Modification of Deoxyribonucleic Acid by a Diol Epoxide of Benzo[*a*]pyrene. Relation to Deoxyribonucleic Acid Structure and Conformation and Effects on Transfectional Activity†

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ABSTRACT: The effects of secondary structure on DNA modification by (±)-7β,9α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(±)BPDE I] were investigated. No differences in the total extent of (±)BPDE I binding to double- and single-stranded calf thymus DNA were found. High-performance liquid chromatography (LC) of the nucleoside adducts obtained from hydrolysates of native and denatured calf thymus, as well as from superhelical and linear plasmid DNA, indicated that in all cases the major adduct (60–80% of total adducts) was formed by reaction of the (+) enantiomer of BPDE I with the N-2 position of dG residues in the DNA. A minor adduct formed from the reaction of the (−) enantiomer with dG residues was also detected and was present in greater amounts in denatured DNA than in native DNA. Small amounts of BPDE I–dA and BPDE I–dC adducts were also detected in both the single- and double-stranded DNAs. Restriction enzyme analysis of BPDE I modified SV40 and phage λ DNA provided evidence that the modification of DNA by this carcinogen is fairly random with respect to nucleotide sequence. Partial hydrolysis of modified plasmid DNA by the single-strand-specific S_1 nuclease and LC analysis of the nucleoside adducts in the digested and undigested fractions of the DNA revealed no preferential excision by the S_1 nuclease of the different BPDE I–deoxynucleoside adducts. Functional changes in BPDE I modified DNA were demonstrated. With increasing extents of modification, there was a decrease in the ability of plasmid DNA to transfect a receptive *Escherichia coli* strain to antibiotic resistance.

Several lines of evidence indicate that, in vivo, the major reactive metabolic intermediate of the chemical carcinogen benzo[*a*]pyrene (BP)[1] is the diol epoxide derivative which is enzymatically formed in two stereoisomeric forms, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (designated BPDE I or "anti") and 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (designated BPDE II or "syn") (Borgen et al., 1973; Sims et al., 1974; Daudel et al., 1975; King et al., 1976; Ivanovic et al., 1976; Weinstein et al., 1976). Both isomers have been chemically synthesized as mixtures of (±) racemic enantiomers (Beland & Harvey, 1976). The various isomers and enantiomers have been shown to be mutagenic in bacteria (Malaveille et al., 1975; Wislocki et al., 1976a,b) and mammalian cells (Huberman et al., 1976; Wood et al., 1977), to transform hamster embryo cells in vitro (Mager et al., 1977), and to be carcinogenic in vivo (Levin et al., 1977). Evidence was presented that BPDE I residues bind covalently predominantly to the N-2 group of guanine residues in RNA (Weinstein et al., 1976) and DNA (Jeffrey et al., 1977). Cytosine and adenosine residues in nucleic acids are also targets, to a smaller extent, both in vitro (Jennette et al., 1977; Meehan et al., 1977; Jeffrey et al., 1979) and in vivo (Jeffrey et al., 1977; Ivanovic et al., 1978), presumably because they also contain an exocyclic amino group.

Due to the multiplicity of nucleoside–BPDE I adducts produced, it was of interest to study whether the type and conformation of DNA exert any effect upon the extent and distribution of the adducts formed. It has been shown previously with the hepatocarcinogen AAF that the conformation of DNA plays an important role in the formation of two different adducts with dG residues (Kriek, 1972; Westra et al., 1976). Interaction of AAF with DNA in vivo results in production of C-8 and N^2 adducts of dG, and in vitro both of these adducts are also formed if the substrate is double-stranded DNA (Kriek, 1972). On the other hand, in vitro modification of single-stranded DNA or RNA leads only to the binding of AAF to the C-8 position of guanine residues (Kriek, 1974). We have found that AAF preferentially binds to the guanine residues in single-stranded regions of DNA as well as tRNA (Levine et al., 1974; Fujimura et al., 1972; Pulkrabek et al., 1974). In the present study, we have examined the extent and type of modification of double- and single-stranded calf thymus, as well as of superhelical and open circular plasmid DNA, by BPDE I. We have also investigated whether different BPDE I adducts induce major differences in the conformation of DNA.

In previous studies, we found that in vitro BPDE I modified calf thymus DNA was impaired in its template activity during in vitro transcription by *Escherichia coli* RNA polymerase. The actual sizes of transcripts were greater, however, than one would expect if the BPDE I residues were randomly distributed along the DNA template and if there was complete interference of chain elongation at every site of modification (Leffler et al., 1977). Therefore, we have explored whether BPDE I modification is a random or a sequence-specific event by measuring the distribution of BPDE I adducts in restriction enzyme fragments of phage λ and SV40 viral DNA. Finally, we have studied the effect of BPDE I modification of plasmid

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[1] Abbreviations used: (±)BPDE I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, also referred to as the "anti" isomer; (±)BPDE II, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, also referred to as the "syn" isomer; BP, benzo[*a*]pyrene; AAF, *N*-2-acetylaminofluorene; LC, high-performance liquid chromatography; pfu, plaque-forming units.

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DNA on its ability to transfect *E. coli* to antibiotic resistance.

Materials and Methods

Chemicals. Calf thymus DNA and DNase I were obtained from Worthington Co., Freehold, NJ; [^{3}H](±)BPDE I (sp act. 223 μCi/μmol) was from the Chemical Repository of the National Cancer Institute, Bethesda, MD; plasmid pSF2124 DNA was a kind gift of Dr. Ray Sweet, Columbia University, New York, NY; plasmid pBR322 DNA was from Bethesda Research Laboratories, Inc., Bethesda, MD; the restriction enzymes were obtained from New England Biolabs, Beverly, MA. Endonuclease S_1 (EC 3.1.4.*X*) from *Aspergillus oryzae* and endonuclease from *Neurospora crassa* were from Miles Laboratories, Inc., Elkhart, IN. Alkaline and acid phosphatases and agarose were from Sigma Chemical Co., St. Louis, MO; snake venom phosphodiesterase and spleen phosphodiesterase were from P-L Biochemicals, Milwaukee, WI. λ DNA was generously supplied by Dr. R. E. Jones, National Institutes of Health, Bethesda, MD.

Preparation of Plasmid DNAs. DNAs from plasmids were obtained in milligram quantities by transformation of *E. coli* HB101 (Rec A$^-$, r$^-$, m$^-$, Str, pro$^-$, leu$^-$, B$_1^-$) according to a published procedure (Cohen & Chang, 1973). *E. coli* cells were grown to A_{590} = 0.6 and made competent for transformation by successive treatment with 10 and 30 mM $CaCl_2$. Competent cells (0.2 mL) were added to 0.1 mL (1 μg) of plasmid DNA and incubated 30 min on ice, followed by 1.5-min incubation at 42 °C. Cells were then incubated in 5 mL of L-broth at 37 °C for 90 min and plated on L-agar containing ampicillin (100 μg/mL). After 24 h at 37 °C, a resistant colony was selected and grown in 1 L of L-broth containing ampicillin (100 μg/mL). When the cells had grown to A_{590} = 1.0, 0.2 mg of chloramphenicol was added to a final concentration of 200 μg/mL for plasmid amplification. Plasmid DNA was isolated by a cleared lysate procedure and purified by CsCl–ethidium bromide centrifugation (Clewell & Helinski, 1969). Plasmid pBR322 DNA was prepared by using an identical procedure, except that, in addition to ampicillin, tetracycline (25 μg/mL) was used in the medium.

Preparation of SV40 DNA. SV40 DNA was purified from BSC-1 cells infected with plaque-purified virus at 5 pfu/cell and harvested after 4 days at 37 °C. Viral DNA was extracted by the method of Hirt (1967), followed by phenol and chloroform extractions. SV40 DNA was banded on CsCl–ethidium bromide gradients and removed by needle puncture. The ethidium bromide was extracted with CsCl-saturated 1-propanol and dialyzed extensively against 0.01 × SSC buffer (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), followed by ethanol precipitation and resuspension in 0.01 × SSC.

Modification of DNA by Benzo[a]pyrene Diol Epoxide. The reaction was carried out at 37 °C for 1 h in the dark, in a solution containing 0.02 M sodium cacodylate (pH 7.1), 33% ethanol or methanol, and designated amounts of [^{3}H](±)-BPDE I. The final concentration of DNA was 0.3 mg/mL. The unreacted BPDE derivatives were removed by repeated (10 times) extraction with water-saturated ethyl acetate, and the DNA was precipitated with 3 volumes of ethanol after the addition of 2 M sodium acetate (pH 5.0) to a final concentration of 0.2 M. In the modified DNA samples, the extent of modification ranged from 0.01 to 0.5% of the total base residues, depending upon the particular experimental conditions.

Hydrolysis of BPDE I Modified DNA. Hydrolysis of modified DNA was performed as previously described (Yamasaki et al., 1977). The incubation mixture (final volume 0.5 mL) contained 10 mM Tris-HCl (pH 7.9), 0.1 M NaCl, 1 mM $MgCl_2$, and modified DNA. The DNA was first heat-denatured by boiling for 3 min, followed by rapid cooling in ice. The denatured samples were then hydrolyzed by *N. crassa* endonuclease (24 units/mL) at 37 °C overnight. Further digestion was achieved by successive incubation at 37 °C with the following enzymes: DNase I (0.2 mg/mL) for 3 h, *E. coli* alkaline phosphatase (8 units/mL) for 3 h, spleen phosphodiesterase (0.5 unit/mL) for 1 h, and crystalline snake venom phosphodiesterase (0.2 mg/mL) for 18 h. This series of enzyme digestions resulted in complete hydrolysis of the DNA.

Analysis of BPDE-Modified Nucleosides. Separation of the modified nucleosides was performed by Sephadex LH-20 column chromatography. The column (0.8 × 20 cm) was first eluted with 50 mL of water and then with 80% methanol. Fractions (1 mL) were collected and radioactivity was counted in 50-μL aliquots in a New England Nuclear Liquid Scintillation Mark II spectrometer using 10 mL of Hydrofluor (National Diagnostics, Somerville, NJ). Radioactive fractions were pooled, evaporated, dissolved in water, and analyzed by high-performance liquid chromatography.

High-Performance Liquid Chromatography (LC). The purified nucleoside adducts were analyzed by using a Du Pont 830 liquid chromatograph with a Waters Associates μBondapak C_{18} column (0.29 × 30 cm) developed with a concave 30–60% methanol gradient. One-minute fractions were collected and counted after adding 10 mL of Hydrofluor.

S_1 Nuclease Digestion of BPDE-Modified Plasmid DNA. Modified plasmid pSF2124 DNA (0.07% modified bases) was digested for various lengths of time according to Vogt (1973). Reaction mixtures contained (final volume 0.16 mL) 50 μg of DNA, 30 mM sodium acetate buffer (pH 4.5), 50 mM NaCl, 1 mM $ZnSO_4$, and 5 units of S_1 nuclease. Incubation was carried out at 46 °C for 30, 60, and 90 min. At each time interval, the incubation mixture was precipitated with 0.45 mL of ethanol in order to separate undigested from digested DNA. The undigested DNA was further hydrolyzed to nucleosides according to the procedure described above. Ethanol supernatants from S_1 nuclease digestion were flash-evaporated to dryness and dissolved in 0.5 mL of water, and free nucleotides were converted to nucleosides by digestion with potato acid phosphatase (1.2 units/mL) at 37 °C for 18 h.

Restriction Enzyme Analysis. Ten micrograms of BPDE I modified or unmodified DNA was digested at 37 °C for 30 min in 0.075 mL of the following mixtures: endonuclease *Hin*dIII, 3 units/μg of DNA; 7 mM Tris-HCl, pH 7.9; 60 mM NaCl; 7 mM $MgCl_2$. Reactions were terminated by adding 6 μL of 0.25 M EDTA and 9 μL of 0.1% bromophenol blue in 50% glycerol and used for agarose gel electrophoresis.

Agarose Gel Electrophoresis. Electrophoresis was performed on slabs (12 × 17 × 0.3 cm) containing 1.0 or 1.2% agarose dissolved in borate buffer (0.09 M Tris-HCl, 2.5 mM EDTA, and 9.0 mM boric acid (pH 9.0)). The samples contained 10% glycerol and 0.1% bromophenol blue and were electrophoresed at 120 V until the dye had migrated at least 10 cm, usually 2.5–3 h. After staining in a buffer containing ethidium bromide (0.5 μg/mL), we photographed the gels under ultraviolet illumination with Polaroid No. 105 film using a red filter. For determination of [^{3}H]BPDE I residues in supercoiled or relaxed DNA, bands were excised from the gel, dissolved in hyamine hydroxide for 1 h at 60 °C, cooled overnight in 10 mL of Hydrofluor, and counted.

Transfection of E. coli by BPDE-Modified Plasmid DNA. For the determination of transfection efficiency, *E. coli* HB101 was transfected to antibiotic resistance with 1 μg of pBR322

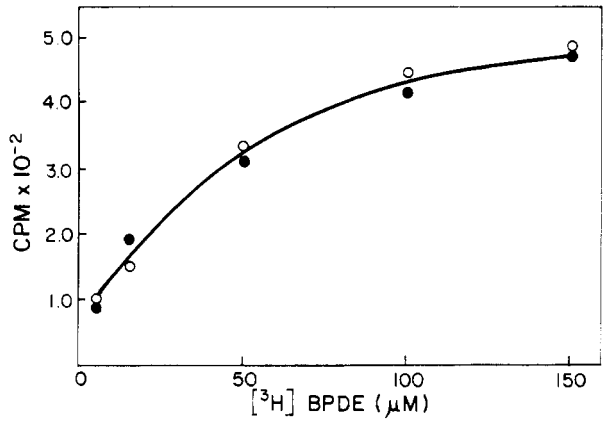

FIGURE 1: Effect of BPDE I concentration on the extent of modification of native and denatured calf thymus DNA. Native and heat-denatured DNA (50 μg) were modified in a final volume of 0.15 mL in a reaction mixture containing 0.02 M sodium cacodylate (pH 7.1), 33% ethanol, and designated amounts of [^{3}H]BPDE I. After 2 h at 37 °C, the unreacted BPDE derivatives were removed by repeated extraction with water-saturated ethyl acetate. DNA was precipitated with 10% trichloroacetic acid, filtered through Millipore filters, and counted. (○) Native DNA; (●) heat-denatured DNA.

previously modified to various extents with BPDE I, as described above. After a 90-min incubation at 37 °C, 0.1-mL aliquots of serial dilutions were plated on L-agar dishes containing tetracycline and ampicillin separately or in combination. After 24 h, each plate was scored for the number of transfected colonies.

Results

Modification of Native and Denatured Linear DNA. Since binding of AAF is accompanied by gross conformational changes involving rotation of the guanine residue about the glycosidic bond from the anti to the syn conformation, denatured DNA is preferentially modified by AAF (Levine et al., 1974). In order to determine whether binding of BPDE I residues to DNA requires similar alterations in conformation of DNA, we have compared the attack of BPDE I residues on native and denatured calf thymus DNA. Heat denaturation was performed by boiling the samples for 3 min, followed by rapid cooling on ice. Both native and denatured DNA were then reacted with [^{3}H](±)BPDE I. The extents of modification of these two types of DNAs are shown in Figure 1. There is no significant difference in the amount of BPDE I moieties bound to the two types of DNA. Since BPDE I forms multiple adducts with DNA, we next examined by LC chromatography whether the conformation of the DNA molecule has any effect on the types of deoxynucleoside adducts formed. Figure 2 shows that the elution profiles of the adducts obtained from both native and denatured DNA are very similar. The major component (peak 3) cochromatographed with a marker whose structure has been previously established to be the adduct formed between (+)BPDE I and dG (Jeffrey et al., 1977). Peak 2 is a second dG adduct formed from the (−) enantiomer of BPDE I. It was found in relatively greater amounts when the denatured DNA was reacted with (±)BPDE I than when native DNA was used. The minor peaks represent dC (peak 1) and dA adducts (peak 4). It was recently established that binding of racemic BPDE I to DNA results in the formation of four diastereomers of dA adducts (Jeffrey et al., 1979) and in all of these adducts, the 10 position of BPDE I is linked to the N^6-amino group of dA residues. This accounts for the complex profile in the region of peak 4. The relative distributions of the individual adducts formed from native and denatured DNA are summarized in Table I. It is evident that

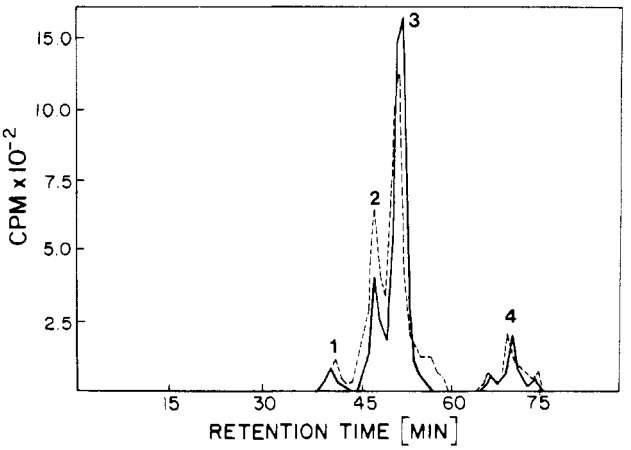

FIGURE 2: LC profiles of deoxynucleoside adducts from hydrolysates of [^{3}H]BPDE I modified native and denatured calf thymus DNA. Modified native and denatured DNA samples were enzymatically hydrolyzed and the [^{3}H]BPDE–nucleosides were separated by Sephadex LH-20 chromatography. The modified adducts were then analyzed by LC as described under Materials and Methods. The numbers indicate positions of elution of the deoxynucleoside adducts: (1) BPDE I–deoxycytidine; (2) (−)BPDE I–deoxyguanosine; (3) (+)BPDE I–deoxyguanosine; (4) BPDE–deoxyadenosines. (—) Hydrolysate of native DNA; (---) hydrolysate of denatured DNA.

Table I: Distribution of Nucleoside Adducts in Native and Heat-Denatured Calf Thymus DNA[a]

adduct	native DNA (%)	heat-denatured DNA (%)
BPDE I-dC	2.2	3.5
(−)BPDE I-dG	12.5	22.0
(+)BPDE I-dG	76.0	63.3
BPDE I-dA[b]	9.3	11.2

[a] The percentages of individual adducts are calculated by integrating radioactivity in the area of each component on the LC profile of Figure 2. [b] Represents total BPDE I-dA adducts eluted in three peaks (see Figure 2).

the only significant difference is in the relative distribution of the dG adducts. In native DNA (+)BPDE I–dG represents 76% of the total adducts and 12.5% is the (−)BPDE I–dG product, in contrast to denatured DNA in which the respective values are 63 and 22%. With both native and denatured DNA, the dC adducts were about 2–3% and the dA adducts were about 9–11% of the total.

Modification of Closed and Open Circular and Linear Forms of Plasmid pBR322 DNA. Another factor which could affect the binding of a carcinogen is whether the DNA is linear or circular. We have compared the effects of using circular and linear forms of plasmid DNA on the distribution of BPDE–nucleoside adducts. The native plasmid contained both covalently closed circular (superhelical) and open circular (nicked) DNA in an approximately 3:1 ratio. Single-stranded DNA was prepared by cleavage of the plasmid DNA with *Eco*RI restriction enzyme and heat denaturation by boiling for 10 min, followed by rapid cooling on ice. After modification of these DNAs with [^{3}H]BPDE I and enzymatic hydrolysis, the BPDE–nucleoside adducts were subjected to LC. Figure 3 shows that, as with calf thymus DNA, the single-stranded plasmid DNA contains somewhat higher amounts of the dG adduct formed from the (−)BPDE I enantiomer than does the native plasmid DNA. Since the distribution of the adducts in the native plasmid DNA is similar to that of native calf thymus DNA (compare Figures 2 and 3), supercoiling and circularity of the DNA do not have an appreciable influence. To prevent the possibility of snapping back and looping of single strands, we performed an experiment in which, in

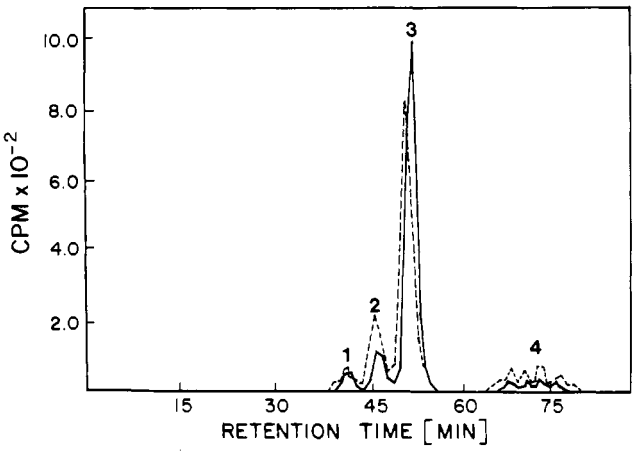

FIGURE 3: LC profile of BPDE I–deoxynucleoside adducts obtained from a complete enzymatic hydrolysate of modified pBR322 DNA. For details, see Materials and Methods. Hydrolysate of native (mixture of closed supercoiled and open circular) DNA (—); hydrolysate of single-stranded (*Eco*RI cleaved and heat-denatured) DNA (---). For the position of authentic nucleoside adducts, see Figure 2.

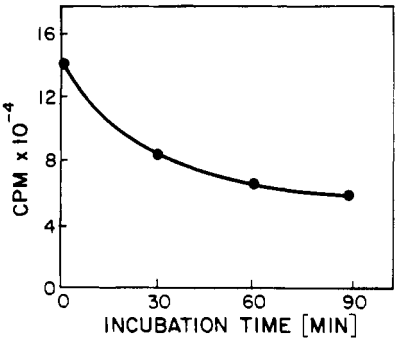

FIGURE 4: Digestion of [^{3}H]BPDE I modified DNA with S_1 nuclease. Native calf thymus BPDE I–DNA (0.25% bases modified) was incubated with S_1 nuclease for the indicated periods of time. The undigested portion of the DNA was precipitated with 10% trichloroacetic acid and filtered through Millipore filters, and radioactivity was counted. For details, see Materials and Methods.

addition to *Eco*RI restriction and heat denaturation, methylmercury hydroxide was added prior to modification. Although the overall modification was inhibited by methylmercury hydroxide by about 60%, the LC profile of BPDE I adducts was similar to that of the heat-denatured sample (data not shown).

S_1 Nuclease Digestion of [^{3}H]BPDE I–Plasmid DNA. It has been previously shown that BPDE I binding to calf thymus DNA causes localized regions of denaturation (Pulkrabek et al., 1977). However, it has not been clearly established as to which of the BPDE I adducts (dG, dA, or dC) contributes to the formation of these denatured regions. Possible contributions of specific modified nucleosides to the formation of localized regions of denaturation were studied by S_1 nuclease digestion of the modified DNA. In Figure 4, the time course of hydrolysis of the modified plasmid DNA by S_1 nuclease is shown. After 30 and 90 min of S_1 nuclease digestion, the nonhydrolyzed DNAs from the supernatant, and those from the precipitates, were subjected to LC after complete enzymatic hydrolysis and purification by Sephadex LH-20 chromatography. Table II, based on LC analysis, shows identical distribution of BPDE I adducts in the S_1 nuclease digested and undigested fractions. These results suggest that there is no preferential excision of specific BPDE I adducts by S_1 nuclease and that all adducts contribute to the formation of localized regions of denaturation in DNA. These results differ from

Table II: Distribution of Nucleoside Adducts in Modified Plasmid DNA and in S_1 Nuclease Hydrolysates[a]

		S_1 nuclease hydrolysis			
		30 min		90 min	
adduct	original[b] DNA (%)	di-gested[c] (%)	undi-gested[c] (%)	di-gested (%)	undi-gested (%)
BPDE I–dC	4.2	3.8	4.5	4.4	4.5
(–)BPDE I–dG	5.4	5.6	5.7	5.8	5.9
(+)BPDE I–dG	80.9	81.8	81.7	82.8	83.1
BPDE I–dA[d]	9.5	8.8	8.1	7.0	6.5

[a] DNA samples digested or undigested by S_1 nuclease were enzymatically hydrolyzed to nucleosides and analyzed by LC as described under Materials and Methods. The numbers represent the percentage of individual adducts calculated by integrating radioactivity in the area of each component. [b] Modified DNA which was not digested with S_1 nuclease but directly enzymatically hydrolyzed to mononucleosides. [c] DNA was digested with S_1 nuclease and precipitated with ethanol, yielding "undigested" (pellet) and "digested" (supernatant) fractions which were further hydrolyzed to mononucleosides. [d] Represents the total BPDE I-dA adducts eluted in four peaks (see Figure 3).

Table III: Restriction Endonuclease *Hin*dIII Digestion of SV40 and Phase λ DNA Modified in Vitro by Benzo[*a*]pyrene Diol Epoxide[a]

fragment	fragment length (kb)[b]	BPDE I residues per unit length of DNA (pmol)[c]	BPDE I residues per 10 kb[d]
Expt 1: 11 BPDE I Residues per SV40 Molecule			
unrestricted	5.2	11.4	22
1	1.8	2.9	16
2	1.2	2.1	18
3	1.1	1.5	13
4	0.5	1.0	20
5	0.4	0.6	15
6	0.2	0.3	14
Expt 2: 36 BPDE I Residues per Phage λ Molecule			
unrestricted	44.2	36	8
A	20.5	14.8	7
B	8.9	6.8	8
C	6.0	4.6	8
D	4.0	3.3	10
E	2.3	2.3	12
F	2.0	2.0	10
G	0.5	0.6	12

[a] Approximately 10 μg (0.3 pmol) of DNA was applied per gel, as described under Materials and Methods. [b] Values were determined on analytical agarose gels by using appropriate molecular weight markers. [c] All values have been normalized to 1 pmol of unrestricted DNA and represent an average of two experiments. [d] Calculated as follows: (pmoles of BPDE I residues bound per unit length of DNA)/[fragment length (kb)] × 10.

those reported by Kakefuda & Yamamoto (1978).

Restriction Enzyme Analysis of [^{3}H]BPDE I Modified λ and SV40 DNA. To establish whether binding of BPDE I residues to DNA is a random or a nucleotide sequence specific event, we modified two well-defined DNAs, linear λ and supercoiled SV40 DNA, to different extents and subjected them to *Hin*dIII restriction enzyme digestion. The distribution of BPDE I residues in individual fragments was then calculated after electrophoresis of the samples on agarose gels, ethidium bromide staining, excision of bands, and counting. Table III presents the distribution of BPDE I residues in SV40 and λ DNA fragments. Six major fragments were obtained with both the modified and nonmodified SV40 DNA. The number and sizes of the cleavage products are in agreement with the

Table IV: Transfection of *E. coli* HB101 by Plasmid pBR322 DNA Modified by Benzo[*a*]pyrene Diol Epoxide

DNA	BPDE I residues per plasmid DNA (mol/mol)	no. of resistant colonies[a]	rel transfection efficiency[b]
native	0	2575	106
mock modified	0	2437	100
BPDE I modified	2	1006	41
BPDE I modified	3	482	20
BPDE I modified	5	4	0.16

[a] Each value represents an average of three separate experiments performed by serial dilution. The colonies were counted after 24 h at 37 °C. Similar numbers of resistant colonies were obtained when the selective medium contained ampicillin, tetracycline, or both. The values given, therefore, are the means of data obtained with all three types of selection. [b] Numbers determined as a percent relative to mock-modified DNA which has been arbitrarily assigned as 100%. The actual efficiency of transfection of *E. coli* HB101 by pBR322 DNA under our conditions is 2.6×10^5 transfectants per μg of DNA. Additional details are given under Materials and Methods.

values reported by Hsu & Berg (1978). The distributions of BPDE I residues in unrestricted DNA and in the separated fragments are expressed as the number of BPDE I residues bound per 10^4 bases. We found that the latter values in all of the fragments and in the unrestricted molecule were similar when studied at the binding of 5 and 11 BPDE residues per SV40 molecule. Data are shown only with the higher modified sample (experiment 1).

Similar experiments were performed with λ DNA modified with [^{3}H]BPDE I to the extent of 4, 36, and 137 residues per molecule. After *Hin*dIII restriction, the number and sizes of the fragments were again in agreement with published values (Thomas & Davis, 1975; Murray & Murray, 1975). The results show that the *Hin*dIII fragments reveal approximately equal distributions of BPDE I residues per 10^4 bases, at all three levels of modification. Table III shows only data with the sample modified at the level of 36 BPDE I residues per molecule (experiment 2). These results suggest that BPDE I modification of SV40 and λ DNA is a fairly random rather than a sequence-specific event.

Transfectional Activity of BPDE I Modified Plasmid DNA. It has been shown previously that the binding of BPDE I to φX174 DNA destroys its infectivity (Hsu et al., 1977a,b). Therefore, it seemed to be of interest to examine the effects of BPDE I modification on the function of plasmid DNA. For this reason, we studied the influence of BPDE modification on the ability of plasmid DNA to transfect a receptive bacterial host, *E. coli* strain HB101, to antibiotic resistance (Cohen & Chang, 1973). Plasmid pBR322, carrying resistance genes to ampicillin and tetracycline, was modified with BPDE I to varying extents and used to transfect *E. coli* HB101. The results in Table IV show that, as the extent of modification increases, the number of transfected colonies decreases. At the level of two BPDE I hits per plasmid molecule, the transfectional activity decreased by 60%. Only 20% of the activity remained when there were three BPDE I hits per plasmid molecule. At an average of five BPDE I hits per plasmid molecule, there was virtually no residual transfection activity. Modification of plasmid DNA by BPDE I had an equal inhibitory effect on the transfection of resistance to ampicillin, tetracycline, or ampicillin plus tetracycline. The latter results suggest that BPDE I modification led to overall inactivation of plasmid activity rather than inactivation of specific markers.

Discussion

The aim of this investigation was to obtain further information on the relation between DNA structure and conformation and DNA modification by an enantiomeric mixture of a specific isomer of BPDE, designated BPDE I (also referred to as the anti isomer). Concerning the structural aspects, our results show that the relative abundance of the different nucleoside adducts formed when (±)BPDE I reacts with DNA is only slightly influenced by the type and state of the DNA. In linear, circular, or superhelical DNA, the major product (about 60–80% of the total adducts) is the adduct formed between the (+) enantiomer of BPDE I and the N-2 position of dG residues. There is a significant increase in the level of binding of the (−) enantiomer to dG residues when one uses denatured DNA rather than native DNA; the values for this adduct in denatured and native DNA are 22 and 12%, respectively, of the total adducts. These results are in the same direction as, but less striking than, those reported by Meehan & Straub (1979). They found that with the double-stranded form of φX174 DNA dG residues reacted almost exclusively with the (+) enantiomer of BPDE I, but with single-stranded φX174 DNA the (+) and (−) enantiomers reacted with dG residues to about equal extents. The latter result may reflect features unique to single-stranded φX174 DNA. At this time, the reasons for stereoselective binding of BPDE to dG residues in DNA are not known. Presumably, both the conformation of the DNA and the stereochemistry of the different enantiomers of BPDE I determine the types of adducts which are formed. It is noteworthy that in all cases the major adduct found, (+)BPDE I–dG, is the same as that formed when human cells are exposed to the parent hydrocarbon BP (Jeffrey et al., 1977).

With regard to the effects of BPDE I modification on DNA conformation, we have concluded, based on heat denaturation, formaldehyde unwinding, and S_1 nuclease susceptibility studies, that double-stranded DNA molecules containing covalently bound BPDE I residues have small localized regions of denaturation (Pulkrabek et al., 1977). The extent of DNA denaturation was much smaller, however, than that produced by equivalent modification with AAF. It is known that AAF modification of DNA causes a major conformational change termed "base displacement" (Grunberger et al., 1970; Nelson et al., 1971; Levine et al., 1974; Yamasaki et al., 1977). Studies utilizing internal and external fluorescence quenchers (Prusik et al., 1979) provide evidence that, in contrast to the situation with AAF, covalently bound BPDE I residues are not intercalated between the base pairs of the helix. Electric linear dichroism studies of BPDE I modified calf thymus DNA (Geacintov et al., 1978) indicate, in contrast to AAF, that the long axis of the bound BPDE I moiety is oriented at an angle of about 35° with respect to the helix axis rather than at 90° which would be the case if it were intercalated. Taken together, the above results suggest that the BPDE I moiety attached to the N-2 position of dG residues lies in the minor groove of the DNA double-stranded helix. Model building supports the feasibility of this conformation [unpublished studies and Beland (1978)].

Our finding that BPDE I, in contrast to AAF, binds equally well to double-stranded and single-stranded DNA is also consistent with the above conclusions. On the other hand, studies by Drinkwater et al. (1978) on the unwinding of supercoiled SV40 DNA induced by BPDE I modification led them to suggest a model of covalent intercalative binding of BPDE I residues with DNA. This model is somewhat similar to the

"base displacement model" described for AAF (Weinstein & Grunberger, 1974; Grunberger & Weinstein, 1976). However, the unwinding of supercoiled DNA is not in itself proof that the compound being studied is intercalated. For example, Cohen et al. (1979) have reported that the antitumor drug *cis*-dichlorodiamineplatinum causes unwinding of the double helix but does not bind to DNA by intercalation.

Another question is whether different BPDE I adducts create different types of changes in the conformation of the modified DNA. Kakefuda & Yamamoto (1978) have reported that S_1 nuclease has a greater ability to digest regions of DNA containing BPDE I modified dA than BPDE I modified dG residues. In the present studies, we were unable to confirm this result with BPDE I modified plasmid DNA. Further studies are required to resolve these differences.

Our finding that BPDE I residues are distributed fairly randomly in both SV40 and λ DNA restriction enzyme fragments is consistent with previous studies on SV40 by Mengle et al. (1978). Thus, although modification of DNA by BPDE I displays considerable base and stereochemical specificity, there does not appear to be appreciable specificity in terms of nucleotide sequence. Since we did not obtain any evidence of clustering of the BPDE I residues in these DNAs, our previous results on in vitro transcription of BPDE-modified DNA (Leffler et al., 1977) must now be interpreted as evidence that with a certain frequency BPDE I adducts in a DNA template can be "bypassed" by RNA polymerase to permit continued chain elongation.

The usefulness of bacterial viruses as model systems for screening biologically active polycyclic aromatic hydrocarbons was reported by Hsu et al. (1977a,b). The present study extends this approach by measuring the effect of carcinogen modification of plasmid DNA on transfection of antibiotic resistance to *E. coli*. Our data on the inactivation of transfection by BPDE I modification of plasmid DNA are consistent with single-hit kinetics. Single-hit kinetics were also found in the study with φX174 phage DNA (Hsu et al., 1977a). In vitro studies also provide evidence that BPDE I modified plasmid DNA and BPDE I modified φX174 DNA are impaired in their template activity for DNA replication (Mizusawa & Kakefuda, 1979; Hsu & Berg, 1978). Because of its simplicity, the plasmid DNA transfection assay described in the present study is well suited for the detection and identification of other toxic chemical substances that bind to DNA.

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Nonionic Nucleic Acid Analogues. Synthesis and Characterization of Dideoxyribonucleoside Methylphosphonates†

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ABSTRACT: A series of dideoxyribonucleoside methylphosphonate analogues, dN*p*N and dN*p*N*p*, which contain a nonionic 3′–5′ methylphosphonyl internucleoside linkage were prepared. The two diastereoisomers, designated isomers 1 and 2, of each dimer differ in configuration of the methylphosphonate group and were separated by column chromatography. The diastereoisomers of each dimer have different conformations in solution as shown by ultraviolet hypochromicity data and their circular dichroism spectra. For example, dA*p*A isomer 1 is more highly stacked than isomer 2, although both isomers are less stacked than the dinucleoside monophosphate, dApA. The circular dichroism spectrum of isomer 1 is very similar to that of dApA, while the CD spectrum of isomer 2 shows a loss of molecular ellipticity, [θ], at 270 nm and a greatly diminished [θ] at 250 nm. These results suggest that the stacked bases of dA*p*A isomer 1 tend to orient in an oblique manner, while those in isomer 2 tend to orient in a parallel manner. This interpretation is verified by the ^{1}H NMR study of these dimers (L. S. Kan, D. M. Cheng, P. S. Miller, J. Yano, and P. O. P. Ts'o, unpublished experiments). Both diastereoisomers of dA*p*A form 2U:1A and 2T:1A complexes with poly(U) and poly(dT), respectively. The higher T_m (T_m of poly(U)–isomer 1, 15.4 °C; T_m of poly(U)–isomer 2, 19.8 °C; T_m of poly(dT)–isomer 1, 18.7 °C; T_m of poly(dT)–isomer 2, 18.4 °C) values of these complexes vs. those of the corresponding dApA–polynucleotide complexes (T_m of poly(U)–dApA, 7.0 °C; T_m of poly(dT)–dApA, 9.2 °C) result from decreased charge repulsion between the nonionic dimer backbone and the negatively charged polymer backbone. The difference in conformations between dA*p*A isomer 1 and dA*p*A isomer 2 is reflected in the T_m of the isomer 1-poly(U) complex which is 4.4 °C lower than that of the isomer 2-poly(U) complex. Since these nonionic oligonucleotide analogues are taken up by cells in culture, they show promise as molecular probes for the function and structure of nucleic acids inside living cells.

Studies on nucleic acid analogues and derivatives possessing modified internucleoside linkages have made important contributions to understanding nucleic acid conformation in solution and have provided materials for various biochemical and biological studies (Jones et al., 1970, 1973; Pitha et al., 1971; Letsinger et al., 1976; Blob et al., 1977; Mungall & Kaiser, 1977; Vosberg & Eckstein, 1977). In a series of seven papers, our laboratory has reported studies on the physical, biochemical, and biological properties of one class of nonionic nucleic acid derivative, the oligonucleotide alkyl phosphotriesters. The physical properties of dinucleotide methyl and ethyl phosphotriesters have been studied by ultraviolet, circular dichroism, infrared, and proton nuclear magnetic resonance spectroscopy (Miller et al., 1971; Deboer et al., 1973). The interaction of deoxyribooligonucleotide ethyl phosphotriesters with sequences complementary to the amino acid accepting stem and anticodon region of transfer RNA has been characterized (Miller et al., 1974), and their inhibitory effects on in vitro aminoacylation have been studied (Barrett et al., 1974). More recently, the inhibitory effect of a 2′-*O*-methyl ribooligonucleotide triester, $G_p^m(Et)G_p^m(Et)U$,[1] on cellular protein synthesis and growth of mammalian cells in culture has been reported (Miller et al., 1977). In addition, selective binding of an octathymidylate ethyl phosphotriester, $[Tp(Et)]_7T$, to poly(deoxyadenylic acid) has been extensively investigated (Pless & Ts'o, 1977).

In this and subsequent papers in this series (Kan et al., unpublished experiments; Cheng et al., unpublished experiments), we describe the synthesis, the interaction with com-

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[1] Abbreviations used: Np(Et)N, an oligonucleotide ethyl phosphotriester; dN*p*N, a deoxyribonucleotide dimer analogue containing a 3′–5′ internucleoside methylphosphonate linkage (in this abbreviation the italic p represents the methylphosphonate linkage); MST, mesitylenesulfonyl tetrazolide; TPSCl, 2,4,6-triisopropylbenzenesulfonyl chloride; DCC, dicyclohexylcarbodiimide. The symbols used to represent protected nucleosides and dideoxyribonucleoside methylphosphonates follow the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (1970).

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