IN VITRO REACTION OF RADIOACTIVE 7β,8α,-DIHYDROXY-9α,10α-EPOXY-7,8,9,10-TETRAHYDROBENZO(A)PYRENE AND 7β,8α-DIHYDROXY-9β,10β-EPOXY-7,8,9,10-TETRAHYDROBENZO(A)PYRENE WITH DNA

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SUMMMARY

The in vitro reaction of bacteriophage T7-DNA with the radioactive diastereomeric benzo(a)pyrene-diol-epoxides, (±) [$^3H_9$,$^3H_{10}$]-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene and (±) [$^3H_9$,$^3H_{10}$]-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, was investigated. Chromatographic analysis of digests of the DNA allowed the distinction of characteristic deoxynucleoside adduct peaks for the two benzo(a)pyrene-diol-epoxides. Our results, together with data from the literature, allow the identification of these adducts as mostly N_2-(10-{7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene}yl)deoxyguanosine and N_2-(10-{7β,8α,9β-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene}yl)deoxyguanosine, respectively. DNA-benzo(a)pyrene adducts with the same chromatographic properties were formed in mouse embryo fibroblasts upon treatment with benzo(a)pyrene.

INTRODUCTION

The formation of DNA-damage may represent an initial step in carcinogenesis. Most chemical carcinogens are electrophilic agents which form covalent adducts with the cellular macromolecules. The ubiquitous environmental pollutant benzo(a)pyrene (B(a)P)has been shown to produce covalent substitution products with RNA and DNA in metabolically active tissue. One major and three minor deoxynucleoside - B(a)P adducts were observed by King et al (1)in DNA digests from baby hamster kidney cells (BHK 21 C13) which had been exposed to radioactive B(a)P by Sephadex LH-20 chromatography using a borate containing eluent. From a comparison with a deoxynucleoside mixture obtained from purine labeled

DNA following reaction in vitro with non-radioactive (+) 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (B(a)P-diol-epoxide I) and (+) 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (B(a)P-diol-epoxide II) it was concluded that the major and a minor Sephadex LH-20 peak represented covalent adducts formed by these two diastereomeric B(a)P-diol-epoxides with the purine bases (1). In studies of the repair of B(a)P-induced DNA lesions, only two major deoxynucleoside adduct peaks were observed in BHK 21/C13 cells and secondary mouse embryo fibroblasts (MEF C57 B1) in our laboratory (2) regardless of whether our own simplified chromatographic procedure or that of King et al (1) was used. The relative heights of the two peaks varied somewhat in different experiments depending on the exact growth conditions of the cell culture. In order to characterize the two adduct peaks obtained in our experiments, we have chemically synthesized racemic B(a)P-diol-epoxide I and II in radioactive form and reacted them in vitro with highly purified bacteriophage T7-DNA. The two diastereomeric B(a)P-diol-epoxides formed distinct DNA adducts which could be readily separated by a simplified Sephadex LH-20 chromatography procedure. Our results, together with data from the literature (1-7,9), allow the assignment of the two major DNA-B(a)P-adducts in mouse embryo fibroblasts and baby hamster kidney cells to N_2-(10-{7β,8α,9α or 9β-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene}yl) deoxyguanosine.

Experiments similar to those reported here were first attempted by Sims et al. (8) However, the DNA adducts could not be structurally identified since the individual diastereomeric B(a)P-diol epoxides were not available and the chromatographic procedures used did not allow the separation of the isomeric DNA adducts at that time.

MATERIALS AND METHODS

(1) Preparation of tritium labeled B(a)P-diol-epoxides I and II

The 9,10 double bond of the dibenzoate of synthetic (+) trans 7,8-dihydroxy-7,8-dihydro-B(a)P was catalytically reduced with tritium gas and this material was converted back to the dihydro-diol which was in turn converted to B(a)P-diol-epoxide I or II as previously described (9,10).

(2) Preparation of 2-^{14}C-thymidine labeled bacteriophage T7-DNA and reaction with the B(a)P-diol-epoxides

Bacteriophage T7 was grown in Escherichia Coli B thy$^-$ in M9 medium with 1.5-2.0μg/ml of cold thymidine and 0.05μCi/ml of 2-^{14}C-thymidine (specific activity 61 mCi/mmole). The phage was purified as described by Yamamoto et

al (11) and the DNA extracted with sodium dodecylsulfate-phenol. The specific activity of the DNA was $4x10^5$ cpm per A_{260} unit. To 288µg of native T7-DNA in 1 ml of $6x10^{-3}$M phosphate buffer pH 7.4 was added 100µl of racemic $^3H_9$, $^3H_{10}$-(B(a)P-diol-epoxide I (0.4 mg, specific activity 2.4 mCi/mmole) in dimethylsulfoxide at 0°. The sample was incubated for 15 min. at 0°, 60 min. at room temperature and then slowly heated to 68°. The sample was passed through a column of Sephadex G50 and the fractions containing ^{14}C-radioactivity were pooled. After addition of 100µg of calf thymus DNA as carrier, the DNA was precipitated twice with cold ethanol. The DNA was enzymatically digested to the nucleoside level essentially according to Baird and Brookes (12) and one quarter of the digested sample was analyzed by Sephadex LH-20 chromatography as described below.

Analogous conditions were used for the reaction of DNA with racemic $^3H_9$, $^3H_{10}$ -B(a)P-diol-epoxide II. To 288µg T7-DNA in 1 ml of $6x10^{-3}$M phosphate buffer pH 7.4 was added 3.6 mg $^3H_9$, $^3H_{10}$-B(a)P-diol-epoxide II (specific activity of 2.4 mCi/mmole) in dimethylsulfoxide in three equal portions of 50µl. Incubation, purification and chromatographic analysis of the DNA was identical to that described above, with the exception that the reaction mixture was extracted four times with n-butanol-ethylacetate (1:4 V/V) (6) before application to the Sephadex G50 column. One quarter of the digested sample was applied to the Sephadex LH-20 column.

Sephadex LH-20 chromatography of DNA digests

The Sephadex LH-20 columns (0.9x25 cm) were first eluted with 30% aqueous methanol and 24 fractions of 1.4 ml were collected. The columns were further eluted with a linear gradient of 30% aqueous methanol-sodium borate to 80% aqueous methanol-sodium borate. The starting solvent of the gradient was prepared by adding 26 ml of methanol to 61 ml of 0.05M sodium borate pH 8.7 and the final solvent contained 80 ml of methanol and 20 ml of 0.05 sodium borate pH 8.7. 76 fractions of 1.4 ml were collected and counted in a Beckman Model LS 233 scintillation system following the addition of 14 ml of Aquasol(2). Recovery of ^{14}C-radioactivity from the columns was always complete while the recovery of tritium was 75 to 85%.

RESULTS AND DISCUSSION

The formation of DNA-adducts by *in vitro* reaction of ^3H-B(a)P-diol-epoxide I and II with native ^{14}C-thymidine-labeled bacteriophage T7-DNA was investigated. After treatment with the B(a)P-diol epoxide the DNA was enzymatically digested to the deoxynucleoside level and the hydrolysate analyzed by Sephadex LH-20 chromatography. Typical elution profiles are

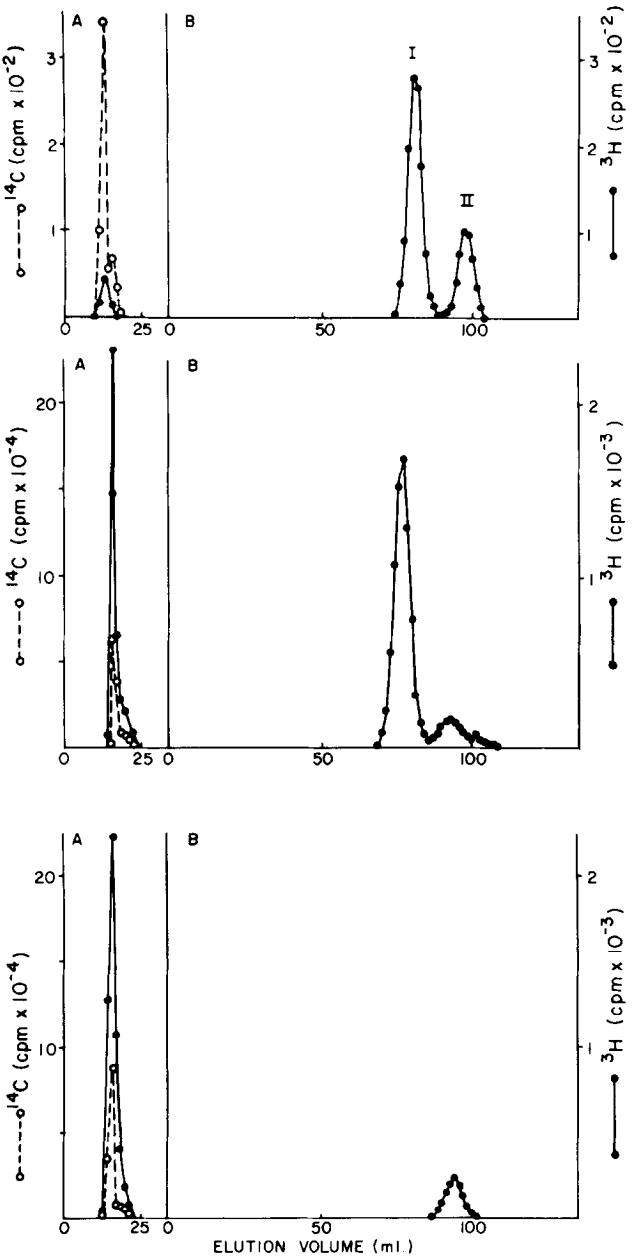

Fig. 1. Sephadex LH-20 chromatography of enzymatic hydrolysates of DNA extracted from ^{3}H-B(a)P treated mouse embryo fibroblasts (MEF C57 B1, upper third) included for comparison (2) and of bacteriophage T7-DNA reacted *in vitro* with ^{3}H-B(a)P-diol-epoxide I (middle third) and of bacteriophage T7-DNA treated in vitro with ^{3}H-B(a)P-diol-epoxide II (lower third). The chromatographic procedure was a modification of that described by King *et al* (1) using an aqueous methanol - sodium borate containing eluent (see under Materials and Methods).

shown in the middle and lower third of the Figure. The profile in the upper third of the figure is included for comparison and shows a profile of DNA which was extracted from mouse embryo fibroblasts which had been treated with ^{3}H-B(a)P (2). All chromatograms are composed of two portions: part A contains the relatively polar, hydrophilic material which is eluted by 30% aqueous-methanol; part B the more hydrophobic material which is eluted by a linear gradient of 30 to 80% methanol-sodium borate buffer. In all cases part A contains a major ^{14}C peak which was readily identified as thymidine by cochromatography with an authentic marker. For mouse embryo fibroblasts only negligible amounts of ^{3}H were eluted in part A while the profiles of ^{3}H-B(a)P-diol-epoxide treated T7-DNA contained significant amounts of ^{3}H in a single, well-defined peak. The identity of this material was not investigated. Similar results have been obtained by other investigators (e.g. refs. 1,8,12). The possibility should be considered that this may be B(a)P-phosphotri ester derivatives (6). While no ^{14}C-radioactivity was contained in part B of the chromatograms a major ^{3}H-peak was obtained in the hydrolysates of ^{3}H-B(a)P-diol-epoxide treated phage DNA. For ^{3}H-B(a)P-diol-epoxide I treated DNA (middle third of figure) the major ^{3}H-peak was eluted from 70 to 84 ml (maximum at 77 ml) and a minor peak from 89 to 100 ml (maximum at 92 ml). For ^{3}H-B(a)P-diol-epoxide II (lower third of figure) the single major peak was eluted from 89 to 100 ml (maximum at 93 ml). It follows that the *in vitro* reaction of DNA with the two diastereomeric B(a)P-diol-epoxides gives rise to two distinct deoxynucleoside adduct peaks on Sephadex LH-20 columns if a borate containing buffer is used as the eluent. Together with evidence from the literature (1-9) which indicates that B(a)P nucleic acid adduct formation mostly involves linkage of the exocyclic amino group of guanine with carbon-10 of the B(a)P ring system, it is concluded that B(a)P-diol-epoxide I leads primarily to the formation of N_2-(10-{7β,8α,8α-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene}yl)deoxyguanosine (abbreviated as dGua-B(a)P I)and B(a)P-diol-epoxide II to N_2-(10-{7β,8α,9β-trihydroxy-7,8,9,10-tetrahydrobenzo-(a)pyrene}yl)deoxyguanosine (abbreviated as dGua-B(a)P II). Osborne *et al* (7) have detected small amounts of deoxyadenosine adducts in B(a)P-diol-epoxide I treated DNA. It is conceivable that such products are also present in our samples but are not separated from the major deoxyguanosine adducts under our chromatographic conditions. Under our reaction conditions, it was calculated that the DNA contained 1 dGua-B(a)P I for every 24 deoxynucleotides following treatment with B(a)P-diol-epoxide I and 1 dGua-B(a)P II for every 190 deoxynucleotides following treatment with B(a)P-diol-epoxide II.

A comparison between the results obtained for DNA which was extracted from ^{3}H-B(a)P treated mouse embryo fibroblasts (2)(upper third of figure)

and the in vitro modified viral DNA shows that peak I corresponds to the major adduct formed in vitro by B(a)P-diol-epoxide I, i.e. dGua-B(a)P-I, and peak II to the adduct formed by B(a)P-diol-epoxide II, i.e. dGua-B(a)P-II. In contrast to mouse embryo fibroblasts and baby hamster kidney cells (1,2) only Gua-B(a)P-I was identified in the RNA of B(a)P treated bovine bronchial tissue (13). Similarly,only dGua-B(a)P-I is detected in vitro upon incubation of DNA with B(a)P-7,8-dihydrodiol, in the presence of rat liver microsomes (1,9,14).

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