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THE INVOLVEMENT OF A NON-'BAY-REGION' DIOL-EPOXIDE IN THE FORMATION OF BENZ(a)ANTHRACENE-DNA ADDUCTS IN A RAT-LIVER MICROSOMAL SYSTEM

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SUMMARY: The metabolic activation of benz(a)anthracene was investigated by incubating [^{3}H]-benz(a)anthracene with DNA, a NADPH-generating system and rat-liver microsomes. When hydrolysates of the DNA were chromatographed on Sephadex LH20 columns, three hydrocarbon-nucleoside adduct peaks were resolved and these were further examined using HPLC. One adduct probably results from the reaction of the non-bay-region diol-epoxide r-8,t-9-dihydroxy-t-10,11-oxy-8,9,10,11-tetrahydrobenz(a)anthracene (anti-BA-8,9-diol 10,11-oxide) with DNA. The other two adducts did not co-chromatograph with adducts formed from any of the four possible isomeric diol-epoxides that can be formed in the 8,9,10,11-ring of benz(a)anthracene.

INTRODUCTION: Polycyclic aromatic hydrocarbons are metabolized in several biological systems to products that include non-K-region dihydrodiols that may be further metabolized to vicinal diol-epoxides. Studies on the formation of hydrocarbon-nucleic acid adducts *in vivo* from benzo(a)pyrene (1) and 7-methylbenz(a)anthracene (2) have shown that these adducts probably arise through the reaction of vicinal diol-epoxides of the bay-region type (3) with nucleic acids. Other studies that involved the incubation of a hydrocarbon with nucleic acids in the presence of rat-liver microsomal preparations (4,5) have shown that some of the hydrocarbon-nucleic acid adducts formed are similar to those that are formed *in vivo* and that are

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Abbreviations: Anti-BA-8,9-diol 10,11-oxide, r-8,t-9-dihydroxy-t-10,11-oxy-8,9,10,11-tetrahydrobenz(a)anthracene; syn-BA-8,9-diol 10,11-oxide, r-8,t-9-dihydroxy-c-10,11-oxy-8,9,10,11-tetrahydrobenz(a)anthracene; anti-BA-10,11-diol 8,9-oxide, t-10, r-11-dihydroxy-t-8,9-oxy-8,9,10,11-tetrahydrobenz(a)anthracene; syn-BA-10,11-diol 8,9-oxide, t-10,r-11-dihydroxy-c-8,9-oxy-8,9,10,11-tetrahydrobenz(a)anthracene .

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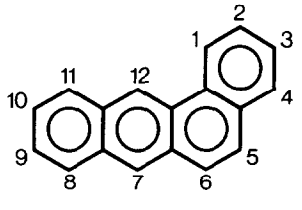
thought to be involved in the metabolic activation of and the carcinogenic effects of the parent hydrocarbon. Further evidence in support of the bay-region theory of metabolic activation of polycyclic hydrocarbons has been obtained from studies on the mutagenic (6,7) and tumourigenic (8,9) activities of dihydrodiols of several hydrocarbons. The results of these studies show that dihydrodiols that may be further metabolized to vicinal diol-epoxides of the bay-region type are more active in these test systems than either the parent hydrocarbons or other dihydrodiols derived from the hydrocarbons. Thus, studies on the tumour initiating (10) and mutagenic activities (11) of benz(a)anthracene (formula shown) and related dihydrodiols have shown that the 3,4-dihydrodiol is highly active in both systems, although the parent hydrocarbon is only weakly carcinogenic. It has recently been found (12) that the major hydrocarbon-nucleic acid adducts that are formed when either mouse skin in vivo or hamster embryo cells in culture were treated with benz(a)anthracene possessed a phenanthrene-like fluorescence spectrum and this is consistent with metabolic activation of benz(a)anthracene occurring in the 8,9,10,11-ring by the formation of a vicinal diol-epoxide that is not of the bay-region type. In agreement with this the results presented here provide evidence showing that a major hydrocarbon-DNA adduct formed when benz(a)anthracene is incubated with DNA in the presence of rat-liver microsomal preparations from rats pretreated with 3-methylcholanthrene probably arises by the reaction of anti-BA-8,9-diol 10,11-oxide with DNA.



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MATERIALS AND METHODS: [^{3}H]-Benz(a)anthracene (15 Ci/mmol) was prepared from unlabelled hydrocarbon by catalytic exchange (Radiochemical Centre, Amersham, Bucks, U.K.). The anti-BA-8,9-diol 10,11-oxide and the syn-BA-8,9-diol

10,11-oxide were synthesized as described (13,14) and the anti-BA-10,11-diol 8,9-oxide and the syn-BA-10,11-diol 8,9-oxide were kindly supplied by Dr.J.N. Keith of the I.I.T. Research Institute, Chicago, Ill., USA through the Chemical Repository of the National Cancer Institute. DNA (salmon testes, type III), that was deproteinized before use, was obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Chemical and enzymic reactions of benz(a)anthracene with DNA. Each of the four diol-epoxides of benz(a)anthracene named above were incubated with DNA and the DNA isolated and hydrolysed to nucleosides exactly as described previously (2). [^{3}H]-Benz(a)anthracene (2 μmol) was incubated at 37°C with DNA (125 mg), NADP, $MgCl_2$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase in the presence of a microsomal fraction (2) prepared from liver (20g) from rats that had been pretreated with 3-methylcholanthrene. After 30 min, the incubation mixture was cooled to 4°C, the microsomes separated by centrifugation (1 h, 100,000 x g, 4°C) and DNA precipitated from the supernatant with ethanol (2 x 1 vol). The DNA was washed with ethanol, deproteinized and hydrolysed to nucleosides as described (2).

Sephadex LH20 column chromatography and high pressure liquid chromatography: Samples of enzymic hydrolysates of DNA that had been incubated with [^{3}H]-benz(a)anthracene in the presence of a rat-liver microsomal fraction were mixed with UV-absorbing amounts of nucleoside adducts obtained from the chemical reaction of one of four diol-epoxides of benz(a)anthracene with DNA and the mixture chromatographed on columns (70 x 1.5 cm) of Sephadex LH20 that were eluted with either water-methanol (15) or with sodium borate (0.05M,pH 8.7)-methanol (16) gradients as described. Eluant fractions (5 ml) were collected and the absorbance (260 nm) and fluorescence (excitation 300 nm, emission 356 nm) determined for each fraction. ^{3}H-Labelled products present in each fraction were determined by liquid scintillation counting of a sample (1 ml) of each fraction in PTX scintillant (10 ml) (Packard Instrument Co., Downers Grove, Illinois, Mo., USA) using a Packard Tricarb 3320 scintillation counter. Fractions that contained hydrocarbon-nucleoside adducts that were to be further investigated were pooled and evaporated to dryness and the residue dissolved in methanol (0.5 ml). Samples (0.1 - 0.25 ml) of these solutions were examined by HPLC at 40°C on columns of Spherisorb 5 ODS (4 x 250 mm; HPLC Technology Ltd., Wilmslow, Cheshire, U.K.) that were eluted at a flow rate of 2 ml/min with a convex gradient, over 45 min, from 0-35% methanol in water and then with a concave gradient, over 45 min, from 35-65% methanol in water using a Waters 660 Solvent Programmer (Waters Associates Ltd., Hartford, Cheshire, U.K.). Fractions (2 ml) of eluate were collected and hydrocarbon-nucleoside adducts detected by fluorescence as described above. Radiolabelled products in samples (1 ml) of each fraction were determined by liquid scintillation counting. In some cases, fractions from Sephadex LH20 columns were pooled, evaporated to dryness and the residues incubated with acetic anhydride-pyridine (1:9, v/v; 0.5 ml, 40h at 4°C). The mixtures were evaporated to 100 μl volume with a stream of nitrogen and the residues examined by HPLC at 40°C on a column of Spherisorb 5 ODS that was eluted with a convex gradient, over 45 min, of 0-60% methanol in water followed by a concave gradient over 45 min, of 60-80% methanol in water, at a flow rate of 2 ml/min. Fractions (2 ml) of eluant were collected and fluorescence and radioactivity determined as described above.

RESULTS AND DISCUSSION: When hydrolysates of DNA that had been incubated with [^{3}H]-benz(a)anthracene and a metabolizing system were chromatographed on Sephadex LH20 columns, the elution profile contained three peaks (products

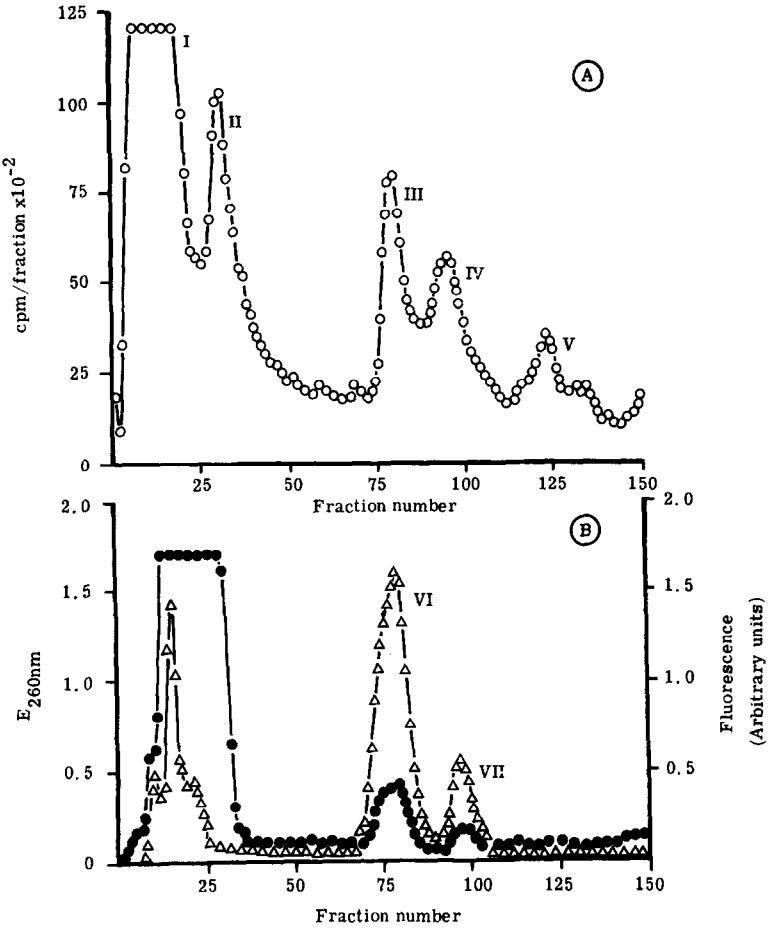

Fig. 1 Sephadex LH20 column chromatography of hydrocarbon-nucleoside adducts. A hydrolysate of DNA that had been incubated with a rat-liver microsomal fraction and ^{3}H-labelled benz(a)anthracene was co-chromatographed with a hydrolysate of DNA that had been incubated with anti-BA-8,9-diol 10,11-oxide. The Sephadex LH20 column was eluted with a water-methanol gradient and radioactivity (O——O, fig. 1A), fluorescence (Δ——Δ, fig. 1B) and absorbance (●——●, fig. 1B) were determined.

III, IV and V fig. 1) that eluted in the region normally occupied by hydrocarbon-nucleoside adducts. The identity of these products was investigated in experiments in which hydrolysates of DNA that had been incubated with microsomes and ^{3}H-labelled benz(a)anthracene were co-chromatographed on Sephadex LH20 columns with hydrolysates of DNA that had been incubated with either anti-BA-8,9-diol 10,11-oxide, anti-BA-10,11-diol 8,9-oxide, syn-BA-8,9-diol 10,11-oxide or syn-BA-10,11-diol 8,9-oxide, the four diol-epoxides that can be formed in the 8,9,10,11-ring of benz(a)anthracene. When the

Sephadex LH20 columns were eluted with water-methanol gradients, ^{3}H-labelled product V did not co-elute with any of the non-labelled hydrocarbon-nucleoside adducts formed from these four diol-epoxides. However, ^{3}H-labelled products III and IV did co-elute with adducts formed from anti-BA-8,9-diol 10,11-oxide (fig. 1) and with adducts formed from the other three diol-epoxides (results not shown).

The identity of products III and IV was further investigated in experiments in which ^{3}H-labelled products and non-labelled adducts that co-eluted

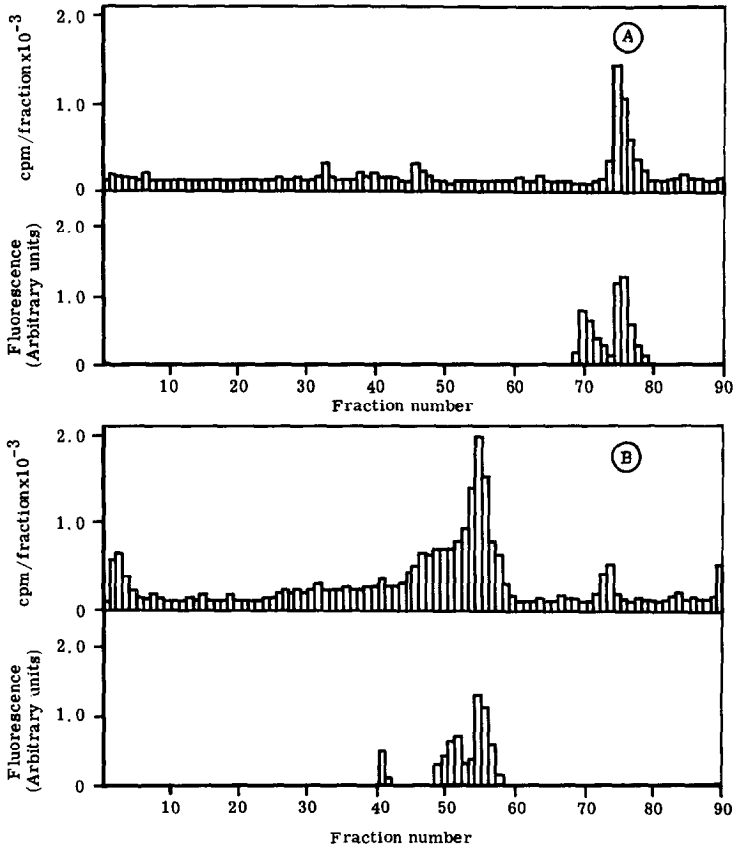

Fig. 2 High pressure liquid chromatography of deoxyribonucleoside-hydrocarbon adducts. ^{3}H-Labelled products present in the hydrolysis of DNA that had been incubated with [^{3}H]-benz(a)anthracene in the presence of a rat-liver microsomal fraction and fluorescent amounts of non-labelled adducts formed from DNA that had been incubated with anti-BA-8,9-diol 10,11-oxide in vitro were co-chromatographed on Sephadex LH20 Eluted fractions containing ^{3}H-labelled product III and non-labelled product VI(fractions 74-90, fig. 1A,B) were pooled and subjected to HPLC on Spherisorb 5 ODS either before (fig. 2A) or after (fig. 2B) acetylation. Eluted fractions were examined for the presence of fluorescent and radioactive material.

from Sephadex LH20 were examined by HPLC on Spherisorb 5 ODS. Using this system, ^{3}H-labelled product IV eluted earlier than and was separated from nucleoside-hydrocarbon adducts formed from each of the four diol-epoxides. In addition, ^{3}H-labelled product III was separated from adducts formed from syn-BA-10,11-diol 8,9-oxide but not from adducts formed from anti-BA-8,9-diol 10,11-oxide (fig. 2A), anti-BA-10,11-diol 8,9-oxide and syn-BA-8,9-diol 10,11-oxide. However, when ^{3}H-labelled products and non-labelled adducts that were co-eluted from Sephadex LH20 columns were acetylated prior to HPLC on Spherisorb 5 ODS, only adducts formed from anti-BA-8,9-diol 10,11-oxide co-eluted with ^{3}H-labelled product III (fig. 2B); product III was separated from adducts formed from anti-BA-10,11-diol 8,9-oxide, syn-BA-8,9-diol 10,11-oxide and syn-BA-10,11-diol 8,9-oxide.

Additional evidence that ^{3}H-labelled product III is not formed from either syn-BA-8,9-diol 10,11-oxide or syn-BA-10,11-diol 8,9-oxide was obtained by examining the behaviour of nucleoside-hydrocarbon adducts on Sephadex LH20 columns eluted with sodium borate (0.05 M, pH 8.7)-methanol gradients. Under these conditions, the adducts formed from the two syn-diol-epoxides eluted in a similar position to that in which they eluted in water-methanol gradients. However, the position of elution of product III changed relative to that of the non-labelled products and,in borate,eluted several fractions earlier.

Products IV and V are probably not formed from any of the four diol-epoxides used in this study because the ^{3}H-labelled products IV and V were separated from the non-labelled adducts formed from these diol-epoxides by HPLC or by Sephadex LH20 chromatography (data not shown). Although no further attempt has been made to identify products IV and V, product V is relatively non-polar and may have arisen by the reaction of a K-region or other simple epoxide with DNA, as has been observed for the reaction of benzo(a)pyrene metabolites with DNA in a similar system (4). The results presented in this study suggest that product III is formed from anti-BA-8,9-diol 10,11-oxide

because a hydrocarbon-nucleoside adduct formed from this diol-epoxide was indistinguishable from product III when examined by HPLC, both before and after acetylation, and by Sephadex LH20 column chromatography. This is consistent with the finding that the major benz(a)anthracene-DNA adduct formed in mouse skin and in hamster embryo cells and which have chromatographic properties similar to those of peak III possess phenanthrene-like fluorescence spectra (12). These findings indicate that major routes of metabolic activation of benz(a)anthracene do not necessarily involve a bay-region diol-epoxide, a suggestion that might explain the discrepancy between the low activity of benz(a)anthracene and the high activity of the related bay-region dihydrodiols and diol-epoxides in mutagenesis (11) and tumour initiation studies (10).

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