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The Metabolism of 3-Methylcholanthrene and some Related Compounds by Rat-Liver Homogenates

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1. A chromatographic investigation of the products of the metabolism of 3-methylcholanthrene by rat-liver homogenates showed the formation of compounds with the properties of 1- and 2-hydroxy-3-methylcholanthrene, *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene and 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene. A glutathione conjugate that is probably *S*-(11,12-dihydro-12-hydroxy-3-methyl-11-cholanthrenyl)glutathione was also detected. 3-Methylcholanthrene-1- and -2-one and -1,2-quinone were also present, but these products may have arisen by the chemical oxidation of the corresponding hydroxy compounds. 2. Other metabolic products were tentatively identified as 9- and 10-hydroxy-3-methylcholanthrene, 4,5-dihydro-4,5-dihydroxy-3-methylcholanthrene and 3-hydroxymethylcholanthrene. 3. 1- and 2-Hydroxy-3-methylcholanthrene were converted by homogenates into the related ketones and into products with the properties of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene: 3-methylcholanthren-1- and -2-one were converted into their related hydroxy compounds and into the isomeric 1,2-dihydroxy compounds. The isomeric 1,2-dihydroxy compounds were each partly converted into the other isomer by these homogenates. All the above substrates also yielded products that appeared to be derivatives of 3-hydroxymethylcholanthrene. 4. 3-Methylcholanthrylene was converted by rat-liver homogenates into products with the properties of *trans*-1,2-dihydroxy-3-methylcholanthrene, 2-hydroxy-3-methylcholanthrene and 3-methylcholanthren-2-one. A small amount of the *cis*-1,2-dihydroxy compound was also formed, together with a glutathione conjugate that is possibly *S*-(2-hydroxy-3-methyl-1-cholanthrenyl)glutathione or its positional isomer. 5. An unidentified product was detected in the metabolism of 3-methylcholanthrene, the monohydroxy compounds, the ketones and the dihydroxy compounds, the formation of which appeared to involve metabolism at the 1,2-bond. 6. 11,12-Epoxy-11,12-dihydro-3-methylcholanthrene was converted by rat-liver homogenates into products with the properties of 11-hydroxy-3-methylcholanthrene (or, less likely, the 12-isomer), 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene and the glutathione conjugate described above. Products with the properties of these compounds were formed when the epoxide was allowed to react with glutathione in an aqueous medium. 7. Mouse-liver homogenate converted 3-methylcholanthrene into products with the chromatographic properties of 1- and 2-hydroxy-3-methylcholanthrene, *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene, 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene, 3-methylcholanthrene-1- and -2-one and -1,2-quinone and the unidentified hydroxy-3-methylcholanthrenes. 8. The syntheses of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene, 3-methylcholanthren-2-one, 2-hydroxy-3-methylcholanthrene, 3-methylcholanthrylene, 11,12-epoxy-11,12-dihydro-3-methylcholanthrene and *trans*-11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene are described.

Although 3-methylcholanthrene (III) has strong cancer-producing properties and has been the subject of many biological experiments, little is known of its fate in the animal body. Harper (1959) examined the faeces of mice that had been injected with the hydrocarbon and detected two metabolites, a phenol that was either 8- or 10-hydroxy-3-methylcholanthrene and a carboxylic acid that was

tentatively identified as cholanthrene-3-carboxylic acid. Evidence was also obtained for the presence of phenolic sulphuric acid and glucuronic acid conjugates. This paper describes the metabolism of 3-methylcholanthrene (III) by rat-liver homogenates.

EXPERIMENTAL

Melting points are uncorrected.

Spectra. Infrared spectra were measured as mulls in Nujol on a Perkin–Elmer Infracord. Ultraviolet-absorption spectra were measured in ethanol, except where indicated below, on a Unicam SP. 500 spectrophotometer or on a Perkin–Elmer model 137 spectrophotometer. The spectra of compounds eluted from paper or from thin-layer chromatograms were usually measured on the latter instrument, solutions being obtained by eluting with methanol containing 1% (v/v) of aq. NH_3 (sp.gr. 0·88) the appropriate bands cut from paper chromatograms, or by eluting with ethanol the appropriate bands removed from thin-layer chromatograms. The positions of the bands were usually determined by the fluorescence seen when the chromatograms were examined by u.v. light.

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

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 10cm. with (a) light petroleum (b.p. 60–80°)–benzene (19:1, v/v), (b) benzene or (c) benzene–ethanol (19:1, v/v). They were examined in u.v. light while still wet, both before and after exposure to NH_3, and were then sprayed with conc. HCl and heated at 80° for 10min. The two-dimensional chromatograms referred to below were developed in the first direction for 10cm. with solvent (c), sprayed with conc. HCl and heated at 100° for 10min. and developed in the second direction for 10cm. with solvent (b). In this way, products arising from the decomposition of acid-labile precursors were detected. The properties on thin-layer chromatograms described below are listed in Table 1.

Table 1. *Properties of compounds related to 3-methylcholanthrene on thin-layer chromatograms*

Details are given in the text.

Compound	R_F in Benzene	R_F in Benzene–ethanol (19:1, v/v)	Fluorescence	Colour after spraying with conc. HCl and heating at 100°
1-Hydroxy-3-methylcholanthrene (X)	0·20	0·62	Violet	Pale brown
1-Acetoxy-3-methylcholanthrene	0·49	0·90	Violet	Pale brown
2-Hydroxy-3-methylcholanthrene (VIII)	0·15	0·55	Violet	Pale brown
2-Acetoxy-3-methylcholanthrene	0·49	0·90	Violet	Pale brown
3-Methylcholanthren-1-one (XIII)	0·46	0·90	Green, turning orange	Pale yellow*
3-Methylcholanthren-2-one (XII)	0·46	0·90	Yellow	Pale yellow*
3-Methylcholanthrene-1,2-quinone (IV)	0·18	0·82	Olive green	Yellow*
cis-1,2-Dihydroxy-3-methylcholanthrene (VII)	0·00	0·28	Violet	Yellow†
cis-1,2-Diacetoxy-3-methylcholanthrene	0·13	0·70	Violet	Yellow†
trans-1,2-Dihydroxy-3-methylcholanthrene (VII)	0·00	0·23	Violet	Yellow†
trans-1,2-Diacetoxy-3-methylcholanthrene	0·18	0·77	Violet	Yellow†
cis- and *trans*-11,12-Dihydro-11,12-dihydroxy-3-methylcholanthrene (V)	0·00	0·23	Dark violet	Brown
Phenol, probably 11-hydroxy-3-methylcholanthrene (I, R=H)	0·49	0·92	Violet (yellow‡)	Brown
11,12-Epoxy-11,12-dihydro-3-methylcholanthrene (II)	0·39	0·92	Dark violet, turning violet (yellow‡)	Brown
3-Methylcholanthrene (III)§	0·95	0·95	Violet	Pale yellow
3-Methylcholanthrylene (XIV)§	0·95	0·95	Dark absorption	Yellow*
2-Acetoxy-3-methylcholanthrylene	0·55	0·90	Dark absorption	Orange, then brown*
Methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-acetate (VI, R=Me)	0·31	0·85	Orange–yellow	Yellow*
Methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-carboxylate (XI, R=Me)	0·35	0·90	Orange	Yellow*

* These colours were seen on untreated chromatograms.
† This colour is due to the formation of 3-methylcholanthren-2-one (XII).
‡ Fluorescence in the presence of NH_3.
§ R_F 0·45 in light petroleum (b.p. 60–80°)–benzene (19:1, v/v).

The small-scale chromic acid oxidations described below were carried out as follows. About 1mg. of solid, or the material obtained by eluting with ether bands removed from thin-layer chromatograms, was heated on the steam bath with 5% (w/v) of $Na_2Cr_2O_7$ in acetic acid (0·1–0·5ml.) for 15min. The mixture was diluted with water and kept at room temperature until solid material separated. This was collected by centrifugation, washed with a little water and suspended in methanol (1ml.). An excess of diazomethane in ether was added and, at the end of the reaction, the solvents were evaporated. The residue was examined on thin-layer chromatograms developed with solvent (b) or (c).

Materials

3-Methylcholanthrene (III). This was obtained from Eastman Kodak Ltd. (Kirkby, Liverpool) and from Roche Products Ltd. (Welwyn Garden City, Herts.). Its light-absorption curve showed $\lambda_{max.}$ at 222, 236, 260, 274, 283, 294, 326, 342, 357, 374 and 391 mμ.

Methyl 9-methylbenz[a]*anthracene-7,12-quinone-8-acetate* (VI, R=Me) *and 8-carboxylate* (XI, R=Me). The parent acid was prepared by heating the hydrocarbon (III) (1g.) under reflux with $Na_2Cr_2O_7$ (5g.) in acetic acid (25ml.) (Cook & Haslewood, 1934). The acid, suspended in methanol, was esterified with diazomethane in ether and the product recrystallized from methanol to give methyl 9-methylbenz[*a*]-anthracene-7,12-quinone-8-acetate (VI, R=Me) (280mg.) as yellow needles, m.p. 217° (Found: C, 76·5; H, 4·65. Calc. for $C_{22}H_{16}O_4$: C, 76·7; H, 4·7%); $\lambda_{max.}$ at 254, 285 and 346 mμ (log ϵ 4·30, 4·70 and 3·70 respectively). Bachmann (1937) reports m.p. 213–214° and Fieser & Hershberg (1938), m.p. 221·5–222° (corr.). The mother-liquors were evaporated and the dry residue was chromatographed in benzene on a silica gel column (20g.) (200–300 mesh; Koch–Light Laboratories Ltd., Colnbrook, Bucks.). The two yellow bands were eluted separately and the eluates evaporated. The residue from the faster-moving band, recrystallized from methanol, gave a product (15mg.) that is probably *methyl 9-methylbenz*[a]*anthracene-7,12-quinone-8-carboxylate* (XI, R=Me) in yellow needles, m.p. 178° (Found: C, 76·3; H, 4·2. $C_{21}H_{14}O_4$ requires C, 76·35; H, 4·3%); $\lambda_{max.}$ at 251, 288 and 338 mμ (log ϵ 4·28, 4·59 and 3·59 respectively). The residue from the slower-moving band, recrystallized from methanol, yielded methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-acetate (VI, R=Me) (30mg.) as yellow needles, m.p. and mixed m.p. 217°.

3-Methylcholanthren-1-one (XIII). This was prepared from (III) and $Na_2Cr_2O_7$ in cold acetic acid by the method of Fieser & Hershberg (1938). The crude ketone was purified by chromatography in benzene on a Florisil (100–200 mesh; Koch–Light Laboratories Ltd.) column. The pale-yellow band was eluted, the solvent evaporated and the residue recrystallized from acetic acid to yield the ketone (XIII) in yellow needles, m.p. 258° [Fieser & Hershberg (1938) report m.p. 262–263° (corr.) and Badger *et al.* (1940) m.p. 256–257°]; $\lambda_{max.}$ at 233·5, 257, 269, 296, 307·5, 367, 387 and 403 mμ (log ϵ 4·64, 4·39, 4·35, 4·66, 4·68, 3·84, 4·02 and 3·99 respectively). The infrared spectrum showed strong carbonyl absorption at 1700cm.$^{-1}$. A little of the ketone (XIII) was oxidized with $Na_2Cr_2O_7$ in acetic acid and the product methylated as described above. Products with the mobilities and properties on thin-layer chromatograms developed with solvents (b) and (c) of the esters (VI, R=Me) and (XI, R=Me) were detected, the former being the major product.

1-Hydroxy-3-methylcholanthrene (X). The ketone (XIII) (100mg.) and lithium tetrahydroaluminate (500mg.), in dry ether (250ml.), were heated under reflux for 6hr. Water (50ml.), followed by 2N-H_2SO_4 (20ml.), was added and the mixture shaken. The ether layer was separated, washed with water, dried over Na_2SO_4 and the ether evaporated. The residue was recrystallized from benzene to yield 1-hydroxy-3-methylcholanthrene (X) (80mg.) in needles, m.p. 211–212° (decomp.) (Found: C, 88·4; H, 6·0. Calc. for $C_{21}H_{16}O$: C, 88·7; H, 5·7%); $\lambda_{max.}$ at 236, 263·5, 273·5, 284, 295, 325, 340, 356, 374 and 391 mμ (log ϵ 4·50, 4·54, 4·78, 4·86, 4·95, 3·64, 3·83, 3·90, 3·73 and 3·01 respectively) and an inflexion at 303 mμ. The melting points (corr.) of the products obtained by Fieser & Hershberg (1938) from the oxidation of the hydrocarbon (III) with lead tetra-acetate and hydrolysis of the acetoxy derivative varied between 207–209° (decomp.) and 221–222° (decomp.). Attempts to obtain the hydroxy derivative (X) by this route were unsuccessful, for, although the corresponding acetoxy derivative was detected on thin-layer chromatograms, chromatography of the reaction mixtures on silica-gel- or on Florisil-packed columns led to extensive decomposition with the formation of coloured products including 3-methylcholanthrylene (XIV) described below.

When a portion of the hydroxy derivative (X) was oxidized with $Na_2Cr_2O_7$ and the product methylated as before, the methyl ester (VI, R=Me) and a small amount of the ester (XI, R=Me) were detected on thin-layer chromatograms.

The acetate, prepared from the hydroxy derivative (X) with acetic anhydride in pyridine, separated from ethanol in needles, m.p. 178° [Fieser & Hershberg (1938) report m.p. 177·5–178·5° (corr.)], $\lambda_{max.}$ at 224, 235, 262, 273, 283, 294, 325, 339, 355, 375 and 391 mμ and an inflexion at 303 mμ.

3-Methylcholanthrylene (XIV). 3-Methylcholanthrene (III) (5g.), in dry carbon tetrachloride (1l.), was cooled to 0° and bromine (1·1ml.) in carbon tetrachloride (50ml.) was added during 15min. Pyridine (50ml.) was added and, after 1hr., the mixture was filtered to remove pyridine hydrobromide. The solvent was removed under reduced pressure and the residue extracted twice with boiling light petroleum (b.p. 80–100°) (150ml.) to leave a brown residue that was not examined. The extract was cooled and the product that separated recrystallized from light petroleum (b.p. 80–100°) to yield *3-methylcholanthrylene* (XIV) (1·7g.) in orange plates with a metallic lustre, m.p. 194° (Found: C, 94·2; H, 5·3. $C_{21}H_{14}$ requires C, 94·7; H, 5·3%); $\lambda_{max.}$ at 262, 292, 314, 384 and 398 mμ (log ϵ 4·61, 4·40, 4·43, 4·04 and 3·99 respectively) and inflexions at 281, 305 and 366 mμ. The hydrocarbon (XIV) (10mg.) was oxidized at 100° for 15min. with $Na_2Cr_2O_7$ (50mg.) in acetic acid (1ml.), and the product esterified with diazomethane as before, to yield methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-carboxylate (XI, R=Me) in yellow needles from methanol, m.p. and mixed m.p. 175–176°.

Oxidation of 3-methylcholanthrene (III) *with lead tetra-acetate.* 3-Methylcholanthrene (III) (3g.), in acetic acid (350ml.), was heated at 100° with lead tetra-acetate (10g.) for 30min. The solution was poured into water (850ml.) and the solid collected and dried and chromatographed in

benzene on a Florisil (200g., 100–200 mesh) column to yield four main fractions. The fastest-moving of these formed an orange band: the eluates containing the material forming this fraction were evaporated and the residue was recrystallized three times from light petroleum (b.p. 80–100°) to yield 3-methylcholanthrylene (XIV) (85mg.) in orange plates with a metallic lustre, m.p. and mixed m.p. 194°.

The second fraction formed a pale-orange band on the column. The band was eluted and the solvent evaporated to yield a red solid that was dissolved in the minimum amount of boiling benzene. The solution was kept overnight at 0° and the red solid (15mg.) that separated was recrystallized from benzene to yield a product that is probably 2-*acetoxy*-3-*methylcholanthrylene* in red plates with a metallic lustre that softened and decomposed at 180–184° (Found: C, 84·7; H, 5·0. $C_{23}H_{16}O_2$ requires C, 85·2; H, 5·0%); $\lambda_{max.}$ at 218, 264, 290, 314, 388 and 410mμ (log ε 4·57, 4·62, 4·46, 4·44, 4·02 and 3·99 respectively) and inflexions at 305 and 365mμ. The infrared spectrum showed carbonyl absorption at 1770cm.$^{-1}$. When a little of the acetate was oxidized with $Na_2Cr_2O_7$ and the product methylated as before, a compound indistinguishable from the ester (XI, R=Me) was detected on thin-layer chromatograms. A little of the acetate was heated to boiling for 2min. with 5% (w/v) KOH in methanol (0·5ml.). A bright magenta solution was obtained, the colour of which was discharged when it was diluted with water and acidified with conc. HCl. An ether extract of this solution was found to contain a number of products, one of which was indistinguishable in its mobility on thin-layer chromatograms developed with solvent (b) and in its fluorescence in u.v. light from 3-methylcholanthren-2-one (XII) described below.

The third fraction, which formed a pale-yellow band, yielded 3-methylcholanthren-1-one (XIII) on evaporation of the eluting solvent, separating from acetic acid in yellow needles (250mg.), m.p. and mixed m.p. 258°.

The fourth fraction was eluted from the column with benzene–ethanol (99:1, v/v) as a dark-coloured band. Evaporation of the solvent yielded a brown gum that appeared to be a mixture of acetoxy derivatives, together with unidentified oxidation products. The gum was heated under reflux with KOH (2g.) in methanol (200ml.) for 15min. and the mixture poured into water (750ml.) and the solution extracted three times with ether (250ml.) to leave a dark-green fluorescent aqueous layer. The residue obtained on evaporation of the ether was chromatographed in benzene–ethanol (99:1, v/v) on a Florisil column (100g.). After the elution of a dark-coloured band that contained unidentified products, two bands, which were detected by their violet fluorescence in u.v. light, were successively eluted, followed by one that was detected by its yellow colour in daylight.

The solution containing the material forming the faster-moving of the fluorescent bands was evaporated: the dark solid was recrystallized three times from aq. ethanol to yield a product (150mg.) that, for reasons discussed below, appeared to be cis-1,2-*dihydroxy*-3-*methylcholanthrene* (VII) in pale-green needles, m.p. 225° (Found: C, 83·7; H, 5·5. $C_{21}H_{16}O_2$ requires C, 84·0; H, 5·4%); $\lambda_{max.}$ at 223·5, 232, 262, 273, 284, 294·5, 324, 338, 355 and 374mμ (log ε 4·72, 4·65, 4·59, 4·64, 4·88, 4·97, 3·67, 3·84, 3·91 and 3·76 respectively). Acetylation with acetic anhydride in pyridine yielded cis-1,2-*diacetoxy*-3-*methylcholanthrene*, separating from ethanol in needles, m.p. 217° (Found: C, 77·8; H, 5·2. $C_{25}H_{20}O_4$ requires C, 78·1; H, 5·2%); $\lambda_{max.}$ at 222·5, 234, 261, 273, 283, 294, 324, 338, 354 and 372mμ (log ε 4·35, 4·46, 4·47, 4·62, 4·90, 5·01, 3·72, 3·90, 3·97 and 3·82 respectively).

The solution containing the material forming the second fluorescent band was evaporated to a yellow solid that was recrystallized three times from aq. ethanol to yield trans-1,2-*dihydroxy*-3-*methylcholanthrene* (VII) (250mg.), in pale-green needles, m.p. 206° (Found: C, 83·5; H, 5·2%); $\lambda_{max.}$ at 223, 232, 262, 272·5, 283, 294, 324, 338, 354·5 and 374mμ (log ε 4·72, 4·66, 4·58, 4·66, 4·90, 4·99, 3·66, 3·82, 3·89 and 3·72 respectively). trans-1,2-*Diacetoxy*-3-*methylcholanthrene* separated from ethanol in prismatic needles, m.p. 202° (Found: C, 77·9; H, 5·3%); $\lambda_{max.}$ at 222·5, 234, 261, 273, 283, 294, 324, 338, 354 and 372mμ (log ε 4·66, 4·49, 4·48, 4·62, 4·89, 5·00, 3·72, 3·90, 3·98 and 3·86 respectively).

When small amounts of the dihydroxy compounds were oxidized and the products methylated as described above, only the ester (XI, R=Me) was detected on thin-layer chromatograms developed with solvent (b) or (c). When tested with the potassium triacetylosmate reagent of Criegee, Marchand & Wannowius (1942), the dihydroxy compound of m.p. 225° rapidly changed the colour of the reagent from blue to purple and then to grey, whereas the dihydroxy compound of m.p. 206° had no effect. This indicates that the former dihydroxy compound probably has the *cis*- and the latter the *trans*-configuration. Supporting evidence for this is discussed below.

Evaporation of the solution containing the material forming the yellow band yielded a solid that was recrystallized from acetic acid to give a product that was probably 3-*methylcholanthrene*-1,2-*quinone* (IV) (45mg.) in pale-orange needles, m.p. 346° (Found: C, 85·1; H, 4·3. $C_{12}H_{12}O_2$ requires C, 85·1; H, 4·1%); $\lambda_{max.}$ at 221, 234, 251, 289, 296, 390 and 410mμ. The infrared spectrum showed strong carbonyl absorption at 1705cm.$^{-1}$. The quinone (IV) was recovered unchanged (mixed m.p.) after an attempted oxidation with $Na_2Cr_2O_7$ in acetic acid.

3-*Methylcholanthren*-2-*one* (XII). When either the *cis*- or the *trans*-isomer of the dihydroxy compound (VII) (100mg.), in acetic acid (1ml.) containing either a few drops of conc. HCl or of water, was heated at 100° for 5min. the solution became yellow. Water was added until the solution was cloudy and it was then allowed to cool. In each case, the crystals (about 70mg.) were recrystallized from acetic acid to yield 3-*methylcholanthrene*-2-*one* (XII) in yellow needles, m.p. 205° (decomp.) (Found: C, 89·0; H, 4·75. $C_{21}H_{14}O$ requires C, 89·3; H, 5·0%); $\lambda_{max.}$ at 245·5, 277, 295, 302, 309·5, 367, 385 and 400mμ (log ε 4·46, 4·59, 4·63, 4·61, 4·67, 3·69, 3·79 and 3·73 respectively). The infrared spectrum of (XII) showed strong carbonyl absorption at 1700cm.$^{-1}$.

3-Methylcholanthren-2-one (XII) (50mg.) and $Na_2Cr_2O_7$ (250mg.), in acetic acid (5ml.), were heated under reflux for 30min. The solution was poured into water and the product suspended in methanol and treated with an excess of diazomethane in ether. The solvents were removed and the residue was recrystallized three times from methanol to yield methyl 9-methylbenz[*a*]anthracene-7,12-quinone-8-carboxylate (XI, R=Me) (25mg.) in yellow needles, m.p. and mixed m.p. 175–176°. The residue obtained on evapora-

tion of the mother-liquors was examined on thin-layer chromatograms developed with solvent (b), when a product, R_F 0·48, with a violet fluorescence in u.v. light, was detected whose u.v. spectrum showed $\lambda_{max.}$ 223, 233, 261, 271, 282, 292, 336, 352, 367 and 394 mμ. The compound was not investigated further, but it could be the dimethyl ester of 9-methylbenz[*a*]anthracene-7,8-dicarboxylic acid, which might be expected from the oxidation of the 1,2-bond of the ketone (XII).

2-Hydroxy-3-methylcholanthrene (VIII). 3-Methylcholanthren-2-one (XII) (100mg.) was reduced with lithium tetrahydroaluminate (500mg.) in ether as described above. The product was recrystallized from benzene to yield *2-hydroxy-3-methylcholanthrene* (VIII) (80mg.) in needles, m.p. 186° (decomp.) (Found: C, 89·0; H, 5·8. $C_{21}H_{16}O$ requires C, 88·7; H, 5·7%); $\lambda_{max.}$ at 223, 237, 262, 276, 284, 295, 304, 324·5, 340, 356·5, 375 and 391 mμ (log ε 4·49, 4·45, 4·48, 4·54, 4·81, 4·91, 4·18, 3·61, 3·79, 3·85, 3·78 and 3·04 respectively). The *acetate*, prepared from the hydroxy compound (VIII) with acetic anhydride in pyridine, separated from ethanol in needles, m.p. 174° (Found: C, 84·7; H, 5·7. $C_{23}H_{18}O_2$ requires C, 84·6; H, 5·6%); $\lambda_{max.}$ at 221, 235, 260, 273, 284, 295, 303, 325, 340, 356, 375 and 391 mμ (log ε 4·89, 4·47, 4·70, 4·70, 4·92, 5·02, 4·15, 3·66, 3·85, 3·91, 3·74 and 3·03 respectively). When a little of the hydroxy compound (VIII) was oxidized with $Na_2Cr_2O_7$ and the product methylated, a compound with the properties of the ester (XI, R=Me) was detected on thin-layer chromatograms. The ester (VI, R=Me) was not detected.

Alternative methods of preparation of the isomeric dihydroxy compounds (VII). 1-Acetoxy-3-methylcholanthrene (100 mg.) and lead tetra-acetate (140mg.), in acetic acid (5ml.), were heated at 100° for 30min. The solution was poured into water and the solid heated under reflux with KOH (100mg.) in methanol (5ml.) for 10min. The solution was poured into water (100ml.) and the mixture extracted three times with ether (25ml.). The ether was evaporated and the residue chromatographed on six thin-layer chromatograms developed in solvent (c). Bands at R_F 0·28 and 0·20, detected by their violet fluorescence in u.v. light, were removed and the absorbed material was eluted with ether. The ether was evaporated and the residues were recrystallized from aqueous ethanol. That derived from the faster-moving bands yielded *cis*-1,2-dihydroxy-3-methylcholanthrene (12mg.) as pale-green needles, m.p. and mixed m.p. 223–225°, and that from the slower-moving band yielded *trans*-1,2-dihydroxy-3-methylcholanthrene (25mg.) as pale-green needles, m.p. and mixed m.p. 205°.

2-Acetoxy-3-methylcholanthrene (10mg.) and lead tetra-acetate (15mg.), in acetic acid (1ml.), were similarly allowed to react. The product was hydrolysed with KOH in methanol and the hydrolysate examined on thin-layer chromatograms developed with solvent (c). Products with the mobilities and properties of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII) were detected, the latter isomer forming the larger spot. Both products had the expected u.v.-absorption spectra.

3-Methylcholanthrylene (XIV) (22mg.), in ethanol (2ml.), was cooled to −5° and an aqueous solution of $KMnO_4$ (5mg.), in aq. 5% (w/v) $MgSO_4$ (0·5ml.), was added over 1·5hr. The mixture was decolorized with SO_2 and extracted with ether (50ml.). The ether was evaporated and the residue examined on thin-layer chromatograms developed with solvent (c). A product with the mobility and properties of *cis*-1,2-dihydroxy-3-methylcholanthrene (VII) was detected, together with a small amount of the *trans*-isomer.

3-Methylcholanthrylene (XIV) (100mg.), in benzene (5ml.), was treated with silver benzoate (200mg.) and iodine (98mg.) according to Hopkins, Brooks & Young (1962) for the preparation of *cis*- and *trans*-1,2-dihydroxyacenaphthene. The benzoates thus obtained formed red gummy solids: they were hydrolysed with KOH in dioxan–methanol and at room temperature as described by Hopkins *et al.* (1962). If the addition reaction was carried out at room temperature, *cis*-1,2-dihydroxy-3-methylcholanthrene (VII) (21mg.) was formed, separating from aq. ethanol in pale-green needles, m.p. and mixed m.p. 225°, whereas if the reaction was carried out under reflux, *trans*-1,2-dihydroxy-3-methylcholanthrene (VII) (15mg.) was obtained, separating from aq. ethanol in pale-green needles, m.p. and mixed m.p. 206°. When the benzoate obtained from the reaction carried out at room temperature was hydrolysed with KOH in boiling methanol a product was obtained that, when examined on thin-layer chromatograms, was found to contain compounds with the properties of both the *cis*- and the *trans*-isomers of (VII).

3-Methylcholanthrene-1,2-quinone (IV) (50mg.), in dry ether (50ml.), was reduced with lithium tetrahydroaluminate (100mg.) as described above. The product was examined on thin-layer chromatograms developed with solvent (c) and found to contain a compound with the mobility and properties of *trans*-1,2-dihydroxy-3-methylcholanthrene, together with small amounts of products with the mobilities and properties of the *cis*-dihydroxy compound and of 1- and 2-hydroxy-3-methylcholanthrene (X and VIII).

Oxidation of 3-methylcholanthrylene (XIV) *with perbenzoic acid*. In an attempt to prepare 1,2-epoxy-3-methylcholanthrene, 3-methylcholanthrylene (XIV) (500mg.) and perbenzoic acid (260mg.), in chloroform (12ml.), were kept at 0° for 48hr. The solid that separated was recrystallized from benzene to yield a *monobenzoate* of *cis*-1,2-dihydroxy-3-methylcholanthrene (220mg.), separating from benzene in needles, m.p. 213° (Found: C, 82·7; H, 5·1. $C_{28}H_{20}O_3$ requires C, 83·15; H, 5·0%); $\lambda_{max.}$ at 225, 232, 263, 274, 284, 295, 325, 341, 357 and 376 mμ. The infrared spectrum showed strong carbonyl absorption at 1730 $cm.^{-1}$. Hydrolysis of the product with KOH in boiling methanol yielded *cis*-1,2-dihydroxy-3-methylcholanthrene (VII), m.p. and mixed m.p. 225°.

The chloroform mother-liquors were examined on thin-layer chromatograms developed with solvents (b) and (c) and found to contain a product with the mobility and fluorescence of 3-methylcholanthren-2-one (XII). A product with the expected properties of the 1,2-epoxy compound was not detected.

cis-*11,12-Dihydro-11,12-dihydroxy-3-methylcholanthrene* (V). This was prepared from the hydrocarbon (III) by the action of OsO_4 as described by Cook & Schoental (1948), when it formed needles from benzene, m.p. 216–217° (decomp.); $\lambda_{max.}$ at 219, 239, 257, 265, 274, 311 and 348 mμ (log ε 4·69, 4·34, 4·60, 4·73, 4·73, 4·15 and 3·28 respectively). Cook & Schoental (1948) report m.p. about 215° (decomp.). When the dihydrodihydroxy compound (V) was heated with HCl in acetic acid as described by these authors, a phenol was obtained that is probably 11-hydroxy-3-methylcholanthrene (I, R=H). This was difficult to purify, but the methoxy derivative (I, R=Me), prepared by the

action of diazomethane in ether on the phenol and purified by chromatography in benzene on Florisil, crystallized from light petroleum (b.p. 80–100°) in yellow needles, m.p. 203–204°; $\lambda_{max.}$ at 225, 264, 285, 295, 309, 344, 357, 360, 368 and 399 mμ. Cook & Schoental (1948) give m.p. 204–205°. The possibility that the phenol (I, R=H) and its derivative are the 12-isomers is not excluded, but is less likely.

3-*Methylcholanthrene*-11,12-*quinone*. *cis*-11,12-Dihydro-11,12-dihydroxy-3-methylcholanthrene (V) (1g.), in acetic acid (50ml.), was heated to 60° and $Na_2Cr_2O_7$ (5g.) was added and the mixture stirred for 15min. The solid that separated was recrystallized from acetic acid to yield 3-*methylcholanthrene*-11,12-*quinone* (850mg.) in red needles with a metallic lustre, decomp. at 238° (Found: C, 84·4; H, 4·7. $C_{21}H_{14}O_2$ requires C, 84·5; H, 4·7%); $\lambda_{max.}$ at 228, 275 and 310 mμ.

trans-11,12-*Dihydro*-11,12-*dihydroxy*-3-*methylcholanthrene* (V). 3-Methylcholanthrene-11,12-quinone (500mg.), in ether (250mg.), was reduced with lithium tetrahydroaluminate (1g.) and the product isolated as before. trans-11,12-*Dihydro*-11,12-*dihydroxy*-3-*methylcholanthrene* (V) (210mg.) separated from benzene in needles, m.p. 193° (Found: C, 83·0; H, 6·1. $C_{21}H_{18}O_2$ requires C, 83·4; H, 6·0%), $\lambda_{max.}$ at 220, 240, 255, 265, 275, 294, 310 and 351 mμ (log ε 4·57, 4·28, 4·45, 4·65, 4·69, 4·04, 4·12 and 3·31 respectively). The compound (V) was hydrolysed with HCl in acetic acid and the product methylated with diazomethane and purified as before to yield 11-methoxy-3-methylcholanthrene (I, R=Me) in yellow needles, m.p. and mixed m.p. 201–202°.

11,12-*Epoxy*-11,12-*dihydro*-3-*methylcholanthrene* (II). To a solution of *cis*-11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V) (1·55g.) in methanol (2l.) and water (300ml.) was added a solution of $NaIO_4$ (4·3g.) in methanol (400ml.) and water (75ml.). The solution was stirred at room temperature for 24hr. and filtered and the filtrate was evaporated to 250ml. under reduced pressure. The solid that separated was recrystallized from light petroleum (b.p. 80–100°) to yield 3-*methyl*-7-*phenylacenaphthene*-2′,8-*dicarboxaldehyde* (800mg.) in pale-yellow crystals, m.p. 151–152° (Found: C, 83·95; H, 5·4. $C_{21}H_{16}O_2$ requires C, 84·0; H, 5·4%). The dialdehyde (500mg.), in dry benzene (5ml.), was heated under reflux with tri(dimethylamino)-phosphine (0·5ml.), following the method of Newman & Blum (1964). After 15min. the solution was cooled in ice and the product that separated was recrystallized from benzene to yield 11,12-*epoxy*-11,12-*dihydro*-3-*methylcholanthrene* (II) (275mg.) in needles that turned orange at 140°, softened and darkened at 222° and melted at 255–265° (Found: C, 88·5; H, 5·4. $C_{21}H_{16}O$ requires C, 88·7; H, 5·7%); $\lambda_{max.}$ at 260, 269, 280, 302, 313, 324, 342 and 359 mμ (log ε 4·52, 4·71, 4·76, 4·09, 4·14, 4·11, 3·83 and 3·82 respectively).

The epoxide (II) was heated under reflux with aq. 50% (v/v) acetone (100ml.) containing conc. HCl (1ml.) for 30min. The acetone was distilled off under reduced pressure and the mixture extracted with ether (50ml.). The residue obtained on evaporation of the ether contained a product that had the same mobility and properties on thin-layer chromatograms as the phenol (I, R=H) described above. The methyl ether, prepared and purified as before, had m.p. 200–202°, undepressed in admixture with the methyl ether (I, R=Me).

The epoxide (II) (100mg.), in aq. 50% (v/v) acetone (250ml.), was heated under reflux for 48hr. The acetone was distilled off under reduced pressure and the solid collected and washed with aq. 2N-KOH (100ml.) and recrystallized twice from benzene to yield *trans*-11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V) (18mg.), m.p. and mixed m.p. 193°. The u.v. spectrum was identical with that of the *trans*-isomer described above. The aqueous layer was acidified with HCl and extracted with ether. The ether extract was examined on thin-layer chromatograms developed with solvent (b) and found to contain a product with the mobility and fluorescence of the phenol (I, R=H).

S-(11,12-*Dihydro*-12-*hydroxy*-3-*methyl*-11-*cholanthrenyl*)-*glutathione* (IX). The epoxide (II) (50mg.), $NaHCO_3$ (100mg.) and glutathione (50mg.), in aq. 50% (v/v) acetone (200ml.), were heated under reflux for 6hr. The acetone was distilled off under reduced pressure and the mixture extracted with ether (50ml.). The ether extract was examined on thin-layer chromatograms developed in solvent (b) or (c) and shown to contain compounds with the mobilities and properties of the phenol (I, R=H) and the dihydrodihydroxy compound (V). The aqueous layer was acidified to pH4 with acetic acid and activated charcoal (British Drug Houses Ltd., Poole, Dorset) (5g.) was added. The charcoal was filtered off and washed with water (250ml.) and the adsorbed material eluted with methanol (500ml.) containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). Evaporation of the solvent yielded a gum that is probably a mixture of the ammonium salts of the diastereoisomeric forms of *S*-(11,12-dihydro-12-hydroxy-3-methyl-11-cholanthrenyl)-glutathione (IX), although the possibility of their being the isomeric 11-hydroxy-12-cholanthrenyl derivatives is not excluded. The u.v.-absorption spectrum, measured in methanol, showed $\lambda_{max.}$ at 222, 268, 277, 310 and 352 mμ. On paper chromatograms, the product had R_F 0·10 and 0·45 in solvents 1 and 2 respectively, had a dark-violet fluorescence in u.v. light and gave a purple colour with ninhydrin and a positive reaction with the platinic iodide reagent. The conjugate was decomposed rapidly with conc. HCl to yield three products, one of which had the u.v.-absorption spectrum, the chromatographic mobility in solvents (a) and (b) and the fluorescence of 3-methylcholanthrene (III) on thin-layer chromatograms. Of the other two products, one had R_F 0·18 and 0·61 on paper chromatograms in solvents 1 and 2 respectively, a bright-blue fluorescence in u.v. light and gave a purple colour with ninhydrin and a weak positive reaction with the platinic iodide reagent: it is probably *S*-(3-methyl-11-cholanthrenyl)glutathione. The other product was indistinguishable from oxidized glutathione on paper chromatograms developed with solvent 2: reduced glutathione was not detected.

Experiments with rat-liver homogenates

Young male rats of the Chester Beatty strain (body wt. approx. 180g.) were used in these experiments, each of which was carried out with the livers of four of these rats. The livers were homogenized in 200ml. of ice-cold 1·15% (w/v) KCl in a Potter & Elvehjem (1936) type of homogenizer with a Teflon pestle. The homogenate was centrifuged at 0° for 20min. at $1480g_{av.}$ in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd., Birmingham) and the supernatant diluted with an equal amount of 0·1M-phosphate buffer, pH7·4, prepared from NaH_2PO_4 and Na_2HPO_4. Nicotinamide (1·76g.) (Roche Products Ltd.),

$NADP^+$ (30mg.) and glucose 6-phosphate (250mg.) (both as the sodium salts from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) were added and the mixture was heated in a water bath to 37° while a slow stream of air was drawn through. The substrate (2–10mg., as indicated below), in ethanol (5ml.), was added and the incubation continued for 30min. The mixture was extracted twice with ether (250ml.): if necessary the organic and the aqueous phase were separated by centrifugation. In experiments in which the aqueous phases were not examined, better yields of the metabolites were obtained if the incubation mixtures were first acidified with conc. HCl.

The ether extract was dried over Na_2SO_4 and evaporated and the residue chromatographed on two thin-layer chromatograms that were developed for 15cm. in solvent (b). Fluorescent bands, seen when the chromatograms were examined in u.v. light, were removed and the adsorbed material was eluted with ether. The fractions thus obtained were each separately chromatographed on fresh thin-layer chromatograms in solvent (a), (b) or (c). The R_F values given below are those obtained when the fractions were rechromatographed. The subsequent treatment of the various fractions is described below: usually only one of these investigations could be carried out on the material obtained from one incubation.

The aqueous phase was heated at 100° for a few minutes to coagulate protein and filtered and the filtrate was acidified to pH4 with acetic acid and charcoal (5g.) was added. The charcoal was filtered off and washed with water (250ml.) and the adsorbed material eluted with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0·88). The solvent was removed under reduced pressure and the residue examined as described below.

Control experiments were carried out in which the rat-liver homogenates were boiled for 5min. and cooled to room temperature before the addition of the factors described above. The mixtures were then incubated at 37° in the presence of the substrates and the mixture was extracted and the products were examined as before. One incubation was carried out with fresh rat-liver homogenate in the absence of a substrate: none of the metabolites described below was detected.

Experiments were also carried out in which 3-methylcholanthrene (III) was incubated with mouse-liver homogenates: the conditions were the same as in the experiments with rat-liver homogenates.

As far as possible all the operations described above were carried out in the dark or in the absence of direct sunlight to reduce the possibility of photochemical oxidations occurring during the incubations and the preparation procedures.

RESULTS

Metabolism of 3-*methylcholanthrene* (III). In each experiment the hydrocarbon (10mg.) was incubated with rat-liver homogenate as described above. The ether-soluble portion yielded ten fractions by chromatography on thin-layer chromatograms.

Fraction 1 was identified as unchanged 3-methylcholanthrene (III) by its mobility in solvents (a) and (b) and by its u.v.-absorption spectrum.

Fraction 2 sometimes contained an unidentified metabolite, which had R_F 0·49 in solvent (b) and a violet fluorescence in u.v. light. Its u.v.-absorption spectrum showed $\lambda_{max.}$ at 232, 263, 272, 284, 295, 302, 325, 355, 374 and 391mμ.

Fraction 3 consisted of a product with the mobility in solvents (b) and (c) and the fluorescence of 3-methylcholanthren-1-one (XIII), which, on oxidation with sodium dichromate and methylation of the product, yielded a compound indistinguishable from the ester (VI, R = Me) on thin-layer chromatograms. Sometimes the fluorescence in u.v. light was yellow–green, indicating the probable presence of 3-methylcholanthren-2-one (XII) as well as the ketone (XIII).

Fractions 4 and 5 contained small amounts of compounds, R_F 0·37 and 0·32 in solvent (b), both of which had a violet fluorescence in u.v. light that changed to pink and green respectively when the chromatograms were exposed to ammonia. The faster-moving compound gave a blue colour when the chromatograms were sprayed with a 0·5% solution of 2,6-dichloroquinonechloroimide in ethanol followed by aq. 10% (w/v) sodium carbonate. The probable structures of these compounds are discussed below.

Fraction 6, R_F 0·20 and 0·62 in solvents (b) and (c) respectively, was identical in its mobility and fluorescence with 1-hydroxy-3-methylcholanthrene (X), and it had the expected u.v.-absorption spectrum. Oxidation with sodium dichromate and methylation of the product yielded two esters: that present in the larger amount had the mobility and properties of the ester (VI, R = Me) and the other those of the ester (XI, R = Me) on thin-layer chromatograms developed with solvents (b) and (c). The u.v.-absorption spectrum of the former product was identical with that of the ester (VI, R = Me). When the metabolite was allowed to stand in air for some hours and examined on thin-layer chromatograms developed with solvent (b) or (c), an additional spot with the mobility and properties of the ketone (XIII) was detected. When the metabolite was treated with acetic anhydride (0·1ml.) in pyridine (0·1ml.) and the product examined on thin-layer chromatograms, it was found to be converted into a product with the mobility in solvents (b) and (c) of 1-acetoxy-3-methylcholanthrene. The u.v.-absorption spectrum of this product showed the expected maxima.

Fraction 7, R_F 0·18 and 0·82 in solvents (b) and (c) respectively, had an olive-green fluorescence in u.v. light and was indistinguishable from 3-methylcholanthrene-1,2-quinone (IV).

Fraction 8, R_F 0·15 and 0·55 in solvents (b) and (c) respectively, was identical in its mobility and properties on thin-layer chromatograms with 2-hydroxy-3-methylcholanthrene (VIII). It had the expected u.v.-absorption spectrum and, after

oxidation with sodium dichromate and methylation of the product, yielded a compound with the mobility and properties on thin-layer chromatograms developed with solvents (b) and (c) of the ester (XI, R = Me). The u.v.-absorption spectrum was the same as that of the synthetic ester. The metabolite was slowly oxidized in air to give a product that was indistinguishable from the ketone (XII) on thin-layer chromatograms. Acetylation as before yielded a product with the mobility on thin-layer chromatograms developed in solvent (b) or (c) and with the u.v.-absorption spectrum of 2-acetoxy-3-methylcholanthrene.

Fraction 9, R_F 0·10 in solvent (b), had a violet fluorescence in u.v. light and its u.v.-absorption spectrum showed $\lambda_{max.}$ at 223, 236, 263, 274, 285, 295, 325, 341, 357 and 375mμ. When the metabolite was treated with acetic anhydride in pyridine, it was converted into a product, R_F 0·49 in solvent (b), whose u.v. spectrum showed the same maxima as that of the parent metabolite. Oxidation of the metabolite with sodium dichromate and methylation of the product did not yield any recognizable products. A possible structure for the metabolite is discussed below.

Fraction 10, which consisted of the material left at the base lines of the original chromatograms, was examined on thin-layer chromatograms developed with solvent (c). Four major products were detected, together with small amounts of several unidentified fluorescent substances. The first metabolite, R_F 0·28, had the chromatographic properties of *cis*-1,2-dihydroxy-3-methylcholanthrene (VII), the second, R_F 0·23, had the properties of 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V), and the third, R_F 0·20, the properties of *trans*-1,2-dihydroxy-3-methylcholanthrene (VII). The fourth product had R_F 0·16.

On the two-dimensional chromatograms described above, it was shown that the metabolites with the mobilities of the isomeric dihydroxy compounds (VII) were each converted into a product with the chromatographic mobility in solvent (b) and the properties of 3-methylcholanthren-2-one (XII). The ketone derived from the *cis*-isomer formed a larger spot on the chromatogram than that derived from the *trans*-isomer, indicating that the *cis*-isomer was the major product. By combining the products from a number of incubations, it was shown that the u.v.-absorption spectrum of the ketone derived from the *cis*-isomer was identical with that of the synthetic product (XII). It was also shown that, when this decomposition product was oxidized with sodium dichromate and the product methylated, a compound with the chromatographic mobility in solvents (b) and (c) of the ester (XI, R = Me) was formed. When the mixture of metabolites in fraction 10 was acetylated as before, products with the mobilities and properties on thin-layer chromatograms of *cis*- and *trans*-1,2-diacetoxy-3-methylcholanthrene were detected.

On these two-dimensional chromatograms, the product of R_F 0·23 was decomposed to yield a phenol with the mobility in solvent (b) and the fluorescence in u.v. light of 11-hydroxy-3-methylcholanthrene (I, R = H), thus providing additional evidence for the presence of the parent compound (V) in the mixture of metabolites.

The product of R_F 0·16 was shown on these two-dimensional chromatograms to yield two products with acid, R_F 0·46 and 0·27 respectively, that appeared to be phenolic in character: both products formed spots with a violet fluorescence in u.v. light, which changed to light-blue and blue respectively when the chromatograms were exposed to ammonia. Neither the phenol nor the parent compound, which appeared to be a dihydrodihydroxy compound, was identified.

The gummy material obtained from the aqueous portion as described above was applied to the base line of a paper chromatogram, which was developed with solvent 1. The paper was dried and examined in u.v. light and the dark-violet fluorescent band at about R_F 0·10 was cut out and the adsorbed material eluted with methanol containing 1% (v/v) of aq. ammonia (sp.gr. 0·88). The solvent was evaporated and the small residue rechromatographed on paper and the product located and eluted as before. The u.v.-absorption spectrum of the material thus obtained showed $\lambda_{max.}$ at 222, 268, 277, 310 and 352mμ. The product was indistinguishable in its chromatographic mobility in solvents 1 and 2, in its fluorescence in u.v. light and in its reaction with ninhydrin and the platinic iodide reagent from the synthetic glutathione conjugate (IX) described above. Three products were formed when the conjugate was acidified with conc. hydrochloric acid. One was identical in its mobility on paper chromatograms in solvents 1 and 2, in its bright-blue fluorescence in u.v. light and in its reaction with ninhydrin and the platinic iodide reagent with the acid-decomposition product of the conjugate (IX) described above. The second product was indistinguishable on paper chromatograms developed with solvent 2 from oxidized glutathione. The third decomposition product was indistinguishable in its u.v. spectrum and in its mobility on thin-layer chromatograms developed in solvents (a) and (b) from 3-methylcholanthrene (III).

None of the products described above was detected in experiments using heated homogenates.

In the experiments with mouse-liver homogenates, metabolites with the chromatographic properties of 1- and 2-hydroxy-3-methylcholanthrene (X and VIII), 3-methylcholanthren-1-one (XIII),

3-methylcholanthrene-1,2-quinone (IV), *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII), 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V) and the unidentified product of R_F 0·10 in solvent (b) were detected. The unidentified phenols and dihydrodihydroxy compound were also found and the aqueous fraction was not examined.

Metabolism of 1- *and* 2-*hydroxy*-3-*methylcholanthrene* (X *and* VIII). Samples (2mg.) of either (X) or (VIII) were used in each experiment, and only the ether-soluble material was examined.

1-Hydroxy-3-methylcholanthrene (X) was converted into a product with the mobility on thin-layer chromatograms developed in solvents (b) and (c), the fluorescence in u.v. light, and the u.v.-absorption spectrum of 3-methylcholanthren-1-one (XIII). Oxidation of this metabolite with sodium dichromate and methylation of the product yielded a compound with the chromatographic mobility in solvents (b) and (c) of the ester (VI, R = Me). 2-Hydroxy-3-methylcholanthrene (VIII) yielded 3-methylcholanthren-2-one (XII), which was similarly identified. A product with the mobility of the ester (XI, R = Me) on thin-layer chromatograms was obtained when the metabolite was oxidized and the product methylated as before.

Both hydroxy compounds (X and VIII) also yielded mixtures of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII), which were identified in the same way as those obtained in the metabolism of the parent hydrocarbon (III). In both cases, more *cis*- than *trans*-isomer appeared to have been formed, as judged from the sizes and the intensities of the spots of the ketone (XII) produced when the products were examined on two-dimensional thin-layer chromatograms as described above.

Both hydroxy compounds yielded a third metabolite, which had R_F 0·01 in solvent (c) and was detected by its violet fluorescence in u.v. light. It was converted by hot conc. hydrochloric acid into a product that had R_F 0·05 in solvent (b) and 0·15 in solvent (c) and an orange–yellow fluorescence in u.v. light.

Products were sometimes detected in these experiments that resembled the unidentified products, R_F 0·49 in solvent (b), detected in the experiments with the hydrocarbon (III), in their mobilities, their fluorescence in u.v. light and their u.v.-absorption spectra. They were not identified.

In experiments with heated homogenates, the hydroxy compounds (X and VIII) yielded the related ketones (XIII and XII), which were identified in the manner already described. The other products formed in the enzymic experiments were not detected.

Metabolism of 3-*methylcholanthren*-1- *and* -2-*one* (XIII *and* XII). Experiments were carried out on 2mg. samples of these compounds, and, in both cases, the unchanged ketones were detected. Both ketones yielded products that were identified as the respective hydroxy compounds (X) and (VIII) by their mobilities on thin-layer chromatograms in solvents (b) and (c), by their u.v.-absorption spectra and by the mobilities of the methyl esters obtained from the products of the oxidations with sodium dichromate, that from the ketone (XIII) yielding mainly the ester (VI, R = Me) and that from the ketone (XII) yielding only the ester (XI, R = Me).

Both ketones were converted by the homogenates into a mixture of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII), which were identified on two-dimensional thin-layer chromatograms as before. The *cis*-isomer appeared to be the major product.

3-Methylcholanthren-1-one (XIII) yielded a product that had R_F 0·05 and 0·10 in solvents (b) and (c) respectively and a bright-green fluorescence in u.v. light. 3-Methylcholanthren-2-one (XII), on the other hand, yielded a product which had the same R_F values as that formed from the 1-isomer, but which had an orange–yellow fluorescence: it resembled the product detected after the acid-decomposition of the slow-moving metabolites of the hydroxy compounds (X and VIII). Possible structures for these products are discussed below.

Metabolites with the properties of the unidentified 3-methylcholanthrene metabolite of R_F 0·49 in solvent (*b*) were sometimes detected in these experiments.

Metabolism of cis- *and* trans-1,2-*dihydroxy*-3-*methylcholanthrene* (VII). Experiments were carried out on 5mg. samples of the isomers, and in both cases products with the mobilities in solvent (b) and the u.v.-absorption spectra of the unidentified 3-methylcholanthrene metabolite of R_F 0·49 were detected.

Although large amounts of the substrates were unchanged in these incubations, some conversions of the *cis*- into the *trans*- and of the *trans*- into the *cis*-isomer occurred, since small spots with the chromatographic mobilities of the second isomers were always detected. These were further identified by their decomposition on two-dimensional thin-layer chromatograms by the formation of products indistinguishable from the ketone (XII). These isomerizations were apparently enzymic in nature because they did not occur either when heated homogenates were used or when the dihydroxy compounds (VII) were each heated at 50° for 2hr. with 0·1M-phosphate buffer.

The *cis*- and the *trans*-dihydroxy compounds (VII) were both converted by homogenates into a product with the mobility on thin-layer chromatograms and the fluorescence in u.v. light of the slow-running metabolite of the hydroxy compounds

(X and VIII), and these were both decomposed by acid into a product with the mobility and fluorescence of the slow-running metabolite of 3-methylcholanthren-2-one (XII).

Occasionally, a product with the mobility in solvents (b) and (c) and the fluorescence of 3-methylcholanthrene-1,2-quinone (IV) was detected in these incubations: this product was also detected in the experiments with heated homogenates.

Metabolism of 3-*methylcholanthrylene* (XIV). In these experiments, which were carried out on 10mg. samples, six fractions were detected in the ether-soluble portion and separated as before. Fraction 1 was identified as 3-methylcholanthren-2-one (XII) by its mobility in solvents (b) and (c), by its fluorescence in u.v. light and by its u.v.-absorption spectrum. Fraction 2 was indistinguishable from the quinone (IV) in its mobility in solvents (a) and (b) and in its fluorescence. Fraction 3 was identified by its mobility in solvents (b) and (c), by its fluorescence and by its u.v.-absorption spectrum as 2-hydroxy-3-methylcholanthrene (VIII). The ester obtained when the product of the oxidation of this metabolite with sodium dichromate was methylated was indistinguishable on thin-layer chromatograms from the ester (XI, R = Me).

Fractions 4 and 5 contained metabolites indistinguishable from *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII): these were identified by their chromatographic mobilities in solvent (c) and by their conversion into products indistinguishable from the ketone (XII) on two-dimensional thin-layer chromatograms. From an inspection of these chromatograms it was evident that the *trans*-compound was the major product.

A metabolite in fraction 6 was identical in its mobility in solvents (b) and (c) and in its fluorescence with the slow-running metabolite of 3-methylcholanthren-2-one (XII).

The aqueous fraction contained a product that had R_F 0·15 on paper chromatograms developed with solvent 1. It was detected by its dark absorption when the chromatograms were examined in u.v. light, and it gave a purple colour with ninhydrin and a positive reaction with the platinic iodide reagent. The product was not further investigated but it is presumed to be *S*-(2-hydroxy-3-methyl-1-cholanthrenyl)glutathione or possibly the isomeric 1-hydroxy-2-cholanthrenyl derivative.

Metabolism of 11,12-*epoxy*-11,12-*dihydro*-3-*methylcholanthrene* (II). The epoxide (II) (10mg.) was incubated with rat-liver homogenate as before, and both the ether-soluble and the aqueous fractions were examined. The ether-soluble fraction contained two products, the first of which was identified by its mobility in solvents (b) and (c), its fluorescence and its u.v.-absorption spectrum as the phenol that probably has the structure (I). The second product had the mobility of 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (V) in solvent (c); additional evidence for its structure was provided by the fact that on two-dimensional thin-layer chromatograms it was decomposed with acid to a product with the mobility and fluorescence of the phenol (I).

The aqueous phase contained a compound with the mobility in solvents 1 and 2 on paper chromatograms of the glutathione conjugate (IX). It was detected as a dark-violet fluorescent spot when the chromatograms were examined in u.v. light and it gave a purple colour with ninhydrin and a positive reaction with the platinic iodide reagent. When the conjugate was acidified with conc. hydrochloric acid, it yielded products with the chromatographic properties of those previously detected in the acid-decomposition of the synthetic conjugate and of that found in the metabolism of the hydrocarbon (III).

In experiments with heated homogenates, products with the chromatographic properties of the epoxide (II) together with relatively small amounts of compounds with the properties of the phenol (I) and the dihydrodihydroxy compound (V) were detected on thin-layer chromatograms. The glutathione conjugate (IX) was not found in the aqueous fraction.

DISCUSSION

The acid (VI, R = H) can be decarboxylated to 8,9-dimethylbenz[*a*]anthracene-7,12-quinone, the structure of which was proved by an unambiguous synthesis (Cook & Haslewood, 1934). Since 1-hydroxy-3-methylcholanthrene (X) and the related ketone (XIII) both yield mainly this acid in the chromic acid oxidation of the hydrocarbon (III) the positions of the hydroxy and oxo groups at C-1 on the molecule are established. The structure of the second oxidation product of the hydrocarbon (III) has not been proved unambiguously, but an acid with the proposed structure (XI, R = H) would be expected if, in the minor oxidative pathway, the primary attack of the oxidizing agent was at C-2 on the hydrocarbon (III). The u.v.-absorption spectrum of the related methyl ester (XI, R = Me) is that expected for a derivative of benz[*a*]anthracene-7,12-quinone.

The position of a hydroxyl group at C-1 of the isomeric dihydroxy compounds (VII) is shown by their syntheses from 1-acetoxy-3-methylcholanthrene. Since only the acid (XI, R = H) was formed in the chromic acid oxidations of these compounds, the second hydroxyl group is probably at C-2, and this is confirmed by the existence of *cis*- and *trans*-isomers. The light-absorption curves are those expected for hydroxy derivatives of 3-methylcholanthrene (III) that are not phenolic.

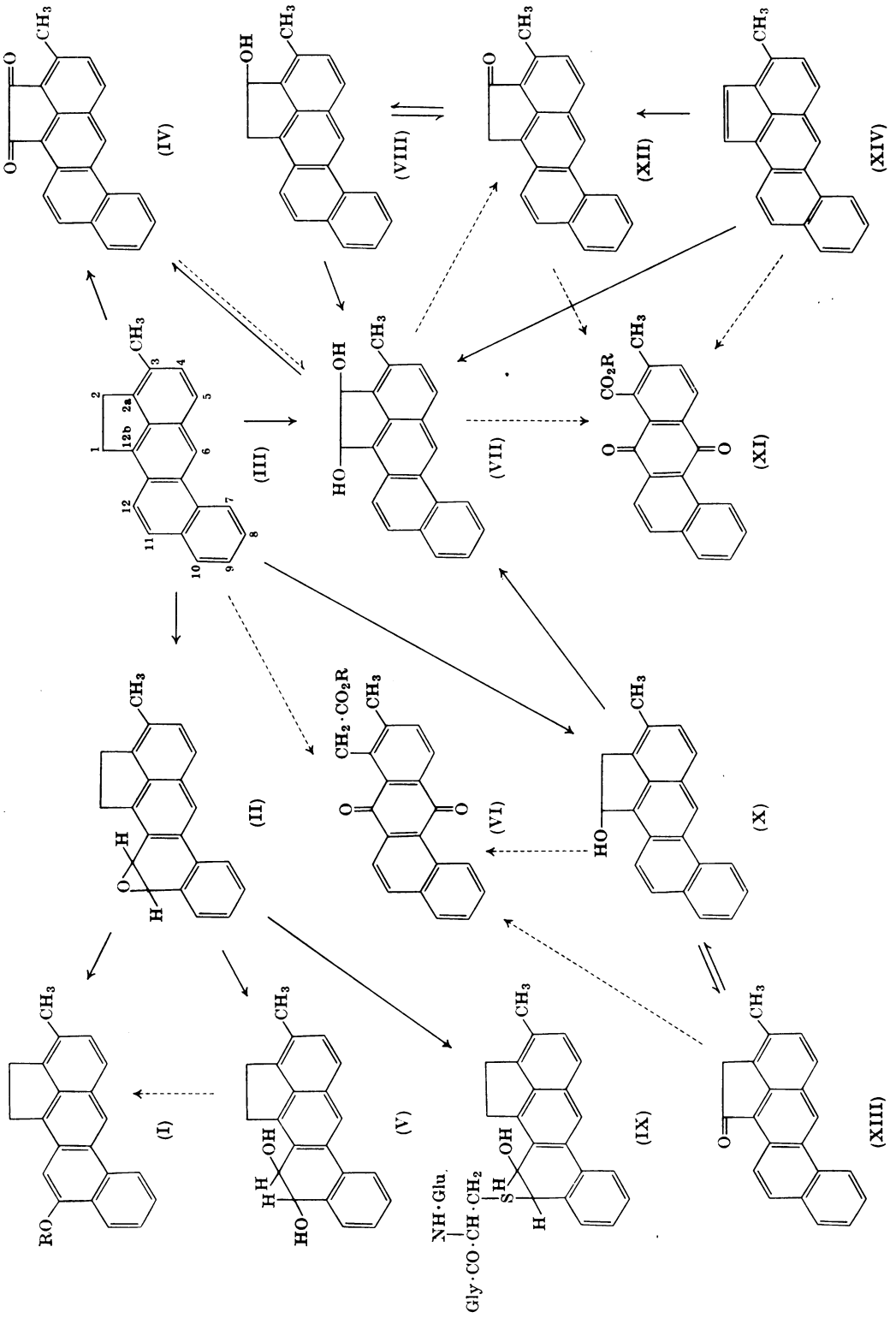
The formation of 3-methylcholanthrylene (XIV) in the lead tetra-acetate oxidation of the hydrocarbon (III) is unexpected and it probably arises from 1-acetoxy-3-methylcholanthrene by loss of acetic acid, probably during the column chromatography. 1-Acetoxyacenaphthene is converted into acenaphthylene on passing through a heated tube (Flowers & Miller, 1947). The bromination of the hydrocarbon (III) presumably leads to the formation of the 1-bromo derivative, which loses hydrogen bromide across the 1,2-positions to yield the hydrocarbon (XIV). The formation of only the acid (XI, R = H) in the chromic acid oxidation of the hydrocarbon (XIV) is additional evidence for its structure.

3-Methylcholanthrene-1,2-quinone (IV) probably arises by oxidation of the isomeric dihydroxy compounds (VII), either during the alkaline hydrolysis of their acetoxy derivatives or on the Florisil column. The formation of the isomeric dihydroxy compounds (VII) in the reduction of the diketone (IV) with lithium tetrahydroaluminate provides evidence for this structure. The reduction also provides confirmatory evidence that the assignment of the *cis*- or *trans*-configuration to the isomers of (VII) on the basis of their reaction with the potassium triacetylosmate reagent is correct, since the isomer believed to have the *trans*-configuration is the major product of the reduction. Acenaphthene-1,2-quinone is similarly reduced to a mixture containing 15% of the *cis*- and 45% of the *trans*-isomer of 1,2-dihydroxyacenaphthene (Trevoy & Brown, 1949). On the other hand, the permanganate oxidation of 3-methylcholanthrylene (XIV) yielded mainly the product believed to be the *cis*-isomer of (VII): the oxidation of olefins with permanganate usually gives *cis*-dihydroxy compounds. The reactions of the hydrocarbon (XIV) with silver benzoate and iodine similarly yielded the expected isomers.

The dehydration of the 1,2-dihydroxy compounds (VII) with acid yields only one ketone, which is not 3-methylcholanthren-1-one (XIII): it must therefore be the 2-isomer (XII). The product obtained from this on reduction with lithium tetrahydroaluminate is therefore 2-hydroxy-3-methylcholanthrene (VIII). *cis*- and *trans*-1,2-Dihydroxyacenaphthene are dehydrated with acid to acenaphthen-1-one (Hopkins *et al.* 1962). The ketone (VII) and the hydroxy compound (VIII) both yielded the expected acid (XI, R = H) on oxidation with chromic acid, showing that the oxo and the hydroxy groups are at C-2 on the nucleus. Additional evidence for the formulation of the hydroxy compound as (VIII) is provided by the fact that the oxidation of the related acetoxy derivative with lead tetra-acetate leads to the formation of the isomers of 1,2-dihydroxy-3-methylcholanthrene (VII).

11,12-Epoxy-11,12-dihydro-3-methylcholanthrene (II) has properties similar to those of the epoxides related to phenanthrene, benz[*a*]anthracene, 7-methylbenz[*a*]anthracene and dibenz[*a*,*h*]anthracene (Newman & Blum, 1964; Boyland & Sims, 1965*b*). It readily rearranges to the phenol (I, R = H), reacts with water to give the *trans*-isomer of the dihydrodihydroxy compound (V) and with glutathione to give the glutathione conjugate (IX). The phenol (I, R = H) and the glutathione conjugate (IX) have been formulated as shown in Scheme 1 by analogy with the structures of the products derived from the other epoxides mentioned above. The decomposition of the conjugate (IX) with acid to yield mainly the parent hydrocarbons is also analogous to the decomposition of conjugates derived from the other epoxides.

It is evident that most of the metabolic activity of 3-methylcholanthrene (III) is centred about the 1- and 2-positions and at the 11,12-bond. The small amounts of the phenols detected in fractions 4 and 5 in the experiments with rat- and mouse-liver homogenates are similar in their properties on thin-layer chromatograms to 4- and 3-hydroxybenz[*a*]anthracene (Boyland & Sims, 1964) and are therefore probably 10- and 9-hydroxy-3-methylcholanthrene respectively. The 10-isomer is possibly that detected by Harper (1959) in the metabolism of 3-methylcholanthrene (III) in mice. The product in fraction 10, which was decomposed by acid to give two unidentified phenols, could, by analogy with benz[*a*]anthracene metabolism, be 4,5-dihydro-4,5-dihydroxy-3-methylcholanthrene, but there is no direct evidence that this is so. The u.v. spectrum of the unidentified metabolite in fraction 9 was similar to that of the parent hydrocarbon (III) and to those of 1- and 2-hydroxy-3-methylcholanthrene (X and VIII) and the metabolite is apparently a hydroxy compound since it reacted with acetic anhydride. It is probable that the metabolite is 3-hydroxymethylcholanthrene: such a metabolite would be expected by analogy with the hydroxylation of the methyl groups of 7,12-dimethylbenz[*a*]anthracene in rat-liver homogenates (Boyland & Sims, 1965*a*). It is also possible that the carboxylic acid detected by Harper (1959) was derived from the metabolite by further oxidation of the hydroxymethyl group. The fact that this and the other unidentified metabolites were not formed when 1- or 2-substituted 3-methylcholanthrene derivatives or the epoxide (II) were incubated with rat-liver homogenates provides further evidence that their formation has involved attack at other positions on the hydrocarbon molecule. The unidentified metabolite in fraction 2, on the other hand, is also formed in the metabolism of 1,2-substituted derivatives of the hydrocarbon (III). The attempted identification of this metabolite was made more



Scheme 1. Reactions of compounds related to 3-methylcholanthrene. Solid arrows indicate reactions occurring in rat-liver homogenate and sometimes chemically; broken arrows indicate chemical reactions only.

difficult by the fact that it was not always formed in the incubations: the reason for this is not apparent.

1- and 2-Hydroxy-3-methylcholanthrene (X and VIII) were clearly formed from the hydrocarbon (III) by an enzymic process, but the related ketones (XIII and XII) could have arisen by the chemical oxidation of the hydroxy compounds during the incubations since they were formed from the respective hydroxy compounds when heated homogenates were used. Similarly the diketone (IV) could have arisen from the dihydroxy compounds (VII) by chemical oxidation. The ketones (XIII and XII) were themselves reduced to their respective hydroxy compounds (X and VIII) by rat-liver homogenates.

The formation of the isomeric 1,2-dihydroxy compounds (VII) is perhaps unexpected since with most substrates only one hydroxyl group is introduced enzymically. Thus, for example, 7,12-dimethylbenz[*a*]anthracene is hydroxylated either on the C-7 or on the C-12 methyl group, but not on both (Boyland & Sims, 1965*a*). Most dihydrodihydroxy compounds derived from aromatic hydrocarbons arise from the hydroxylation of double bonds by a mechanism that probably involves epoxide formation. Hydroxylation at C-1 and C-2 of acenaphthene has not been reported, but Chang & Young (1943) have shown that in whole animals these positions are oxidized to yield 1,8-naphthalic anhydride. With 3-methylcholanthrene (III), the hydroxyl groups are probably introduced in two stages since 1- and 2-hydroxy-3-methylcholanthrene (X and VIII) are themselves hydroxylated by rat-liver homogenates. Although it might be expected that random hydroxylations of this type would yield equal amounts of the two isomers, an inspection of the chromatograms showed quite clearly that the *cis*-isomer was the major product. In this respect the enzymic hydroxylation of the hydrocarbon (III) differed from its oxidation by lead tetra-acetate, where, both chromatographically and by isolation of the products, it was shown that the *trans*-isomer was the major product: it is possible that isomerization of the *cis*- to the *trans*-isomer occurred during the alkaline hydrolysis of the acetates. Similar relationships exist between the enzymic hydroxylations of 1- and 2-hydroxy-3-methylcholanthrene (X and VIII) and the oxidations of their acetates with lead tetra-acetate. The reason for these differences is not clear since, in the metabolism of the hydroxy compounds in rat-liver homogenates, both isomers behave in the same way with respect to their further metabolism.

The isomerizations of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (VII) by rat-liver homogenates resemble those of *cis*- and *trans*-1,2-dihydroxyacenaphthene and *cis*- and *trans*-1,2-dihydroxyindane, all of which are partly converted into the related isomer when injected into rats (Hopkins, Lewis & Young, 1964). Like the hydroxy compounds (VII), these isomers are stable when incubated by themselves in phosphate buffer. The mechanisms involved in these isomerizations are not known: possibly one of the hydroxy groups is oxidized to an oxo group, which is then reduced back to the hydroxyl. It is significant that these isomerizations have not been observed with dihydrodihydroxy compounds formed by the hydroxylation of aromatic double bonds; in these cases the ketones are tautomeric with, and should readily rearrange to, the corresponding dihydroxy compounds, which are known metabolites of the dihydrodihydroxy compounds.

In the metabolism of 3-methylcholanthrylene (XIV), the *trans*-isomer of 1,2-dihydroxy-3-methylcholanthrene (VII) is the predominant isomer formed. Clearly a different mechanism is in operation here, probably involving the formation of an epoxide at the 1,2-double bond. The glutathione conjugate detected in the metabolism of the hydrocarbon (XIV) presumably arises from the reaction of an epoxide of this type with glutathione, although the possibility of a reaction occurring on the K region is not excluded. 3-Methylcholanthren-2-one (XII), which is also formed in the metabolism of the hydrocarbon (XIV), probably arises either by rearrangement of 2-hydroxy-3-methylcholanthrylene, formed by monohydroxylation at C-2 or by rearrangement of the 1,2-epoxide. It is possible that an isomerization of the 1,2-epoxide to the 2-ketone (XII) occurred during the chemical oxidation of the hydrocarbon (XIV) with perbenzoic acid: the formation of the monobenzoate of the *cis*-1,2-dihydroxy compound (VII) is not unexpected (Swern, 1953). Ketones do not appear to be metabolites of indene (Brooks & Young, 1956) or of acenaphthylene (Hopkins *et al.* 1962) in whole animals, although *cis*- and *trans*-dihydrodihydroxy derivatives are formed from both hydrocarbons.

It is probable that the slow-running metabolites of the hydroxy compounds (X and VIII) and of the isomeric dihydroxy compounds (VII) are the same compound, possibly 1,2-dihydroxy-3-hydroxymethylcholanthrene. The product formed from this compound with acid should, by analogy with the decomposition of the dihydroxy compounds (VII), be 3-hydroxymethylcholanthren-2-one, which might also be expected to arise by the hydroxylation of the methyl group in 3-methylcholanthren-2-one (XII). In fact, a compound with the chromatographic properties of the acid-decomposition product is formed as a metabolite of the ketone (XII). 3-Methylcholanthren-1-one (XIII), on the other hand, forms a different metabolic product, which is probably 3-hydroxymethylcholanthren-1-one. Compounds with the suggested structures

have not been synthesized. These metabolites were not detected as products of the metabolism of 3-methylcholanthrene (III) itself, possibly because smaller amounts were formed.

The metabolic products of 3-methylcholanthrene (III) formed at the K region are similar to those reported for other hydrocarbons, such as benz[*a*]anthracene and dibenz[*a*,*h*]anthracene (Boyland & Sims, 1965*b*). The configuration of the dihydrodihydroxy compound (V) is presumed to be *trans* by analogy with the related products formed in the metabolism of these hydrocarbons. The formation of (V) and of the glutathione conjugate (IX), both from the hydrocarbon (III) and from the epoxide (II), suggests that, as with other hydrocarbons, the formation of an epoxide, in this case (II), is the initial metabolic step at the K region. The absence of the phenol (I, R = H), which could have arisen by hydroxylation of the K region of the hydrocarbon (III), is also in agreement with earlier observations that phenols of this type are not formed in the metabolism of aromatic hydrocarbons. The formation of the phenol (I, R = H) in the metabolism of the epoxide (II) is, however, in agreement with observations on the metabolism of other epoxides of this type: a probable explanation for this has been given (Boyland & Sims, 1965*b*).

There was no evidence for any metabolic action at the 6- or 12b-positions (the L region) of 3-methylcholanthrene (III). Chemically these positions are active, as indicated by the chromic acid oxidations described above, by the oxidation with perbenzoic acid (P. Sims, unpublished work) and by ozonolysis (Moriconi & Taranko, 1963), all of which yielded the acid (VI, R = H). With ozone there was also evidence for attack, to a smaller extent, at the K region and at the 2a,3- and 4,5-bonds. In the ascorbic acid–Fe^{2+}–oxygen system, the only identified products were 1- and 2-hydroxy-3-methylcholanthrene (X and VIII), their related ketones (XIII and XII) and the quinone (VI) (P. Sims, unpublished work).

The relationship between the metabolic products of the hydrocarbon (III) and its carcinogenic properties is not clear. Both 1-hydroxy-3-methylcholanthrene (X) and the related ketone (XIII) have carcinogenic properties when injected subcutaneously into mice (Shear & Leiter, 1941; Badger *et al.* 1940) and tumours have been obtained in mice injected subcutaneously with the epoxide (II) (E. Boyland & P. Sims, unpublished work): other metabolites have not been tested.

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REFERENCES

Bachmann, W. E. (1937). *J. org. Chem.* **1**, 347.
Badger, G. M., Cook, J. W., Hewett, C. L., Kennaway, E. L., Kennaway, N. M., Martin, R. H. & Robinson, A. M. (1940). *Proc. Roy. Soc.* B, **129**, 439.
Boyland, E. & Sims, P. (1964). *Biochem. J.* **91**, 493.
Boyland, E. & Sims, P. (1965*a*). *Biochem. J.* **95**, 780.
Boyland, E. & Sims, P. (1965*b*). *Biochem. J.* **97**, 7.
Brooks, C. J. W. & Young, L. (1956). *Biochem. J.* **63**, 264.
Chang, L. H. & Young, L. (1943). *J. biol. Chem.* **151**, 87.
Cook, J. W. & Haslewood, G. A. D. (1934). *J. chem. Soc.* p. 428.
Cook, J. W. & Schoental, R. (1948). *J. chem. Soc.* p. 170.
Criegee, R., Marchand, B. & Wannowius, M. (1942). *Liebigs Ann.* **550**, 99.
Fieser, L. F. & Hershberg, E. B. (1938). *J. Amer. chem. Soc.* **60**, 2542.
Flowers, R. G. & Miller, H. F. (1947). *J. Amer. chem. Soc.* **69**, 1388.
Harper, K. H. (1959). *Brit. J. Cancer*, **13**, 732.
Hopkins, R. P., Brooks, C. J. W. & Young, L. (1962). *Biochem. J.* **82**, 457.
Hopkins, R. P., Lewis, D. A. & Young, L. (1964). *Biochem. J.* **90**, 7P.
Moriconi, E. J. & Taranko, L. B. (1963). *J. org. Chem.* **28**, 2526.
Newman, M. S. & Blum, S. (1964). *J. Amer. chem. Soc.* **86**, 5598.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Shear, M. J. & Leiter, J. (1941). *J. nat. Cancer Inst.* **2**, 241.
Swern, D. (1953). In *Organic Reactions*, vol. 7, p. 378. Ed. by Adams, R. New York: John Wiley and Sons Inc.
Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
Trevoy, L. W. & Brown, W. G. (1949). *J. Amer. chem. Soc.* **71**, 1675.