, which had been pretreated with one of the compounds listed in Table 2 or 3, were pooled and homogenized, and the homogenate was centrifuged as before. The supernatant was divided into four equal portions, two of which were heated to 37°. The substrate (250 μg.) in ethanol (0·5 ml.) was added and the mixtures were immediately diluted with 2 vol. of acetone. Nicotinamide (0·22 g.) and the sodium salts of glucose 6-phosphate (31 mg.) and $NADP^+$ (4 mg.) were added to each of the other portions and the mixtures were heated to 37°. The substrate was added as before and the mixtures were incubated for 30 min. and diluted with 2 vol. of acetone. The four portions were extracted first with 150 ml. and then with 50 ml. of ethyl acetate, and the combined extracts were washed with water (150 ml.) and dried over Na_2SO_4, and the solvent was evaporated. Each residue was applied to the base line of a thin-layer chromatogram that was developed for 15 cm. with solvent (*b*). The regions of the chromatograms containing the hydrocarbon and the monohydroxymethyl derivatives were located by their fluorescence in u.v. light. The bands were removed and the absorbed material

was eluted from the silica gel by shaking with ethanol (10ml.). The silica gel was removed by centrifugation and the eluted material was estimated by the fluorescence at 410mμ when the activating wavelength was 360mμ. There was a straight-line relationship between concentration and intensity for concentrations up to 3μg./ml. so that where necessary the solutions were diluted. Recoveries of the substrates from the unincubated mixtures were about 70% of the amounts added; higher recoveries were possible if the mixtures were made strongly alkaline as described by Conney, Miller & Miller (1957) in the estimation of benzo[*a*]-pyrene hydroxylase, but under these conditions extensive decomposition of the hydroxymethyl derivatives occurred. The substrates metabolized were calculated from the differences between amounts extracted from the unincubated and from the incubated mixtures. As far as possible all the operations were carried out in the dark or in light-shielded vessels to avoid photodecomposition.

RESULTS

Identification of metabolites. The chromatographic properties of the metabolites are recorded in Table 1. The criteria used in the identification of the various metabolic products are described below.

7-Hydroxymethyl-12-methylbenz[*a*]anthracene, 12-hydroxymethyl-7-methylbenz[*a*]anthracene and 7,12-dihydroxymethylbenz[*a*]anthracene were identified by means of their absorption and fluorescence spectra and by direct comparison with the synthetic compounds on thin-layer chromatograms.

Two phenolic products previously recognized as metabolites of 7,12-dimethylbenz[*a*]anthracene (Boyland & Sims, 1965*a*) could not be further characterized because the small amounts present did not allow the spectra to be measured. Neither of the phenols was chromatographically identical with that obtained by the acid treatment of 5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]-anthracene, but the close resemblance between the chromatographic properties of these phenols and the benz[*a*]anthracene metabolites, 3- and 4-hydroxybenz[*a*]anthracene, suggested that the 7,12-dimethylbenz[*a*]anthracene metabolites were also the 3- and 4-hydroxy compounds.

Phenolic products were also formed as metabolites of 7- and 12-hydroxymethylbenz[*a*]anthracene, and products with identical chromatographic properties were also formed from 7,12-dimethylbenz[*a*]anthracene itself. The two phenolic products present in the largest amounts resembled those described above in the characteristic change in the fluorescence in u.v. light when thin-layer chromatograms were exposed to ammonia, so that they were tentatively identified as 3- and 4-hydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene when the 7-hydroxymethyl derivative was used as substrate and 3- and 4-hydroxy-12-hydroxymethyl-7-methylbenz[*a*]anthracene when the 12-hydroxymethyl derivative was used as substrate. Presumably mixtures of the isomers were formed with 7,12-dimethylbenz[*a*]anthracene as substrate.

12-Methylbenz[*a*]anthracene-7-carboxaldehyde, which could be identified on thin-layer chromatograms by its characteristic fluorescence in u.v. light (see Table 1) and by its yellow colour on untreated chromatograms, was never detected as a metabolite of the hydrocarbon or of the related hydroxymethyl derivative. 12-Methylbenz[*a*]anthracene-7-carboxylic acid did not run on thin-layer chromatograms as a distinct spot, but when present it could usually be recognized by its distinctive u.v. spectrum when the material near the base lines of chromatograms developed in solvent (*c*) or (*d*) were examined. Confirmatory evidence was provided by treating the material from the base lines of these chromatograms with diazomethane in ether and examining the products on thin-layer chromatograms developed with solvent (*b*) for the presence of the methyl ester. The isomeric 7-methylbenz[*a*]-anthracene-12-carboxaldehyde and carboxylic acid were not prepared, but it was expected that their properties on thin-layer chromatograms would be similar to those of the corresponding 12-methyl derivatives. In a few experiments the incubated homogenates were acidified to pH 1 with concentrated hydrochloric acid before extraction with ethyl acetate: no increases in the amounts of the carboxylic acids recovered were detected.

The substance that appears to be the major metabolic product of 7,12-dimethylbenz[*a*]anthracene under the conditions used in this work behaves like a dihydrodihydroxy compound, since it yielded two phenols and small amounts of a number of other fluorescent products with acid when examined on two-dimensional thin-layer chromatograms. Of the phenols, the major product was that described by Boyland & Sims (1965*a*), whereas the minor product (seen only when large amounts of the dihydrodihydroxy compound were chromatographed) formed a pink fluorescent spot when the chromatograms were examined in u.v. light in the presence of ammonia. These phenols were not chromatographically identical with those described above. The dihydrodihydroxy compound was tentatively identified as 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[*a*]anthracene (Boyland & Sims, 1965*a*). It has been difficult to obtain further evidence for this structure, but a metabolic product of benz[*a*]anthracene with similar chromatographic properties possessed a u.v. absorption curve ($\lambda_{max.}$ at 265, 292, 305, 319, 344 and 358mμ and an inflexion at 276mμ) similar to that of the 7,12-dimethylbenz[*a*]anthracene metabolite ($\lambda_{max.}$ at 268, 304, 317, 331, 358 and 376mμ and an inflexion at 284mμ) except that with the latter compound the peaks were shifted to longer wavelengths. The structure of the benz[*a*]anthracene metabolite is

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

Details are given in the text. The R_F values varied slightly for each chromatogram: those quoted are typical.

Compound	R_F in Benzene	R_F in Benzene–ethanol (19:1, v/v)	R_F in Benzene–ethanol (9:1, v/v)	Fluorescence in u.v. light: Immediate	Fluorescence in u.v. light: After exposure to NH_3
7,12-Dimethylbenz[*a*]anthracene	0·95*	0·98	0·98	Violet	Violet
7-Hydroxymethyl-12-methylbenz[*a*]anthracene	0·22	0·62	0·72	Violet	Violet
12-Hydroxymethyl-7-methylbenz[*a*]anthracene	0·40	0·78	0·85	Violet	Violet
7,12-Dihydroxymethylbenz[*a*]anthracene	0·00	0·28	0·45	Violet	Violet
12-Methylbenz[*a*]anthracene-7-carboxaldehyde	0·62	0·98	0·98	Yellow–green	Yellow–green
12-Methylbenz[*a*]anthracene-7-carboxylic acid	0·00	0·00	0·05	Violet	Violet
Methyl 12-methylbenz[*a*]anthracene-7-carboxylate	0·69	0·98	0·98	Blue	Blue
cis-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene†	0·00	0·23	0·38	Dark violet	Dark violet
trans-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene†	0·00	0·20	0·32	Dark violet	Dark violet
cis-5,6-Dihydro-5,6-dihydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene	0·00	0·05	0·25	Dark violet	Dark violet
cis-7-Acetoxymethyl-5,6-dihydro-5,6-dihydroxy-12-methylbenz[*a*]anthracene	0·00	0·25	0·38	Dark violet	Dark violet
8,9-Dihydro-8,9-dihydroxy-7,12-dimethylbenz[*a*]anthracene‡	0·00	0·16	0·30	Dark violet	Dark violet
9,9-Dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene‡§	0·00	0·05	0·21	Dark violet	Dark violet
3-Hydroxy-7,12-dimethylbenz[*a*]anthracene‡	0·28	0·70	0·79	Violet	Yellow–green
4-Hydroxy-7,12-dimethylbenz[*a*]anthracene‡	0·38	0·76	0·82	Violet	Pink
3-Hydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene‡§	0·00	0·22	0·39	Violet	Yellow–green
4-Hydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene‡§	0·00	0·26	0·42	Violet	Pink

* R_F 0·54 in light petroleum (b.p. 80–100°)–benzene (19:1, v/v).

† These products were also characterized by the acid-decomposition products formed on two-dimensional chromatograms as described in the text.

‡ These compounds were only obtained as metabolic products: the evidence for their structure is discussed in the text.

§ The isomeric 12-hydroxymethyl-7-methyl derivatives had identical properties.

established because it yielded 8- and 9-hydroxybenz[*a*]anthracene with acid (Boyland & Sims, 1964). The fluorescence spectrum of the 7,12-dimethylbenz[*a*]anthracene metabolite (activation maximum at 330mμ, fluorescence maximum at 390 and 410mμ) was similar to that of the unidentified metabolite A described by Conney & Levin (1966) (activation maximum at 320mμ, fluorescence maximum at 388mμ). The 7- and 12-hydroxymethyl derivatives similarly yielded products whose absorption and fluorescence spectra were similar to those of the hydrocarbon metabolite. These products are presumed to be 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene ($\lambda_{max.}$ 269, 303, 316, 330, 358 and 373mμ and an inflexion at 284mμ) and 8,9-dihydro-8,9-dihydroxy-12-hydroxymethyl-7-methylbenz[*a*]anthracene ($\lambda_{max.}$ 270, 304, 317, 331, 358 and 373mμ and an inflexion at 284mμ) respectively. Both compounds had activation maxima at 330mμ and fluorescence maxima at 390 and 410mμ. Their formation clearly represents the major metabolic routes of the hydroxymethyl derivatives.

The search in the reaction products for dihydrodihydroxy compounds formed on the 5,6-bonds (the 'K regions') of the hydrocarbon and the 7-hydroxymethyl derivative was carried out in two ways. First, attempts were made to find fluorescent bands in thin-layer chromatograms that had both the chromatographic properties and the characteristic u.v. spectra of the synthetic compounds. Secondly, with the hydrocarbon, the reaction products were examined on two-dimensional thin-layer chromatograms for the phenol (presumably the 5-hydroxy compound) that should arise from the acid decomposition of the dihydrodihydroxy compound. No indication of the presence of the dihydrodihydroxy compounds was found in experiments

with homogenates from either treated or untreated animals, thus confirming earlier observations (Boyland & Sims, 1965*a*).

Metabolism of 7,12-dimethylbenz[a]*anthracene.* The results in Table 2 show that 7,12-dimethylbenz[*a*]anthracene is metabolized about four times as fast in liver homogenates from rats treated with 3-methylcholanthrene or with Sudan III as in homogenates from oil-treated controls. The large-scale experiments showed that the metabolic route mainly affected was that yielding the compound believed to be 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[*a*]anthracene. The results of several experiments showed wide variation in the amounts of this compound formed, but increases of at least 300% and sometimes as much as 900% over the amounts formed in control experiments were always obtained. The reasons for these wide variations are not apparent. The results also showed that the amounts of the phenolic derivatives from homogenates of treated animals were larger than those from homogenates from the controls, as judged by a comparison of the intensities of the fluorescent bands on chromatograms of the respective products.

In contrast with the increases in the amounts of the ring-hydroxylated products described above, it is shown in Table 2 that the amounts of the monohydroxymethyl derivatives were much decreased as compared with the control when homogenates from animals treated either with 3-methylcholanthrene or with large amounts of Sudan III were used. In most large-scale experiments with animals treated with these inducers the amounts of the hydroxymethyl derivatives were much decreased and sometimes the derivatives were not detected in the reaction products. Occasionally, however, there appeared to be an increase rather than a decrease in the amount of the 7- (but not of the 12-) hydroxymethyl derivative: the reason for this is not known. 7,12-Dihydroxymethylbenz[*a*]anthracene was not detected as a metabolite in the homogenates from control animals, but was present in small amounts in those from treated animals.

The amounts of the carboxylic acids detected in the large-scale experiments were small, indicating that little of the added hydrocarbon was metabolized in this way. A comparison of the u.v. spectra of the metabolite from treated and control animals showed that twice as much was present in the homogenates from the treated than from the control animals. 8,9-Dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene and the isomeric 12-hydroxymethyl derivative could not be detected in the experiments with oil-treated animals,

Table 2. *Effect of pretreatment on the metabolism of* 7,12-*dimethylbenz*[a]*anthracene by rat-liver homogenates*

The compounds used for the pretreatments were administered in arachis oil (0·5ml.) as described in the text. Unless otherwise indicated, the homogenates were incubated with the hydrocarbon as described in the text 40hr. after the pretreatment. Mean values ± S.E.M. are given.

Pretreatment	Dose (mg.)	No. of expts.	Amount of 7,12-dimethylbenz[*a*]-anthracene metabolized (μg./g. of liver/30min.)	Amount of derivative extracted from the homogenate derived from 1g. of liver at the end of 30min. incubation: 7-Hydroxymethyl-12-methylbenz[*a*]-anthracene (μg.)	12-Hydroxymethyl-7-methylbenz[*a*]-anthracene (μg.)
Arachis oil	—	8	9±3	2·1±0·3	4·0±0·4
3-Methylcholanthrene	5	6	36±5	0·4±0·2	0·2±0·1
Sudan III	10	2	33±3	0·8±0·2	0·3±0·1
Sudan III	1	2	18±4	1·2±0·3	1·0±0·2
Sudan III	0·1	2	9±4	1·9±0·2	4·5±0·4
Phenobarbitone	12*	2	15±2	3·5±0·3	6·3±0·5
Metyrapone	10†	2	19±4	3·4±0·2	5·4±0·2
7,12-Dimethylbenz[*a*]anthracene	5	2	18±2	2·2±0·3	3·1±0·3
Benz[*a*]anthracene	5	2	21±3	1·1±0·2	0·7±0·3
11,12-Epoxy-11,12-dihydro-3-methylcholanthrene	5	2	20±4	1·0±0·2	0·8±0·2
1-Hydroxy-3-methylcholanthrene	5	2	10±4	2·0±0·2	4·2±0·4
cis-1,2-Dihydroxy-3-methylcholanthrene	5	2	9±5	2·1±0·3	3·9±0·4

* A second dose was administered 20hr. before the homogenates were incubated.

† Four doses were given at 24, 20, 16 and 44hr. before the homogenates were incubated.

but small amounts were present in the reaction products of experiments with animals treated with 3-methylcholanthrene and Sudan III. The large-scale experiments also indicated that there were increases in the amounts of the phenols derived from the hydroxymethyl derivatives when homogenates from treated animals were used. These increases were difficult to measure because sharp u.v. spectra could not be obtained, but they appeared to be about fourfold.

Pretreating rats with either phenobarbitone or with metyrapone increased the rates of metabolism of 7,12-dimethylbenz[*a*]anthracene and also increased slightly the yields of the monohydroxymethyl derivatives as compared with the corresponding values from control animals (see Table 2). In the large-scale experiments small increases in the amounts of the dihydroxymethyl derivative and of the carboxylic acid as compared with the controls were detected with both compounds. There was no indication of any significant increases in the amounts of the other metabolites formed from animals pretreated with phenobarbitone, but small increases in the amounts of the 8,9-dihydro-8,9-dihydroxy compound in animals pretreated with metyrapone were detected: metyrapone was, however, less effective than 3-methylcholanthrene and Sudan III in the induction of the dihydro-dihydroxy compound.

A few experiments were carried out in which rats were pretreated with 7,12-dimethylbenz[*a*]anthracene and benz[*a*]anthracene, both of which will protect rats against adrenal necrosis, although they are less effective than 3-methylcholanthrene (Huggins & Fukunishi, 1964). The results in Table 2 show that both hydrocarbons cause increases in the amounts of 7,12-dimethylbenz[*a*]anthracene metabolized, and decreases in the amounts of the hydroxymethyl derivatives formed as compared with the controls, but that the effects are less than with 3-methylcholanthrene.

Several derivatives of 3-methylcholanthrene were also examined. 11,12-Epoxy-11,12-dihydro-3-methylcholanthrene was about half as effective as the parent hydrocarbon: the others were without effect (see Table 2).

Metabolism of 7-hydroxymethyl-12-methyl- and 12-hydroxymethyl-7-methyl-benz[a]*anthracene.* The results in Table 3 show that the hydroxymethyl derivatives were metabolized about four times as fast by homogenates from animals treated either with 3-methylcholanthrene or, for the 7-hydroxymethyl derivative, with large doses of Sudan III. Smaller doses of this inducer were less effective. The large-scale experiments showed that there were large increases in the amounts of the 8,9-dihydro-8,9-dihydroxy derivatives and smaller increases in the amounts of the phenolic derivatives formed from the hydroxymethyl compounds. There were also small increases in the amounts of the dihydroxymethyl derivative and of the carboxylic acids.

The results in Table 3 also show that the hydroxymethyl derivatives were metabolized faster in homogenates from animals treated with phenobarbitone than from those from controls. The large-scale experiments indicated that the increases were due to increases in the amounts of the dihydroxymethyl derivative and of the carboxylic acids: the amounts of the other derivatives were not affected.

Metabolism of 12-methylbenz[a]*anthracene-7-carboxaldehyde.* Qualitative examinations of the products from the incubations of the aldehyde with homogenates from control rats and from rats pretreated with 3-methylcholanthrene showed that both contained products resulting either from the

Table 3. *Effect of pretreatment on the metabolism of the hydroxymethyl derivatives of 7,12-dimethylbenz*[a]*anthracene by rat-liver homogenates*

Details are given in Table 2. Substrate A is 7-hydroxymethyl-12-methylbenz[*a*]anthracene; substrate B is 12-hydroxymethyl-7-methylbenz[*a*]anthracene.

Substrate	Pretreatment	Dose (mg.)	No. of expts.	Amount of hydroxymethyl derivative metabolized (μg./g. of liver/30 min.)
A	Arachis oil	—	4	13±4
A	3-Methylcholanthrene	5	4	55±5
A	Sudan III	10	2	52±5
A	Sudan III	1	2	32±4
A	Sudan III	0·1	2	18±4
A	Phenobarbitone	12*	2	22±4
B	Arachis oil	—	2	15±4
B	3-Methylcholanthrene	5	2	52±6
B	Phenobarbitone	12*	2	23±2

* A second dose was given 20 hr. before the homogenates were incubated.

reduction or from the oxidation of the aldehyde group. Products with the properties of 7-hydroxymethyl-12-methylbenz[*a*]anthracene, its 8,9-dihydro-8,9-dihydroxy derivative and the phenolic derivative described above were detected, together with 12-methylbenz[*a*]anthracene-7-carboxylic acid. A comparison of the spectra of the acid obtained in experiments with the aldehyde in homogenates from control and from 3-methylcholanthrene-treated animals with those obtained in similar experiments with the 7-hydroxymethyl derivative showed that about ten times as much acid was formed from the former than from the latter substrate.

Metabolism of 7-acetoxymethyl-12-methylbenz[a]*anthracene.* The metabolism of this compound was investigated in liver homogenates from rats pretreated with 3-methylcholanthrene, mainly to determine if hydroxylation occurred on the 5,6-bond (the 'K region') of the molecule. No metabolite with the chromatographic properties of the synthetic 7-acetoxymethyl-5,6-dihydro-5,6-dihydroxy compound was found. Instead, a large proportion of the acetoxy compound was deacetylated to yield small amounts of 7-hydroxymethyl and the 7,12-dihydroxymethyl derivative together with the compound believed to be 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene.

Metabolism of trans-5,6-*dihydro*-5,7-*dihydroxy*-7,12-*dimethylbenz*[a]*anthracene.* This experiment was carried out to see whether the dihydrodihydroxy compound was destroyed on incubation with rat-liver homogenates from animals pretreated with 3-methylcholanthrene. Large amounts of the compound were recovered unchanged and no metabolic products were identified.

DISCUSSION

The effect of pretreating rats with either 3-methylcholanthrene or with Sudan III was to increase the ability of their liver homogenates to metabolize both 7,12-dimethylbenz[*a*]anthracene and the monohydroxymethyl derivatives. The metabolic routes mainly affected appear to be those yielding 8,9-dihydro-8,9-dihydroxy compounds and phenols, but there are also small increases in products arising from the oxidation of methyl and hydroxymethyl groups. It is not clear from the experiments whether the decreases in the amounts of the hydroxymethyl derivatives are due to further oxidation of these derivatives or whether hydrocarbon that would normally be converted into the hydroxymethyl derivative is converted instead into the 8,9-dihydrodihydroxy derivative and the phenols. Although the incubations were carried out in the presence of large excesses of the hydrocarbon, presumably only small amounts of it are available for hydroxylation at any one moment because of its low solubility in the reaction medium. The 8,9-dihydro-8,9-dihydroxy derivatives are the major metabolic products of the hydroxymethyl compounds, but only relatively small amounts of the dihydrodihydroxy compounds were detected in the products of the incubations of the hydrocarbon with the homogenates from treated animals.

The pretreatment of rats with phenobarbitone, a compound that, when given repeatedly, acts as a protector against adrenal necrosis by 7,12-dimethylbenz[*a*]anthracene (the 7-hydroxymethyl derivative has not been tested) (Huggins & Fukunishi, 1964), did not have a profound effect on the metabolism of either the hydrocarbon or the 7-hydroxymethyl derivative by their liver homogenates. The small increases in the metabolic rate found appear to be due to increased hydroxylation of the methyl groups and the further oxidation of these products without any dramatic increases in ring-hydroxylated products. Presumably the conversion of the monohydroxymethyl derivative into 7,12-dihydroxymethylbenz[*a*]anthracene and into the carboxylic acid would have the same effect as ring hydroxylation in protecting the adrenal glands. The apparent differences in the metabolic routes stimulated by phenobarbitone and by 3-methylcholanthrene indicate that different enzymes are involved in ring and methyl group hydroxylation. In the metabolism of biphenyl, 4-hydroxylation is moderately stimulated by phenobarbitone and 2-hydroxylation is stimulated by 3-methylcholanthrene (Creaven & Parke, 1966).

The pretreatment of rats with metyrapone increased the rate of metabolism of 7,12-dimethylbenz[*a*]anthracene by liver homogenates, apparently by stimulating the formation of the mono- and di-hydroxymethyl derivatives of the hydrocarbon, with only small alterations in the amounts of the ring-hydroxylated products formed. Currie, Helfenstein & Young (1962) and Dao & Tanaka (1963) showed that metyrapone will inhibit adrenal necrosis induced by 7,12-dimethylbenz[*a*]anthracene and Wheatley *et al.* (1966*b*) that it will inhibit adrenal necrosis induced by the 7-hydroxymethyl derivative. Metyrapone is an inhibitor of the 11β-hydroxylation of steroids and therefore it decreases the concentrations of corticosterone and cortisol in the adrenal gland. It has been shown by Morii & Huggins (1962) that there is a correlation between damage to the adrenal gland by 7,12-dimethylbenz-[*a*]anthracene and high concentration of corticosterone in the gland. It is therefore possible that metyrapone acts in this way rather than as an enzyme stimulator.

The experiments now described, together with those of Conney & Lewin (1966) and of Jellinck & Goudy (1966), indicate that the pretreatment of

animals with enzyme inducers stimulates the metabolism of 7,12-dimethylbenz[*a*]anthracene and its hydroxymethyl derivatives, so that, if these reactions are related to the metabolism of the compound in the animal's body, they should have the effect of decreasing by one means or another the effective concentration of the 7-hydroxymethyl derivative. It is probable that this derivative is the active intermediate in the induction of adrenal necrosis in rats by 7,12-dimethylbenz[*a*]anthracene (Boyland, Sims & Huggins, 1965; Wheatley *et al.* 1966*b*), so that any treatment that either decreases the amount of this product formed in the body or causes further metabolism to occur should prevent the adrenal necrosis. It is difficult to decide the significance of the amounts of the hydroxymethyl derivatives found at the end of the incubation, since the derivatives clearly are intermediates in the metabolic pathways. A small decrease in the amounts formed in the experiments *in vitro* might well mean that little or none is formed in the whole animal. It seems clear from the experiments of Wheatley *et al.* (1966*a*) that the 7-hydroxymethyl derivative is formed in the liver and not in the adrenal gland itself, since liver damage or partial hepatectomy protects the animal against adrenal damage by 7,12-dimethylbenz[*a*]anthracene. The doses of the hydrocarbon and of its hydroxymethyl derivative required to induce adrenal necrosis are critical. Thus 2·5mg. of 7,12-dimethylbenz[*a*]-anthracene injected intravenously induces adrenal necrosis in 100% of the animals whereas 1mg. similarly injected is without effect (Huggins & Morii, 1961), and 5mg. of the hydroxymethyl derivative given orally was 100% effective whereas 2mg. doses were without effect (Boyland *et al.* 1965). This means that a small alteration in the amount of the 7-hydroxymethyl derivative formed from the hydrocarbon or a small increase in the rate of metabolism of the 7-hydroxymethyl derivative in the body could radically alter the ability of the injected compounds to induce adrenal necrosis. Huggins & Pataki (1965) showed that Sudan III was effective as a protector at doses as low as 0·01mg. and Huggins & Fukunishi (1964) that 3-methylcholanthrene was effective at doses of 0·25mg. The present work shows that doses of 1mg. of the dye were required to produce measurable effects on the metabolism of 7,12-dimethylbenz[*a*]anthracene and the 7-hydroxymethyl derivative by liver homogenates and doses of 10mg. were required to produce effects comparable with those produced by 5mg. of 3-methylcholanthrene. Conney & Levin (1966) detected small increases in the formation of their unidentified metabolites with low doses of the two enzyme inducers. If, then, the action of the 'protectors' is to induce hydroxylating enzymes, then the small effective doses of Sudan III must be sufficient to decrease the 7-hydroxymethyl derivative in the body to a non-active concentration: the protective effect of Sudan III against the 7-hydroxymethyl derivative itself has not been examined. Similarly, although only small effects were detected in liver homogenates from animals treated with phenobarbitone, it might be that in the body these would be sufficient to decrease the concentration of the 7-hydroxymethyl derivative.

Dao & Varela (1966) have shown that actinomycin D, puromycin and DL-ethionine inhibit the 3-methylcholanthrene-induced stimulation of the enzyme, benzo[*a*]pyrene hydroxylase, but that only puromycin and DL-ethionine abolish the adrenal protection afforded by the hydrocarbon. 3-Methylcholanthrene, as well as stimulating hydroxylating enzymes, inhibits corticosterone synthesis (Dao, Flaxman & Lonergan, 1963).

Although both 7-hydroxymethyl-12-methyl- and 12-hydroxymethyl-7-methyl-benz[*a*]anthracene are equally effective in producing sarcomata when given by subcutaneous injection into mice, only the 7-hydroxymethyl derivative will induce mammary tumours when given to rats (Boyland *et al.* 1965). The metabolism of both derivatives in rat-liver homogenates followed identical pathways, so that no indication was obtained as to the reasons for these differences in reactivity. The parent hydrocarbon was more effective in inducing mammary cancer than the 7-hydroxymethyl derivative, but it is noteworthy that compounds that protect against adrenal necrosis can also inhibit to some degree the induction of mammary tumours by the hydrocarbon (Huggins, Grand & Fukunishi, 1964).

The products of the metabolism in rat-liver homogenates of 7,12-dimethylbenz[*a*]anthracene itself and of the hydroxymethyl derivatives were similar and consisted of mixtures of ring- and methyl group-hydroxylated products. Only small proportions of the substrates were oxidized to carboxylic acids. The absence of hydroxylation at the 5,6-bond (the 'K region') of all substrates is difficult to explain, since closely related compounds such as benz[*a*]anthracene and dibenz[*a*,*h*]anthracene (Boyland & Sims, 1965*b*) and 7- and 12-methylbenz[*a*]anthracene (P. Sims, unpublished work) all yield 5,6-dihydro-5,6-dihydroxy derivatives with rat-liver preparations. It therefore seems possible that reaction at the 'K region' is not important in the carcinogenic action of these compounds.

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