# Electric Linear Dichroism Study on the Orientation of Benzo[*a*]pyrene-7,8-dihydrodiol 9,10-Oxide Covalently Bound to DNA†

N. E. Geacintov,* A. Gagliano, V. Ivanovic, and I. B. Weinstein

ABSTRACT: The electric linear dichroism spectra of native calf thymus DNA modified to a small extent (1 hydrocarbon residue per 1000 bases) by reaction with (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), which binds covalently mainly to the 2-amino group of guanosine residues, and physical complexes of benzo[*a*]pyrene (BP) and proflavin (PF) with DNA were measured. The linear dichroism $\Delta A$ of the covalent BPDE-DNA complex in the wavelength region of the absorption of the pyrene-like BPDE chromophore is positive. In contrast, $\Delta A$ is negative in the absorption region of the DNA bases, as well as in the absorption region of the BP and PF molecules physically bound to DNA. It is concluded that the orientation of the BPDE moiety is not of the intercalation type as is the case for the physical BP and PF complexes. The reduced linear dichroism is wavelength dependent for the BPDE-DNA and BP·DNA complexes which indicates that there is a heterogeneity of binding sites with different orientations. The quantitative analysis of such results is discussed in detail and it is concluded that there is one major type of oriented BPDE covalently bound to DNA. The long axis of the pyrene-like chromophore of BPDE lies on the surface of a cone whose axis is that of the DNA helix and whose angle, with respect to this axis, is 35° or less. As expected for intercalation-type complexes, the planes of BP and PF are found to be nearly parallel to the planes of the DNA bases, according to this analysis.

Polycyclic aromatic hydrocarbon[1] (PAH) carcinogens are known to bind covalently to nucleic acids in vivo. In living cells the relatively inert PAH molecules are metabolically converted to a variety of hydroxy and epoxy derivatives (Jerina & Daly, 1974). The major reactive intermediate involved in nucleic acid binding in the case of benzo[*a*]pyrene (BP) is a 7,8-dihydrodiol 9,10-oxide metabolite (Borgen et al., 1973; Sims et al., 1974; Daudel et al., 1975; King et al., 1976; Ivanovic et al., 1976; Weinstein et al., 1976) and the structure of its (±)-7β,8α-dihydroxy-9α,10α-epoxy isomer (BPDE) is shown in Figure 1.

The covalent binding of the bulky PAH residue to native DNA alters the local structure of the DNA (Pulkrabek et al., 1977) and its template activity in vitro (Leffler et al., 1977; Hsu et al., 1977). In order to understand how the function of the modified DNA differs from that of normal DNA, it is desirable to have a knowledge of the complete physicochemical structure and properties of regions of DNA containing the covalently bound BPDE.

In this work we have investigated the orientation of the BPDE chromophore (with respect to those of the DNA bases) in BPDE-DNA complexes. In the present study we have modified native calf thymus DNA to a small extent (one hydrocarbon residue per 1000 bases) by in vitro reaction with BPDE using the methods described elsewhere (Pulkrabek et al., 1977; Prusik et al., 1978). The complete structure and absolute stereochemistry of the guanosine-BPDE adduct formed by in vitro reaction with RNA and DNA has been elucidated (Weinstein et al., 1976; Jeffrey et al., 1976, 1977; Nakanishi et al., 1977; Koreeda et al., 1976). The 10 position of the BPDE moiety is linked to the 2-amino group of guanine. Furthermore, this appears to be the prinicpal DNA reaction product in vitro (Prusik et al., 1978), and it has the same chemical characteristics as the major BP-DNA adduct formed in vivo, when human and bovine bronchial explants are exposed to BP (Jeffrey et al., 1977).

Based on fluorescence quenching studies we have provided evidence that the BPDE moiety in these covalent BPDE-DNA complexes is located on the outside of the DNA helix (Prusik et al., 1978). Using the electric field induced linear dichroism technique (Fredericq & Houssier, 1973), we show here that the long axis of the pyrene-like chromophore (Figure 1) is *not* parallel to the planes of the DNA bases as in the case of intercalation-type complexes such as physically bound complexes of DNA and BP, or proflavin (PF).

In the electric field induced linear dichroism technique a voltage pulse is applied to an aqueous buffer solution of either the covalent BPDE-DNA complex or the physical BP·DNA or PF·DNA complexes. The electric field thus produced within the solution tends to orient the DNA and the polycyclic molecules which are either covalently or physically attached to the DNA molecule. Using polarized light, the absorbance is determined with the electric field vector of the polarizer oriented either vertically or parallel with respect to the electric field. The *relative* orientations of the transition moment vectors, which lie within the planes of the DNA bases and the polycyclic molecules, are then compared. The results indicate that there is a heterogeneity of binding sites with respect to the orientation angles of the BPDE chromophore, and the interpretation of linear dichroism spectra under these conditions is discussed in detail. It is shown that the usual straightforward approach (Chang et al., 1974; Fuchs et al., 1976) to calculate orientation angles from linear dichroism spectra must be modified to take this heterogeneity into account. It is shown under certain conditions, such as in the case of the covalent BPDE-DNA complexes, that a range for the most probable angle of orientation of the BPDE chromophore can be estimated.

† From the Chemistry Department and Radiation and Solid State Laboratory, New York University (N.E.G. and V.I.), New York, New York 10003, Departement de Biologie, Centre d'Etudes Nucleaires de Saclay (N.E.G. and A.G.), 91190 GIF sur Yvette, France, Universite Paris XI, Institut Universitaire de Technologie (A.G.), Cachan, France, and the Division of Environmental Sciences and Institute of Cancer Research (V.I. and I.B.W.), Columbia University, New York, New York 10032. *Received April 28, 1978.* This investigation was supported by Grant No. CA-20851-01 to N.E.G. and Grant CA-21111-01 to I.B.W. awarded by the National Cancer Institute, Department of Health, Education and Welfare. Partial support from a Department of Energy contract at the Radiation and Solid State Laboratory is gratefully acknowledged.

[1] Abbreviations used: PAH, polycyclic aromatic hydrocarbon; BP, benzo[*a*]pyrene; BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; AAF, *N*-acetoxy-*N*-2-acetylaminofluorene; ED, electric dichroism; DNA-BPDE, covalent complex of DNA and BPDE; PF·DNA and BP·DNA, physical complex between DNA and proflavin or benzo[*a*]pyrene, respectively.

0006-2960/78/0417-5256$01.00/0 

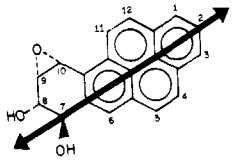

FIGURE 1: Structure of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE). The heavy arrow indicates the transition moment vector of the 310-350-nm absorption band.

## Experimental Procedures

### *Materials*

BPDE was kindly provided by Dr. Ronald G. Harvey of the University of Chicago. Native calf thymus DNA was purchased from Worthington Co., Freehold, N.J., and had the following characteristics: protein content 0.1%, hyperchromicity 42%, and $s_{20,w} = 22$.

### *Methods*

*Preparation of DNA Complexes.* The proflavin-DNA complex was an aqueous solution containing sodium cacodylate buffer, pH 7.1 ($5 \times 10^{-3}$ M), DNA ($1.5 \times 10^{-3}$ M), and proflavin ($1 \times 10^{-5}$ M). At such low concentrations of PF, this dye is fully solubilized by DNA. The intercalated PF showed the characteristic 18-nm red shift in the absorption spectrum. The physical complex between BP and DNA was prepared as previously described (Geacintov et al., 1976).

In the previous electric dichroism studies, controversial results have been obtained, due to the denatured state of DNA and/or extensive modification of DNA by *N*-acetoxy-*N*-2-acetylaminofluorene (AAF) (Chang et al., 1974; Fuchs et al., 1976). Therefore in the present study, special care has been taken in preparing the in vitro BPDE-DNA samples. The modification of DNA with BPDE was essentially the same as described by Pulkrabek et al. (1977). BPDE ($10^{-4}$ M) and DNA (10 $A_{260}$ units/mL) were incubated in 95:5 (v/v) water:ethanol containing $5 \times 10^{-3}$ M sodium cacodylate buffer, pH 7.1. The modified DNA samples contained 1 residue of covalently bound BPDE per 1000 nucleotide residues, as detected by UV measurements and confirmed in separate studies using [$^3$H]BPDE (Ivanovic, V., unpublished studies). Electric dichroism measurements were performed at 20 °C on BPDE-DNA samples in aqueous solutions containing $5 \times 10^{-3}$ M sodium cacodylate buffer, pH 7.1, with concentrations of DNA in the range 1-10 $A_{260}$/mL, with no detectable concentration effects in this range on the *relative* reduced linear dichroism values measured at 260 nm (DNA absorption) and at 345 nm (BPDE absorption). There is, however, an effect of DNA concentration on the reduced linear dichroism within the absorption band of DNA, as previously reported by Yamaoka & Charney (1973).

*Chemical Characterization of the DNA-BPDE Sample.* The base specificity of the binding of BPDE was determined by enzymatic hydrolysis of the modified DNA and high-pressure liquid chromatography of the modified deoxyribonucleosides. The same stereoselective and chemically homogeneous binding of BPDE to native DNA was observed (Prusik et al., 1978) as reported earlier for human and bovine bronchial explants (Jeffrey et al., 1977). The single BPDE-nucleoside adduct obtained results from trans addition of the 2-amino group of guanine to the 10 position of the 7*R* enantiomer of BPDE or (+)-BPDE derived from (−)-BP-7,8-dihydrodiol.

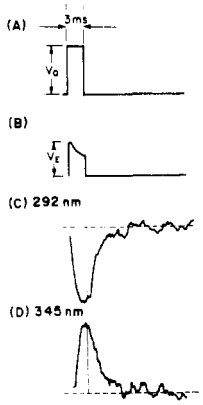


FIGURE 2: Typical voltages and linear dichroism signals (oscilloscope tracings). (A) Voltage $V_0$ without cell connected (3-ms pulse duration). (B) Effective voltage ($V_E$) with cell connected. At the beginning of the pulse there is a 30% voltage drop ($V_E \approx 0.7V_0$); the effective voltage decreases further to $V_E = 0.40V_0$. The effective voltages $V_E$ given in Figure 3 represent the values taken approximately halfway between the maximum and minimum values of $V_E$. Sample: covalent BPDE-DNA complex, 0.5% modification, $A_{260/mL} = 4.1$ in 5 mM sodium cacodylate buffer, pH 7.1. Cell resistance: 300 Ω. (C) Linear dichroism signal at 292 nm (due to DNA). $\Delta A$ is negative. Same sample as in B; $V_E \approx 200$ V. (D) Linear dichroism signal at 345 nm (due to the pyrene-like chromophore attached to the DNA). Same sample as in B. Dichrograph sensitivity is five times greater than in B above.

*Apparatus.* The voltage source was a specially constructed transistorized power supply which is capable of delivering unipolar square wave pulses of up to 4000 V with a duration variable from 10 μs to 10 ms over a 1000-Ω load. The sample holder consisted of a 1 × 1 cm quartz cuvette containing the electrodes mounted on a Teflon block similar to the one described by Fredericq & Houssier (1973). The electrodes consisted of two parallel platinum strips (2 × 1 cm wide) which were held 3 mm apart by Teflon spacers. This entire assembly was immersed in the cuvette containing the sample. A thermocouple was inserted into a hole in one of the Teflon spacers and was used to monitor the temperature of the solution.

The linear dichroism was measured using a dichrograph constructed by Breton et al. (1973) at Saclay. In this apparatus, light from a xenon lamp is passed through a grating monochromator followed by a Glan polarizer. A photoelastic modulator (PEM 3, Morvue, Tigard, Ore.) whose driving voltage was automatically adjusted to produce a half-wave retardation at each wavelength was used to produce vertically and horizontally polarized light at a frequency of 100 KHz. The light beam, thus modulated, then passes through the sample cuvette and is incident onto a photomultiplier tube. The 100-KHz component of the photomultiplier tube output is demodulated using a lock-in amplifier which delivers a signal whose magnitude is proportional to the linear dichroism of the sample. Absolute calibration of the apparatus was obtained by using two polarizers. The sensitivity of the apparatus is such that a difference in the alignment of the two polarizers by only 5 min of arc (corresponding to a linear dichroism of $6 \times 10^{-5}$, or 60 ppm) could be readily detected.

The demodulated linear dichroism signal was fed into an oscilloscope for viewing. When necessary, it was fed into an Intertechnique Didac 800 multichannel analyzer for signal averaging. Typically, 3-5 scans were used to obtain a satisfactory signal/noise ratio. The duration of the square unipolar voltage pulses was 3 ms (apparatus response time 1 ms). Typical signals are shown in Figure 2. The wavelength dependence of the linear dichroism was recorded point by point.

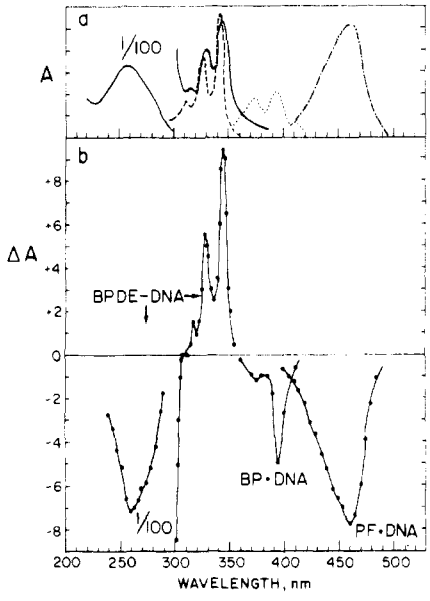


FIGURE 3: Absorption and linear dichroism spectra of PAH–DNA complexes. (A) Absorption spectra (A) of covalent BPDE-DNA complex (BPDE-DNA) (—); BPDE in ethanol (- - -); physical complex of benzo[*a*]pyrene in DNA (BP·DNA) (• • •); proflavin·DNA complex (— • —). (B) Linear dichroism ($\Delta A$) spectra; 700 V cm$^{-1}$; 5 mM cacodylate buffer solution. (In both A and B, the BPDE–DNA solutions were diluted in order to make measurements in the DNA absorption region of 240–300 nm.) BPDE-DNA; $A_{260}$ = 10 units/mL (1 BPDE/1000 bases), BP·DNA; $A_{260}$ ≃ 6.0 units/mL (~1 BP/1000 bases), PF·DNA; $A_{260}$ ≃ 10.0 units/mL (~7 PF/1000 bases). All samples were dissolved in 5 mM cacodylate buffer. $V_E$ ~ 200 V (the effective voltage is defined in the legend of Figure 2.) Cell resistance: 300 Ω.

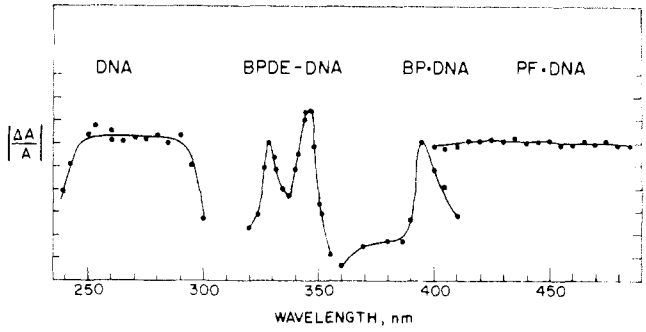


FIGURE 4: Reduced linear dichroism spectra for the DNA complexes shown in Figure 3; $\Delta A/A$ is in relative units.

## Results

*1. Qualitative Aspects of ED Measurements.* The absorption spectra, $A$, and the linear dichroism spectra, $\Delta A$, of the covalent BPDE-DNA and the physical BP·DNA and PF·DNA complexes are shown in Figure 3.

The linear dichroism $\Delta A$ is defined by

$$\Delta A = A_{\parallel} - A_{\perp} \qquad (1)$$

where $A_{\parallel}$ is the absorbance of the sample with the polarization vector of the light oriented parallel to the electric field $\vec{\mathbf{E}}$ and $A_{\perp}$ refers to the absorption when the polarization vector is perpendicular to $\vec{\mathbf{E}}$. As usual, the absorbance $A$ is defined by log $(I_0/I)$, where $I_0$ is the intensity of the incident light and $I$ is the intensity of the light after passing through the sample.

The absorption spectrum of the physical complex between proflavin and DNA within the absorption region of the proflavin chromophore, is shown in Figure 3A. The linear dichroism spectrum $\Delta A$, is shown in Figure 3B. The sign of the linear dichroism signal is negative. Within the absorption band of the DNA bases between 240 and 290 nm, $\Delta A$ is also negative as is demonstrated in Figure 3B. In order to observe this linear dichroism, it was necessary to dilute the samples to bring the optical density into the range of ~0.10. The contribution of the proflavin molecules to the total absorbance at 255–260 nm was negligible at these concentrations. Since the transition moment vectors of both the PF and the DNA bases are oriented within the planes of the aromatic rings, a negative $\Delta A$ (Figure 3B) is expected for both PF and the DNA bases if the PF residues are bound via intercalation since the axis of the helix tends to align itself parallel to the electric field.

BP, when physically bound to DNA, is believed to form an intercalation complex (Arcos & Argus, 1968). The linear dichroism spectrum for BP in the BP·DNA complex follows the absorption spectrum of BP only qualitatively (Figure 3). There is a sharp peak at 394 nm and a weaker one at 374 nm, corresponding to the absorption bands of BP bound to DNA. The transition moment vector in the 390–400-nm absorption region of BP lies within the plane of the aromatic ring. Therefore, the negative ED signal observed for DNA·BP (Figure 3B) is expected for the intercalation model proposed for this physical complex.

The absorption spectrum of the BPDE-DNA complex (Figure 3A) reveals the typical pyrene-like absorption spectrum (Jennette et al., 1977), with peaks at 330 and 347 nm and a shoulder at 325 nm. The BPDE moiety bound covalently to DNA displays a 2–3-nm red shift and a wider line width when compared with free BPDE in ethanol solution (Figure 3A). The broader line width is indicative of inhomogeneous broadening. Such a broadening is due to the overlapping of different absorption bands, slightly displaced with respect to each other in wavelength, due to different physicochemical microenvironments of the chromophore. This observation will be further discussed below (see also Appendix).

The linear dichroism spectrum $\Delta A_\lambda$, presented on Figure 3B of the BPDE chromophore is positive, which is in contrast to the negative sign displayed by the DNA bases. The absorption of the pyrene-like chromophore in the 310–350-nm region corresponds to an excitation to the second excited electronic state. This transition is polarized along the long symmetry axis of the pyrene chromophore which passes through the central carbon atoms and is oriented within the plane of the aromatic ring system (Becker et al., 1963; and Figure 1). These results indicate that the BPDE-DNA covalent complex is not of the intercalation type and that the plane of the aromatic moiety is not perpendicular to the long axis of the DNA helix.

*2. Quantitative Aspects of ED Measurements.* a. Reduced Linear Dichroism Spectra. The reduced linear dichroism, $\Delta A/A$, for all three complexes studied is shown in Figure 4. This quantity is constant with wavelength λ for the PF·DNA complex, but varies strongly as a function of λ for the BPDE-DNA and BP·DNA complexes. Before proceeding to a discussion of these results, we shall examine in detail the relationships between the linear dichroism and the angle of orientation of the transition moment vector of the chromophores. This is necessitated by the fact that the $\Delta A/A$ spectrum of the BPDE-DNA complex varies with wavelength which, as is shown below, is due to a heterogeneity of orientation angles and physical binding sites.

b. Reduced Linear Dichroism for a Single Oriented Species. If the chromophore is oriented in such a way that its transition moment vector makes an angle $\theta$ with the electric field $\vec{\mathbf{E}}$, then this angle can, in principle, be calculated from the reduced

linear dichroism $\Delta A/A$ (Fredericq & Houssier, 1973):

$$\frac{\Delta A}{A} = \frac{3}{2}(3\cos^2\theta - 1)f(\vec{E}) \qquad (2)$$

$A$ denotes the absorbance of light by the sample in the absence of the orienting electric field. The reduced linear dichroism has a value of $+3f(\vec{E})$ for $\theta = 0°$, $-1.5f(\vec{E})$ for $\theta = 90°$, and is equal to zero for the angle ~55°. When $\Delta A/A$ is positive, it can be concluded that the angle $\theta \lesssim 55°$.

The degree of orientation of the molecule or particle depends on the factor $0 < f(\vec{E}) < 1.0$, which in turn depends on the electric field strength and the temperature (Fredericq & Houssier, 1973). For complete orientation, which has not been achieved for DNA solutions even at field strengths of 8000 V $cm^{-1}$ (Fredericq & Houssier, 1973; Yamaoka & Charney, 1973), this orientation function is unity. In our experiments, since we used a concentrated buffer solution, we utilized very low electric fields (the effective voltage was typically 200 V, corresponding to an electric field strength of 700 V $cm^{-1}$). We were limited to these low voltages in order to minimize deleterious heating effects; thus, in these experiments $f(\vec{E}) \ll 1.0$. The orientation angle $\theta$ of a chromophore bound to DNA can, nevertheless, be estimated by comparing the reduced linear dichroism $\Delta A/A$, in the wavelength region of absorption by the chromophore, with the $\Delta A/A$ values at 250–260 nm, where absorption is due to the transition moments of the DNA bases. The transition moment vectors at 250–260 nm are oriented within the planes of the purine and pyrimidine bases, and it is assumed that $\theta$ would be 90° for complete orientation of DNA. (However, an angle of $\theta = 73°$ has been recently proposed by Hogan et al. (1978). This would modify the quantitative conclusions made in this paper to a slight extent as discussed below.) The DNA tends to align itself with the helix axis parallel to the applied electric field (Fredericq & Houssier, 1973; Yamaoka & Charney, 1973), with the planes of the bases perpendicular to $\vec{E}$. This approach was utilized by Chang et al. (1974) and by Fuchs et al. (1976) to deduce the orientation angle $\theta$ of the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene (AAF) bound covalently to DNA. Using the ratio of the reduced linear dichroism for DNA and AAF, where

$$\frac{(\Delta A/A)}{(\Delta A/A)_{DNA}} = \frac{3\cos^2\theta - 1}{3\cos^2 90° - 1} = 1 - 3\cos^3\theta \qquad (3)$$

the orientation factor $f(\vec{E})$ cancels and the orientation angle $\theta$ for AAF was calculated to be 60 ± 4° by Chang et al. (1974) and 82° by Fuchs et al. (1976).

Equation 2 predicts that, within the absorption band of the chromophore, $\Delta A/A$ should be constant and independent of wavelength. This is an important criterion for the interpretation of linear dichroism results according to eq 2 and 3. In such cases the calculation of the orientation angle is straightforward. In the Appendix, we discuss the factors which give rise to a variation of the reduced linear dichroism $\Delta A/A$, as a function of wavelength, and what kind of information can be obtained in such cases.

c. Estimation of the Orientation Angle of the PAH Moiety in the PAH–DNA Complexes. Before proceeding to a full discussion of the results obtained with the BPDE–DNA complexes, we first show, using the physical PF·DNA complex, that the general approach of calculating average orientation angles using eq 3 is justified. The reduced linear dichroism for the PF·DNA complex is shown in Figure 4. Within experimental error, $\Delta A/A$ is constant as a function of wavelength, which is in agreement with the results of Ramstein et al. (1973). According to the discussion presented in the Appendix,

TABLE I: Values of Reduced Linear Dichroism $\Delta A/A$; ~700 V $cm^{-1}$, 5 mM Cacodylate Buffer, ~$10^{-3}$ M DNA Phosphate Concentration, Room Temperature.

| sample | wavelength | $\Delta A/A$ |
|---|---|---|
| DNA | 260 | $-2.8 \times 10^{-2}$ |
| BPDE-DNA[a] | 345 | $+2.7 \times 10^{-2}$ |
| PF·DNA[b] | 460 | $-2.8 \times 10^{-2}$ |
| BP·DNA[c] | 394 | $-2.6 \times 10^{-2}$ |

[a] BPDE-DNA, covalent complex formed by reacting BPDE in vitro with a modification of 0.1% (one BPDE molecule bound per 1000 nucleotides). The reproducibility with three other samples was found to be within 10% of the value given. [b] PF·DNA, physical proflavin–DNA complex. [c] BP·DNA, physical benzo[*a*]pyrene–DNA complex.

this indicates that either case I or II applies. The average orientation angle $\theta_{PF}$ can then be calculated directly using eq 3. This angle should equal 90° for the intercalation model of binding of proflavin to DNA. The absolute values of $\Delta A/A$ for both PF and the DNA bases are given in Table I. Within experimental error (ca. ±5%), these two values are the same, which indicates that the transition moment vectors of PF and the DNA bases are parallel to each other. Thus $\theta_{PF} = 90°$ (or 73° if the base tilt angle of Hogan et al. (1978) is used) which is in agreement with the prediction based on the intercalation model.

As previously mentioned, the BPDE moiety covalently bound to DNA reveals a broader line width in the absorption spectrum due to the overlapping of different absorption bands. We identify these different overlapping absorption bands with the different species characterized by the absorption coefficients, $\epsilon_i$, and concentrations, $C_i$, as discussed in the Appendix. Since $\Delta A_\lambda/A_\lambda$ is wavelength dependent as shown in Figure 4, we assume that case III (see Appendix) applies to the covalent BPDE–DNA complex. In contrast to the wavelength dependence in the 300–350-nm region corresponding to the BPDE chromophore, $\Delta A_\lambda/A_\lambda$ is constant within the DNA absorption wavelength region of 250–290 nm; this latter result is in qualitative agreement with the data obtained by Yamaoka & Charney (1973), and the decreases in $\Delta A/A$ below 250 and above 290 nm have been discussed in detail by these workers.

Since the linear dichroism is positive in the absorbance region of the BPDE moiety for the BPDE–DNA complex, it is possible to conclude unambiguously that $\theta_{BPDE}$ is less than ~55°. However, utilizing the absolute values of the reduced linear dichroism in Table I and the appropriate expressions based on the discussion presented in the Appendix (case III), it is possible to narrow down the possible range of the orientation angle still further.

The narrowness of the vibronic bands in the $\Delta A$ spectrum of BPDE bound to DNA, which compares well with the width of the absorption bands of free BPDE in a homogeneous solution, suggests that the linear dichroism is due to one major spectroscopic species of BPDE bound to DNA. The wavelength variation of $\Delta A_\lambda/A_\lambda$ for BPDE–DNA thus arises because of the presence of other absorbing species of BPDE which contribute less to the linear dichroism spectrum, but give rise to a broadening of the absorption spectrum. Furthermore, the total absorbance measured in the 310–350-nm region contains contributions due to the DNA itself. While the absorbance of DNA itself is weak in this wavelength region, there is considerable light scattering because of the relatively large size of the DNA molecules. This light scattering effect contributes to the

total effective absorbance of the sample, particularly at shorter wavelengths, since the light scattering power is proportional to $\lambda^{-4}$. These two effect, inhomogeneous broadening of the BPDE spectrum due to multiple sites (each displaying different orientation angles), as well as the DNA scattering background, thus contribute to the wavelength dependence of $\Delta A/A$. Referring to the numerator of eq A-1 presented in the Appendix, it appears that, if there is one dominant species, the linear dichroism spectrum $\Delta A_\lambda$ will resemble its absorption spectrum. We assume that this is the case for the $\Delta A_\lambda$ spectrum of the BPDE chromophore in Figure 3B because of the narrowness of the $\Delta A_\lambda$ spectrum which has the same width as the BPDE absorption spectrum in a homogeneous solution; we shall label the orientation angle of this dominant species by $\theta_1$, its absorbance by $A_1$, and its extinction coefficient by $\epsilon_1(\lambda)$. The contributions of the other terms in the numerator of eq A-1 may be small for either of two reasons: (1) the orientation angles $\theta_i$ of the species $i = 2, 3, 4 \ldots$ are close to 55°; for this angle the orientation function is close to zero; (b) the species $i = 2, 3, 4 \ldots$ are randomly oriented. The linear dichroism technique cannot distinguish between these two possibilities. With these assumptions, the reduced linear dichroism is given by

$$\frac{\Delta A_\lambda}{A_\lambda} = \frac{3}{2}\frac{(3\cos^2\theta_1 - 1)\epsilon_1(\lambda)C_1 f_1(\vec{E})}{\Sigma\epsilon_i(\lambda)C_i} = \left(\frac{\Delta A_1}{A_1}\right)\frac{A_1}{\sum_i A_i} \quad (4)$$

Under these circumstances $\Delta A_\lambda$ will reproduce the absorption spectrum of the oriented species due to the presence of the term $\epsilon_1(\lambda)$ in the numerator. Furthermore, the observed reduced linear dichroism $\Delta A_\lambda/A_\lambda$ will be a lower limit to $\Delta A_1/A_1$ since

$$\frac{A_1}{\sum_i A_i} < 1.0 \quad (5)$$

and we can thus write

$$\frac{\Delta A_1}{A_1} > \frac{\Delta A_\lambda}{A_\lambda} \quad (6)$$

Therefore, expression 3, together with eq 6, can be utilized to estimate upper or lower bounds of the angle $\theta_1$. Rearranging eq 3, we obtain the following:

$$\cos^2\theta_1 = \frac{1}{3}\left\{1 - \frac{(\Delta A_\lambda/A_\lambda)_{345}}{(\Delta A_\lambda/A_\lambda)_{DNA}}\right\} \quad (7)$$

where the reduced linear dichroism values for the BPDE and DNA are taken at 345 and 260 nm, respectively, and are presented in Table I. The value at 345 nm is chosen since the oriented species of BPDE gives a maximum contribution to $\Delta A$ at this wavelength and this value is therefore most reflective of the true orientation angle. It should thus be noted that, when there is a heterogeneity of absorbing species, the angles should be evaluated at the highest absorption maxima of the oriented species, rather than at different wavelengths, as is usually done (Chang et al., 1974; Fuchs et al., 1976) when $\Delta A_\lambda/A_\lambda$ is assumed to be constant as a function of wavelength.

Using the absolute values for the reduced linear dichroism for BPDE and DNA given in Table I, the angle of $\theta_1 \approx 35°$ is obtained. (If a DNA base tilt angle of 73° is assumed (Hogan et al., 1978), instead of 90°, $\theta$ is calculated to be 41°.) Keeping in mind the inequality in eq 6, and the fact that the reduced linear dichroism values at 345 and at 260 nm have opposite signs, the value of $\cos^2\theta_1$ calculated in eq 7 is a lower bound and thus the calculated angle is an upper bound. We therefore conclude that the angle of orientation of the long, in-plane symmetry axis of the pyrene chromophore (Figure 1) is given by

$$\theta_{BPDE} = \theta_1 \lesssim 35° \quad (8)$$

The planes of the aromatic chromophore thus tend to be aligned parallel to the surface of a cone whose axis is that of the DNA helix and whose angle is 35° or less. This result was reproducible with four different samples of BPDE-DNA with the concentration of the BPDE moiety varying between 0.1 and 0.5% (based on the nucleotide concentration).

In order to further verify the validity of our approach to calculate the range of orientation angles for the BPDE-DNA complex, we used the same method to estimate the orientation angle of the PAH in BP·DNA complex. The $\Delta A_\lambda/A_\lambda$ spectrum of this complex shown in Figure 4 is wavelength dependent, indicating here also a heterogeneity of absorbing species, and it appears to be more complex than that of the covalent BPDE-DNA complex. Interference from light scattering by the DNA molecules may give rise to relatively large errors in the determination of $\Delta A_\lambda/A_\lambda$ for the physical BP·DNA complex, since the absorbance due to the BP chromophore is quite small in the 350–400-nm region ($A \approx 0.01$–$0.03$) and is thus difficult to determine accurately. Furthermore heterogeneity of binding sites (case III), and possible differences in the polarization of the absorption bands of BP bound physically to DNA, may all contribute to the complexity of the reduced linear dichroism of the BP·DNA complex. The relative contributions of each of these factors is not known and their elucidation is beyond the scope of this work. We will confine ourselves to a semiquantitative analysis of the results in which we utilize the maximum observed $\Delta A_\lambda/A_\lambda$ value (at 394 nm), where the contribution of the BP chromophore to the overall linear dichroism is the highest. As expected for an intercalation model, the sign of $\Delta A_\lambda$ for the BP·DNA is negative. This immediately indicates that $\theta_{BP} > 55°$. Using the approach embodied in eq 7 and the data in Table I, the possible range of angles can be narrowed down still further. We obtain

$$\theta_{BP} \gtrsim 81° \quad (9)$$

Keeping in mind that the value of $\Delta A_\lambda/A_\lambda$ at 394 nm is a lower bound and that the signs of the reduced linear dichroism at 260 and at 394 nm are the same, the angle of 81° is a *lower* bound value, as indicated in eq 9. (If a DNA base tilt angle of 73° is assumed (Hogan et al., 1978) instead of 90°, this angle comes out to be $\theta_{BP} \gtrsim 71°$.) Since such an angle is expected for the intercalation model of the physical binding of BP to DNA, this result supports the validity of the calculations for BPDE bound covalently to DNA.

## Discussion

The electric linear dichroism spectrum of BPDE covalently bound to DNA shows that the pyrene ring system is not intercalated between the base pairs of DNA. A quantitative analysis of the data indicates that the plane of the pyrene-like BPDE chromophore lies on the surface of a cone, whose axis is that of the DNA helix and whose angle is 35° or less.

The validity of the quantitative analysis of the electric linear dichroism data, on which the above results are based, was verified by examining the linear dichroism spectra of the intercalation type complexes that are formed when either proflavin or benzo[*a*]pyrene are physically bound to DNA. The same method of approach yields an angle of 90° for the PF and $\gtrsim 81°$ for the BP transition moment vectors, with respect to the long axis of the DNA helix. Since these transition moment vectors lie within the planes of the aromatic ring systems, these

results are totally consistent with the intercalation model of binding for these physical complexes. These results are in striking contrast to those obtained when the identical method of linear dichroism and data analysis are applied to the covalent BPDE–DNA complex.

The variation of the reduced linear dichroism $\Delta A_\lambda/A_\lambda$ with wavelength is an important criterion for establishing whether or not there is more than one species of chromophores whose absorption spectra, while different from each other, overlap in the same wavelength region. If there is only one oriented species among these, then the linear dichroism spectrum $\Delta A_\lambda$ reveals the absorption spectrum of this species. Under these conditions, only upper or lower bounds of the orientation angles can be determined.

In summary, examination of a model of double-stranded DNA with Watson-Crick geometry indicates that the 2-amino group of deoxyguanosine is relatively exposed in the minor groove of the helix. Concerning the orientation angle of 35° or less for the BPDE chromophore bound to DNA, model building suggests that this chromophore is not oriented perpendicular to the long axis of the macromolecule. This orientation would be expected if PAH were intercalated, such as BP or PF, or had a base-displacement conformation similar to that of covalently bound AAF residues. Our data are more consistent with the location of BPDE in the minor groove, which would cause little distortion of the native DNA conformation. This conclusion is supported by the findings of Pulkrabek et al. (1977) that the regions of denaturation associated with BPDE modification are considerably smaller than those associated with AAF modification.

There is a second possibility, however, that base pair hydrogen bonds are destabilized at sites of BPDE modification. This interference in the usual hydrogen bonding of deoxyguanosine to deoxycytidine may favor the rotation of BPDE modified guanosine which would give rise to an exposed position exterior to the DNA helix. According to the Dreiding molecular model of the backbone, this rotation would not necessitate altering the conformation of the deoxyribose residues immediately adjacent to the modified guanosines. The latter model is consistent with the findings on accessibility of the covalently bound pyrene chromophore to an external fluorescence quencher ($I^-$) but not the internal quenchers ($Ag^+$ and $Hg^{2+}$) (Prusik et al., 1978). Further studies are required to distinguish between these two possibilities.

Acknowledgments

The authors thank Dr. R. G. Harvey for providing samples of BPDE I. We are grateful to Dr. J. Breton at Saclay for placing his dichrograph at our disposal and for stimulating discussions. We also wish to acknowledge helpful discussions with Drs. G. Paillotin, C. E. Swenberg, and M. Pope.

## Appendix: Reduced Linear Dichroism in the Presence of More than One Chromophore

We suppose that the total absorbance $A$ of a sample is the sum of absorbances $A = A_1 + A_2 + \ldots A_i = \Sigma_i A_i$, where the $A_i$ terms represent the contribution of each chromophore at a given wavelength. We further suppose that each species has a characteristic orientation angle $\theta_i$ and thus contributes to the overall reduced linear dichroism at a particular wavelength. The reduced linear dichroism is then given by the formula:

$$\frac{\Delta A_\lambda}{A_\lambda} = \frac{3}{2}\frac{\sum_i A_i(3\cos^2\theta_i - 1)f_i(\vec{\mathbf{E}})}{\sum_i A_i} \qquad \text{(A-1)}$$

Allowance has been made for the possibility that the sample is polydisperse and that each oriented species has associated with it a different orientation factor $f_i(\vec{\mathbf{E}})$. Since this factor is independent of the wavelength $\lambda$, it is evident that a wavelength dependence can arise only through a noncancellation of the $A_i$ terms in the numerator and denominator of eq A-1. The absorbance $A_i$ of each species is given by Beer–Lambert's law $A_i = \epsilon_i(\lambda)C_i l$, where $C_i$ is the concentration of species $i$, $\epsilon_i(\lambda)$ is its extinction coefficient, and $l$ is the optical pathlength.

We now consider several different possibilities.

Case I: The extinction coefficients of all of the species have the same wavelength dependence, but their orientation angles are different ($\epsilon_1(\lambda) = \epsilon_2(\lambda) = \epsilon_3(\lambda) = \ldots$, and $\theta_1 \neq \theta_2 \neq \theta_3$ etc.).

Since $\epsilon_i(\lambda)$ is the same for each species, eq A-1 reduces to:

$$\frac{\Delta A_\lambda}{A_\lambda} = \frac{3}{2}\frac{\sum_i C_i(3\cos^2\theta_i - 1)f_i(\vec{\mathbf{E}})}{\sum_i C_i} \qquad \text{(A-2)}$$

which is independent of wavelength. This case is experimentally indistinguishable from the case in which there is only one species as in eq 2, which points out the inherent limitation of all linear dichroism techniques. The experimental results do not provide an answer as to whether there is one or more oriented species. When $\Delta A_\lambda/A_\lambda$ is independent of wavelength, it is justifiable to utilize eq 2 to calculate the orientation angle. However, in view of eq A-2, this calculated angle reflects some weighted average of the orientation function $3/2(3\cos^2\theta_i - 1)$.

Case II: The wavelength dependences of the absorption coefficients are different, but their orientation angles are the same ($\epsilon_1(\lambda) \neq \epsilon_2(\lambda) \neq \epsilon_3(\lambda) = \ldots$, and $\theta_1 = \theta_2 = \theta_3 = \ldots$ etc.).

In this case the reduced linear dichroism becomes

$$\frac{\Delta A_\lambda}{A_\lambda} = \frac{3}{2}(3\cos^2\theta - 1)\frac{\sum_i \epsilon_i(\lambda)C_i f_i(\vec{\mathbf{E}})}{\sum_i \epsilon_i(\lambda)C_i} \qquad \text{(A-3)}$$

Case IIa: We further distinguish two different possibilities. If the electric field dependent orientation factors are all the same ($f_1(\vec{\mathbf{E}}) = f_2(\vec{\mathbf{E}}) = f_3(\vec{\mathbf{E}}) \ldots$), then eq A-3 is also independent of wavelength and this case is experimentally indistinguishable from case I.

Case IIb: For polydisperse systems, when $f_1(\vec{\mathbf{E}}) \neq f_2(\vec{\mathbf{E}}) \neq f_3(\vec{\mathbf{E}}) = \ldots$ etc., a wavelength dependence in eq A-3 may arise if one or more terms in the numerator dominate over the others. Such a situation could arise, for example, if there were present in the sample particles of different sizes, each displaying a different $f_i(\vec{\mathbf{E}})$ value at a given field strength, and each site a different $\epsilon_1$. Since both the $f_i$ and $\epsilon_i$ values must differ from each other at the same time, we consider this to be a highly specialized case which most likely does not apply to our experiments.

It is well known that DNA solutions behave in a polydisperse manner in the presence of electric fields (Fredericq & Houssier, 1973). However, it is unlikely that the $\epsilon_i(\lambda)$ coefficients of the bound polycyclic aromatic molecules would also vary with the size of the DNA segments. It has already been established that DNA does not undergo chain scissions when the level of in vitro modification with BPDE is lower than 1.5%

(Pulkrabek et al., 1977). Therefore this highly specialized case ($f_1 \neq f_2 \neq f_3$) is not likely to apply to our experiments where the modification of BPDE-DNA samples was very low (0.1%).

Case III: Both the wavelength dependence of the absorption coefficients and the orientation angles differ from each other ($\epsilon_1(\lambda) \neq \epsilon_2(\lambda) \neq \epsilon_3(\lambda) \ldots$, and $\theta_1 \neq \theta_2 \neq \theta_3 = \ldots$ etc.).

In this case, there is no cancellation of the wavelength dependent $\epsilon_i(\lambda)$ terms and the reduced linear dichroism is given by eq A-1. If the $\epsilon_i(\lambda)$ coefficients are strongly different from each other, i.e., if the chromophores have completely different absorption bands, for example, both the $\Delta A_\lambda$ and the $\Delta A_\lambda/A_\lambda$ spectra may be quite complex. In such cases it is very difficult to interpret the results quantitatively. Fortunately, at least for the most important case of the BPDE-DNA complex considered in this work, the wavelength dependence of the linear dichroism $\Delta A$ is nearly the same as the absorption spectrum of BPDE dissolved in ethanol (cf. Figures 3A and 3B). It is therefore possible to interpret the data quantitatively.

In general, a wavelength dependence of $\Delta A_\lambda/A_\lambda$ can arise if there is only one chemically distinct chromophore (in our case, for example, this corresponds to the single major binding product which is observed when BPDE binds covalently to DNA). There are at least two causes which are (a) light scattering due to the DNA macromolecules, and (b) inhomogeneous broadening.

(a) Light Scattering. When this effect is important, a wavelength dependence in $\Delta A_\lambda/A_\lambda$ can arise even if there is only one physically distinct chromophore present (i.e., no inhomogeneous broadening, with the absorbance of this species equal to $A_1$, with $A_2$, $A_3$, $A_4$ . . . etc., in eq A-1 all equal to zero). Nevertheless, the light scattering is equivalent to the presence of an "apparent" absorbance term $A_s$ in the denominator and an apparent linear dichroism term in the numerator of eq A-1. We have observed that this $A_s$ term can easily contribute as much or even more to the apparent absorbance of our DNA solutions than the chromophores which are bound to the DNA. Furthermore, both the contribution of the scattering to the linear dichroism in the numerator of eq A-1, due to the orientation of the DNA molecules, and the $A_s$ term display a smooth wavelength dependence (the light scattering power is proportional to $\lambda^{-4}$). The overall $\Delta A_\lambda/A_\lambda$ spectrum should then display the general appearance of the absorption spectrum of the chromophore due to the presence of the $A_1$ term in the numerator of eq A-1. Such light-scattering effects should be less pronounced for those chromophores which absorb at long wavelengths, and also when $A_1 > A_s$.

(b) Inhomogeneous Broadening. A chemically homogeneous chromophore may be distributed among physically distinct sites, each site differing slightly from the other with respect to the position in wavelength of the absorption maximum. Thus, an overall broadening of an absorption band occurs; this seems to be the case when BPDE binds to DNA, since the absorption spectrum of the pyrene-like chromophore is broader in the DNA than in ethanol solution (Figure 3). These physically distinct sites with $\epsilon_1(\lambda) \neq \epsilon_2(\lambda) \neq \epsilon_3(\lambda) \ldots$ etc., may also display different orientation angles, thus giving rise to a noncancellation of terms in the numerator and denominator of eq A-1 and thus to a wavelength dependence of $\Delta A_\lambda/A_\lambda$. The appearance of the wavelength dependence of the reduced linear dichroism spectrum should reflect the absorbance of the species with the highest concentration and the largest angular orientation factor.

It is believed that both effects a and b are important in the analysis of the data for the covalent BPDE–DNA and physical BP-DNA complexes. A method for estimating orientation angles in such cases is discussed within the main text.

References


Arcos, J. C., & Argus, M. F. (1968) *Adv. Cancer Res. 11,* 305.

Becker, R. S., Singh, I. S., & Jackson, E. A. (1963) *J. Chem. Phys. 38,* 2144.

Borgen, A., Darvey, H., Castagnoli, N., Crocker, T. T., Rasmussen, R. E., & Wang, T. Y. (1973) *J. Med. Chem. 16,* 502.

Breton, J., Michel-Villaz, M., & Paillotin, G. (1973) *Biochem. Biophys. Acta 314,* 42.

Chang, C. T., Miller, S. J., & Wetmur, I. G. (1974) *Biochemistry 13,* 2142.

Charney, E., Milstein, J. B., & Yamaoka, K. (1970) *J. Am. Chem. Soc. 92,* 2657.

Daudel, P., Duquesne, M., Vigny, P., Grover, P. L., & Sims, P. (1975) *FEBS Lett. 57,* 250.

Fredericq, E., & Houssier, C. (1973) *Electric Dichroism and Electric Birefringence,* Clarendon Press, Oxford.

Fuchs, R. P. P., Lefevre, J. F., Pouyet, J., & Daune, M. P. (1976) *Biochemistry 15,* 3347.

Geacintov, N. E., Prusik, T., & Khosrofian, J. M. (1976) *J. Am. Chem. Soc. 98,* 6444.

Hogan, M., Dattagupta, N., & Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 195.

Hsu, W. T., Lin, E. J. S., Harvey, R. G., & Weiss, S. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 3335.

Ivanovic, V., Geacintov, N. E., & Weinstein, I. B. (1976) *Biochem. Biophys. Res. Commun. 70,* 1172.

Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Weinstein, I. B., Beland, F. A., Harvey, R. G., Kasai, H., Miura, I., & Nakanishi, K. (1976) *J. Am. Chem. Soc. 98,* 5714.

Jeffrey, A. M., Weinstein, I. B., Jennette, K. W., Grzeskowiak, K., Nakanishi, K., Harvey, R. G., Autrup, H., & Harris, C. (1977) *Nature (London) 269,* 348.

Jennette, K. W., Jeffrey, A. M., Blobstein, S. H., Beland, F. A., Harvey, R. G., & Weinstein, I. B. (1977) *Biochemistry 16,* 932.

Jerina, D. M., & Daly, J.W. (1974) *Science 185,* 573.

King, H. W. S., Osborne, M. R., Beland, F. A., Harvey, R. G., & Brookes, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2679.

Koreeda, M., Moore, P. D., Yagi, H., Yeh, J. C., & Jerina, D. M. (1976) *J. Am. Chem. Soc. 98,* 6720.

Leffler, S., Pulkrabek, P., Grunberger, D., & Weinstein, I. B. (1977) *Biochemistry 16,* 3133.

Nakanishi, K., Kasai, H., Cho, H., Harvey, R. G., Jeffrey, A. M., Jennette, K. W., & Weinstein, I. B. (1977) *J. Am. Chem. Soc. 99,* 258.

Prusik, T., Geacintov, N. E., Tobiasz, C., Ivanovic, V., & Weinstein, I. B. (1978) *Photochem. Photobiol.* (in press).

Pulkrabek, P., Leffler, S., Weinstein, I. B., and & Grunberger, D. (1977) *Biochemistry, 16,* 3127.

Ramstein, J., Houssier, C., & Leng, M. (1973) *Biochem. Biophys. Acta 335,* 54.

Sims, P., Grover, P. L., Swaisland, A., Pal, K., & Hewer, A. (1974) *Nature (London) 252,* 326.

Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H., & Nakanishi, K. (1976) *Science 193,* 592.

Yamaoka, K., & Charney, E. (1973) *Macromolecules 6,* 66.