Photochemical Binding of Anthracene and Other Aromatic Hydrocarbons to Deoxyribonucleic Acid with Attendant Loss of Tritium

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Anthracene can be bound covalently to calf thymus deoxyribonucleic acid (DNA) by means of long-wavelength u.v. irradiation to a greater extent than any other of seven polycyclic aromatic hydrocarbons investigated. ^3H is incorporated into the photochemical product resulting from the irradiation of doubly labelled hydrocarbons and DNA to a lesser extent than is ^{14}C. In the case of anthracene, the ratio of incorporation of the two isotopes is *ca.* 3:4 and is independent of the extent of irradiation or of hydrocarbon binding. The relevance of these results to the mechanism of binding of hydrocarbons to DNA and to the photodynamic carcinogenicity of anthracene is discussed briefly.

COVALENT binding or polycyclic aromatic hydrocarbons to the deoxyribonucleic acid (DNA) of mammalian skin *in vivo* was first observed by Heidelberger and Davenport [1] in the case of dibenz[*a,h*]anthracene and was later established as providing a satisfactory correlation with carcinogenicity for a variety of such hydrocarbons.[2]

The mechanism of such covalent attachment is not known, although much recent activity has centred on the possible role of aromatic hydrocarbon epoxides [3] as potential ultimate carcinogens since such compounds are known intermediates in the conversion of the aromatic hydrocarbons into the corresponding dihydrodiols by liver preparations.[4-7] Several model systems have been developed to effect covalent binding of these hydrocarbons to native and to denatured DNA *in vitro*. These methods include radiation by u.v.,[8] *X*-ray [9] and γ [10] sources, iodine,[11,12] hydrogen peroxide,[11,13] and ascorbic acid [11] oxidising systems, and also some combination of these.[14]

In general, the results from these models have been interpreted to show that the more carcinogenic hydrocarbons could be bonded to DNA to a greater extent than their non-carcinogenic isomers.[8,11,14] In addition, the majority of these results have relied on a radioisotope assay involving the incorporation of tritiated aromatic hydrocarbon into the DNA. On the other hand, the elucidation of structures for the photoproducts formed between benzo[*a*]pyrene and 1-methylcytosine [15] and thymine [16] has shown that they arise from an oxidative coupling between hydrocarbon and nucleic acid base which necessarily involves the loss of hydrogen from the hydrocarbon. Accordingly, there must be some uncertainty about the reliability and accuracy of the tritium-incorporation assay.

This paper reports a re-evaluation of the extent of photochemical binding of seven polycyclic aromatic hydrocarbons to both native and denatured DNA using both carcinogenic and non-carcinogenic compounds in conjunction with isotopic labelling by both ^3H and ^{14}C. It presents original, detailed evidence for the binding of anthracene to DNA *in vitro* by u.v. irradiation.

EXPERIMENTAL

Materials.—Tritium-labelled hydrocarbons, anthracene, benzo[*e*]pyrene, benzo[*a*]pyrene, dibenz[*a,c*]anthracene and 3-methylcholanthrene were obtained from the Radiochemical Centre, Amersham, and in each case were described as generally-labelled. [6-^{14}C]-3-Methylcholanthrene was purchased from New England Nuclear, Frankfurt, W. Germany and [9-^{14}C]anthracene, [3,6-$^{14}C_2$]benzo[*a*]pyrene, [9-^{14}C]dibenz[*a,c*]anthracene, and 7,12-dimethyl[12-^{14}C]-benz[*a*]anthracene were supplied by the Radiochemical Centre, Amersham. Commercially available, unlabelled aromatic hydrocarbons were crystallised prior to use while chromatographic examination of the isotopically-labelled hydrocarbons established their radiochemical purity at $>98\%$ and they were therefore used as supplied in benzene solution. [9-^{14}C]Dibenz[*a,c*]anthracene was purified by chromatography on neutral alumina (5 g). The initial 15 ml of benzene eluate were concentrated and further purified by preparative t.l.c. on alumina plates developed with n-hexane–benzene (6 : 1; v/v). The blue fluorescent band at R_F 0·3 was collected and extracted with benzene to give the pure product with a specific activity of 2·5 mCi mmol^{-1}.

DNA was obtained from Sigma Chemicals, London (calf thymus; type V; average molecular weight 10^6 daltons) and standard solutions were prepared by its slow dissolution at 4 °C in 10^{-2}M-phosphate buffer at pH 6·8 to give a concentration of 2·5 mg ml^{-1}. All solutions contained 5×10^{-4}M-EDTA. Solutions of denatured DNA were prepared by heating the above solution on a boiling water-bath for 10 min and rapidly cooling the solution in ice for 30 min and the procedure repeated.

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Sephadex G-200 powder for gel filtration chromatography was purchased from Pharmacia. Unisolve 1 (Koch–Light) was employed for liquid scintillation counting and efficiencies determined using [1-^{14}C]-n-hexadecane and [1,2(n)-$^{3}H_2$]-n-hexadecane from the Radiochemical Centre as standards. U.v. spectra were recorded using a Unicam SP 1800 or Gilford 240 spectrophotometer and a Radiometer PHM 26 in conjunction with standard calomel and G202B glass electrodes was used to measure pH.

Methods.—DNA–hydrocarbon solutions. Sufficient standard benzene solution of the radioactive hydrocarbon to give 50 μCi of ^{3}H (10 μCi for ^{14}C) was evaporated in a stream of dry nitrogen and solid, carrier hydrocarbon added to a total of 4 μmol. The whole was then dissolved in pure dioxan (0·5 ml) and this solution diluted with standard DNA solution (40 ml). The mixture was incubated at 0—4 °C for 3 days in the dark and then filtered through a selected glass sinter or, more usually, through Whatman No. 1 paper. The hydrocarbon content of the filtrate was assayed by diluting 0·1 ml with 0·9 ml of the standard DNA solution and 15 ml Unisolve 1 prior to counting the clear, homogeneous solution in a Packard Tricarb scintillation counter. The DNA content was determined spectrophotometrically.

Covalent binding of hydrocarbons to native calf thymus DNA (number of hydrocarbons per 10^4 nucleotide bases)

Hydrocarbon	Iball index [a]	U.v. irradiation[11]	Iodine in aqueous ethanol[12]	Iodine and u.v. irradiation [b]	Present work: ^{14}C assay	Present work: ^{3}H assay
7,12-Dimethylbenz[*a*]anthracene	151		3		1·3 ± 0·2	
3-Methylcholanthrene	80		3·6	10—50	5·0 ± 0·5	3 ± 1
Benzo[*a*]pyrene	75	3–5	9	10—50	2·8 ± 0·5	2 ± 1
Dibenz[*a,h*]anthracene	26	0·5		10—50		0·5 ± 0·2
Benz[*a*]anthracene			0·9	1		
Benzo[*e*]pyrene		1–2	0·65			0·5 ± 0·2
Dibenz[*a,c*]anthracene		0·4			0·45 [c]	0·6 [c]
Anthracene				0 [d]	5·4 ± 1	4 ± 2

[a] J. Iball, *Amer. J. Cancer*, 1939, **35**, 188. [b] *M. lysodeikticos* DNA.[14] [c] Single experiment. [d] Not detectable.

For experiments using doubly-labelled hydrocarbons, the appropriate volumes of the ^{3}H and ^{14}C labelled hydrocarbon solutions in benzene were admixed, evaporated in a stream of nitrogen, and the procedure continued as above.

Irradiation and counting procedure. The filtered DNA–hydrocarbon solution (3 ml) was placed in a 1 cm path-length quartz cuvette at 25 cm from the point source of a Hanovia HA100 high pressure mercury arc fitted with filters to restrict transmission to the 310—400 nm band and using the standard concave mirror and quartz lens provided to focus the radiation onto an area of *ca.* 8 mm diameter. Since preliminary experiments showed that the continuous bubbling of water-saturated nitrogen through the solutions during irradiation resulted in no change in the extent of radioisotope incorporation into the DNA, such degassing was not routinely adopted.

After irradiation for the prescribed time, the solution was diluted with ammonium acetate (10 g l^{-1}) in 95% ethanol at 0 °C and the DNA precipitate washed exhaustively with 95% ethanol and with ether, as described by Ts'o and Lu.[8] The DNA pellet was air-dried and transferred to a scintillation bottle by dissolution in two 0·5 ml portions of 10^{-2}M-phosphate buffer (pH 6·8) and then admixed with Unisolve 1 (15 ml) to give a clear, homogeneous solution for counting.

In certain experiments with anthracene, this procedure was modified in two respects. A series of irradiations was performed on solutions which contained 10^{-2}M-tetramethylammonium chloride, 10^{-3}M-phosphate at pH 6·8, and EDTA as usual. In a second, separate group of irradiations, a standard solution of native DNA and [^{14}C]-anthracene in 10^{-2}M-phosphate buffer (40 ml) was incubated with a solution of iodine (4 mol. equiv.) in 95% ethanol (10 ml) at 0 °C for 2 days in the dark. Aliquot portions (3 ml) were then withdrawn and irradiated for periods up to 4 h and the DNA precipitated and counted as before.

Gel filtration chromatography. A standard solution of native DNA and [^{14}C]anthracene (3 ml) was irradiated for 4 h. Part of this solution (1 ml) was layered on a column of Sephadex G-200 (30 × 1·5 cm) and eluted with 10^{-2}M-phosphate buffer. The eluate was continuously monitored at 254 nm and fractions (1 ml) collected for radioactive assay.

The DNA in a similar portion of the same solution was precipitated with ethanolic ammonium acetate and washed free of unbound hydrocarbon as usual, then redissolved in 0·14M-sodium chloride solution (1·15 ml). A part of this solution (1 ml) was subjected to chromatography on Sephadex G-200 as before.

Sucrose gradient centrifugation. A sample of the foregong, washed DNA–anthracene complex (0·15 ml) was layered on 5 ml of a sucrose density gradient (5—20%; w/v) in a Spinco model L centrifuge using an SW 50 rotor. The rotor was operated at 50,000*g* ($r_{av.}$ 7·5 cm) for 20 h at 5 °C. Fractions (20 drops) were collected and monitored at 260 nm in 1 mm path-lengths before dilution to 1 ml with 10^{-2}M-phosphate buffer and radioactive assay as before.

RESULTS

Direct Photochemical Binding of Hydrocarbons to DNA.—For all seven hydrocarbons investigated, the extent of binding to DNA increases with time and usually reaches a maximum after some 3—4 h irradiation. The extent of photochemical binding, gauged by the difference between maximal incorporation of radioactivity after irradiation and that inseparable from the DNA before the commencement of irradiation, is shown in the Table. A significant variation of the extent of this incorporation was observed in successive experiments performed under apparently identical conditions; accordingly, median values are indicated with upper and lower limits.

The amount of added hydrocarbon which could not be separated from the DNA by precipitation and washing was <0·8% of that added initially and usually <0·4%, as observed by Ts'o and Lu.[8] In every case, more extensive binding of each hydrocarbon was observed for denatured than for native DNA. This increase was usually some 3—4 fold though in one experiment the binding of anthracene to denatured DNA exceeded 3 molecules of hydrocarbon per 1000 bases and corresponded to 25% incorpor-

ation of the total hydrocarbon added. An independent increase in the level of hydrocarbon binding was observed to result from the inclusion of 10^{-2}M-tetramethylammonium chloride in the DNA solution. This stimulated an approximately five-fold increase in photochemical binding to native DNA but had a much smaller effect on denatured DNA.

Irradiations of doubly-labelled hydrocarbon–DNA solutions were performed for anthracene, benzo[*a*]pyrene and 3-methylcholanthrene. All showed a similar time-dependent uptake of both ^{3}H and ^{14}C, as illustrated for

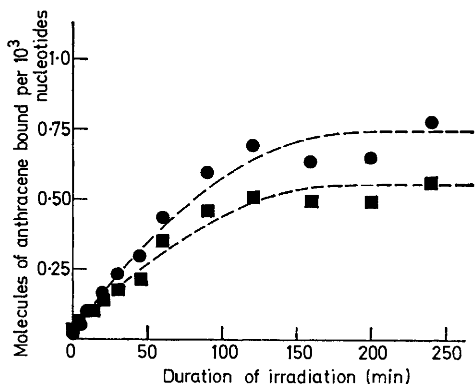

FIGURE 1 Photochemical binding of anthracene to denatured DNA: incorporation of ^{14}C, ●; incorporation of ^{3}H, ■

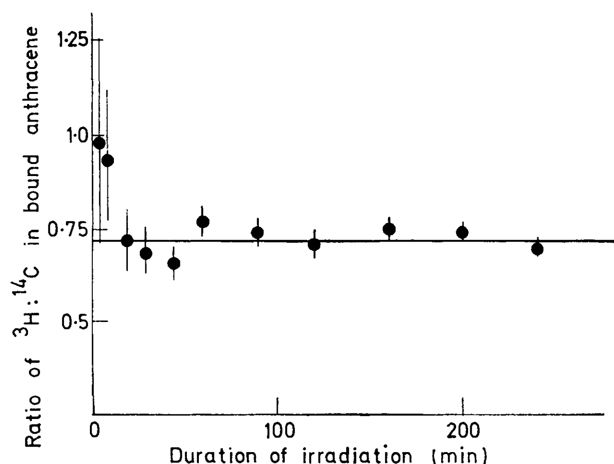

FIGURE 2 Ratio of $^{3}H : ^{14}C$ incorporated into denatured DNA using doubly-labelled anthracene. The horizontal line is the average value for the ratio of the net photochemical incorporation of ^{3}H to that of ^{14}C weighted in inverse proportion to the magnitude of the errors represented by the vertical bars

anthracene (Figure 1), but reveal some difference in the ratio of bound $^{3}H : ^{14}C$ with time. For anthracene the ratio of photochemical incorporation appears to be independent of the duration of irradiation and the data provides a weighted average ratio for $^{3}H : ^{14}C$ incorporation of 0·72 in the case of denatured DNA (Figure 2). A similar effect was apparent in the binding to native DNA.

While the extent of dark-binding for anthracene is too small to permit of a significant comparison of isotopic incorporation into DNA, this is not the case for the other two hydrocarbons used. The $^{3}H : ^{14}C$ ratios for overall incorporation of benzo[*a*]pyrene and 3-methylcholanthrene to native DNA apparently decrease from unity and tend towards limiting values of 0·6 for the former and 0·7 for the latter with prolonged irradiation (Figure 3).

Irradiation of DNA–Anthracene Solution with Iodine.— The iodine-containing solution of anthracene and native DNA exhibited an orange-red fluorescence which persisted

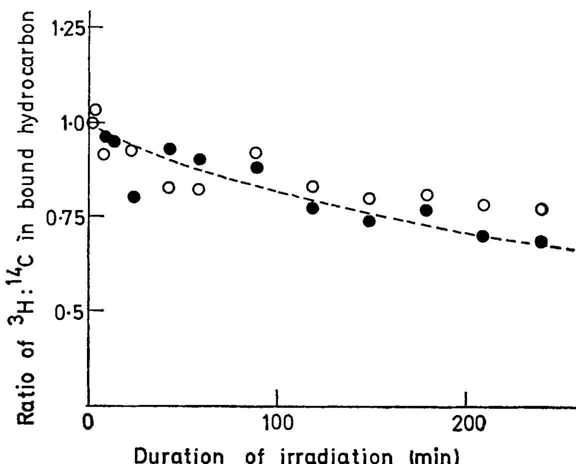

FIGURE 3 Ratio of $^{3}H : ^{14}C$ incorporated into native DNA using doubly-labelled benzo[*a*]pyrene and 3-methylcholanthrene. Symbols represent the ratio of total uptake of ^{3}H to ^{14}C including dark-binding: benzo[*a*]pyrene, ●; 3-methylcholanthrene, ○. The broken line is computed for first-order kinetic behaviour for the benzopyrene data

for 1·5—2 h irradiation and then was discharged to be replaced by the characteristic green-blue fluorescence of the hydrocarbon. At the same time, the last traces of iodine colour in the DNA solution were bleached. The standard ^{14}C assay showed that the amount of anthracene incorporated into the DNA remained virtually constant at 0·7 mmol anthracene per mol phosphate during this period and then rose sharply to a final binding level of 1·6 mmol mol^{-1} after some 2 h further irradiation (Figure 4).

Gel Filtration Chromatography and Sucrose Gradient Centrifugation.—The complex resulting from irradiation of anthracene and native DNA was characterised by gel filtration and by sucrose gradient centrifugation to show that it was free of low-molecular weight, radioactive

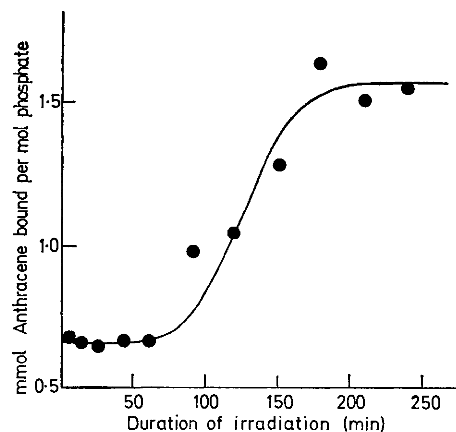

FIGURE 4 Photochemical binding of anthracene to native DNA in the presence of iodine. Iodine concentration initially 0·8 μmol in 20% (v/v) ethanol

contaminants which might not be removed by washing of the complex in organic solvents. The results of gel filtration of an irradiated complex before washing with

organic solvents show that DNA and anthracene are eluted together from the column at void volume and are later followed by a second, larger anthracene fraction (Figure 5). The proportion of hydrocarbon which is eluted with the DNA

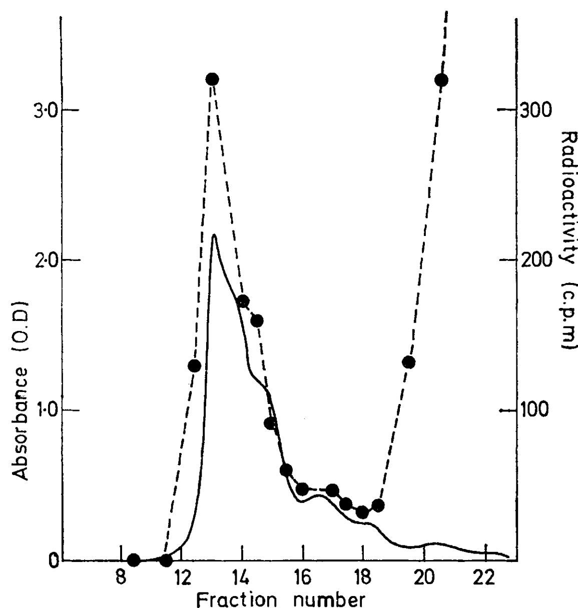

FIGURE 5 Gel filtration chromatography of an unwashed photoproduct of anthracene–native DNA on Sephadex G-200

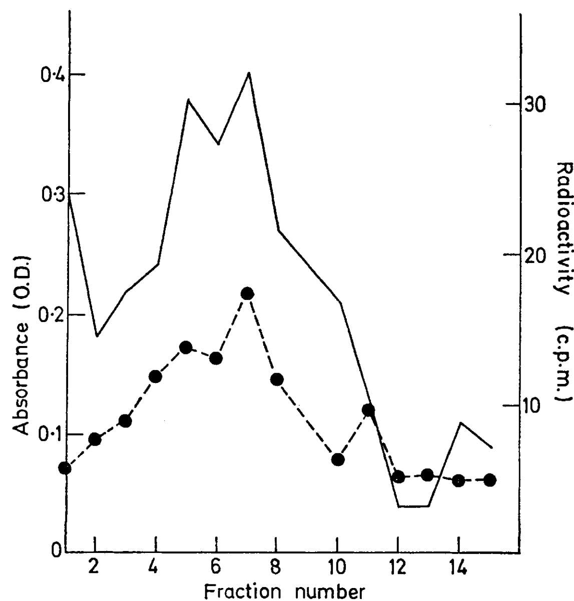

FIGURE 6 Sucrose gradient centrifugation pattern of a washed DNA–anthracene photoproduct

corresponds closely to that which is inseparable from the DNA by the precipitation and washing routine and amounted, in independent experiments, to 12—19% of the total hydrocarbon added to the DNA. Gel filtration of this complex after precipitation and washing with organic solvents revealed only a single peak of radioactivity which was eluted in company with the DNA. The same, washed DNA–anthracene photoproduct was examined by sucrose gradient centrifugation (Figure 6). While there is evidence of inhomogeneity of the DNA, which is a likely consequence of its irradiation or precipitation, it is apparent that the major part of the radioactivity sediments identically with the DNA and is thus closely associated with it.

DISCUSSION

Anthracene has been identified as one of the polycyclic aromatic hydrocarbons which is most extensively incorporated into a purely physical complex with DNA [17] but no covalent binding between these two species has been detected hitherto.[14] The results presented here firmly establish that physical complexes of anthracene with either native or denatured DNA can be converted photochemically into chemical complexes in which the hydrocarbon is covalently linked to the DNA. Moreover, the extent of this covalent binding, in terms of mole fraction, is significantly greater than that achieved for the carcinogenic hydrocarbons examined under strictly comparable conditions (Table). The proportion of conversion of physically-bound into chemically-bound anthracene of up to 25% also compares well with the most efficient conversion for benzo[*a*]pyrene of 50%.[18]

The criteria which have been used to establish the chemical integrity of complexes between DNA and carcinogenic hydrocarbons [18] are also satisfied by the behaviour of the DNA–anthracene photoproduct.* Both gel filtration chromatography and sucrose gradient centrifugation show that the movement of photochemically-bound [^{14}C]anthracene is coincident with that of DNA (Figures 5 and 6) while [^{14}C]anthracene which is only physically-complexed is readily separable either by gel filtration (Figure 5) or by precipitation and washing of the DNA.

Iodine also influences the binding of anthracene to DNA. The dose-dependence of incorporation on irradiation of a DNA–anthracene complex in the presence of iodine shows a high level of ' dark-binding ' followed by an induction period which lasts until the molecular iodine has been reduced (Figure 4). It thus appears that anthracene is bound to DNA by the action of iodine in the dark, that photochemical binding is markedly inhibited by the presence of molecular iodine, and that subsequent photochemical binding of anthracene to DNA is some twofold greater than that experienced in the absence of iodine. It is, however, possible that this increase is simply due to partial denaturation of the DNA by the action of iodine or the ethanol in the solvent.

That Pascal failed to detect any anthracene binding to DNA as a result of the joint action of iodine and u.v.

* Although there is no direct evidence for the site of attachment of the hydrocarbon to the DNA, preliminary observations indicate that partial hydrolysis of the washed DNA–anthacene complex leaves a part of the anthracene linked to the apurinic acid while the remainder is released in association with the diffusable purine fraction.[19]

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[18] S. A. Lesko, H. D. Hoffman, P. O. P. Ts'o, and V. M. Maher, *Molecular and Subcellular Biology*, 1971, **2**, 347.
[19] P. Taussig, M.Sc. Thesis, Sheffield University, 1973.

radiation [14] must be attributed either to the use of a predominantly organic solvent system or, more probably, to the insensitivity of the fluorescence assay method adopted.

Nonetheless, the radioisotope assay developed by Ts'o [8] is also suspect as long as its use is restricted to ^{3}H-labelled hydrocarbons since ^{3}H exchange or loss must always be anticipated in aromatic substitution processes. In principle, such ^{3}H exchange can be manifested as a consequence of three separate phenomena. These correspond to the photochemical activation of ^{3}H (i) prior to the covalent attachment of the hydrocarbon to the DNA, or (ii) in the course of that attachment, or (iii) as a consequence of reactivation of the hydrocarbon subsequent to its primary bonding to the DNA. It is clear that only the second of these patterns of behaviour can result in the specific activity of ^{3}H in the incorporated hydrocarbon remaining independent of the duration of irradiation while both the first and last of them should be manifest in a declining specific activity of ^{3}H with increasing duration of irradiation. It seems reasonable that the first of them should show a constant specific activity for ^{3}H during further irradiation after attainment of maximal incorporation of the hydrocarbon into the DNA while the last of them should show a continuing decline of that activity during such irradiation.

The results obtained from the experiments using doubly-labelled aromatic hydrocarbons appear to illustrate two of these three phenomena. In the case of anthracene, the extent of photochemical incorporation of the hydrocarbon into native or denatured DNA usually attains a maximum after 3—4 h irradiation, as gauged by the uptake of ^{14}C (Figure 1). The ratio of $^{3}H:^{14}C$ in the washed photoproduct is sensibly independent of the extent of hydrocarbon uptake or of the duration of irradiation, showing a constant value of 0·72 of the ratio of these two isotopes present in the DNA–anthracene mixture prior to irradiation (Figure 2). This behaviour is consistent only with the displacement of ^{3}H from anthracene simultaneously with hydrocarbon bonding to the DNA. It thus strongly indicates that the mechanism of such attachment is an aromatic substitution process of the same kind as that involved in the formation of photoproducts between benzo[*a*]pyrene and 1-methylcytosine [15] and benzo[*a*]pyrene and thymine.[16] Clearly the further use of specifically-tritiated anthracenes should be capable of enabling identification of the position on the hydrocarbon which becomes bonded to the DNA.

In the case of benzo[*a*]pyrene, the $^{3}H:^{14}C$ ratio falls with increasing duration of irradiation (Figure 3). Moreover this decline appears to continue as irradiation is extended beyond the period of 2—3 h required to achieve maximal incorporation of this hydrocarbon into DNA. A similar, though less marked, pattern of behaviour is shown by 3-methylcholanthrene. These results suggest that little or no ^{3}H is lost from these two carcinogenic hydrocarbons in the course of dark-binding to DNA, that a significant proportion of the tritium is lost during the course of photochemical linkage to the DNA, and that a small, continuing decline in the $^{3}H:^{14}C$ ratio is indicative of a secondary photochemical process involving the activation of hydrocarbon after its attachment to the DNA. In this context it is noteworthy that cross-linking of DNA has been reported to result from the binding to it of benzo[*a*]pyrene or of 7,12-dimethylbenz[*a*]anthracene [14,20] and that photochemical cross-linking would be manifest in a further decline in the specific activity of tritium similar to that observed. It may thus emerge that the carcinogenicity of polycyclic aromatic hydrocarbons is associated with their potential for cross-linking DNA rather than with their capacity for simple covalent binding to DNA.

Although anthracene is normally considered to be non-carcinogenic, Heller showed that topical application of anthracene ointment in conjunction with non-carcinogenic, long-wavelength u.v. irradiation produced malignant tumours with high efficiency in mice.[21] He described this photodynamic activity of anthracene as 'a new cancer-inducing principle.' Anthracene, however, has been shown to possess but a small photodynamic activity with respect both to the immobilisation of *Paramecium caudatum* [22] and to the lethal effects of light on cultured mouse tumour cells.[23] It must, therefore, be considered a serious possibility that the tumours produced in Heller's experiments arose not from a photosensitising effect of anthracene but from its covalent binding to DNA *in vivo*. Experiments to explore this possibility are in progress.[19]

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