

Structural Effects on the Circular Dichroism of Ethidium Ion-Nucleic Acid Complexes[†]

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ABSTRACT: Binding of the frameshift mutagen ethidium bromide to eight dinucleoside phosphates of different complementary base sequences, to trinucleoside diphosphates with mismatched bases capable of forming bulged structures (CpUpG and GpUpG + CpC), and to calf thymus DNA at different counterion concentrations was studied by optical methods, notably UV-visible spectroscopy, circular dichroism (CD), and fluorescence-detected circular dichroism (FDCD). The ethidium ion intercalated between the base pairs of the minihelix formed by the complementary dimers and displaced the mismatched bases to form bulged structures with the trimers. The stoichiometry of the complexes was 2:1 oligonucleotide:dye (or 1:1:1 for non-self-complementary species). Equilibrium constants for complex formation showed a general preference for dye binding to complementary sequences in the order $\text{Py}(3'-5')\text{Pu} > \text{Pu}(3'-5')\text{Pu} \approx \text{Py}(3'-5')\text{Py} > \text{Pu}(3'-5')\text{Py}$

Ethidium bromide is shown by the Ames test to be a weak frameshift mutagen; it intercalates in double-stranded regions of both DNA (LePecq & Paoletti, 1967) and RNA (Douthart et al., 1973). An extensive number of intercalation studies of the ethidium ion (EI) with nucleic acids have been done; some of the features which make the ethidium ion so useful in these studies are its water solubility, a low tendency to self-aggregate (Reinhardt & Krugh, 1978), a large absorption shift in the visible spectrum of the dye upon binding to nucleic acids (Waring, 1965), a marked fluorescence intensity increase upon dye binding (LePecq & Paoletti, 1967), and the acquisition of an induced optical activity in the intercalated dye's transitions (Aktipis & Martz, 1970). These spectroscopic features of the dye have been seen upon binding to polymers, as well as when it is bound to complementary nucleic acid fragments as small as two base pairs (Krugh & Reinhardt, 1975; Krugh et al., 1975; Reinhardt & Krugh, 1978). Sobell and co-workers (Tsai et al., 1977; Jain et al., 1977) were able to obtain refined crystal structures at atomic resolution of ethidium ion complexes with 5-iodo-UpA and 5-iodo-CpG.

The dye's induced CD spectrum in complexes with dinucleoside phosphates (and dinucleotides), together with nuclear magnetic resonance chemical shifts of the dye protons, led to the conclusion that an ethidium ion was intercalated between the two base pairs of the minihelix in the complexes (Krugh & Reinhardt, 1975; Krugh et al., 1975). The structures obtained from the X-ray data showed that the phenanthridinium ring of one dye molecule was stacked between the

where Py = a pyrimidine base and Pu = a purine base. Complexes with ribodinucleoside phosphates had larger formation constants than their deoxyribose analogues. Above 300 nm, where only the dye absorbs light, the induced CD spectra of the complexes had bands at 510, 375, 330, and 307 nm, with the magnitude of the 307-nm band, per bound dye, being the largest and sequence dependent. The CD spectra down to 220 nm of some complexes were obtained by FDCD measurements; between 220 and 300 nm, both the positions and the magnitudes of the bands were sequence dependent. At very low ionic strength, complexes of calf thymus DNA plus ethidium ion in different salt concentrations showed that the 307-nm CD magnitude increased with decreasing counterion concentration. Implications of the results for theories of the induced CD spectrum of the ethidium ion are discussed.

base pairs in each minihelix, and a second dye was stacked outside of one base pair for a 2:2 dimer:dye stoichiometry. On the other hand, evidence from solution studies pointed to a 2:1 dinucleoside phosphate:dye complex as long as an excess amount of dinucleoside phosphate was maintained relative to the ethidium ion (Krugh & Reinhardt, 1975).

The magnitude of the CD bands induced per bound dye, $\Delta\epsilon_{\text{bound}}$, at 307 and 330 nm depended strongly upon the ratio of bound dye to phosphate in the DNA-EI complexes and increased as dye bound:phosphate (r) increased (Dalglish et al., 1971; Aktipis & Kindelis, 1973; Aktipis & Martz, 1974; Williams & Seligy, 1974). This finding was reproducible, within experimental error, for dye binding to nucleic acids of different base contents (Dalglish et al., 1971; Aktipis & Martz, 1974; Williams & Seligy, 1974), and for dye binding to DNA through a wide range of added monovalent counterion concentrations (Aktipis & Kindelis, 1973; Houssier et al., 1974). The 510-nm band behaved differently; it remained relatively constant with increasing binding ratios (Houssier et al., 1974).

Dalglish and co-workers (Dalglish et al., 1969), in a study of the induced optical activity of aminoacridine dyes bound to DNA, proposed two qualitative mechanisms which could account for variations in the induced CD magnitudes per bound dye with increasing binding ratios. In one, interactions between bound dye ligands were responsible for the variation, since the chances of such interactions rose with the number of bound molecules. In the other, the changes in DNA conformation with dye binding altered the ligands' environments, giving rise to the variations in $\Delta\epsilon_{\text{bound}}$ with r . Similar mechanisms for the induced CD of the aminoacridines in DNA were presented by Jackson & Mason (1971). For the 510-nm band of the ethidium ion, which is independent of binding ratio, there can be no strong dye-dye interaction, and the CD is not dependent on a change of dye environment. However, the 307-nm band, which changes markedly with binding ratio, is obviously affected by either or both of the two mechanisms.

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In this paper, we describe experiments which investigate the origins of the induced CD spectrum of the complexed ethidium ion more closely. We use complexes of the dye with excess amounts of dinucleoside phosphates (dimers) and trinucleoside diphosphates (trimers) as small analogues of the polymer binding sites.

We first use titrations and equilibrium sedimentation methods to confirm the 2:1 binding stoichiometry and obtain binding constants for complex formation in the presence of a large excess of complementary oligonucleotides. Induced CD studies of these complexes on a per bound dye basis show that the molar CD magnitudes near 307 nm are quite large, indicating that the contribution to the CD from the inherent asymmetry of the binding site is sizable. The CD spectra of these complexes below 300 nm are measured via fluorescence-detected circular dichroism (FDCD), a technique sensitive only to signals from the bound dye in the complexes. Sequence dependencies in these spectra are quite evident. Lastly, the induced CD of calf thymus DNA-ethidium ion complexes are examined as a function of salt concentration; these show signs that conformational and/or electrostatic changes in the macromolecule affect the 307-nm band magnitude.

Experimental Procedures

Materials. The ribodinucleoside phosphates and ethidium bromide were purchased from Sigma Chemical Co. The deoxyribodinucleoside phosphates were purchased from Collaborative Research. All dinucleoside phosphates were used as received except for UpA and dTpA, which were first purified by paper chromatography. The ethidium bromide was dissolved in doubly distilled water and lyophilized several times. Stock solutions of the dye (0.4–2.5 mM) in doubly distilled water were kept cool and in the dark. The concentration of ethidium bromide was determined spectrophotometrically; the extinction coefficient at 480 nm was taken as 5600 L/(mol·cm) (Waring, 1965). Stock solutions of dinucleoside phosphates were prepared in a buffer of 0.18 M NaCl, 8.0 mM Na₂HPO₄, 20 mM NaH₂PO₄, and 0.1 mM Na₂EDTA, pH 7.0. Stock concentrations were determined optically by using the extinction coefficients in the *Handbook of Biochemistry, Selected Data for Molecular Biology* (1975).

The trinucleoside phosphates, GpUpG and CpUpG, were obtained from Boehringer Mannheim; however, most of the CpUpG used in this study was synthesized enzymatically with polynucleotide phosphorylase by standard methods (Martin et al., 1971). These trimers were dissolved in the same buffer as the dimers to form stock solutions. The extinction coefficients were calculated, with the assumption of nearest-neighbor interactions only, by using the extinction coefficients of the dimers and monomers (Bloomfield et al., 1974).

Calf thymus DNA was purchased from Worthington Biochemical. A stock solution (1 mg/mL) was prepared by dissolving the DNA in the cold in a 100 mM NaCl, 1 mM tris(hydroxymethyl)aminomethane (Tris), and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, buffer. The extinction coefficient for the DNA as phosphate was taken as 6600 L/(mol·cm) at 260 nm (Mahler et al., 1964).

Optical Titrations. Solutions containing variable amounts of the dimers or trimers and a constant dye concentration were diluted to a fixed volume with buffer. Ethidium ion concentrations were typically 0.03–0.04 mM, with the total dimer (trimer) concentration in excess by at least 8-fold. For complexes with the non-self-complementary dimers, nearly equal amounts (within 10%) of the two dimers were added to each solution. Complex formation was monitored by measuring the

shift of the band in the visible spectrum (Waring, 1965), with spectra measured with either a Cary 118 or a Gilford 250 spectrophotometer with scanning option. Spectra were digitized and stored for later use via interfaced Pet minicomputers. Temperatures of the sample cells were maintained to $\pm 0.4^\circ\text{C}$ by a Neslab bath on the Cary 118 and to $\pm 0.1^\circ\text{C}$ by a Gilford 2527 thermoelectric temperature programmer on the Gilford 250. All spectra were measured in 1-cm path-length quartz microcells (Precision Cells).

Equilibrium Sedimentation. Equilibrium sedimentation studies were made in a Beckman Model E ultracentrifuge equipped with an A_nF rotor. Samples were run at 5°C and a rotor speed of 42000 rpm. Equilibrium was established after 24 h in all cases.

Absorbance. ΔH° and ΔS° values for complex formation were determined by van't Hoff plots of $\ln K$ vs. T^{-1} by using data points provided by the optical titrations; other points were obtained from absorbance vs. temperature measurements of known dimer-dye solutions. The melting temperature of the complex, T_m , was defined as the midpoint of the transition, and was measured from the dye absorbance at 465 nm.

Circular Dichroism Spectra. CD spectra of the complexes from 450 to 290 nm were measured on a Cary 60 spectropolarimeter equipped with a Cary 6001 accessory. Cell temperature was maintained at 0°C ($\pm 0.2^\circ\text{C}$) with a thermoelectrically cooled temperature jacket (Allen et al., 1972). Quartz microcells of 1.0 and 0.5 cm were used. Solutions prepared for the optical titrations were also used in the CD study. A base-line spectrum on an equal amount of total dimer was subtracted from each spectrum. Spectra were digitized and stored by using a PDP 8/E minicomputer. The molar CD on a per bound dye base was calculated by using

$$\Delta\epsilon_{\text{bound}} = \theta / (32.98 l C_{\text{complex}}) \quad (1)$$

where θ is the measured ellipticity in degrees, l is the path length in centimeters, and C_{complex} is the concentration of the ethidium ion in the complex.

Fluorescence-Detected CD Spectra. Solutions for FDCD studies were prepared as above. A base-line measurement was made with a solution of α -naphthylamine (Sigma) in buffer. This material is an OSHA-classified carcinogen, and proper safeguards were taken during its use.

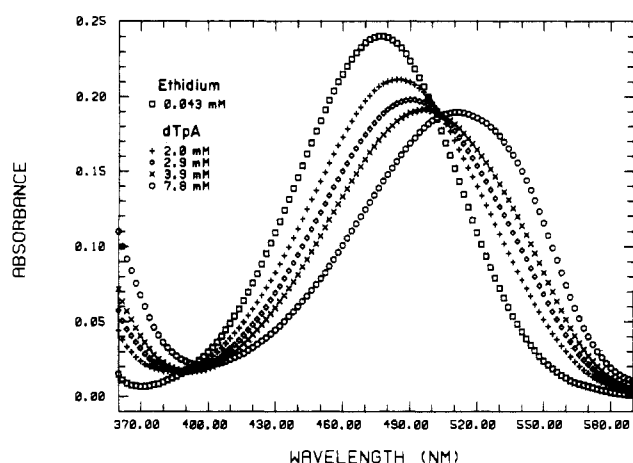
A spectrometer capable of measuring FDCD spectra was prepared from the Cary 60/6001 by the scheme of Turner et al. (1974). Cooling of the cells was performed with an external bath (Neslab). All spectra were run at $0.0 \pm 0.5^\circ\text{C}$. Strain-free quartz fluorescence cells (Precision Cells) of 2- and 3-mm path lengths were used in the measurements. A Schott KV 408 interference filter was used to distinguish emitted light from scattered light. No photoselection (Tinoco et al., 1977), as measured with a linear polarizer (Polaroid), was evident in any of the cases studied. Multiple (five to nine) scans of the spectra were run; these were averaged and smoothed to a 13-point cubic equation (Savitsky & Golay, 1964). CD and absorbance spectra of the same solutions were run as above.

Salt Studies. The high salt solution, composed of 100 mM NaCl–1 mM tris(hydroxymethyl)aminomethane (Tris), and the low salt solution, composed of 0.1 mM NaCl–1 μM Tris, were prepared with doubly distilled water in prerinsed glassware. The pHs of these solutions were 7.0 ± 0.5 . Dilutions of these stock solutions in the preparation of the DNA-EI mixtures, combined with the calculated Na⁺ contribution from the DNA aliquot, gave final Na⁺ concentrations of 86 and 0.45 mM, respectively.

Calf thymus DNA stock solutions were dialyzed to remove excess counterions following the approach of Record (1975).

Table I: Thermodynamic Parameters of Ethidium Ion Complexes

complex	temp (°C)	$K \times 10^{-5} (M^{-2})$	$\epsilon_b - \epsilon_f [L/(\text{mol} \cdot \text{cm})]$	$\Delta H^\circ (\text{kcal/mol})$	$\Delta S^\circ (\text{cal mol}^{-1} \text{K}^{-1})$
CpG-CpG-EI	0	890 ± 200	-3400 ± 200	-32	-84
	5	420 ± 150	-3200 ± 300		
UpA-UpA-EI	0	7.2 ± 1.0	-3600 ± 200		
CpA-UpG-EI	0	150 ± 20	-3500 ± 100	-32 ± 1	-86 ± 4
	5	46 ± 7	-3500 ± 200		
	10	19 ± 5	-3200 ± 400		
dCpG-dCpG-EI	0	65 ± 10	-3500 ± 200	-29	-69
	5	15 ± 2	-3300 ± 100		
dTpA-dTpA-EI	0	1.3 ± 0.3	-2900 ± 400		
ApA-UpU-EI	0	3.0 ± 0.4	-2900 ± 100	-12 ± 2	-18 ± 7
	5	1.9 ± 0.3	-2600 ± 200		
	10	1.4 ± 0.3	-2300 ± 200		
ApG-CpU-EI	0	4.9 ± 1.2	-3000 ± 300	-13 ± 3	-22 ± 12
	5	2.7 ± 0.6	-2800 ± 300		
	10	2.1 ± 0.6	-2400 ± 400		
ApU-ApU-EI	0	0.5 ± 0.1	-2500 ± 200		
CpUpG-CpUpG-EI	0	10 ± 3			
GpUpG-CpC-EI	0	<1			



quences. The other complex, GpUpG-CpC-EI, with only one bulged base, was less stable than the (CpUpG)-EI complex which has bulges on both strands.

Stoichiometry of the Complexes. Krugh and co-workers (Krugh & Reinhardt, 1975; Krugh et al., 1975; Reinhardt & Krugh, 1978) found complexes of complementary dimers with the ethidium ion had stoichiometries of 2:1 dimer:dye under conditions where an excess of the dimers was present. Further support for this stoichiometry (and the equivalent 1:1:1 stoichiometry for the non-self-complementary dimers) is provided by our failure to obtain satisfactory fits of the titration data in this work to alternate stoichiometries (1:1 or 2:2) by either the Benesi-Hildebrand or the Peacocke (Blake & Peacocke, 1968) methods (Pardi, 1980).

As the stoichiometry cannot distinguish 2:1 complexes from 4:2 complexes, equilibrium sedimentation studies of the CpG + EI system were done at 5 °C to determine if aggregates of the complexes formed in the mixture. The weight-average molecular weight of the complex was determined, taking into account charge effects, by use of the following equation (Tanford, 1961):

$$M_{\text{complex}} = \frac{1}{2} M_{\text{NaCl}} Z \left(\frac{1 - \bar{V}_{\text{NaCl}} \rho}{1 - \bar{V}_{\text{complex}} \rho} \right) + 2RT \left[\frac{d \ln C / d r^2}{(1 - \bar{V}_{\text{complex}}) \omega^2} \right] \quad (2)$$

where Z is the charge on the complex, \bar{V}_{complex} and \bar{V}_{NaCl} are the partial specific volumes of the complex and sodium chloride, respectively, ρ is the density of the buffer solution, C is the concentration of the complex at a distance r from the axis of rotation, and ω is the angular velocity of the rotor. \bar{V}_{complex} was calculated by using the assumption that it was the weight average of the components' partial specific volumes:

$$\bar{V}_{\text{complex}} = \frac{2M_{\text{CpG}} \bar{V}_{\text{CpG}} + M_{\text{EI}} \bar{V}_{\text{EI}}}{2M_{\text{CpG}} + M_{\text{EI}}} \quad (3)$$

where \bar{V}_{CpG} and \bar{V}_{EI} were obtained by sedimentation runs on the isolated species and were 0.58 and 0.75 cm³/g, respectively. The molecular mass of the complex was found to be 1800 g/mol under conditions similar to those used in the optical studies and at a concentration where all the dye was bound in the complex (Pardi, 1980). The molecular mass of a 2:1 complex is 1570 g/mol; a 2:2 complex (1920 g/mol) is ruled out by the stoichiometry, as mentioned above. The measured molecular mass does show that higher aggregates (such as 4:2 etc.) do not occur for the concentrations used in our work.

Thermodynamics of Complex Formation. Additional equilibrium constants for complex formation were obtained from optical melting with solutions of known dimer-dye composition. A typical melting curve is displayed in Figure 3.

At the melting temperature, T_m , half the ethidium ions were bound in the complex while the other half were free in solution. These data, in combination with the equilibrium constants obtained from the optical titrations, provided estimates of ΔH° and ΔS° for formation of the complexes, by using van't Hoff plots of $\ln K$ vs. T_m^{-1} . A second estimate of ΔH° was obtained from the slopes at T_m of the melting curve by the method of Applequist & Damle (1965); both methods were used for the CpG and dCpG mixtures and were in close agreement.

The enthalpy and entropy of complex formation are presented in Table I. Once again, there was a clear distinction between the results for the different types of sequences. The Py(3'-5')Pu sequences possessed larger enthalpies than the

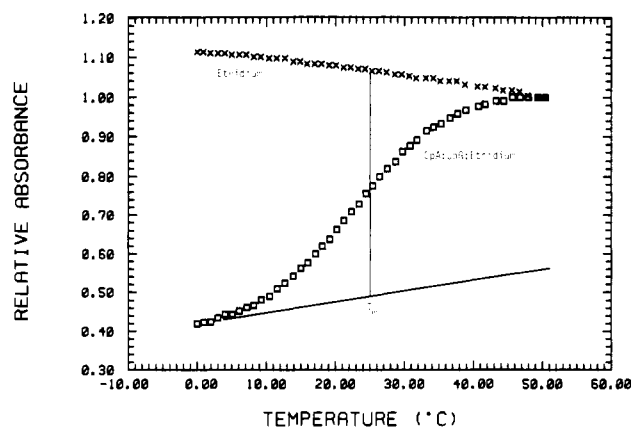
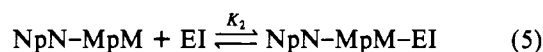


FIGURE 3: Optical melt of the CpA-UpG-EI complex monitored at 465 nm in a 1-cm cell. Concentrations are 0.039 mM for ethidium ion, 3.5 mM for CpA, and 3.5 mM for UpG.

Pu(3'-5')Pu sequences examined. The formation of the complexes in each case was enthalpy driven, since the entropies are all negative. Davanloo & Crothers (1976) obtained a ΔH° of -27 to -30.6 kcal/mol for the formation of a (dpCpG)₂-EI complex; this compares favorably with our value of -29 kcal/mol for the (dCpG)₂-EI complex. The overall reaction for complex formation can be written in terms of two composite reactions:



where $K = K_1 K_2$. The actual mechanism of complex formation between the ethidium ion and complementary nucleic acid dimers is quite different from these equations (Davanloo & Crothers, 1976), which are used only to evaluate the relative importance of various contributions to the overall stability of the complex. Equilibrium constant K_1 for reaction 4 was measured between 0 and 5 °C by using NMR by Krugh and co-workers (Young & Krugh, 1975; Krugh et al., 1976). The complementary dimers dpCpG, dpGpC, CpG, GpC, and GpU plus ApC all had minihelix formation equilibrium constants on the order of 10 M⁻¹ or less. Since our overall constants, K , are at least 2 orders of magnitude larger, the minihelix stability is but a minor fraction of the overall stability. Much of the stability, and undoubtedly the specificity, for the ethidium ion binding to these sequences must arise from interactions covered in the second reaction (eq 5).

Induced CD Spectra. The induced circular dichroism spectra from 440 to 290 nm were measured for all the complexes listed in Table I except for ApU-ApU-EI, where no significant CD signal above the ApU base line could be observed, even at the highest dimer:dye ratio (11:0.040 mM). The molar CD per bound dye may be very low for this complex. For GpC + EI, we obtained the same spectrum as that obtained by Krugh et al. (1975), but a suspended precipitate was observed in the cell. We believe the spectrum was largely due to aggregation in this sample.

Representative induced CD spectra for ethidium ion plus dimers are presented in Figure 4. The largest induced CD band was between 300 and 310 nm, and the maximum position varied slightly with the base sequence. These values of λ_{max} remained quite constant throughout the range of dimer-dye concentrations studied for each sequence. Krugh & Reinhardt (1975), using solutions in which all ethidium ion was bound in the complex, also obtained $\Delta \epsilon_{\text{bound}}$ values for some of the same sequences. Their results compared favorably with ours

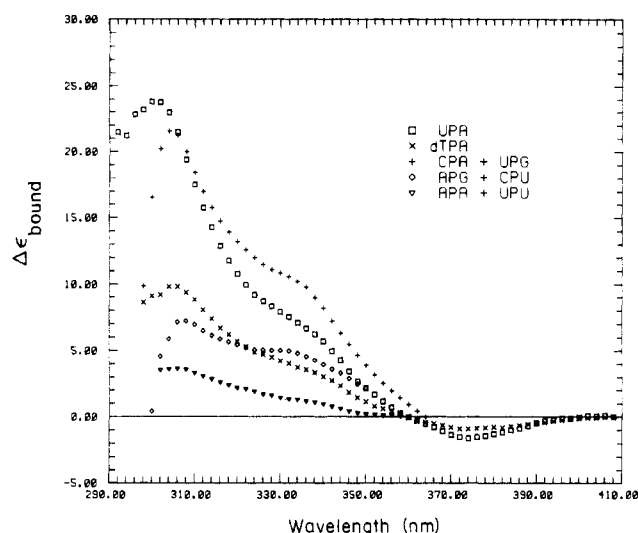


FIGURE 4: Induced CD spectra per bound ethidium ion at 0 °C for 2:1 dimer:dye complexes.

Table II: Induced CD per Bound Ethidium Ion in Minihelical Complexes

complex at 0 °C	wavelength (nm)	$\Delta\epsilon_{\text{bound}}$ [L/(mol·cm)]
CpG-CpG-EI	307	22 ± 3
	307	20^a
UpA-UpA-EI	301	23 ± 4
CpA-UpG-EI	305	22 ± 4
	305	21.4^a
dCpG-dCpG-EI	307	15 ± 3
dTpA-dTpA-EI	305	10 ± 5
ApA-UpU-EI	306	3.7 ± 0.8
	303	9.0^a
ApG-CpU-EI	308	7.4 ± 0.7

^a Data from Krugh & Reinhardt (1975).

in most cases (Table II). As these induced CD spectra were measured for single 2:1 complexes (the equilibrium sedimentation study demonstrated that larger aggregates of the complexes did not form), it is clear that dye-dye interactions are not responsible for the large induced CD spectrum of bound ethidium ion.

The induced CD spectra for ethidium ion binding to the singly bulged (GpUpG + CpC + EI) and doubly bulged (CpUpG + EI) minihelices are very similar in shape to the CD spectra found for the dye-dimer complexes shown in Figure 4. This indicates that whatever interactions were responsible for the induced CD of the dye with perfect minihelices probably occurred within the bulged complexes as well. The evidence from NMR studies (Lee & Tinoco, 1978) indicated that the G-C base pairs were stacked upon either side of the intercalated dye, leaving the uridine residues unstacked to form the bulges. Thus, interactions between the G-C base pairs and the dye are responsible for the characteristic induced CD spectra in these cases.

FDCD Spectra. The CD spectra of the dimer-ethidium ion mixtures below 290 nm largely resembled those of the dimers, which were present in excess over any complex formed. On the other hand, fluorescence-detected circular dichroism (FDCD) spectra of the same solutions measured CD signals derived *only* from the complex, because only chiral, fluorescent species contribute to the FDCD. In FDCD, a sample is excited with circularly polarized light and the intensity of emission is measured as a function of the incident beam's polarization sense (Turner et al., 1974). The signal from an FDCD measurement (one fluorescent, optically active species; no

photoselection) on the Cary 60 recorder is given by

$$\theta_F = -14.32(\Delta\epsilon_F/\epsilon_F - R) \quad (6)$$

$$R = \Delta A \left(\frac{1}{A} - \frac{2.303}{10^A - 1} \right) \quad (7)$$

where θ_F is the ellipticity in degrees, $\Delta\epsilon_F = (\epsilon_L - \epsilon_R)_F$ is the molar CD of the fluorophore in liters per moles per centimeter, ϵ_F is the fluorophore's molar extinction coefficient, $\Delta A = A_L - A_R$ is the CD of the entire sample, and A is its absorbance (Tinoco & Turner, 1976). So that $\Delta\epsilon_F/\epsilon_F$, which is proportional to the complex's CD, could be obtained, the FDCD (θ_F), the CD (ΔA), and the absorbance (A) spectra of the dimer-dye mixtures were measured and substituted into eq 6 and 7. The factor $\Delta\epsilon_F/\epsilon_F$ is the Kuhn anisotropy factor of the fluorophore.

The Kuhn anisotropy factors for the dimer-EI complexes are shown in Figure 5. All spectra show a region of positive anisotropy above 300 nm; most show a negative region above 360 nm. These regions correspond to the CD spectra seen in Figure 4, which show two positive bands between 300 and 360 nm and a negative band near 372 nm. The measured FDCD of the complex is thus consistent with the measured CD of the complex in this wavelength region, where both can be measured. Below 300 nm, the CD of the complex cannot be measured directly, because the excess dinucleoside phosphate dominates the spectrum. However, the FDCD measurement gives directly the positions and signs of the CD bands as seen in Figure 5.

The assignments of all the bands are difficult. Clearly, the bands above 300 nm are all dye transitions. The feature seen at 295 nm in all the spectra was also assigned to a dye transition, because of the strong absorption of the ethidium ion at 285 nm which red shifts in the complex. Below 290 nm, the observed bands can be either ethidium ion transitions or nucleic acid base transitions which transfer energy to the ethidium ion.

The sequence dependence of the spectra below 300 nm is of interest. These spectral differences will depend on the nature of the bases surrounding the ethidium ion and the orientation of the ethidium ion relative to the bases in the complex. We note that CpG-CpG-EI has a spectrum very similar to that of dCpG-dCpG-EI; this indicates a similar structure for the two complexes. However, keeping the base composition of the complex constant, but changing the sequence, has a large effect on the spectra as seen in Figure 5b (UpA vs. ApA + UpU) and Figure 5c (CpA + UpG vs. ApG + CpU). In the X-ray structures of ethidium ion complexed with 5-iodo-UpA and 5-iodo-CpG (Tsai et al., 1977; Jain et al., 1977), the intercalated dye molecule in each complex overlapped roughly equal amounts with the base pairs, and the substituents on the phenanthridinium ring were in the minor groove of the minihelix. Thus, the differences seen here in the anisotropies of the different complexes are likely due to the different electronic properties of the bases.

Salt Effects on the CD Spectra. The DNA molecule, a polyanion, is conformationally sensitive to cation concentration. The ethidium ion, a cation, is sensitive to the salt concentration when binding to DNA (LePecq & Paoletti, 1967; Aktipis & Kindelis, 1973; Houssier et al., 1974). Furthermore, in addition to the normal intercalation binding with DNA, there is a weaker, outside binding associated with ethidium-DNA electrostatic interactions (Waring, 1965).

The induced CD of ethidium ion bound to DNA as a function of added salt concentration has been previously studied (Aktipis & Kindelis, 1973; Houssier et al., 1974). We

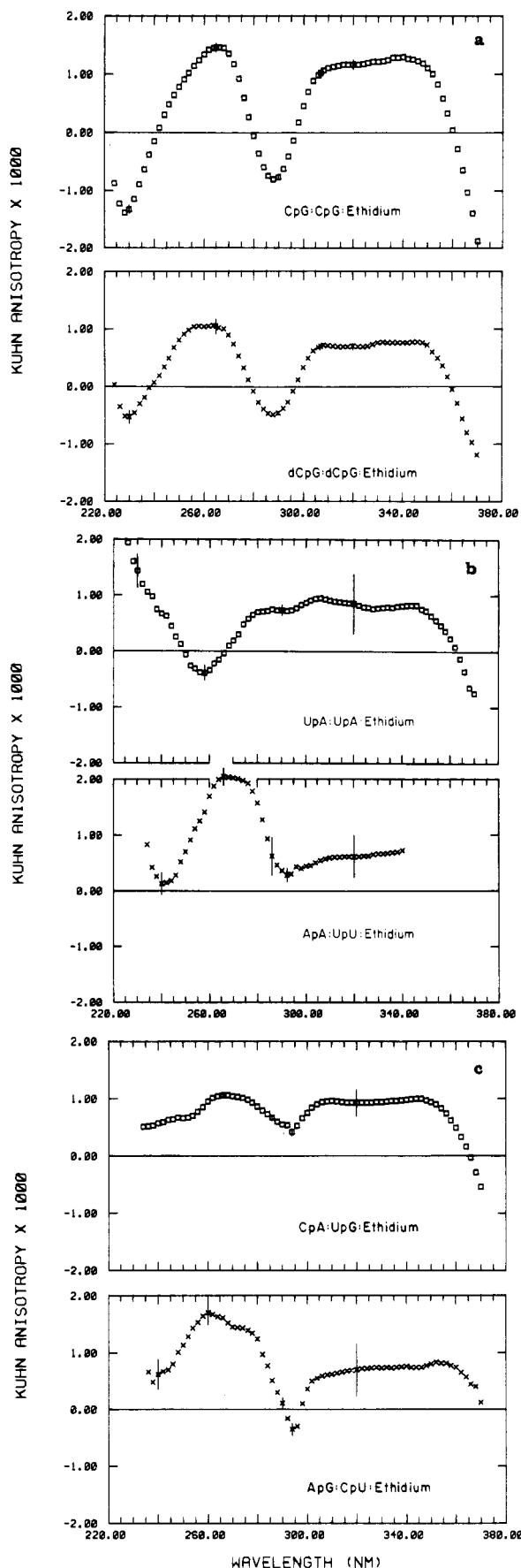


FIGURE 5: (a) Kuhn anisotropy spectra for CpG-CpG-EI (top) and dCpG-dCpG-EI (bottom). Estimated errors are represented by vertical lines. (b) Kuhn anisotropy spectra for UpA-UpA-EI (top) and ApA-UpU-EI (bottom). Estimated errors are represented by vertical lines. (c) Kuhn anisotropy spectra for CpA-UpG-EI (top) and ApG-CpU-EI (bottom). Estimated errors are represented by vertical lines.

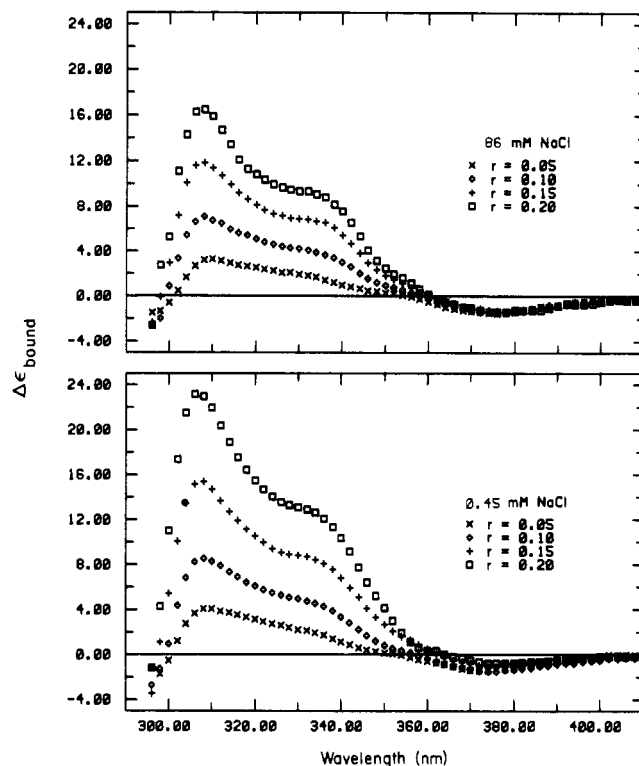


FIGURE 6: Induced CD spectra of DNA-EI complexes at different binding ratios and salt concentrations at 25 °C.

did a series of experiments to further investigate the ionic strength dependence of the ethidium ion's induced CD. The equilibrium constants for ethidium ion binding to calf thymus DNA were determined by Scatchard analysis of optical titration data (Waring, 1965). For the high salt case (86 mM Na^+), a least-squares fit to the binding data yielded an equilibrium constant of $(9.7 \pm 0.7) \times 10^5 \text{ M}^{-1}$ and 0.20 ± 0.02 binding sites/phosphate at 25 °C. This result was in fair agreement with LePecq & Paoletti's (1967) result in 90 mM Na^+ at 23 °C, where $K = 6.6 \times 10^5 \text{ M}^{-1}$. In the low salt case (0.45 mM Na^+), we found that essentially all dye present in the mixture, for total dye:phosphate ratios of 0.25 or less, was bound in the complex, so that an equilibrium constant was impossible to determine by this method. Previous workers (LePecq & Paoletti, 1967; Aktipis & Kindelis, 1973; Houssier et al., 1974) also found that the equilibrium constant for ethidium ion binding to DNA increased as the counterion concentration decreased. For our purposes, it was sufficient to know that all dye was bound to the nucleic acid at dye:phosphate ratios below 0.25 in the low salt solutions.

The induced CD spectra of ethidium ion bound to calf thymus DNA in the two salt solutions are shown in Figure 6. One sees that at the same binding ratio, r , $\Delta\epsilon_{\text{bound}}$ at 307 and 330 nm is larger in 0.45 mM Na^+ than in 86 mM Na^+ . The increase in $\Delta\epsilon_{\text{bound}}^{307}$ at 307 nm with increasing r found for the high salt case (Figure 7) is in close agreement with data from previous studies, which had counterion concentrations of 40 mM or greater (Dalgleish et al., 1971; Aktipis & Kindelis, 1973; Aktipis & Martz, 1974; Houssier et al., 1974). Our low salt curve directly conflicts with the previous studies, where no change in the $\Delta\epsilon_{\text{bound}}^{307}$ vs. r curve with salt concentration was seen (Aktipis & Kindelis, 1973; Houssier et al., 1974).

We believe past failures to observe an effect upon $\Delta\epsilon_{\text{bound}}^{307}$ in different salt concentrations can be traced to the conditions used to vary r in those studies: constant dye and variable DNA concentrations, which necessarily alter the effective counterion

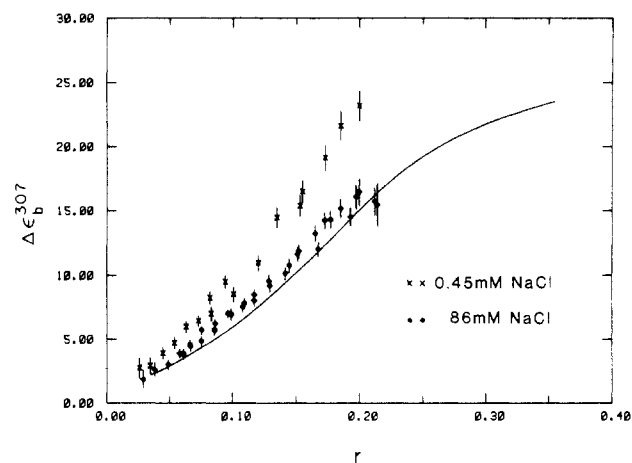


FIGURE 7: Variation of the magnitude of the induced CD per bound dye at 307 nm with the extent of ethidium ion binding, r , and counterion concentration. Vertical lines represent estimated errors. The solid line is taken from Dalglish et al. (1971) and Aktipis and co-workers (Aktipis & Kindelis, 1973; Aktipis & Martz, 1974) and represents a synthesis of results in different salt concentrations and with different nucleic acid sequences.

concentration. At lower salt concentrations, these changes can be sizable, often increasing the counterion concentration manyfold. When we measured the induced CD of DNA-EI mixtures used for the low salt Scatchard analysis (constant dye-variable DNA), the $\Delta\epsilon_{\text{bound}}^{307}$ vs. r curve (not shown) closely resembled our high salt results, especially at lower r values, where the amount of added DNA (and hence, added Na^+) was larger. Only when we kept the DNA concentration constant were we sure that the Na^+ counterion concentration was constant throughout our range of r values. The fact that the dye bound completely to the DNA under low salt conditions allowed us to take this approach. At higher salt concentrations, 10 mM or greater, the variation of DNA concentrations in solutions from Scatchard analyses is less of a problem; the Na^+ contribution from the DNA aliquot is a smaller fraction of the total counterion concentration.

Discussion

In this study, we have considered oligonucleotide-ethidium ion complexes with the intent of determining what features of these complexes contribute to the induced CD spectra, and to use this knowledge to understand DNA-ethidium ion complexes as well. The induced CD spectrum of ethidium ion in DNA complexes contains two types of bands: those with constant magnitudes per bound dye as the binding ratio, r , rises (the 510-nm band) and those whose magnitudes per bound dye increase with more binding (the 307-nm band). The standard explanation for these phenomena (Aktipis & Kindelis, 1973; Houssier et al., 1974) is that the CD of the 510-nm band is induced by the asymmetry of the binding site and that this asymmetry is independent of binding ratio. The CD of the 307-nm band is caused by this mechanism at low binding ratios, but at high ethidium ion binding, dye-dye interaction increases the CD per bound dye from $\Delta\epsilon$ near 2 L/(mol-cm) to over 20 L/(mol-cm). The large effect at 307 nm and the negligible effect at 510 nm could be rationalized by the larger transition moment at 307 nm. Another proposal was that the induced CD was caused by the site asymmetry but that this asymmetry changed when dye binding occurred at adjacent sites (Lee et al., 1973).

Effect of Site Asymmetry. Our data for 2:1 complexes of dinucleoside phosphate-ethidium ion complexes yield large CD magnitudes [$\Delta\epsilon \geq 20$ L/(mol-cm) at 307 nm] in the absence

of dye-dye interaction. Obviously, site asymmetry by itself can induce CD values as large as are found in DNA-ethidium ion complexes at high binding ratios. Furthermore, the FDCD spectra show (Figure 5) that all the oligonucleotide complexes have a negative band near 295 nm. This band must be site induced; it cannot be due to dye-dye interaction. However, the positive band at 307 nm and the negative band at 295 nm seen in DNA-ethidium ion complexes had previously been interpreted as an exciton band (Aktipis & Kindelis, 1973; Balcerski & Pysh, 1974). We conclude that the induced CD for ethidium ion bound to DNA, and its change with binding ratio, can be explained simply in terms of the structure (asymmetry) of the binding site.

Effect of Charge. We found that the magnitude of the 307-nm CD band per bound dye increased with decreasing ionic strength of the solution at low salt concentration (Figure 7). Furthermore, the magnitude of the CD band of the DNA alone decreased by about 10% from high salt (86 mM Na^+) to low salt (0.45 mM Na^+) concentration. This suggests some sensitivity of DNA conformation to ionic effects in this concentration range.

Using the minihelix complexes (2 dinucleoside phosphates:1 ethidium ion) as our model for the DNA-ethidium ion complex, we find that its CD agrees with the CD of the DNA complex at high binding ratio, but not at low binding ratio. This implies a similarity of site geometries between the DNA with many bound ethidium ions and an ethidium ion bound to only two base pairs. A possible reason for this similarity is the lower charge density on the DNA at high binding ratios. An intercalating cation, such as the ethidium ion, lowers the charge density in two ways: first, by placing a positive charge in the negatively charged array of backbone phosphates, and second, by lengthening the helix by the width of the dye. According to polyelectrolyte theory (Manning, 1978), these combined effects would reduce the number of condensed counterions (e.g., Na^+) required per phosphate to maintain stability and so increase each phosphate's fractional charge. Both the conformational and electrostatic changes accompanying dye intercalation would thus change the site structure, and possibly make it more like a minihelix. Reducing the salt concentration (Figure 7) would have a similar effect.

To understand in detail the induced CD of an intercalated dye in either DNA or a minihelix will require calculations taking into quantitative account the contributions of dye-base, dye-sugar, and dye-phosphate interactions.

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