Binding of Ethidium Ion to Left-Handed Z-RNA Induces a Cooperative Transition to Right-Handed RNA at the Intercalation Site†

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ABSTRACT: The equilibrium binding of the ethidium cation (Etd^+) to the right-handed A-form of poly[r(C-G)], the B-form of poly[d(C-G)], and the left-handed Z-forms of Br-poly[r(C-G)] and Br-poly[d(C-G)] was investigated in 0.22 M NaCl by optical methods. Scatchard analysis indicates that Etd^+ intercalates into right-handed forms of poly[r(C-G)] and poly[d(C-G)] in a noncooperative manner. Correlation of Etd^+ absorbance binding isotherms and polynucleotide circular dichroism data indicates that drug binding to Br-poly[r(C-G) and Br-poly[d(C-G)] results in cooperative conversion from left-handed Z-forms to right-handed intercalated conformations. Approximate stoichiometries necessary to induce the left- to right-handed transitions are 1 Etd^+/9 base pairs (bp) for Z-RNA and 1 Etd^+/6 bp for Z-DNA. The apparent limiting binding stoichiometries are approximately 1 Etd^+/3 bp for RNA and 1 Etd^+/2 bp for DNA. The equilibrium binding constants for binding to the right-handed forms decrease in the order Br-poly[d(C-G)], Br-poly[r(C-G)], poly[d(C-G)], and poly[r(C-G)]. Thermodynamic parameters are obtained by van't Hoff analysis of Etd^+ absorbance thermal dissociation data. Enthalpy values for all four polynucleotides are negative and of similar magnitude. Negative entropy values indicate that the binding processes are primarily enthalpically driven. Circular dichroism spectra for the complexes formed between Etd^+ and the Z-forms of poly[r(C-G)] in 6 M $NaClO_4$ buffer (Z_R-RNA) and 4 M $MgCl_2$, pH 5.75 (Z_D-RNA), change in a manner consistent with transition to right-handed intercalated conformations; however, the Etd^+ binding affinities are approximately 10-fold lower in the concentrated $NaClO_4$ and $MgCl_2$ solutions due to the elevated ionic strength.

The binding of ethidium ion (Etd^+)[1] to both natural and synthetic polynucleotides has been investigated by a variety of physical and biochemical means in order to develop a better understanding of the relation between structure, thermodynamics, kinetics, and function in intercalator–nucleic acid systems [for reviews see Berman and Young (1981) and Neidle and Abraham (1984)]. The drug exhibits a wide range of detrimental biological activities including inhibition of replication (Henderson, 1963), transcription (Richardson, 1973), recombination (Seto & Tomasz, 1977), self-splicing (Tanner & Cech, 1985), and ribosome function (Burma et al., 1978). The molecule also has potent antiviral activity (Vilagines, 1966) probably due in part to its ability to inhibit retroviral reverse transcription (Sarih et al., 1980) and possibly RNA methylation (Liau et al., 1977).

The ethidium molecule is composed of the planar tricyclic phenanthridium ring and a secondary phenyl group perpendicular to the primary ring system. The primary mode of binding to right-handed double-stranded polynucleotides is by intercalating between adjacent base pairs (Neidle & Abraham, 1984; Lybrand & Kollman, 1985). This process has been characterized by a high binding constant ($\sim 10^4$–10^7 M^{-1}) and rapid kinetics (milliseconds) and has been shown to have the following sequence preference in both ribo- and deoxyribo-oligonucleotides: pyrimidine-(3′–5′)-purine > purine-(3′–5′)-purine > purine-(3′–5′)-pyrimidine (Kastrup et al., 1978, and references cited therein).

Only a few comparisons of the binding of Etd^+ to RNA, DNA, and hybrid duplexes of the same sequence have been done due to the limited availability of double-stranded RNAs. Bresloff and Crothers (1981) compared the equilibrium binding of Etd^+ to poly[r(A)·r(U)], poly[d(A)·d(T)], the hybrids poly[r(A)·d(T)] and poly[d(A)·r(U)], and the inosine-containing duplexes poly[r(I)·r(C)] and poly[d(I)·d(C)] using absorbance methods. They found a higher binding constant for the RNA and hybrid duplexes relative to the DNA in the poly[A·U(T)] series [see also Chou et al. (1987)], while the binding constants for the RNA and DNA in the poly[I·C] series were about equal. The higher binding constants for Etd^+ binding to the r(A)·r(U) series relative to that for the d(A)·d(T) series was also observed in oligonucleotides by Nelson and Tinoco (1984). Baguley and Falkenhaug (1978) found that Etd^+ binding constants are ranked as follows: r(A)·r(U) > d(C-G) > d(A-C)·d(G-T) > d(A-T) > d(G)·d(C) > d(A-G)·d(C-T) > d(A)·d(T). A reduced affinity of Etd^+ for d(A)·d(T) in natural DNAs was recently detected by DNase I footprinting of Etd^+-bound restriction fragments (Fox & Waring, 1987). The high relative affinity of Etd^+ for poly[r(A)·r(U)] suggests that these sequences may not be as highly discriminated against in RNA. A surprising result noted by Bresloff and Crothers (1981) was that Etd^+ binds to DNA with a neighbor-exclusion parameter of 2 (1 Etd^+/2 bp), while RNA has a value of 3.

Pohl and Jovin (1972) initially observed a highly cooperative salt-induced conformational change in poly[d(C-G)] that was subsequently shown to correspond to a change from the right-handed B-form to the left-handed Z-form [reviewed by Rich et al. (1984)]. Pohl et al. (1972) demonstrated that Etd^+ acts as a cooperative antagonist to the B to Z transition in poly[d(C-G)]. These studies were extended by Mirau and Kearns (1983), who showed that the effectiveness of several

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[1] Abbreviations: bp, base pairs; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; Etd^+, ethidium ion; IgG, immunoglobulin G; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride.

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intercalating drugs in inhibiting the rate of the B to Z transition correlates better with their binding kinetics than their binding constants. In discussing their results, they emphasized the effects of the drugs on the nucleation and propagation steps of the transition. Walker et al. (1985) showed that Etd$^+$ binding cooperatively induces regions of right-handed DNA at and near the intercalation site in the following Z-DNAs: poly[d(C-G)] stabilized by 4.4 M NaCl or 40 μM [Co$(NH_3)_6]^{3+}$ and poly[d(m^{5}C-G)] in 2 mM $MgCl_2$. The stoichiometries required to induce the left- to right-handed transition were shown to range between 1 Etd$^+$/4 bp and 1 Etd$^+$/25 bp depending on the method used to stabilize the Z conformation.

Hall et al. (1984a) showed that poly[r(C-G)] is stabilized in the left-handed Z conformation in 6 M $NaClO_4$ buffer at 45 °C. A much higher ionic strength is required to stabilize the Z conformation in RNA than in DNA, i.e., >2.7 M NaCl for poly[d(C-G)] (Pohl & Jovin, 1972). Subsequently, it was shown that 4 M $MgCl_2$ also stabilizes a left-handed Z conformation in poly[r(C-G)] (Cruz et al., 1986a). In 4 M $MgCl_2$ solution the polynucleotide adopts a conformation with a Z-DNA-like circular dichroism spectrum. In 6 M $NaClO_4$ or NaBr buffers poly[r(C-G)] has a circular dichroism spectrum distinctly different from Z-DNA in the 240–300-nm region. On the basis of this difference, these conformations have been termed Z_D- and Z_R-RNA, respectively (Cruz et al., 1986a). As shown with poly[d(C-G)] (Möller et al., 1984), chemical bromination of guanine and cytosine residues stabilizes the Z-form of poly[r(C-G)] (Hardin et al., 1987). However, the level of modification required to stabilize the RNA in the Z conformation (49% br^{8}G, 43% br^{5}C) is higher than is required to facilitate the B → Z transition in the DNA under the same conditions (38% br^{8}G, 18% br^{5}C). It was also demonstrated that anti-Br-poly[d(C-G)] antibodies specifically recognize a Z-DNA-like determinant present in Br-poly[r(C-G)] (Hardin et al., 1987). However, there are similarities and important differences when one compares the right- to left-handed transitions in RNA and DNA [see also Cruz et al. (1986b) and Hardin et al. (1988)].

Ethidium interactions with nucleic acids provide a general model of the biological activities of a number of intercalative drugs. Ethidium has also been established as a useful probe of left- and right-handed conformational equilibria in DNA (Walker et al., 1985). Here we study the structural and thermodynamic properties of ethidium binding to alternating (C-G) sequence RNA and DNA in right- and left-handed conformations. The studies compare the left- and right-handed equilibria of the two polynucleotides and show the general ability of ethidium to discriminate between DNA and RNA and right- and left-handed helical conformations.

Materials and Methods

Poly[r(C-G)] was prepared by transcription using a poly[d(I-C)] template and *Escherichia coli* RNA polymerase as described by Hall et al. (1984b). The enzyme was kindly provided by Professor Michael Chamberlin (Department of Biochemistry, University of California, Berkeley). Poly[d(I-C)] and poly[d(C-G)] were purchased from Sigma. Poly[d(C-G)] was dialyzed extensively against 10 mM sodium phosphate (pH 7), 1 mM EDTA, and then H_2O prior to use. Chemical bromination of poly[r(C-G)] and poly[d(C-G)] was achieved as described previously (Hardin et al., 1987). The extent of bromination was determined by HPLC analysis of the neutralized perchloric acid hydrolysates (Hardin et al., 1987; Möller et al., 1984). Br-poly[r(C-G)] contained 49% br^8 G and 43% br^{5}C, and Br-poly[d(C-G)] contained 35% br^8 G and 19% br^{5}C. Brominated polynucleotides were protected from unnecessary light during all manipulations. Molar extinction coefficients per base pair at 260 nm (M^{-1} cm^{-1}) are as follows: poly[r(C-G)], 13 120 (Gray et al., 1981); Br-poly[r(C-G)], 11 540 (Hardin et al., 1987); poly[d(C-G)], 14 200 (Walker et al., 1985, supplementary material); Br-poly[d(C-G)], 9500 (Möller et al., 1984). Ethidium bromide was purchased from Sigma; no detectable impurities were observed by ^{1}H NMR. Unless otherwise specified all experiments were performed in 40 mM Tris-HCl (pH 7.5) buffer containing 220 mM NaCl and 4 mM EDTA.

Circular Dichroism Measurements. CD spectra were recorded on a Jasco J500C spectropolarimeter in a 1-cm Teflon-stoppered quartz cuvette. The temperature was maintained at 25 °C (standard conditions) by a Zeiss Model P/N 80 thermoelectric unit.

Optical Titrations. Binding isotherm data were collected by using a Cary 118 UV–vis spectrophotometer at 25 °C. Sufficient stock polynucleotide solution was added to buffer in 1-cm path length cuvettes to yield a concentration of 35–50 μM (bp). Successive aliquots from a stock Etd$^+$/buffer solution were added to the cuvette at 15-min intervals. The A_{480} was recorded following mixing and equilibration for 10 min. The polynucleotide concentration was calculated at each r value; the decrease in polynucleotide concentration as a result of dilution was <15%. The extinction coefficients used for free (ϵ_f) and bound (ϵ_b) ethidium (in M^{-1} cm^{-1} at 480 nm) were 5680 and 2800, respectively (Walker et al., 1985).

Thermal Dissociation Measurements. Thermal dissociation of Etd$^+$ from the polynucleotides was monitored by using a Gilford Model 250 UV–vis spectrophotometer at 480 nm; the heating rate was maintained at 0.25 °C/min and monitored by a Gilford Model 2527 thermoprogrammer. Absorbance versus temperature data were accumulated on an Apple IIe microcomputer interfaced to the instrument. The temperature was increased from 0 to 90 °C and returned to 0 °C to check for evaporation and hysteresis; absorbance changes due to these factors were less than 1%. These experiments were performed in 10 mM sodium phosphate (pH 7.5) and 220 mM NaCl buffer in place of Tris-HCl and NaCl due to the strong temperature dependence of the pK_a of Tris-HCl. The binding constants were the same within experimental error in both buffers at 25 °C.

Enthalpy values for Etd$^+$ binding were obtained from van't Hoff analysis with intrinsic equilibrium binding constants for the right-handed forms (K_R) calculated at each temperature according to the binding equation for interacting ligands (Crothers, 1968; McGhee & von Hippel, 1974)

$$r/c_f = K(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1} \quad (1)$$

where r is the bound ethidium to base pair ratio, c_f is the free ethidium concentration, and n is the neighbor-exclusion limit.

Results

CD of the Etd$^+$–Polynucleotide Complexes. The CD spectra of A-form poly[r(C-G)] are shown in Figure 1A as a function of the amount of Etd$^+$ bound. The spectrum of poly[r(C-G)] is altered significantly upon Etd$^+$ binding (Figure 1A). Large changes occur in the 280–350-nm range, where the CD goes from negative values to positive values upon Etd$^+$ intercalation. Bound Etd$^+$/bp stoichiometries for the poly[r(C-G)] (A-RNA) spectra corresponding to 0, 1/6.6, and 1/3.3 were monitored concurrently by the Etd$^+$ absorbance change at 480 nm due to intercalation. Another significant change in the CD spectrum occurs at wavelengths below 250 nm, where the CD becomes more negative upon intercalation.

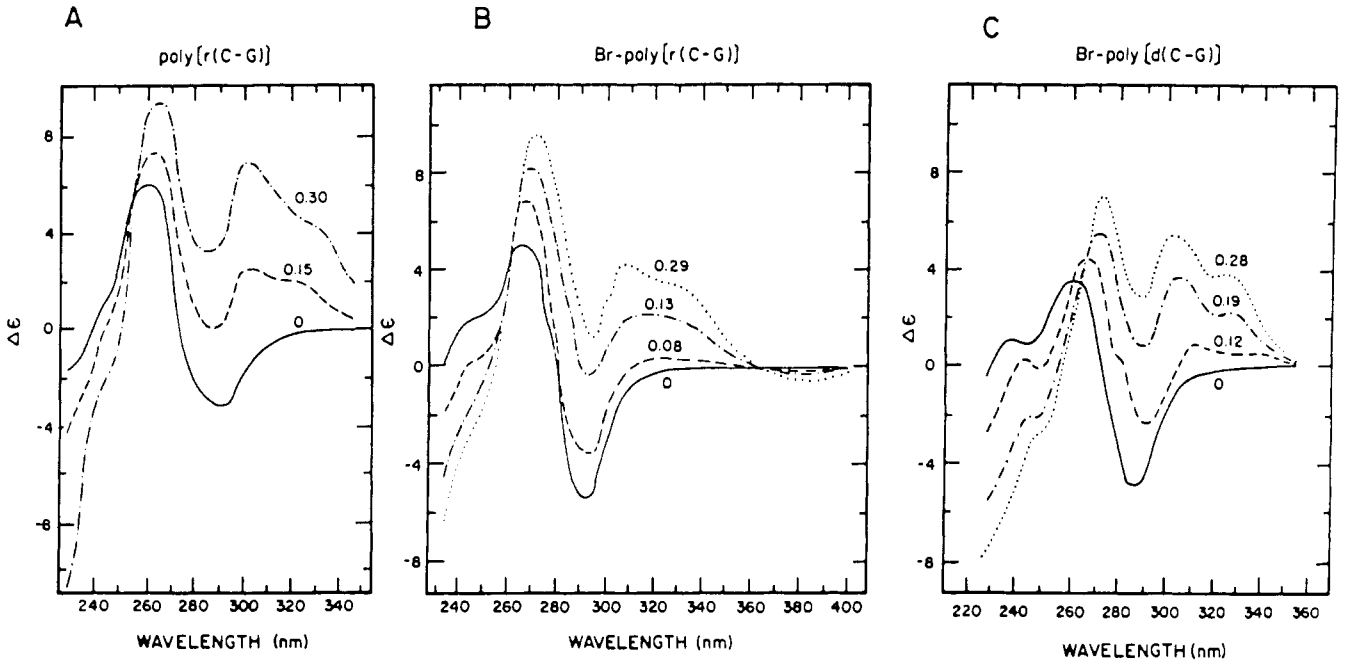

FIGURE 1: Circular dichroism spectra of Etd^+–polynucleotide complexes in 40 mM Tris-HCl (pH 7.5), 4 mM EDTA, and 220 mM NaCl buffer at 25 °C. (A) Poly[r(C-G)] (A-RNA) upon addition of Etd^+ to the indicated bound Etd^+:polynucleotide ratios (r). (B) Br-poly[r(C-G)] (Z-RNA) at the indicated r values. (C) Br-poly[d(C-G)] (Z-DNA) at the indicated r values. The r values were obtained from concurrent absorbance titration experiments.

All CD spectra corresponding to right-handed alternating (C-G) sequence polynucleotides and oligonucleotides measured to date have negative CD in the 240-nm region, while all left-handed molecules of this family have positive CD in this region regardless of the type of modification or solution conditions required to induce the Z-form (Pohl & Jovin, 1972; Uesugi et al., 1984; Tinoco et al., 1986; Hardin et al., 1987; Behe & Felsenfeld, 1981; Wu & Behe, 1984). Parts B and C of Figure 1 show the effect of ethidium binding on the spectra of Br-poly[(C-G)] and Br-poly[d(C-G)] in 220 mM NaCl buffer, respectively. Previous studies have shown that both polynucleotides are in the Z-form in the absence of ethidium under these conditions (Hardin et al., 1987; Möller et al., 1984). In both cases, the positive band at 240 nm becomes negative upon Etd^+ binding, and the negative bands at 295 nm become positive. The qualitative effects of Etd^+ binding to Br-poly[d(C-G)] were reported by Möller et al. (1984); our results agree with the previous study. The spectra in Figure 1B correspond to 1/12.5 and 1/7.7 bound Etd^+/bp of Br-poly[r(C–G)], while those in Figure 1C are for ratios of 1/8.3 and 1/5.3 bound Etd^+/bp of Br-poly[d(C-G)].

Circular dichroism values at 295 nm are plotted as a function of r for Etd^+ binding to A-RNA and brominated Z-RNA in Figure 2A and to brominated Z-DNA in Figure 2B. Values corresponding to the unmodified right-handed polynucleotides in 220 mM NaCl buffer in the absence of Etd^+ are also shown. Previous studies have shown that in the range r = 0–0.15, $\Delta\epsilon_{295}$ values for B-form poly[d(C-G)] are essentially constant (Walker et al., 1985). Similar data plotted in Figure 2A for poly[r(C-G)] show that $\Delta\epsilon_{295}$ changes only slightly for r = 0–0.15. A comparison of $\Delta\epsilon_{295}$ values show that at a stoichiometry of 1 Etd^+/9 bp, $\Delta\epsilon_{295}$ values for intercalated Br-poly[r(C-G)] approach that of A-RNA; at 1 Etd^+/6 bp, $\Delta\epsilon_{295}$ values for intercalated Br-poly[d(C-G)] approach that of B-DNA. At corresponding values of $r > 0.15$, the CD spectra for all three types of polynucleotide are quantitatively similar in the 230–350-nm region (Figure 1) and closely resemble the corresponding spectra of intercalated poly[d(C-G)] (B-DNA) in low-salt buffer (Walker et al., 1985). We conclude from these data that all four Etd^+ complexes (with Z-RNA, A-RNA, Z-DNA, and B-DNA) are in similar conformations in the range $r \geq 0.15$.

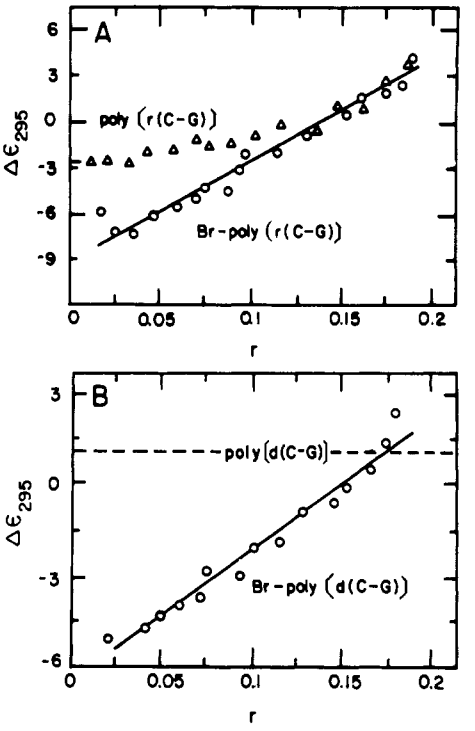

FIGURE 2: CD values at 295 nm as a function of r for Etd^+–polynucleotide complexes. Data are shown for Br-poly[r(C-G)] (○) and poly[r(C-G)] (△) in (A) and for Br-poly[d(C-G)] in (B). These data were obtained under the conditions described in Figure 1 and are plotted as a function of r values obtained from concurrent absorbance binding isotherm data. CD values corresponding to unmodified B-DNA in the absence of Etd^+ (Walker et al., 1985) are also shown in (B).

Thermodynamics for Etd^+ Intercalation into Alternating (C-G) Sequence Polynucleotides. The absorption maximum in the visible spectrum of Etd^+ shifts from 480 to about 520

Table I: Thermodynamic Parameters for the Binding of Ethidium to Alternating (C-G) Series Polynucleotides in 220 mM NaCl Buffer

polynucleotide (conformation)	K_L[a] ($\times 10^{-4}$ M^{-1})	K_R[b] ($\times 10^{-4}$ M^{-1})	limiting binding stoichiometry[c]	ΔG_R[d] (kcal/mol)	ΔH_R[e] (kcal/mol)	ΔS_R[f] [cal/(mol·K)]
poly[r(C-G)](A-RNA)	*f*	3.6 ± 0.5[g]	2.6 ± 0.3[g]	−6.2	−9.3 ± 1.5[g]	−11
poly[d(C-G)](B-DNA)	*f*	9.4 ± 0.4	1.9 ± 0.1	−6.8	−13.1 ± 0.8	−21
Br-poly[r(C-G)]	0.6	9.4 ± 0.3	2.9 ± 0.2	−6.8	−10.6 ± 1.1	−13
Br-poly[d(C-G)]	2.2	21.3 ± 0.5	3.0 ± 0.4	−7.3	−11.7 ± 1.2	−22

[a] Binding constant for the interaction of Etd$^+$ with an isolated Z-form site; obtained as r/c_f at $r = 0$. [b] Binding constant for intercalation of Etd$^+$ into an isolated right-handed site obtained by using eq 1; values for r/c_f and $r(\geq 0.15)$ were used for Br-poly[r(C-G)] and Br-poly[d(C-G)]. [c] Obtained as $1/r$ at $r/c_f = 0$. [d] Obtained from $\Delta G_R^\circ = -RT \ln K_R$. [e] Obtained by van't Hoff analysis. [f] Right-handed polynucleotide in 220 mM NaCl buffer. [g] Standard deviation in duplicate analyses.

nm and decreases in intensity when the ion binds to both right- and left-handed polynucleotides (Lepecq & Paoletti, 1967; Pohl et al., 1972). This hypochromic shift at 480 nm can be analyzed to obtain the concentrations of both free and bound Etd$^+$ as a function of the binding stoichiometry. These data can then be used to construct the binding isotherm for the system. Scatchard (1949) plots are shown in Figure 3A,B for Etd$^+$ binding to each of the right- and left-handed alternating (C-G) sequence polynucleotides investigated in this study.

Ethidium binds in a noncooperative manner to poly[r(C-G)] and poly[d(C-G)] as evidenced by the negative slopes in the Scatchard plots (Figure 3A). Limiting binding stoichiometries and intrinsic binding constants (K_R, where R and L refer to right- and left-handed polynucleotides, respectively) are listed in Table I for A-RNA and B-DNA. Etd$^+$ binds with a higher affinity to DNA than to RNA in alternating (C-G) polynucleotides in contrast to d(A)·d(T) and r(A)·r(U) series oligo- and polynucleotides where the order is reversed (Nelson & Tinoco, 1984; Bresloff & Crothers, 1981; Baguley & Falkenhaug, 1978; Chou et al., 1987). In agreement with the results of Bresloff and Crothers (1981) 1 Etd$^+$ apparently binds to 3 bp of A-RNA, while 1 Etd$^+$ binds per 2 bp of B-DNA.

Scatchard plots for Etd$^+$ binding to the Z-forms of RNA and DNA show a change from positive to negative slope at $r \sim 0.1$, indicating positive cooperativity in the binding reactions (Figure 3B). These values closely correspond to the r values at which the CD spectra of these brominated polynucleotides approach the spectra of the respective right-handed unmodified polynucleotides (Figures 1 and 2). Similar results were obtained by Walker et al. (1985) in their study of Etd$^+$ binding to the Z-forms of poly[d(C-G)] induced by 4.4 M NaCl or 40 μM $[Co(NH_3)_6]^{3+}$ and the Z-form of poly[d(m^{5}C-G)] induced by 2 mM $MgCl_2$. These results indicate that Etd$^+$ binding to the left-handed forms of Br-poly[r(C-G)] and Br-poly[d(C-G)] results in cooperative conversion to right-handed intercalated forms of RNA and DNA at Etd$^+$:polynucleotide stoichiometries well below the limiting binding stoichiometries. Thus, tracts contiguous to the Etd$^+$-binding site in both Z-RNA and Z-DNA are affected in an allosteric manner by the intercalation event.

Large uncertainties in bound ethidium concentrations at high r and the asymptotic behavior of Scatchard plots at high r values due to neighbor exclusion (Crothers, 1968; McGhee & von Hippel, 1974) create difficulties in estimating limiting binding stoichiometries. Thus, although we report values of 3 for the limiting binding stoichiometries for intercalation of Etd$^+$ into A-RNA, Z-DNA, and Z-RNA and a value of 2 for B-DNA, errors of ±1 in the neighbor exclusion range are clearly possible.

A model for induced allosteric changes in DNA proposed by Crothers and collaborators (Dattagupta et al., 1980; Bresloff & Crothers, 1981) was used to analyze the above binding data. The model allows for drug binding to two forms

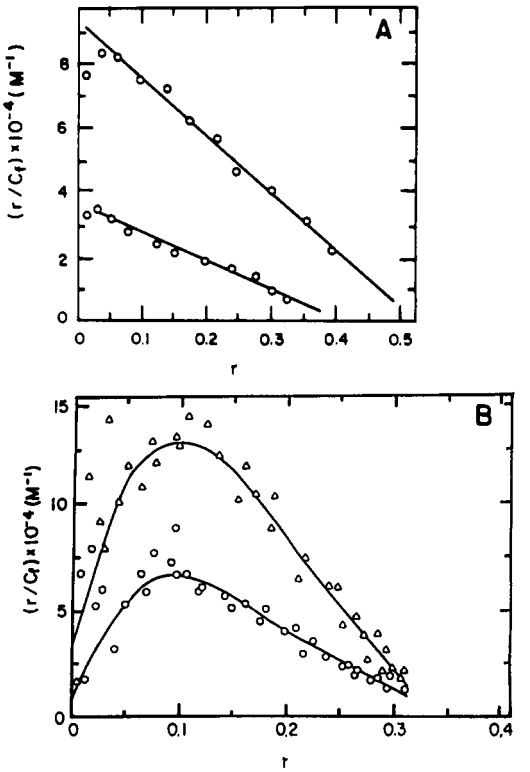

FIGURE 3: Equilibrium binding isotherms for the interaction of Etd$^+$ with (A) poly[r(C-G)] and poly[d(C-G)] and (B) Br-poly[r(C-G)] and Br-poly[d(C-G)]. Etd$^+$ absorbance data were obtained under the conditions described in Figure 1 and were analyzed according to the method of McGhee and von Hippel (1974). Binding constants (r/c_f at $r = 0$) and limiting binding stoichiometries ($1/r$ at $r/c_f = 0$) were obtained by linear regression analysis of these data in the range $r \geq 0.14$. Theoretical fits to the data in (B) were generated by using the allosteric binding model of Bresloff and Crothers (1981). Parameters used in the case of Br-poly[r(C-G)] were $K_R/K_L = 100$, $n_L = n_R = 3$, $\tau_L = \tau_R = 1$, $S = 0.85$, and $\sigma = 0.05$; all parameters were the same in the case of Br-poly[d(C-G)] except $S = 0.9$ and $n_R = 2$.

of a nucleic acid. In the present case, left- and right-handed conformations of a polynucleotide are represented by form L and form R, respectively. Adjustable parameters for the two forms in the model include intrinsic binding constants K_L and K_R, neighbor-exclusion ranges n_L and n_R, and cooperativity parameters τ_L and τ_R, where $K_i\tau_i$ is the binding constant for the intercalator bound the minimum distance from a contiguous Etd$^+$ molecule. Other adjustable parameters include S, which is the equilibrium constant for the conversion of a base pair from form L to form R at the interface between the two forms, and σ, where $\sigma^2 S$ is the equilibrium constant for nucleating the formation of form R within a tract of form L polynucleotide. The data were successfully fit by using the model (Figure 3B) and the parameters listed in the legend for Figure 3. Similar results were obtained for all values of K_R/K_L

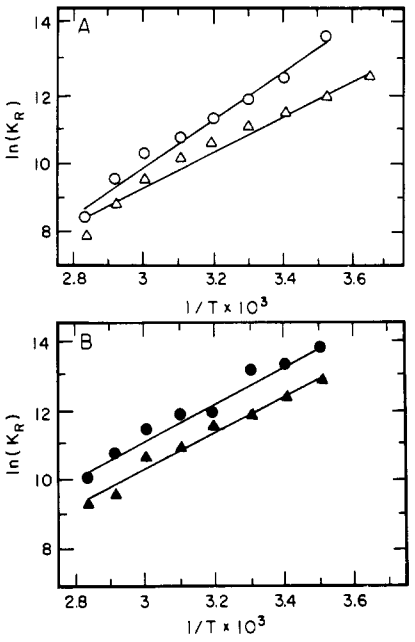

FIGURE 4: Effect of temperature on the binding of Etd^+ to (A) poly[r(C-G)] (△) and poly[d(C-G)] (○) and (B) Br-poly[r(C-G)] (▲) and Br-poly[d(C-G)] (●). Initial polynucleotide:bound Etd^+ ($1/r$) were as follows: poly[r(C-G)], 10; poly[d(C-G)], 11; Br-poly[r(C-G)], 12; Br-poly[d(C-G)], 7. Binding constants were obtained from absorbance data measured as a function of temperature under the conditions described in Figure 1. Equilibrium binding enthalpies were obtained by van't Hoff analysis as described under Materials and Methods and are listed in Table I.

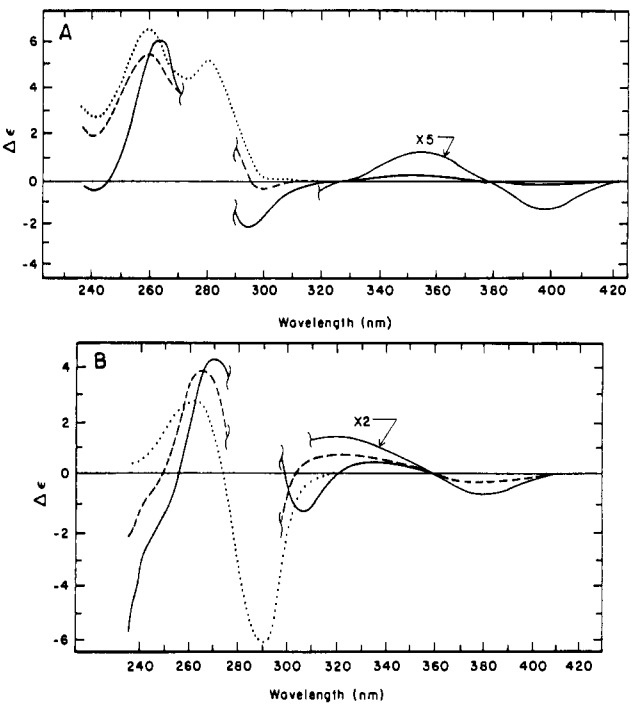

FIGURE 5: Circular dichroism spectra of Etd^+ complexes with unmodified left-handed Z-forms of poly[r(C-G)]. (A) Spectra of poly[r(C-G)] (12 μM in bp) in 10 mM sodium phosphate (pH 7), 1 mM EDTA, and 6 M sodium perchlorate (Z_R-form) upon addition of 0 (···), 150 μM (---), and 250 μM (—) Etd^+. (B) Spectra of poly[r(C-G)] (12 μM in bp) in 4 M $MgCl_2$, pH 5.75 (Z_D-form) upon addition of 0 (···), 200 μM (---), and 250 μM (—) Etd^+. CD curves are also shown at the indicated increased sensitivities in the 320–420-nm range to emphasize the Etd^+-induced CD bands in this region.

in the range 10–1000. This analysis supports an agreement between the polynucleotide CD results and Etd^+ absorbance binding data in showing the presence of induced allosteric transitions from left- to right-handed intercalated conformations.

The enthalpies for Etd^+ binding to each of the four polynucleotides were determined by van't Hoff analysis using binding constants obtained from eq 1 (Figure 4, Table I). Some curvature is present in the van't Hoff plots for the right-handed polynucleotides; however, since a linear fit is well within the error limits of the data, they were analyzed by linear regression. The $\Delta H_R°$ contributions are ordered as follows: B-DNA (−13.1 kcal mol^{-1}) < Z-DNA (−11.7) < Z-RNA (−10.6) < A-RNA (−9.3). Entropies (ΔS_R) for Etd^+ binding to the four polynucleotides were determined by using $\Delta H_R°$ and $\Delta G_R°$ (see below) values listed in Table I. These results demonstrate that the binding of Etd^+ to all four types of polynucleotide is enthalpically driven.

The analysis of Chaires (1986) illustrates the fact that a key quantity is required in order to assess the individual binding constants for the interaction of Etd^+ with right- and left-handed polynucleotides. This quantity is the free energy for the transition that couples the nonintercalated left- and right-handed conformations, $\Delta G°(Z \rightarrow A/B)$. Since the three equilibria (i.e., $Z \leftrightarrow A/B$, $Z \leftrightarrow Z{\cdot}Etd^+$, $A/B \leftrightarrow A/B{\cdot}Etd^+$) are coupled, a thermodynamic cycle can be constructed as shown previously (Chaires, 1986). From these relationships we can obtain reasonable estimates for the $\Delta G°$ contributions due to binding of Etd^+ to left- and right-handed sites. Values for $\Delta G°(Z \rightarrow A/B)$ are obtained from the relationship $\Delta G°(Z \rightarrow A/B) = -RT \ln S$, where values of S for the transitions in Br-poly[r(C-G)] and Br-poly[d(C-G)] are listed in the legend of Figure 3: $\Delta G°(Z \rightarrow A) = +0.1$ kcal/mol, and $\Delta G°(Z \rightarrow B) = +0.06$ kcal/mol. Thus, using values from Table I, we calculate $\Delta G°(Z \rightarrow Z{\cdot}Etd^+) = -5.2$ kcal/mol and $\Delta G°(Z \rightarrow A{\cdot}Etd^+) = -6.7$ kcal/mol for Br-poly[r(C-G)] and $\Delta G°(Z \rightarrow Z{\cdot}Etd^+) = -5.9$ kcal/mol and $\Delta G°(Z \rightarrow B{\cdot}Etd^+) = -7.2$ kcal/mol for Br-poly[d(C-G)]. As observed by Chaires (1986) with the intercalator daunomycin, these results show that the conformational transitions between right- and left-handed polynucleotides are driven by coupling to the energetically favorable transitions between free Z-forms and Etd^+-bound A- or B-forms. Also, note that since the enthalpies for Etd^+ binding to the brominated polynucleotides were obtained with r values (at 25 °C) that are >0.15, the $\Delta H_R°$ listed in Table I will closely approximate $\Delta H°(A/B \rightarrow A/B{\cdot}Etd^+)$ for these polynucleotides.

The $\Delta G_R°$ values for Etd^+ intercalation into the A- and B-form polynucleotides obtained by using eq 1 (as described above) are listed in Table I. Note that Etd^+ has a higher affinity for the brominated polynucleotides despite the additional free energy required for the left- to right-handed transitions. These elevated affinities may be due to the strong positive electrostatic energy component for Etd^+ binding and the polarizable nature of the bromine atom. Similar relatively minor effects have also been seen in studies of the protein-binding properties of brominated polynucleotides (Hardin et al., 1987, 1988). More importantly, the higher binding constant for DNA relative to RNA with the right-handed polynucleotides is maintained with the brominated Z-form polynucleotides; thus, this phenomenon is independent of the chemical modification. The K_R values are in good quantitative agreement with the previously measured value for poly[d(C-G)] (Winkle et al., 1982) and data for a variety of other polynucleotides when differences in buffer conditions are accounted for (Bresloff & Crothers, 1981; Walker et al., 1985; Chou et al., 1987).

Binding of Etd^+ to Unmodified Z-RNA. Panels A and B of Figure 5 show the CD spectra for poly[r(C-G)] in 6 M

$NaClO_4$ buffer (Z_R-RNA) and in 4 M $MgCl_2$ (Z_D-RNA), respectively, before and after adding Etd$^+$ to the solutions. The elevated ionic strength of these buffers reduces the binding affinity of Etd$^+$ approximately 10-fold compared to binding in 220 mM NaCl. Consequently, the high Etd$^+$ concentrations required and the limited solubility of the drug in these high ionic strength buffers did not allow us to record CD spectra near the Etd$^+$ UV absorbance band (270–290 nm). The CD spectra change upon Etd$^+$ binding in a manner consistent with conversion to right-handed intercalated forms. For example, CD values at 230 nm become negative, and a spectrum corresponding to bound ethidium is observed in the 420–320-nm range.

Discussion

The availability of poly[r(C-G)] (Hall et al., 1984b) has allowed the first direct comparison of the properties of right- and left-handed alternating (C-G) sequence polynucleotides of RNA and DNA [e.g., Cruz et al. (1986a,b) and Lamos et al. (1986)]. Since high ionic strength conditions are required to stabilize the Z-forms of both DNA and RNA (Pohl & Jovin, 1972; Hall et al., 1984a), their drug-binding properties could not be compared directly with those of the right-handed forms. However, partially chemically brominated poly[r(C-G)] and poly[d(C-G)] were recently shown to be in similar left-handed Z conformations at low ionic strength (Hardin et al., 1987; Möller et al., 1984), allowing the first direct comparison of the drug-binding properties of right- and left-handed RNA and DNA under the same solution conditions.

In agreement with previous studies with the Z-forms of poly[d(C-G)] and poly[d(m^5C-G)] (Pohl et al., 1972; Walker et al., 1985), we find that binding of Etd$^+$ to the Z-forms of Br-poly[r(C-G)] and Br-poly[d(C-G)] results in cooperative transitions to right-handed intercalated polynucleotides. The transitions occur in an allosteric manner at stoichiometries of approximately 1 Etd$^+$/9 bp for Z-RNA and 1 Etd$^+$/6 bp for Z-DNA (Figures 2 and 3B). The CD results suggest that a right-handed bound conformation is formed at the intercalation sites. Moreover, quantitative similarities between the CD spectra in the range $r \geq 0.15$ suggest that the brominated and unmodified polynucleotide all adopt similar Etd$^+$-bound conformations.

Since the data for the Z-form polynucleotides were fit successfully to the allosteric binding model of Crothers and collaborators (Dattagupta et al., 1980; Bresloff & Crothers, 1981) using $K_R/K_L \geq 10$, there is at least a 10-fold higher preference for Etd$^+$ intercalation into the right-handed forms of the polynucleotides. It is important to note that this is an equilibrium binding preference and does not preclude a binding mechanism involving transient intercalation into Z-form polynucleotides (Shafer et al., 1984; Gupta et al., 1983).

The present studies show similarities and important differences in the binding of Etd$^+$ to alternating (C-G) sequence polynucleotides and other sequence classes of RNA and DNA. Thermodynamic results for the binding of Etd$^+$ to all four types of polynucleotide are quantitatively similar. Values for $\Delta G_R°$, $\Delta H_R°$, and $\Delta S_R°$ are all negative (Table I) and in the range of values measured previously for Etd$^+$ binding to other A- and B-form poly- and oligonucleotides (Bresloff & Crothers, 1981; Chou et al., 1987; Nelson & Tinoco, 1984; Jones et al., 1986). The drug binds about 2-fold more strongly to alternating (C-G) sequence B-DNA (Z-DNA) than to A-RNA (Z-RNA) (Table I). This is in contrast to results obtained with [d(A)·d(T)] and [r(A)·r(U)] sequence nucleic acids where the opposite order of affinities was found, i.e., K_R(RNA) > K_R(DNA) (Baguley & Falkenhaug, 1978; Bresloff & Crothers, 1981; Chou et al., 1987; Nelson & Tinoco, 1984).

Jaworski et al. (1987) recently demonstrated that catalysis of DNA methylation by *Eco*RI methylase is inhibited both in vitro and in vivo by the presence of superhelical stress-induced Z-DNA inserts in plasmids. They discussed their results in the context of the concept of "microconformational heterogeneity". They showed that an induced conformational change can act as a mechanism for altering the biological compatibility of an enzymatic recognition site on a nucleic acid. Thus, while we are beginning to understand the range of possible conformations adopted by DNA [e.g., see Lilley et al. (1987), Diekmann and Zarling (1987), and Henderson et al. (1987)], it is clear that the solutions to many biological questions involving nucleic acids will require consideration of structure–function relationships such as illustrated with the *Eco*RI methylase/Z-DNA system.

The results in this paper show that Etd$^+$ binding occurs in very different ways with right- and left-handed polynucleotides, i.e., noncooperatively in the former case and cooperatively in the latter. Thus, the detailed effects of intercalator-induced conformational changes in DNA and RNA (and their corresponding effects on potential biological activities) are dependent on both sequence and initial conformational state [see also Chou et al. (1987) and Jones et al. (1986)].

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Registry No. Etd$^+$, 3546-21-2; poly[r(C-G)], 49846-05-1; poly[d(C-G)], 36786-90-0.

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