Carcinogen-induced Alteration of DNA Structure*

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We have prepared covalent complexes between defined length DNA fragments and a diol epoxide derivative of the carcinogenic polycyclic aromatic hydrocarbon, benzo(a)pyrene. We have studied the structure of these complexes, using transient electric dichroism, circular dichroism, fluorescence quenching, thermal denaturation, polyacrylamide gel electrophoresis, and nuclease digestion.

Our observations suggest that the covalently bound carcinogen is intercalated within the helix, forming a wedge-shaped complex. Binding of the carcinogen distorts the structure of the DNA over a region extending beyond the immediate binding site. The most striking aspect of this distortion is that it produces a bend in the helix.

Benzo(a)pyrene is a polycyclic aromatic hydrocarbon which is a potent mutagen and carcinogen. Because of its widespread occurrence as an environmental pollutant, the chemistry, metabolism, and biological effects of BP have been studied extensively (1, 2). BP is chemically inert; however, during its metabolic processing by microsomal enzymes within the cell, it can be activated to electrophilic derivatives which bind covalently to cellular macromolecules (3–6). One such metabolite, the diol epoxide 7,8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE), shown in Fig. 1, is the “ultimate” carcinogenic form of BP (7–12). The structures of the DNA adducts formed when intact cells are exposed to BP are identical with those formed when purified DNA is exposed to BPDE. In these situations, more than 80% of the adducts result from the covalent modification of N-2 of guanine (13–19).

On the assumption that the carcinogenic effect of BP is due to its covalent binding to DNA, the structure of the carcinogen-DNA complex has been widely studied. Some investigations have produced apparently contradictory results. For example, Pulkrabek et al. (20) suggest that the binding of BPDE to DNA enhances the susceptibility of the helix to denaturation by heat or formaldehyde and produces an enhanced susceptibility of the modified DNA to digestion by S1 nuclease. In contrast, other workers find considerably less of an effect on the susceptibility of modified DNA to S1 nuclease (21). Some workers, who studied BPDE-modified calf thymus DNA using transient linear dichroism techniques and fluorescence quenching measurements, conclude that the carcinogen binds to the outside of the helix, lies in the minor groove, and produces only a minor distortion of B-DNA geometry (22, 23). However, other workers, using biochemical techniques, report that the binding of BPDE to closed circular DNA results in a substantial distortion and unwinding of the DNA helix (24, 25). In one case, these observations were interpreted to mean that the carcinogen intercalates into DNA, forming a stable double-stranded complex (24); in the second case, the results were thought to indicate that a local denaturation of the helix had occurred at the BPDE binding site (25).

We undertook the studies reported here in order to resolve the apparent contradictions in the previous reports and to determine in more detail the structure of the carcinogen-DNA complex. Our results indicate that the covalent modification of DNA by BPDE produces DNA bending and a distortion of the helix involving an extended region of DNA. We find no physical or biochemical evidence for a local denaturation of the helix. The bound carcinogen is quite inaccessible to solvent; therefore, we believe that it is intercalated within the helix, as suggested previously (24), but in an atypical configuration. Our experiments also provide a plausible explanation for many of the apparent contradictions found in earlier reports, particularly those in which the DNA was not well characterized; preferential modification of denatured DNA by BPDE can introduce substantial artifacts into studies of the covalent carcinogen-DNA complex.

MATERIALS AND METHODS

DNA Preparation—As we will show, the purity of the DNA samples is crucial to this work. Therefore, we have prepared fractionated, nearly monodisperse fragments of DNA in two separate ways.

DNA fragments were prepared by a combination of sonication and enzyme digestion as previously described (26). Briefly, calf thymus DNA (Sigma) was dissolved in 0.18 m NaCl, 7 mm Na2HPO4, 2 mm NaH2PO4, 3 mm Na2EDTA, and 5 mm cacodylate, pH 7.0 (Buffer A), and then sonicated under a nitrogen atmosphere for 30 min at 4 °C. Next, the DNA was digested for 4 h at 37 °C with 0.25 mg/ml of ribonuclease A (Sigma) and then dialyzed into 0.1 m NaOAc, 30 mm Tris-Cl, and 1 mm ZnSO4, pH 4.8, and digested overnight at 37 °C with 200 units/mg of S1 nuclease (Sigma). The reaction was stopped by adding EDTA to 10 mm. DNA was then digested with 0.5 mg/ml of bacterial proteinase (Sigma) for 4 h at 37 °C. The pH was adjusted to 7.0, the ionic strength was increased to 1 m with NaCl, and the DNA was extracted three times with ice-cold buffered phenol. Residual phenol was extracted with cold diethyl ether, and the aqueous phase was dialyzed against Buffer A. DNA was fractionated using a Sepharose 4B gel (Pharmacia). The size of the DNA fragments was determined by electrophoresis in 5% polyacrylamide gels, using Hae III restriction enzyme fragments of pX174 (New England Biolabs) as standards.

The length distributions of the DNA fragments prepared by this method were 400 ± 100 base pairs, 190 ± 20 base pairs, and 160 ± 15 base pairs.

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§ The abbreviations used are: BP, benzo(a)pyrene; BPDE, benzo(a)pyrene diol epoxide; BPT, benzo(a)pyrene tetrol; H, furan, tetrahydrofuran; bp, base pairs; BND cellulose, benzoylated, naphtholylated DEAE-cellulose.
base pairs. The length distributions were estimated from the width of the DNA bands on polyacrylamide gels stained with ethidium bromide and represent the distributions estimated to contain 75% of the DNA.

DNA fragments prepared by this procedure had an alkaline hyperchromicity at 260 nm of 1.32 ± 0.01 and a thermal hyperchromicity at 260 nm of 1.39 ± 0.02. Electrophoresis in polyacrylamide gels under both native and denaturing conditions revealed no evidence of single strand nicks or terminal single-stranded regions.

One hundred forty-five-base pair DNA fragments were isolated from nucleosome core particles which had been prepared from chicken erythrocyte chromatin, following a procedure described elsewhere (27). DNA was purified from core particles by digestion with bacterial proteinase, followed by phenol extraction and dialysis as described above.

Covalent Modification of DNA with BPDE—We have developed a method for condensing DNA with BPDE under relatively mild conditions. DNA fragments were dialyzed into 0.2 M NaCl, 30 mM Tris-Cl, and 3 mM Na₂EDTA, pH 7.8 (TE buffer). A concentrated aliquot of BPDE in tetrahydrofuran was then added directly to the DNA at 4°C in the dark. The DNA concentration was kept above 1 mM to maximize binding, and in all cases, the total concentration of tetrahydrofuran in the reaction mixture was kept below 10%.

After reaction for 2 h, the noncovalently bound benzo[a]pyrene tetraol (BPT) was removed in one of two ways. In one procedure, the reaction mixture was passed through a 20-cm column of Sepharose 6B (Pharmacia) at 4°C using TE as the eluting buffer. DNA eluted in the excluded volume and was well separated from BPT. After two consecutive fractionations, the component eluting in the excluded volume no longer contained ethyl-ether-extractable material which absorbed at 346 nm. The BPDE-DNA complex was then dialyzed for 24 h in the dark against TE, pH 7.0.

In the second method, after the reaction of DNA with BPDE, the ionic strength of the mixture was increased to 1 M with NaCl. The mixture was then ethanol precipitated three times at −20°C and dialyzed against TE, pH 7.0, for 48 h at 4°C in the dark. BPDE-DNA complexes prepared this way were free of ethyl-ether-extractable material absorbing at 346 nm.

Regardless of DNA length and the final extent of covalent modification, both extraction procedures produced complexes which had identical physical properties (fluorescence yield, gel mobility, and linear dichroism). After either procedure, we could not detect low molecular weight material on denaturing polyacrylamide gels, suggesting that neither procedure produces single-stranded nicks in the DNA.

The extent of covalent modification was determined optically, using for the carcinogen an extinction coefficient, εₚₐ₇₃ = 2.95 × 10⁴ M⁻¹ cm⁻¹ (16) and for DNA, εₚₐ₇₃ = 1.29 × 10⁵ M⁻¹ cm⁻¹ (measured in base pairs). Typically, the extent of condensation has been expressed as the ratio r = bound carcinogen/DNA base pairs.

Analytical S1 Nuclease Digestion—S1 nuclease (Sigma) was used to assay for possible single-stranded regions in the covalent complex between DNA and BPDE. DNA or BPDE-DNA complexes were dialyzed at 4°C in the dark against 0.2 M NaCl, 30 mM Tris-Cl, and 0.5 mM Na₂EDTA, pH 6.8. The DNA concentration was adjusted to 8.7 × 10⁻⁴ M, the concentration of Zn⁺⁺ was raised to 2.2 mM, 620 units/ml of S1 nuclease was added, and the digestion was allowed to proceed for 20 min at 25°C; the reaction was stopped by increasing the concentration of EDTA to 11 mM. The resulting digest was fractionated on Sepharose 6B at 4°C, eluting with TE, pH 7.0. Undigested DNA eluted in the excluded volume of the column, well separated from smaller digestion products. Material absorbing at 346 nm which eluted in the late fractions was not extractable with diethyl ether and, therefore, remained covalently bound to the digestion products.

The amounts of native and digested DNA were determined optically assuming for denatured DNA εₚₐ₂₆₀ = 1.8 × 10⁴ M⁻¹ cm⁻¹. The extinction coefficient of the carcinogen in the late eluting fractions was assumed to be identical with that measured for the limit digest produced by the combined action of DNase I and S1 nuclease; that value was measured to be εₚₐ₂₆₀ = 0.5 units/ml of S1. After X = 3 × 10⁻³ M, the activity of S1 nuclease toward single-stranded DNA was assayed by using DNA which had been heat denatured and cooled rapidly in an aceton-dry ice bath.

DNase I + S1 Nuclease Digestions—DNase I produces single strand nicks in DNA (28). S1 nuclease cleaves DNA opposite nicks and rapidly digests single-stranded DNA (29). When both S1 and S1 nuclease are present together (with S1 in excess), they first attack single-stranded regions and then digest the rest of the helix by making double-stranded cuts. At the site of a double-stranded cut, two base pairs unstack, resulting in an increase in absorbance at 260 nm.

Under the experimental conditions used here (where DNA is in great excess over covalently bound carcinogen), the rate at which the absorbance at 260 nm increases reflects the rate of digestion of unmodified DNA. As shown later, the fluorescence of covalently bound carcinogen increases by a factor of about 2.5 when the modified DNA is digested to small fragments. Therefore, the rate of increase of fluorescence reflects the rate of digestion of modified DNA.

Modified DNA was dialyzed into 0.2 M NaCl, 30 mM Tris-Cl, and 0.5 mM EDTA, pH 7.0. The DNA concentration was adjusted to 1.07 × 10⁻⁴ M and ZnSO₄ was added to 2.2 mM. DNase I and S1 nuclease were then added to a final concentration of 140 units/ml of DNase I and 600 units/ml of S1. The DNA absorbance at 260 nm was measured at 24°C on a Cary 219 spectrophotometer; the rate of change of fluorescence, with excitation at 346 nm and emission at 398 nm, was measured on a Perkin-Elmer 512 spectrophotometer, also at 24°C.

Circular Dichroism Measurements—Circular dichroism spectra were measured on a Jasco-J40 spectropolarimeter made available by the Stanford Chemistry Department. Molar ellipticities θ were calculated using an extinction coefficient at 260 nm of 1.29 × 10⁵ cm⁻¹ liter⁻¹ (mole base pairs). Spectra were measured at 24°C and were corrected by subtracting a buffer blank.

Fluorescence Measurements—Steady state fluorescence measurements were made on a Perkin-Elmer 512 spectrophotometer. For ethidium bromide or acrylamide tetrations, small aliquots of concentrated ethidium bromide or acrylamide solutions were added directly to the BPDE-modified DNA. The observed fluorescence changes were corrected for dilution. Light-scattering corrections were not necessary.

BPDE Reactivity Measurements—The preference of BPDE for binding to double-stranded or single-stranded DNA was measured by adding BPDE directly to a mixture of native and heat-denatured DNA, separating the complexes by affinity chromatography, and then measuring the amount of bound carcinogen in the DNA and alkaline-DNA forms. One-hundred ninety-base pair DNA in 0.2 M TE was heat denatured at 100°C and cooled in a dry ice bath. The ratios of its thermal and alkaline hyperchromicity before and after denaturation were 1.39/1.20 (thermal) and 1.32/1.16 (alkaline). We calculate from the thermal hyperchromicity that the fraction of single-stranded DNA in the denatured sample is 49%; the alkaline hyperchromicity yields a value of 50%. Such values are typical for heat denaturation under relatively high salt conditions. 0.1 ml of the heat denatured DNA was added to 0.1 ml of undenatured DNA [(native) = (denatured) = 1.8 × 10⁻⁴ M], and 10 μl of [1CPDE (1.15 × 10⁻³ M) in tetrahydrofuran was added and allowed to react for 2 h at 4°C in the dark. The reaction mixture was then applied directly to a 4-ml column of benzoylated, naphthoylated DEAE-cellulose (BND cellulose) (Serva, Heidelberg). The carcinogen-native DNA complex was separated from the carcinogen-denatured DNA complex as previously described (30). The column was first washed with 10 ml of column buffer (0.3 M NaCl, 20 mM Tris-Cl, and 0.10 mM Na₂EDTA, pH 7.4). The carcinogen-native DNA complex was eluted by increasing the NaCl concentration to 1 M. The carcinogen-DNA complex was then eluted by adding 1% caffeine to the high salt buffer. BPDE tetrations were removed from the eluted fractions by ethanol precipitation three times at −20°C, followed by dialysis against 0.2 M TE for 48 h in the dark.

Column fractions were then denatured by adding a 1/50th volume of a saturated NaOH solution. The DNA concentration in each fraction was determined from its absorbance at 290 nm. Each fraction was
then neutralized by addition of concentrated HCl, and the amount of bound carcinogen was measured by scintillation counting. The specific activity of the BFPE was 6.2 × 10^12 cpm/mol.

The reactivity of BPDE toward single-stranded versus double-stranded DNA was determined by comparing the ratio (bound BFPE/DNA bases) of the carcinogen-denatured DNA complex to that of the carcinogen-native DNA complex.

Analytical Polyacrylamide Gel Electrophoresis—The electrophoretic mobilities of native DNA-carcinogen complexes were measured on 5% polyacrylamide slab gels using a Tris/borate/EDTA buffer system. The mobilities of denatured complexes were measured by heat denaturing the modified DNA samples in 7 M urea and electrophoresing them in 8% polyacrylamide gels containing 7 M urea using TBE buffer. Gels were stained with ethidium bromide and illuminated with ultraviolet light. The mobilities of the samples were determined from photographs of the stained gels.

Electric Dichroism Measurements—Details of the electric dichroism technique and the device which we have used for measuring dichroism have been described at length elsewhere (31–33). Briefly, a linear electric field gradient between 5 and 35 kV/cm is applied across a solution of modified DNA complexes, causing the molecules to orient parallel to the fields. After orientation, the absorbance of plane-polarized light by these complexes is altered when compared to the absorbance before orientation. The preferential absorption of parallel polarized light which occurs in an electric field E is defined as the linear dichroism p(E) where

\[ p(E) = \frac{A_{E}}{A_{0}} \]  

\[ A_{0} \] is defined as the absorbance by the sample of light polarized parallel to the electric field; \( A_{E} \) refers to its absorbance in the absence of orientation.

Under the conditions which we have used, modified DNA complexes become highly oriented in the electric field, allowing an accurate extrapolation of measured values p(E) to the limiting dichroism at complete orientation p(0).

\[ p = 3/4 \left( \frac{A_{0} - A_{45}}{A_{45}} \right) \]  

where \( \gamma \) is the angle between a helical segment and the axis of orientation and \( A_{E} \) is the angle formed by the local helix axis and the optical transition moment responsible for absorption. For DNA bases, the optical transition moment is directed in the base plane and is roughly parallel to the short base pair axis (31). For pyrene-type chromophores such as BPDE, the structures of two DNA complexes are defined at 346 nm by the base axis of orientation and the average orientation of the helix with respect to the axis of orientation of the complex in the field, i.e.

\[ p = 3/2(\cos y - 1)(\cos^2 \frac{\gamma}{2} - 1) \]  

Native DNA fragments shorter than 200 base pairs behave as if they were rods (31, 35); therefore, the local helix axis and the axis of molecular orientation coincide. As a result, \( \gamma = 0 \) and

\[ p = 3/2(\cos y - 1) \]  

The DNA helix remains linear when ordinary intercalating compounds, such as ethidium bromide, bind to DNA (33). Consequently, the observed dichroism of DNA or of the bound intercalator does not change with increasing amounts of bound intercalator (33). In contrast, if the helix is bent as the result of binding of a ligand to DNA, then the observed dichroism of DNA will decrease continuously with increasing amounts of bound ligand. When bending occurs, equation 2 can be used to calculate an average angle of binding \( \gamma \) in the complex. For smooth superhelical distortion, \( \gamma \) is identical with the average squared cosine of the angle \( \gamma \) formed by helical segments in the modified complexes and the average axis of orientation (i.e., \( \gamma = 0 \) for unmodified DNA).

By comparing the limiting DNA dichroism at \( r = 0.04 \) to that of unmodified DNA (Fig. 2), we calculate, using equation 2, that \( \gamma = 29^\circ \), i.e., when, on the average, less than 7 molecules

| \( r_{DNA} \) | \( r_{BPDE} \) |
|----------------|----------------|
| 0.01 | 0.09 |
| 0.02 | 0.19 |
| 0.03 | 0.29 |
| 0.04 | 0.39 |

**RESULTS AND DISCUSSION**

**Electric Dichroism Measurements**

We prepared covalent complexes between BPDE and DNA and measured the electric field dependence of the dichroism \( p(E) \) of these complexes at both 265 nm (where DNA contributes ≥ 98% of the dichroism) and 346 nm (where the dichroism is due to the pyrene moiety of the bound carcinogen). The limiting dichroism \( p \) was extrapolated from \( p(E) \) as previously described (31).

Fig. 2 indicates the limiting DNA dichroism at 265 nm \( (p_{DNA}) \) and the limiting pyrene dichroism at 346 nm \( (p_{BPDE}) \) as a function of the degree of modification \( r \). The DNA dichroism of the complexes is negative, while the pyrene dichroism is positive at all \( r \) values, which indicates that the molecules of bound carcinogen do not lie in a plane which is parallel to the DNA bases. Furthermore, both \( p_{BPDE} \) and \( p_{DNA} \) decrease markedly as the amount of bound carcinogen increases. For example, \( p_{DNA} \) has dropped to two-thirds of the value for native DNA when only 1 BPDE molecule is bound/25 base pairs \( (r = 0.04) \); \( p_{BPDE} \) drops by one-half between \( r = 0.011 \) and \( r = 0.040 \). Other measurements (described below) show that the structure of the BPDE-DNA binding site does not change as more BPDE binds to the DNA. Therefore, the simultaneous decrease in \( p_{DNA} \) and \( p_{BPDE} \) which occurs with increasing \( r \) must reflect bending of the DNA helix.

BPDE presumably condenses at random with guanosine residues in DNA. Therefore, bends which occur as a result of binding will be distributed randomly along the helix. Because of this random binding, the calculation of the amount of bending per molecule of bound carcinogen is more complicated than if the helix were bent at regular intervals. We can, however, use equation 2 to calculate an average angle of bending which occurs when BPDE binds covalently to the DNA helix.

We assume that at the low levels of modification used in this study, the structure of the DNA in the complexes can be represented by segments of native (linear) helix separated by bends. Under these circumstances, bending is the only factor which can reduce the limiting DNA dichroism. Therefore, we calculate from equation 2 that

\[ \rho_{D} = \frac{\rho_{p}}{\rho_{0}} = \frac{\cos\gamma_{p}}{\cos\gamma_{0}} \]  

Where \( \rho_{p} \) refers to the limiting DNA dichroism at the degree of modification \( r \) and \( \rho_{0} \) to the limiting dichroism of unmodified DNA. (\( \cos\gamma_{p} \)) is the average squared cosine of the angle \( \gamma \) formed by helical segments in the modified complexes and the average axis of orientation (i.e., \( \gamma = 0 \) for unmodified DNA).

By comparing the limiting DNA dichroism at \( r = 0.04 \) to that of unmodified DNA (Fig. 2), we calculate, using equation 2, that \( \gamma = 29^\circ \), i.e., when, on the average, less than 7 molecules

**FIG. 2.** Electric dichroism of BPDE-modified DNA. One hundred sixty-base pair DNA was modified with BPDE to the extent indicated on the abscissa. Electric dichroism was measured at 5°C in 5 mM Tris·Cl and 0.5 mM Na₂EDTA, pH 7.0 and the limiting dichroism was determined by extrapolation, as described under "Materials and Methods." D, Dichroism at 265 nm; C, dichroism at 346 nm.
The limiting dichroism at 346 nm decreases more rapidly than does the limiting DNA dichroism with increasing degrees of modification (Fig. 2). We believe that at these low degrees of modification, this difference is due to the way in which dichroism signals are averaged. For example, when dichroism is measured at r = 0.011 (i.e. 1.7 molecules of carcinogen/160 base pairs), some DNA molecules are likely to be unmodified. Therefore, unmodified DNA will contribute to the average dichroism at 265 nm and will mask the changes produced as a result of DNA modification. In contrast, the average dichroism at 346 nm always reflects modified DNA complexes and is not affected by unmodified DNA even at low levels of DNA modification.

At the highest levels of modification (i.e. r = 0.04, reflecting 6.4 molecules of bound carcinogen/160 base pairs), we assume that nearly all DNA molecules have been modified and, as a result, \( r_{PBDPE} \) and \( r_{DNA} \) reflect identical populations of bent DNA complexes. We can then use the average bending angle \( \gamma \) calculated from the DNA dichroism to calculate the average angle \( \alpha \) formed by the carcinogen and the helix axis of the (linear) DNA segments adjacent to the binding site. Substituting into equation 2 \( \gamma = 29^\circ \) and the measured pyrene dichroism at \( r = 0.04 \) \( \rho_{PBDPE} = +0.6 \), we calculate that \( \alpha = 43^\circ \). Therefore, these calculations suggest that, on the average, the long axis of the bound carcinogen is tipped by 47° from being perpendicular to the DNA axis of unmodified DNA regions. However, since the carcinogen binding site is itself a bent region, the orientation of the carcinogen with respect to DNA at the binding site cannot be determined from the dichroism data alone.

### Circular Dichroism Measurements

We measured the circular dichroism (CD) of carcinogen-DNA complexes in order to further characterize the structural changes which occur as a result of the modification of the nucleic acid by BPDE. Fig. 3 shows the CD spectra of the complexes as a function of increasing degrees of modification. The molar ellipticity at 275 nm increases and the peak ellipticity is blue shifted even at the lowest degree of modification. The molar ellipticity at 245 nm decreases and the ellipticity minimum is also blue shifted as a result of BPDE binding; thus, the symmetry of the CD spectrum of DNA is altered. It is important to note that under these conditions, the absorbance of the carcinogen at 260 nm is less than 8% of DNA and that its molar ellipticity at 346 nm is too small to be measured (Fig. 3).

Fig. 4 shows that the changes in molar ellipticity at 245 nm and 275 nm are linear with respect to increasing degrees of modification and that the changes are very large (\( \theta_{25} \) increases by 13% when only 1 molecule of carcinogen is bound, 28 base pairs). Changes of that magnitude cannot be due to contributions from “hidden” BPDE bands (the molar ellipticity at 275 nm of carcinogen in the complex would need to be 3.6 \( \times 10^5 \), even though the molar ellipticity of the tetraol at 275 nm is negligible, data not shown). Thus, these CD changes must reflect structural changes in the DNA produced by the covalent binding of BPDE.

These CD changes do not indicate what specific structural alteration has occurred. They do, however, place limits on the change which could occur at the carcinogen binding site. 1) Peak DNA ellipticity in the modified complex is blue shifted and is increased with respect to that of unmodified DNA. Such changes are not consistent with the idea that the carcinogen binding site is a locally denatured region; denatured DNA has an ellipticity which is lower than that of native DNA and has a red-shifted spectrum (36, 37). 2) Ellipticity at 275 nm increases by approximately 25,000 units/mole of modified base pairs; a change of this magnitude suggests that the region of altered DNA extends beyond the immediate site of covalent modification.

The altered DNA structures which are consistent with these CD changes involve a tipping of several DNA base planes with respect to the helix axis. The effects of tipping have been predicted from CD theory (38) and have been observed experimentally in at least one instance (29). The steroid irrehi-

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**Fig. 3.** Circular dichroism of BPDE-modified DNA. Four hundred-base pair DNA was modified with BPDE to varying extents and the circular dichroism was measured at 24 °C in 10 mM Tris buffer. DNA concentration was 1.2 \( \times 10^{-4} \) M, measured in base pairs. ---. Unmodified DNA, \( r = 0 \); –. – , modified DNA, \( r = 0.018 \); . – . , modified DNA, \( r = 0.036 \); –. – , modified DNA, \( r = 0.037 \).
amine A binds to DNA by wedging itself into the helix, producing a bent DNA region (40, 41). As a result of binding, irethidiamine produces both a blue-shifted CD spectrum and an increased peak ellipticity, changes which are qualitatively similar to those we have observed here (39). The irethidiamine-induced CD changes are, however, quantitatively smaller per bound molecule than are the BPDE-induced effects, perhaps because irethidiamine binding alters only a two base pair domain (40, 41).

**Electrophoretic Mobilities**

BPDE is uncharged and binds to uncharged sites on the helix (primarily N-2 of guanosine and N-6 of adenine). If, at low degrees of modification, the carcinogen-DNA complexes remain unbent, then their electrophoretic mobilities should remain nearly unchanged when compared to the mobility of unmodified DNA. In contrast, if modification produces bending of the helix, this should have a substantial effect on the electrophoretic mobilities of the complexes. In general, the electrophoretic mobility (\(U\)) of a DNA complex can be expressed as a product of ionic and hydrodynamic terms

\[
U = \frac{ZeX}{f}
\]

where \(Ze\) is the net charge on the complex and \(X\) is a complicated function of ionic strength, related to a modification of the applied electrical field due to condensed counterion. \(f\) is the translational frictional coefficient which is a function of particle shape, solvent viscosity, and temperature. The term \(X\) is difficult to calculate unambiguously. Therefore, electrophoresis is not, in general, a useful method for measuring the hydrodynamic properties of a macromolecular complex. The BPDE-modified DNA complex is a possible exception to that generalization. Using the optical methods described above, we have measured substantial changes in the structure of modified complexes under conditions where only a few molecules of carcinogen are bound per 100 base pairs of DNA and where the net charge on the complex is (presumably) constant. In the experiments which we describe now, we make the assumption that, at these low degrees of modification, the counterion atmosphere at the surface of the helix is not measurably changed as a result of BPDE binding. In that instance, the electrophoretic mobilities of the carcinogen-DNA complexes relative to that of unmodified DNA depend only on changes in their shape, i.e.

\[
\frac{U_r}{U_o} = \left(\frac{f_r}{f_o}\right)^{-1}
\]

where \(U_r\) and \(f_r\) are the mobility and frictional coefficient, respectively, of modified complexes at a particular \(r\) value, and \(U_o\) and \(f_o\) are the values for unmodified DNA.

Fig. 5 shows the electrophoretic mobilities of several different modified DNA complexes as a function of the degree of modification. Mobilities reflect the numerical average of three independent experiments. The mobilities of the undenatured (double-stranded) complexes decrease linearly with increasing degrees of modification; in contrast, the mobilities of the urea-denatured (single-stranded) complexes do not change. The unaltered mobilities of the denatured complexes are important controls because they indicate that carcinogen-acrylamide interactions do not contribute to the mobilities. They also show that, at least for single-stranded DNA, covalently bound carcinogen has no measurable effect on the ion atmosphere term \(X\).

To the extent that our assumptions are correct, the ratios \(U_r/U_o\) are direct measures of changes in the frictional coefficients of these complexes. As has been shown (40), moderate bending of a linear DNA helix can increase its effective hydrodynamic volume significantly, thereby increasing \(f\) (a few small angle bends in a long rod increase its effective radius enormously, but do not shorten it very much). Thus, the increase in frictional coefficient which we calculate from gel mobilities is consistent with the idea that the modified complex is bent.

**Ethidium Bromide Fluorescence Measurements**

When ethidium bromide intercalates into B-form DNA, its fluorescence increases more than 20-fold. However, when bound to the outside of the helix or to single-stranded DNA, its fluorescence remains relatively weak (42). We have used this selective fluorescence enhancement to study the altered structure of BPDE-modified DNA. Fig. 6 shows the titration with ethidium bromide of modified \((r = 0.054)\) and unmodified DNA, performed under identical buffer conditions with the DNA concentration held constant. The results indicate that the maximum ethidium bromide fluorescence attainable with the modified complex is substantially lower than that attainable with unmodified DNA. The absorption and emission maxima of intercalated ethidium bromide differ by 200 nm from those of pyrene. Therefore, energy transfer or other nonradiative interactions between bound carcinogen and ethidium bromide cannot be responsible for this fluorescence decrease.

If we define \(F_r\) as the maximum ethidium bromide fluorescence in a modified complex and \(F_o\) as the maximum fluorescence in unmodified DNA, then \(1 - (F_r/F_o)\) represents the fraction of the total potential ethidium bromide fluorescence which is missing from the modified complex. We have measured \(1 - (F_r/F_o)\) as a function of the degree of modification of DNA. The results (Fig. 7) show that the amount of ethidium bromide fluorescence which is missing is directly proportional to the amount of carcinogen bound to the helix. The slope of this line can be used to estimate the minimum number of DNA base pairs which are influenced as a result of covalent modification. For simplicity, we assume that in a region around the carcinogen binding site, ethidium bromide cannot bind to DNA to yield a fluorescent complex and that beyond this region, ethidium bromide binding and fluorescence are identical with that which occurs with unmodified DNA. With these assumptions, the slope of the line in Fig. 7 is a measure of the length of the nonfluorescing region. The calculated
There have been enhanced susceptibility to digestion by dependent changes in volume. At a DNA concentration DNA at the carcinogen binding site is, in effect, single stranded sense, the secondary structure of the BPDE-modified site is melting. Therefore, we conclude that in a thermodynamic sigmoidal shape of both curves reveals no evidence of pre-
tuations on the abscissa. The midpoints of the transitions indicate that the melting temperature of both modified and unmodified regions is 58.5 °C. Measurements are expressed as the extent of reaction, θ, and have been corrected for temperature-dependent changes in volume. Absorbance at 260 nm; ○, fluorescence (λex = 343 nm, λem = 398 nm).

**Thermal Denaturation Studies**

The work of others apparently indicates that the binding of BPDE to DNA destabilizes the helix and reduces the thermal hyperchromicity of DNA in the modified complex. Therefore, others have suggested that the DNA region around the carcinogen binding site is denatured (20). We decided to examine this possibility in more detail since the CD spectra and electrophoretic mobilities of the modified complexes were not consistent with the idea that the complexes contained regions of denatured DNA. Our preliminary melting experiments revealed that the thermal denaturation of BPDE-modified DNA was accompanied by a 4-fold increase in pyrene fluorescence. Therefore, we could use this fluorescence change to measure the helix-coil transition around the carcinogen binding site. Furthermore, at low degrees of modification, the absorbance at 260 nm of the carcinogen is negligible compared to that of DNA. Therefore, to a good approximation, by monitoring the changes in absorbance at 260 nm, we measure the helix-coil transition of unmodified DNA regions.

Fig. 8 shows the melting of a modified complex, monitoring both pyrene fluorescence changes (λex = 346, λem = 398) and DNA absorbance changes. To allow a direct comparison, we have expressed both the absorbance and fluorescence changes as an extent of reaction θ versus temperature (38). The results indicate that the melting transition measured by fluorescence is identical with that measured by absorbance. The midpoints of the transitions indicate that the melting temperature of both modified and unmodified regions is 58.5 ± 1 °C. The sigmoidal shape of both curves reveals no evidence of pre-

**Enzyme Digestion Studies**

Others have reported that BPDE-modified DNA complexes have an enhanced susceptibility to digestion by S1 nuclease. Such observations have been interpreted to mean that the DNA at the carcinogen binding site is, in effect, single stranded

![Graph](https://via.placeholder.com/150)

**Fig. 6 (left). Ethidium bromide fluorescence of BPDE-modified DNA.** Four hundred-base pair DNA was modified with BPDE to an extent ρ = 0.054. Modified and unmodified samples were titrated with ethidium bromide to the extent indicated on the abscissa, and the fluorescence of the ethidium bromide-DNA complexes was measured at 24 °C in TE buffer (λex = 520 nm, λem = 600 nm). The DNA concentration was 8.2 × 10⁻⁸ M. O, Modified DNA; ○, unmodified DNA.

**Fig. 7 (center). Limiting ethidium bromide fluorescence as a function of extent of DNA modification by BPDE.** Four-hundred-bp DNA was modified with BPDE to the extents indicated on the abscissa, and the limiting ethidium bromide fluorescence of these complexes was determined as described in the legend to Fig. 6.

**Fig. 8 (right). Thermal denaturation of BPDE-modified DNA.** One hundred ninety-base pair DNA was modified with BPDE to an extent ρ = 0.011. Samples were degassed and sealed prior to melting. Thermal denaturation was in 10 mM Tris-Cl and 1 mM Na₂EDTA, pH 7.0, at a DNA concentration of 6.2 × 10⁻⁸ M. Measurements are expressed as the extent of reaction, θ, and have been corrected for temperature-dependent changes in volume. O, Absorbance at 260 nm; ○, fluorescence (λex = 343 nm, λem = 398 nm).

**Fig. 9. Digestion of BPDE-modified DNA with S1 nuclease.** Samples were digested with S1 nuclease and fractionated using Sepharose 6B as described under “Materials and Methods.” A, Heat-denatured DNA; B, native DNA; C, BPDE-modified DNA (ρ = 0.011); ●, absorbance at 260 nm; ○, absorbance at 343 nm.

(20). Again, since using other techniques we had been unable to detect any evidence for denatured DNA in the modified complexes, we studied the susceptibility of BPDE-modified DNA to digestion with S1 nuclease. To minimize potential artifacts, we performed the digestions under mild, relatively physiological conditions and fractionated the digest by gel filtration rather than by precipitation.

Fig. 9 shows the Sepharose 6B elution profiles of modified complexes which have been digested under identical conditions. The top panel indicates that unmodified DNA which has been heat denatured is almost completely digested, yielding small polynucleotides which elute at 16 to 18 ml. The
middle panel shows that approximately 5% of unmodified, native DNA is digested under the same conditions. Presumably, this reflects digestion of the ends of the DNA fragments. The bottom panel shows the elution profile of an S1 nuclease digest of BPDE-modified DNA. The profile of absorbance at 260 nm is essentially identical with that of unmodified DNA. More important, the profile of absorbance at 340 nm reveals no evidence for either selective depletion of carcinogen from the undigested material or enrichment of carcinogen in the digested fractions. When corrected for DNA hyperchromicity, the r value in both fractions remains 0.011. These results indicate that when digestion is performed with well characterized complexes under mild conditions, the carcinogen binding site is not recognized by S1 nuclease as being single stranded.

We confirmed this observation by measuring the rate at which a mixture of DNase I and S1 nuclease digested the carcinogen-DNA complex. Our preliminary digestion experiments revealed that the complete digestion of modified complexes was accompanied by a 2.4-fold increase in pyrene fluorescence. Therefore, by analogy to our thermal denaturation experiments, we used the rate at which this fluorescence change occurred to measure the rate at which the DNA at the carcinogen binding site was digested. Again, we used the absorbance change at 260 nm to measure the rate at which unmodified DNA was digested. Fig. 10 shows the time course of a DNase I + S1 nuclease digestion of BPDE-modified DNA. The results indicate that pyrene fluorescence increases at the same rate as does the absorbance at 260 nm. Thus, these observations again argue against the idea that the binding of BPDE to DNA produces a local denaturation of the helix.

Fluorescence Quenching Studies

Acrylamide—Other workers have proposed that after binding of BPDE to DNA, the carcinogen remains near the surface of the helix, lying in the small groove (22, 23). This conclusion is based upon studies which apparently indicate that the fluorescence of covalently bound carcinogen can be quenched by interaction with small molecules or with other molecules of unmodified DNA (23). However, our melting experiments (Fig. 8) and nuclease digestion studies (Fig. 10) suggested to us that the bound carcinogen might actually be buried within the helix. Therefore, we have performed experiments designed to measure the accessibility of the bound carcinogen to solvent.

Acrylamide is a compound whose fluorophores produce quenching by a simple collisional process (43). For collisional quenching, the inverse of the fluorescence intensity decrease (F/F₀)⁻¹, which occurs in the presence of the quencher, is proportional to the concentration of the quencher. Therefore, when acrylamide is used as the quencher,

\[ F₀/F = 1 + K_q[Ac] \]

(7)

where \( K_q \) is defined an acrylamide quenching constant and is equal to \( N \tau_0 / \tau_\alpha \) where \( \tau_0 \) is the lifetime of the fluorescing species in the absence of quenching, \( k \) is the translational diffusion constant of the quencher, and \( \lambda \) is an accessibility parameter which is related to the surface area of the fluorophore accessible to solvent (44). \( \lambda \) depends only on the solvent viscosity and the properties of the quencher. Therefore, the relative accessibility of two fluorophores, \( a \) and \( b \), under identical solution conditions, can be determined from the ratio of their quenching constants. That is,

\[ K^a_q/K^b_q = (\lambda^a \tau_\alpha^a) / (\lambda^b \tau_\alpha^b) \]

(8)

Fig. 10. Nuclease digestion of BPDE-modified DNA. DNA was modified with BPDE to an extent \( r = 0.011 \) and was digested with DNase I and S1 nuclease as described under "Materials and Methods." At the indicated times, absorbance and fluorescence measurements were made and are expressed as a fraction of the starting values. ○, Absorbance at 260 nm; ●, fluorescence (\( \lambda_m = 343 \) nm, \( \lambda_{em} = 358 \) nm).

Fig. 11 shows the quenching effect of acrylamide on the fluorescence of several different carcinogen-DNA complexes. The bottom curve shows the quenching of undenatured, undigested complexes (\( r = 0.011 \)). The initial slope of the curve is low, and the curve plateaus at a value near 2. Such curvature suggests that there may be at least two classes of covalently bound carcinogen molecules; the initial slope specifies a quenching constant, \( K_q = 3.8 \pm 0.4 \), and the final slope specifies a quenching constant, \( K_q < 1 \). Neither class is sensitive to digestion by S1 nuclease. Additional experiments (not shown) indicate that these constants do not vary either with the length of the DNA or with the degree of modification of the nucleic acid.

Fig. 11 also shows the quenching by acrylamide of heat-denatured and nuclease-digested carcinogen-DNA complexes. The initial slopes of the curves are much higher than that of the undenatured, undigested complex. At high acrylamide concentrations, both curves plateau, again suggesting the presence of two classes of bound carcinogen. For the heat-denatured complexes, the initial slope specifies a constant, \( K_q \gtrsim 30 \); for the digested complexes, \( K_q \gtrsim 70 \). Together, these fluorescence data indicate that BPDE binds to native DNA to form a complex which is very different from the complexes which it forms with either denatured or digested DNA.

The accessibility (\( \lambda \)) of bound carcinogen to acrylamide can be calculated unambiguously only if the fluorescence lifetimes of the complexes are known. However, since the increase in fluorescence lifetime which occurs upon denaturation or digestion will, in general, be less than or equal to the increase in steady state fluorescence, we can use our steady state quenching measurements to place a lower limit on the relative accessibility of the carcinogen in the denatured or digested complex.

We know that independent of DNA length or degree of modification, the thermal denaturation of BPDE-modified DNA leads to an increase in fluorescence intensity of 3.8 ± 0.2 (Fig. 8); nuclease digestion of the complexes leads to an increase of 2.4 ± 0.2 (Fig. 10). Here, we assume that these changes reflect increases in fluorescence lifetimes; using this assumption, we calculate from equation 8 that the carcinogen in the nuclease-digested complex is at least 2-fold more accessible to acrylamide than it is in the heat-denatured complex and at least 7-fold more accessible than in the undenatured, undigested complex.
Carcinogen-induced Alteration of DNA Structure

Fig. 11. Fluorescence quenching by acrylamide. Measurements were made at 24 °C in TE buffer at the indicated concentrations of acrylamide (λex = 343 nm, λem = 398 nm). •, BPDE-modified DNA, r = 0.011; ◦, BPDE-modified DNA, r = 0.011; ◇, BPDE-modified DNA, r = 0.013, heat denatured; □, BPDE-modified DNA, r = 0.011, digested with DNase 1 and S1 nuclease as described in the legend to Fig. 10; ▽, BP tetraol.

Fluorescence Quenching Studies

DNA—Others have reported that the fluorescence of BPDE-modified DNA is susceptible to quenching by other molecules of unmodified DNA. These findings were interpreted to mean that covalently bound carcinogen is positioned on the surface of the helix (23). Fig. 12 shows the fluorescence quenching which occurs when DNA is added to either undenatured or heat-denatured carcinogen-DNA complexes. The results indicate that the undenatured complexes show no measurable change in fluorescence even when unmodified DNA is added in great excess. In contrast, the fluorescence of heat-denatured complexes is strongly quenched by added DNA. Again, these results suggest that in the undenatured complexes, the bound carcinogen is located within the helix, rather than at its surface.

BPDE Binding to Native and Denatured DNA

Our observations on the fluorescence quenching of modified DNA complexes suggested to us that, in some instances, previous studies may have been complicated by the presence of denatured material in the starting DNA. Therefore, we measured the relative affinity of BPDE for both native and denatured DNA. We prepared a mixture containing both native DNA and an equal amount of heat-denatured DNA which was approximately 50% single stranded. We reacted the mixture with [14C]BPDE and separated native from denatured DNA using BND cellulose (30). The specific activity of each fraction was calculated from the ratio of radioactivity to absorbance at 260 nm. Fig. 13 shows that about 2.2-fold more BPDE reacted with the denatured DNA than with the native DNA. Since only 50% of the denatured sample was single stranded at the time of modification, the preference factor of BPDE for denatured over native DNA is about 3.6; that is when BPDE is confronted with equal concentrations of native and denatured DNA, 3.6 times more carcinogen will react with the denatured material.

This preference is an important consideration in the interpretation of BPDE-DNA binding experiments. For example, if BPDE were allowed to modify a DNA sample containing 10% denatured material as a contaminant, about 29% of the carcinogen in the resulting complex will be bound to denatured regions (at low levels of modification); 29% of the bound carcinogen would be rapidly digested by S1 nuclease and would contribute at least 50% of the total fluorescence, since the fluorescence yield of denatured complexes is increased at least 2.4-fold (Fig. 8). As we have shown, such fluorescence would be very sensitive to quenching by both acrylamide and added DNA. Therefore, we conclude that contamination of DNA samples by relatively small amounts of denatured material can alter greatly the average properties of BPDE-modified DNA.

A Possible Model for the Structure of the BPDE Binding Site

The results of our physical and biochemical experiments indicate that the covalent binding of BPDE to DNA alters the structure of the nucleic acid in such a way that the helix behaves as if it were bent. The altered structure is similar to native DNA with respect to its thermal stability and suscep-
Carcinogen-induced Alteration of DNA Structure

This information is not sufficient to describe in detail the structure of the carcinogen binding site. However, when our findings are combined with the stereochemical observations made by others, they do suggest a range of plausible structures. The primary adduct (80% or more of total adducts) formed when racemic anti-BPDE modifies DNA results from the modification of the N-2 position of guanine by (+) anti-BPDE (13-19). Therefore, we have considered here only this preferred complex.

There are only two ways that (+) anti-BPDE can bind to the N-2 of guanine to produce a complex which remains double stranded and which at the same time protects the bound carcinogen from interactions with the solvent; both are intercalation type complexes of the sort proposed by Drinkwater et al. (24). If BPDE binds on the 3' side of guanine, the pyrene moiety is positioned over cytosine with the three hydroxyl groups on the carcinogen facing outside into the small groove (Fig. 14, A and B). If BPDE binds on the 5' side of guanine, the pyrene moiety is positioned more nearly over guanine, with the triol moiety facing into the side of the small groove and perhaps making contact with the sugar-phosphate backbone (Fig. 14, C and D). Both the 3' and 5' geometrics produce a wedge-shaped complex; the plane of the carcinogen is inclined to an angle of 25° to 35° with respect to the plane of the purine ring in the 3' complex or at about 50° with respect to the purine ring in the 5' complex. The 3' complex appears to be a less distorted structure. Therefore, we consider the 3' overlap model (Fig. 14, A and B) between BPDE and guanosine to be the more likely representation of the local structure of the carcinogen-DNA binding site.

The structure has several interesting features. DNA base pairing can be maintained within the two base pairs immediately adjacent to the bound carcinogen (with the exception of the single hydrogen bond lost as a result of modification at N-2). The pyrene moiety is in relatively close contact with cytosine at the site of modification. With minor alterations of sugar pucker and phosphate backbone geometry (similar to those which have been proposed for more ordinary intercalated complexes), the base pair forming the 3' half of the complex can stack parallel to the pyrene moiety (not shown). Such a structure is, therefore, consistent with the thermal stability and double-stranded character of the complex and also explains the inaccessibility of bound carcinogen to interaction with acrylamide or added DNA. The 3' complex is wedge-shaped. Even if the unmodified DNA regions remain linear at either side of the binding site, the wedge shape of the complex would bend the helix, as suggested by the electric dichroism measurements and gel mobility changes.

In this simple model, we have considered only the two base pairs adjacent to the bound carcinogen. However, our CD and ethidium bromide fluorescence measurements suggest that DNA structure is altered over a region which may be several base pairs long. The 3' complex which we have described is a distorted DNA region which must interface with regions of unmodified DNA on either side of the complex. The magnitudes of the CD and ethidium fluorescence changes suggest that this interfacial region extends over several DNA base pairs.

Drinkwater et al. (24) reported that the covalent binding of BPDE to SV40 DNA alters the supercoil density of the nucleic acid in a fashion similar to that of ethidium bromide. Therefore, they proposed that the covalently bound carcinogen is intercalated into DNA. The general features of that model are

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Fig. 14. Molecular models of BPDE-DNA complexes. For description, see text. A, 3' complex, side view; B, 3' complex, top view; C, 5' complex, side view; D, 5' complex, top view.
similar to the 3' complex we have proposed here; the major difference is that our observations suggest that the helical unwinding which occurs as a result of BPDE binding may be due to both bending and untwisting of the helix over a domain substantially larger than the classical two base pair intercalation binding site.

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