Covalent Complexes of DNA and Two Stereoisomers of Benzo(a)pyrene 7,8-Dihydrodiol-9,10-epoxide Studied by Fluorescence and Linear Dichroism

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ABSTRACT

Fluorescence and absorption spectroscopy and linear dichroism have been used to study the covalent adducts of calf thymus DNA with the two stereoisomers of benzo(a)pyrene 7,8-dihydrodiol-9,10-oxide, (±)-7,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (anti-BPDE; a strong carcinogen) and (±)-7,8α-dihydroxy-9α,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (syn-BPDE; a weak carcinogen). Each stereoisomer gives rise to two types of complexes, I and II, with characteristic spectral properties: type I with light absorption and fluorescence excitation maxima at 322, 337, and 354 nm, and type II with the corresponding maxima at 316, 330, and 345 nm. In anti-BPDE-DNA, the type II component dominates and the type I component amounts to <10%. In syn-BPDE-DNA, approximately 35%, of the adduct is of type II and approximately 65% of type I. From linear dichroism, it is concluded that the type II component of anti-BPDE-DNA has the plane of the chromophore molecule nearly parallel to the helix axis. The type I component of syn-BPDE-DNA has a very different geometry with the chromophore probably intercalated between the DNA bases. This is also in accord with the large wavelength shift of the light absorption and the weak quenching of the fluorescence by O₂ for the type I complex. The properties of complex type II of anti-BPDE-DNA are in agreement with a wedge-type geometry at the binding site. The Stern-Volmer quenching curves are bent, and the fluorescence decays are not monoexponential, which demonstrates that there is heterogeneity in the microenvironment of the chromophore. From the dynamic quenching constants with O₂, it is found that different subcategories of the chromophore are differently exposed to the medium. Addition of Ag⁺ to anti-BPDE-DNA (type II complexes) leads to increased fluorescence and longer decay times. The Ag⁺-induced effects are probably due to a conformational change of DNA when Ag⁺ is bound, causing the anti-BPDE adducts to interact less strongly with the DNA. Component type I of syn-BPDE-DNA is not affected by Ag⁺ in a similar way. Instead, a weak quenching is observed.

Upon denaturation, both anti- and syn-BPDE-DNA give a type of single-stranded complex with light absorption and fluorescence excitation maxima at 316, 332, and 351 nm. The chromophores are probably sandwiched between the DNA bases.

INTRODUCTION

BP, a ubiquitous environmental contaminant, is a potent mutagen and carcinogen in various experimental systems and is also believed to be involved in the etiology of certain human tumors (14, 40).

BP is not carcinogenic per se but must undergo biological transformation to specific electrophilic intermediates (11) that subsequently interact with cellular constituents, such as DNA (45).

Of particular importance for BP carcinogenesis is the formation of BP 7,8-oxide, the epoxide hydrolase catalyzed hydrolysis to trans-7,8-dihydroxy-7,8-dihydro-BP and the subsequent activation to syn and anti isomers of trans-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-BP (11, 45). Both isomers bind to DNA and exhibit biological activity. The anti isomer and in particular its (±)-enantiomer has, however, been shown to be the most active form (1, 17, 39, 41, 46).

The (±)-anti-BPDE has been shown to preferentially bind to native DNA when incubated with racemic anti-BPDE (22). The selectivity is abolished when native DNA is replaced by denatured DNA (22, 27). Accordingly, the structure and conformations of DNA and the stereochemistry of the reacting electrophile seem to be factors of fundamental importance in determining the final pattern of BP intermediates bound to DNA, and of their abilities to undergo biochemical repair reactions (30).

Several nucleophilic sites in DNA have been shown to react with BPDE (21, 28, 31). Quantitatively, the major binding site is the 2-amino group of guanine, which reacts with BPDE through its C-10 position (45).

The structure of the DNA carcinogen complex(es) seems to be crucial for the carcinogenic effect, and structural features have therefore been studied widely, with varying results (5, 7–9, 15, 19, 20, 24, 34–37, 42, 46, 47). Some studies have suggested that the BPDE molecule binds to the outside of the DNA helix with the bulky pyrene moiety in the minor groove of the DNA (7–9, 24, 34, 35). Others have proposed an intercalated complex, e.g., Drinkwater et al. (5), who based their proposal on the unwinding of supercoiled SV40 DNA modified with BPDE. Meehan and Straub (27) argued that the specific binding of BPDE to

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DNA could reflect a specific interaction, as would be expected for intercalation. A wedge-shaped intercalated carcinogen DNA complex has been proposed by Hogan et al. (15) in a study using DNA fragments of defined length. The heterogeneity of the binding has been stressed in studies using normal DNA, and different classes of bound BPDE have been characterized (15, 20, 42).

In this investigation, we have used physical techniques to study the complexes formed when DNA reacted with either racemic anti-BPDE or racemic syn-BPDE, a diastereomer of anti-BPDE. The 2 diastereomeric BP diol-epoxides are depicted in Chart 1. In spite of the structural similarity between the 2 BPDE isomers, the syn-BPDE isomer is a weak carcinogen compared to the anti isomer (2). The aim of this study was to elucidate a possible structural difference between the DNA complexes of these 2 BP derivatives.

The studies reported here include absorbance and fluorescence spectroscopic measurements on the native and denatured forms of BPDE-modified calf thymus DNA, as well as LD measurements on the native modified DNA oriented by flow. Fluorescence quenching agents have been used to test the accessibility of the DNA-bound chromophores. We also report results from fluorescence decay measurements.

MATERIALS AND METHODS

Materials. DNA (Sigma calf thymus type I) was purchased from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification. anti-BPDE and syn-BPDE were obtained through the Cancer Research Program, Division of Cancer Cause and Prevention, National Cancer Institute (Bethesda, Md.). The purity of the diol-epoxides was checked by thin-layer chromatography (tetrahydrofuran:hexan:methanol:triethylamine, 75:25:5:1; 0.2-mm silica gel prespotted with triethylamine, and L0 — A (370 nm) transitions.

In order to remove noncovalently bound hydrocarbons, the reacted DNA solutions were extracted 7 to 8 times with 4 volumes of ethyl acetate to remove formaldehyde was added to 1%. After the heating procedure, it was necessary to make 1 or 2 extractions with ethyl acetate to remove adducts which were released during heating.

In some preparations, the DNA was precipitated with NaCl-saturated ethanol and stored at —20°C.

Denatured DNA samples were prepared by heating for 5 min to 100°C and rapid cooling in ice water. To prevent reassociation when cooling, formaldehyde was added to 1%. After the heating procedure, it was necessary to make 1 or 2 extractions with ethyl acetate to remove adducts which were released during heating.

Spectroscopic measurements. All spectroscopic measurements were made on BPDE-DNA in 10 mM sodium cacodylate buffer adjusted with HNO3 to pH 7.0 at 20°C.

Absorption spectra were recorded on a Cary 219 spectrophotometer. The bandwidth used was 1 nm. The fluorescence excitation spectra were recorded on a Shimadzu RF510 spectrofluorometer equipped with a quantum counting compensator. The bandwidth used was 3 nm. The fluorescence quenching studies were carried out on a spectrofluorometer described elsewhere (18) but now equipped with a Jobin-Yvon double monochromator on the excitation side. The excitation bandwidth used was 2 nm, with the emission monochromator set at 420 and 40 nm spectral bandwidth. All quenching studies were performed at 20 ±1°C.

The fluorescence quenching by oxygen was measured in a pressurized cell, as described by Lakowicz et al. (23). The solution was slowly bubbled with the oxygen at the desired pressure to speed up the equilibrium. Oxygen concentrations were calculated using Henry’s law, and the oxygen solubility was 1.44 mm per atm of oxygen pressure at 20°C (23).

Appropriate background spectra, recorded with unmodified DNA, were always subtracted point by point in a computer from the sample fluorescence spectra.

LD of Flow-oriented BPDE-DNA. The LD technique makes it possible to measure the orientation of transition dipole moments of oriented chromophores. Using polarized light, the absorbance of light-polarized parallel A, or perpendicular A, to an orientation direction is measured and compared by forming $LD = A - A_\perp$.

In this study, the DNA molecules were oriented by flow in a Couette cell. The DNA solution is introduced into the narrow space (0.5 mm) between 2 concentric quartz cylinders. By rotating the inner cylinder, a velocity gradient is generated in the solution. Hence, the DNA molecules are affected by shear forces which will tend to stretch and align the DNA molecules along the flow direction. The LD measurements were made along a radial direction through the cylinders with $A_\parallel$ parallel to the flow direction (43).

DNA is a flexible chain molecule. It has been shown by statistical theory that short segments of the long DNA molecule are orientated as uniaxial particles in the flow field (29). The flow dichroism can be analyzed by the equation

$$LD = \frac{3}{2} (3 \cos^2 \alpha - 1) \cdot S$$

(A)

where A is the absorbance of the isotropic sample (no flow), $\alpha$ is the angle which the transition dipole moment forms with the local helix axis of the DNA segment, and S is an orientation function. For perfect orientation with all DNA segments parallel to the flow direction, $S = 1$; for an isotropic sample, $S = 0$. The reduced LD, $LD/\alpha$, factorizes into an optical part $\frac{3}{2} (3 \cos^2 \alpha - 1)$, depending only on the orientation of the chromophores relative to the local DNA helix axis, and S, which relates only to the properties of the DNA in the flow field.

If n absorbing chromophores with different absorbance $A_\lambda(\lambda)$ and transition dipole moments forming angles $\alpha$ with the DNA helix axis are present, then Equation A becomes...
When BPDE is reacted with DNA, in addition to the covalent BPDE-DNA complexes, the BP tetrol BPT is formed (16). BPT binds noncovalently to DNA. The intercalative binding is accompanied by a 10-nm shift toward longer wavelengths in the light absorption of BPT (16). The DNA samples were extracted several times with ethyl acetate to remove noncovalently bound hydrocarbons.

The unbound BPDE derivatives were readily removed from the reaction mixture of anti-BPDE and DNA. The DNA adducts of anti-BPDE thus prepared were apparently stable for 7 to 8 hr at room temperature.

The DNA adducts of syn-BPDE were much less stable. About 1% of the adducts were released during 1 hr at room temperature. Exposure to UV increased the release of adducts. The released BP derivative showed a BPT-like spectrum.

For the subsequent studies, we used freshly extracted samples and kept the UV illumination of the samples at a minimum, e.g., by using small spectral bandwidths in the spectroscopic measurements.

Absorption Spectroscopic Measurements. The absorption spectra of anti-BPDE-DNA and syn-BPDE-DNA are presented in Chart 2a. The absorption spectrum of anti-BPDE-DNA (full line) is similar to the spectrum of BPT in water (16) except for a slight shift of 2 nm toward longer wavelengths and a spectral broadening by approximately a factor of 2 in apparent line width.

The syn-BPDE-DNA absorption spectrum in Chart 2a (— — —) may be described as composed of 2 spectral components. The spectral properties of these 2 components correspond approximately to the BPT spectra obtained from 2 types of physical binding Sites I and II in DNA (16). We have adopted the same nomenclature for the 2 syn-BPDE components. One component (I) is a BPT-like spectrum shifted 11 nm toward longer wavelengths, while the other component (II) is very much like the anti-
BPDE-DNA spectrum. Resolution and sensitivity do not permit detection of possible minor components.

Visual inspection of the 2 spectra suggests that the anti-BPDE-DNA spectrum consists of a fairly pure type II component, whereas the syn-BPDE-DNA spectrum is a mixture of the 2 components, with type I as the major one. A quantitative estimation of the relative contributions of the 2 components in these spectra was made in the following way. First, we observed that the shapes of the spectra of anti- and syn-BPDE-DNA on the long-wavelength side are nearly identical. Hence, as a first approximation, the recorded anti-BPDE-DNA spectrum is taken as representative of pure Component II, and the same spectrum shifted 9 nm toward longer wavelengths as representative of Component I. Using the absorbances of syn-BPDE-DNA at 345 and 354 nm, it is easy to calculate that this spectrum is composed of 64% type I and 36% type II components. As the next approximation, we assumed that the recorded anti-BPDE-DNA spectrum contained a 10% contribution of the type I spectrum and corrected the 2 component spectra accordingly. With the new pair of type I and type II spectra, the composition of the syn-BPDE-DNA spectrum was again calculated and found to be the same as given above. It was observed for the newly corrected type II spectrum that the slope on the long-wavelength side was significantly altered, showing that it was not realistic to try with larger contributions of Component I in the anti-BPDE-DNA spectrum. The relative contributions of the 2 spectral components should correspond to concentrations of components if their extinction coefficients are identical.

Fluorescence Excitation Spectra. The fluorescence excitation spectra of the BPDE-DNA samples are shown in Chart 3.

The excitation spectrum of anti-BPDE-DNA resembles the absorption spectrum closely. On the other hand, the excitation spectrum of syn-BPDE-DNA is significantly different from the absorption spectrum. This is evidence of at least 2 species of chromophores with different quantum yields.

To explain the properties of the absorption and excitation spectra of the syn-BPDE-DNA complex, we therefore will describe them as the sum of 2 spectral components (corresponding to the components discussed in the absorption spectroscopy section above): spectrum type I (resembling the spectrum of BP in water but broadened and shifted 11 nm toward longer wavelengths) with peaks at 322, 337, and 354 nm, and spectrum type II of similar shape but with peaks at 316, 330, and 345.5 nm. The peaks of the 2 components are indicated by bars above the syn-BPDE-DNA absorption spectrum in Chart 2A and the excitation spectra in Chart 3.

LD Measurements. The LD spectra, \( \Delta \lambda = \lambda_1 - \lambda_2 \), of the BPDE-DNA samples are shown in Chart 2B together with the reduced LD, LD/A, in Chart 2C.

The positive sign of the LD at 345 nm of the anti-BPDE-DNA indicates that, for the majority of chromophores, the angle between the DNA helix axis and the transition dipole moment of the chromophore is less than 55°. With the simple first assumption that there is only one chromophoric species, it is possible to estimate the binding angle. \( S \) in Equation A is first obtained from the LD at 258 nm (\( S = 0.043 \)). Together with the LD/A at 345 nm (corresponding to maximum optical absorption), this gives the value \( \alpha = 35° \). This is an upper limit, since minor chromophoric components (see below), whether unoriented or with \( \alpha > 35° \), will tend to give an apparently too large angle.

In the case of syn-BPDE-DNA, the negative LD indicates a binding angle which is larger than 55°. A calculation under the same assumptions as for anti-BPDE-DNA but at 354 nm (also at the maximum optical absorption) gives a transition dipole moment angle of 65° relative to the DNA helix axis as a lower limit.

In Chart 2C, it is seen that, for both anti- and syn-BPDE-DNA, the reduced LD spectra are not constant over the entire absorption band assigned to a single transition. This observation deserves further attention. In reduced LD spectra, a single transition, which is either ordered uniquely or has several \( \alpha \)'s, should give a constant value LD/A over the whole absorption spectral range of the transition. In the case of components with different absorbances (\( \Delta \lambda \)) but with the same orientation angle (\( \alpha \)), we also expect a constant LD/A over the total spectral range. All other cases (e.g., 2 spectral components with different \( \Delta \lambda \) and different \( \alpha \)) will give a nonconstant reduced LD (8). In our case, the presence of an additional minor species with different LD properties would be expected to show up in the total reduced LD spectrum as peaks or troughs approximately at the peaks of its absorption spectrum.

The reduced LD spectrum of anti-BPDE-DNA has troughs overlaying its positive constant LD component at about 335 and 360 nm, close to the absorption peaks of Component I. The reduced LD spectrum of syn-BPDE-DNA has peaks overlaying its negative constant LD component at about 314, 327, and 346 nm, which coincide with the absorption peaks of Component II.

Taken together with the absorption and fluorescence spectral data, the reduced LD spectra of both anti- and syn-BPDE-DNA are consistent with the 2 types of Spectra I and II in different proportions. The anti-BPDE-DNA is dominated by Component II, which is nearly parallel with the helix axis (\( \alpha < 35° \)), whereas the syn-BPDE-DNA has Component I as its major part, which has its plane more perpendicular to the helix axis (\( \alpha > 65° \)).

Denaturation of BPDE-DNA With Heat and Formaldehyde. In denatured DNA, the anti-BPDE-DNA adduct was more stable than was the syn-BPDE-DNA adduct.

The resulting absorbance and fluorescence excitation spectra from anti-BPDE-DNA are shown in Chart 4. Both are similarly shifted toward longer wavelengths and have peaks at 316, 334, and 351 nm (spectrum type III). The intensity of the fluorescence spectrum increased by a factor of 2.3 to 2.6 compared to the undenatured sample for both the anti- and syn-BPDE-DNA complexes.
Titrations With Ag+. Ag+ ions at [Ag+]/[DNA phosphate] ratios below 0.5 bind preferentially to the DNA bases and have been used as a probe (internal quencher of fluorescence) for chromophores which form classical intercalative complexes with DNA (10). A conformational change of the DNA accompanies the binding of Ag+ to DNA (3).

To monitor the conformational changes caused by Ag+ on pure DNA, we measured the LD and CD of DNA with and without added Ag+ to a concentration corresponding to 0.5 Ag+ per DNA phosphate. The results are shown in Chart 5. The LD spectrum shows that the DNA is still oriented in the flow field; thus, the DNA does not aggregate upon addition of silver ions. The CD signal completely changed its shape and increased about 10 times upon addition of silver, indicating an internal conformational change (3).

We previously found a significant increase in the fluorescence of anti-BPDE-DNA after addition of Ag+ (42). We now recorded fluorescence excitation spectra at different Ag+ concentrations for anti-BPDE-DNA, syn-BPDE-DNA, and denatured anti-BPDE-DNA. For the following evaluation, we have assumed that the spectra of both native complexes are composed of Components I and II and have adopted a similar mode of analysis as described for the light absorption spectra.

Chart 6 depicts the calculated fluorescence intensities of the major spectral components II of anti-BPDE-DNA and I of syn-BPDE-DNA, respectively. The measurements on the denatured anti-BPDE-DNA are also included in the chart. With increasing concentrations of Ag+ added to the anti-BPDE-DNA, the type II spectrum increases significantly. Addition of silver to the syn-BPDE-DNA sample also results in increased intensity of the type II spectrum (data not shown). The type I spectral component of syn-BPDE-DNA is slightly decreased by Ag+ (Chart 6). The spectrum of denatured anti-BPDE-DNA is strongly quenched by Ag+. The denatured syn-BPDE-DNA shows the same properties (data not shown).

The fluorescence of the DNA samples with added Ag+ remained unchanged after 2 extractions with ethyl acetate. These measurements show that the type II spectral component must be strongly quenched by native DNA at its binding site, since the change of DNA conformation induced by Ag+ can cause the observed large increase in fluorescence intensity. The chromophores giving the type III spectrum must be in close contact with the single-stranded DNA bases, since here, Ag+ acts as an internal fluorescence quencher.

Fluorescence Quenching By O2. The fluorescence quencher O2 has been used as a probe for externally DNA-bound chromophores (23). O2 does not penetrate into or bind to the DNA helix; therefore, mainly chromophores bound on the outside of DNA will be affected. The rate of quenching can be evaluated using the Stern-Volmer equation (32)
where $F$ and $F_0$ are the measured fluorescence intensities with and without added quencher $Q$ and $K$ is the Stern-Volmer quenching constant.

Chart 7 shows Stern-Volmer plots for $O_2$ quenching of Components I and II of syn-BPDE-DNA and anti-BPDE-DNA, respectively, calculated as described above from measurements at the excitation wavelengths 354 and 345 nm (see Ag$^+$ titrations). The plot for Component II of anti-BPDE-DNA is curved for the $O_2$ quenching, indicating the existence of chromophores with different quenching constants $K$, although with the same spectra. Component I of syn-BPDE-DNA is similarly quenched by $O_2$. The fluorescence quenching of denatured anti-BPDE-DNA is strong for $O_2$. Again, the curvature indicates that several chromophoric species with different quenching constants are present.

It is not possible from the quenching results alone to draw any conclusions about the accessibility of the quencher to the DNA-bound chromophores. The fluorescence lifetimes of the chromophores have to be known, since the Stern-Volmer quenching constant is proportional both to the encounter frequency between the quencher and the chromophore and to the lifetime of excited state of the chromophore.

Fluorescence Decay Measurements. With the aim to further characterize different species of DNA-bound BPDE and evaluate the $O_2$ quenching experiments, we measured the fluorescence decay of anti-BPDE-DNA and syn-BPDE-DNA. The decay data were recorded with an apparatus using the single-photon counting technique. Chart 8 shows the results of a typical decay measurement and evaluation of anti-BPDE-DNA. The decay time and amplitude parameters are presented in Table 1.

For syn-BPDE-DNA, the excitation was at 355 nm, which is close to the absorption maximum of the type I spectrum. Two exponential terms were sufficient to make a good fit between the experimental decay and the model. The fast-decaying component with an estimated lifetime of 2.7 nsec is responsible for about 80% of the absorption at the excitation wavelength 355 nm, assuming there is no static quenching of the DNA-bound chromophores. Since the lifetime of the free chromophore (BPT) in water solution is about 200 nsec, this component represents chromophores which are strongly quenched by the DNA.

The fluorescence decay curve of anti-BPDE-DNA after excitation at 335 nm could not be fitted satisfactorily with 2 exponential terms ($\chi^2 = 3.04$). Three terms were needed to obtain a good fit ($\chi^2 = 1.11$). This indicates that there are at least 3 different fluorescence species in the anti-BPDE-DNA. There are 2 major components with lifetimes of 1.6 and 7 nsec, respectively. The third component, which is less quenched by the DNA, represents only a minor fraction of the chromophore molecules seen by fluorescence. Its relative amplitude varied from 3 to 12% in different preparations, whereas its contribution to the total steady-state fluorescence was in the range of 20 to 80%.

In the decay measurements on anti-BPDE-DNA with added silver ions, the component with the shortest decay time disappears completely and the amplitude of the longest lifetime component increases about 10 times. Evidently, the chromophores experience different microenvironments at the binding sites on DNA upon addition of silver ions.

Although no lifetime measurements could as yet be made on samples quenched with oxygen, we could make rough estimates of the limiting quenching constants for the oxygen quenching by combination of the results from the steady-state fluorescence.

![Chart 7](chart7.png)

**Chart 7.** Stern-Volmer plots of oxygen quenching of the fluorescence of BPDE-DNA samples. Native anti-BPDE-DNA Component I ( ): native anti-BPDE-DNA Component II ( ) ; denatured anti-BPDE-DNA ( ). The fully drawn curves in Chart 7 represent theoretical Stern-Volmer quenching curves, calculated as described in the text. The $\alpha_3$ (see text) of anti-BPDE-DNA was somewhat higher than given in Table 3 for the preparation used in the $O_2$ quenching measurements; therefore, $\alpha_3 = 0.12$ was used in the calculation. The samples were prepared and the fluorescence was measured as described in the text, with $\Delta A_{380} = 0.66$ and approximately 1 BPDE residue per 250 DNA bases. $F_0/F$ is the ratio between the intensities without and with the quencher. The excitation wavelength was 345 or 354 nm, and the emission was observed at 420 nm.

![Chart 8](chart8.png)

**Chart 8.** Fluorescence decay curves after excitation with a short flash. Computed decay of anti-BPDE-DNA in 10 mM cacocylate buffer, pH 7.0 ( ), with $\Delta A_{380} = 0.66$ and approximately 1 BPDE residue per 250 DNA bases. The parameters used in the computation are indicated in Table 1. The fluorescence decay of 0.5 $\mu$m POPOP in benzene ( ). This decay curve was used to generate the apparatus response function, as described in "Materials and Methods." Top curve, weighted residuals. The excitation wavelength was 335 nm, and the emission was observed at 402 nm. The counts for the POPOP sample have been divided by 10.
Typical fluorescence decay parameters for BPDE-DNA samples

The samples were prepared and the decay parameters estimated as described in "Materials and Methods." Amplitudes ($a_i$) are normalized to unity. A confidence interval on the 95% significance level is given for those parameters that did not correlate strongly with each other (see also "Materials and Methods"). Repeated measurements on different preparations of the same system (anti-BPDE-native DNA, 4 samples; anti-BPDE-native DNA plus Ag⁺, 3 samples; anti-BPDE-denatured DNA, 3 samples; and syn-BPDE-native DNA, 1 sample) gave results with parameter values typically within ±30% of one another within each group.

<table>
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<th>System</th>
<th>$a_1$ (nsec)</th>
<th>$a_2$ (nsec)</th>
<th>$a_3$ (nsec)</th>
<th>$x^2$</th>
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<tr>
<td>BPT in H₂O</td>
<td>0.52 ± 0.04</td>
<td>0.42 ± 0.04</td>
<td>0.35</td>
<td>1.0</td>
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<tr>
<td>anti-BPDE-native DNA</td>
<td>1.6 ± 0.3</td>
<td>7.0 ± 0.6</td>
<td>0.27 ± 0.02</td>
<td>199 ± 1</td>
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<tr>
<td>anti-BPDE-native DNA + Ag⁺</td>
<td>0.35</td>
<td>17</td>
<td>9.2 ± 1</td>
<td>42</td>
</tr>
<tr>
<td>anti-BPDE-denatured DNA</td>
<td>1.1 ± 0.2</td>
<td>0.24 ± 0.04</td>
<td>0.24</td>
<td>67</td>
</tr>
<tr>
<td>syn-BPDE-native DNA</td>
<td>2.7 ± 0.4</td>
<td>31 ± 2</td>
<td>0.24</td>
<td>1.15</td>
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</table>

* Mean ± S.D.
* At an [Ag⁺]/[DNA phosphate] ratio of 0.5.

Fluorescence and Linear Dichroism of BPDE-DNA

Our LD measurement on syn-BPDE-DNA, which gives a negative type I spectrum and $\alpha > 65^\circ$, is further evidence of an intercalated complex. Comparison of Charts 2A and 2B reveals for syn-BPDE-DNA an apparent absence in the LD spectrum of the type II spectral component which is present in the light absorption spectrum. It is calculated that about 35% of the bound chromophores contribute as spectral Component II to the light absorption spectrum. If these syn-BPDE molecules are bound with $\alpha \leq 55^\circ$, the peaks at 327 and 346 nm in the LD and reduced LD spectra of syn-BPDE-DNA are well explained. Since the type II absorption spectrum also contributes at 354 nm, it follows that the $\alpha$ value of 65° calculated for the type I chromophore is an underestimate.

The absorption spectrum of anti-BPDE-DNA is shifted and broadened in comparison with a spectrum from BPT in aqueous solution. This broadening is indicative of a heterogeneity in the binding sites.

Also, in the case of anti-BPDE-DNA, the reduced LD is not constant over the absorption range of the A → L' transition. The significance of the troughs at 335 and 360 nm in the reduced LD is difficult to assess, but they could arise from a minor species of DNA-bound BPDE molecules which display a type I spectrum and are oriented nearly parallel to the DNA bases. The magnitude of the reduced LD at 345 nm indicates, however, that the majority of chromophores are bound with the long symmetry axis of the pyrene moiety at an angle of less than 35° to the DNA helix axis.

We may conclude that in the covalent reaction product(s) of syn-BPDE and DNA, the spectral Component I is dominating, with a significant contribution also of Component II, whereas the anti-BPDE-DNA complex is an almost pure Component II type, with only a minor contribution of Component I. The spectral Component I may be due to classic intercalation, with the molecular plane of the chromophore being close to perpendicular to the DNA helix axis. Component II has a very different geometry,
with the transition moment vector of the chromophore forming only a small angle with the DNA helix axis.

As has been pointed out by Hogan et al. (15), the calculated angles $\alpha$ need not be the angle between the DNA helix axis and the transition moment vector of the pyrene moiety at the binding site, since the DNA may be bent at the binding site. The $\alpha$ value may rather represent an average of angles between straight DNA segments and the chromophoric transition moment vectors.

Heat denaturation of the DNA with bound chromophores changes the spectral properties and the fluorescence intensities considerably. The type III spectrum, which results from both the anti- and syn-BPDE-DNA, is consistent with a complex in which changes the spectral properties and the fluorescence intensities considerably. The type III spectrum, which results from both the anti- and syn-BPDE-DNA, is consistent with a complex in which the chromophore is sandwiched between bases on a single DNA strand. Such a structure was proposed by Frenkel et al. (6) for a BPDE-dinucleotide monophosphate complex.

The spectral types of BPDE-DNA complexes identified by their light absorption, fluorescence excitation, and LD spectra are given in Table 3.

Next, we consider the titrations with silver ions and the fluorescence quenching by oxygen ions together with the results of lifetime measurements. We have earlier reported that the addition of silver ions to anti-BPDE-DNA causes an increased fluorescence intensity (42). The data presented here on the Component I spectrum of anti-BPDE-DNA is in full agreement with the previous observations. The fluorescence increase was interpreted in terms of a DNA conformational change induced by silver ions which forced the BPDE chromophoric moiety from a binding with strong quenching interaction with the DNA bases to a geometry with less such quenching.

That the fluorescent chromophores in the presence of silver ions ([Ag$^+$]:[DNA phosphate] ratio, 0.5) really are exposed on the outside of the DNA was confirmed in a separate $O_2$ quenching experiment (data not shown). The chromophores are easily accessible with $k_q \approx 5 \times 10^9$ M$^{-1}$ sec$^{-1}$, which is only a factor of 2 below the value expected for free diffusion-controlled quenching.

The small change in light absorption of DNA itself caused by addition of silver ions (Chart 5) is indicative of the persistence of a double-stranded structure after complexing with Ag$^+$. The strong LD observed shows that good orientation occurs in the flow field and suggests that the structure must still be rather extended and rigid. In presence of Ag$^+$, the LD becomes positive below 260 nm, and the CD spectrum shows a very pronounced trough at 270 nm. This is best explained as caused by a tilt of the bases induced by the binding of silver ions, as also suggested by Dattagupta and Crothers (3). The tilt would orient some of the transition moment vectors of the bases at an angle less than 55° to the DNA helix axis, thereby giving a positive LD in the spectral region of the transitions. The tilt would also facilitate a coupling of oscillators along the DNA helix, giving the increased rotatory strength we observed for the DNA:Ag$^+$ complex.

The model proposed for the effect of Ag$^+$ complexing to anti-BPDE-DNA is further supported by the measurements of fluorescence lifetimes of the bound chromophores (Table 1). In the presence of Ag$^+$, the fastest decaying component is completely abolished, and the lifetimes of the other 2 components are increased considerably. The only spectral change observed in the presence of Ag$^+$ is a sharpening of the lines and an increase in intensity of Component II. Also, in the case of syn-BPDE-DNA, the addition of Ag$^+$ leads to an increase of the type II component in the fluorescence excitation spectrum, which most likely is also due to unquenching of chromophoric species when DNA changes conformation. The decrease in intensity of the type I component is not significant enough to permit safe conclusions concerning quenching by Ag$^+$ ions in this case.

The Stern-Volmer plots showing the fluorescence quenching of anti-BPDE-DNA by oxygen are curved. This fact indicates the existence of 2 or more chromophoric species which display different quenching constants.

The estimated bimolecular quenching constants from the oxygen quenching of the steady-state fluorescence (Table 2) reveal that both for anti-BPDE-DNA and syn-BPDE-DNA, there are chromophoric species which are relatively inaccessible to oxygen. Since oxygen is a small uncharged quencher, these chromophoric species must be sterically protected from collisions with the oxygen molecules, e.g., by insertion into the DNA helix. This is most pronounced for syn-BPDE, where about 80% of the chromophores show a quenching which is 7-fold lower than the diffusion-controlled limiting quenching (cf. Table 1). Only the long lifetime component of anti-BPDE-DNA seems to be freely quenched by oxygen. The denatured anti-BPDE-DNA shows an intermediate quenching of the 2 longer lifetime components. It should be noted that for the shortest lifetime components, the estimated $k_q$ values are uncertain due to the low fluorescence yield from these components.

For the major type II component of anti-BPDE-DNA, fluorescence decay measurements reveal at least 3 components. About 50% of the chromophores are strongly quenched by the DNA. The interaction causing this strong quenching is probably different from the interaction shifting the absorption spectrum, since the quenched chromophores are of type II that has only a small shift. The nature of this interaction is still obscure. One possibility would be that it is related to the strong electric field gradient...
from the phosphate groups of DNA. Another alternative would be that this fraction of DNA-bound chromophores has a wedge-like geometry in a bent DNA helix, as suggested by Hogan et al. (15). This would give an intermediate form of interaction between the bases and the chromophore, which could explain the strong fluorescence quenching that the native DNA exerts on the chromophore. At the same time, this would also be in accord with the positive LD spectrum of anti-BPDE-DNA.

For syn-BPDE-DNA, the major type I component is, in agreement with the proposed intercalation, much less accessible to quenching by O₂ than is the type II component of anti-BPDE-DNA. The reason for the small effect of Ag⁺ on the type I component of syn-BPDE-DNA is, however, not clear.

Upon heat denaturation, both anti- and syn-BPDE-DNA give rise to type III spectra. To obtain the long-wavelength shift observed, the chromophores most probably would be sandwiched between bases of the DNA. The strong fluorescence quenching by Ag⁺ is in accord with such a structure. Because of its size, a sandwiched chromophore is still exposed and rather well accessible to O₂ quenching, which is consistent with the fairly large quenching constants (Table 2).

The present investigation demonstrates that it is possible and meaningful to describe anti- and syn-BPDE-DNA complexes in terms of 2 spectral types I and II and further subcategories according to fluorescence lifetime components and their response to various quenching agents. This classification is not claimed to be rigorous, since each component is far from homogeneous. Since anti-BPDE is much more carcinogenic than syn-BPDE (2), the properties of the anti-BPDE-DNA complexes and their differences relative to the syn complexes or of the corresponding single-stranded species, possibly mimicked by the heat-denatured BPDE-DNA, might be most relevant, depending on the level at which the interference with normal physiological activity takes place.

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