Differences in unwinding of supercoiled DNA induced by the two enantiomers of *anti*-benzo[a]pyrene diol epoxide

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ABSTRACT

The unwinding of supercoiled ϕ X174 RFI DNA induced by the tumorigenic (+) and non-tumorigenic (–) enantiomers of *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) has been investigated by agarose slab-gel and ethidium titration tube gel electrophoresis. The differences in adduct conformations were verified by flow linear dichroism techniques. Both enantiomers cause a reversible unwinding by the formation of noncovalent intercalative complexes. The effects of covalently bound BPDE residues on the electrophoretic mobilities of the RF I DNA form in agarose gels were investigated in detail in the range of binding ratios $r_b \approx 0.0 - 0.06$ (covalently bound BPDE residues/nucleotide). In this range of r_b values, there is a striking difference in the mobilities of (+)-BPDE- and (–)-BPDE-adducted ϕ X174 DNA in agarose slab-gels, the covalently bound (+)-BPDE residues causing a significantly greater retardation than (–)-BPDE residues. Increasing the level of covalent adducts beyond $r_b \approx 0.06$ in the case of the (+)-BPDE enantiomer, leads to further unwinding and a minimum in the mobilities (corresponding to co-migration of the nicked form and the covalently closed relaxed modified form) at $r_b 0.10 \pm 0.01$; at still higher r_b values, rewinding of the modified DNA in the opposite sense is observed. From the minimum in the mobility, a mean unwinding angle (per BPDE residue) of $\theta = 12 \pm 1.5^\circ$ is determined, which is in good agreement the value of $\theta = 11 \pm 1.8^\circ$ obtained by the tube gel titration method. Using this latter method, values of $\theta = 6.8 \pm 1.7^\circ$ for (–)-BPDE- ϕ X174 adducts are observed. It is concluded that agarose slab gel techniques are not suitable for determining unwinding angles for (–)-BPDE-modified ϕ X174 DNA because the alterations in the tertiary structures for $r_b < 0.06$ are too small to cause sufficiently large changes in the electrophoretic mobilities. The major *trans* (+)-BPDE-

N²-guanosine covalent adduct is situated at external binding sites and the mechanisms of unwinding are therefore different from those relevant to noncovalent intercalative BPDE-DNA complexes or to classical intercalating drug molecules; a flexible hinge joint and a widening of the minor groove at the site of the lesion may account for the observed unwinding effects. The more heterogeneous (–)-BPDE-nucleoside adducts (involving *cis* and *trans* N²-guanosine, and adenosine adducts) are less effective in causing unwinding of supercoiled DNA for reasons which remain to be elucidated.

INTRODUCTION

Benzo[a]pyrene and related polycyclic aromatic hydrocarbons (PAH) are metabolized *in vivo* to potent mutagenic and tumorigenic diol epoxide derivatives [1]. These hydrophobic, electrophilic, and highly reactive compounds can bind both noncovalently and covalently to DNA in aqueous solutions. The formation of non-covalent complexes is important in determining the chemical reaction kinetics of these diol epoxide derivatives with nucleic acids [2], while the covalent binding to DNA is a critical step in the expression of their mutagenic and tumorigenic potentials [3]. The ultimate biologically active metabolite of benzo[a]pyrene, is the bay region 7,8-dihydroxy-9,10-epoxy-derivative. Among the four different stereoisomers of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, the (+)-enantiomer of the diastereomer *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene ((+)-BPDE, known also as (+)-*anti*-BPDE, or (+)-BPDE 2) is the most active tumorigen and displays the highest mutagenic activity in mammalian cells [4–8]. In contrast, the (–)-enantiomer, (–)-BPDE (or (–)-*anti*-BPDE, or (–)-BPDE 2) is nontumorigenic [6,7], and its mutagenicity is quite different from that of the (+)-enantiomer [4,5,8]. Differences in covalent adduct conformations and extent of alteration of the local DNA structure,

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are believed to be two of the critical factors which distinguish the biological activities of (+)-BPDE and (–)-BPDE [2,4,9,10]. The two enantiomers of BPDE are characterized by differences in their chemical binding patterns with DNA [4, 11–13], and the conformations of the chemical DNA adducts which are formed [reviewed in 2,9,10]. Most recently, the solution conformations of the most abundant, stereochemically defined *trans*-adducts derived from the binding of (+)- and (–)-BPDE to the exocyclic amino group of guanosyl moieties in oligonucleotides has been determined by high resolution NMR methods; the bulky pyrenyl residues derived from these two stereoisomers are positioned in the minor groove of DNA, and are oriented in nearly opposite directions with respect to the 5'–3' strand polarity; all hydrogen bonds are intact at 5°C and the B-DNA structure is maintained near and at the lesion site [14,15].

Differences in changes in the tertiary structures of DNA induced by the two enantiomers of BPDE, for example bends or flexible hinge joints at the site of the lesion, and unwinding of supercoils in closed circular DNA, could also play a role in defining the differences in the biological effects induced by these two isomers. The unwinding of supercoiled DNA by racemic BPDE ((±)-BPDE) via noncovalent complex [16,17] and covalent adduct [18–21] formation, has been investigated previously. In this work, the effects of the resolved enantiomers (+)-BPDE and (–)-BPDE are described for the first time, and unwinding angles θ are estimated by different methods.

MATERIALS AND METHODS

Preparation of BPDE-DNA adducts

The (+)-BPDE and (–)-BPDE enantiomers were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. Supercoiled ϕ X174 RFI DNA was purchased from Bethesda Research Laboratories (Gaithersburg, MD). The covalent binding reactions of the BPDE enantiomers with DNA were carried out in 5 mM Tris (Tris(hydroxymethyl)aminomethane) buffer containing 1 mM sodium ethylenediaminetetraacetate (EDTA) at pH 7.9 (TE buffer). Varying amounts of a concentrated BPDE-tetrahydrofuran solution (2–4 mM BPDE) were added to 500 μ l aliquots of buffer solutions containing 12 μ g of supercoiled DNA (7.5×10^{-5} M in concentration of nucleotides) to initiate the chemical binding reaction; the concentration of tetrahydrofuran did not exceed 3% by volume in any of the experiments. The samples were placed on a shaker for four hours at room temperature. Subsequently, the reaction mixtures were exhaustively dialyzed against TE buffer to remove the 7,8,9,10-tetrahydroxytetrahydro-benzo[a]pyrene (BPT) hydrolysis products.

Determination of extent of modification and product distribution by HPLC analysis

Spectroscopic methods based on the UV absorbance of the adducts in the 346–354 nm region were employed in most cases to estimate the level of covalent binding [22,23]; the accuracy of this rapid and convenient method was verified by enzymatically degrading the covalent BPDE-DNA adducts to nucleosides and quantitatively determining the fraction of unmodified and BPDE-modified nucleosides by reverse phase high performance liquid chromatography (HPLC) by established methods [13] and used routinely in our laboratory [24,25]. Briefly, the BPDE-supercoiled DNA adducts were first digested with DNase I in

40 mM Tris–HCl, 10 mM $MgCl_2$, and 12 mM $CaCl_2$ (pH = 7.8) buffer solution at 37°C. The adducts were further digested with snake venom phosphodiesterase I and alkaline phosphatase (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) in 100 mM Tris–HCl, 100 mM NaCl and 15 mM $MgCl_2$ solution for 24 hours at 37°C. The HPLC elution times of the different BPDE-DNA digests were compared with those of BPDE-N²-dG and BPDE-N⁶-dA standards using reverse phase HPLC methods as described elsewhere [24,25]. Using molar extinction coefficients of 29,000 $M^{-1} cm^{-1}$ at the 346 maximum [22] for (+)-BPDE-DNA adducts, and 15,000 M^{-1} at 352–354 nm for (–)-BPDE-DNA adducts (the absorption maxima are broader and less well defined [23,26]), agreement between the HPLC and spectrophotometric methods was within $\pm 20\%$. The lower extinction coefficients for adducts with absorption maxima at 352–354 nm is in agreement with recent results obtained with site-specific and stereospecific BPDE-oligonucleotide adducts [25,27].

Gel electrophoresis

The electrophoretic mobilities of unmodified and BPDE-modified supercoiled DNA were compared using 1% agarose wedge-shaped slab-gels in 89 mM Tris base, 89 mM sodium borate, and 2 mM EDTA solution (TBE buffer). The final dimensions of the gels were 15×20 cm with the thick and thin ends about 11–15 and 3–5 mm thick, respectively. Before the samples were added to the gels, a loading buffer (1 mM EDTA, 2.5% Ficoll, and 0.005% bromophenol blue) was added. Electrophoresis was performed (thick → thin direction) using an LKB 2012 Maxiphor Submarine electrophoresis unit (Pharmacia/LKB, Piscataway, NJ) connected to a Buchler Model No. 3-1500 power supply (Haakebuchler, Saddle River, NJ) at 40–45 V and 24 mA for 22 hours. The gels were then stained with ethidium bromide (0.75 μ g/ml) for 30 min, and destained for 30 min with water, photographed under UV illumination, and the film negatives were then subjected to densitometry analysis. The densitometer was a Fisher Scientific (Springfield, NJ) Model EC910, manufactured by E-C Apparatus Corporation (St. Petersburg, FL), and was coupled to a computer system. In the range of DNA concentrations utilized, it was verified that the areas under the densitometer peaks were proportional to the amount of DNA added to the gels (40 ng DNA in 10 μ l, per well).

In order to relate the decreases in mobilities of the partially relaxed supercoiled DNA bands to the number of superhelical turns removed by the covalently bound BPDE residues, different topoisomer distributions of ϕ X174 DNA were prepared. Reaction mixtures were prepared containing 3.2 μ g of ϕ X174 DNA and 30 units of topoisomerase I ((Bethesda Research Laboratories) in 50 mM Tris–HCl, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 30 μ g bovine serum albumin. The reactions were allowed to proceed at 37°C for time intervals of 1, 2, 4, 6, 10 and 18. The reactions were stopped by adding a 25:24:1 solution of phenol: chloroform: isoamyl alcohol, mixing and subsequent extraction with this solution (twice), and one extraction with chloroform.

Tube gel method: titration of superhelical turns with ethidium bromide

This approach [28,29] is particularly useful for determining the titratable superhelical density in carcinogen-modified DNA [30]. Briefly, agarose tube gels were prepared by pouring hot (60°C) 1% agarose solutions in 0.018 M NaCl TBE buffer containing

different amounts of ethidium bromide (0.001–0.10 $\mu\text{g/L}$) into 20 cm-long glass tubes with one of their ends closed with parafilm. After 45 min, the solidified tubular gels were cut to a length of 18 cm and inserted into a vertical tube gel apparatus with the upper and lower chambers filled with 0.018 M NaCl TBE buffer solution. About 200 ng of modified or unmodified supercoiled DNA in 10 μL of solution containing 4.5% ficoll and 2.4 μM ethidium bromide (EB/(DNA nucleotide) ratio ≈ 0.2) were added to the tops of the tubes. Electrophoresis was performed at 95–100 V (2.5 mA/tube, up to 16 tubes at a time) for 3 hours at 20°C. After electrophoresis, the gels were extruded into separate large test tubes and the DNA bands were developed by adding a 0.7 $\mu\text{g/L}$ EB solution for 30 min with shaking, and were subsequently photographed under UV illumination.

Flow linear dichroism measurements of relative adduct conformations

Linear dichroism (LD) measurements provide information on the relative orientations of the long axis of the pyrenyl residue relative to the average orientations of the planes of the DNA bases [31]. Briefly, the aqueous DNA solutions are placed within the annular space of a Couette cell consisting of two concentric suprasil cylinders, with the outer cylinder (23 mm inner diameter) remaining stationary, while the inner one (22 mm outer diameter) is rotated at speeds of 400 RPM. The resulting hydrodynamic flow gradient (900 s^{-1}) causes a partial orientation of the DNA bases perpendicular to the flow direction. The linear dichroism signal is defined as $\text{LD} = A_{\parallel} - A_{\perp}$ where $A_{\parallel}$ and $A_{\perp}$ are absorbances measured with the linearly polarized light E-vector oriented parallel and perpendicular with respect to the flow direction, respectively. Additional details concerning the LD apparatus may be found elsewhere [32,33].

RESULTS

Superhelical density

The superhelical density of the ϕX174 DNA used in these experiments can be most conveniently evaluated from ethidium bromide (EB) titration-unwinding data [34]. Because the hydrodynamic volume of supercoiled DNA molecules increases as the degree of superhelicity is decreased, the flow linear dichroism method can be used to monitor the degree of unwinding

Figure 1. Linear dichroism signal measured within the DNA absorption band at 260 nm as a function of the ethidium bromide concentration expressed in terms of the molar ratio [EB added]/[DNA nucleotide]. The DNA concentration was 10^{-4} M, the buffer solution was identical to the one employed in the tube gel experiments (see Materials and Methods), the temperature was 24°C.

as the concentration of EB is increased [17,35]. A typical EB titration curve obtained by the flow linear dichroism method is shown in Fig. 1. The minimum in the LD signal, corresponding to a superhelical density of zero [17,35], is observed at the critical ratio of $r_C = (\text{EB added})/(\text{DNA nucleotide}) = 0.048 \pm 0.003$. Assuming that $\sim 95\%$ of the EB molecules are bound to the DNA at this equivalence point [36], the critical binding ratio v_C (bound EB molecules/nucleotide) is equal to $\approx 0.95r_C = 0.046 \pm 0.003$. Defining the superhelical density σ in terms of the unwinding angle $\theta = 26^\circ$ (per bound EB molecule) according to the formula $\sigma = -(v_C/18)\theta$ [37], we obtain a value of $\sigma = -0.066 \pm 0.004$ under our own conditions of ionic strength (0.071) and temperature (24°C) which, after correcting for the differences in temperature and ionic strength, is close to the standard published values of -0.057 ± 0.010 at 37°C and 0.2 M NaCl concentration [37]. Since the number of base pairs in ϕX174 DNA is 5386 [38], this value of σ corresponds to 35 ± 3 superhelical turns per molecule at 24°C.

Unwinding due to formation of noncovalent, unstable BPDE-DNA complexes

When (+)-BPDE (or (–)-BPDE) molecules are added to an aqueous solution of ϕX174 DNA, there is an immediate increase in the magnitude of the LD signal which returns approximately to its initial value after some 12–15 min with exponential decay kinetics (Fig. 2). The first-order decay constant under the experimental conditions used here is 0.0020 s^{-1} . Because of the tendency of the planar DNA bases to orient with their normals parallel to the flow lines, the linear dichroism signal of the supercoiled DNA solution is negative in sign below 300 nm [35].

Linear dichroism characteristics and conformations of covalent BPDE-DNA adducts

After removing the BPT molecules from the equilibrated reaction mixtures by exhaustive dialysis, the LD spectra of covalent adducts shown in Fig. 3 are obtained. These spectra are analogous to those obtained with linear DNA [22,23,26,39–41], thus indicating that the adduct conformations in supercoiled DNA are similar to those observed in linear DNA.

The (–)-BPDE- ϕX174 covalent adducts (Fig. 3B) display a red-shifted (relative to BPDE or BPT in DNA-free aqueous solution) negative LD spectrum resembling in shape the LD (and

Figure 2. Kinetic trace of the linear dichroism signal measured at 260 nm (LD) reflecting the unwinding and rewinding of the supercoiled DNA (7.5×10^{-5} M) after the addition (at time $t \approx 100$ s) of (+)-BPDE (8.6 μM). Similar effects were observed in the case of the (–)-enantiomer (data not shown). The structure of the (+)-BPDE enantiomer is also shown.

Figure 3. Linear dichroism spectra of covalent (+)-BPDE- ϕ X174 and (-)-BPDE- ϕ X174 adducts in solution (after exhaustive dialysis of the equilibrated reaction mixtures). In (A), the initially added BPDE concentration was as in the legend to Fig. 2, whereas in (B) all experimental conditions were as in (A), except that the initial reaction mixture contained 26×10^{-6} M (-)-BPDE.

absorption spectrum) of noncovalently bound intercalated DNA [23,32]. These types of quasi-intercalative binding sites have been designated as site I [2,9,10,39].

The LD spectrum of the (+)-BPDE- ϕ X174 DNA adducts is mostly positive in sign above 300 nm and is characteristic of external site II adducts, although a negative LD signal due to the contribution of smaller fractions of site I adducts is also visible (Fig. 3A); in site II adducts, the pyrenyl residues are at least partially exposed to the aqueous environment [26,42,43] and are tilted ($> 50^\circ$) away from the planes of the DNA bases [22,26,32,39,40].

Distribution of reaction products

Using initial reaction ratios r_i ([initial BPDE]/[DNA nucleotide]) in the range ≤ 0.5 , it was found that $22 \pm 4\%$ of the initially added (+)-BPDE binds covalently to DNA. This fraction tends to decline with increasing values of $r_i \geq 0.5$. In the case of (-)-BPDE, 3.5–6.6% of the diol epoxide molecules were converted to covalent DNA adducts in the range of $r_i = 0.3$ –1.5, the highest fraction being obtained at the lower r_i values. In the case of the (+)-BPDE-DNA adducts, only the *trans*-BPDE-N²-dG adduct was detectable ($> 90\%$ of all adducts), consistent with previous findings using racemic BPDE and linear [4,11,13] and supercoiled [19,44] DNA. In the case of the (-)-BPDE-DNA adducts, the adduct distribution is more heterogeneous [4,11,13]. The (-)-*trans* N²-dG, (-)-*cis* N²-dG, (-)-*trans* N⁶-dA, and other, unidentified adducts, accounted for 35 ± 5 , 18 ± 2 , 29

Figure 4. Photographs of ethidium-stained agarose slab gels comparing distances of migration of covalently modified BPDE- ϕ X174 DNA samples at different levels of r_b (covalently bound BPDE residues/nucleotide) (A) (+)-BPDE- ϕ X174 DNA adducts, (B) (-)-BPDE- ϕ X174 DNA adducts; r_b values: (A) lanes: (1) 0.0, (2) 0.036, (3) 0.054, (4) 0.10, (5) 0.12, (6) 0.12, (7) 0.14, (8) 0.16. (B) lanes: (1) 0.0, (2) 0.020, (3) 0.030, (4) 0.038, (5) 0.045, (6) 0.053. All values of r_b are within $\pm 20\%$.

± 6 , and $18 \pm 3\%$ of all covalent BPDE products. However, only overall adduct levels are reported here since, in any event, the effects of different types of adducts on the unwinding of supercoiled DNA cannot be resolved in our experiments.

Gel electrophoresis

Photographs of ethidium bromide-stained agarose slab-gels showing some typical electrophoresis data obtained with covalent (+)-BPDE- ϕ X174 and (-)-BPDE- ϕ X174 DNA adducts are shown in Fig. 4. Typical densitometer tracings obtained from the negatives are displayed in Fig. 5. The relative proportions of the supercoiled RF I and relaxed (nicked) RF II DNA forms were somewhat variable from sample to sample.

The electrophoretic mobilities of the supercoiled DNA covalently modified with (+)-BPDE are markedly slowed relative to the unmodified DNA; approximate co-migration of the supercoiled DNA and the nicked form is observed for relatively high binding ratios $r_b = 0.09$ –0.11 ($10 \pm 1\%$ of the bases modified). This behavior is attributed to the removal of left-handed superhelical turns and the concomitant increase in the overall hydrodynamic size and lower electrophoretic mobilities of the BPDE-DNA molecules.

The mobilities of covalent adducts derived from the (-)-enantiomer are barely affected in the range of $r_b = 0.00$ –0.06 (because of the low reactivities of the (-)-BPDE enantiomer with DNA, solubility problems at $r_i > 1.5$, as well as the relative scarcity and high cost of the BPDE enantiomers, no attempts were made to obtain adducts with higher levels of modification with this isomer).

Values of the relative mobilities as a function of the number of (+)-BPDE residues per genome are shown in Fig. 6; the data points represent the maxima in the distributions which are observed in the densitometer tracings (Fig. 5). At high (+)-BPDE adduct levels, these distributions are quite broad, especially beyond the value of $r_b = 0.10$; above this value of r_b , rewinding of the closed circular DNA gives rise to positive superhelicity, and is reminiscent of the effects observed with EB (Fig. 1).

In contrast to the behavior of the supercoiled band, the electrophoretic mobility of the slower, nicked band, increases somewhat upon modification with (+)-BPDE (Fig. 5). In the case of the (-)-BPDE- ϕ X174 DNA adducts, the mobility of the nicked band does not seem to change visibly as a function of r_b .

Figure 5. Densitometer tracings of photograph negatives (same data as in Fig. 4).

Comparisons of electrophoretic mobilities of (+)-BPDE- ϕ X174 DNA adducts with mobilities of unmodified topoisomers

Samples of untreated supercoiled DNA were partially relaxed with topoisomerase I for varying amounts of time, and these solutions were then subjected to gel electrophoresis. Typical photographs of slab-gels are shown in Fig. 7A. In our ϕ X174 RF I samples, we counted 19 bands, including the fully relaxed RF II and supercoiled RF I bands. This approach is not capable of revealing all of the bands corresponding to molecules with different number of superhelical turns, since some of the highest and lowest mobility bands cannot be resolved using agarose slab-gels; however, the resolved adjacent bands differ from one another by one superhelical turn [45]. The relationship between the band number (starting from the fastest bands) and relative electrophoretic mobilities (measured as a fraction of the distance on the slab-gels between the RF I and RF II bands in the unmodified samples) is plotted in Fig. 7B.

The relative mobilities of the (+)-BPDE- ϕ X174 DNA adducts (Fig. 6) were then matched with those in Fig. 7B, and the corresponding band positions were plotted as a function of the number of covalently bound (+)-BPDE residues/ ϕ X174 DNA molecule (Fig. 8) as previously described for other bulky adducts [30,46]. A reasonably good straight line with a slope of $\Delta\tau/\Delta[\text{BPDE}] = 0.017 \pm 0.001$ turns/adducts is obtained ($[\text{BPDE}] = \text{adducts/genome}$); the effective unwinding angle determined by this method is $360^\circ \times \Delta\tau/\Delta[\text{BPDE}] = 6.2 \pm 0.5^\circ$.

Figure 6. Relative mobilities of BPDE-supercoiled DNA adducts in electrophoretic agarose slab-gels as a function of the covalent modification level r_b . $\square$(+)-BPDE- ϕ X174 DNA adducts; $\blacksquare$(-)-BPDE- ϕ X174 DNA adducts. The values provided correspond to maxima in the mobility distributions evaluated from densitometer tracings.

Figure 7. (A) Electrophoretic slab-gel separation of topoisomerase I-treated unmodified ϕ X174 DNA. Lanes: (1) reference, untreated DNA, (2) treatment for 2 min, and (3) 4 min. (B) Positions of the different bands observed upon treating ϕ X174 DNA with topoisomerase I. The distance of each band was measured relative to the distance between the fast RF I and slow RF II bands of untreated DNA measured on the same gels.

The experimental data points corresponding to $40 < [\text{BPDE}] < 200$ adducts/genome lie well below the straight line in Fig. 8. Apparently, at low adduct levels, it is difficult to detect any changes in the mobilities due to removal of the first few superhelical turns.

Figure 8. Comparisons of band positions of (+)-BPDE- ϕ X174 DNA adducts with those of topoisomerase I-treated unmodified ϕ X174 DNA as a function of covalently bound BPDE residues per DNA molecule (see text).

Unwinding measured by the tube gel method

Changes in electrophoretic mobilities of carcinogen-modified supercoiled DNA on agarose slab-gels may, in principle, also reflect alterations in the tertiary structures of the molecules other than those produced by changes in the number of superhelical turns. Other effects may include bends [47] or flexible hinge joints [2,23,31,41] at the site of binding. The adducts derived from the binding of (–)-BPDE appear to be predominantly stacked (or site I, quasi-intercalated [22,40]) with neighboring bases as judged from linear dichroism experiments with linear DNA [22,23,26,40,41] and with supercoiled DNA (Fig. 3), while (+)-BPDE residues appear to be situated predominantly at external site II binding sites [22,23,26,32,40–43]; thus, we initially expected to find more unwinding in the case of (+)-BPDE- than in the case of (–)-BPDE- ϕ X174 DNA adducts; however, the exact opposite conclusion can be derived from the agarose slab gel results (Figs. 4 & 5). Therefore, another method was sought to determine the losses of superhelical turns. The tube gel technique is convenient and suitable for this purpose. In this method [28,29], samples of the same DNA adduct solution are subjected to agarose gel electrophoresis in individual tubes, each containing EB molecules at different concentrations, thus producing different degrees of unwinding in each tube. The number of superhelical turns can be estimated from the EB concentration C' in the tube in which the fully relaxed covalently closed circular DNA molecules co-migrate with the nicked form, using the equation [28,29]:

$$\nu_C = \nu_m KC' / (1 + KC') \quad (1)$$

where K is the equilibrium binding constant of EB and ν_m is the maximum molar ratio of bound EB/nucleotide. Since the magnitude of K depends on the ionic strength and other factors, we determined its value under our own experimental conditions using standard equilibrium dialysis methods. A Scatchard plot was constructed (data not shown), which yielded values of $K = (3.0 \pm 0.1) \times 10^6 \text{ M}^{-1}$ and $\nu_m = 0.18$, similar to those reported by other workers [28,29]. The superhelical density is then calculated from the values of ν_C using the relationship $\sigma = -1.44\nu_C$. In these tube gel experiments, the levels of covalent BPDE adducts were held below $r_b = 0.06$, since the association constant for EB binding was not measurably affected in this range of BPDE binding levels (data not shown).

The tube gel experiments were run in two stages: (1) a coarse scale of incremental increases of the EB concentration C in each

gel was employed in order to determine the approximate value of C' , (2) the experiments were then repeated with the same DNA sample using a narrower range of C near the value of C' estimated from the first experiment. In this way, a more accurate value of C' was obtained.

Examples of such experiments are shown in Fig. 9. Using a coarse range of C values, the value of C' for unmodified ϕ X174 RF I DNA is found to be in the range of 0.040–0.050 $\mu\text{g/ml}$ (Fig. 9A); using a narrower range of C values, C' is found to lie in the range of 0.042–0.046 $\mu\text{g/ml}$; the corresponding value of ν_C is 0.046 ± 0.002 , yielding values of $\sigma = -0.066 \pm 0.003$ and 35 ± 2 superturns/DNA molecule, in excellent agreement with the data obtained from the linear dichroism EB-titration curve (Fig. 1).

Examples of similar experiments with (+)-BPDE- ϕ X174 and (–)-BPDE- ϕ X174 DNA adducts are shown in Figs. 9B and 9C, respectively. In both cases, rewinding of the DNA in the opposite sense is observed at ethidium concentrations $C > C'$. In the case of the (+)-BPDE adduct with a level of modification of $r_b = 0.05$, the value of C' is $0.021 \pm 0.003 \mu\text{g/ml}$ (Figs. 9B), which is considerably smaller than in the case of the unmodified DNA. In the case of the (–)-BPDE- ϕ X174 DNA sample with a similar level of modification ($r_b = 0.045$), $C' = 0.030 \pm 0.003 \mu\text{g/ml}$. Thus, both enantiomers, upon binding covalently with DNA, give rise to measurable degrees of unwinding as measured by the tube gel method.

Particularly striking are the apparently small effects of the covalently bound (–)-BPDE residues on the mobilities of the modified DNA as measured by the agarose slab-gel method (Figs. 4 and 5), and the definite unwinding effect detected in the same range of binding by the gel method (Fig. 9C); the loss of superhelical turns increases with increasing levels of (–)-BPDE modification, and values of θ in the range of $6-8^\circ$ are obtained (Table I).

DISCUSSION

Unwinding of supercoiled DNA by noncovalent binding of BPDE

Noncovalent BPDE-DNA complexes are formed on time scales of milliseconds after the addition of the PAH diol epoxides [2], and cause the rapid initial increase in the magnitude of the linear dichroism signal at 260 nm (LD_{260}) shown in Fig. 2. The decay kinetics of the LD_{260} signal are identical to the kinetics of disappearance of intercalated PAH diol epoxide molecules [17]. The LD_{260} signal decays because the BPT hydrolysis products are characterized by a ≈ 6 times smaller binding affinity (noncovalent complex formation) than BPDE [48]; the overall number of noncovalently bound intercalated molecules thus decreases with time.

Both enantiomers of BPDE cause the unwinding of supercoiled DNA by noncovalent complex formation. Most [16,23,48–50] though not all [49] non-covalent PAH diol epoxide—and other PAH metabolite—DNA [51] complexes appear to be intercalative in nature. Meehan et al. [16] have shown that the unwinding angle associated with ($\pm$)-BPDE intercalation is only 13° , about one half of the value observed with EB [37,52].

Slab-gel electrophoretic mobilities

Supercoiled form (RF I). There is a striking difference in the electrophoretic mobilities of adducts derived from the binding of (+)-BPDE and (–)-BPDE to ϕ X174 DNA at similar values of r_b (≤ 0.06 , Figs. 4–6). At the highest level of modification

Figure 9. Photographs of tube gels of (A) untreated Φ X174 DNA, (B) (+)-BPDE- Φ X174 DNA adducts, $r_b = 0.05$, and (C) (-)-BPDE- Φ X174 DNA adducts, $r_b = 0.045$. Left: coarse Ethidium bromide concentration intervals; right: finer intervals. EB concentrations in $\mu\text{g/ml}$: (A) Left: (1) 0.001, (2) 0.005, (3) 0.010, (4) 0.020, (5) 0.025, (6) 0.030, (7) 0.035, (8) 0.040, (9) 0.050, (10) 0.060, (11) 0.070, (12) 0.090, (13) 0.090, (14) 0.110, (15) and (16) 0.00. (A) Right: (1) 0.022, (2) 0.026, (3) 0.030, (4) 0.034, (5) 0.038, (6) 0.042, (7) 0.046, (8) 0.050, (9) 0.054, (10) 0.058, (11) 0.062, (12) 0.066, (13) 0.070, (14) 0.074, and (15) and (16) 0.000. (B) Left: (1) 0.001, (2) 0.005, (3) 0.010, (4) 0.015, (5) 0.020, (6) 0.025, (7) 0.030, (8) 0.035, (9) 0.040, (10) 0.045, (11) 0.050, (12) 0.060, (13) 0.070, (14) 0.080, and (15) 0.00, and (16) 0.00, unmodified DNA. (B) Right: (1) 0.010, (2) 0.012, (3) 0.014, (4) 0.016, (5) 0.018, (6) 0.020, (7) 0.022, (8) 0.024, (9) 0.026, (10) 0.028, (11) 0.030, (12) 0.032, (13) 0.034, (14) 0.036, and (15) 0.00, and (16) 0.00, unmodified DNA. (C) Left: (1) 0.005, (2) 0.010, (3) 0.015, (4) 0.020, (5) 0.025, (6) 0.030, (7) 0.035, (8) 0.040, (9) 0.045, (10) 0.050, (11) 0.055, (12) 0.060, (13) 0.065, (14) 0.070, and (15) 0.00, and (16) 0.00, unmodified DNA. (C) Right: (1) 0.018, (2) 0.021, (3) 0.024, (4) 0.027, (5) 0.030, (6) 0.033, (7) 0.036, (8) 0.039, (9) 0.042, (10) 0.045, (11) 0.048, (12) 0.051, (13) 0.054, (14) 0.057, and (15) 0.00, and (16) 0.00, unmodified DNA.

with (-)-BPDE, the average mobility of adducts is decreased by only $\approx 7\%$, while in the case of the adducts derived from (+)-BPDE the mobility is decreased by $\approx 45\%$ (Fig. 6). The covalent adducts arising from the binding of (+)-BPDE are more effective than (-)-BPDE in decreasing the mobilities of covalent BPDE- supercoiled DNA adducts. Thus, decreases in the electrophoretic mobilities of racemic *anti*-BPDE-SV40 supercoiled DNA adducts previously observed in agarose gels [18–21], are predominantly associated with the (+) enantiomer.

Nicked form (RF II). The increase in the mobility of the nicked closed circular RF II band with increasing r_b for both BPDE isomers (Fig. 4) have also been observed with racemic *anti*-BPDE-DNA adducts [19,20]. The higher mobility of the modified form II DNA was attributed to a flexible hinge-like behavior at

Table I. Unwinding angles determined for covalent (-)-BPDE- Φ X174 DNA adducts at different values of r_b .

$r_b^{(a)}$ (1)	$C'^{(b)}$ ($\mu\text{g/ml}$) (2)	$\theta_C^{(c)}$ (3)	Unwinding angle (4)
0	0.044	0.046 ± 0.0015	$26 \pm 2^\circ$
0.019 ± 0.002	0.038	0.041 ± 0.0017	$6.8 \pm 0.9^\circ$
0.030 ± 0.002	0.035	0.039 ± 0.0018	$6.1 \pm 0.7^\circ$
0.038 ± 0.003	0.033	0.037 ± 0.0019	$6.2 \pm 0.7^\circ$
0.046 ± 0.003	0.030	0.034 ± 0.0020	$6.8 \pm 0.7^\circ$
0.053 ± 0.004	0.026	0.030 ± 0.0021	$7.8 \pm 0.9^\circ$

^aCovalently bound BPDE residues/nucleotide.

^bEthidium bromide concentration at which the covalently closed and nicked DNA co-migrate. Central value taken from the higher resolution tube gel experiments; the maximum uncertainties in these values is $\pm 0.002 \mu\text{g/ml}$ (see text).

^cCritical EB concentration ratio which leads to the complete relaxation of supercoiled modified DNA; the uncertainties reflect the extreme ranges of C' .

the alkylation sites [19]. The observed reductions in the mobilities of short linear DNA fragments [47], and decreases in the degrees of alignment of high molecular weight linear DNA molecules modified with racemic BPDE and (+)-BPDE in hydrodynamic flow gradients [23,31,41], are consistent with this hypothesis.

Estimates of unwinding angle

Slab- gels. The unwinding angle θ can be estimated using the relationship [37]:

$$\theta = (\Delta\tau \times 360^\circ) / (r_b \times 10,772) \quad (2)$$

where $\Delta\tau$ is the number of superhelical turns removed at a given value of r_b , while 10,772 is the number of bases per genome of ϕ X174. In principle, unwinding angles in carcinogen-modified superhelical DNA can be determined by comparing the changes in electrophoretic mobilities (Fig. 6) with those observed with the unmodified topoisomers (Fig. 7). However, only a lower limit of the unwinding angle θ can be obtained in this way, as is shown here. From Fig. 4, using r_b values of 0.05, the relative mobility of (+)-BPDE-DNA adducts is estimated to be $\approx 55\%$, while for the (-)-BPDE-DNA adducts it is $\approx 93\%$, corresponding to a loss of ≈ 10 and ≈ 1.5 superhelical turns respectively. The fastest mobility RF I band is known to consist of several highly wound topoisomers [45]. The loss of the first few N superturns is not expected to exhibit any noticeable changes in the observed average electrophoretic mobilities. Thus, at binding levels of $r_b = 0.05$, $\Delta\tau = N + 10$, or $= N + 1.5$, for (+)-BPDE- and (-)-BPDE- ϕ X174 DNA adducts, respectively. Since the values of $\Delta\tau = 10$ and 1.5 represent lowest limits for the numbers of superhelical turns removed when $r_b = 0.05$, unwinding angles of $\theta > 7^\circ$ and $\theta > 1^\circ$ for (+)-BPDE- ϕ X174 DNA and (-)-BPDE- ϕ X174 DNA adducts, respectively, are estimated by this method and Eq. 2.

The observance of a minimum in the relative mobilities as a function of r_b in the case of adducts derived from (+)-BPDE (Fig. 6) provides yet another method for estimating the average unwinding angle per bound BPDE residue. With $v_c = 0.10 \pm 0.01$, and using Eq. 2 with $\tau = 35 \pm 3$ (complete unwinding), yields a value of $\theta = 12 \pm 1.5^\circ$.

Comparisons of unwinding angles θ determined by different techniques. The results are summarized in Table II. The average value of θ for (-)-BPDE- ϕ X174 DNA adducts obtained by the tube gel method is $6.8 \pm 1.7^\circ$. Good agreement is obtained in the case of (+)-BPDE- ϕ X174 DNA adducts by the complete unwinding-agarose slab gel method (column 4) and the tube gel method (column 5); the average value of θ is $11.5 \pm 2.4^\circ$. This value is, however, about twice as large as the value of θ estimated by the band counting method (column 2). This latter value ($6.2 \pm 0.5^\circ$) was obtained by comparing the slab-gel mobilities of (+)-BPDE- ϕ X174 DNA adducts with those of unmodified isomers with the number of superturns/molecule between N and N+18 (Fig. 7). The value of N can be estimated from Eq. 2 using $\theta((+)-BPDE) = 11.5 \pm 2.4^\circ$, and $\theta((-)-BPDE) = 6.8 \pm 1.7^\circ$ and $\Delta\tau = N+10$ and $\Delta\tau = N + 1.5$ for (+)-BPDE-DNA and (-)-BPDE-DNA adducts, respectively. Values of $N = 7.2 \pm 2.7$ and $N = 8.7 \pm 2.5$ are obtained which are in reasonable agreement with one another, and also with the expected number of unresolvable fast electrophoretic bands [45].

In the case of (-)-BPDE- ϕ X174 DNA adducts, the changes in the relative electrophoretic mobilities observed on agarose slab gels are too small to reflect the value of θ measured by the

Table II. Summary of unwinding angles obtained by different methods.

ADDUCT FROM:	Slab Gel, $\Delta\tau/\Delta N^{(a)}$	Slab Gel, Relative Mobilities ^(b)	Slab Gel, Complete unwinding ^(c)	Tube Gels
(1)	(2)	(3)	(4)	(5)
(+)-BPDE	$6.2 \pm 0.5^\circ$	$> 7^\circ$	$12 \pm 1.5^\circ$	$11 \pm 1.8^\circ$
(-)-BPDE	—	$> 1^\circ$	—	$6.8 \pm 1.7^\circ$

^(a)From topoisomer experiments, Fig. 8.

^(b)Estimated from data in Fig. 6, see text.

^(c)Estimated from the minimum in Fig. 6 at $r_b = 0.010 \pm 0.001$.

ethidium bromide titration tube gel method. This suggests that the slab gel electrophoresis method is not suitable for estimating unwinding angles in those cases in which the unwinding angle/ligand is small, and the degree of modification is also relatively low. In these cases, the removal of the first few superhelical turns does not appear to be sufficient to cause any significant changes in the electrophoretic mobilities in agarose slab gels.

The value of θ estimated from the data in Fig. 7 in the range of superhelicities between N and N+18 (Table II, column 2) is smaller than the overall, mean value of θ obtained by other methods (Table II, columns 4 & 5). This suggests that the unwinding angle/[BPDE] residue must be higher than the average value $\theta = 11.5 \pm 2.4^\circ$ for highly supercoiled ϕ X174 isomers. Such a phenomenon was observed by Gamper et al. [19] for racemic BPDE-SV40 DNA adducts, who reported decreasing unwinding angles with increasing levels of modification in the range of $330^\circ - 30^\circ$, the highest values being obtained for highly supercoiled topoisomers. These values (or the inferred mean value) are substantially higher than those obtained by us with (+)-BPDE using a different kind of supercoiled DNA (Table II). Gamper et al. [19] worked with a 10% dimethyl sulfoxide (DMSO)-90% buffer mixture, while the organic solvent concentration (THF) in our reaction mixtures was less than 3%. However, we found that the levels of binding of (+)-BPDE to ϕ X174 DNA (and the relative electrophoretic mobilities) were the same in the absence or presence of 10% dimethylsulfoxide during the preparation of the adducts (data not shown). Therefore, differences in reaction conditions do not appear to be the source of this discrepancy.

There is a difference in the adduct level (r_b) thresholds at which unwinding is first observed in our experiments on agarose slab gels and those of Gamper et al. [19]. These latter authors, based on the retardation of individual topoisomer bands induced by ($\pm$)-BPDE in a different gel system, observed significant decreases in mobilities of SV40 DNA at levels of modification of only 2–40 BPDE residues per genome. Drinkwater et al. [18] stated that the binding ratio r_b in agarose slab gel should be at least 0.015 (> 160 adducts/genome) in order to observe changes in electrophoretic mobilities on agarose slab gels. In our experiments, changes in electrophoretic mobilities of modified ϕ X174 DNA are not observed unambiguously on slab-gels unless there are at least ≈ 200 (+)-BPDE adduct residues/genome ($r_b \geq 0.02$, Fig. 6), which is more consistent with the threshold established by Drinkwater et al. than the one found by Gamper et al. In these latter two investigations, racemic BPDE (rather than the resolved enantiomers) were used, and the sources of the supercoiled DNA were different from ours. It seems unlikely, though not certain, that these factors are the sources of the differences.

Our value of the mean unwinding angle for (+)-BPDE-DNA adducts ($11.5 \pm 2.4^\circ$) is smaller than the value of $22 \pm 3^\circ$ published by Drinkwater et al. [18]. Because the (+)-isomer of BPDE is about 3–4 times more reactive with respect to supercoiled DNA than the (–) enantiomer, the unwinding angle should be, all other things being equal, somewhat higher in the case of (+)-BPDE than in the case of racemic BPDE. This discrepancy is traceable to the differences in the values of r_b at which minima in the electrophoretic mobilities are observed (r_b (min) = 0.05 ± 0.008 in the case of Drinkwater et al., and r_b (min) = 0.10 ± 0.01 , this work). Given the $\pm 20\%$ uncertainty in our values of r_b , the discrepancy is not large. Inaccuracies in adduct level determinations could account for this difference. Another possible source of error is the absolute superhelical density which is used to calculate θ based on the experimentally determined values of r_b (min). In this work, we have made efforts to reduce the errors associated with these problems by (1) determining the superhelical density of the ϕ X174 DNA samples under our own experimental conditions, and (2) basing adduct level determinations on two different methods.

Adduct conformations and mechanisms of unwinding

Classical intercalation involves the insertion of planar aromatic molecules between adjacent DNA base pairs, which leads to an extension of the phosphodiester backbone, an untwisting of base pairs, and thus to unwinding [53]. Flow linear dichroism studies indicate that the noncovalent binding of (+)-BPDE and (–)-BPDE to DNA leads to adduct conformations consistent with those of intercalative complexes [23]; however, noncovalent racemic BPDE-SV40 supercoiled DNA complexes are characterized by unwinding angles of 13° [16], which is much smaller than the value of 26° [37,52] observed for the classical intercalator ethidium bromide. While the unwinding of supercoiled DNA by covalently bound (+)-BPDE is also close to 13° (Table II), the mechanisms of unwinding must be different in the noncovalent complex and covalent adducts since their conformations are quite different from one another [23,32]. While the unwinding of supercoiled DNA by drug molecules is often taken as evidence for an intercalative binding mechanism, Bauer has emphasized that the demonstration of duplex unwinding should not be considered as incontrovertible evidence for intercalation [37]. Other causes of duplex unwinding not involving intercalative binding have been documented, including the effects of covalently bound *cis*- and *trans*-dichlorodiammineplatinum (II) [54,55], dehydration [56], the binding of histones [57] and metal ions [58], and photochemical thymine dimer formation [59].

In the case of (+)-BPDE, over 90% of the adducts formed involve *trans*-addition (at the C10 position) to the exocyclic amino group of guanine in native [11–13] and in supercoiled [44] DNA. Recent high-resolution NMR experiments with stereospecific and site-specific (+)-BPDE-deoxyoligonucleotide adducts have shown that the pyrenyl residue is situated in a widened minor groove of B-DNA as gauged from the larger than normal distances between adjacent negatively charged phosphate moieties [14,15]. Possible mechanisms of unwinding based on this effect can be discussed in terms of a model recently proposed by Wilson et al. [53] for classical intercalators: because of the lowered repulsive interactions between neighboring phosphate groups due to the minor groove widening, an untwisting of the base pairs may occur which increases the favorable van der Waals contacts due to better overlap between adjacent base pairs. This mechanism of unwinding could be further complemented by a

flexible hinge joint at the site of binding. Flow linear dichroism experiments [23,31,41] and molecular dynamic simulation studies [60] suggest that such effects are predominantly associated with (+)-BPDE, rather than with (–)-BPDE. Furthermore, using ligation and gel electrophoresis techniques [61] with covalent oligonucleotide adducts derived from the *trans* and *cis* addition of both (+)- and (–)-BPDE, we have shown that only the (+)-*trans*-BPDE-N²-dG adduct gives rise to a flexible hinge joint at the site of binding (B.Mao, B.Li and N.E.Geacintov, in preparation).

Adducts derived from the binding of (–)-BPDE are chemically more heterogeneous. Little is known about the conformations of BPDE-dA adducts at this writing. The (–)-*trans*-BPDE-N²-dG adducts are also situated in the minor groove, but their orientation are different from those of the analogous (+)-adducts [15]; unwinding due to the mechanism discussed above is therefore also expected. However, the absence of a prominent hinge joint in (–)-*trans*-BPDE-N²-dG-oligonucleotides suggests that the overall unwinding may be lower than in the case of the structurally related (+)-*trans* adducts. The (–)-*cis* adducts appear to be situated inside the helix and are at least partially base-stacked with neighboring bases [25,27]. However, their conformation may be different from those of classical intercalative structures: the modified guanosyl residues may be displaced by the pyrenyl moieties in a quasi-intercalative carcinogen-base-stacked conformation, as proposed recently by Singh et al. based on potential energy minimization studies of analogous, higher energy *trans*-adducts [60]. The degree of unwinding associated with such conformations may be lower than for classical intercalation complexes.

Collectively, the heterogeneous (–)-BPDE adduct species give rise to a smaller unwinding angle than (+)-BPDE-N²-dG lesions (Table II). The reasons for these differences are presently not well understood and must await further developments in the structural determinations of site-specific and stereospecific BPDE-oligonucleotides adducts [14,15], and the analysis of the associated structural effects involving unwinding of superhelical turns and other alterations in the tertiary structure of DNA.

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