(5 ml) the inorganic precipitate was removed by filtration (Dicalite), and the resulting clear solution was evaporated under reduced pressure. The residue was extracted with acetone (75 ml) which after treatment with decolorizing carbon was removed under reduced pressure. The residue was extracted with diethyl ether (50 ml) from which (*S*:*S*)-**17** (0.6 g) separated when kept at room temperature overnight. The melting point was 112–113.5°, $[\alpha]^{20}D$ +21.2° (*c* 2, ethanol).

Anal. Calcd for $C_{16}H_{30}N_2O_6$: C, 45.44; H, 7.63; N, 10.60. Found: C, 45.20; H, 7.84; N, 10.50.

The material (3.2 g) which remained undissolved after the ether extraction was recrystallized from acetone to give 1.4 g of (*S*:*S*)-**18**, mp 141–143°, $[\alpha]^{20}D$ +39.3° (*c* 2, ethanol).

Anal. Calcd for $C_8H_{14}N_2O_5$: C, 44.03; H, 6.47; N, 12.84. Found: C, 43.86; H, 6.66; N, 12.91.

(2*S*:3*S*)-N,N′-Dicarbethoxy-1,4-diamino-2,3-butanediol 2,3-Bismethanesulfonate](*S*:*S*)-2]. (a)—To a suspension of (*S*:*S*)-**14**·$2CH_3SO_3H$[1] (9.4 g) in pyridine (75 ml), ethyl chloroformate (9.5 ml) was added dropwise over a period of about 40 min while stirring at 0–5°. After additional stirring for about 30 min the mixture was poured into ice–2.5 *N* HCl (300 ml). The precipitate was washed with water and dried *in vacuo* yielding crude (*S*:*S*)-**2** (7.3 g). After recrystallization from tetrachloroethane the melting point was 162.5–163.5°, $[\alpha]^{20}D$ −12.6° (*c* 2, DMF).

(b)—To a solution of (*S*:*S*)-**17** (0.3 g) in pyridine (3 ml), methanesulfonyl chloride (0.3 ml) was added dropwise over a period of about 20 min while stirring at 0–5°. After additional stirring for about 30 min the mixture was poured into ice–2.5 *N* HCl (12 ml). The precipitate was washed with water and dried *in vacuo* yielding crude (*S*:*S*)-**2** (0.45 g), melting at 155–157°. Recrystallization from tetrachloroethane raised the melting point to 162–163°, $[\alpha]^{20}D$ −13.7° (*c* 2, DMF). The infrared spectrum (KBr) and the analysis were identical with those of the material prepared as in part a.

Anal. Found: C, 34.35; H, 5.70; N, 6.55; S, 15.22.

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Syntheses of Metabolites of 7,12-Dimethylbenz[*a*]anthracene. 4-Hydroxy-7,12-dimethylbenz[*a*]anthracene, 7-Hydroxymethyl-12-methylbenz[*a*]anthracene, Their Methyl Ethers, and Acetoxy Derivatives[1a]

JAMES W. FLESHER,[1b] SOEKENI SOEDIGDO,[1c] AND DONALD R. KELLEY

Department of Pharmacology, College of Medicine, University of Kentucky, Lexington, Kentucky

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The synthesis of 4-hydroxy-7,12-dimethylbenz[*a*]anthracene (V) was accomplished by a scheme starting with the preparation of known 4-methoxybenz[*a*]anthracene (I). This was oxidized to 4-methoxybenz[*a*]anthraquinone (II) which was treated with methylmagnesium iodide and the Grignard complex slowly added to HI in methanol affording 4-methoxy-7-iodomethyl-12-methylbenz[*a*]anthracene (III). This was reduced to 4-methoxy-7,12-dimethylbenz[*a*]anthracene (IV) which was cleaved with HBr–AcOH to give 4-hydroxy-7,12-dimethylbenz[*a*]anthracene (V). 7-Hydroxymethyl-12-methylbenz[*a*]anthracene (X) was synthesized from 7-iodomethyl-12-methylbenz[*a*]anthracene (IX). This was conveniently prepared from benz[*a*]anthraquinone (VII) by the Grignard method. A dioxane solution of IX was converted to the corresponding hydroxy compound. 4-Hydroxy-7,12-dimethylbenz[*a*]anthracene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, and 7,12-dihydroxymethylbenz[*a*]anthracene were identified in rat liver homogenates as metabolites of 7,12-dimethylbenz[*a*]anthracene. Preliminary data on carcinogenic activity of these metabolites are presented.

Although many chemical carcinogens are believed to exert their remarkable effects only after being transformed to active forms *in vivo*, in the case of polycyclic aromatic hydrocarbons it is not clear whether the parent compound or one or more metabolites are responsible for producing cancers in test animals. Since polycyclic aromatic hydrocarbons are, according to present evidence,[2a] metabolized to hydroxy derivatives by rats, it is conceivable that the active forms of the hydrocarbon are hydroxylated metabolic products.[2b,c] The synthesis of hydroxylated derivatives of the potent carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) and related compounds was therefore undertaken to provide authentic metabolites for carcinogenic testing and for direct comparison with compounds isolated in animal studies. This paper is the first of a series describing the synthesis and biological properties of metabolites of polycyclic hydrocarbons.

Initial effort was directed toward the synthesis of 4-hydroxy-7,12-dimethylbenz[*a*]anthracene which was earlier identified as a metabolite of DMBA by its fluorescence spectrum after conversion to the methoxy derivative.[3] The sequence of reactions is outlined in Scheme I. The 4-methoxybenz[*a*]anthraquinone (II) required for this synthesis was prepared by oxidation of known 4-methoxybenz[*a*]anthracene (I). This compound was prepared from 4-hydroxybenz[*a*]anthracene[4a] by methylation with dimethyl sulfate according to Sempronj.[4b] 4-Methoxybenz[*a*]anthraquinone was condensed with methyl Grignard, the Grignard solution

(1) (a) Aided, in part, by Grant E-307, from the American Cancer Society. A preliminary report was presented at the Southeastern Regional Meeting of the American Chemical Society, Louisville, Ky., Oct 29, 1966, Abstracts of Papers A53, No. 192. (b) To whom inquiries should be addressed. (c) Indonesian Government Predoctoral Fellow.

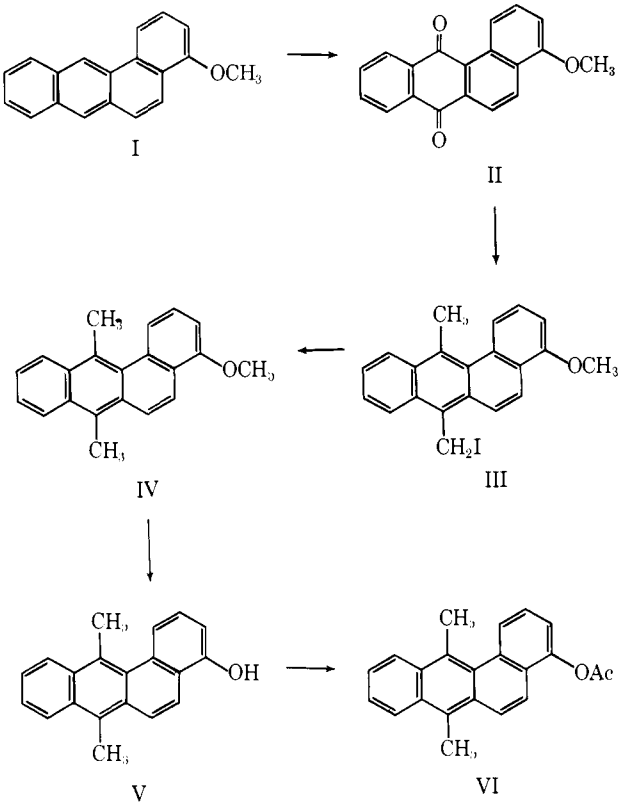
(2) (a) E. Boyland, *Brit. Med. Bull.*, **20**, 121 (1964). (b) Boyland[2a] has suggested epoxides as intermediates in the metabolism of polycyclic aromatic hydrocarbons. These are alkylating agents and would be expected to react with cellular constituents. (c) M. S. Newman and S. Blum, *J. Am. Chem. Soc.*, **86**, 5598 (1964), have shown that epoxides of aromatic hydrocarbons are sensitive to acid and are converted to hydroxy derivatives.

(3) (a) F. Dickens, *Brit. Empire Cancer Campaign*, 55 (1945). (b) After this synthesis was completed we learned (private communication) that the preparation of 4-methoxy-7,12-dimethylbenz[*a*]anthracene was accomplished by Professor Dickens using essentially the same scheme.

(4) (a) J. Cason and L. F. Fieser, *J. Am. Chem. Soc.*, **62**, 2681 (1940); (b) A. Sempronj, *Gazz. Chim. Ital.*, **69**, 448 (1939).

was poured into methanol–HI, and 4-methoxy-7-iodomethyl-12-methylbenz[*a*]anthracene (III) was isolated. The iodo derivative was found to react smoothly with stannous chloride in dioxane containing HCl, which after dilution with water afforded 4-methoxy-7,12-dimethylbenz[*a*]anthracene (IV). Cleavage of the 4-ether was difficult and, although 4-hydroxy-7,12-dimethylbenz[*a*]anthracene (V) was easily detected on thin layer chromatograms[5] following treatment with HI or HBr, the free compound was very difficult to purify. We found, however, that cleavage of IV with HBr under N_2 gave 4-hydroxy-7,12-dimethylbenz[*a*]anthracene (V) which could be separated from VIII by chromatography.

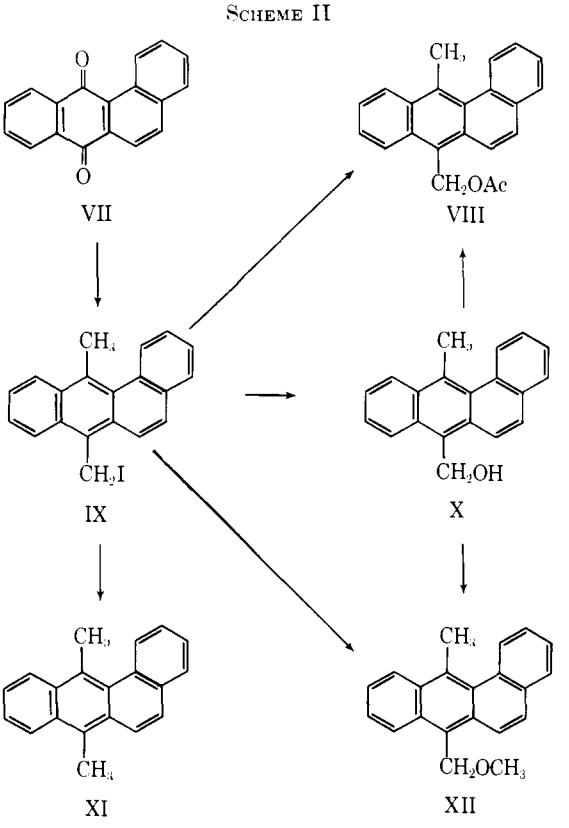
SCHEME I



Our attention turned next to the synthesis of 7-hydroxymethyl-12-methylbenz[*a*]anthracene (X). This compound is of interest because the recent findings of Boyland and Sims[6] demonstrates conversion of DMBA to monohydroxymethyl derivatives by rat liver homogenates. Boyland and co-workers[7] found that X, but not 7-methyl-12-hydroxymethylbenz[*a*]-anthracene, was able to induce mammary cancer in rats following a single feeding of the hydrocarbon dissolved in sesame oil. Interestingly, they also found it produced adrenal necrosis in smaller doses than DMBA.

The synthesis of X has been reported[6] using limited amounts of lead tetraacetate. The procedure is difficult and leads to a mixture of compounds which must be separated by chromatography. For this reason a new synthesis was devised which employed 7-iodomethyl-12-methylbenz[*a*]anthracene as shown in Scheme II. The iodo compound IX, previously described by Sandin and Fieser[8] as an intermediate in the synthesis of DMBA, provides a versatile intermediate for further transformations. Thus these investigators converted IX to 7-methoxymethyl-12-methylbenz[*a*]-anthracene (XII). Further we found IX also served as a convenient intermediate for the introduction of hydroxy (X) and acetoxy (VIII) groups. In addition to the formation of VIII and XII from IX, these compounds were prepared from X.

SCHEME II



Animal Studies.—The metabolism of DMBA was studied in rat liver homogenates, essentially as described by Boyland and Sims.[6] All studies were conducted using the female Sprague–Dawley rat, since this strain has been shown to be most susceptible to mammary cancer induction with DMBA.[9] A single oral administration of the hydrocarbon dissolved in sesame oil has been shown by Huggins, *et al.*, to be a rapid method for determining carcinogenic activity.[10] This method was employed in the present study.

Results

Identification of Metabolites in Rat Liver Homogenates.—Rat liver homogenates were extracted with ether and the ether-soluble extract was chromatographed on thin layer chromatograms (0.5-mm thickness) and divided into eight fractions under ultraviolet light. The fractions were removed from the plate, and

(5) Glass plates coated with silica gel G (Brinkman Instrument Co.) of 0.25-mm thickness were developed with benzene or benzene containing 5% (v/v) ethanol and examined under ultraviolet light.

(6) E. Boyland and P. Sims, *Biochem. J.*, **95**, 780 (1965).

(7) E. Boyland, P. Sims, and C. Huggins, *Nature*, **207**, 816 (1965).

(8) R. B. Sandin and L. F. Fieser, *J. Am. Chem. Soc.*, **62**, 3098 (1940).

(9) K. L. Sydnor, O. Butenandt, F. P. Brillantes, and C. Huggins, *J. Natl. Cancer Inst.*, **29**, 805 (1962); E. Boyland and K. L. Sydnor, *Brit. J. Cancer*, **16**, 731 (1962).

(10) C. Huggins, G. Briziarelli, and H. Sutton, *J. Exptl. Med.*, **109**, 25, (1959); C. B. Huggins, L. C. Grand, and F. P. Brillantes, *Nature*, **189**, 204, (1961).

TABLE I

R_f VALUES OF HYDROXYLATED DERIVATIVES OF DMBA AND RELATED COMPOUNDS

Benz[a]anthracene	R_f[a] System A	System B
DMBA	0.71	0.68
4-Hydroxy-7,12-dimethyl-	0.44	0.14
7-Hydroxymethyl-12-methyl-	0.37	0.04
7,12-Dihydroxymethyl-	0.17	0.00
4-Acetoxy-7,12-dimethyl-	0.65	0.22
7-Acetoxymethyl-12-methyl-	0.66	0.18
7,12-Diacetoxymethyl-	0.61	0.05
4-Methoxy-7,12-dimethyl-	0.72	0.58
7-Methoxymethyl-12-methyl-	0.66	0.19
7,12-Epidioxy-7,12-dimethyl-	0.65	0.48

[a] System A = benzene–EtOH 95:5), system B = benzene.

the compounds adsorbed to the silica gel were eluted with ethanol. The ethanolic solutions were rechromatographed in parallel with authentic compounds prepared by synthesis.

Table I presents the R_f values of the compounds described in this report.

Fraction 1 was identified as DMBA by its behavior on thin layer chromatograms. Fraction 2 contained a compound indistinguishable from 7,12-epidioxy-7,12-dimethylbenz[*a*]anthracene. This compound was found only in trace amounts and undoubtedly was obtained during manipulation of the sample in the ordinary light of the laboratory. In so far as possible, experiments were conducted in darkness or in a minimum amount of light.

Fraction 3 contained a metabolite which corresponds to 7-methyl-12-hydroxymethylbenz[*a*]anthracene previously described by Boyland and Sims.[6] When this compound was acetylated with pyridine and acetic anhydride its mobility on tlc was identical with that of 7-acetoxymethyl-12-methylbenz[*a*]anthracene, which has the same mobility as 7-methyl-12-acetoxymethyl-[*a*]anthracene according to these authors.

Fraction 4 contained large amounts of 4-hydroxy-7,12-dimethylbenz[*a*]anthracene which on oxidation with chromic acid yielded a product indistinguishable on tlc from that obtained when the authentic compound was treated in an identical manner. The products of microchemical acetylation and methylation, respectively, were indistinguishable from products found when the authentic compound was run in parallel with the metabolite.

Fraction 5 was identical in its behavior with 7-hydroxymethyl-12-methylbenz[*a*]anthracene. On acetylation it formed the expected acetate. Fraction 6 was identical in its mobility and properties on tlc with 7,12-dihydroxymethylbenz[*a*]anthracene. When the compound was acetylated, a product whose behavior on tlc was identical with 7,12-diacetoxymethylbenz[*a*]-anthracene was obtained.

Fraction 7 contained an unknown compound, R_f 0.08 in system A, that had a blue fluorescence in ultraviolet light. The compound apparently arose from 4-hydroxy-7,12-dimethylbenz[*a*]anthracene since samples of this compound exposed to air contained a similar product. Fraction 8, R_f 0.04 (system A), contained an unknown compound which was also detected in control rat liver homogenates incubated in the absence of DMBA.

TABLE II

INDUCTION OF CANCER BY ORAL ADMINISTRATION OF HYDROCARBONS[a]

Benz[a]anthracene	Dose, mg	Animals with tumors, no. of animals	Appearance of tumors, days Range	Mean
7-Hydroxymethyl-12-methyl-	20	6/8	83–151	120
7-Methoxymethyl-12-methyl-	20	1/10	110	
7-Acetoxymethyl-12-methyl	20	2/10	110–114	112
7,12-Dihydroxymethyl-	10	0/10	(180 days of observation)	
4-Hydroxy-7,12-dimethyl-	20	0/10	(180 days of observation)	
7,12-Epidoxy-7,12-dimethyl-	20	0/10	(180 days of observation)	
7,12-Dimethyl-	20	6/6	64–160	87

[a] The hydrocarbon, dissolved in 1 ml of sesame oil was administered by gastric instillation to female Sprague-Dawley rats, age 50 days. The animals were examined for appearance of palpable tumors twice weekly.

Induction of Cancer Following a Single Feeding of Hydroxylated Derivatives of DMBA and Related Compounds.—The preliminary results of carcinogenic activity of metabolites of DMBA and related compounds are presented in Table II.

7-Hydroxymethyl-12-methylbenz[*a*]anthracene, 7-methoxymethyl-12-methylbenz[*a*]anthracene, and 7-acetoxymethyl-12-methylbenz[*a*]anthracene all possess carcinogenic properties. 7-Hydroxymethyl-12-methylbenz[*a*]anthracene is approximately as carcinogenic as the parent hydrocarbon, although the latent period is longer. 4-Hydroxy-7,12-dimethylbenz[*a*]anthracene, tested for the first time, is devoid of carcinogenic activity under these conditions. 7,12-Dihydroxymethylbenz[*a*]anthracene was also without activity. Thus, of the metabolites of DMBA so far examined for carcinogenic properties only 7-hydroxymethyl-12-methylbenz[*a*]anthracene has been shown to have carcinogenic properties in the rat. Interestingly, the isomeric 7-methyl-12-hydroxymethylbenz[*a*]-anthracene is carcinogenic in mice.[7]

Discussion

Dickens detected 4-hydroxy-7,12-dimethylbenz[*a*]-anthracene in rat feces and this was identified by its fluorescence spectrum after conversion to 4-methoxy-7,12-dimethylbenz[*a*]anthracene.[3a] Boyland and Sims tentatively suggested that the metabolite was present as a product of DMBA metabolism by rat liver homogenates. We have established the compound as a metabolite of DMBA, and no doubt the slower moving product observed by Boyland and Sims in fraction 4 was 4-hydroxy-7,12-dimethylbenz[*a*]anthracene. The monohydroxymethyl derivatives, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, and 7-methyl-12-hydroxymethylbenz[*a*]anthracene were also identified, confirming the findings of Boyland and Sims. These investigators failed to observe any 7,12-dihydroxymethylbenz[*a*]anthracene as a metabolite in rat liver homogenates although they made a careful search for this

compound. Jellinck and Goudy[11] detected a radioactive zone which corresponded to 7,12-dihydroxymethylbenz[*a*]anthracene on thin layer chromatograms when ^{14}C-DMBA was incubated with rat liver homogenates. We have identified this compound in the present work. It is of interest that this compound was not found by Boyland and Sims in the strain of rats (Chester Beatty) studied and this suggests that strain differences in metabolism probably account for the fact that the compound is not present in the Chester Beatty rat strain, but is present in the Sprague–Dawley strain.

Earlier experiments of Huggins had demonstrated the high potency of DMBA in inducing mammary cancer following a single feeding of the hydrocarbon in sesame oil.[10] It was also shown that the monohydroxymethyl derivatives possess carcinogenic properties in certain tests.[7]

The fact that the hydroxymethyl, the methoxymethyl, and the acetoxymethyl derivatives have carcinogenic properties is of interest. It may be that these compounds are themselves lacking in carcinogenic properties, but must be converted to the carcinogenic 7-hydroxymethyl-12-methylbenz[*a*]anthracene. It appears that the alcoholic hydroxyl group, in appropriate positions, is more favorable for conferring carcinogenic potency than the phenolic hydroxyl group. It must be emphasized that compounds which do not produce tumors in a single test are not necessarily to be regarded as noncarcinogenic. Similarily, additional study is required to establish the carcinogenic potency of the compounds which are reported as carcinogenic in this study.

The present experiments confirm and extend the results of other investigators. Hydroxylation reactions occur in both the ring and on the methyl groups. Both monohydroxymethyl and dihydroxymethyl derivatives are present as products in rat liver homogenates indicating that drug metabolizing enzymes can attack the hydrocarbon in several locations. The primary point of attack appears to be the 7-methyl position, but whether the resulting product is the proximate carcinogen remains to be established. When hydroxylation occurs on both the 7 and 12 positions or in the 4 position, no cancers were observed, whereas 7-hydroxymethyl-12-methylbenz[*a*]anthracene appeared to be as carcinogenic as the parent compound in this assay. Studies on the distribution of hydrocarbons in rat tissues clearly indicate that only a small fraction of the dose can be found in the tissues following oral administration.[12] The variability in the amount of carcinogen absorbed would be expected to influence the tumor yield. Further work will be required to determine the concentration of hydrocarbon metabolites in the target tissue.

Experimental Section[13]

4-Methoxybenz[*a*]anthraquinone (II).—4-Methoxybenz[*a*]-anthracene (I) (2.0 g) dissolved in AcOH (60 ml) was treated with $K_2Cr_2O_7$ (2.45 g in 15 ml of water). After refluxing for 30 min, the cooled reaction mixture was poured into ice water (800 ml), and a red solid was collected by filtration and thoroughly washed with ice water. The product was dried *in vacuo* to give 2.0 g of crude material. Chromatography on alumina (benzene) and recrystallization with benzene gave pure II, mp 220–221°, yield 1.7 g (79%).

Anal. Calcd for $C_{19}H_{12}O_3$: C, 79.15; H, 4.34. Found: C, 79.32; H, 4.41.

4-Methoxy-7-iodomethyl-12-methylbenz[*a*]anthracene (III).—4-Methoxybenz[*a*]anthraquinone (2 g) was suspended in benzene (100 ml) and MeMgI (from 2.5 g of Mg and 8 ml of MeI in 70 ml of ether) was added with vigorous stirring over 1 hr. The olive drab solution was slowly poured into a solution of 70 ml of methanol and 25 ml of 57% HI maintained at −5 to 0°. Addition of AcOH (150 ml) and standing overnight (−10°) gave a crystalline product containing iodine (III), mp 105° dec, yield 2.0 g (74%).

4-Methoxy-7,12-dimethylbenz[*a*]anthracene (IV).—The iodo compound III (1.425 g) was dissolved in dioxane (100 ml) containing 5 ml of concentrated HCl. This solution was added to a mixture of $SnCl_2$ (20 g), dioxane (90 ml), and 54 ml of concentrated HCl and brought to reflux for 15 min. The cooled solution was added to water (1.5 l.) and the suspension was allowed to stand overnight (0°). The solid was collected by filtration, washed with water, and dried. Chromatography on alumina (Alcoa-F-20, 100 g) in benzene gave 1.0 g (96%) of IV, mp 121°.

Anal. Calcd for $C_{21}H_{18}O$: C, 88.07; H, 6.33. Found: C, 87.96; H, 6.29.

4-Hydroxy-7,12-dimethylbenz[*a*]anthracene (V).—4-Methoxy-7,12-dimethylbenz[*a*]anthracene (0.5 g) was dissolved in acetic acid (20 ml) and HBr (48%) (1 ml) was added under N_2. After 2 hr HBr (0.5 ml) was again added. Refluxing was continued under N_2 for a total of 7 hr. The cooled solution was poured into ice water and a yellow solid separated immediately. The solid was collected by centrifugation and washed (H_2O, $NaHCO_3$, H_2O). Chromatography on silica gel (benzene) gave three fractions. The first fraction (50 ml) contained starting material. The second fraction (50 ml) contained a mixture of starting material and product and the final fraction (250 ml) contained V, yield 70%. The product was recrystallized from benzene; mp 164–165°.

Anal. Calcd for $C_{20}H_{16}O$: C, 88.30; H, 5.90. Found: C, 88.37; H, 5.89.

4-Acetoxy-7,12-dimethylbenz[*a*]anthracene (VI).—A mixture of 4-hydroxy-7,12-dimethylbenz[*a*]anthracene (50 mg), pyridine (0.5 ml), Ac_2O (0.25 ml), and fused sodium acetate (0.1 g) was refluxed 45 min. The cooled solution was poured into ice water (10 ml) and stored in the refrigerator overnight. The solid was collected by centrifugation and washed several times with water to remove pyridine. Crystallization from ethanol–benzene gave cream-colored plates (30 mg), mp 180–181°.

7-Hydroxymethyl-12-methylbenz[*a*]anthracene (X).—7-Iodomethyl-12-methylbenz[*a*]anthracene (4 g), prepared by the method of Sandin and Fieser,[8] was dissolved in dioxane (200 ml) on gentle warming. To the bright yellow solution, a suspension of Ag_2CO_3 (4 g) and Na_2CO_3 (4 g) in water (5 ml) was then added. The reaction mixture was refluxed for 1 hr, while stirring vigorously. The precipitated salts gradually turned gray and finally black. At the end of the reaction period the supernatant liquid was almost colorless. After filtration to remove the salts and washing several times with dioxane, the combined solutions and washings were evaporated under reduced pressure until nearly dry. The residue was dissolved in hot benzene (50 ml) to give a clear light yellow solution. Light yellow needles separated on standing at room temperature, mp 162°, lit.[6] 162°, yield 2.9 g (96%).

Anal. Calcd for $C_{20}H_{16}O$: C, 88.30; H, 5.90. Found: C, 88.35; H, 6.0.

7-Methoxymethyl-12-methylbenz[*a*]anthracene (XII). Method A.—To 1 g of X suspended in 200 ml of methanol was added 2.5 ml of concentrated H_2SO_4 in 40 ml of methanol. After refluxing for 2.5 hr, the mixture was concentrated under reduced pressure to 50 ml and poured into 300 ml of water. Sodium bicarbonate solution was added until pH 7. The mixture was allowed to stand overnight in an ice bath and was then filtered off and dried, yield 0.6 g (57%); crystallized from methanol, mp 120–121°, lit.[8] 120–121°.

Method B.—Sodium (200 mg) was dissolved in 15 ml of absolute methanol. 7-Iodomethyl-12-methylbenz[*a*]anthracene (200 mg) was then added and refluxed 1 hr while stirring vigor-

(11) P. H. Jellinck and B. Goudy, *Science*, **152**, 1375 (1966).

(12) J. W. Flesher and K. L. Sydnor, *Proc. Soc. Exptl. Biol. Med.*, **104**, 776 (1960); F. G. Bock and T. L. Dao, *Cancer Res.*, **21**, 1024 (1961); E. B. Gammal, K. K. Carroll, B. H. Muhlstock, and E. R. Plunkett, *Proc. Soc. Exptl. Biol. Med.*, **119**, 1086 (1965).

(13) Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn. Melting points were taken on a Fisher-Johns block and are uncorrected.

ously. The solvent was removed under reduced pressure until the volume was reduced to 1–2 ml, and the mixture was diluted with 10 ml of water. After cooling at 5° for several hours the yellow crystalline solid which separated was filtered off and dried in air, 125 mg (85%), mp 120–121°.

7-Acetoxymethyl-12-methylbenz[*a*]anthracene (XIII). Method A.—A mixture of X (3.0 g), pyridine (30 ml), fused sodium acetate (3 g), and Ac_2O (15 ml), was refluxed for 1 hr and stirred overnight at room temperature. It was poured in ice water (300 ml) and the solid was removed by filtration. Crystallized from benzene–ethanol it formed feather-like crystals (3 g, 84%), mp 145–146°.

Method B.—A mixture of X (0.25 g), Ag_2CO_3 (0.25 g), and Ac_2O (1.5 ml) was refluxed 0.5 hr. After evaporation under reduced pressure, the mixture was diluted with 50 ml of ice water and allowed to stand overnight in the refrigerator. The product was filtered off and crystallized from benzene–methanol, mp 145–146°, 0.15 g (80%) of yellow needles.

7,12-Dihydroxymethylbenz[*a*]anthracene was prepared by the method of Badger and Cook,[14] and **7,12-epidioxy-7,12-dimethylbenz[*a*]anthracene** by the method of Cook and Martin.[15]

Experiments with Rat-Liver Homogenates.—Female Sprague–Dawley rats, age 50 days, were used. Liver homogenates were prepared as described by Boyland and Sims.[8]

A 10% homogenate was prepared in ice-cold 1.15% (w/v) KCl. The homogenate was centrifuged for 20 min at 1475*g* (0°) and the supernatant was diluted with an equal volume of 0.1 *M* sodium phosphate buffer, pH 7.4. To 120 ml of the diluted supernatant was added niacinamide (450 mg), $NADP^+$ (10 mg), glucose 6-phosphate (60 mg), and DMBA (2 mg), dissolved in 1 ml of ethanol. The mixture was incubated 1 hr at 37° under O_2 in a Dubnoff metabolic shaker, then cooled, acidified with 1 *N* HCl to pH 3, and extracted with three 50-ml portions of ether. The ether extract was dried (Na_2SO_4) and evaporated under N_2 to a small volume and chromatographed by tlc. The chromatogram was divided into zones under ultraviolet light. The zones were removed from the plate, and the compounds adsorbed to the silica gel were eluted with ethanol and rechromatographed.

Identification of Metabolites.—Metabolites and their derivatives, prepared by microchemical reactions carried out in parallel with authentic compounds, were identified by their mobility on thin layer chromatograms.

Microchemical Reactions.—Metabolites, purified by thin layer chromatography, were scraped from the thin layer plates and eluted from the silica gel with ethanol.

1. **Acetylation.** The metabolite, dissolved in 0.1 ml of pyridine, was added to an equal volume of Ac_2O and the mixture was heated at 60–70° for 1 hr. The solvent was evaporated with N_2 and the residue was dissolved in benzene and chromatographed (tlc).

2. **Oxidation with CrO_3.**—The sample, dissolved in 0.5 ml of AcOH, was treated with 0.25 ml of CrO_3 (2%) and allowed to stand 1 hr. Water (10 ml) was added and the mixture was extracted with two 5-ml portions of ether, washed ($NaHCO_3$, H_2O), and dried (Na_2SO_4). The product was examined on tlc.

Methylation with Dimethyl Sulfate.—The zone corresponding to 4-hydroxy-DMBA was scraped from the plate and eluted with ethanol. To this ethanolic solution (0.1 ml) was added dimethyl sulfate (0.1 ml) and 20% NaOH (0.1 ml). After standing in the water bath (50°) for 20 min, 0.05 ml of dimethyl sulfate and 0.1 ml of 20% NaOH were added with stirring and the mixture was returned to the bath for 15 min. The mixture was cooled, 20% NaOH (0.5 ml) was added and, after a few minutes, extracted with CH_2Cl_2 (5 ml). The extract was washed with two 0.5-ml portions of water, 1 drop of AcOH was added, and the solution was concentrated under N_2 and examined on tlc.

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Derivatives of Fluorene. XXIII.[1] New Thiofluorenes Related to Metabolism of the Carcinogen N-2-Fluorenylacetamide

T. LLOYD FLETCHER, MOSES J. NAMKUNG, AND HSI-LUNG PAN

Chemistry Research Laboratory of the Department of Surgery, University of Washington School of Medicine, Seattle, Washington 98105

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Synthesis of N-2-(3-methylthiofluorenyl)acetamide (I) by two routes is described. This compound is identical with one which has been isolated by alkaline degradation of the *in vitro* reaction product of N-acetoxy-N-2-fluorenylacetamide, a highly potent carcinogen, and methionine or methionylglycine or proteins. I is also identical with a compound isolated from rat liver tissue after the rat has been fed N-2-fluorenylacetamide. For comparative studies, N-2-(7-fluoro-3-methylthiofluorenyl)acetamide was prepared and its structure was confirmed by an alternate route. In the initial preparation of this compound, we first used alcoholic sodium methyl sulfide which had been standing for some time, and a series of 3-ethoxyfluorene derivatives resulted instead of the expected 3-methylthiofluorenes. A number of new fluorene derivatives are reported and infrared spectral data are presented and discussed.

N-Hydroxy-N-2-fluorenylacetamide (N-HO-FAA) has been shown by the Millers and their associates[2] to be both a metabolite of the carcinogen N-2-fluorenylacetamide (FAA) and a more potent carcinogen than the latter. In addition, higher levels of hepatic protein and nucleic acid bound derivatives were found after administration of the N-hydroxy compound than after the parent amide or amine.[3] This pointed to the likelihood that N-HO-FAA or a derivative is involved in the binding reaction; indeed, it has now been shown that certain esters of N-HO-FAA react with methionine and its peptides at physiological pHs.[4] N-HO-FAA itself has not been observed to react with proteins or nucleic acids *in vitro*, although N-hydroxyfluoren-2-amine (N-HO-FA) reacts with guanine in nucleic acids at an acid pH.[5] More recently, the Millers, *et al.*,[6,7] have observed a reaction between N-acetoxy-N-2-fluorenylacetamide (N-AcO-FAA) and nucleic acid guanine at pH 7. Certain metabolic esters of N-HO-

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