Formation and Excision of Covalent Deoxyribonucleic Acid Adducts of Benzo[*a*]pyrene 4,5-Epoxide and Benzo[*a*]pyrenediol Epoxide I in Human Lung Cells A549†

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ABSTRACT: The formation and repair of covalent DNA adducts induced by the two ultimate benzo[*a*]pyrene (BP) metabolites 4,5-dihydro-4,5-epoxybenzo[*a*]pyrene (BPE) and 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene (BPDE I) were studied in human epithelioid lung cells A549. The covalent DNA adducts of BPE were characterized by reacting native DNA specifically labeled in a single deoxynucleoside with racemic BPE in vitro. From the chromatographic analysis of enzymatic hydrolysates of BPE-treated DNA, it was concluded that adducts to deoxyguanosine (BPE–dG) and deoxyadenosine (BPE–dA) were formed. No adducts to the pyrimidine nucleotides were detected. DNA adducts with the same chromatographic properties were formed upon treatment of intact A549 cells with BPE. Treatment of A549 cells with racemic BPDE I mostly produced (7*R*)-N^2-(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-10-yl)deoxyguanosine (BPDE I–dG). The efficiency of formation of covalent DNA adducts in intact A549 cells was much lower for BPE than for BPDE I. The initial concentration of all adducts was considerably higher in micrococcal nuclease sensitive DNA fractions of A549 chromatin. The time course of the excision from high molecular weight DNA of the covalent BPE and BPDE I adducts was determined from 0- to 35-h posttreatment incubation of A549 cells. The rates of excision decreased according to the sequence BPE–dA > BPE–dG > BPDE I–dG. While the removal of BPE–dA was carried to completion, approximately 20% of BPE–dG and BPDE I–dG persisted in the DNA when the excision process had come to a halt or slowed down considerably. The low efficiency of formation and the high excisability of BPE adducts in comparison to BPDE I adducts may explain the lower cytotoxicity of BPE relative to BPDE I for A549 cells.

Until recently, the K-region epoxide of benzo[*a*]pyrene, 4,5-dihydro-4,5-epoxybenzo[*a*]pyrene (BPE),[1] was considered the most likely ultimate metabolite responsible for benzo[*a*]pyrene (BP) carcinogenicity. However, differences between the DNA adducts formed upon in vitro reaction of DNA with BPE and those produced from BP in intact rodent and human cells (Sims et al., 1974; Baird et al., 1975), as well as comparative studies of the mutagenicity of different classes of BP metabolites, focused the attention on the BP-diol epoxides, i.e., 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-BP (Wislocki et al., 1976a,b; Wood et al., 1976; Newbold & Brookes, 1976; Huberman et al., 1976). Of all BP metabolites tested the (+) enantiomer (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-BP [(+)-BPDE I] possessed the highest mutagenicity in V79 Chinese hamster cells (Wood et al., 1977). In V79 cells the mutagenic efficiency, i.e., the mutagenicity per lethal event, was considerably higher for BPDE I at high survival than for the isomeric BPDE II, i.e., 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydro-BP, BPE, and several other arylating agents (Newbold et al., 1979). BPDE I was also considerably more potent than BPE in the in vitro transformation of golden hamster embryo cells (Mager et al., 1977) and M2 mouse fibroblasts (Marquardt et al., 1977) and possessed highest activity in carcinogenicity tests in mice (Kapitulnik et al., 1978; Buening et al., 1978; Slaga et al., 1979).

Work with fibroblasts from normal individuals and patients with Xeroderma pigmentosum (XP) which are characterized by deficiencies in excision repair indicates the importance of DNA repair for the biological potencies of several ultimate metabolites of polycyclic aromatic hydrocarbons (PAH) including BPE and BPDE I. It is particularly interesting that the mutagenic efficiencies, i.e., the mutagenicity per lethal event, were comparable for different ultimate PAH metabolites (Maher et al., 1977, 1978). The important question of whether individual unexcised lesions induced by different but structurally related ultimate DNA damaging agents possess the same biological potencies has not yet been answered, however.

The repair of DNA adducts of BPDE I and BPDE II in human cells has been studied by a number of different methods and found to resemble the type of repair induced by far-ultraviolet light (UV) (Day et al., 1978; Waters et al., 1978; Cerutti et al., 1978; Feldman et al., 1978). There is evidence that the repair of the BPE-induced lesions occurs by a different pathway, on the other hand (Regan et al., 1978).

Our present work represents a step toward the goal to relate the efficiency of formation and the repairability of individual, structurally characterized PAH–DNA adducts to the biological potencies of the ultimate metabolites by which they are induced. We have compared the ultimate BP metabolites BPE and BPDE I because of the well documented and substantial differences in their biological potencies. First, the DNA adducts formed by BPE were further characterized. It was shown that four major adducts to guanine and adenine were formed. We have found earlier that BPDE I preferen-

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[1] Abbreviations used: BP, benzo[*a*]pyrene; BPE, 4,5-dihydroxy-4,5-epoxybenzo[*a*]pyrene; BPDE I, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BPDE II, 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydro[*a*]pyrene; BPE–dA, covalent adduct of BPE to deoxyadenosine; BPE–dG, covalent adduct of BPE to deoxyguanosine; BPDE I–dG, N^2-(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-10-yl)deoxyguanosine; XP, xeroderma pigmentosum; PAH, polycyclic aromatic hydrocarbons; UV, far-ultraviolet light; LC, high-pressure liquid chromatography; Me_2SO, dimethyl sulfoxide.

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tially produced N^2-(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-10-yl)deoxyguanosine (BPDE I–dG) in human lung cells A549 (Cerutti et al., 1978). The efficiency of DNA adduct formation in A549 cells was much lower for BPE than for BPDE I. In a comparative study of the excisability and persistence of these BP–DNA adducts in A549 cells, we found that the BPE adducts were removed more rapidly and more completely from the DNA than BPDE I–dG. The higher excisability of the BPE lesions and the lower yield of their formation relative to BPDE I induced lesions undoubtedly are factors which contribute to the substantial difference in the biological potencies of these ultimate BP metabolites.

Experimental Procedure

[^{3}H]-(±)-BPDE I and [^{3}H]-(±)-BPE (sp act. 267 or 438 mCi/mmol, respectively) were obtained from Midwest Research Institute, Kansas City, MO, through the auspices of Dr. D. Longfellow of the Chemistry Operational Unit, Carcinogenesis Branch of the National Cancer Institute. After evaporation of the tetrahydrofuran–triethylamine solvent, the samples were dissolved in a small amount of anhydrous dimethyl sulfoxide (Pierce Chemical, Rockford, IL). [2-^{14}C]-Thymidine (sp act. 61 mCi/mmol), deoxy[U-^{14}C]guanosine 5′-triphosphate (sp act. 534 mCi/mmol), deoxy[8-^{3}H]-guanosine 5′-triphosphate (sp act. 15 Ci/mmol), deoxy[U-^{14}C]cytidine 5′-triphosphate (sp act. 414 mCi/mmol), deoxy[8-^{3}H]adenosine 5′-triphosphate (sp act. 14.2 Ci/mmol), and [^{3}H]BP (sp act. 24–40 Ci/mmol) were purchased from Amersham/Searle, Arlington Heights, IL, and Zurich, Switzerland. All enzymes used for DNA digestion as well as DNA polymerase I from *Escherichia coli* were purchased from Worthington Biochemical, Bedford, MA, and Millipore, Switzerland.

Characterization of Covalent DNA Adducts of BPE. DNA labeled selectively in a single nucleoside was prepared by polymerization of three nonradioactive deoxynucleotide triphosphates plus one radioactive deoxynucleotide triphosphate with DNA polymerase I from *E. coli* in the presence of calf thymus DNA according to Richardson et al. (1964). The DNA was purified by phenol extraction and three consecutive precipitations with cold ethanol. Approximately (6–10) × 10^6 cpm of [^{14}C]deoxycytidine- or [^{14}C]deoxyguanosine-labeled DNA in 0.5 mL of 0.005 M phosphate buffer, pH 7.4, was reacted at 37 °C with 0.1 mg of [^{3}H]-(±)-BPE dissolved in 0.2 mL of dimethyl sulfoxide. Analogous conditions were used in experiments with [^{3}H]deoxyadenosine- or [^{3}H]deoxyguanosine-labeled DNA but the BPE was nonradioactive. After 2 h of incubation the samples were extracted 12 times with isoamyl alcohol (equilibrated with phosphate buffer), and, after addition of 50 μg of calf thymus DNA as carrier, the DNA was precipitated twice with cold ethanol. The DNA samples were then enzymatically hydrolyzed and the nucleoside mixtures were chromatographed on Sephadex LH-20 as described previously (Shinohara & Cerutti, 1977) with the following modification. After 106 mL of the 30–80% methanol–sodium borate gradient had passed through the column, elution was continued with 80% aqueous methanol–sodium borate until an additional 75 mL of eluant had been collected.

The BPE–deoxynucleoside adduct peaks from the Sephadex LH-20 chromatograms were analyzed by high-pressure liquid chromatography (LC). LC was accomplished with a μBondapak C_{18} reverse-phase column on a Waters ALC200 liquid chromatograph equipped with a U6K injector system and a Model 6000A solvent delivery system. The column was eluted with a linear 50–100% water–methanol gradient at ambient temperature with a pump speed of 2 mL/min. Fractions of 1 mL were collected and the radioactivity of each fraction was determined.

Cytotoxicity of BPE and BPDE I for Human Lung Cells A549. The origin and the culturing conditions for human epithelioid lung cells A549 have been described by Lieber et al. (1976). A549 cells were seeded into Falcon T25 flasks containing 5 mL of medium, and the cultures were allowed to grow for 72 h and then treated with 0–1.5 μM (±)-BPE or 0–1.5 μM (±)-BPDE I for 30 min at 37 °C. The cultures were then washed and trypsinized, and the cells were suspended in fresh medium. Appropriate numbers of cells (200–1000) were seeded into T25 flasks. After 7–10 days of incubation, cells were fixed with methanol and stained with 2% crystal violet. Colonies were counted and the plating efficiency was determined. Colony-forming ability is calculated from the plating efficiencies of the treated samples relative to those of untreated controls (which had a plating efficiency of 40–50%). Each experimental point represents the mean of nine replica plates. The data were plotted in the usual semilogarithmic fashion. Straight lines were fit by the method of least squares through all the points along the exponential portions of the curves. Statistical comparisons of these straight lines were performed with standard techniques (Diem, 1968). Correlation coefficients of 0.94 and 0.95 were obtained for the BPE and the BPDE I curves, respectively. D_0 values were obtained from the exponential portion of each curve and represent the doses necessary to reduce colony-forming ability from any point to 37% of that point.

Removal from DNA of Covalent Adducts Induced by BPE and BPDE I in Human Lung Cells A549. A549 cells were cultured in T150 Corning flasks and prelabeled in their DNA with [2-^{14}C]thymidine as described previously (Feldman et al., 1978). For treatment with the ^{3}H-labeled BP metabolites, the old medium was replaced with 12.5 mL of fresh complete media. [^{3}H]-(±)-BPDE I in 100 μL of Me_2SO was added to a final concentration of 1.1 μM; [^{3}H]-(±)-BPE in 100 μL of Me_2SO was added to a final concentration of 53.4 μM. The cultures were incubated for 30 min at 37 °C on a rocker platform and washed twice before addition of 30 mL of complete media for posttreatment incubation. After different lengths of incubation, the cells were harvested, the DNA was extracted by a modified Kirby procedure and digested enzymatically, and the resulting nucleoside mixtures were chromatographed on Sephadex LH-20 as described previously (Shinohara & Cerutti, 1977). In the experiments with [^{3}H]-(±)-BPE the chromatographic procedure was modified as described in the previous paragraph. All ^{14}C radioactivity from the Sephadex LH-20 columns was eluted by the 30% aqueous methanol wash and represented [^{14}C]thymidine. These fractions contained only small amounts of tritium, the major tritium-containing adduct peaks being eluted much later by the 30–80% methanol–sodium borate gradient. The ratios of ^{3}H radioactivity contained in each fraction to total ^{14}C radioactivity eluted from the column were determined. From these data the adduct concentrations were calculated as a function of posttreatment incubation.

Distribution of Covalent BP–DNA Adducts between Micrococcal Nuclease Resistant and Sensitive DNA in Human Lung Cells A549 Treated with BPE and BPDE I. A549 cells were cultured, prelabeled with [2-^{14}C]thymidine, and treated with the ultimate ^{3}H-labeled BP metabolites as described above. Immediately after treatment the cells were collected and frozen as pellets. Nuclei were prepared from the thawed cells by homogenization in 5 mL of 0.25 M sucrose, 0.01 M

Tris (pH 8.0), 0.001 M Mg, and 0.5% Triton X-100 with a Dounce homogenizer using the "B" pestle. Nuclei were washed and resuspended at 2×10^7 mL^{-1} in 0.25 M sucrose, 0.001 M Tris (pH 8.0), and 0.0001 M Ca^{2+}. Half of the nuclei of each sample was digested with micrococcal nuclease at 5 μg mL^{-1} for 50 min at 37 °C according to Söllner-Webb & Felsenfeld (1975). Excess EDTA was added and the DNA was precipitated with 95% ethanol to stop the reaction. Under these conditions approximately 50% of the DNA had been rendered alcohol soluble. The integrity of the nucleosomal cores after digestion with micrococcal nuclease was ascertained by centrifugation on linear 5–20% (w/v) sucrose gradients at 25 000 rpm for 24 h at 6 °C in a Spinco SW 27 rotor. The ethanol-precipitable core DNA was extracted and enzymatically hydrolyzed, and the nucleoside mixture was chromatographed on Sephadex LH-20 as described above. The other half of the nuclei preparation received the same treatment with the exception that the micrococcal nuclease step was omitted.

All ^{14}C radioactivity from the Sephadex LH-20 columns was eluted by the 30% aqueous methanol wash and represented [^{14}C]thymidine. These fractions contained only small amounts of tritium, the major tritium-containing adduct peaks being eluted much later by the 30–80% methanol–sodium borate gradient (see Figures 2 and 3). The ratios of ^{3}H radioactivity contained in each fraction to total ^{14}C radioactivity eluted from the column were determined. From these data the relative adduct concentrations in the micrococcal nuclease resistant and sensitive DNA were calculated in the following manner. All samples were normalized for differences in the yields of DNA extraction from the recovery of the ^{14}C radioactivity, and normalized ^{3}H over ^{14}C ratios, representing lesion concentrations, were computed for the micrococcal nuclease resistant and total DNA. Assuming an average core size of 135 base pairs and an average linker size of 47 base pairs for A549 cells [cf. Lohr et al. (1977)], the lesion concentration in the linker DNA, x, was calculated from the equation $x = 3.87y - 2.87z$, where y represents the average lesion concentration in the total DNA and z represents the lesion concentration in the core DNA (i.e., in the micrococcal nuclease resistant DNA).

Results

Cytotoxicity of BPE and BPDE I for Human Lung Cells A549. The effect of the exposure to (±)-BPE and (±)-BPDE I on the colony-forming ability of A549 cells was determined. As shown in Figure 1, colony-forming ability decreased exponentially with increasing concentrations of both ultimate BP metabolites. A shoulder at low concentrations is discernible in the BPE survival curve. The D_0 concentrations determined from the exponential portions of the curves are 1.83 μM for (±)-BPE and 0.30 μM for (±)-BPDE I.

Characterization of Covalent Adducts Formed by BPE with DNA. DNA specifically labeled in either deoxyguanosine, deoxyadenosine, deoxycytidine, or thymidine was prepared with an *E. coli* polymerase I nick translation system. Calf thymus DNA was used as the template, and three nonradioactive and one labeled deoxynucleoside triphosphate were polymerized as described by Richardson et al. (1964). The [^{14}C]deoxyguanosine-labeled DNA and [^{14}C]deoxycytidine-labeled DNA were reacted with [^{3}H]-(±)-BPE in phosphate buffer, pH 7.4, at a final Me_2SO concentration of 28%. [^{3}H]Deoxyadenosine-labeled DNA was reacted with nonradioactive (±)-BPE. Figure 2 shows the hydrophobic regions of Sephadex LH-20 chromatograms of deoxynucleoside mixtures obtained by enzymatic hydrolysis of the BPE-treated DNA preparations. Figure 2A contains the results of the reaction

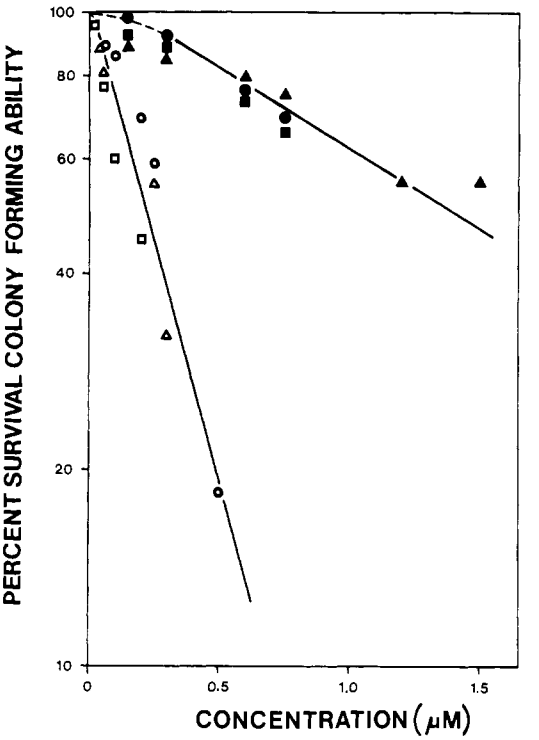

FIGURE 1: Effect of (±)-BPE and (±)-BPDE I on the colony-forming ability of human lung cells A549. Experimental points are means of nine replica plates, and straight lines were fit by the method of least squares through all points along the exponential portions of the curves (for experimental conditions see Experimental Procedure). (△, □, ○) Three independent experiments with (±)-BPDE I; (▲, ■, ●) three independent experiments with (±)-BPE.

of [^{14}C]deoxycytidine-labeled DNA with [^{3}H]-(±)-BPE. Two major ^{3}H-containing peaks with peak fractions at 140- and 160-mL elution volume are discernible. Both peaks are free of ^{14}C radioactivity, and it is evident that no detectable amounts of BPE–deoxycytidine adduct are formed. A small amount of ^{14}C radioactivity eluting earlier has not been identified. An analogous negative result was also obtained from the reaction of [^{14}C]thymidine-labeled DNA with [^{3}H]-(±)-BPE (not shown). Figure 2B contains the result of the reaction of DNA–[^{14}C]deoxyguanosine with [^{3}H]-(±)-BPE. The ^{3}H elution profile is analogous to that shown in Figure 2A, but now the earlier eluting ^{3}H peak coincides with the only ^{14}C peak in this part of the chromatogram. From the specific activities of the ^{14}C- and ^{3}H-labeled compounds used in the reaction mixture, it follows that the earlier eluting peak consists of a 1:1 adduct of BPE to deoxyguanosine which is referred to as BPE–dG. Figure 2C gives the chromatogram resulting from the reaction of [^{3}H]deoxyadenosine-labeled DNA with nonradioactive (±)-BPE. Only a single ^{3}H peak with a peak fraction at 158 mL is observed corresponding to a BPE–deoxyadenosine adduct (BPE-dA) and most likely to the later eluting major ^{3}H peaks in the chromatograms shown in Figure 2A,B. The ratio of the amounts of BPE–dG over BPE–dA formed was between 2 and 3 in several experiments. The BPE–dG and BPE–dA peaks from the Sephadex LH-20 columns were further analyzed by LC on a μBondapak C_{18} column. The chromatographic characteristics are given in Table I. Both Sephadex LH-20 peaks were further resolved into two components which may correspond to position or stereoisomers.

Removal from DNA of Human Lung Cells A549 of Covalent Adducts Formed upon Treatment with BPDE I and BPE. A549 cells were prelabeled with [^{14}C]thymidine, treated

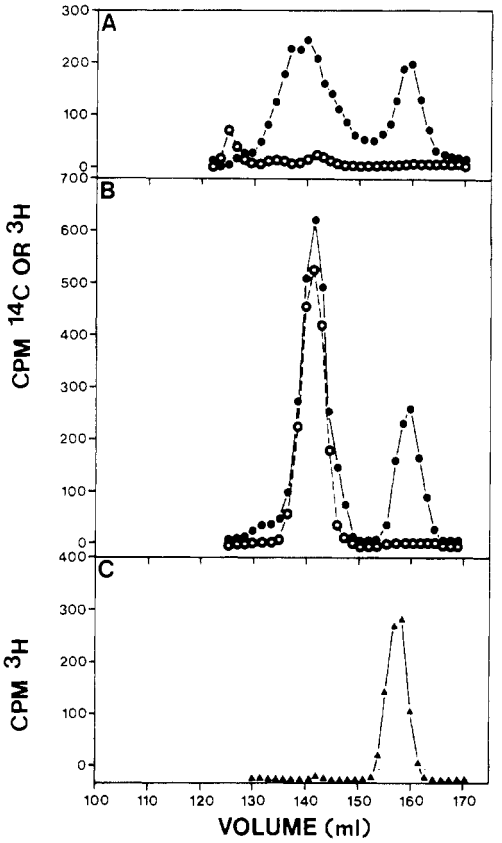

FIGURE 2: Hydrophobic regions of Sephadex LH-20 chromatograms of digests of DNA labeled in a single nucleoside and reacted with (±)-BPE. DNA labeled in a single nucleoside was prepared by polymerization of three nonradioactive and one radioactive deoxynucleoside triphosphates by *E. coli* polymerase I and reacted with (±)-BPE. (A) Hydrolysates of [^{14}C]deoxycytidine-labeled DNA reacted with [^{3}H]-(±)-BPE. (●) ^{3}H radioactivity; (○) ^{14}C radioactivity. (B) Hydrolysates of [^{14}C]deoxyguanosine-labeled DNA reacted with [^{3}H]-(±)-BPE. (●) ^{3}H radioactivity; (○) ^{14}C radioactivity. (C) Hydrolysates of [^{3}H]deoxyadenosine-labeled DNA reacted with nonradioactive (±)-BPE. (▲) ^{3}H radioactivity.

Table I: LC Analysis[a] of Hydrophobic Sephadex LH-20 Peaks of Digests of [^{3}H]dG-DNA or [^{3}H]dA-DNA Reacted with (±)-BPE

Sephadex LH-20 peak	retention time (min)	% yield
from [^{3}H]dG-DNA	14.0	48
	15.5	52
from [^{3}H]dA-DNA	18.0	72
	20.5	28

[a] μBondapak C_{18} column; solvent, linear gradient of 50–100% water–methanol over a 40-min period; 1-mL fractions were collected.

with 1.1 μM [^{3}H]-(±)-BPDE I, and incubated at 37 °C for different lengths of time before the DNA was extracted and enzymatically hydrolyzed to the deoxynucleoside level. The BPDE I adduct region of Sephadex LH-20 chromatograms for 0- and 29-h incubation is given in Figure 3A. As has been shown previously (Cerutti et al., 1978), the reaction of A549 cells with (±)-BPDE I produces preferentially N^2-(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-10-yl)deoxyguanosine (BPDE I–dG). According to LC data ~70% of BPDE I–dG corresponds to *trans*-10-(7*R*)-N^2-(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-10-yl)deoxyguanosine and most of the remaining material corresponds to the enantiomeric 7S adduct, in agreement with the observations of Brown et al. (1979). The data in Figure 3A are expressed as ratios of the ^{3}H content of each fraction

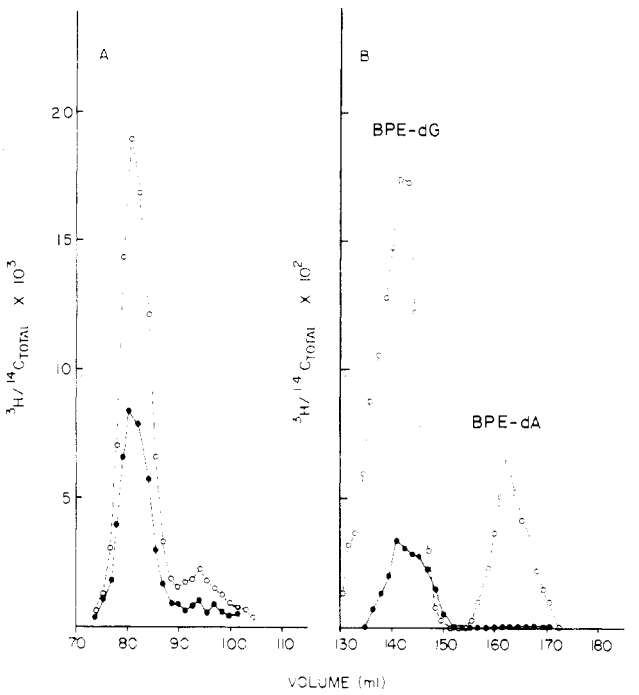

FIGURE 3: Hydrophobic regions of Sephadex LH-20 chromatograms of digests of DNA extracted from [^{14}C]thymidine-labeled human lung cells A549 which had been treated with [^{3}H]-(±)-BPDE I or [^{3}H]-(±)-BPE, respectively. The ratios of ^{3}H radioactivity contained in each fraction to total ^{14}C radioactivity eluted from the column are plotted (note that all ^{14}C radioactivity corresponds to [^{14}C]thymidine and is eluted in earlier fractions of the chromatogram which are not shown). (A) Digests of DNA from A549 cells treated with [^{3}H]-(±)-BPDE I and incubated for 0 and 29 h. The major peak corresponds to BPDE I–dG. (○) 0-h posttreatment incubation; (●) 29-h posttreatment incubation. (B) Digests of DNA from A549 cells treated with [^{3}H]-(±)-BPE and incubated for 0 and 26 h. The earlier eluting peak most likely consists of BPE–dG; the later eluting peak most likely consists of BPE–dA. (○) 0-h posttreatment incubation; (●) 26-h posttreatment incubation.

of the BPDE I–dG adduct peak over the total ^{14}C radioactivity contained in the entire chromatogram. The ^{3}H over ^{14}C ratio is a measure of the adduct concentration. At 0-h incubation the concentration of BPDE I–dG was 28.5 μmol/mol of DNA-P, and after 29 h it was 14.1 μmol/mol of DNA-P; i.e., 49% of the adducts had been removed from high molecular weight DNA in this time period. Kinetics of BPDE I–dG removal during 72-h incubation are given in Figure 4. It is evident that the rate of adduct removal had slowed down considerably after 72 h, leaving approximately 20% of the adducts unexcised.

The removal from DNA of A549 cells of BPE adducts following treatment with 53.4 μM [^{3}H]-(±)-BPE was studied in analogous experiments. As shown in Figure 3B, (±)-BPE produced two deoxynucleoside adduct peaks in the hydrophobic portion of the chromatogram, the first eluting from 130–150 mL and the second eluting from 154–170 mL, analogous to the adducts formed in the corresponding in vitro reaction with free, native DNA (see Figure 2). Therefore, the first peak most likely consists of the covalent adducts of BPE with deoxyguanosine, i.e., BPE–dG; the second peak most likely contains the BPE adducts to deoxyadenosine, i.e., BPE–dA. The initial concentration of BPE–dG was 8.7 μmol/mol of DNA-P and of BPE–dA was 2.5 μmol/mol of DNA-P. Figure 3B also contains a chromatogram after 26-h posttreatment incubation. It is evident that the concentration of BPE–dG had decreased by 80% to 1.7 μmol/mol of DNA-P and BPE–dA had disappeared entirely from the DNA. Kinetics of the removal of the BPE adducts from 0- to 35-h incubation are

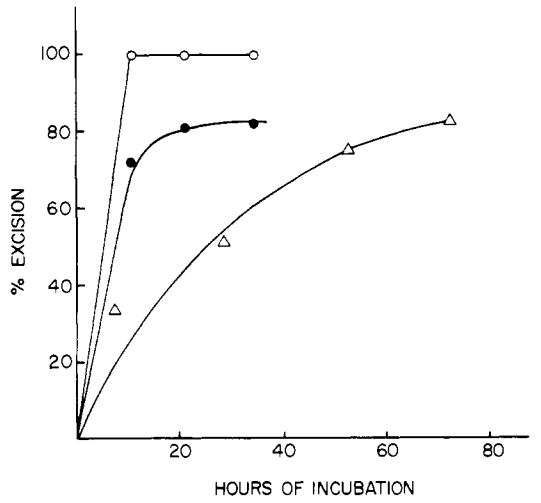

FIGURE 4: Kinetics of removal from DNA of major adducts formed by BPDE I and BPE in human lung cells A549. These data are derived from Sephadex LH-20 chromatograms of DNA digests of the type shown in Figure 3. The initial adduct concentrations were as follows: BPDE I–dG, 28.5 μmol/mol of DNA-P; BPE–dG, 8.7 μmol/mol of DNA-P; BPE–dA, 2.5 μmol/mol of DNA-P. (△) Removal of BPDE I–dG; (●) removal of BPE–dG; (○) removal of BPE–dA.

shown in Figure 4. BPE–dA had been removed very rapidly and completely within 10 h of incubation while removal of BPE–dG came to a halt after approximately 20-h incubation, leaving 20% residual adducts unexcised.

Initial Distribution of Covalent BPE and BPDE I Adducts between Micrococcal Nuclease Sensitive and Resistant DNA in A549 Cells. Nuclei were prepared from A549 cells which had been labeled with [^{14}C]thymidine and treated with [^{3}H]-(±)-BPE or [^{3}H]-(±)-BPDE I as described in the previous paragraph. From half of each nuclei preparation, the DNA was extracted and analyzed for its adduct content by Sephadex LH-20 chromatography; the second half of each sample was first treated with micrococcal nuclease according to the conditions of Söllner-Webb & Felsenfeld (1975) until 49–50% of the DNA could no longer be precipitated with ethanol. The precipitable DNA fractions were then analyzed for their adduct content by Sephadex LH-20 chromatography. These data yielded the initial average adduct concentration in total DNA and in micrococcal nuclease resistant DNA. The adduct concentrations in the micrococcal nuclease sensitive DNA were computed as outlined under Experimental Procedure. The data are given in Table II. It is evident that the initial adduct concentration was considerably higher for all adducts in the micrococcal nuclease sensitive DNA. The most extreme case was found for BPE–dA which was virtually absent from the micrococcal nuclease resistant DNA.

Table II: Initial Distribution of Covalent BPE and BPDE I Adducts between Micrococcal Nuclease Sensitive and Resistant DNA in A549 Cells

	μmol of adduct per mol of DNA phosphate		
DNA adduct	total DNA	micrococcal nuclease[a] resistant DNA	micrococcal nuclease[b] sensitive DNA
BPDE I–dG	28.5	8.5	85.9
BPE–dG	8.7	2.0	27.9
BPE–dA	2.5	0.27	8.9

[a] At 49–50% solubilization of DNA in nuclei upon treatment with micrococcal nuclease. [b] Calculated from the concentration in total DNA and micrococcal nuclease resistant DNA as described under Experimental Procedure.

Discussion

An important aim in molecular carcinogenesis research is to relate the cytotoxicity, mutagenicity, and transforming potency of a particular DNA damaging agent to the initial concentration of specific lesions and to the repairability of these lesions [see, e.g., Newbold et al. (1979) and Cerutti (1978)]. A prerequisite for such a correlation is the characterization of the individual lesions. In the present work the formation and repairability of the lesions induced by two classes of BP epoxides, i.e., BP 4,5-epoxide (BPE) and BP-diol epoxide I (BPDE I), were compared since much information was already available about their differential biological activities. The structures of the DNA lesions induced by BPDE I are well characterized. We have found earlier (Cerutti et al., 1978) and further corroborated in the present work that (±)-BPDE I produces mostly BPDE I–dG, i.e., N^2-(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-10-yl)deoxyguanosine, in A549 cells. In agreement with the results of Brown et al. (1979) with mouse embryo fibroblasts $10T^1/_2$, the 7*R* enantiomer is formed preferentially, but the stereoselectivity of the reaction is apparently less pronounced than for the in vitro reaction of (±)-BPDE I with native DNA (Meehan & Straub, 1979). High stereoselectivity was found for the formation of (7*R*)-N^2-(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-10-yl)deoxyguanosine from BP in actively metabolizing A549 cells (Feldman et al., 1978) and mouse embryo fibroblasts $10T^1/_2$ (Brown et al., 1979), on the other hand.

The formation of covalent adducts of BPE with synthetic polynucleotides and DNA in vitro has been demonstrated, and evidence was obtained that the reaction occurred mostly with guanine and to a lesser degree with adenine (Jennette et al., 1977). It has also been observed that poly(riboguanylic acid) catalyzed the formation of 5-OH-BP from BPE probably via the intermediate formation of an unstable adduct to the N^7 position of guanine (Murray et al., 1976). Our results with native DNA specifically labeled on either deoxyguanosine, deoxyadenosine, or deoxycytidine and Sephadex LH-20 chromatography, followed by LC, indicate that four major covalent adducts are formed with (±)-BPE, two at guanine and two at adenine. Since these adducts were stable to lengthy enzymatic hydrolysis and chromatographic analysis, it is likely that they involve substitution at the exocyclic amino groups of guanine and adenine [cf. King et al. (1979)]. Whether linkage occurs at the 4 or 5 position of BP cannot be decided although the poly(guanylic acid)-catalyzed formation of 5-OH-BP suggests that nucleophilic attack may occur preferentially at the 4 position of BPE. Treatment of A549 cells with [^{3}H]-(±)-BPE yielded two major ^{3}H-containing DNA adduct peaks in the hydrophobic region of the Sephadex LH-20 chromatogram with mobilities which were identical with those of the adducts formed in the reaction of BPE with native DNA in vitro. The two BPE adducts formed in A549 cells most likely correspond to BPE–dG and BPE–dA, therefore. The radioactivity content of the adduct peaks from A549 DNA was insufficient for further analysis by LC.

An important observation for the understanding of the low biological potency of BPE relative to BPDE I is the low yield of formation of BPE adducts in the reaction with the DNA in vitro and in the intact cell. The initial concentration of total BPE adducts in A549 cells was 11.2 μmol/mol of DNA-P at 53 μM (±)-BPE while the initial concentration of BPDE I–dG was 28.5 μmol/mol of DNA-P at 1.1 μM (±)-BPDE I. Differences in the reactivity of the two classes of BP epoxides with the weakly nucleophilic centers of the purine bases, in the rates of the abortive reactions with the N^7 position of

guanine (Murray et al., 1976; King et al., 1979) relative to the reactions leading to stable adducts, as well as differences in solubility and solvation properties of the two metabolites are important factors which determine adduct yields. The low yield of BPE–DNA adduct formation in A549 cells may represent a major factor in explaining the low cytotoxicity of BPE for these cells.

The removal of the BPE–purine adducts from high molecular weight DNA was faster than the removal of BPDE I–dG (Figure 4). The following alternatives for the interpretation of this observation should be considered. (1) Different enzymes may be involved in the removal of the adducts. Support for this possibility comes from work on the repair of alkylation damage in XP fibroblasts. It was shown that O^6-ethylguanine but not the other ethylated bases remained unexcised in XP fibroblasts of complementation group A (Bodell et al., 1979a,b; Goth-Goldstein, 1977; Altamirano-Dimas et al., 1979). (2) The same enzymes are responsible for the removal of the BPE and BPDE I adducts, but the excision system in the experiments with BPDE I, but not with BPE, were reaching saturation. This appears unlikely since the amounts of BPDE I–dG removed during posttreatment incubation increased substantially when the initial adduct concentration was further increased (data not shown). (3) The same enzymes are involved but the differences in the initial distribution of the adducts in chromatin (see Table II) are responsible for the observed rate differences. It should be noted that BPE–dA which possessed the highest relative concentration in micrococcal nuclease sensitive DNA was removed most rapidly and BPDE I–dG with the lowest relative concentration in micrococcal nuclease sensitive DNA was removed least rapidly. Evidence for increased repair activity in nuclease sensitive regions of chromatin has been obtained by several investigators (Bodell, 1977; Ramanathan et al., 1976; Cleaver, 1977; Smerdon & Lieberman, 1978; Cox, 1979). (4) The same enzymes are involved in the removal of the BPE and BPDE I adducts but the rates of removal differ because of the structural differences of the individual lesions. The extent of the local disruption of the DNA helix by the lesions may be a factor influencing their excisability (Cerutti, 1975). Evidence for more extensive local single strandedness in DNA treated with BPE relative to DNA treated with BPDE I has been presented, but quantitation on a per lesion basis was not given (Heflich et al., 1977). Higher excisability of BPE adducts relative to BPDE I adducts, as observed in the present work, might be expected on the basis of these results. It should be noted that the adenine arylalkylation product produced by 7-(bromomethyl)benzanthracene was also removed more rapidly from the DNA of HeLa cells than the arylalkylation product of guanine (Dipple & Roberts, 1977).

The completeness of adduct removal also varied for the different BP–purine adducts. While BPE–dA had disappeared completely within 10-h incubation, ~20% of the initial BPE–dG and BPDE I–dG remained in the DNA at later incubation times when BPE–dG removal had come to a halt and BPDE I–dG occurred only at a very low rate. These results were derived from Sephadex LH-20 chromatograms which did not resolve the subcomponents of BPE–dA and BPE–dG. The amounts of radioactivity recovered were insufficient for LC analysis. Therefore, it is not known whether the persistent fractions of BPE–dG and BPE I–dG contained the initial adduct composition or were enriched in a particular component. The higher rate of removal of the BPE relative to the BPDE I adducts as well as the completeness of the removal of BPE–dA may contribute to the lower cytotoxicity of BPE relative to BPDE I for A549 cells.

In earlier work we have studied the repair of BPDE I–dG induced via cellular metabolism of BP in A549 cells (Feldman et al., 1978) while the ultimate metabolites BPDE I and BPE were used in the present work. The fraction of lesions persisting in the DNA at prolonged incubation was considerably higher in the experiments with BP, i.e., 40–55% persistent adducts relative to 20% in the present work. This may be explained by differences in the initial distribution of the adducts in chromatin. While the adduct concentration was similar in micrococcal nuclease resistant and sensitive DNA following 48-h incubation with BP, it was 10 times higher in the micrococcal nuclease sensitive DNA following treatment for 30 min with the highly reactive BPDE I (see Table II). Adducts located at the chemically more reactive sites of the nuclease sensitive DNA of chromatin may be more accessible to repair enzymes and removed more completely. The observation of differences in initial adduct distribution and adduct persistence between experiments with the procarcinogen BP and the ultimate carcinogen BPDE I which produce structurally identical lesions may have important implications. The extrapolation of results of in vitro mutagenesis and transformation using ultimate metabolites to the in vivo situation where, in general, procarcinogens are metabolized in the target organ have to be done with caution. Different distribution and repairability of structurally identical lesions may mean different biological potency of the respective inducing agents.

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Existence of an Extended Series of Antitumor Compounds Which Bind to Deoxyribonucleic Acid by Nonintercalative Means†

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ABSTRACT: Viscometric titrations of bacteriophage PM2 closed circular DNA, in addition to spectrophotometric and fluorometric methods, were used to investigate the mode of DNA binding of a number of antitrypanosomal and antitumor compounds. Several classes of compounds were identified which failed to unwind PM2 DNA, which appeared to have a large DNA binding site of at least 4 base pairs and which often showed considerable selectivity of binding to poly[d(AT)] as opposed to poly[d(GC)]. The classes included the antiviral antibiotics distamycin and netropsin, bisamidines such as the trypanocidal drug berenil, phthalanilide bisamidines, aromatic bis(guanylhydrazones), and the bisquaternary ammonium heterocycles. It is proposed that the compounds all bind in the minor groove of the DNA double helix.

In recent years, Cain and co-workers (Cain et al., 1969, 1971) have synthesized a large number of compounds termed bisquaternary ammonium heterocycles [reviewed by Denny et al. (1979)]. Many of these compounds are highly active against the murine L1210 leukemia in mice, and in structure they resemble another group of antiprotozoal and antitumor compounds termed the phthalanilides (Bennett, 1965; Cain et al., 1969). On the basis of model building studies, Cain et al. (1969) proposed that these compounds bind to the minor groove of the DNA double helix, but experimental evidence was not advanced. Waring (1970) reported that the trypanocidal drug berenil did not unwind closed circular DNA, and subsequently several workers studying the antibiotics netropsin and distamycin concluded that they bound, without intercalation, in the minor groove of double-stranded DNA (Wartell et al., 1974; Zimmer, 1975). Since netropsin, like the bisquaternary ammonium heterocycles and the phthalanilides, contains terminal basic functions separating a largely coplanar aromatic skeleton, it seemed worthwhile to examine the DNA binding properties of these other classes. The viscometric analysis of closed circular DNA (Revet et al., 1971) has been

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