The Preparation of some Specifically Tritium Labelled Carcinogenic Hydrocarbons and their Covalent Binding to DNA

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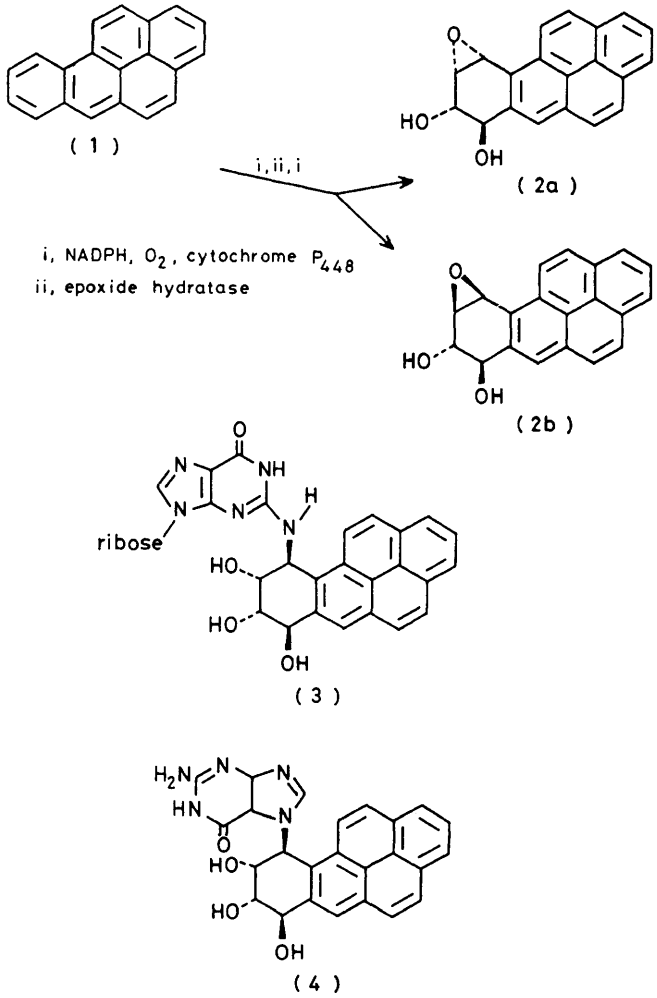
Syntheses are described for the preparation of [9(10)-^{3}H]anthracene, [6-^{3}H]benzo[*a*]pyrene, and [7-*Me*-$^3H_1$]-7,12-dimethylbenz[*a*]anthracene. The nature and extent of covalent binding of these hydrocarbons to calf thymus DNA resulting from ultraviolet or gamma irradiation, from oxidative processes involving iodine or hydrogen peroxide, or from oxygen in conjunction with NADPH and a rat liver microsomal preparation is investigated. The displacement of tritium on hydrocarbon bonding to DNA identifies position-6 in benzo[*a*]pyrene and position-9 in anthracene as sites for DNA binding by irradiation processes or by chemical oxidation. Metabolic binding of benzo[*a*]pyrene to DNA shows an altered regioselectivity while that for 7,12-dimethylbenz[*a*]anthracene appears to to involve the 7-methyl group.

DIBENZ[*ah*]ANTHRACENE was isolated by Kennaway's group in 1932 as the first pure chemical compound capable of causing cancer in animals and three years later benzo[*a*]pyrene was identified as the first naturally occurring chemical mutagen.[1,2] Following these discoveries, a wide range of polycyclic aromatic hydrocarbons has been isolated or synthesised of which many have proved to be carcinogens or photocarcinogens. Anthracene is photocarcinogenic in mice treated topically with the hydrocarbon and then irradiated by atmospherically transmitted ultraviolet radiation,[3] though usually more extensive polycyclic ring systems are identified with the manifestation of chemical carcinogenicity. This phenomenon is currently associated with metabolic transformation of hydrocarbon by oxidising enzymes that gives electrophilic products, often epoxides, which can alkylate DNA bases and other biological targets. A major pathway for benzo[*a*]pyrene (1) metabolism involves oxidation to the 7,8-dihydrodiol 9,10-oxides (2) which [4] are attacked by nitrogen (and oxygen) nucleophiles of the DNA bases, principally guanine.[5] This leads to covalent products *in vitro* and *in vivo*, for some of which structures have been provided by microchemical analysis.[6,7]

The major isomer resulting from such bonding of the *anti*-epoxide (2a) to RNA in cultural bronchial mucosa has been identified [8] as the product (3) of linking guanine *N*-2′ to *C*-10 of the epoxide (2a). An isomeric product has been identified (4) which is hydrolytically more labile.[9]

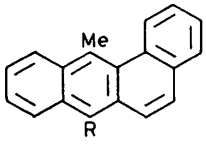
While these oxidative processes involving positions-7 to -10 for benzo[*a*]pyrene (1) have been a major focus of attention, this hydrocarbon is, in fact, metabolically oxidised to some nine primary products: the 9,10-, 7,8-, and 4,5-dihydrodiols; the 1,6-, 3,6-, and 6,12-quinones; and the 3-, 6-, and 9-phenols.[10] In particular, 6-hydroxybenzo[*a*]pyrene is an active metabolite which may be involved with binding to DNA and carcinogenesis.[11]

In the case of methylated hydrocarbons, oxidative metabolism involves attack on the side-chains as well as at annular positions.[12,13] Some studies [13,14] have indicated that the carcinogenic character of these hydrocarbons may be associated with methyl metabolism, others have investigated arene oxides for, *e.g.* 7,12-dimethylbenz[*a*]anthracene (5), and three minor hydrocarbon–RNA adducts, accounting for some 10% of total binding, have been identified [15,16] as resulting from the interaction of guanosine and the *K*-region 5,6-epoxide (6). However, data from fluorescence studies [17] have

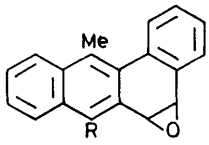


indicated that (5) binds to DNA in mouse embryo cells *via* a dihydrodiol epoxide formed at positions 1—4, while other studies have shown that the 8,9-dihydrodiol of (5) is more active than the parent hydrocarbon in effecting malignant transformation of cell cultures.[18]

In the light of this great variety of metabolically founded, structural information on oxygenated hydrocarbon products and fragments of DNA-linked species, it appeared opportune to assess the use of specific tritium labelling of selected aromatic hydrocarbons as a means of providing information concerning the loci involved in chemical and metabolic bonding of such carcinogens to DNA. We here describe results obtained for anthracene, benzo[*a*]pyrene, and 7,12-dimethylbenzo[*a*]anthracene. Some parts of this work have appeared in preliminary form.[19,20]



(5) R = Me
(7) R = CH_2I



(6)

EXPERIMENTAL

Materials.—[9(10)-^{14}C]Anthracene, [7,10-$^{14}C_2$]benzo[*a*]pyrene, 7,12-dimethyl-[12-^{14}C]benz[*a*]anthracene, and tritiated water (5 Ci/ml) were obtained from the Radiochemical Centre, Amersham, Bucks. DNA (Calf thymus, Type V, average molecular weight 10^6 a.m.u.) was supplied by Sigma Chemicals, London, and standard solutions were prepared by slow dissolution at 4 °C in phosphate buffer (10^{-2}M, pH 6.8, and containing 5×10^{-4}M EDTA) to a final concentration of 2.5 mg/ml unless otherwise specified. NADH, NADPH, and enzymes used were obtained from Sigma Chemicals, London. Sephadex LH20 powder was supplied by Pharmacia Ltd., Uppsala, Sweden, and Unisolve 1 liquid scintillation fluid by Koch Light Ltd., Colnbrook, Bucks. AnalaR grade solvents and glass-distilled water were used throughout.

General Methods.—Melting points were determined using a Kofler hot-stage apparatus and are otherwise uncorrected. ^{1}H N.m.r. spectra were recorded at 100 MHz using a Varian HA100 or at 220 MHz using a Perkin-Elmer R34 instrument with chloroform solvent and tetramethylsilane internal reference. Low-resolution mass spectra were obtained using an AEI MS12 machine. Scintillation counting was performed in glass vials using a Packard 3385 machine with automatic external standardisation: counting efficiencies were determined by means of standard solutions containing ^{3}H- or ^{14}C-toluene standards variously quenched by carbon tetrachloride.

[9(10)-^{3}H]*Anthracene.*—9,10-Dibromoanthracene (0.5 g) was stirred as a yellow suspension in dry ether (20 ml) under dry nitrogen at −78 °C while a solution of n-butyl-lithium in ether (20 ml, 5 mmol) was added to give a brown solution.[21] This solution was stirred 40 min at room temperature then cooled to 0 °C and quenched with tritiated water (100 mCi in 100 μl) followed after 10 min by D_2O (1 ml). The mixture was evaporated *in vacuo* and the solid residue washed with water, dried, and thrice crystallised from toluene to give [9(10)-^{3}H]anthracene, m.p. 215—218 °C (71 mg, 38%, 6.34 mCi/mmol). This material had spectroscopic properties and t.l.c. behaviour identical with those of an authentic sample of anthracene.

[6-^{3}H]*Benzo*[a]*pyrene.*—6-Iodobenzo[*a*]pyrene [22] (200 mg) in pure tetrahydrofuran (50 ml) was added to a solution of n-butyl-lithium in ether (1 ml, 1.5 mmol) diluted with tetrahydrofuran (10 ml) at −78 °C under dry nitrogen. After 1 h, tritiated water (250 mCi, 50 μl) dissolved in tetrahydrofuran (2 ml) was added when the colour of the solution changed from purple to yellow. The solution was brought to room temperature and D_2O (0.5 ml) added. Solvent was evaporated under reduced pressure and the residue dissolved in ether (20 ml), washed with dilute HCl and water, and dried ($MgSO_4$). The product obtained on evaporation of solvent was purified by preparative t.l.c. using silica plates and cyclohexane–benzene (9 : 1 v/v) to give [6-^{3}H]benzo[*a*]pyrene, m.p. 196—198 °C (lit.,[23] m.p. 197—198°) (93 mg, 70%, specific activity 28 mCi/mmol).

Bromination of [6-^{3}H]*Benzo*[a]*pyrene.*—A sample of the above [6-^{3}H]benzo[*a*]pyrene (40 μCi) was co-chromatographed with [6-^{14}C]benzo[*a*]pyrene (1.0 μCi) and carrier hydrocarbon (200 mg) and a sample removed for radioassay. The remainder was dissolved in carbon tetrachloride (25 ml) and heated under reflux with *N*-bromosuccinimide (100 mg) for 3 h and the solution cooled and evaporated under reduced pressure. The crude product was purified by chromatography on neutral alumina with benzene elution, and recrystallised from acetone to give 6-bromobenzo[*a*]pyrene as yellow needles (100 mg, 40%), m.p. 220 °C (lit. [24] m.p. 223—224°); ^{3}H/^{14}C ratio (d.p.m.): benzo[*a*]pyrene, 40.8 ± 0.1; 6-bromobenzo[*a*]pyrene, 2.93 ± 0.05.

[7-Me-$^3H_1$]-7,12-*Dimethylbenz*[a]*anthracene.*—A suspension of freshly prepared 7-iodomethyl-12-methylbenz[*a*]anthracene [25] (100 mg) in dioxan (5 ml) and dimethyl sulphoxide (5 ml) was added to a well-stirred solution of sodium boro[^{3}H]hydride (25 mCi, 19.8 mg) in pure dimethyl sulphoxide (5 ml). On completion of the reduction, water (100 ml) was added and the mixture extracted with cyclohexane (3 × 50 ml) and benzene (2 × 50 ml). The combined extracts were dried and evaporated to give a pale yellow solid which was purified by preparative t.l.c. on silica using cyclohexane–benzene (9 : 1 v/v). The principal band (R_f 0.5) was eluted to give the product as a bright yellow solid which was crystallised from ethanol, m.p. 120.5—122 °C (lit.,[25] m.p. 122—123 °C) (24 mg, 36%), $\lambda_{max.}$ (ethanol) 382 (ε 6.13 × 10^4), 363 (6.35 × 10^4), and 332 nm (3.78 × 10^4), specific activity 9.74 mCi/mmol.

9,10-*Dideuterioanthracene.*—The foregoing procedure was employed with quenching using only D_2O (1 ml). The product was obtained as a yellow solid, m.p. 215—218 °C (72.7 mg, 27%), *m/e* 180 (M^+); δ 7.45 (4 H, dd, ^3J 7 Hz, ^4J 3 Hz) and 7.99 (4 H, dd, ^3J 7 Hz, ^4J 3 Hz).

6-*Deuteriobenzo*[a]*pyrene.*—The foregoing procedure was employed with quenching using only D_2O (0.4 ml). The product, obtained as a yellow solid, was purified by preparative t.l.c. and by crystallisation from benzene–isopropyl alcohol (1 : 1 v/v), m.p. 196—198 °C (50 mg, 38%), *m/e* 253 (M^+); δ 7.75 (2 H, m, H-8, H-9), 7.89 (2 H, dd, ^3J 7 Hz, H-4, H-5), 7.94 (1 H, m, H-2), 7.98 (1 H, m, H-3), 8.14 (1 H, d, ^3J 7 Hz, H-1), 8.20 (1 H, d, ^3J 7 Hz, H-7), 8.30 (1 H, d, ^3J 7 Hz, H-12), and 8.90 (2 H, m, H-10, H-11).

Acetoxylation of [6-^{3}H]*Benzo*[a]*pyrene.*—The above [6-^{3}H]benzo[*a*]pyrene (*ca.* 10 μCi) and recrystallised benzo[*a*]-

pyrene (30 mg) were co-chromatographed by preparative t.l.c. and dissolved in benzene (3 ml). Aliquots (3 × 20 μl) were removed for radioassay. The benzene solution was treated with lead(IV) acetate (47 mg) in glacial acetic acid (1.3 ml) and set aside 1 h at 20 °C. The benzene was then evaporated *in vacuo* and the solution diluted with water at 0 °C. The crude product was filtered off and purified by preparative t.l.c. using silica and cyclohexane–benzene (3 : 7, v/v) to give 6-acetoxybenzo[*a*]pyrene as a yellow solid (28 mg, 76%), m.p. 210 °C (lit.,[26] m.p. 210 °C). Specific radioactivities: benzo[*a*]pyrene, 61.95 ± 1.7 mCi/mol; 6-acetoxybenzo[*a*]pyrene, 0.90 ± 0.02 mCi/mmol.

Oxidative Demethylation of [7-Me-$^3H_1$]-7,12-*Dimethylbenz*[a]*anthracene*.—A portion of the above sample of [7-*Me*-$^3H_1$]-7,12-dimethylbenz[*a*]anthracene (0.25 mg, 9.5 μCi) admixed with 7,12-dimethyl[12-^{14}C]benz[*a*]anthracene (1.84 μCi) and carrier hydrocarbon (200 mg) were co-chromatographed (preparative t.l.c.) as before and dissolved in glacial acetic acid (5 ml). This solution was heated under reflux while a solution of sodium dichromate (750 mg) in glacial acetic acid (2.5 ml) and water (0.5 ml) was added slowly.[26] After 1 h, the solution was poured into water and the solid residue washed with dilute alkali and water and dried. After chromatography on neutral alumina (benzene–ether, 1 : 1 v/v) the product was recrystallised from ethanol to give 7,12-benz[*a*]anthraquinone (33 mg), m.p. 169—170 °C, undepressed on admixture with an authentic sample. The radioactivity of this material was determined using internal standardisation to correct for colour quenching: [7-*Me*-$^3H_1$]-7,12-dimethylbenz[*a*]anthracene, 12.1 μCi/mmol, ^{14}C 2.35 μCi/mmol; 7,12-benz[*a*]-anthraquinone, no detectable tritium activity, ^{14}C-specific activity 2.32 μCi/mmol.

DNA–Hydrocarbon Physical Complexes.—These complexes were prepared as described previously [27] using synthetic ^{3}H-labelled hydrocarbons admixed with ^{14}C-labelled and carrier hydrocarbon which had been co-chromatographed (preparative t.l.c.) on alumina before use to give a DNA concentration of 2.5 mg/ml.

Preparation of Covalently Bound DNA–Hydrocarbon Complexes.—(*i*) *Long-wavelength u.v. irradiation*. Aliquots (3 ml) of the filtered DNA–hydrocarbon physical complex solutions were irradiated in 10 mm Pyrex cuvettes with radiation of 310—400 nm from a 100-W Hanovia HA100 high-pressure mercury lamp.[28] The DNA was precipitated by the addition of saturated ethanolic ammonium acetate (12 ml) and collected by centrifugation. The pellet was washed thrice with absolute ethanol, twice with ether, air dried, and dissolved in phosphate buffer (pH 6.8, 2 ml) prior to counting.

(*ii*) *γ-Radiolysis*. This was performed using a 1065 Ci cobalt-60 source providing doses up to 50 krad in phosphate buffer solutions purged with nitrous oxide. The DNA was then precipitated, washed, and counted as above.

(*iii*) *Hydrogen peroxide and iodine oxidations*. Hydrogen peroxide was added to solutions of DNA–benzo[*a*]pyrene physical complexes to a final concentration of 30mM and the solutions were then incubated at 37 °C in the dark for up to four days. In selected reactions, additions of sodium citrate (to 15mM) or ferrous sulphate (to 3mM) were made prior to incubation. Aliquots (3 ml) were removed periodically for DNA precipitation and radiochemical assay as described above.

Oxidative binding of hydrocarbon to DNA using iodine was carried out as previously described.[28]

Binding using Rat Liver Microsomes.—Rat liver microsomes were prepared [29] from male Wistar rats induced with 3-methylcholanthrene 24 h before sacrifice. The pellet obtained after centrifugation at 10^5 × g for 1 h was suspended in 0.1M-sucrose solution at a concentration of protein of 2 mg/ml and stored at −170 °C prior to use. NADPH (6 mg) was added to a solution of EDTA (0.003M; 0.3 ml), $MgCl_2$ (0.05M; 0.3 ml), potassium phosphate buffer (0.5M; 1.2 ml, pH 7.5), distilled water (0.6 ml), hydrocarbon (0.5 μmol, 1.2 μCi ^{14}C, 1.2 to 12 μCi ^{3}H), native calf thymus DNA solution (2.5 mg/ml; 3 ml), and microsomal protein (2 mg protein/ml; 0.3 ml). The solution was incubated for 2.5 h at 37 °C with shaking and aeration and then extracted with phenol (6 ml); the DNA was precipitated with ethoxyethanol (6 ml) and collected by centrifugation at 1 000 × g. The DNA pellet was washed thoroughly with ethoxyethanol–water (1 : 1 v/v), twice with ethanol at 20 °C, then twice with absolute ethanol at 70 °C, and lastly twice with ether. The DNA pellet was redissolved in water (3 ml), reprecipitated with ethoxyethanol, and again washed as before. The pellet was finally dissolved in phosphate buffer (2 ml, pH 6.8) prior to counting and hydrolysis.

Radioactive Assay of DNA–hydrocarbon Complexes.—Aliquots of washed DNA–hydrocarbon covalent complexes prepared variously by the above methods (3.75 mg, 1 ml) were treated with HCl (1 ml; 8M) and heated at 120 °C in sealed glass tubes for 1 h. The cooled contents were neutralised with concentrated ammonia solution (33% v/v; 1 ml) and aliquots (1 ml) added to Unisolve 1 (10 ml) in glass scintillation vials and shaken to give a clear solution. The counts recorded on such solutions did not vary over several days.

Investigation of ^{3}H *and* ^{14}C *Counting as a Function of DNA Concentration*.—[*Methyl*-^{3}H]Thymine and [2-^{14}C]thymine were admixed to give a ^{3}H/^{14}C (d.p.m.) ratio of *ca.* 8 in solutions with DNA concentrations from 0.1 to 5.5 mg/ml. Aliquots (1 ml) were counted without hydrolysis by dilution into Unisolve 1 (10 ml).

RESULTS AND DISCUSSION

Radioactive Counting Methods.—Counting data for a standard amount of ^{3}H- and ^{14}C-labelled thymine in the presence of a variable amount of DNA (up to 5.5 mg/ml) using the Triton X based scintillation fluid, Unisolve 1, are presented (Table 1). There is a small but consistent decline in the response to tritium decay, not seen for carbon-14, which results in a fall of some 8% in the ratio of tritium : carbon-14 at the highest concentration of DNA investigated.

Although it has been argued [30] that tritium cannot be estimated accurately in the presence of undegraded DNA, these results show that the quenching of tritium counts by DNA is small and is comparable to the errors encountered in ^{3}H/^{14}C isotope ratios at the concentrations of DNA (0.8 to 2.5 mg/ml) used in this and in previous work.[27,31] In any event, the use of unhydrolysed DNA in radioactivity assays will lead to a small *underestimate* of the loss of tritium from dual-labelled hydrocarbon species.

Nonetheless, the effect of DNA on the counting data can be further examined by comparing results on other-

wise identical samples with and without vigorous acid hydrolysis of the DNA. Counting data are presented (Table 2) for the binding of [G-^{3}H]anthracene to calf thymus DNA mediated by ultraviolet irradiation as described previously.[27] These results show that the change in the measured tritium activity (d.p.m.) of anthracene covalently bonded to DNA varies by no more than 5% between acid-hydrolysed and unhydrolysed samples. This result is in agreement with other

TABLE 1

Variation in the ^{3}H/^{14}C ratio (d.p.m.) for thymine as a function of DNA concentration

DNA concentration [DNA] (mg/ml)	^{3}H (d.p.m.)	^{14}C (d.p.m.)	^{3}H/^{14}C	Ratio (%)
0.125	22 643	2 746	8.25	100
0.725	23 072	2 825	8.17	99.0
1.925	22 838	2 809	8.13	98.5
3.125	21 911	2 780	7.88	95.5
4.325	21 926	2 791	7.86	95.2
5.525	20 923	2 769	7.56	91.2

work showing a change of less than 3% in the ^{3}H/^{14}C ratio on acid hydrolysis for a range of covalently bonded DNA–hydrocarbon adducts, though larger increases in this ratio were observed on hydrolysis of DNA–hydrocarbon physical complexes.[32]

oxide gave 9,10-dideuterioanthracene in modest yield. The mass spectrum showed the incorporation of two deuterium atoms and the n.m.r. spectrum identified their location at positions-9 and -10. Repetition of the experiment using tritiated water of specific activity 18 Ci/mol afforded 9(10)-tritio-anthracene of specific activity 6.34 Ci/mol. On the basis of capture of only one tritium atom per water molecule, this gives a primary kinetic isotope effect of 1.42.

The need for specific tritium-labelling of benzo[*a*]pyrene at position-6 stemmed from photochemical studies in model systems which have established that position as the site of attachment to pyrimidine bases [34,35] and by the reactivity of the benzo[*a*]pyrene cation-radical towards substitution at that position.[36,37] 6-Deuteriobenzo[*a*]pyrene was obtained in satisfactory yield *via* lithiation of 6-iodobenzo[*a*]pyrene [22] and quenching with deuterium oxide. The mass spectrum showed incorporation of one deuterium atom and the n.m.r. spectrum was essentially identical with that for benzo[*a*]pyrene minus the sharp singlet [38] for H-6 at δ 8.40. The experiment was repeated using tritiated water of specific activity 90 Ci/mol to give benzo[*a*]pyrene of specific activity 28 Ci/mol in good yield. As before, this corresponds to a primary kinetic isotope effect of 1.61.

TABLE 2

Binding of [G-^{3}H]anthracene to DNA by ultraviolet radiation

Duration of irradiation (min)	Unhydrolysed DNA d.p.m.*	Unhydrolysed DNA d.p.m.*	Hydrolysed DNA d.p.m.*	Hydrolysed DNA d.p.m.*	Ratio (d.p.m.) Hydrolysed / Unhydrolysed
0	3 194	9 275	3 072	9 185	0.99
5	3 086	8 921	3 061	9 137	1.02
10	4 148	11 920	4 174	12 496	1.05
30	5 769	16 690	5 719	16 877	1.01
75	11 115	31 993	10 315	30 851	0.96
120	10 973	31 988	10 592	32 060	1.00
200	11 773	34 057	11 029	32 629	0.96
275	13 031	37 866	13 376	39 776	1.05

* All samples contain 0.84 mg DNA.

This evidence suggests that criticism levelled at previous binding experiments based on dual labelling ratios is unfounded. Nonetheless, in order to obviate this criticism, all DNA samples in the remainder of the present work have been subjected to acid hydrolysis prior to determination of their radioactivity. In our hands, a more serious source of error arises from inaccuracies in correcting for background radiation in the case of weakly radioactive samples which are amplified in calculating ^{3}H/^{14}C ratios.

Syntheses of Specifically Tritiated Hydrocarbons.—Previous studies [27] on the photochemical binding of anthracene and benzo[*a*]pyrene to DNA employed generally tritiated hydrocarbons. For anthracene, the major tritium content (60 *percent*) was located [33] at positions-9 and -10 which thus directed our efforts to the synthesis of anthracene specifically labelled at these positions.

Quenching of 9,10-dilithioanthracene with deuterium

Proof of the location of the tritium in this material was achieved by its conversion into 6-acetoxybenzo[*a*]pyrene [26] by lead tetra-acetate oxidation. The tritium-labelled hydrocarbon was admixed with carrier benzo[*a*]pyrene and the mixture co-chromatographed before oxidation. The specific activity of the benzo[*a*]pyrene, 61.95 mCi/mol, fell to 0.90 mCi/mol in the 6-acetoxybenzo[*a*]pyrene product showing that 98.5% of the tritium content of the hydrocarbon is located at position-6. In a cognate experiment, ^{3}H- and ^{14}C-labelled benzo[*a*]pyrene was converted into 6-bromobenzo[*a*]pyrene [24] with a change in ^{3}H/^{14}C ratio (d.p.m.) from 40.8 to 2.93. This corresponds to a loss of 93% of tritium. A similar isotope ratio analysis of the conversion of benzo[*a*]pyrene into 6-acetoxybenzo[*a*]pyrene gave a loss of 97% of tritium.

It is apparent that the synthesis of 6-tritiobenzo[*a*]pyrene gives material of at least 93% isotopic purity and this figure may well be higher (97—98%) if there is

some scrambling of tritium in the course of bromination of the hydrocarbon. In the event, the interpretation of the data obtained in using this material in DNA-binding experiments is not significantly dependent on the choice between these alternatives. We note in passing that the preparation employed is comparable to the Grignard route used by Warshawsky and Calvin [22] though in our hands the latter proved to be experimentally more exacting. The hydrocarbon product has a lower specific activity than that achieved by catalytic reduction of 6-bromo-benzo[*a*]pyrene [39] though it seems likely that catalytic hydrogen exchange at positions-4 and -5 impairs the regioselectivity of tritium incorporation in that process. In both syntheses described in the present work, the observed primary kinetic isotope does not significantly discriminate against tritium which indicates that higher specific activities could be achieved by the use of tritiated water of higher specific activity than that employed here.

Specific tritiation of 7,12-dimethylbenz[*a*]anthracene in the 7-methyl group was chosen as a means of differentiating between metabolic processes [40] involving epoxidation of annular positions and hydroxylation of methyl groups as the mechanism of binding of (5) to DNA. Fieser's early work on the synthesis of 7,12-dimethylbenz[*a*]anthracene (5) had utilised [25] reduction of 7-iodomethyl-12-methylbenz[*a*]anthracene (7) as the final step. The ease of reduction of primary alkyl halides with sodium borohydride in dimethyl sulphoxide [41] thus offered a facile synthesis of the desired tritium-labelled hydrocarbon. In practice, the reduction was effected in 36% chemical yield with a primary kinetic isotope effect of only 1.24 (assuming only one of the four hydrogens to be transferred from the borohydride). The oxidation of a sample of the tritiated product gave 7,12-benz[*a*]anthraquinone devoid of tritium, thus showing that no tritium had entered any annular position of the hydrocarbon.

Radiation-promoted Binding of Hydrocarbons to DNA.—The ultraviolet-induced covalent binding of anthracene to DNA was examined for [9(10)-^{3}H]-anthracene admixed with [9-^{14}C]anthracene by the ^{3}H/^{14}C ratio method. The data (Table 3) show an increasing level of covalent bonding with increasing duration of irradiation that tends to a limit close to 40 μmol anthracene per DNA base pair (after correction for non-irradiated controls). While a higher binding level of 150 μmol per base pair was achieved for a single 50 krad dose of γ-radiation from a cobalt-60 source, both of these binding levels are lower than those described previously.[27] They illustrate a general observation in this work that the extent of covalent binding of hydrocarbons to DNA by ultraviolet irradiation is directly related to the amount of hydrocarbon associated with the DNA in the physical complex formed initially and that this, in turn, can be regulated by the quantity of hydrocarbon available and the precise conditions employed in the formation of the physical complex.

In the photochemical binding, the ratio of tritium/carbon-14 in the covalent complex falls with increasing radiation dose, though a change in the ratio for residual, unbound anthracene was not observed. It thus appears that there is a 40% loss of tritium from 9(10)-tritioanthracene on photochemical binding to DNA and a 30% loss on γ-radiation induced-binding. Ultraviolet binding thus appears to involve displacement of tritium from either position-9 or -10 with a primary kinetic isotope effect of 1.25. Alternatively, it can be argued that DNA binding involves loss of hydrogen from *both* positions-9 and -10 with an isotope effect in excess of 2.5. Since no significant primary kinetic isotope effect is seen in the case of 6-tritiobenzo[*a*]pyrene, where this ambiguity does not exist, we favour the former interpretation strongly. At this time there is no evidence for, or against, the co-existence of two mechanisms for anthracene binding to DNA, the major involving substitution of the hydrocarbon at position-9 and a minor process involving no displacement of tritium.

The simplest interpretation of the limited data available for binding of anthracene to DNA by γ-radiolysis is that bonding to position-9 is associated with a modest primary kinetic isotope or accompanied by a minor process not involving hydrogen displacement from C-9(10).

In the case of benzo[*a*]pyrene, photochemical binding also increases progressively with ultraviolet dose (Table 4) but there is no indication that saturation of binding has been achieved even after 4 h irradiation. Double-

TABLE 3

Radiation-promoted bonding of [9(10)-^{3}H]anthracene and [9(10)-^{14}C]anthracene to native calf thymus DNA by irradiation

Radiation–Dose	^{3}H (d.p.m.)	^{14}C (d.p.m.)	^{3}H/^{14}C	^{3}H (%)	Net anthracene bound (μmol/mol base pair)
γ-Radiation 50krad	1 006	1 312.5	0.77	69	150
Ultraviolet 5 min	139	130	1.07	95	4.7
Ultraviolet 10 min	180	168	1.07	95	9.3
Ultraviolet 30 min	224	214	1.05	93	15
Ultraviolet 45 min	197	217	0.91	81	15
Ultraviolet 60 min	251	265	0.95	85	21
Ultraviolet 120 min	331	372	0.89	79	34
Ultraviolet 190 min	277	402	0.69	61	38
Control	123	92	1.34	119	
Physical complex	5 135	4 597	1.12	100	

isotope label studies were carried out with admixed [6-^{3}H]- and [7,12-^{14}C]-benzo[*a*]pyrene. The loss of tritium on binding of (1) to DNA is accurately shown by the change in ratio of ^{3}H/^{14}C for bound relative to unbound hydrocarbon and increases to a stable value of 88% after 3—4 h irradiation. The data fits well a calculated profile which combines no loss of tritium for the small, residual ' dark ' binding of hydrocarbon with a 92% loss of tritium for photochemical binding to DNA (Figure 1).

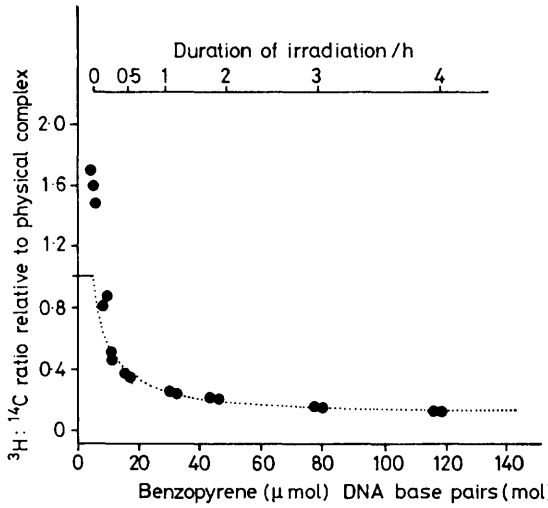


FIGURE 1 ^{3}H : ^{14}C Ratio (d.p.m.) for benzo[*a*]pyrene covalently bound to native calf thymus DNA by ultraviolet irradiation as a function of irradiation duration (top) and of net binding to DNA (bottom) (calculated on carbon-14 association to DNA). Theoretical curve calculated for 92% loss of tritium from hydrocarbon photochemically bound to DNA and no loss of tritium from ' dark ' hydrocarbon-binding.

It is thus clear that photochemical binding of benzo[*a*]-pyrene to DNA *in vitro* involves position-6 and is thus comparable to photochemical binding of this hydrocarbon to pyrimidines in model systems.[34,35] Taken in conjunction with the location of some 97—98% of tritium at this position, either there exists a primary kinetic isotope effect of 1.05 *cr* some 5% of the photochemical binding of (1) to DNA proceeds by an alternative route, not involving displacement of hydrogen from C-6. There is also evident an apparent accretion of tritium into the DNA–hydrocarbon covalent complex which represents irradiation-independent association of benzo[*a*]pyrene with DNA *in vitro*. This anomaly seems to be an artefact arising from the difficulty of making accurate corrections for background radiation at low counting levels for dual-labelled samples, as discussed above.

In similar experiments using [7-Me-$^3H_1$]-dimethylbenz[*a*]anthracene, admixed with 7,12-dimethyl[12-^{14}C]benz[*a*]anthracene, the ^{3}H/^{14}C ratio fell from 2.67 in the physical complex to 1.90 in the covalently bound hydrocarbon giving a loss of tritium of 29%. In the absence of other evidence, this result indicates that photochemical binding of (5) to DNA involves loss of hydrogen from the 7-methyl position and allows for an isotope effect of only 1.15.

Binding of Hydrocarbons to DNA by Chemical Oxidation.—The results for the binding of [6-^{3}H]benzo[*a*]-pyrene to DNA as a result of oxidation with hydrogen peroxide or with iodine are shown (Table 5). The data is provided for plateau levels of binding which were usually attained after incubation for 24 h. It is evident that hydrogen peroxide is most effectively augmented by ferrous sulphate while sodium dihydrogen citrate appears to have a suppressive effect on the covalent binding of benzo[*a*]pyrene to DNA. Iodine oxidation is also a potent means of binding this hydrocarbon to DNA and levels of attachment achieved are comparable with those for radiation-induced binding.

In all experiments, there is a dramatic loss of tritium from the [6-^{3}H]benzo[*a*]pyrene attendant on its linkage to DNA. At the highest levels of binding of the hydrocarbon (some one hydrocarbon for every 10^4 base pairs) and both in the case of hydrogen peroxide–ferrous sulphate and for iodine-promoted binding, the 95% loss of tritium leaves no doubt that these processes both involve activation of position-6 of the hydrocarbon (1) which, in turn, leads to displacement of H-6 by a DNA residue. In the absence of conclusive structural information, the chemical nature of this process remains obscure. However, the involvement of a cation radical, as demonstrated in the anodic oxidative coupling of benzo[*a*]pyrene to pyridine,[37] must be a strong candidate.

For 7,12-dimethylbenz[*a*]anthracene, binding to DNA mediated by hydrogen peroxide was observed to increase with time even after 4 days incubation (Figure 2) and binding levels up to 1 molecule of hydrocarbon per 10^3 base pairs could be achieved. Based on a ratio for

TABLE 4

Ultraviolet irradiative binding of [6-^{3}H]benzo[*a*]pyrene * to native calf thymus DNA

U.v. dose (min)	^{3}H (d.p.m.)	^{14}C (d.p.m.)	^{3}H/^{14}C	^{3}H (%)	Net binding benzo[*a*]pyrene (μmol/mol base pair)
5	2 439	180	13.55	83	3.6
10	1 842	246	7.48	46	6.7
30	1 994	348	5.73	35	11.7
60	2 569	669	3.83	23	27.3
120	3 084	925	3.33	20	39.7
180	3 476	1 643	2.12	13	74.2
240	4 867	2 402	2.03	12	111.3
Control	2 779	107	25.9	158	0
Physical complex	21 995	1 343	16.38	100	

* Admixed with carbon-14 labelled and carrier hydrocarbon.

TABLE 5

Binding of [6-^{3}H]- and [7,10-^{14}C]-benzo[*a*]pyrene to native calf thymus DNA by chemical means

Sample/oxidant	^{3}H (d.p.m.)	^{14}C (d.p.m.)	^{3}H/^{14}C	^{3}H (%)	Net benzo[*a*]pyrene binding to DNA (μmol/mol base pair)
H_2O_2 (30mM)	290	336	0.86	11.3	19.1
H_2O_2 (30mM) + Citrate (15mM)	231	169	1.37	17.9	3.3
H_2O_2 (30mM) + $FeSO_4$ (3mM)	647	1 651	0.39	5.1	144
Iodine (10mM)	563	1 218	0.46	6.0	103
Physical complex	53 316	6 965	7.65	100	

^{3}H/^{14}C of 3.26 in the physical hydrocarbon–DNA complex and of 2.30 in the washed, covalent complex, there is a 30% loss of tritium on hydrogen peroxide mediated binding of [7-Me-$^3H_1$]-7,12-dimethylbenz[*a*]-anthracene to DNA. This figure accords well with that observed for photochemical binding and shows non-selective loss of one hydrogen from the 7-methyl group on covalent attachment of (5) to DNA.

A study of the enzymatic hydrolysis of dual-labelled DNA–hydrocarbon covalent complexes was attempted to facilitate analysis of nucleoside–hydrocarbon adducts. The general procedure described by Baird and Brookes [42] was adopted and employed with covalent complexes of DNA with anthracene and benzo[*a*]pyrene formed by photochemical and oxidative means. Successive treatment of DNA–hydrocarbon complexes with deoxyribonuclease I, snake venom phosphodiesterase, and alkaline phosphatase was followed by chromatography of the products on Sephadex LH 20 gel-filtration medium using a water–methanol gradient. While this resulted in the liberation of much nucleoside material (Figure 3, ultraviolet trace), the majority of the radioactivity (usually near 90%) was eluted with the exclusion volume. It is thus evident that the DNA–hydrocarbon complexes produced *in vitro* with high levels of hydrocarbon attachment are largely resistant to enzymatic digestion and that only a small proportion of the radioactivity is eluted by the gradient at a position characteristic of nucleoside–hydrocarbon adducts.

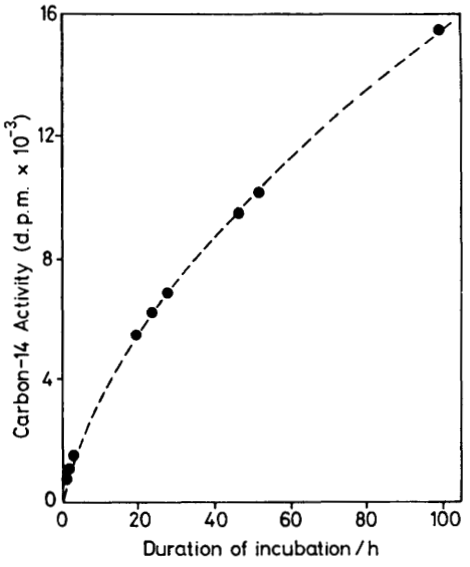


FIGURE 2 Covalent binding of 7,12-dimethylbenz[*a*]anthracene to native calf thymus DNA by hydrogen peroxide (30mM) as a function of duration of incubation.

Microsomally Induced Bonding of Hydrocarbons to DNA.—Benzo[*a*]pyrene was covalently bonded to native calf thymus DNA using well-described procedures [29] and with microsomes obtained from 3-methylcholanthrene-induced, male, Wistar rats. Typical binding data are provided (Table 6) which show

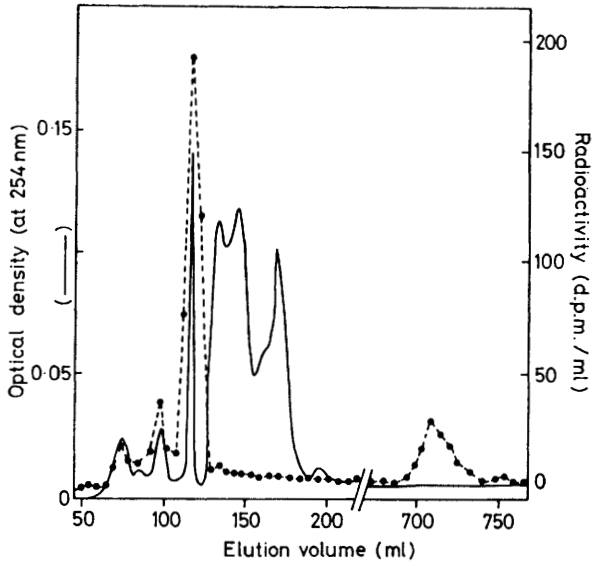


FIGURE 3 Gel filtration chromatographic analysis on Sephadex LH 20 of an enzyme digest of a DNA–benzo[*a*]pyrene covalent complex formed by ultraviolet irradiation (4 h).

that a good level of covalent binding of hydrocarbon to DNA was achieved. An admixture of [6-^{3}H]- and [7,12-$^{14}C_2$]-benzo[*a*]pyrene gave an initial ^{3}H/^{14}C ratio (d.p.m.) of 12.22. This fell to a ratio of 9.81 in the washed, covalent complex, corresponding to a loss of 19% of the tritium from the hydrocarbon which became bonded to DNA. Since chemical degradation has identified 93 to 98% of this tritium to be located at position-6, this result corresponds to some 19.5 to 20.5% of binding of benzo[*a*]pyrene to DNA *via* position-6 for microsomal oxidation. It must be emphasised that these data were analysed under conditions where the isotope ratio analytical method works well.[30]

Ts'o has estimated [43] that 20% of total benzo[*a*]pyrene metabolism in rat liver involves hydroxylation at position-6 and has detected a radical species by e.s.r. that can lead on to the 1,6- 3,6-, and 6,12-benzo[*a*]-pyrenediones—themselves capable of producing chemical

TABLE 6

Binding of [6-^{3}H]benzo[*a*]pyrene * and [7-*Me*-$^3H_1$]-7,12-dimethylbenz[*a*]anthracene * to native calf thymus DNA in the presence of rat liver microsomes

Sample	^{3}H (d.p.m.)	^{14}C (d.p.m.)	^{3}H/^{14}C	^{3}H (%)	Net hydrocarbon binding to DNA (μmol/mol base-pair)
Benzo[*a*]pyrene					
Physical complex	97 698	7 993	12.22	100	
Covalent complex	14 757	1 504	9.81	80.3	141
7,12-Dimethylbenz[*a*]anthracene					
Physical complex	32 084	7 516	4.27	100	
Covalent complex	3 913	888	4.41	103.3	355

* Admixed with carbon-14 labelled and carrier hydrocarbon.

changes in DNA.[44] It thus appears that a sensible interpretation of the present results can combine predominant (80%) binding of benzo[*a*]pyrene to DNA by a process of metabolic activation that does not involve tritium loss from position-6, *e.g. via* the well-established dihydro-diol epoxide processes,[4] with a minor process (20%) that involves tritium displacement from position-6. The fact that 6-hydroxybenzo[*a*]pyrene is, at best, only a weak mutagen and a non-carcinogen [45] may indicate that a liver metabolic process activates benzo[*a*]-pyrene at position-6 and can lead on either to formation of 6-hydroxybenzo[*a*]pyrene or to its covalent binding to DNA.

The results of metabolic binding studies using 7-tritiated 7,12-dimethylbenz[*a*]anthracene are also presented in Table 6. The level of attachment of hydrocarbon (5) to DNA is even higher than that observed for benzo[*a*]pyrene (1) and the data show no loss of tritium on binding 7,12-dimethylbenz[*a*]anthracene to DNA. This result is in sharp contrast to those presented above for chemical and photochemical binding of this hydrocarbon to DNA. It is consistent either with metabolic binding *via* the 5,6-epoxide [17] or through the formation of a dihydrodiol epoxide [17,46] and appears to exclude a bonding process that involves metabolic activation of the 7-methyl group.[13,14,47]

The results described in this study show clearly the value of employing specifically tritiated aromatic hydrocarbons in covalent binding studies with DNA. Sharp distinctions emerge between processes involving chemical activation of hydrocarbons and those using metabolic transformation to a reactive intermediate species. In particular, the use of isotope ratio analysis appears to have particular potential for the identification of minor metabolic binding processes which may escape detection by means of structural analysis of nucleoside–hydrocarbon adducts.

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