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THE INTERACTION OF ETHIDIUM BROMIDE WITH NUCLEIC ACIDS

by

WILLIAM WARREN MARTZ

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF LOYOLA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

The Interaction of Ethidium Bromide with Nucleic Acids

Ethidium bromide (EB) is a phenanthridinium derivative which interferes with nucleic acid metabolism in a variety of organisms apparently by forming a strong primary complex in which the planar phenanthridinium ring system is intercalated between adjacent base pairs of the DNA double helix. Primary binding occurs up to a bound EB to DNA nucleotide ratio (r) of 0.20-0.25. At higher ratios a weaker secondary binding process occurs.

The interaction of EB with DNA results in the induction of circular dichroic (CD) bands throughout the spectral region from 220 nm to 550 nm. One positive band centered near 307 nm reflects primary binding, whereas a negative band centered near 500 nm appears to reflect both primary and secondary binding. The molar circular dichroism (E_L-E_R) at 307 nm, calculated on the basis of bound EB, increases from zero at r = 0 to +25.0 at r = 0.30, corresponding to the saturation of primary binding sites. The increase in E_L-E_R with increasing r indicates that the CD band at 307 nm results from nearest-neighbor interactions between EB molecules bound to adjacent primary binding sites. In the EB-ice quenched heat denatured DNA complex, E_L-E_R is +12.89 at r = 0.10 compared to an E_L-E_R of +7.50 for EB-native DNA at

the same ratio. At an added EB/P ratio of 0.10, E_L-E_R increases from +5.00 to 32.00 between 75° and 96°C, temperatures sufficient for DNA strand separation. Between pH 11.3 and 11.7 DNA at EB/P equal to 0.15, undergoes partial alkaline denaturation and E_L-E_R of the EB-DNA complex increases from 12.18 to 26.29. This increase in E_L-E_R under conditions at which DNA is partially denatured may be regarded as further evidence that nearest-neighbor effects are responsible for the 307 nm circular dichroism.

In contrast to the results at 307 nm, the E_L-E_R at 500 nm is constant at -0.76 for r values up to 0.52, about twice that required to saturate primary binding sites (0.25) indicating that induced asymmetry at this wavelength results from the binding of EB within the asymmetric environment of the DNA polymer.

Strong induced CD, similar to that noted for the EB-DNA complex, was observed for EB complexes with poly (A + U). A similar induced CD was not observed, however, in EB complexes with either double-stranded poly (A + I), which possesses an unusual hydrogen-bonded structure, or with single-stranded poly C. These results indicate EB may form primary complexes similar to the EB-DNA complex only with double-stranded polynucleotides in which the hydrogen-bonded base pairing is similar to that usually found in DNA or RNA.

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LIFE

The author was born in Chicago, Illinois on March 15, 1940. He attended Bremen Community High School in Midlothian, Illinois and was graduated in 1958. He continued his education at Southern Illinois University from 1958-1961 majoring in chemistry. He attended Northwestern University from 1961-1965, receiving a Bachelor of Philosophy in Chemistry in 1965.

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The author would like to express his sincere appreciation to Dr. Stelios Aktipis without whom the opportunity to perform this research would not have been afforded. A debt of thanks is owed to the many colleagues at the Loyola University Medical Center who have provided council and assistance throughout the course of this research. Special gratitude is extended to Dr. N. C. Melchior, Dr. S. Keresztes-Nagy, M. C. Cress, P. J. Fabri, A. J. Iammartino and A. L. N. Rao. Finally I express the utmost appreciation to my wife, Nancy Joy and daughters, Jan Renae and Heidi Lyn for years of faith and patience.

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CHAPTER I: INTRODUCTION

A. BIOCHEMICAL AND ANTIMICROBIAL PROPERTIES OF ETHIDIUM BROMIDE

Ethidium bromide[1] (3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide)[2] is an effective trypanocidal drug first synthesized by Watkins in 1952 (114).

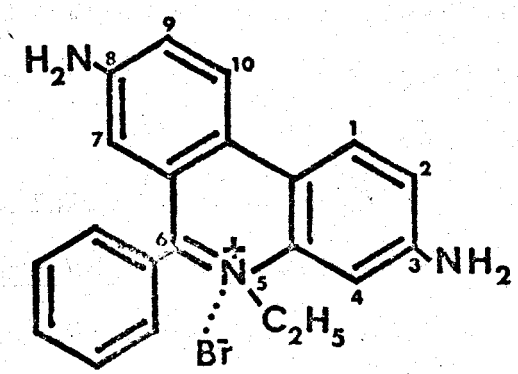


Fig. 1. Ethidium Bromide

Ethidium bromide (EB) is a potent inhibitor of the growth of *Trypanosoma congolense* in cattle (115). Trypanosomes are flagellates which are the cause of trypanosomiasis in man

---

[1]Ethidium bromide and various other aromatic ring systems are often classified as "dyes." This name refers to their intense color and to the use of these compounds as histological stains.

[2]This chemical name is based on the numbering system instituted by the IUPAC in 1957. 2, 7-Diamino-9-phenyl-10-ethylphenanthridinium bormide and 2, 7-diamino-10-ethyl-9-phenylphenanthridinium bromide are alternative names based on a different numbering system. The synonym homidium bromide was used extensively in the earlier literature.

and domestic stock. The *in vivo* trypanocidal activity of this drug is noted only about 24 hours after administration. During this period, from three to seven cell divisions may take place (40). Furthermore, if trypanosomes which have been exposed to ethidium bromide *in vivo* for one hour, are transferred to an untreated animal, infection does not take place (40). This suggests that EB causes some irreversible damage to the trypanosomes during the first hour of contact, which ultimately inhibits their growth. Ormerod (80), on the basis of cell morphology studies, postulated that ethidium combines with nucleoprotein and noted that EB acts to prevent cell division. It has also been demonstrated that the presence of ethidium reduces both the RNA and DNA content of *Crithida oncopelti* and decreases the incorporation of ^{14}C-adenine into RNA (76,77). These results strongly suggest that ethidium prevents or inhibits normal nucleic acid synthesis.

Ethidium bromide exhibits a similar inhibitory effect on nucleic acid systhesis in a variety of other cell systems. These include mammalian tissue cultures of HeLa (119,124), and Ehrlich ascites tumor cells (51); bacteria such as *B. subtilis* (87), *E. coli* (36), and disrupted staphylococci (36); as well as polyoma (21) and other viruses (79,106).

The antibacterial (94) and antiviral (26) properties of EB can be suppressed by the addition of exogenous DNA. *In vitro* DNA synthesis, catalyzed by a partially purified

DNA-dependent DNA-polymerase (EC 2.7.7.7) is also inhibited at EB concentrations comparable to those required to retard the growth of E. coli (31). The data accumulated from these studies give support to the idea that the antimicrobial activity of ethidium bromide is due to its ability to form complexes with DNA and RNA, thus inhibiting synthesis of these nucleic acids (79,94).

B. THE SCOPE OF THIS DISSERTATION

In this dissertation, we will examine the interaction of ethidium bromide with DNA by circular dichroism (CD). As discussed in Chapter III, binding of EB to DNA causes the appearance of several induced CD bands. This investigation will be mostly limited to the spectral region above 300 nm. In this region, DNA itself does not exhibit absorption and, therefore, the induced circular dichroism consists solely of ethidium bromide contributions.

The examination of the circular dichroism of the ethidium bromide-DNA complex with respect to the location of predominant bands and the dependence of the molar circular dichroism of these bands on bound EB permits band assignments to transitions characteristic of various types of EB-polynucleotide complexes. The nature of the EB-DNA interaction in these complexes can be investigated further by examination of the influence on the molar circular dichroism of such factors as complex concentrations, inorganic cations, pH and elevated temperature.

Polynucleotide structural requirements for formation of complexes with specific geometries will also be examined. These studies will be carried out by examination of the circular dichroism of ethidium bromide complexes with synthetic single and double stranded polynucleotides whose secondary structures are well established.

Some current ideas on the nature of the interaction of ethidium bromide with nucleic acids as well as information on the circular dichroism properties of DNA complexes with other dyes whose structural and biological properties are similar to those of ethidium bromide are reviewed briefly in the following sections. This information is of value as a background for discussing the results included in this dissertation.

C. INTERACTION OF ETHIDIUM BROMIDE WITH NUCLEIC ACIDS

1. Formation of Ethidium Bromide-DNA Complexes

The interaction of ethidium bromide with DNA and RNA has been extensively studied by Waring (111). Ethidium forms complexes with DNA and RNA (108) in which the ethidium is strongly bound to the polynucleotide. The DNA-bound EB cannot be removed by dialysis but may be dissociated from DNA at high sodium chloride concentrations or by chromatography on cation exchange resins (83).

Ethidium causes pronounced changes in the physical properties of DNA. Upon interaction with EB, an increase

in intrinsic viscosity and a decrease in the sedimentation coefficient of DNA are observed (57). The binding of ethidium bromide to nucleic acids also produces a metachromic shift in the absorption spectrum of the dye near 500 nm (Fig. 2). The absorption maximum for bound EB (Fig. 2a) varies slightly with each polynucleotide. However, the metachromic shift is similar in each case.

Free EB (Fig. 2b, spectrum A) exhibits an absorption maximum at 479 nm with a molar extinction coefficient of 5600. Increasing concentrations of DNA progressively shift the 479 nm maximum towards 517 nm and decrease the extinction coefficient to 3900. At EB/DNA ratios below 0.14 no further changes in the spectrum are noted. Ethidium in the fully complexed form exhibits a characteristic spectrum (E) with a maximum near 517 nm. Spectra of EB containing various intermediate amounts of DNA cross at an isosbestic point near 510 nm. This indicates that the spectrum for each intermediate ratio is the result of superimposition of the characteristic absorption spectra of free and bound EB. Thus the properties of free and bound dye at each ratio may be determined from these spectra. Obviously most accurate results can be obtained from measurements at the wavelengths at which the difference between the spectra of free and bound EB is the largest (81,111).

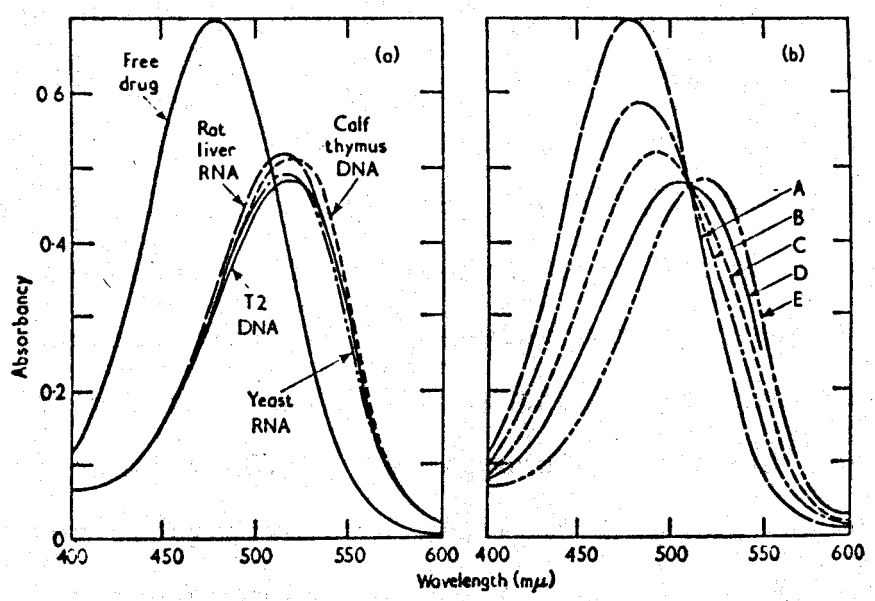


Fig. 2. Effect of nucleic acids on the absorption spectrum of ethidium bromide. Solutions contained 1.25 x 10^{-4} M ethidium and the absorbancy was measured using a 1-cm light path. In panel (a) the concentration of each nucleic acid was 1.5 x 10^{-3}M. In panel (b) T2 DNA was present at the following concentrations: A, zero; B, 1.5 x 10^{-4}M; C, 3 x 10^{-4}M; D, 5 x 10^{-4}M; E, 1.2 x 10^{-3}M. (M. J. Waring, J. Mol. Biol. (1965) 13, 269).

2. The Relationship between DNA-Bound Ethidium Bromide and DNA-Polymerase Inhibition

A direct correlation between the amount of EB bound to template DNA and the degree of inhibition of *E. coli* DNA-dependent RNA-polymerase (EC 2.7.7.6) has been reported by Waring (109). The properties of DNA templates containing varying amounts of EB, expressed as the ratio (r) of bound EB per nucleotide residue of DNA, were compared with EB-free templates. A linear decrease in RNA polymerase activity was observed for increasing ratios between 0 and 0.10. Nearly total inhibition of the enzyme was observed at a ratio equal to 0.11, corresponding to about one EB molecule for every five base pairs in DNA. These results suggest that the pharmacological properties of EB may depend on the formation of EB-DNA complexes with nucleic acids.

3. Binding Parameters for the Ethidium Bromide-DNA Interaction

(a) Spectrophotometric Methods

The metachromic shift observed upon the binding of EB to DNA and RNA permits the detailed examination of stoichiometric relationships involved in the ethidium-DNA interaction. The Scatchard method of studying ligand binding (92) has been employed in the investigation of EB-DNA (111) and EB-transfer RNA (6) binding. On the assumption that all nucleic acid sites for dye binding are equivalent

and that each behaves independently of the others, the binding process may be described by the Scatchard equation:

$$k = \frac{c\ (n-r)}{r} \qquad\qquad \frac{r}{c} = \frac{n}{k} - \frac{r}{k}$$

Where r is the concentration of dye bound per nucleotide, n is the number of binding sites per nucleotide, c is the molar concentration of free dye and k is the intrinsic dissociation constant of the complex which may be defined as:

$$k = \frac{\text{(unoccupied sites) x (free ligand)}}{\text{(occupied sites)}}$$

A plot of r/c versus r yields a straight line with a slope of 1/k. The intercept on the r-axis is equal to n. Obviously, if more than one type of binding site is involved or if the binding on one site affects the interaction at neighboring sites the r/c versus r plot would be expected to deviate from linearity.

The interaction of ethidium bromide with calf thymus DNA yields the Scatchard plot shown in Fig. 3 (111). The binding parameters for DNA's with G + C content varying from 35 to 72% determined by this method are essentially independent of G + C content. The average values for n and k are respectively 0.20 and 5.5×10^{-7} M. The magnitude of k indicates that the binding is very strong. Saturation of primary sites occurs at an n of 0.20. The deviation from linearity in the relation between r/c and r, which occurs

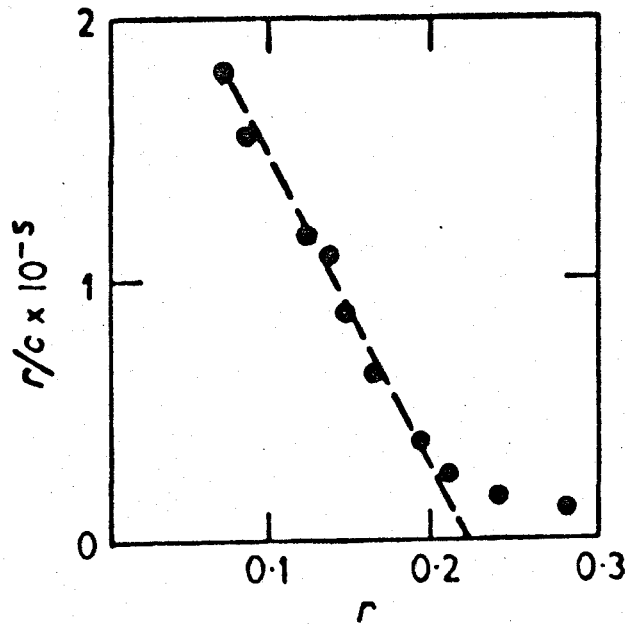


Fig. 3. Scatchard plot of the binding of ethidium bromide to calf thymus DNA. Measurements were made on solutions containing 2.5 x 10^{-4} M ethidium bromide. A 4 cm light path cell was used. (M. J. Waring, J. Mol. Biol. (1965) 13, 269).

above an r of 0.20 (Fig. 3), suggests the development of weaker secondary binding interactions. Comparable values of n equal to 0.19 and k equal to 4×10^{-7} M have been obtained for the interaction between EB and transfer RNA (6).

(b) Fluorescence Measurements

The interaction of ethidium bromide with polynucleotides has also been investigated by fluorescence methods. The binding of ethidium to DNA or RNA cause a marked increase in the fluorescence increment of ethidium bromide (6,56). The excitation spectra (Fig. 4) indicate a considerable increase in the fluorescent intensity of EB upon binding to t-RNA, with the fluorescence enhancement being somewhat greater in the ultraviolet than it is in the visible region of the spectrum.

Shifts are observed in the maxima at 281 and 475 nm noted for free EB to 298 and 515 nm for bound EB. The fluorescence enhancement in both spectral regions is dependent on the amount of bound EB and may be used to measure the binding (56). The values of n equal to 0.20 and k, 4×10^{-7} for the EB-DNA complex agree well with those calculated by the absorption method (111). Thus, the results obtained by absorption and fluorescence methods indicate that at low dye to nucleotide ratios, binding of EB to DNA is strong and initially occurs up to an r of 0.20 to 0.25. Above this ratio weaker secondary interactions take place.

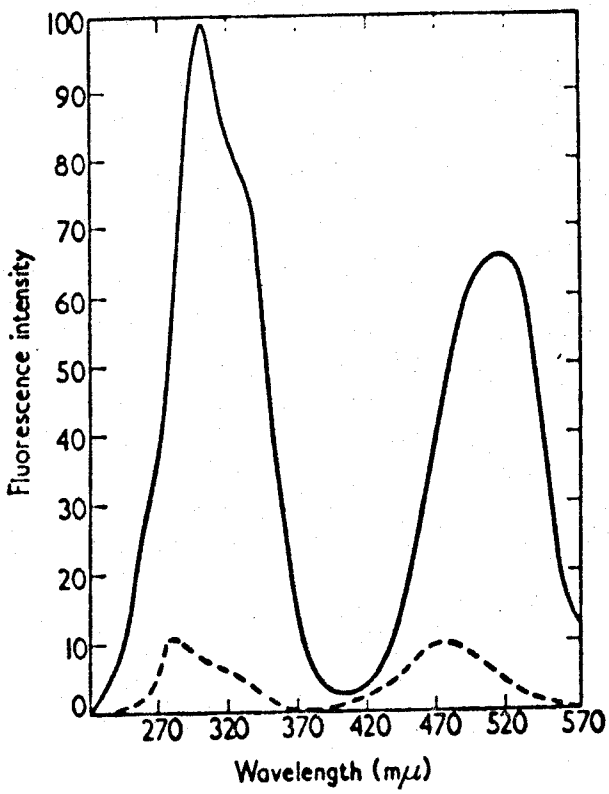


Fig. 4. Apparent excitation spectra of EB, and EB in the presence of t-RNA for emission of 586 nm. Solutions were made in sodium phosphate (0.01 M), socium chloride (3×10^{-3} M), disodium ethylenediaminetetraacetic acid (1×10^{-4} M), pH 7.0 and contained 1.3×10^{-5} M EB. The concentration of t-RNA was 3.1×10^{-4} M._____, EB in the presence of t-RNA -----, EB in the absence of t-RNA. (R. Bittman, J. Mol. Biol. (1969) 46, 251).

The initial strong interaction is described as primary binding (111). Since almost complete inactivation of RNA polymerase is observed for DNA templates containing ethidium at a dye to nucleotide ratio of 0.11 (109), it may be that primary binding is responsible, at least in part, for the biological activity of ethidium bromide.

D. INTERACTION OF DNA WITH ACRIDINE DYES

The acridines (Fig. 5) are a family of compounds which exhibit antibacterial properties and a low degree of trypanocidal activity (4,41). The ability of the acridines to combine with nucleic acids forms the basis for the use of these compounds as histological stains (69). The well-investigated interaction of acridine derivatives with DNA may provide a model applicable to the study of the ethidium-DNA complex.

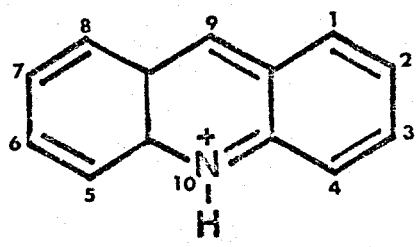


Fig. 5. Acridine

The biological activity of proflavine (3,6-diaminoacridine) (Fig. 6) depends upon the binding of the drug to DNA (68). The interaction between DNA and proflavine has been examined by absorption (81) and fluorescence (117)

spectroscopy. Proflavine interacts at primary DNA binding sites up to a dye to nucleotide ratio of 0.20 (81). A weaker secondary binding process occurs at higher ratios.

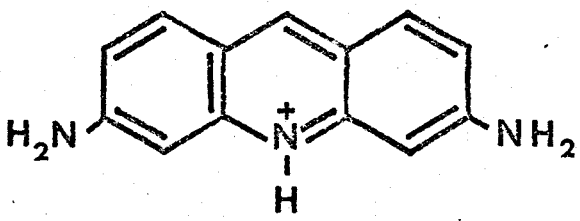


Fig. 6. Proflavine

The interaction between proflavine and DNA has been investigated more extensively than the EB-DNA interaction. Proflavine and ethidium bromide exert similar effects on the physical properties of DNA and exhibit similar binding behavior toward DNA, native or denatured. Proflavine and ethidium also share certain common structural features in that both are diamine cations with a planar ring system consisting of three fused aromatic rings. The similarities in the behavior of proflavine and ethidium bromide toward DNA and the structural similarities between these dyes may permit analogies to be drawn between available information on the proflavine-DNA and ethidium bromide-DNA complexes.

E. GEOMETRY OF THE ETHIDIUM BROMIDE-DNA COMPLEX

1. Proposed Model

Binding of ethidium bromide to primary sites on DNA apparently occurs by insertion of the planar phenanthridinium ring-system between adjacent base-pairs of the double helix (Fig. 7). Fuller and Waring (35) have postulated that the phenanthridinium ring is completely inserted between base pairs. This model is based on X-ray crystallographic data indicating that the EB-DNA complex has a smaller average molecular diameter than DNA. It was further noted that the 3.4 Å reflection corresponding to the stacking of base-pairs is retained. The complete insertion of the ethidium ring would retain the 3.4 Å reflection, even though the base pairs on either side of the EB would be separated by about twice the normal distance, since the EB molecule itself would provide the reflection. To accommodate the inserted EB, local uncoiling of the DNA may be required, resulting in the smaller average molecular diameter of DNA noted in the presence of EB.

2. Effect of Ethidium Bromide Binding on DNA Secondary Structure

The intercalation of ethidium molecules between adjacent base pairs results in separation of these pairs by about twice the normally occurring distance and overall elongation of the polynucleotide. Separation of adjacent base-pairs in a manner accommodating the EB molecule requires back

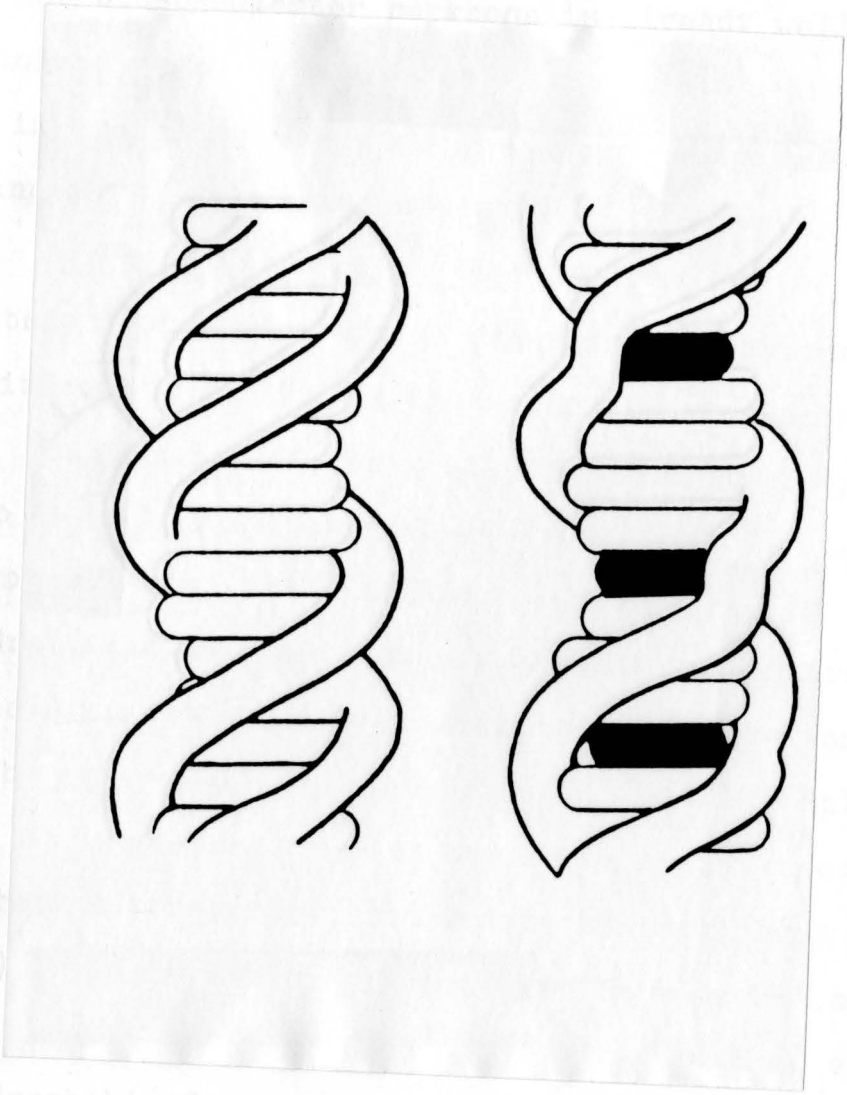


Fig. 7. Sketches representing the secondary structure of normal DNA (left) and DNA containing intercalated proflavine molecules (right). The helix is drawn as viewed from a remote point, so that the base-pairs and the intercalated ethidium bromide appear only in edgewise projection, and the phosphate-deoxyribose backbone appears as a smooth coil. (M. J. Waring, Nature (1968) 219, 1320).

rotation between respective base-pairs since in the normal helix the phosphodiester backbone is already well extended. The minimum angle of back rotation of the phosphodiester bonds in opposite DNA strands has been estimated from model building at 12° per bound molecule (35).

3. Evidence Supporting Intercalation

Intercalation of aromatic rings has become increasingly recognized as occurring in the mechanism of drug action (113). The concept of intercalation is supported, in addition to the previously mentioned X-ray data, by numerous other experimental observations.

Hydrophobic interactions between DNA base pairs and aromatic ring systems are expected to stabilize complexes formed by intercalation, including the DNA-EB complex (35). Ethanol or isopropanol, which are known to interfere with hydrophobic binding (96), dissociate EB from these complexes (47,111). The formation of complexes between DNA and polynuclear aromatic hydrocarbons is also regarded as evidence that hydrophobic forces are involved in the intercalation process (73). In the case of phenanthrene (Fig. 8) for instance, which resembles the ethidium ring system but does not contain the hetero-nitrogen cation, flow dichroism experiments indicate that the phenanthrene ring in phenanthrene-DNA complexes is oriented in a direction parallel to that of the stacked base pairs. Since the phenanthrene ring systems cannot accommodate a positive charge, as in the case

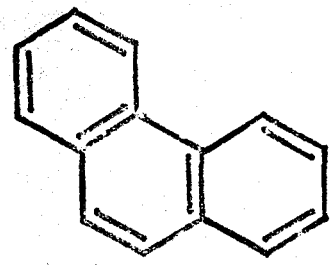


Fig. 8. Phenanthrene

with ethidium bromide, ionic forces may not be involved in the phenanthrene-DNA interaction. Hydrophobic forces, however, undoubtedly contribute to the interaction of the phenanthrene ring with DNA.

Ethidium and proflavine have similar effects on the physical properties of DNA. In the presence of these dyes, DNA exhibits increased intrinsic viscosity and decreased sedimentation coefficient (57,109). These observations are consistent with the expected elongation of DNA upon insertion of dye molecules between base pairs. An increase in the length of the double helix has also been observed directly by autoradiography of ^{3}H - T2 DNA in the presence of proflavine (15). The binding of ethidium produces a pronounced decrease in the sedimentation coefficient of supercoiled closed circular DNA. This property, incidentally, is used for the separation of circular DNA from linear DNA molecules (5,21,124). Elongation of the DNA helix as described in Chapter I, Section E2, cannot occur with circular DNA, to the extent that it does with linear

DNA. The pronounced changes in the physical properties of closed circular DNA at very low dye to nucleotide ratios (r = 0.04) emphasize the influence of ethidium on the secondary structure of DNA. These results are considered to be strong evidence for the intercalation of ethidium and concomitant local uncoiling of the DNA helix.

4. Secondary Binding

The binding of ethidium to secondary sites in DNA is considerably weaker than primary binding, and is generally believed to be the result of electrostatic interactions between phosphate groups and the ethidium cations (56,111). Ethidium molecules bound in this manner would be located on the external surface of the DNA helix. Progressively increasing binding of EB to DNA results first in saturation of primary binding sites at an r of 0.20 to 0.25. The interaction between EB and DNA at higher dye to nucleotide ratios can also be followed spectrophotometrically. Binding continues to take place up to an EB/DNA ratio of 1.0. At high DNA concentrations, precipitation of the 1:1 EB-DNA complex is observed (111). The EB induced changes in sedimentation and viscosity become maximal at EB to DNA ratios corresponding to saturation of primary binding sites and remain nearly constant at the higher ratios. Fluorescence enhancement reaches a maximum at the same ratios (6,56). These findings suggest that the weaker secondary binding does not involve further intercalation of EB molecules.

5. Ethidium Bromide Interaction with Polyvinylsulfate

Since secondary binding apparently occurs by electrostatic interactions, the interaction of EB with polyanions, with which primary binding cannot occur, may give information regarding the nature of secondary binding (111). The polyvinylsulfate DNA complex may serve as a model for secondary interaction.

The interaction between ethidium bromide and polyvinylsulfate (PVS) (Fig. 9) produces a metachromic shift in the absorption spectrum of EB similar to that observed in the presence of DNA (56,111). Both primary and secondary interaction between EB and DNA produce this metachromic shift, but only primary interactions result in increased fluorescence (56). Increased fluorescence for EB is not observed in the presence of polyvinylsulfate indicating that secondary interactions do not contribute to fluorescence enhancement noted for the EB-DNA interaction.

The polyvinylsulfate-induced metachromic shift on the spectrum of ethidium is reduced at high sodium chloride concentrations or in the presence of divalent metal ions. Divalent metal ions such as magnesium (II) and manganese (II), interact principally with phosphate groups in DNA and decrease the secondary interactions. The effect of these metals on primary binding is less pronounced (6,56,111). These observations support the concept that secondary binding of EB to DNA occurs by electrostatic interaction between the DNA phosphate and the ethidium cation.

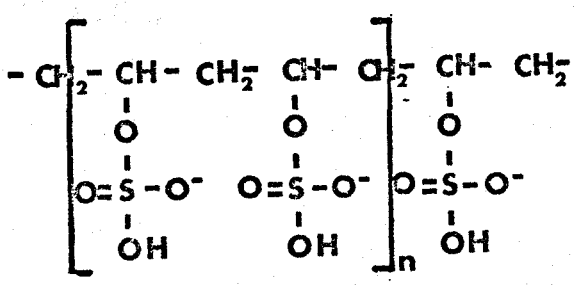


Fig. 9. Polyvinylsulfate

6. Geometry of the EB-DNA Interaction

There is general agreement that primary binding of ethidium to DNA occurs by intercalation. However, two schools of thought exist regarding the orientation of the dye molecules in relation to the base pairs and phosphate groups. Fuller and Waring (35), on the basis of steric considerations, place the ethyl and phenyl groups of the intercalated ethidium cation within the large groove of the double helix (Fig. 10). Clearly, the EB-DNA complex may be stabilized not only by hydrophobic forces, but also by hydrogen bonding between the basic amino groups on EB and the DNA phosphate residues. Examination of the intermolecular distances between the ethidium amino groups and the phosphate moieties of the opposite DNA strands (Fig. 11) indicates that the relative geometry of an intercalated EB molecule favors such hydrogen bonding.

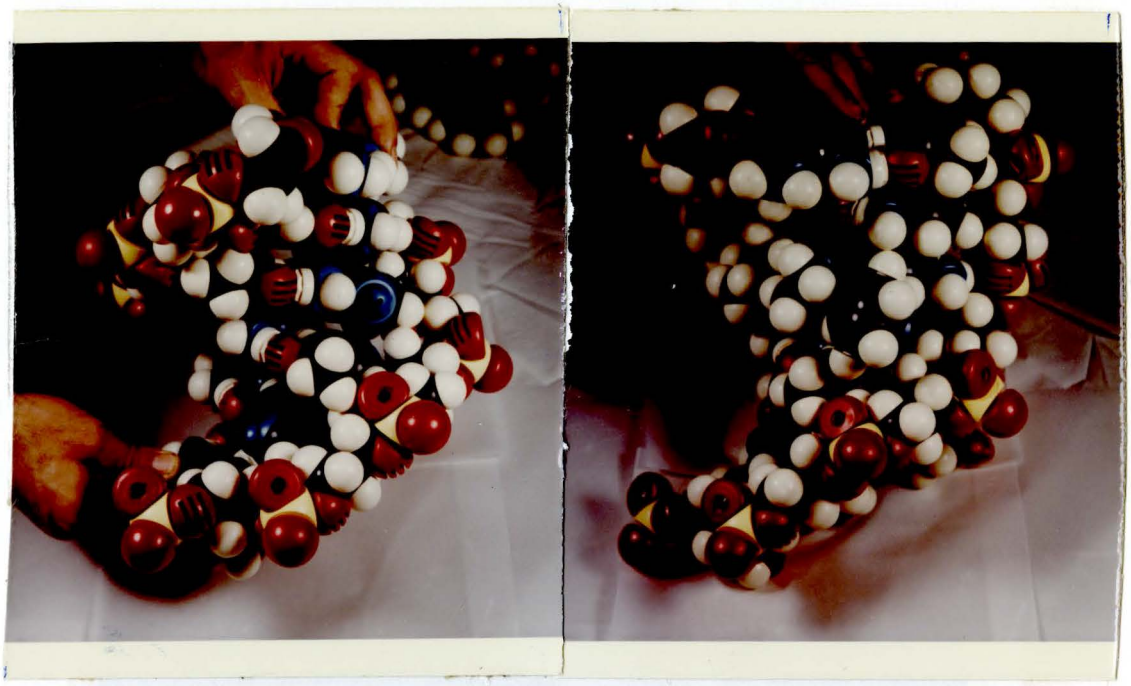


Fig. 10. CPK space filling molecular model of ethidium bromide-DNA complex. Left: view of a DNA double helix section. Right: EB-DNA complex with ethyl and phenyl groups of EB placed within the large groove of the helix.

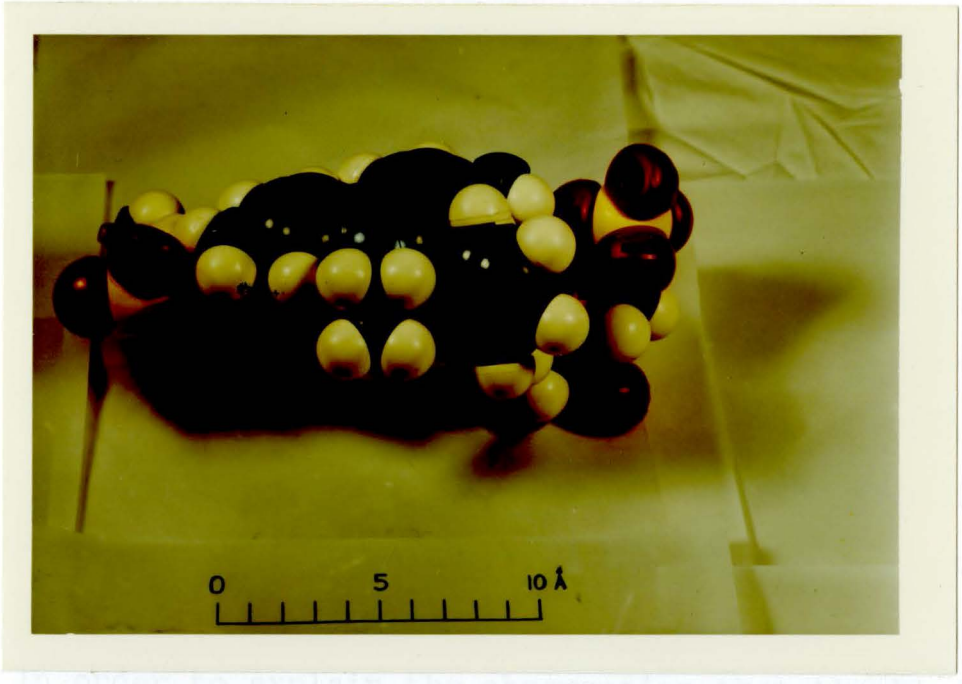


Fig. 11. Top view of spatial relationships between ethidium bromide amino groups and the opposite strand phosphate groups of DNA.

7. Modified Intercalation Model

The involvement of hydrogen bonding in intercalation is supported by studies of the ability of various proflavine analogs to form primary complexes with DNA (23,37,62). Since investigations of similar nature have not yet been reported for ethidium bromide, comparison with the results obtained with proflavine may be useful.

The intramolecular distances between the respective amino groups in ethidium and proflavine are identical (Fig. 12). According to the proposed models for the respective primary complexes, the orientation of the amino groups within the primary binding site would also be the same for EB and proflavine. Since the number of sites at which primary binding occurs decreases upon denaturation of DNA, primary binding of EB to the denatured polynucleotide is also expected to diminish.

In order to explain the observation that the binding of aminoacridines is not affected by denaturation of DNA (29,61), Peacocke proposed a modified intercalation model (82). This model describes the interaction of acridine with DNA as occurring by insertion of the acridine ring system between adjacent base pairs of the same polynucleotide chain (Fig. 13) rather than between hydrogen bonded base pairs of opposite strands (Fig. 7). A slight rotation of the negatively charged oxygen of the phosphate moiety toward the interior of the helix would favor interaction with the

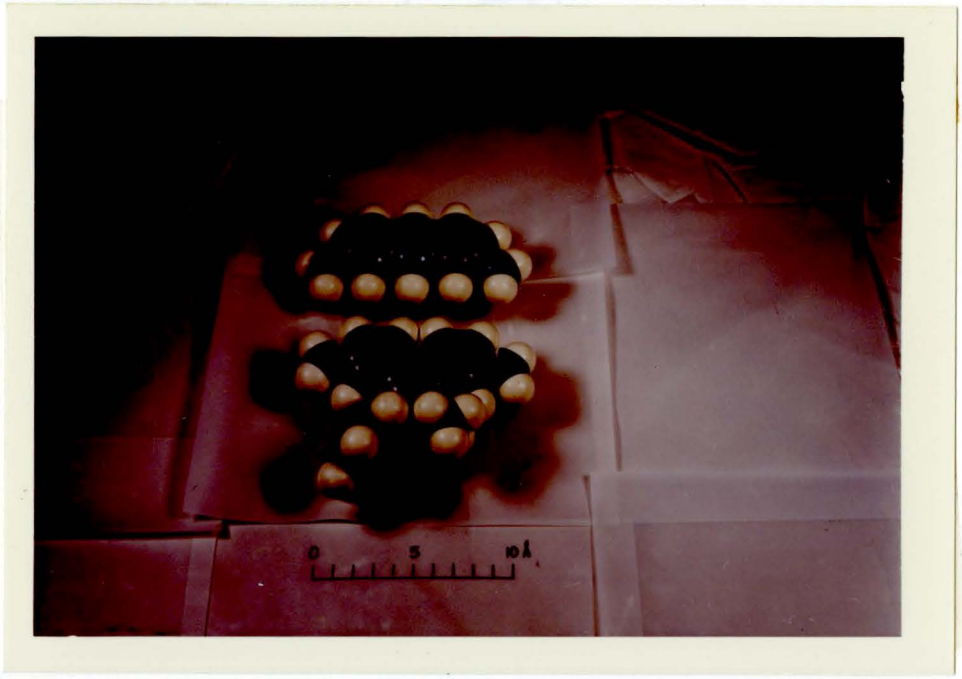


Fig. 12. CPK space filling models of proflavine (top) and ethidium bromide (bottom).

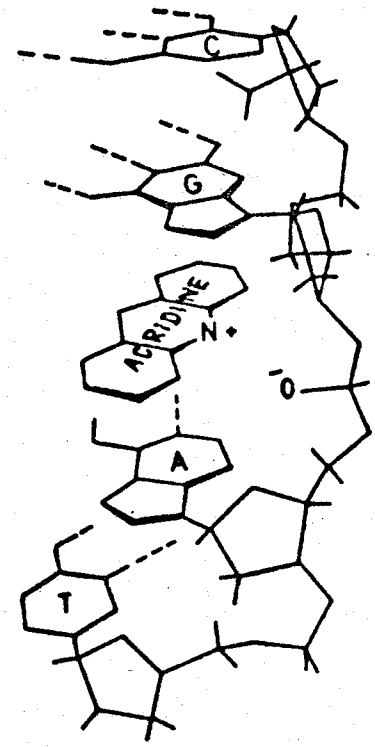


Fig. 13. Perspective view of the modified intercalation model showing an acridine molecule oriented between adjacent bases on a single polynucleotide chain; the other chain has been omitted for clarity. In this diagram, the spacing between A and G represents 6.8 Å, although this distance is not critical to the model. The actual bases shown are examples only. (A. R. Peacocke *et al*. *Nature* (1966) *212*, 1360).

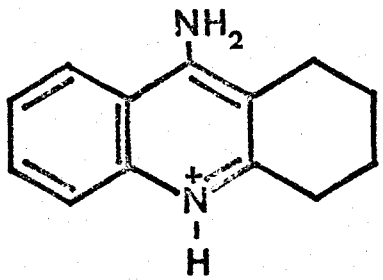
nitrogen cation of the acridine ring. This model is consistent with Lerman's X-ray diffraction data (57). It is also supported by the observation that 9-amino-tetrahydroacridine (Fig. 14) binds less strongly while acranil and atebrin bind as strong as proflavine (29). On the basis of Lerman's model the non-planar ring systems of 9-amino-tetrahydroacridine could not intercalate within primary sites. In the modified intercalation model, however, the geometry of the ring may not interfere substantially with binding.

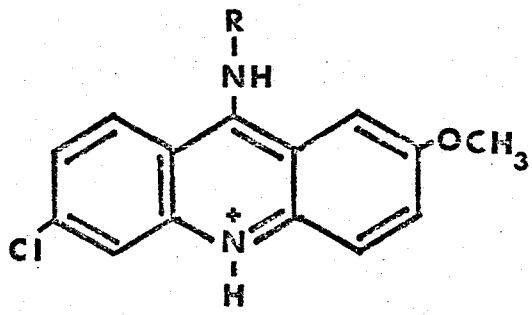
These conditions are based on the assumption that all acridines bind in the same manner. However, the geometry and orientation of the primary complex between polynucleotides and acridines or ethidium bromide may depend on both the secondary structure of the polynucleotide as well as the dye structure.

8. Ethidium Bromide-Denatured DNA Interaction

Scatchard plots for the binding of proflavine and EB to "native" DNA are linear up to a dye to DNA ratio of 0.20 (111). Plots for the interaction of EB with ice-quenched heat-denatured DNA on the other hand, deviate from linearity beginning at an EB to nucleotide ratio as low as 0.10 (111). This deviation indicates that the binding of EB to DNA and denatured DNA may involve different geometries.

The decrease in double stranded regions in DNA appears to parallel the decrease in the number of primary binding





9-amino-1,2,3,4,-tetrahydroacridine

$R=CH_2CH(OH)CH_2N(C_2H_5)_2$; Acrinil

$R=CH(CH_3)(CH_2)_3N(C_2H_5)_2$; Atebrin

Fig. 14. Structures of Acridine Derivatives

sites. The relatively strong binding that occurs above an EB to DNA ratio of 0.10 with denatured DNA may be the result of partial intercalation similar to that proposed by Peacocke for the acridine-DNA complex. The maximum fluorescent intensity observed with EB-denatured DNA complex is about one-half that of the EB-DNA complex. This is consistent with the decrease in the number of primary binding sites in denatured DNA (56).

9. Influence of Dye Structure on Binding

The effect of dye structure may be another important factor in the formation of dye-polynucleotide primary complexes. It should be noted that primary binding occurs up to a dye to nucleotide ratio of 0.25 for proflavine and up to a ratio of 0.125 for 9-amino-tetrahydroacridine at low ionic strength (29). Furthermore, the ability of various proflavine analogs to form strong primary complexes with DNA varies with the specific chemical structure of the dye.

These observations indicate that the orientation of the acridine ring system within the primary binding site may depend on the specific structure of the ring (23,37,62). For example, in the case of unsubstituted acridine, it is likely that the positive charge is located principally at the ring nitrogen while in proflavine the amino substituents may share a portion of this charge (58). Calculations based on Linear Combinations of Atomic Orbital - Molecular Orbital theory are consistent with this view (52).

In a molecule, such as acridine, in which a major portion of the positive charge is located at the hetero nitrogen, the ring nitrogen may be oriented toward the phosphate residues in a manner similar to that described by the modified intercalation model. In the case of proflavine, however, both the ring nitrogen and the amino substituents are expected to interact with phosphate groups. The results of ring nitrogen alkylation experiments appear to also support the idea that the orientation of the acridine and proflavine molecules in the DNA-dye complex may be different (62). The binding of acridine decreases with alkylation and is dependent on aliphatic chain length. However, alkylation of the ring nitrogen of proflavine actually enhances binding regardless of the chain length of the alkylating group. Furthermore, the reactivity of the proflavine amino groups toward nitrous acid diazotization is considerably reduced in DNA-bound proflavine (59). This would be expected if intercalation of proflavine results in steric shielding of the amino substituents. Thus, the reactivity of the amino groups and the effect of ring nitrogen alkylation support Lerman's model for primary binding of proflavine to DNA.

In conclusion, the strong binding of acridine dyes and ethidium bromide appears to occur by intercalation. The ability of polynuclear hydrocarbons to bind by intercalation (Chapter I, E3) (73) suggests that this interaction is

stabilized by hydrophobic forces. In the case of cationic acridine and phenanthridinium dyes, the orientation of the ring system in the DNA-dye complex appears to depend on the type and location of ring substituents. For unsubstituted acridines, the ring nitrogen is probably oriented toward the phosphate (Fig. 13), while for proflavine (3, 6-diaminoacridine) and ethidium bromide (Fig. 11) the amino groups may be hydrogen bonded to the phosphate groups of each polynucleotide strand.

To date, binding studies on analogs of ethidium bromide have not been reported. The structural similarities between ethidium and proflavine, however, and the similarities in the binding behavior of these dyes toward DNA suggest that the structures of the EB-DNA and proflavine-DNA complexes may be similar. The information obtained by X-ray diffraction methods and the influence of these dyes on the physical properties of DNA suggest that the orientation of the dye molecule within the primary binding site may be the same.

F. POLYNUCLEOTIDE STRUCTURAL REQUIREMENTS FOR PRIMARY BINDING

1. Effect of Polynucleotide Structure on Ethidium Bromide Binding

On the basis of the intercalation models proposed by Fuller for ethidium (35) and Lerman for proflavine (57), strong primary binding requires a double stranded

polynucleotide. However, in the model proposed by Peacocke *et al*. (83) primary binding is postulated to occur by insertion of the dye ring system between base pairs in only one of the two strands of the double helix. Therefore on this basis, single stranded polynucleotides may also be expected to interact strongly with dyes.

The interaction of ethidium bromide with single and double stranded synthetic polynucleotides has been investigated by absorption (112) and fluorescence (56) spectroscopy. The results of these studies give support to the concept that binding requires a double stranded structure.

2. The Possibility of EB Interaction with Single-Stranded Regions

Scatchard plots for denatured DNA and yeast RNA indicate that strong binding occurs between EB and these nucleic acids (111). The relationship between r/c and r however is not linear above dye to nucleotide ratios of 0.10. As previously mentioned, this behavior may be interpreted as indicating either that more than one binding site is available or that the binding of EB to one binding site affects the affinity of a nearby binding site for the dye. In either case, a different behavior would be expected for denatured DNA and yeast RNA than for "native" DNA.

Investigation of the interaction of EB with transfer-RNA by stopped-flow and temperature-jump methods indicates the existence of at least three distinct first order rate

constants for the formation of the EB-t-RNA complex (6). These rate constants fall in the ranges of between 140 to 165, 60 to 80 and 15 to 25 sec^{-1}. Since the secondary structure of transfer RNA consists of both single and double stranded regions (27), the possibility exists that these rate constants reflect different affinities of EB for polynucleotide regions of different secondary structures. The three rate constants may apply to the interaction of EB with double stranded polynucleotides, single stranded polynucleotides and DNA phosphate groups respectively.

3. Interaction with Synthetic Polynucleotides

The Scatchard plot (112) and the fluorescence enhancement (55) observed with the double stranded polynucleotide, poly (A + U), upon interaction with EB, are similar to those noted for the EB-DNA complex. Other double stranded polynucleotides such as poly (I + C) and poly (A + I) exhibit weaker binding than DNA or poly (A + U). In those instances higher EB to nucleotide ratios are required for spectrophotometric measurement of the interaction (112).

Single stranded poly A, poly I and poly U interact with EB very weakly as measured by either absorption or fluorescence techniques. Strong binding may thus occur only between EB and double-stranded polynucleotides. Apparently a single stranded stacked structure such as poly A is not sufficient for strong binding.

G. A COMPARISON OF ABSORPTION AND FLUORESCENCE METHODS FOR STUDYING THE ETHIDIUM BROMIDE-DNA INTERACTION

1. Absorption Methods

As it may be apparent from the preceding discussion, the interaction of ethidium bromide with nucleic acids has been studied principally by absorption and fluorescence techniques. Each of these techniques has inherent advantages and limitations. The absorption method is based on the metachromic shift in the absorption maximum of free EB at 479 nm to 518 nm noted for the bound dye. The shift is observed upon interaction of EB with DNA or RNA but it is not specific for polynucleotides. The binding of EB to polymers with structures not related to polynucleotides, such as polyvinylsulfate, produces a similar shift (56,111).

Absorption measurements, nevertheless, are useful for the determination of rate constants for the formation of EB-DNA and EB-RNA complexes. Scatchard plots for native DNA and t-RNA are linear up to a dye to nucleotide ratio of 0.20 (6,110), but those for denatured DNA and various other single and double stranded polynucleotides exhibit considerable deviation from linearity (110). The deviation indicates that the behavior of EB toward RNA and denatured DNA is different from that toward native DNA or transfer RNA, but the non-specific nature of the metachromic shift makes it difficult to determine what the exact differences are.

The metachromic shift has also been employed in temperature-jump and stopped flow kinetic studies of the binding of EB to t-RNA. These studies have indicated the involvement of at least three different binding processes (6). Transfer RNA consists of both single and double stranded regions and, therefore, the results obtained for the EB-t-RNA complex may not be directly applicable to the EB-DNA complex. Similar kinetic studies have not as yet been reported for DNA, denatured DNA, or synthetic polynucleotides of known secondary structure. The results of such studies could lead to the assignment of individual rate constants for the formation of polynucleotide structure-dependent EB-polynucleotide complexes.

2. Fluorescence Methods

While absorption techniques measure total binding of ethidium bromide to polynucleotides, fluorescence methods may be specific for primary binding determination. Fluorescence occurs as the result of energy transfer between EB and DNA bases (56). The quantum efficiency of the fluorescence is much greater for double-stranded polynucleotides and as a result the technique may be useful for determining primary binding (56).

The results of fluorescence (55) and absorption (112) measurements differ regarding the binding of ethidium to poly (A + U), poly (I + C) and poly A. Fluorescence methods indicate that primary binding occurs between EB and poly

(I + C) or poly (A + U). The binding parameters k and n for these complexes are reported to be identical ($k = 15 \times 10^{-7}$ M and n = 0.15). On the other hand, absorption spectroscopy indicates that poly (A + U) and DNA behave similarly towards EB while poly (I + C) exhibits weaker interactions. Fluorescence also indicates that interaction between EB and poly A does not occur (56).

The differences in the results obtained by spectrophotometry and fluorescence are probably due to inherent limitations in each of these methods. Fluroescence enhancement occurs as a result of energy transfer between EB and the nitrogen bases of DNA. Thus, this technique apparently reflects only primary binding. The EB-single stranded poly A complex which is stabilized only by secondary interaction does not exhibit increased fluorescence. The absorption method on the other hand is sensitive to the binding of EB within the environment of the polymer and reflects the interaction of the dye with either primary or secondary binding sites.

The different results obtained for poly (A + U) and poly (I + C) by absorption and fluorescence may simply be due to the differences in experimental conditions. Absorption results were obtained in a 0.04 M Tris-HCl solution while fluorescence measurements were carried out in 0.20 M NaCl - 0.20 M Tris-HCl. These large differences in ionic strength may be responsible for the differences in the n and

k for the EB-poly (A + U) and EB-poly (I + C) complexes. Double stranded homopolymer complexes such as poly (A + U) and poly (I + C) are sensitive to ionic strength with respect to their secondary structure. High ionic strength can affect stacking interactions between base pairs. This may affect the ability of a given polynucleotide to form complexes with EB. It is difficult to ascertain whether or not the different results obtained by fluorescence and absorption techniques are real unless comparisons are made under comparable ionic strength conditions by these same methods.

H. CIRCULAR DICHROISM STUDIES OF DNA-DYE COMPLEXES

Induced optical activity is observed upon the interaction of acridine orange or proflavine with DNA (7,8,9,23, 37,60,74). These interactions have been investigated by optical rotatory dispersion (ORD) and circular dichroism (CD) methods. Proflavine, which as a free dye is optically inactive, exhibits upon binding to DNA a strong positive Cotton effect in the spectral region near 465 nm (8,23). The induced optical activity disappears under conditions which denature DNA, such as low pH and elevated temperatures (37,74). The molar circular dichroism ($E_L - E_R$) of bound proflavine is markedly dependent on the ratio (r) of bound proflavine per nucleotide residue (23) and decreases rapidly at very low r values. The induced optical activity is apparently specific for primary bound proflavine and it

reaches a maximum at r values corresponding to the saturation of primary binding sites. This dependence of the molar circular dichroism on r has been interpreted as indicating that induced optical activity results from nearest-neighbor interactions between proflavine molecules bound to adjacent primary binding sites (23).

Thus, circular dichroism provides a good method for the study of dye-polynucleotide primary interactions. The advantage of this method over fluorescence or absorption techniques is that it is sensitive to nearest neighbor interactions between DNA bound dye molecules. Nearest-neighbor interactions, in turn, are sensitive to the geometry of the dye molecules within the binding site (23). Circular dichroism can therefore provide detailed information on the nature of the primary complex not available by other methods.

CHAPTER II:

MATERIALS, PROCEDURES AND EXPERIMENTAL METHODS

A. MATERIALS

1. Chemicals

Calf thymus deoxyribonucleic acid, Worthington Biochemicals.

Dialysis tubing, cellulose, 19 mm diameter, pore size 24 Å, Fisher Scientific Company.

Ethidium bromide, Lot No. 800304, Calbiochem.

Magnesium chloride, analytical grade, J. T. Baker Chemical Co.

Polydeoxyriboadenylate-thymidylate, Lot No. 6-4317, Miles Laboratories.

Polydeoxyriboguanidylate-polydeoxyribocytidylate, Lot No. 4318, Miles Laboratories.

Polyriboadenylate, Type I, Lot 1990, Sigma Chemical Company.

Polyribocytidylate, Type I, Lot No. 119B-0280, Sigma Chemical Company.

Polyribinosinate, Type I, Lot No. 10C-0390, Sigma Chemical Company.

Polyribouridylate, Lot No. M-3, Biopolymers Inc.

Polyvinylsulfate, Lot No. 88254, General Biochemical Company.

Tris(hydroxymethyl)aminomethane, Primary Standard, Fisher Chemical Company.

Yeast RNA, Type XI, Lot No. R-3650, Sigma Chemical Company.

2. Analytical Instruments

Beckman TM Analyzer, Beckman Instrument Inc.

Cary 15 Recording Spectrophotometer, Cary Instrument Company.

Durrum-Jasco ORD/CD/UV-5 Recording Spectropolarimeter, Durrum Instrument Company.

Moseley Model 7035B X-Y Recorder, Hewlett Packard Corporation.

B. PROCEDURES

1. Circular Dichroism Measurements

Circular dichroism measurements were carried out on a Durrum-Jasco ORD/CD/UV-5 Recording Spectropolarimeter using fused silica rectangular cells (1.0-10 mm path length, Opticel Company), cylindrical cells (0.10-0.50 mm path length, Opticel Company and 20, 50 and 100 mm path length, Beckman Instruments) as well as a low volume demountable cylindrical cell (50 mm path length, 3.0 ml volume, Durrum Instrument Company). The circular dichroism scale was standardized with a solution of d-10-camphorsulfonic acid (1.0 mg/ml obtained from Durrum Instrument Company). Differences in the absorbances for right and left circularly polarized light (Δ Absorbance) were recorded versus wavelength. The standard solution has a Δ Absorbance of +0.0093 at 290 nm. The spectropolarimeter was calibrated so that the solution causes a +9.30 cm deflection on the 0.01 scale.

Measurements of circular dichroism were carried out at polynucleotide concentrations of 0.20, 0.60 or 5.0 μM/ml as specified. Optical path lengths of the cells used were selected so that in each instance the resulting optical density did not exceed a limit of 2.5. Optical artifacts may occur at optical densities exceeding this limit.

Circular dichroism measurements were obtained at chart speeds of 2 nm per minute at scale sensitivity settings of 0.001 for DNA, poly dAT, poly dG:dC, poly (A+U), poly (I+C) and poly (A+I) and at a scale sensitivity of 0.005 for poly A, poly C and poly I. Because the baseline of the spectropolarimeter is not flat, the spectra were replotted by transferring Δ Absorbance readings with a pair of dividers. Points were generally plotted at intervals of 5 nm and at intervals of 2 nm for regions of maximum slope change. The difference in extinction coefficients between left and right circularly polarized light (E_L-E_R) was obtained from the following equation:

$$E_L - E_R = \frac{\Delta \text{ Absorbance}}{C \times l}$$

Δ Absorbance is obtained directly from the recorder chart. The concentration, C, is expressed as moles per liter and l, the optical path length of the cell is expressed in centimeters.

a. General Calculations

The interaction of EB to polynucleotides results in a metachromic shift in the absorption spectrum of EB near 500 nm as previously noted (Chapter I, C1) (111). In our experiments, the concentration of bound ethidium bromide was followed at 460 nm and calculated as follows:

If A represents the total ethidium bromide concentration of a solution, ϵ_f and ϵ_b are respective molar extinction coefficients for ethidium bromide in the free and bound forms at 460 nm respectively, and A_c is the absorbance at 460 nm of a ethidium-DNA complex; then the absorbances of free (A_f) and bound (A_b) EB may be calculated from the respective molar extinction coefficients as $A_f = [EB]_t \times \epsilon_f$ and $A_b = [EB]_t \times \epsilon_b$. The difference between the absorbances of free and bound ethidium may be expressed as $A_{f-b} = A_f - A_b$. The fraction of ethidium in the bound form (β) is thus equal to $\frac{A_f - A_c}{A_{f-b}} = \beta$ and the concentration of bound EB, $[EB]_b$ equal to $\beta \times [EB]$ total.

The molar ratios of bound ethidium per DNA phosphate, r, is calculated from molar concentrations of bound ethidium divided by the molar concentration of DNA nucleotide phosphate. The molar circular dichroism, $E_L - E_R$, per bound ethidium (EB_b) may be then calculated from the equation; $E_L - E_R = \frac{\Delta \text{ Absorbance}}{[EB]_b \times 1}$. Δ Absorbance is measured directly from the recorder of the spectropolarimeter.

Both free EB and the EB-DNA complex may contribute to the circular dichroism in the region near 307 nm. Since we are only interested in examining the complex, it is necessary to correct the final E_L-E_R values for contributions from free EB which may be present in the solution under examination. At 307 nm free ethidium, EB_f, possesses a molar circular dichroism of 0.50 and its contribution to the Δ Absorbance, ΔA_f, measured on the spectropolarimeter may be expressed as:

$$\Delta A_f = (E_L - E_R)_f \times [EB]_f$$

The final equation from which the molar circular dichroism of the EB-DNA complex is calculated may be obtained by algebraic substitution as shown below:

$$(E_L - E_R)_c = \frac{\Delta A_t - \Delta A_f}{[EB]_b \times l}$$

In this equation, E_L-E_R complex, $(E_L-E_R)_c$, is the corrected molar circular dichroism at 307 nm, Δ Absorbance total, ΔA_t, is measured directly from the spectropolarimeter, and l is the optical path length. The other parameters; $[EB]_b$ and ΔA_f are defined in the text.

A SCM Model 1016 PR calculator was programed to perform calculations according to this equation so that when the appropriate numbers for the following parameters are entered into the program;

1. EB total in μM/ml, $[EB]_t$
2. A 460 nm of EB-DNA complex
3. Δ Absorbance as read directly from the spectropolarimeter

values are printed out for;

1. percent DNA-bound EB
2. Concentration of bound-EB in μM/ml, $[EB]_b$
3. r
4. r^2
5. E_L-E_R
6. E_L-E_R corrected for free EB, $(E_L-E_R)_c$

b. Calculation of E_L-E_R of the EB-DNA complex at 500 nm.

The calculation of E_L-E_R at 500 nm is made in a different manner. Free EB exhibits a positive CD maximum at 500 nm (Fig. 24)(Chapter III, C), whereas EB in the bound form exhibits a positive maximum near 540 nm and a negative extremum near 500 nm. The CD of bound EB may result from two overlapping bands, one originating from DNA-bound EB between 440 and 540 nm superimposed on the EB circular dichroism centered around 500 nm. The Δ Absorbance of bound EB at 500 nm at a given EB concentration (ΔA_t) can be determined from the algebraic sum of the Δ Absorbances of free and bound EB at this wavelength. The equation for calculating the Δ Absorbance at 500 nm is:

Program For SCM Calculator

M # 4 . 8 3 7 5	Enter $[EB]_t$ in I Molar Extinction Coefficient of EB_f at 460 nm, ϵ_f
X R =	Recall I $4.8375 \times [EB]_t$ = A Free EB at 460 nm, A_f
W 3 M	Store in III and IV
W 4 M	
1 . 4 3 7 5	Molar Extinction Coefficient Of Bound EB at 460 nm, ϵ_b
X W 1 R =	Recall I $1.4375 \times [EB]_t$ = A Bound EB at 460 nm, A_b
W 3 A	$A_f - A_b$ in III
W 4 K A R ÷	Enter A 460 EB-DNA, A_c Recall IV $A_f - A_c$
W 3 R = # X	Recall III $\frac{A_f - A_c}{A_{f-b}} = \beta$:PRINT
W 1 R = #	Recall I $\beta \times [EB]_t = [EB]_b$:PRINT

A	$[EB]_t - [EB]_b = [EB]_f$ in I
W	
5	Store $[EB]_b$ in V
M	
÷	
0	
.	
2	$\frac{[EB]_b}{[DNA]} = r$: PRINT
0	
0	
=	
#	
X	
=	$r \times r = r^2$: PRINT
#	
K	Enter ΔA
÷	Recall V
R	$\Delta A/[EB]_b = E_L - E_R$: PRINT
=	
#	
.	
0	$E_L - E_R$ $EB_f/10$
5	
X	
W	Recall I, $[EB]_f$
1	$.05 \times [EB]_f = \Delta A$ EB_f
R	
=	
-	
K	$\Delta A_t - \Delta A_f = \Delta A_c$
+	
T	
÷	
W	Recall V, $[EB]_b$
5	
R	ΔA EB-DNA/$[EB]_b = E_L - E_R$ Corrected : PRINT
=	$\Delta A_c/[EB]_b = (E_L - E_R)_c$: PRINT
#	
W	Recycle
1	
E	Space
K	Stop

In this program, the Roman Numerals I through V refer to the registers of the SCM 1016 calculator. The vertical list of symbols represents the order in which the keys are depressed to enter the program.

$$\Delta A_t = \Delta A - [(E_L - E_R)_f \times [EB]_t]$$

Where ΔA is read directly from the spectropolarimeter, $(E_L-E_R)_f$ is the molar circular dichroism at 500 nm of free EB, and $[EB]_t$ is the total ethidium bromide concentration. This equation yields negative values for the circular dichroism at 500 nm. The molar circular dichroism may be calculated from the equation $E_L - E_R = \frac{\Delta A_t \ 500 \text{ nm}}{[EB]_b \times 1}$.

2. Elevated Temperature Experiments

a. Instrumentation

The effect of elevated temperature on the interaction between EB and DNA was examined utilizing a modified Beckman Tm Analyzer. For circular dichroism measurements at elevated temperatures the analyzer was connected to the Durrum-Jasco Spectropolarimeter. This arrangement provides for automatic recording of CD versus temperature at wavelengths between 190 and 700 nm.

The Beckman Tm Analyzer consists of three basic parts; an electrically heated sample chamber, an automatic temperature programmer and a temperature bridge connected to a platinum probe which measures the temperature of the sample. The cell compartment (Fig. 15) encloses an electrically heated copper core (c) consisting of two parts which are held together by spring release clamps (sc) and a lid

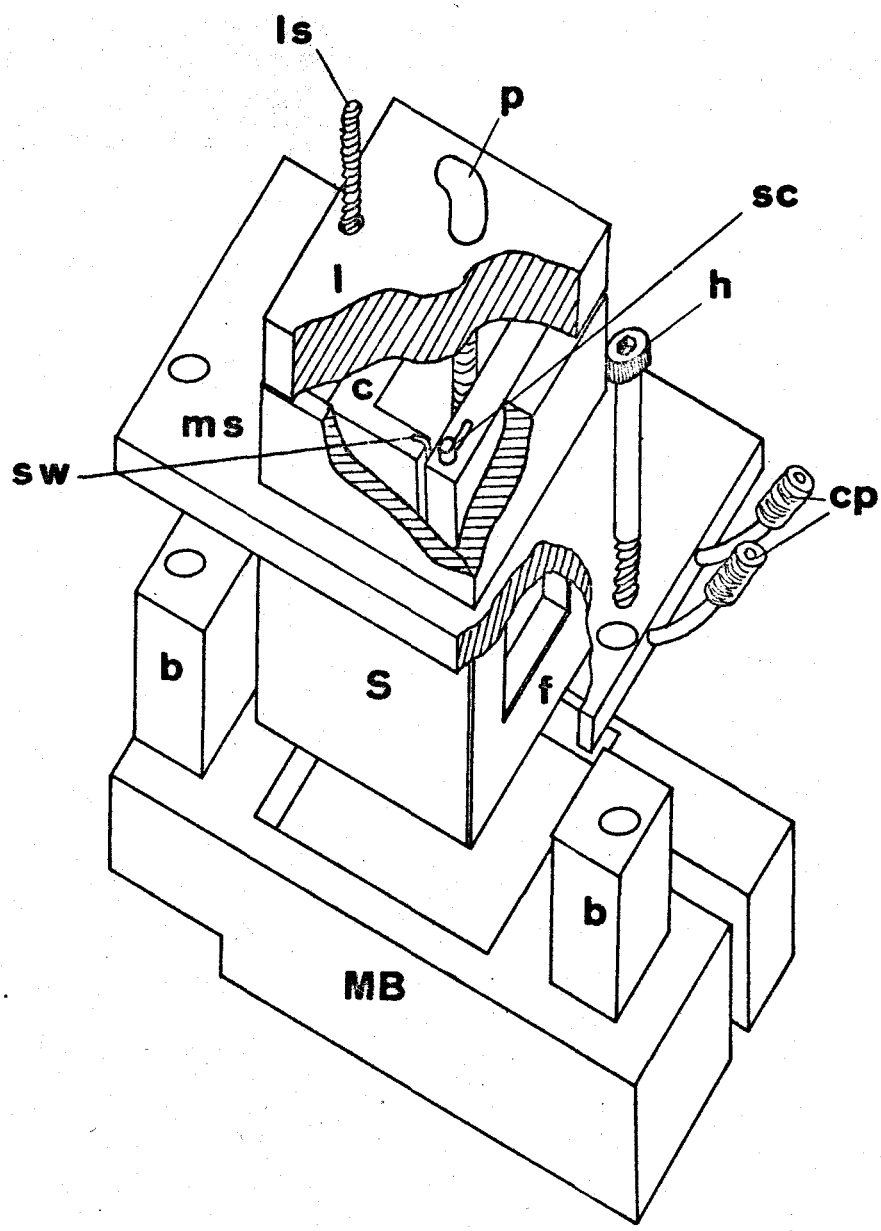


Fig. 15. Exploded, cutaway view of mounted Tm heating compartment: MB, mounting block; S, electrically heated sample chamber; b, block spacers; c, copper core; cp, coolant ports; f, face plate; h, mounting screw (1 7/16, 10-32 allen head cap screw); l, sample compartment lid; ms, mounting shelf; p, platinum sample probe; ls, lid tightening screw; sc, spring clamps; sw, spacer wire (20 gauge).

(l) that can be tightened by appropriately positioned screws (ls). This core is surrounded by a metal jacket through which coolant is circulated. The rate of temperature increase can be controlled by a proportional heater. Both the time of heating (5-2500 minutes) and the temperature range (0-120°C) can be regulated.

The cell compartment of the Tm assembly has been adapted so that it can fit in the CD light path of the spectropolarimeter by removal of the spectropolarimeter cell holder guide and use of a specially machined mounting block (Fig. 16). Some modification of the Beckman Tm heating assembly was also required for proper fitting and alignment of this compartment within the spectropolarimeter. The face plate (f) was removed to eliminate interference with the light path, the length of the mounting shelf (ms) was shortened to 4½ inches and the coolant ports (cp) bent upward.

For recordings of CD at a specific wavelength versus temperature an X-Y recorder was utilized with the X-axis calibrated to read temperature and the Y-axis to indicate spectropolarimeter pen position. The X-axis signal is obtained directly from the platinum probe of the Tm Analyzer via the temperature calibration bridge which converts the temperature-dependent resistance of the probe to a linear output signal. The signal to the Y-axis is obtained from a potentiometer installed on the pen-drive shaft of the

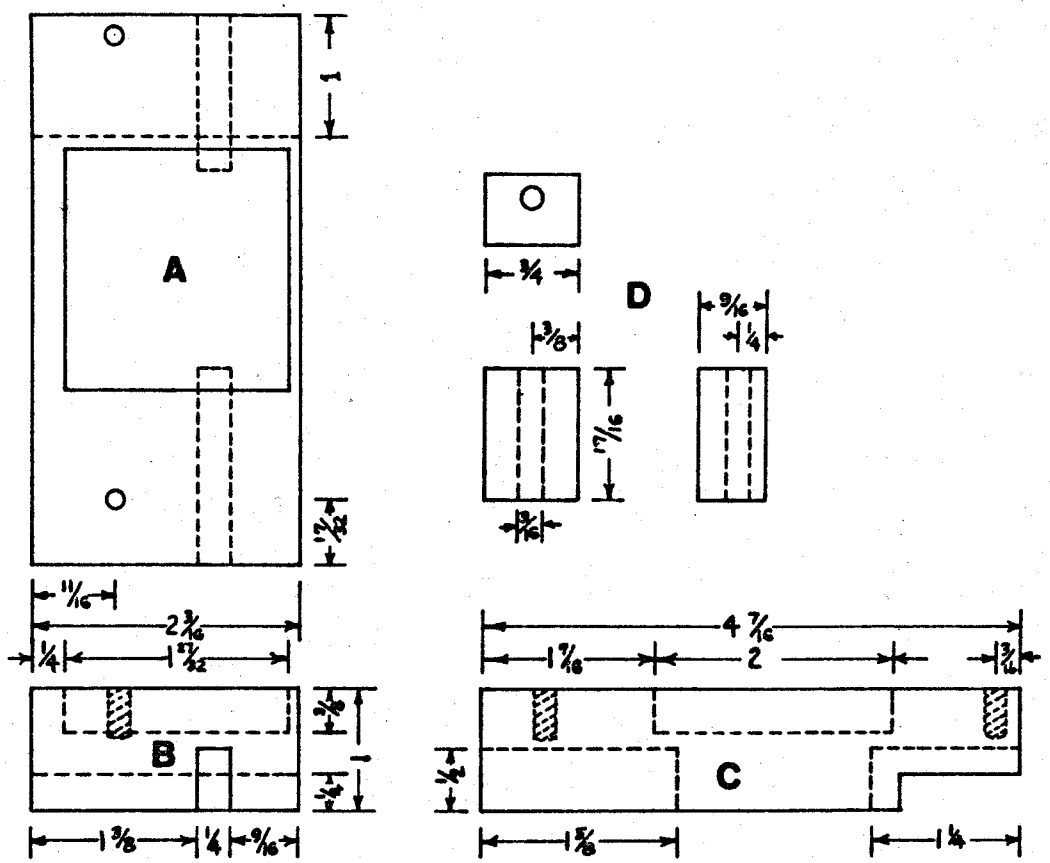


Fig. 16. Diagram of mounting block and block spacer: A, B and C top, front, and side views of the mounting block; D shows similar views of the block spacer. The dimensions are in inches.

spectropolarimeter. This signal was mediated by a custom-made calibration box (Fig. 17) which provides for expansion and baseline adjustment of the scale. This is necessary because the signal is proportional to the Jasco recorder pen position and is not influenced by the sensitivity setting of the spectropolarimeter. The trimmer potentiometers (T) and selector switch (ss) permit frequently used scale expansions to be conveniently interchanged.

Certain precautions were followed in the use of the modified spectropolarimeter to avoid inducing stress on cell windows that may result in optical artifacts. The heating compartment lid was permitted to rest freely on the assembly. Pressure exerted by the cell block was also controlled by the insertion of a spacer wire (sw) between the two parts of the copper core.

b. Temperature-Optical Density Profiles

For optical density measurements at elevated temperatures, the Tm analyzer was connected to a Cary 15 spectrophotometer. A mounting block, similar to that used for the Durrum-Jasco, was used to adapt the Tm Analyzer cell compartment to that of the Cary 15.

The binding of EB to DNA at elevated temperatures was measured by monitoring the optical density at 460 nm, throughout the temperature range under investigation. A Tris-HCl (0.04M) buffer, pH 7.9 blank was used for these studies.

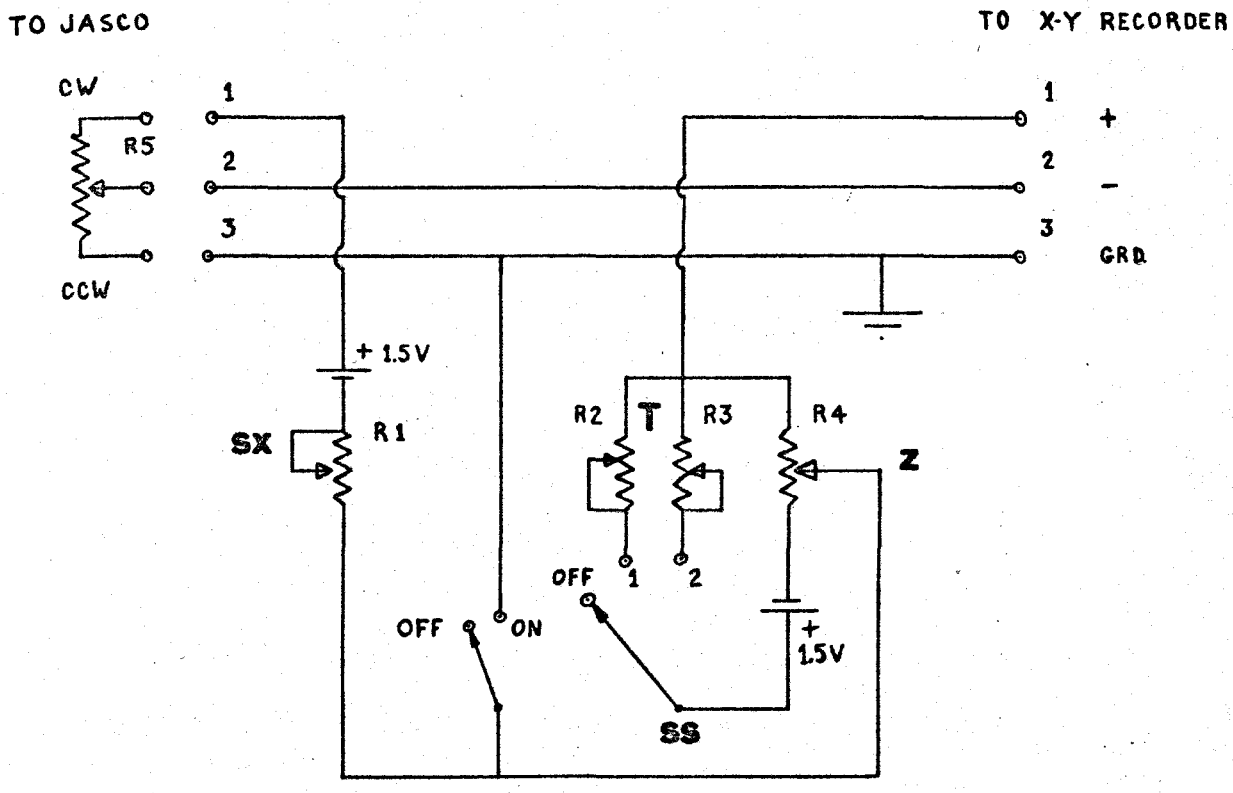


Fig. 17. Schematic diagram of wiring for calibration box: SX, scale expansion; SS, expansion selector switch; T, trimmer potentiometers; Z, zero adjustment; R1, 20KΩ 10 turn potentiometer; R2 and R3, 50KΩ trimmer potentiometers; R4, 10KΩ 10 turn potentiometer; R5, 10KΩ one turn potentiometer on Jasco pen-drive shaft.

The effect of EB on the temperature-absorbance profile of DNA was monitored at the absorption maximum of DNA at 260 nm. Blanks containing a second sample identical to the one being heated were employed. The results obtained from the X-Y recorder were replotted as hyperchromicity (i.e. the absorbance at temperature t divided by the absorbance at 25°) versus temperature. The temperature at which one-half of the total increase in absorbance is reached is usually referred to as the melting temperature (Tm). We will be referring to the temperature at which the initial rise in absorbance is observed as the Ti.

c. Measurements of Bound EB at Elevated Temperatures

The spectrophotometric measurement of the concentration of bound ethidium bromide at elevated temperatures is complicated by the effect of temperature on the absorption spectrum of free ethidium. The free EB exhibits at elevated temperature a small red shift (Fig. 18). The absorption maximum shifts from 480 nm at 25° to 490 nm at 90° with the extinction coefficients decreased from 5600 to approximately 5470.

The extinction coefficient of free ethidium bromide at 460 nm is required for calculation of the amount of bound ethidium bromide. The value of the extinction coefficient though, continually changes with increasing temperature (Fig. 19) and can be conveniently obtained from

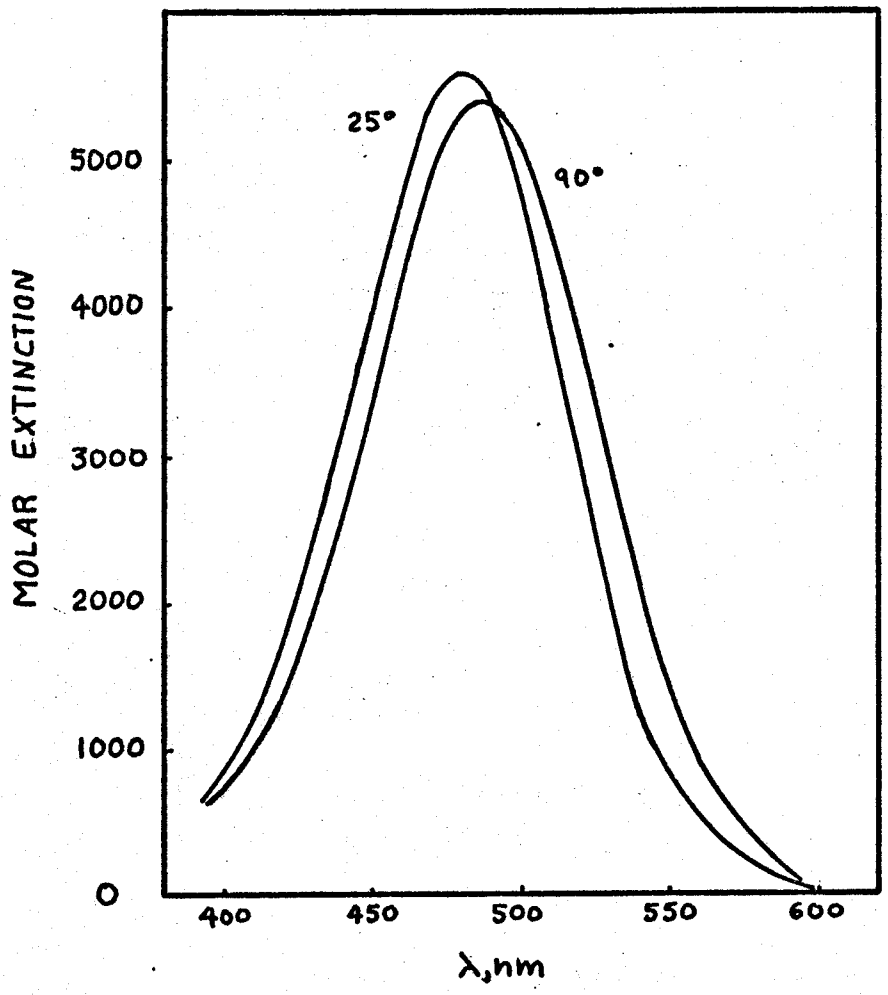


Fig. 18. Effect of elevated temperatures on the absorption spectrum of ethidium bromide. Absorption spectra of EB ([EB] = 0.10 μM/ml) were obtained in 0.04M Tris-HCl pH 7.9 in a 10.0 mm cell. The spectrum at 90° is not corrected for thermal expansion. This would effect the spectrum at 90° by about 2 percent and would not significantly alter the quantitative determinations from these spectra.

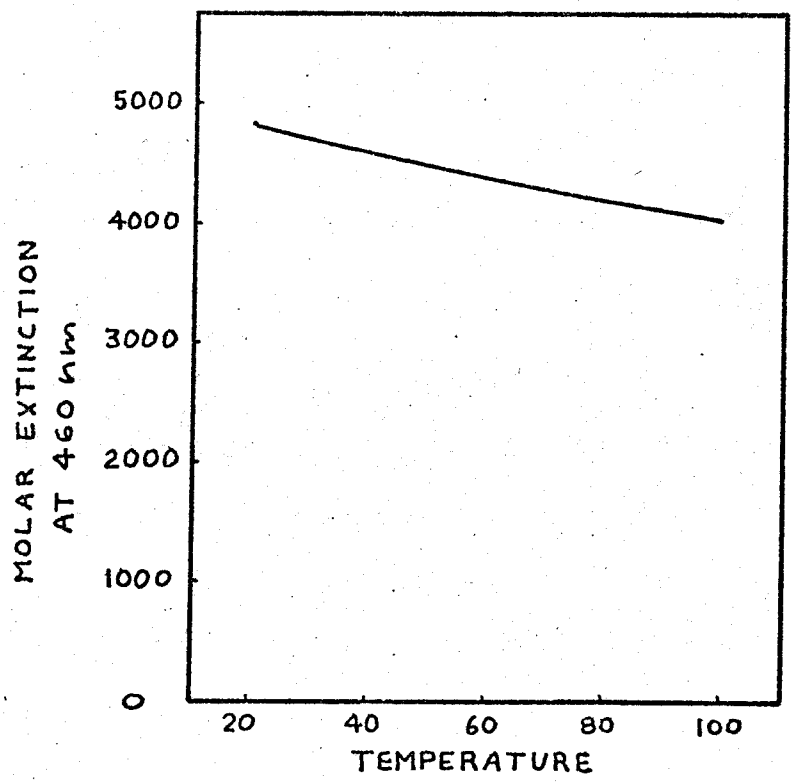


Fig. 19. Dependence of the apparent molar extinction of EB at 460 nm on temperature. [EB] = 0.10 μM/ml in 0.04 M Tris-HCl, pH 7.9, in a 10.0 mm cell.

a tracing of the optical density of free EB at 460 nm versus temperature. The determination of bound EB concentration at elevated temperatures by the spectrophotometric method were found to correlate well with binding determinations by dialysis techniques described in the following section. The spectrophotometric method possesses the inherent advantage, over dialysis, of being considerably more rapid and convenient. Furthermore, it provides for a continuous monitoring of the binding and it therefore was the method that was generally used in these studies.

3. Dialysis Experiments

Dialysis experiments were carried out to measure the binding of ethidium bromide to DNA in order to confirm the results of the measurements obtained spectrophotometrically. The dialysis tubing employed (cellulose, 19 mm diameter, pore size = 24Å) was washed repeatedly in boiling water to remove plasticizer used in manufacturing of the tubing.

Aliquots (5.0 ml) were dialyzed once for 18-24 hours against a fixed volume (25.0 ml) of buffer (0.04 M Tris-HCl, pH 7.9). For experiments conducted at elevated temperatures, the dialysis was carried out inside a centrifuge tube (50 ml capacity) equipped with a screw top. The tube was placed in a water bath that was maintained at the desired temperature. At the end of the dialysis period, the concentration of ethidium bromide remaining bound to DNA was

determined spectrophotometrically from the optical density of the dialysate at 480 nm. A dialysate obtained by dialysis of DNA containing no ethidium bromide was used as a blank.

The concentration of EB remaining bound to DNA may be determined as:

$$[EB]_b = [EB]_t - [EB]_f$$

and by substitution for $[EB]_f$;

$$[EB]_b = [EB]_t - \left[\frac{A480\ (\text{dialysate})}{5.6\ A/\mu M/ml} \times \frac{30\ ml}{5\ ml} \times \frac{1}{5.0\ cm\ (\text{light path})}\right]$$

and the bound EB to DNA nucleotide molar ratio calculated as $\frac{[EB]_b}{[DNA]} = r$.

This method was used to verify the results of the determination of EB binding at elevated temperatures obtained spectrophotometrically as indicated in Chapter II, B 2 c. For example, a complex prepared by addition of EB to DNA ([P] = 0.60 μM/ml, [EB] = 0.09 μM/ml) was dialyzed for 20 hours at 96°C and the amount of EB remaining bound determined as:

$$[EB]_b = 0.09\ \mu M/ml - \left[\frac{0.344}{5.6} \times \frac{30\ ml}{5\ ml} \times \frac{1}{5\ cm}\right] = 0.016\ \mu M/ml$$

The molar ratio of bound EB to nucleotide ratio is therefore:

$$r = \frac{0.016\ \mu M/ml}{0.60\ \mu M/ml} = 0.027$$

This value for r is in good agreement with that of 0.028 (Fig. 40) determined spectrophotometrically.

C. EXPERIMENTAL METHODS

1. Preparation of Polynucleotide Stock Solutions

DNA calf thymus was dissolved (4mg/ml) in Tris-HCl buffer (0.04 M), pH 7.9, by slow stirring for about 48 hours. The solution was centrifuged and dialyzed twice versus buffer of the same concentration.

The synthetic polynucleotides were received as lyophilized powders. The stock solution (8 mg/2 ml) of polyriboadenylic, polyribocytidylic, polyriboinosinic, polyribouridylic acids and yeast RNA were prepared in the same manner as the DNA stock solution. Polydeoxyriboadenylic-deoxyribothymidylic acid and polydeoxyriboguanidylic-polydeoxyribocytidylic acid were prepared by dissolving the powders in buffer so that the desired concentrations were obtained without further dilution. The lyophylized powder containing 10 absorbance units was usually dissolved in about 4 ml of buffer. One absorbance unit gives an absorbance of one when dissolved in one ml of solvent. The concentrations of the polynucleotide stock solutions were

determined spectrophotometrically from the extinction coefficients listed in Table 1.

TABLE 1

Polynucleotide Extinction Coefficients

Polynucleotide	Molar Extinction Coefficient at 260 nm	
Calf thymus DNA	6600	(66)
Polyriboadenylic Acid (poly A)	9000	(12)
Polyribocytidylic Acid (poly C)	6300	(32)
Polyriboinosinic Acid (poly I)	10,300	(84)
Polydeoxyadenylate-thymidylate (poly dAT)	6700	(48)
Polydeoxyguanidylate-polydeoxycytidylate (poly dG:dC)	7400[1]	
Yeast RNA	6100[2]	

1. Supplied by Miles Laboratories
2. Calculated from phosphorous content supplied by Sigma Chemical Co.

2. Preparation of Ethidium Bromide Stock Solution

Ethidium bromide (120 mg) was dissolved (4 mg/ml) in Tris-HCl (0.04 M) buffer (30 ml), pH 7.9, and stirred for about 16 hours. The exact concentration of the ethidium bromide stock solution was determined spectrophotometrically from the molar extinction coefficient at 480 nm (111).

3. Preparation of Ethidium Bromide-Polynucleotide Samples

a. EB-DNA at a DNA concentration of 0.20 μM/ml

A polynucleotide solution (0.40 μM/ml) and an ethidium bromide solution (2.0 μM/ml) were prepared by appropriate dilutions of the corresponding stock solutions in Tris-HCl (0.04 M) buffer, pH 7.9. Solutions containing ethidium and DNA (0.20 μM/ml) at total EB to DNA molar ratios varying between 0.02 and 2.00 were prepared as shown in Table 2.

b. EB-DNA at a DNA Concentration of 5.00 μM/ml

Solutions of ethidium bromide-DNA complexes at a DNA nucleotide phosphate concentration of 5.00 μM/ml and a variety of EB/P molar ratios were prepared as indicated in Table 3.

c. The Influence of Magnesium (II) Ions on the Ethidium Bromide-DNA Interaction

The effect of magnesium (II) ions on the interaction of ethidium bromide with DNA was examined in a manner similar to that described for the EB-DNA interaction. DNA solutions (0.40 μM/ml) were prepared by diluting an appropriate aliquot of DNA stock solution in Tris-HCl (0.04 M)-$MgCl_2$ (4×10^{-3} M) buffer, pH 7.9. Ethidium bromide (2.0 μM/ml) was prepared by dissolving ethidium (1 mg/ml) in the same buffer. The aliquots of the DNA and ethidium solutions used are shown in Table 4.

TABLE 2

Ethidium Bromide-DNA Complexes at Varying EB/P Ratios (DNA concentration of 0.20 μM/ml)

Ethidium Bromide		EB/P
ml	μM/ml	Molar Ratio
0.00	0.000	0.00
0.02	0.004	0.02
0.04	0.008	0.04
0.06	0.012	0.06
0.08	0.016	0.08
0.10	0.020	0.10
0.12	0.024	0.12
0.14	0.028	0.14
0.16	0.032	0.16
0.18	0.036	0.18
0.20	0.040	0.20
0.25	0.050	0.25
0.30	0.060	0.30
0.40	0.080	0.40
0.50	0.100	0.50
0.75	0.150	0.75
1.00	0.200	1.00
1.30	0.260	1.30
1.70	0.340	1.70
2.00	0.400	2.00

DNA (5.0 ml of a 0.400 μM/ml solution) and ethidium bromide (2.0 μM/ml) solution were added to a 10.0 ml volumetric flask and diluted to the mark with Tris-HCl (0.04 M) buffer, pH 7.9.

TABLE 3

Ethidium Bromide-DNA Complexes at Various EB/P Ratios (DNA Concentration of 5.0 μM/ml)

Ethidium Bromide		EB/P
ml	μM/ml	Molar Ratio
0.25	0.25	0.05
0.50	0.50	0.10
0.75	0.75	0.15
1.00	1.00	0.20
1.25	1.25	0.25
1.50	1.50	0.30
2.00	2.00	0.40
2.90	2.90	0.58
3.85	3.85	0.77

DNA (5.0 ml of a 10 μM/ml solution) and ethidium bromide (10 μM/ml) were added to a 10.0 ml volumetric flask and diluted to the mark with Tris-HCl (0.04 M) buffer, pH 7.9.

TABLE 4

EB-DNA Complexes at Various

EB/P Ratios in the Presence of Magnesium

Ethidium Bromide		EB/P
ml	μM/ml	Molar Ratio
0.00	0.000	0.00
0.02	0.004	0.02
0.04	0.008	0.04
0.06	0.012	0.06
0.08	0.016	0.08
0.10	0.020	0.10
0.12	0.024	0.12
0.14	0.028	0.14
0.16	0.032	0.16
0.18	0.036	0.18
0.20	0.040	0.20
0.25	0.050	0.25
0.30	0.060	0.30
0.40	0.080	0.40
0.50	0.100	0.50
0.75	0.150	0.75
1.00	0.200	1.00
1.30	0.260	1.30
1.70	0.340	1.70
2.00	0.400	2.00

DNA (5.0 ml of a 0.40 μM/ml solution) and ethidium bromide (2.0 μM/ml) were added to a 10.0 ml volumetric flask and diluted to the mark with Tris-HCl (0.04 M)-$MgCl_2$ (0.004 M) buffer, pH 7.9.

d. Interaction of Ethidium Bromide With Denatured DNA

The interaction of ethidium bromide with ice-quenched heat denatured DNA was examined in a manner identical to that described for native DNA in Chapter II, D3a. Complexes were prepared as shown in Table 5. Denatured DNA was prepared by heating native DNA (100 ml, [P] = 0.40 μM/ml) in boiling water for 30 minutes, and subsequently immersing the DNA-containing flask in ice water at 0°C. The degree of denaturation was determined from the hyperchromicity at 260 nm as discussed in Chapter II, B2b. The obtained hyperchromicity was usually in excess of 25 percent.

e. Effect of Ethidium Bromide Addition to DNA at 95°C

The ability of ethidium bromide to bind to DNA at temperatures at which DNA strands have separated was examined by the following method. A DNA solution (3.33 ml, [P] = 0.20 μM/ml) was heated to 95° and maintained in the cell compartment of the Tm Analyzer at this temperature for 20 minutes. A small aliquot of ethidium bromide (25 μl, 8.00 μM/ml) was then introduced while the temperature was maintained constant and the circular dichroism was examined at 95° as well as after cooling to 25°C. All solutions were prepared in Tris-HCl (0.04 M) buffer, pH 7.9. The final concentrations were [P] = 0.20 μM/ml and [EB] = 0.060 μM/ml giving an EB to DNA phosphate ratio of 0.30.

TABLE 5

Ethidium Bromide-Denatured DNA Complexes at Various EB/P Molar Ratios

Ethidium Bromide		EB/P
ml	μM/ml	Molar Ratio
0.02	0.004	0.02
0.04	0.008	0.04
0.06	0.012	0.06
0.08	0.016	0.08
0.10	0.020	0.10
0.12	0.024	0.12
0.14	0.028	0.14
0.16	0.032	0.16
0.18	0.036	0.18
0.20	0.040	0.20
0.25	0.050	0.25
0.30	0.060	0.30
0.40	0.080	0.40
0.50	0.100	0.50
0.60	0.120	0.60
0.70	0.140	0.70
0.80	0.160	0.80
0.90	0.180	0.90
1.00	0.200	1.00
1.20	0.240	1.20
1.40	0.280	1.40
1.60	0.320	1.60
1.80	0.360	1.80
2.00	0.400	2.00

DNA (5.0 ml of a 0.40 μM/ml solution) and ethidium bromide (2.0 μM/ml) solution were added to a 10.0 ml volumetric flask and diluted to the mark with Tris-HCl (0.04 M) buffer, pH 7.9.

f. Dependence of Circular Dichroism on Temperature

The samples used for examination of the dependence of the circular dichroism on temperature were prepared as follows. A DNA solution (1.20 μM/ml) and a ethidium bromide solution (2.0 μM/ml) were prepared by appropriate dilutions of the corresponding stock solutions with Tris-HCl (0.04 M) buffer, pH 7.9. Solutions containing DNA (0.60 μM/ml) and EB at various EB to DNA phosphate ratios were prepared by adding DNA (1.20 μM/ml) and EB (2.0 μM/ml) into a 10 ml volumetric flask and diluting with Tris-HCl (0.04 M) buffer, pH 7.9 to the mark (Table 6).

g. Effect of pH on the Formation of the EB-DNA Complex

The examination of the effect of pH on the formation of the EB-DNA complex was carried out in the following way. The variation of E_L-E_R at 307 nm with pH was examined for an added EB to DNA phosphate ratio of 0.15. DNA (0.40 μM/ml) and ethidium (2.00 μM/ml) solutions were prepared at each pH by dilution of the corresponding stock solution in the proper buffer. Buffers used for the various pH ranges were: Acetate (0.04 M), pH 2.5-6.0, Tris-HCl (0.04 M), pH 6.0-9.0 and Diethanolamine (0.04 M), pH 9.0-12.0. Solutions containing DNA (0.20 μM/ml) and ethidium (0.03 μM/ml) were prepared by adding 5.0 ml DNA (0.40 μM/ml and 0.15 ml ethidium (2.0 μM/ml) to a 10 ml volumetric flask and diluting to the mark with buffer.

TABLE 6

EB-DNA Complexes of Various EB/P Ratios at Elevated Temperatures

Ethidium Bromide		EB/P
ml	µM/ml	Molar Ratio
0.15	0.03	0.05
0.30	0.06	0.10
0.45	0.09	0.15
0.60	0.12	0.20
0.75	0.15	0.25
0.90	0.18	0.30
1.20	0.24	0.40
3.00	0.60	1.00
4.50	0.90	1.50

DNA (5.0 ml of a 1.20 µM/ml solution) and ethidium bromide (2.0 µM/ml) were added to a 10.0 ml volumetric flask and diluted to the mark with Tris-HCl (0.04 M) buffer, pH 7.9. The final DNA concentration for all samples was 0.60 µM/ml.

The examination of the dependence of the molar circular dichroism at 307 nm on the bound EB to nucleotide ratios at pH 11.3 and 11.7 was carried out in a manner similar to that used for experiments at pH 7.9 (Chapter II, C3) with the exception that the buffers appropriate for these pH's were used. DNA solutions (0.40 μM/ml) and ethidium bromide (2.00 μM/ml) were prepared by dilution of the corresponding stock solutions in diethanolamine (0.04 M) buffer, pH 11.3 or 11.7. Solutions containing a total EB to DNA molar ratios varying between 0.015 and 2.00 were prepared at both pH's as shown in Table 7.

h. Interaction of Ethidium Bromide with Polyvinylsulfate

Solutions of ethidium bromide-polyvinylsulfate complex were prepared in the following manner. Polyvinylsulfate (5.00 ml; [S] = 10 μM/ml) and ethidium bromide (0.20 ml, 8 μM/ml) solutions were introduced into a volumetric flask (10.0 ml) and diluted to the mark with Tris-HCl (0.04 M) buffer, pH 7.9. The final concentrations were [S] = 5.00 μM/ml and [EB] = 0.16 μM/ml resulting in an EB to sulfate ratio of 0.032.

TABLE 7

Ethidium Bromide-DNA Complexes at Alkaline pH

Ethidium Bromide		EB/P
ml	μM/ml	Molar Ratio
0.015	0.003	0.015
0.020	0.004	0.020
0.040	0.008	0.040
0.060	0.012	0.060
0.080	0.016	0.080
0.100	0.020	0.100
0.120	0.024	0.120
0.140	0.028	0.140
0.160	0.032	0.160
0.180	0.036	0.180
0.200	0.040	0.200
0.250	0.050	0.250
0.300	0.060	0.300
0.400	0.080	0.400
0.500	0.100	0.500
0.600	0.120	0.600
0.700	0.140	0.700
0.800	0.160	0.800
0.900	0.180	0.900
1.000	0.200	1.000
1.200	0.240	1.200
1.400	0.280	1.400
1.600	0.320	1.600
1.800	0.360	1.800
2.000	0.400	2.000

DNA (5.0 ml of a 0.40 μM/ml solution) and ethidium bromide (2.0 μM/ml) solutions were added to a 10.0 ml volumetric flask and diluted to the mark with diethanolamine (0.04 M) buffer. Separate sets of solutions were prepared at pH 11.3 and pH 11.7.

CHAPTER III: RESULTS

A. THE APPEARANCE OF INDUCED CIRCULAR DICHROISM UPON INTERACTION OF ETHIDIUM BROMIDE WITH DNA

1. The CD of the EB-DNA Complex

The effect of ethidium bromide on the circular dichroism of DNA is shown in Fig. 20. Increased circular dichroism is observed near 250 and 275 nm. New CD bands also appear near 290, 307 and 370 nm and a shoulder near 335 nm. In the presence of EB and at an added molar ratio of dye to DNA nucleotide (EB/P ratio) of 0.30, an increase in E_L-E_R for DNA is observed from -2.0 to -4.0 at 250 nm and from +2.5 to +5.0 at 275 nm.

Since circular dichroism bands below 300 nm may originate from three different sources, DNA, EB-DNA complex and free ethidium bromide, transitions are difficult to analyze. For this reason, subsequent measurements were limited primarily to the wavelength region above 300 nm. In this region, circular dichroism is likely to arise from ethidium bromide only, either free or bound.

2. Absorption and Difference Spectra

The absorption spectrum of EB (Fig. 21) exhibits maxima at 275 and 480 nm and a shoulder near 310 nm. Maxima near 310 and 335 nm with broad bands in the region near 500 nm are also observed in the differential absorption spectrum

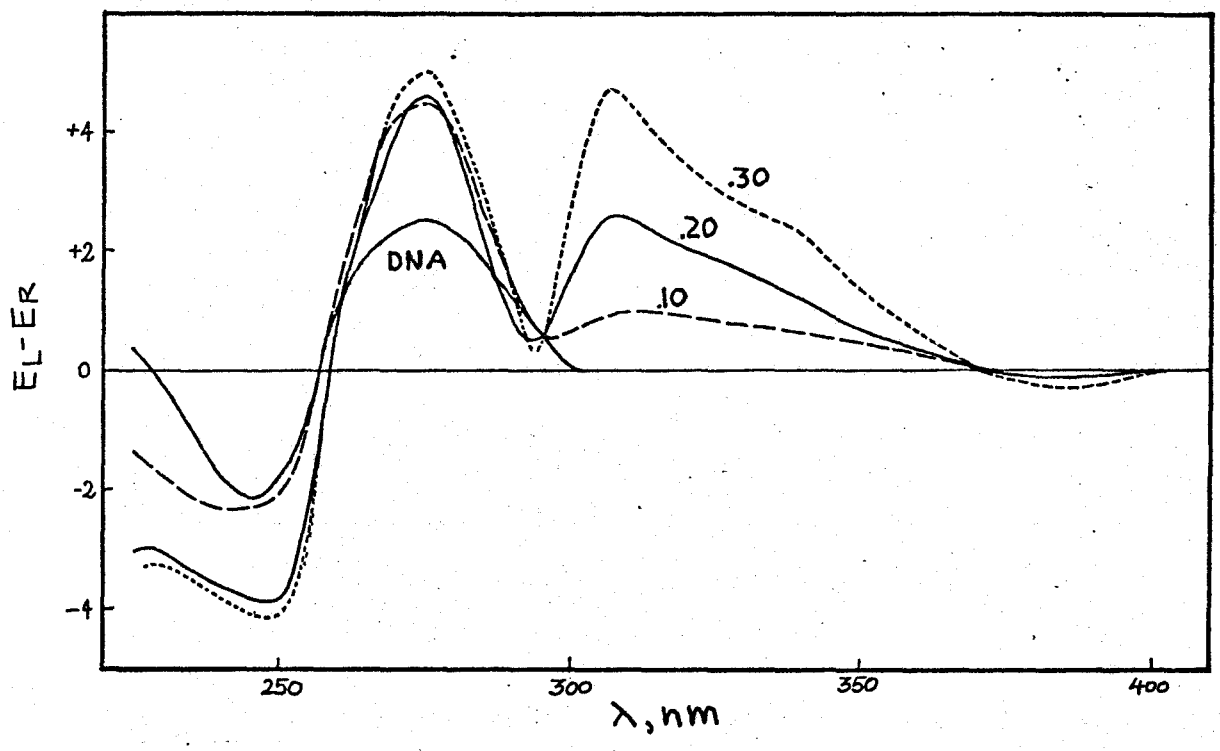


Fig. 20. Circular dichroism of DNA and EB-DNA in 0.04 M Tris-HCl, pH 7.9 at varying molar ratios of added-EB per nucleotide phosphate. DNA concentration 0.20 μM/ml. The measurements were carried out in a 10 mm cell. E_L-E_R is determined on the basis of DNA phosphate concentration.

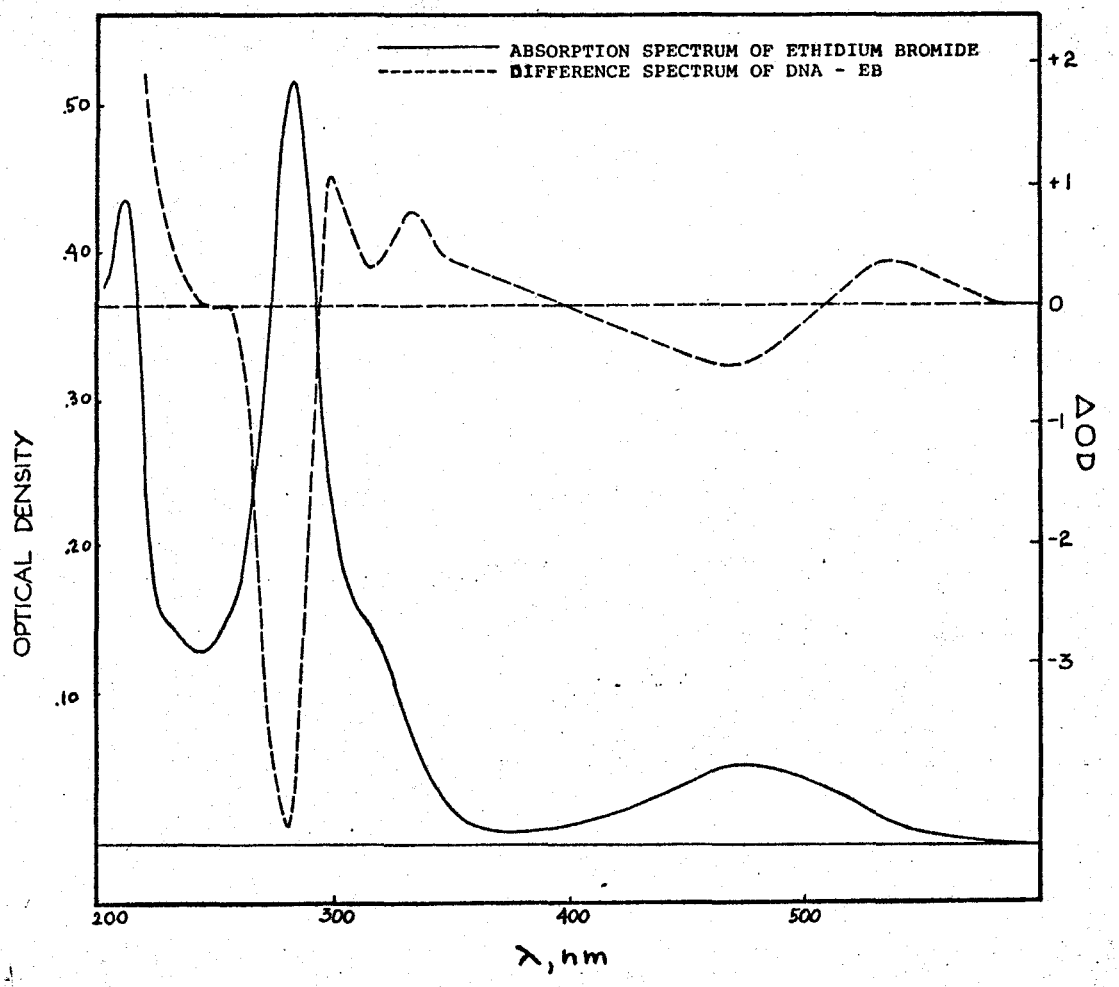


Fig. 21. Absorption and difference spectra of ethidium bromide. The absorption spectrum was obtained at an EB concentration of 0.01 μM/ml in a 10.0 mm cell. The difference spectrum was obtained by using four 10.0 mm cells, two each in the sample and reference compartments of the spectrophotometer. In the sample compartment, one cell contained buffer and the other EB-DNA ([EB] = 0.01 μM/ml [DNA (P)] = 0.20 μM/ml). The cells in the reference compartment contained respectively EB and DNA at the same concentrations as those present in the sample compartment cell. Solutions prepared in 0.04 M Tris-HCl, buffer, pH 7.9.

of the EB-DNA complex. The difference spectrum is obtained by subtracting the spectrum of free EB and free DNA from the spectrum of the EB-DNA complex. The appearance of bands near 500 nm in the difference spectrum agrees with previous observations (112).

DNA does not exhibit any absorption above 300 nm while ethidium does. Since circular dichroism is a manifestation of optical activity within the vicinity of an absorption band, the induced CD at wavelengths above 300 nm must originate from ethidium bromide transition. The observed correlation between the wavelengths of maxima for the circular dichroic and difference spectra is consistent with the concept that the EB-DNA complex contributes to the circular dichroism above 300 nm. A maximum near 310 nm is observed in the CD spectrum of free EB (Fig. 22c). This band, however, is of low intensity and may make only minor contributions to the circular dichroism of the ethidium bromide-DNA complex.

3. <u>The Relationship Between Free and DNA-Bound EB</u>

In the spectra that have been examined so far, the EB-DNA complexes were characterized by small molar EB to DNA ratios. Under these conditions, all added EB binds to DNA and no EB remains free in solution. This, however, is not the case at higher ratios. It is, therefore, necessary to define two terms. First, the molar ratio of total added ethidium bromide per nucleotide will be referred to as the

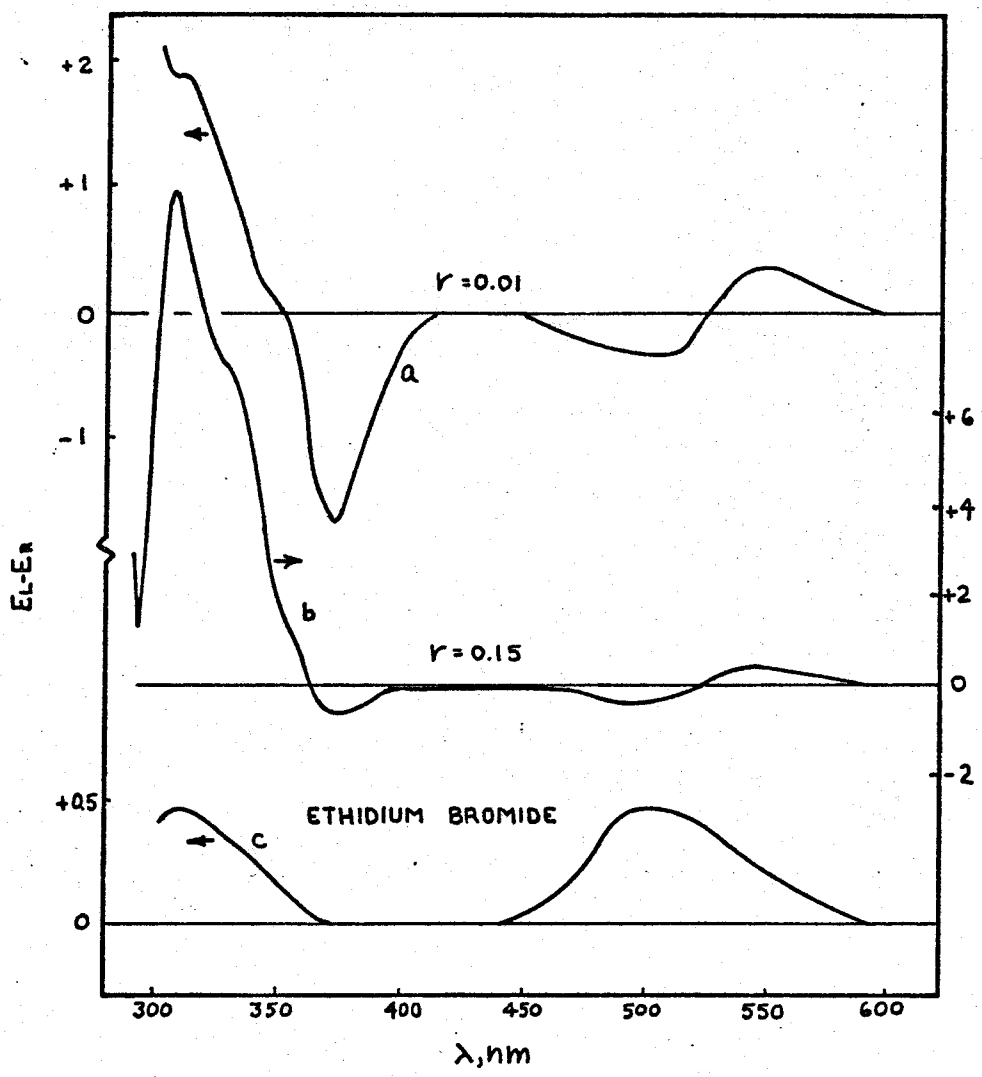


Fig. 22. Circular Dichroism of EB-DNA and EB in 0.04 M Tris-HCl pH 7.9. (a) DNA-EB in a 50.0 mm cell ([P] = 5.0 μM/ml, EB = 0.05 μM/ml) (b) DNA-EB in a 10.0 mm cell ([P] = 0.60 μM/ml EB = 0.09 μM/ml) (c) EB in a 10.0 mm cell (0.50 μM/ml); a and c left ordinate; b right ordinate. E_L-E_R for the EB-DNA complex was determined on the basis of the concentration of bound EB.

added dye to nucleotide ratio and will be abbreviated as EB/P. The molar ratio of bound ethidium bromide per nucleotide residue, on the other hand, will be referred to as the bound dye ratio and will be abbreviated as r. The values of r were determined spectrophotometrically for each sample. The relation between EB/P and r is summarized in Table 8 and illustrated in Fig. 23. At EB/P ratios below 0.15 these terms are equivalent since the dye is present only in the bound form. However, at EB/P ratios above 0.20, a considerable portion of the added ethidium remains free. For example, at EB/P ratios of 0.60 and 1.00, r is equal to 0.26 and 0.32 respectively. At these higher ratios, therefore, the presence of free EB must be taken into consideration whenever quantitative interpretations of the CD spectra are made. The relationship illustrated in Fig. 23 represents a single DNA concentration. The relationship between EB/P and r would of course be different for other DNA concentrations.

B. THE DEPENDENCE OF CIRCULAR DICHROISM UPON THE RATIO OF DNA-BOUND EB TO DNA PHOSPHATE

1. CD Variation with Increasing EB Concentration

The magnitude of the circular dichroism of the EB-DNA complex (Fig. 20) in the 300 to 360 nm range increases considerably with increasing EB concentrations. At 307 nm, the E_L-E_R values are 1.1, 2.8 and 4.6 at EB/P ratios of 0.10, 0.20 and 0.30 respectively. This apparent non-linear

TABLE 8

Relationship Between "Added" and "Bound" EB to Nucleotide Ratios at a DNA Concentration of 0.20 μM/ml

EB	Ratios	
μM/ml	EB Added / EB/P	EB Bound / r
0.000	0.000	0.000
0.004	0.020	0.020
0.008	0.040	0.040
0.012	0.060	0.060
0.016	0.080	0.080
0.020	0.100	0.100
0.024	0.120	0.120
0.028	0.140	0.140
0.030	0.150	0.150
0.032	0.160	-----
0.036	0.180	0.163
0.040	0.200	0.186
0.050	0.250	0.203
0.060	0.300	0.216
0.080	0.400	0.237
0.100	0.500	0.263
0.120	0.600	0.278
0.150	0.800	0.296
0.200	1.100	0.306
0.280	1.400	0.332
0.360	1.800	0.406
0.420	2.100	0.528

Solutions were prepared in Tris-HCl (0.04 M), pH 7.9.

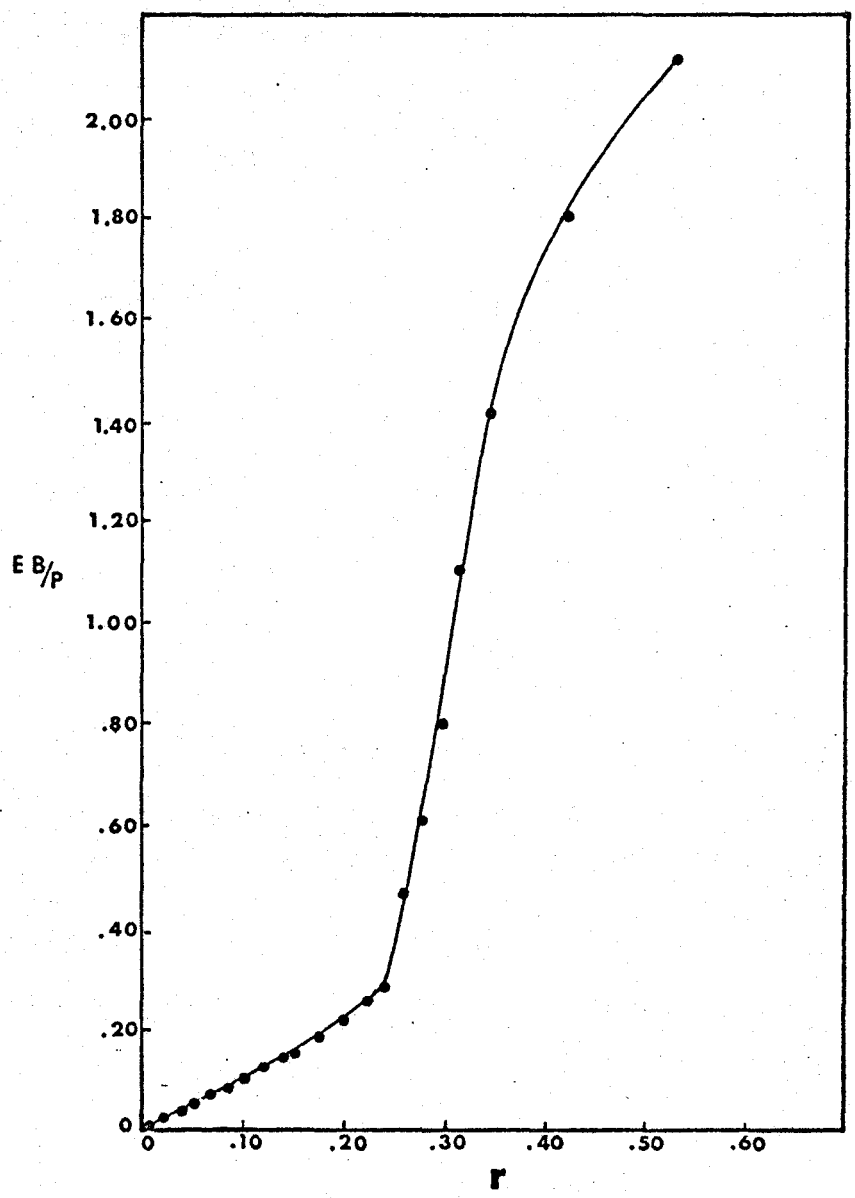


Fig. 23. Relationship between total added (EB/P) and bound (r) EB to nucleotide phosphate ratios. DNA concentration was constant at 0.20 μM/ml and EB concentrations vary from zero to 4.00 μM/ml. Tris-HCl buffer (0.04 M), pH 7.9 was used for all solutions. The relationship between EB/P and r would, of course, be different at other DNA concentrations.

increase in circular dichroism cannot be studied quantitatively from spectra in which EB to DNA ratios are expressed in terms of total added EB rather than DNA-bound EB. Furthermore, the molar circular dichroism, E_L-E_R, is calculated in these spectra in terms of DNA phosphate rather than EB-DNA complex concentrations. The latter concentration which is equivalent to that of bound EB, may be determined spectrophotometrically from the metachromic shift in the EB absorption spectrum near 500 nm (Chapter I, C2 and II, B2a) (111).

2. The CD of EB-DNA at Two Ratios of Bound EB to DNA

The circular dichroism of free EB from 300 to 600 nm is compared to that of the EB-DNA complex at two ratios of bound EB per DNA nucleotide (r) in Fig. 22. Free ethidium bromide exhibits weak positive circular dichroism below 370 nm and between 450 and 590 nm. The maxima near 307 and 500 have approximately equal E_L-E_R values of 0.50. For each of the above ratios ethidium is present almost entirely (> 97%) in the bound form. The CD spectra of the EB-DNA complex at the two ratios exhibit extrema at the same wavelengths with positive maxima at 307 and 540 nm and negative maxima at 370 and 500 nm. However, a large difference in the magnitude of the molar circular dichroism for the two ratios is apparent at 307 nm. E_L-E_R is 11.0 at an r of 0.15 whereas at an r of 0.01 it is only 1.9. This behavior is somewhat surprising if it is assumed that each ethidium

bound to DNA contributes equally to the dichroism at 307 nm. However, this apparently is not the case. The significance of this finding is discussed in Chapter III, E3.

The negative CD band near 370 nm actually exhibits a decrease in E_L-E_R at higher r. At r values of 0.01 and 0.15 E_L-E_R is -1.80 and -0.66 respectively. The decreased ellipticity probably results from the overlap of the strong positive CD bands between 300 and 360 nm with a relatively weak band centered around 370 nm.

C. THE CIRCULAR DICHROISM OF THE ETHIDIUM BROMIDE-DNA COMPLEX IN THE 400-600 nm REGION

In contrast to the results obtained in the spectral region between 300 and 350 nm, the magnitudes of the CD extrema near 500 nm are comparable for bound dye to nucleotide ratios of 0.01 and 0.15. This region is examined in more detail in Fig. 24. As previously indicated, free EB exhibits a positive circular dichroism from 440 to 590 nm with a maximum E_L-E_R of 0.50 at 500 nm. In these experiments, the EB/DNA ratio is varied by increasing the concentration of DNA while maintaining the concentration of EB constant.

At first glance it appears that the development of the negative CD band at 500 nm associated with bound EB is inversely dependent on the EB/P ratics. However, examination of Table 9 shows that the 500 nm band depends on the extent to which ethidium is present in the bound form. As

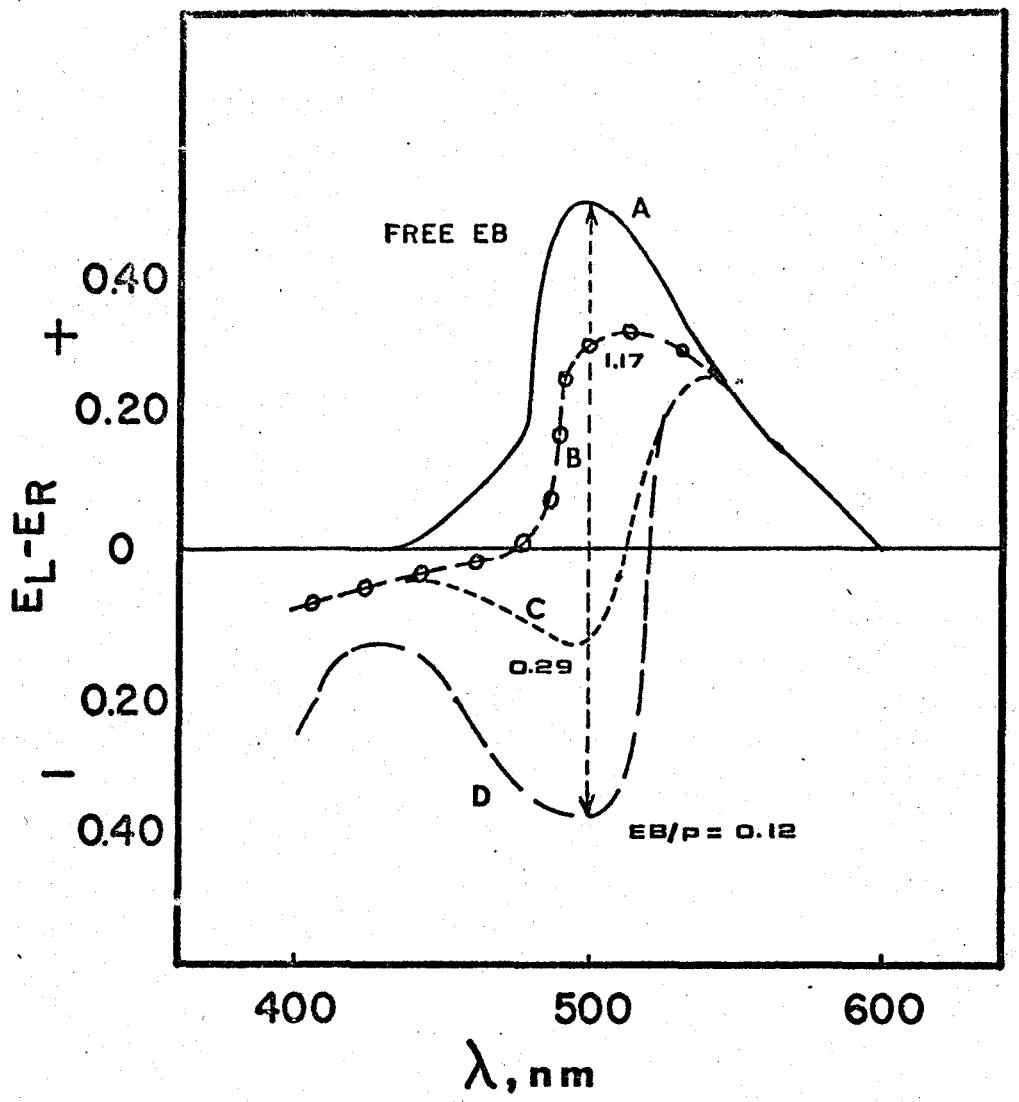


Fig. 24. Circular dichroism of EB and EB-DNA from 400 to 600 nm. DNA concentrations are listed in Table 9. The symbols A, B, C, and D refer to spectra as listed in the same table. The values 0.12, 0.29 and 1.17 refer to the added EB to nucleotide ratios.

TABLE 9

Circular Dichroism of EB-DNA

Mixtures with Varying Degrees of EB Binding

Spectrum	(DNA) μM/ml	EB/P	% EB Bound	r
A	0.00	---	0	---
B	0.09	1.16	17.5	0.21
C	0.39	0.28	64.6	0.18
D	0.90	0.12	100.0	0.12

EB concentration was 0.108 μM/ml in 0.04 M Tris-HCL, pH 7.9.

the amount of DNA-bound EB increases from 17.5 to 100%, the circular dichroism centered near 500 nm becomes increasingly more negative.

The magnitude of this band reaches a limit at DNA concentrations at which 100% of the ethidium is in the bound form. Under these conditions, a positive extremum near 540 nm and a negative extremum near 500 nm are observed with E_L-E_R values of +0.26 and -0.38 respectively. Examination of these spectra indicates that the CD of bound EB consists of a negative component between 440 and 540 nm, which is superimposed on the positive dichroic band centered near 500 nm characteristic of EB. The magnitude of the negative CD at 500 nm is the algebraic sum of the spectra of free and bound EB at this wavelength. The E_L-E_R of bound EB (spectrum D) for example, indicated by the broken line, is -0.82.

D. THE CIRCULAR DICHROISM OF THE EB-POLYVINYLSULFATE COMPLEX

The interaction of ethidium bromide with polyvinylsulfate (PVS) with which only secondary binding is possible (56,111) may permit the assignment of specific CD bands observed with the EB-DNA complex to either primary or secondary interactions. The CD spectra from 290 to 600 nm of ethidium bromide and ethidium bromide-polyvinylsulfate complex (EB/S = 0.032) are shown in Fig. 25. Induced circular dichroism is not observed between 300 and 360 nm

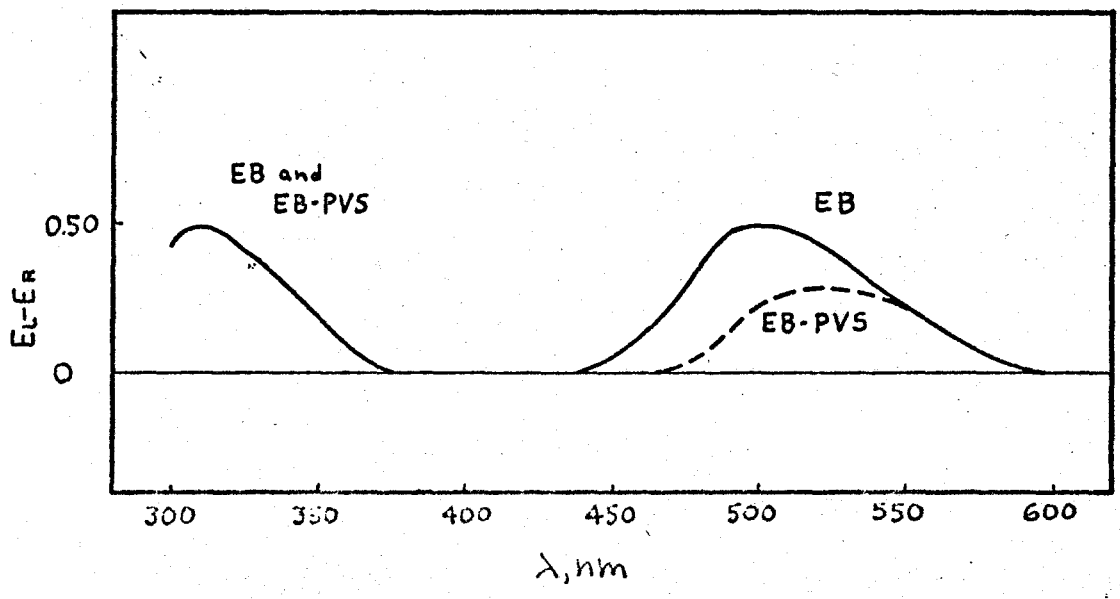


Fig. 25. Effect of polyvinylsulfate on the circular dichroism of ethidium bromide. [(EB) = 0.16 μM/ml and [S] = 5.00 μM/ml; in 0.04 M Tris-HCl, pH 7.9, path length = 10.0 mm.]

for EB-PVS as it is in the case of EB-DNA complexes (Figs. 20 and 22). However, a decrease in ellipticity of the EB is observed between 450 and 550 nm.

The absence of induced CD in the 300-360 nm spectral region is not surprising since CD in this region is apparently a reflection of primary binding (Chapter III, B2) and primary interactions involving intercalation of EB cannot occur between EB and polyvinylsulfate.

The reduction in the ethidium bromide ellipticity near 500 nm in the presence of polyvinylsulfate indicates that secondary interactions contribute to induced circular dichroism in this region. In contrast, the failure of polyvinylsulfate to modify the CD spectrum of EB between 300 and 350 nm suggests that the CD of the EB-DNA complex in this region originates from interactions different from those involved in secondary binding.

E. DEPENDENCE OF CIRCULAR DICHROISM ON BOUND ETHIDIUM BROMIDE TO NUCLEOTIDE RATIO

1. Molar Circular Dichroism at 500 nm

We have noted that the binding of ethidium bromide to DNA results in the appearance of several induced CD bands between 290 and 600 nm. The dependence of the molar circular dichroism at 307 and 500 nm on the bound ethidium to nucleotide ratio (r) is compared in Fig. 26 which is constructed from the data in Table 10.

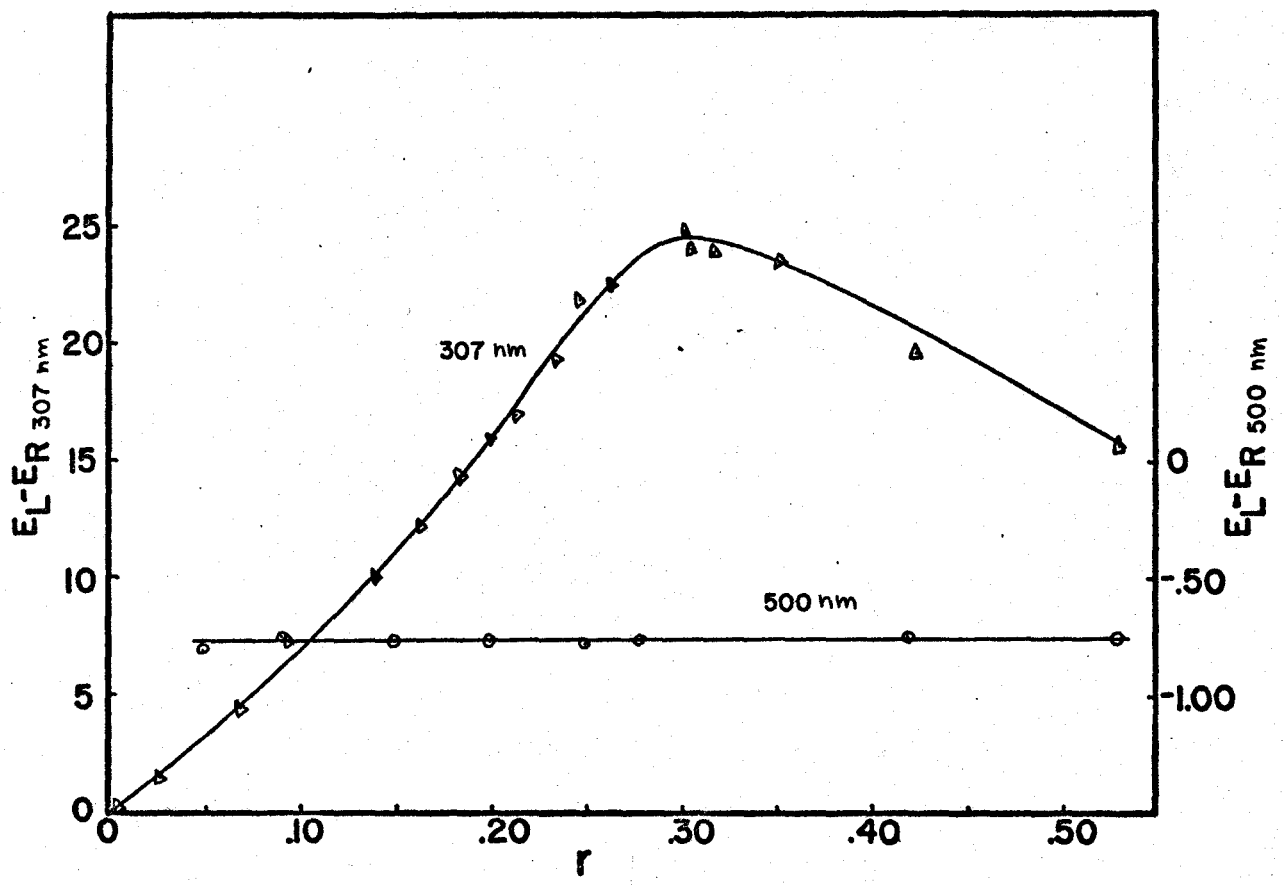


Fig. 26. Dependence of molar circular dichroism at 307 nm and 500 nm on the molar ratio (r) of bound-EB to DNA phosphate. [[DNA] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9] EB concentrations are listed in Table 2.

TABLE 10

Dependence of Molar Circular Dichroism at 307 nm on Bound EB to Nucleotide Ratio at a DNA Concentration of 0.20 μM/ml

r	Molar Circular Dichroism 307 nm	Molar Circular Dichroism 500 nm
0.023	2.44	------
0.047	3.40	-0.71
0.070	4.35	------
0.093	7.28	-0.76
0.116	8.65	------
0.140	9.93	------
0.163	12.13	-0.75
0.186	14.47	------
0.203	15.85	-0.76
0.216	16.90	------
0.237	19.34	------
0.243	22.08	-0.81
0.260	22.96	------
0.280	23.19	-0.75
0.275	25.37	------
0.298	25.06	------
0.313	24.18	------
0.292	25.46	------
0.305	26.62	------
0.305	24.06	------
0.422	20.03	-0.74
0.528	16.10	-0.76

Solutions prepared in Tris-HCl (0.04 M) buffer, pH 7.9. E_L-E_R values were corrected for contributions from free EB as described in Chapter II, B1a.

E_L-E_R values at 500 nm, obtained as previously mentioned in Chapter II, B1b and III, C, are constant at -0.76 for r between 0.05 and 0.52. The induced negative CD at 500 nm apparently does not discriminate between primary and secondary binding since, although primary binding sites are saturated at an r of about 0.25, E_L-E_R remains constant above this ratio.

As previously noted the binding of ethidium to DNA affects a metachromic shift in the EB absorption spectrum (Chapter I, C1). The 500 nm CD is analogous to the metachromic shift in the absorption spectrum which is linearly dependent on the amount of EB in the bound form and is dependent equally upon the extent to which EB binds to either primary or secondary sites.

2. Molar Circular Dichroism at 307 nm

The dependence of circular dichroism on r at 307 nm contrasts sharply with that at 500 nm. At 307 nm, E_L-E_R values increase from zero at very low r values (0.005) to 25.0 at an r of 0.30. At r higher than 0.30 the molar circular dichroism gradually decreases reading 16.1 at an r of 0.52.

The maximum E_L-E_R noted at an r of 0.30 indicates that at this ratio primary binding sites in DNA are saturated. The increase in molar circular dichroism at 307 nm with increasing r may indicate that the induced CD at this wavelength is the result of nearest-neighbor interactions

between EB molecules bound to adjacent primary sites (Chapter I, G). The value of r for primary site saturation obtained by CD is slightly higher than the generally accepted r range of 0.20-0.25 for completion of primary binding obtained by spectrophotometric and spectrofluorometric methods (56,111).

The relatively higher values of r calculated from CD data may be due to the limited accuracy of the spectrophotometric techniques in differentiating between EB bound to primary and secondary sites. The molar circular dichroism, E_L-E_R, of the complex is calculated on the basis of total bound EB (Chapter II, B1a). If, as reported (111), the number of primary binding sites per DNA nucleotide is 0.20-0.25, and secondary binding begins to occur at an r of about 0.15, the apparent saturation of primary binding sites by CD is expected to take place at an r higher than 0.20 or 0.25, since both primary and secondary sites are occupied at a measured r of 0.30.

It should also be noted that the results expressed in Fig. 26 are based on the tacit assumption that the 307 nm CD maximum originates from an isolated band. However, the CD in the 290 to 360 nm region actually appears to consist of several overlapping bands (Fig. 20). The magnitude of the 307 nm band may actually be influenced by a negative band near 290 nm and/or a positive band with a maximum near 335 nm manifesting itself as a shoulder at this wavelength.

The relative circular dichroism of these bands may be dependent on r. The CD band near 290 nm is of relatively low intensity but it becomes more pronounced with increasing EB/P ratios.

The overlap of the 290 and 307 nm bands and the relative dependence observed at higher EB/P ratios suggests that the 290 nm band may contribute to the high value of r at which apparent primary binding saturation occurs. An overlap between the 290 nm band and the 307 nm band may cause a decrease in the CD at 307 nm. This effect may become more evident at increasing EB/P ratios.

3. Dependence of Δ Absorbance at 307 nm on Bound EB-DNA Ratio

The decrease observed in E_L-E_R at ratios (r) higher than 0.30 in Fig. 26 may be explained on the basis of the method of calculating E_L-E_R. The measured difference between the absorbance of left and right circularly polarized light (ΔA) reaches a maximum value near r = 0.30 (Table 11) as illustrated in Fig. 27, but the concentration of EB-DNA complex continues to increase up to an r of 0.52 due to secondary binding. Since circular dichroism is expressed in terms of ΔA per mole of ENA-bound EB, an apparent decrease in E_L-E_R for the EB-DNA complex may be expected at r values exceeding 0.30.

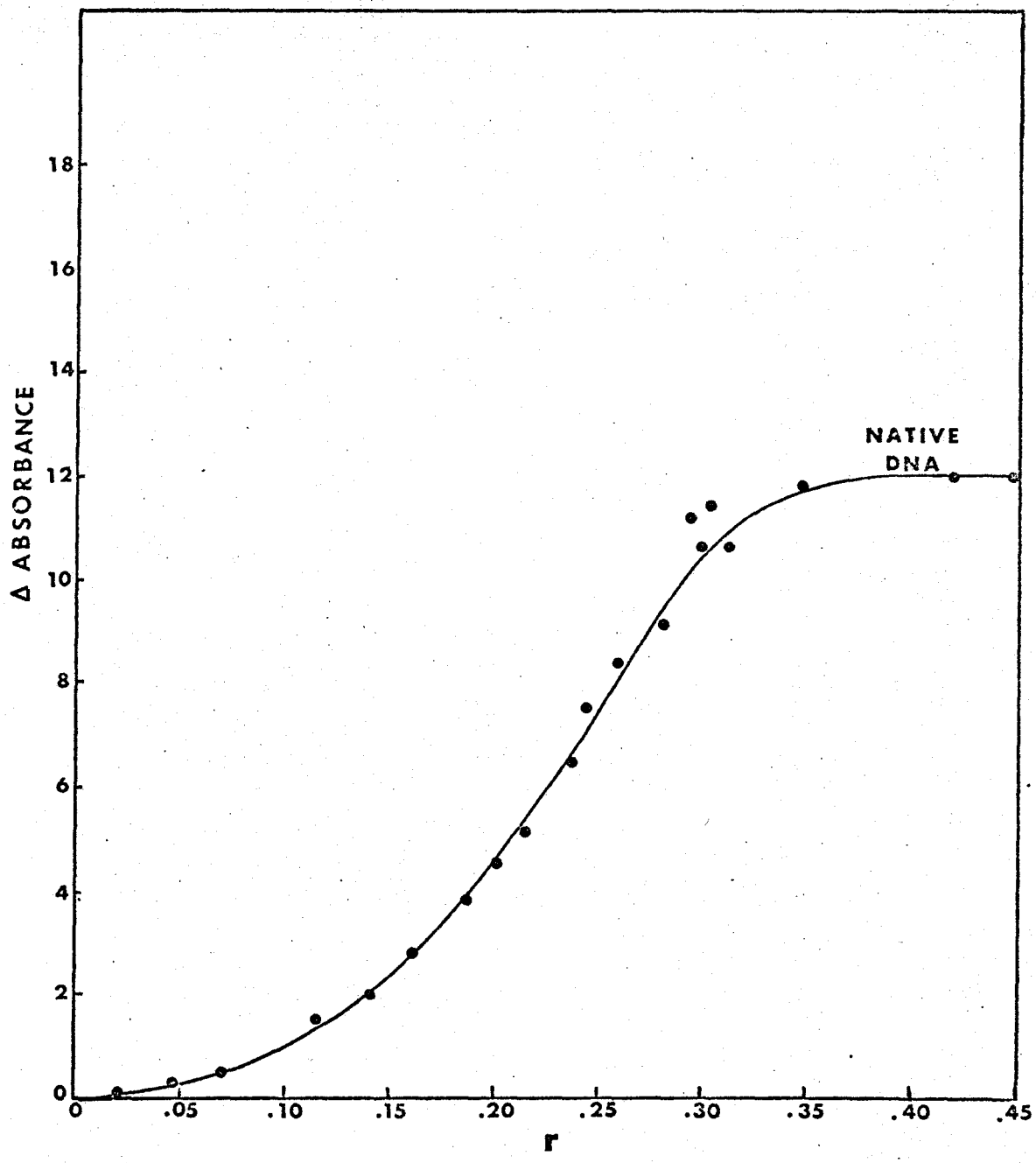


Fig. 27. Dependence of Δ Absorbance for the EB-DNA complex at 307 nm on r. (DNA = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9)

TABLE 11

Dependence of Δ Absorbance at 307 nm on Bound Dye to Nucelotide Ratio

r bound	Δ Absorbance at 307 nm
0.023	0.08
0.047	0.22
0.070	0.43
0.093	0.96
0.116	1.42
0.140	1.95
0.163	2.78
0.186	3.79
0.203	4.52
0.216	5.13
0.237	6.50
0.243	7.56
0.260	8.40
0.280	9.19
0.275	9.80
0.298	10.58
0.313	10.64
0.292	11.20
0.305	11.44
0.350	11.86
0.422	11.90
0.528	11.98

In Tris-HCl (0.04 M) buffer, pH 7.9.

F. DEPENDENCE OF MOLAR CIRCULAR DICHROISM ON COMPLEX CONCENTRATION

For accurate experimental determination of E_L-E_R at low r ($r < 0.05$) increased concentration of the complex must be used. Under these conditions, a strong (111) EB-DNA primary complex may be formed rapidly upon addition of ethidium to DNA. This apparently is also the case for the interaction of EB and transfer RNA (6) in which the first order rate constant for complex formation is in the range of about 10-100 sec^{-1}.

Therefore, if, after initial interaction with DNA, the DNA-bound EB is not re-distributed among all available binding sites, both the method of mixing and the concentration of the components would be expected to influence the nature of the resulting complex. Increased concentrations of DNA and ethidium bromide at a constant r might increase the probability that an added EB molecule will bind to a primary site adjacent to another bound EB. This, in turn, may be expected to enhance the molar circular dichroism at a given r. However, if EB distribution along the helix is determined only by r, E_L-E_R at 307 nm would be independent of complex concentration.

The dependence of circular dichroism at 307 nm on r for two different DNA concentrations is compared on Table 12 and illustrated in Fig. 28. The results obtained at [P] = 0.20 μM/ml are repeated from Fig. 26. At a DNA concentration

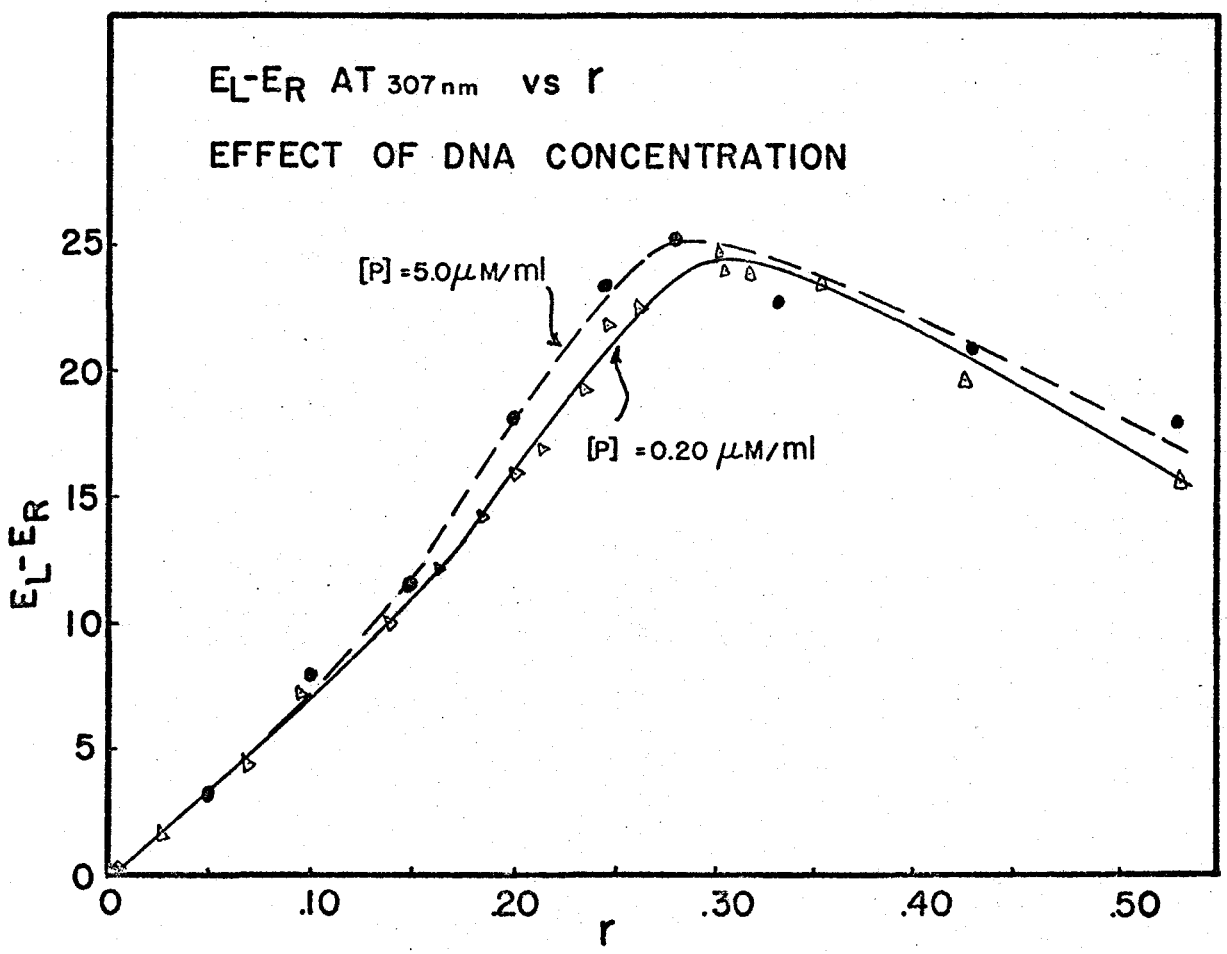


Fig. 28. Dependence of the molar circular dichroism at 307 nm on r compared at DNA concentrations of 0.20 and 5.00 μM/ml in 0.04 M Tris-HCl, pH 7.9.

TABLE 12

Dependence of Molar Circular Dichroism at 307 nm on Bound Ethidium Bromide to Nucleotide Ratio at a DNA Concentration of 5.0 μM/ml

r bound	Molar Circular Dichroism 307 nm
0.050	3.16
0.100	7.86
0.150	13.68
0.200	18.02
0.245	23.35
0.280	26.03
0.333	22.72
0.430	21.05
0.532	18.60

In Tris-HCl (0.04 M) buffer, pH 7.9.

of 5.00 μM/ml, E_L-E_R increases from zero to 26.0 at an r of 0.28. Further increases in r result in a gradual decrease in E_L-E_R to about 18.6 at an r of 0.53. By comparison, the maximum E_L-E_R at [P] = 0.20 is 25.0 at an r of 0.30. The measurements at each DNA concentration are corrected for contributions from free EB (Chapter II, B1a). E_L-E_R values at [P] = 5.00 appear slightly, but not decisively, higher than those at [P] = 0.20 for all values of r above 0.10.

The close agreement in r values corresponding to maximum E_L-E_R and the dependence of E_L-E_R on r for widely varying DNA concentrations indicates that the circular dichroism of the EB-DNA complex is dependent only on r and not on total complex concentration.

G. INFLUENCE OF MAGNESIUM CHLORIDE ON THE CIRCULAR DICHROISM OF THE ETHIDIUM BROMIDE-DNA COMPLEX

1. The Circular Dichroism of the EB-DNA Complex in Magnesium (II)

Increased concentrations of inorganic cations have been reported to interfere with the binding of ethidium to DNA and RNA (6,56,111). Magnesium (II) ions in particular are in this respect nearly 100 times as effective as sodium ions (111). At least two types of binding, primary and secondary, occur between ethidium and DNA and it may be that magnesium interferes selectively with one or the other type of binding. Examination of the influence of magnesium (II)

ions on the CD of the EB-DNA complex may permit a distinction between these two possibilities.

The circular dichroism of EB-DNA in the presence of 4×10^{-3} M $MgCl_2$ is shown in Fig. 29. Extrema appear below 250 nm and near 275, 307 and a shoulder near 335 nm. In contrast to the results observed in the absence of magnesium, however, the relatively weak band near 290 nm is not observed in the presence of magnesium. The extrema at 245 and 275 nm exhibit increased CD at increased EB/P ratios. For example, at an EB/P ratio of 0.20, E_L-E_R increases from -2.5 to 4.0 and from +2.5 to 5.0 respectively. These E_L-E_R values are of the same magnitude as those noted in the absence of magnesium for the same ratio of 0.20 (Fig. 20).

In contrast to the results noted at the extrema at 245 and 275 nm, the CD between 300 and 360 nm decreases in the presence of magnesium. At 307 nm, E_L-E_R at EB/P ratios of 0.20 and 0.30 decreases from 2.8 and 4.6 to 1.8 and 3.9 respectively. This decrease is clearly attributed to the decrease in EB binding in the presence of magnesium (II) ions (111). Under these conditions, a lower bound dye to nucleotide ratio (r) is expected for the same added EB to nucleotide ratio.

2. <u>The Effect of Magnesium (II) on Molar Circular Dichroism</u>

The dependence of E_L-E_R on r is not appreciably changed in the presence of magnesium (II) as shown in Table 13 and Fig. 30. The molar circular dichroism at 307 nm of

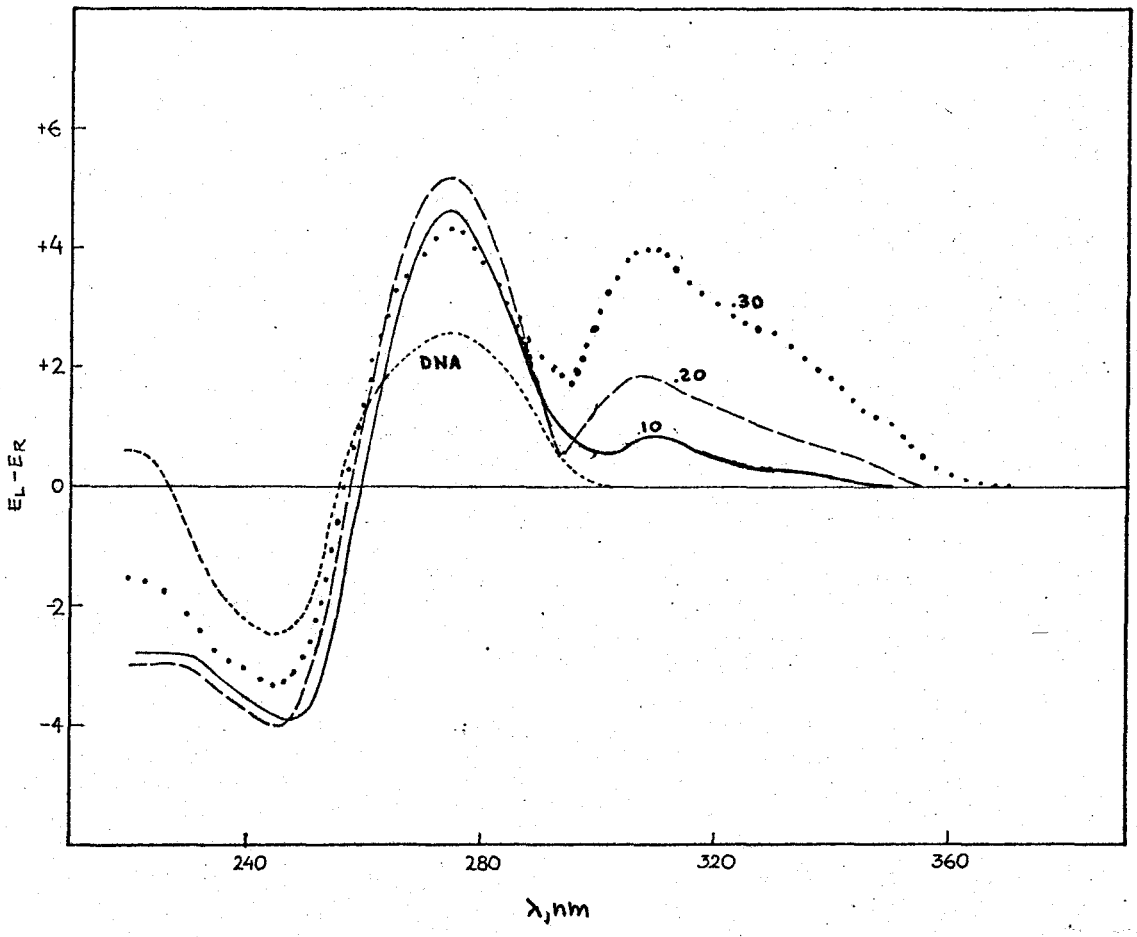


Fig. 29. Circular dichroism of EB-DNA in the presence of 4 x 10^{-3} M $MgCl_2$. Ratios refer to added EB to nucleotide ratios. [DNA] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9. Path length = 10.0 mm. E_L-E_R was calculated on the basis of DNA phosphate concentration.

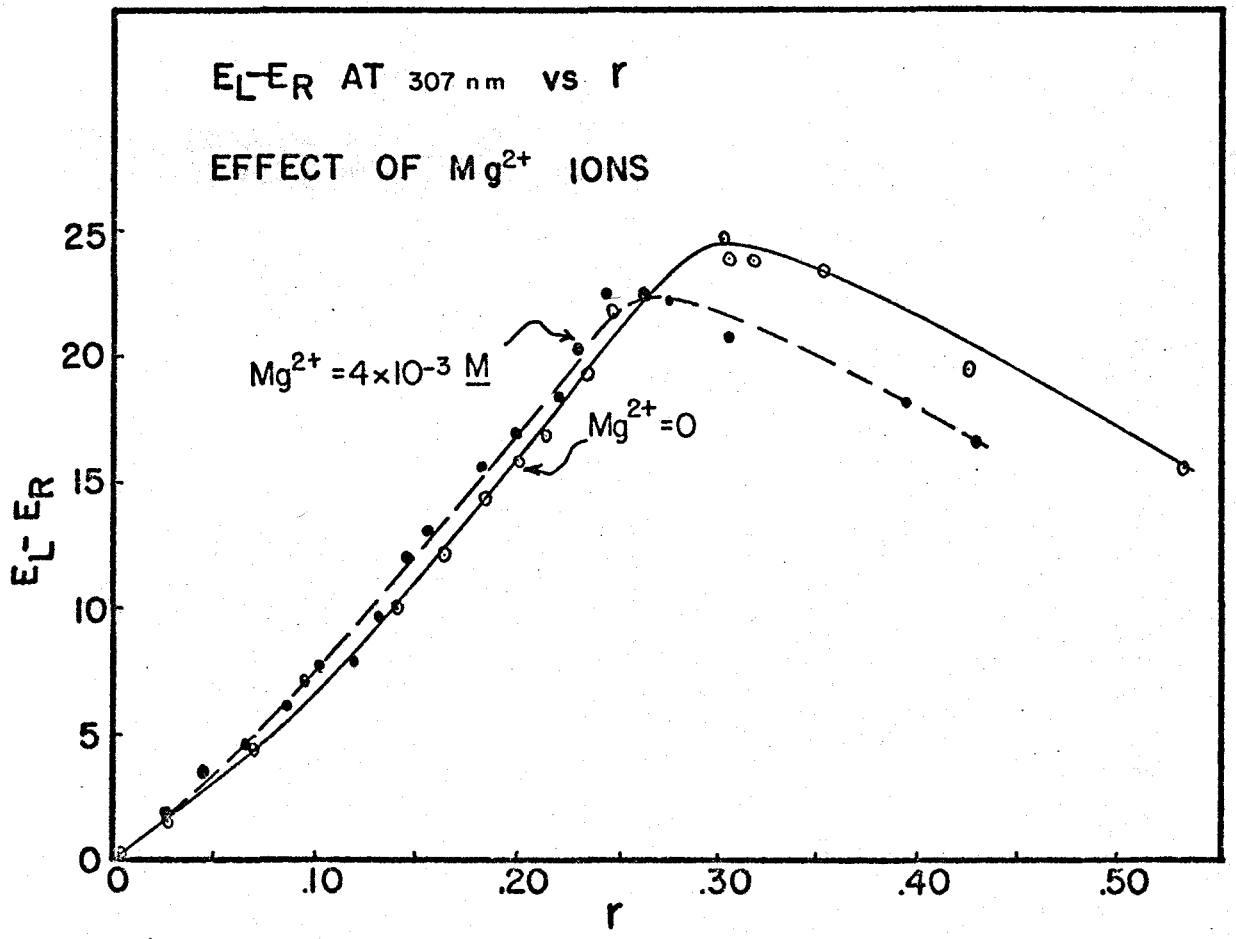


Fig. 30. Dependence of molar circular dichroism on bound EB to nucleotide ratio in 4 x 10^{-3} M $MgCl_2$. [DNA] = 0.20 μM/ml in 0.04 M Tris-HCl.

TABLE 13

Dependence of Molar Circular Dichroism at 307 nm on Bound EB to Nucleotide Ratio in the Presence of Magnesium (II)

r bound	E_L-E_R 307 nm
0.023	4.55
0.047	3.48
0.067	4.53
0.087	6.12
0.102	7.71
0.120	7.98
0.132	9.69
0.146	12.00
0.157	13.09
0.157	14.51
0.183	15.66
0.199	16.97
0.222	18.38
0.230	20.30
0.244	22.48
0.255	22.38
0.277	22.21
0.306	20.73
0.297	22.40
0.321	22.14
0.395	18.01
0.429	16.59

In Tris-HCl (0.04 M) buffer, pH 7.9.

the EB-DNA complex at 4×10^{-3} M magnesium chloride increases from zero to 22.5 at an r of 0.25. At r above 0.25 a gradual decrease in E_L-E_R is observed to about 16 at an r of 0.43. The behavior of the complex for r up to 0.25 is very similar to that noted in the absence of magnesium ions. As previously discussed, the induced circular dichroism at 307 nm reflects nearest-neighbor interactions between EB molecules bound to adjacent primary binding sites (Chapter I, H). These interactions apparently are not affected in the presence of magnesium.

Magnesium (II) ions, which bind preferentially to phosphate groups rather than the nitrogen bases of DNA (33, 98), have been reported to interfere principally with secondary binding (111). It has been reported that at low r values (r = 0.07), at which only primary binding occurs, magnesium does not cause dissociation of the EB-t-RNA complex as measured spectrophotometrically (6). Also the number of primary binding sites (~0.23) obtained by absorption techniques does not change in the presence of magnesium (II) ions.

The CD data in the presence of magnesium (II) indicate that saturation of primary binding sites occurs at an r of 0.25 which is in good agreement with the reported value of 0.23 for calf thymus DNA calculated spectrophotometrically (111). Since primary binding sites are saturated at an r of about 0.20-0.25, the binding of EB in the presence of

magnesium up to an r of 0.43 must be the result of secondary binding.

Secondary binding in the absence of magnesium is reported to occur before primary binding sites are completely saturated (111). In the presence of 4×10^{-3} M magnesium, however, secondary binding may not occur until nearly all primary binding sites are saturated. Under these circumstances, primary sites would be filled at a lower r, and since primary binding is the main contributor to the circular dichroism at 307 nm; the molar circular dichroism would be expected to reach a maximum value at a lower r.

In the presence of magnesium (II), the E_L-E_R maximum is 22.5 compared with 25.0 in the absence of magnesium. This is difficult to rationalize since E_L-E_R in the two systems corresponds closely up to an r of 0.25. If the difference between these two systems is only in secondary interaction as previously suggested, and these interactions do not contribute to the circular dichroism at 307 nm, E_L-E_R would be expected to be greater at an r of 0.25 in magnesium.

It is difficult to interpret this paradox given the present state of understanding of the properties of EB-DNA complex. It should be noted, however, that in the proflavine-DNA system as well (8), a high sodium ion concentration is reported to decrease the electronic interaction of the dye with the bases. The reason for this decrease is not clear.

H. THE CIRCULAR DICHROISM OF EB-THERMALLY DENATURED DNA COMPLEX

1. The CD of the EB-Denatured DNA Complex

Ethidium bromide is reported to form strong complexes with heat-denatured DNA (56,111). The binding of either ethidium or proflavine to denatured DNA is apparently not diminished as compared to native DNA (29,111). This is somewhat surprising if a double stranded polynucleotide is required for primary interactions to occur (111,112) and since denatured DNA contains less hydrogen bonded regions than native DNA (27,34).

The circular dichroism of the EB-denatured DNA complex is shown in Fig. 31. The addition of ethidium bromide to denatured DNA results in the appearance of induced CD from 220 to about 360 nm with maxima at 250, 275, 290, 307 and a shoulder at 335 nm. The presence of EB at an added EB/P of 0.10 results in an increase in E_L-E_R at 250 and 275 nm from -1.5 to -2.5 and from +2.5 to +3.0 respectively. At 307 nm, EB at EB/P ratios of 0.10, 0.20 and 0.30 results in E_L-E_R values of 1.6, 3.7 and 5.4 in the same sequence. E_L-E_R values at this wavelength are somewhat larger than 1.1, 2.8 or 4.6 noted for the EB-native DNA complex at the same ratios. In addition to the differences in ellipticity at 307 nm between the EB complexes formed with native and denatured DNA, differences are also apparent below 300 nm.

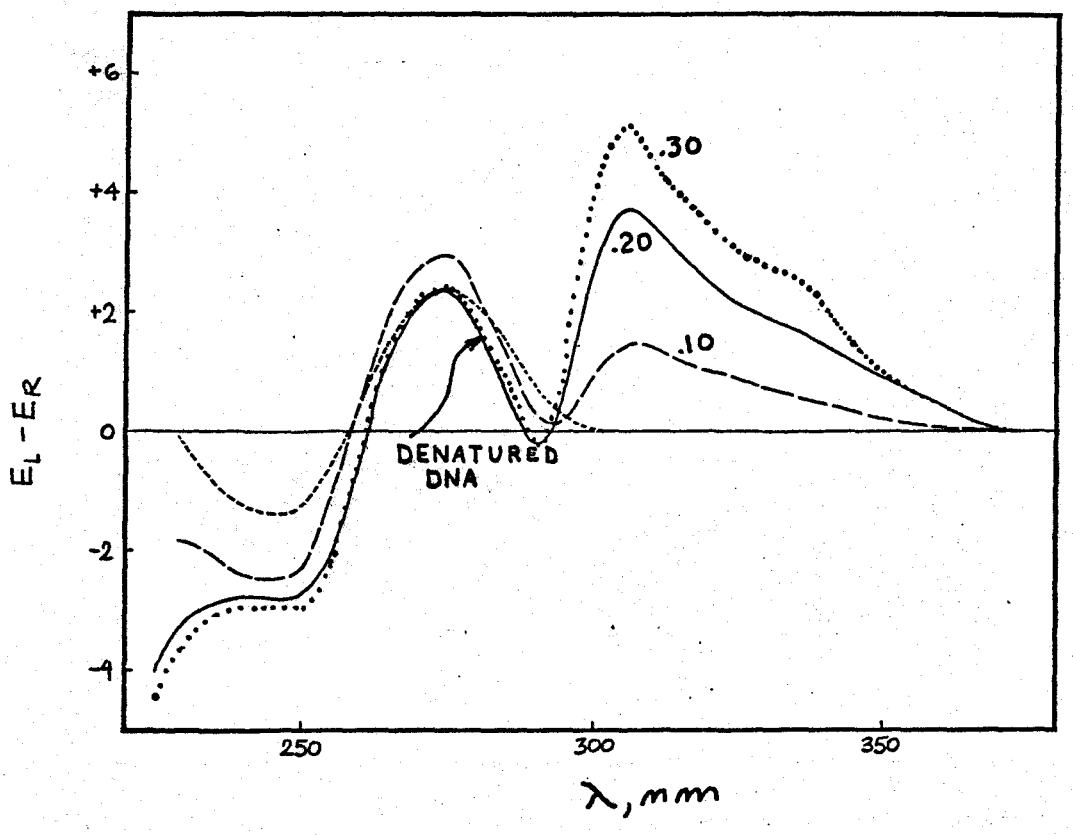


Fig. 31. Circular dichroism of EB-"denatured" DNA at several ratios of added-EB to DNA phosphate. [DNA = 0.20 μM/ml in 0.04 M Tris HCl, pH 7.9. Path length = 10.0 mm.] E_L-E_R is calculated on the basis of DNA phosphate concentration.

As previously indicated (Chapter III, A1) quantitative interpretations in this region are difficult because several molecular species may contribute to the circular dichroism. Nevertheless, some useful comparisons can be made. The spectra of DNA and denatured DNA do not differ appreciably in the 260 to 300 nm region. However, significant differences in the magnitude of the respective maxima near 275 and 290 nm are noted in the presence of ethidium bromide. At 275 nm, for example, an increase in E_L-E_R from +2.5 to about +4.5 occurs for native DNA for every EB/P ratio. In contrast, the 275 nm band with denatured DNA is little affected by the presence of ethidium at EB/P ratios up to 0.30. The CD band near 290 nm, which is hardly apparent in the EB-native DNA complex, appears more distinct with denatured DNA and becomes increasingly apparent at higher EB/P ratios.

2. <u>Dependence of Molar Circular Dichroism at 307 nm on Bound EB to Nucleotide Ratio for Denatured DNA</u>

The dependence of the molar circular dichroism at 307 nm on r is shown in Fig. 32 which is constructed from the data listed in Table 14. E_L-E_R increases from 8.0 at an r equal to 0.02 to 26.0 at an r equal to 0.36. Extrapolation of the E_L-E_R versus r line, however, does not cross the coordinates at zero as is the case with native DNA. At r values above 0.36 a gradual decrease in E_L-E_R is observed to a value of 18.0 at an r of 0.60.

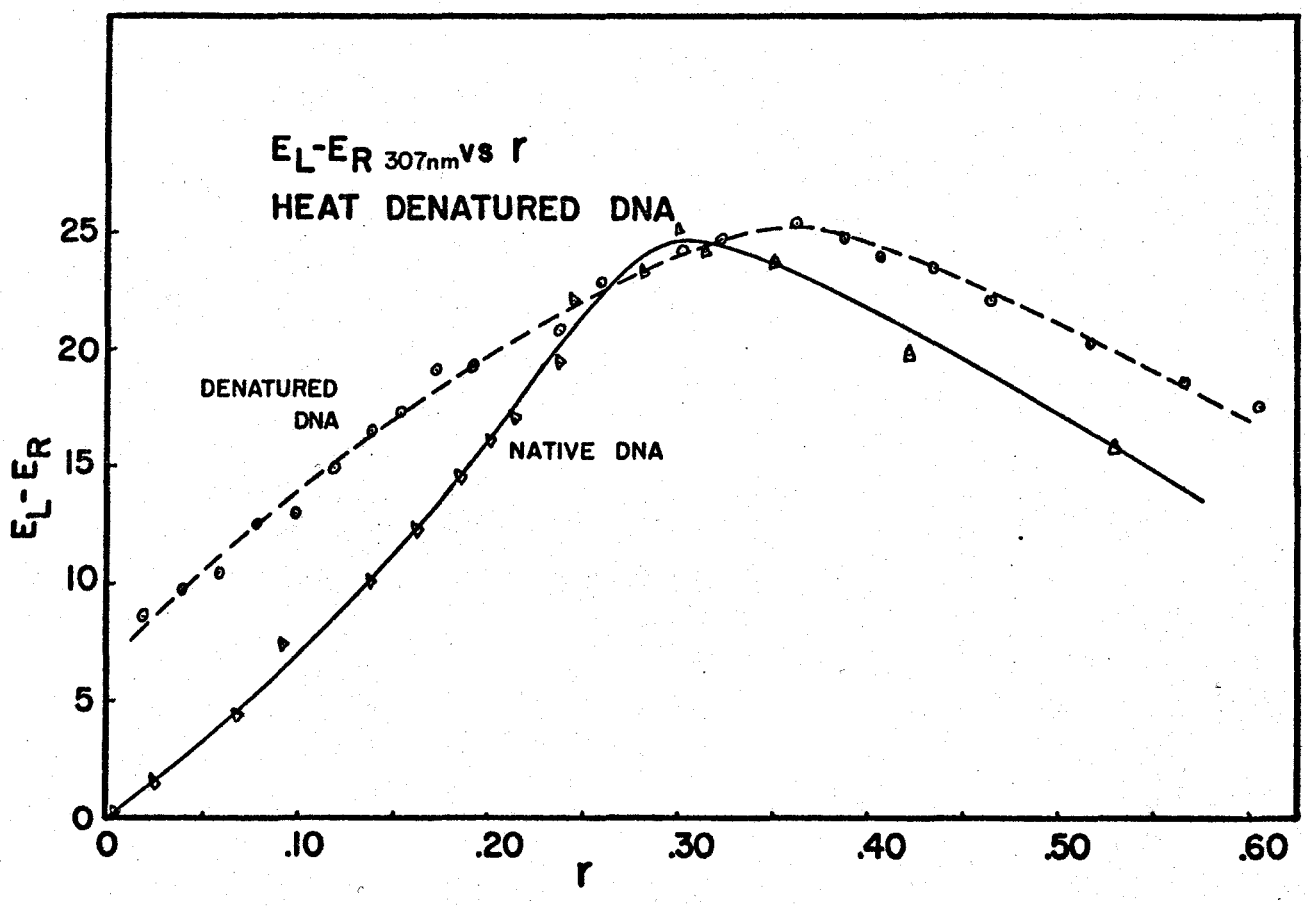


Fig. 32. Dependence of molar circular dichroism on the ratio of bound EB to DNA phosphate for denatured DNA. ([DNA] = 0.20 μM/ml in 0.04 M Tris-HCl.)

TABLE 14

Molar Circular Dichroism at 307 nm

for Ethidium Bromide-Denatured DNA Complex

r bound	Molar Circular Dichroism 307 nm
0.00	0.00
0.020	8.49
0.040	9.74
0.060	10.24
0.080	12.45
0.100	12.89
0.120	14.82
0.140	16.41
0.150	17.33
0.173	18.08
0.193	19.08
0.238	20.69
0.261	22.81
0.303	24.15
0.325	24.45
0.364	24.94
0.383	24.50
0.406	23.93
0.431	23.73
0.436	23.43
0.465	22.08
0.515	20.37
0.560	19.20
0.600	18.00

In Tris-HCl (0.04 M) buffer, pH 7.9.

The circular dichroism for denatured DNA is higher for every r from 0.02 to 0.25 and the maximum E_L-E_R attained for EB-DNA and EB-denatured DNA is 25.0 and 26.0 respectively. Since induced CD at 307 nm may be the result of nearest-neighbor interactions, the agreement noted in the maximum values attained for E_L-E_R for native and denatured DNA may indicate that similar primary complexes are formed.

The increase in E_L-E_R for denatured DNA as compared with native DNA at r between zero and 0.25 suggests enhanced nearest-neighbor interaction in the former. Information on the secondary structure of denatured DNA may illuminate the origin of this enhancement.

Ice-quenched heat-denatured DNA is reported to contain regions of hydrogen-bonded secondary structure (27,34) which is required for primary binding (56,110). However, the coexistence of single and double stranded regions obviously decreases the total number of primary binding sites. This decrease in binding sites has been substantiated by Le Pecq (56) by fluorescence spectroscopy. The maximum fluorescence enhancement of EB, which in native DNA depends directly on the amount of EB bound to primary sites, is decreased by one half for EB-denatured DNA.

For r values between zero and 0.20, the total binding of EB, determined spectrophotometrically, is not dependent on the tertiary structure of DNA. Thus, a decrease in the available primary binding sites in denatured DNA would

increase the probability that an added EB molecule will be bound adjacent to another one occupying a primary binding site. This, in turn, would increase nearest-neighbor interactions which are reflected by the circular dichroism at 307 nm.

The r at which the maximum circular dichroism occur is 0.36 for denatured DNA and 0.30 for native DNA. This paradox of the apparent increase in the number of primary binding sites accompanying the decrease in the double stranded regions in denatured DNA may be partially resolved if the CD spectra of EB-denatured DNA are examined. The negative CD band centered near 290 nm appears more pronounced at increasing EB/P ratios (Fig. 31) and at 0.30 appears centered near 285 nm. This band may overlap with the 307 nm band. Since the 290 nm band shifts toward lower wavelengths at increased EB/P ratios, some decrease in overlap between these bands may occur at the higher ratios resulting in the increased molar circular dichroism at 307 nm observed for denatured DNA.

3. Dependence of the Difference in Absorption of Left and Right Circular Polarized Light on Bound EB to Nucleotide Ratio

The data summarized in Table 15 and presented in Fig. 33 indicate the existence of differences between the CD of the EB-denatured DNA complex and that of the EB-native DNA complex. The dependence of the difference in the

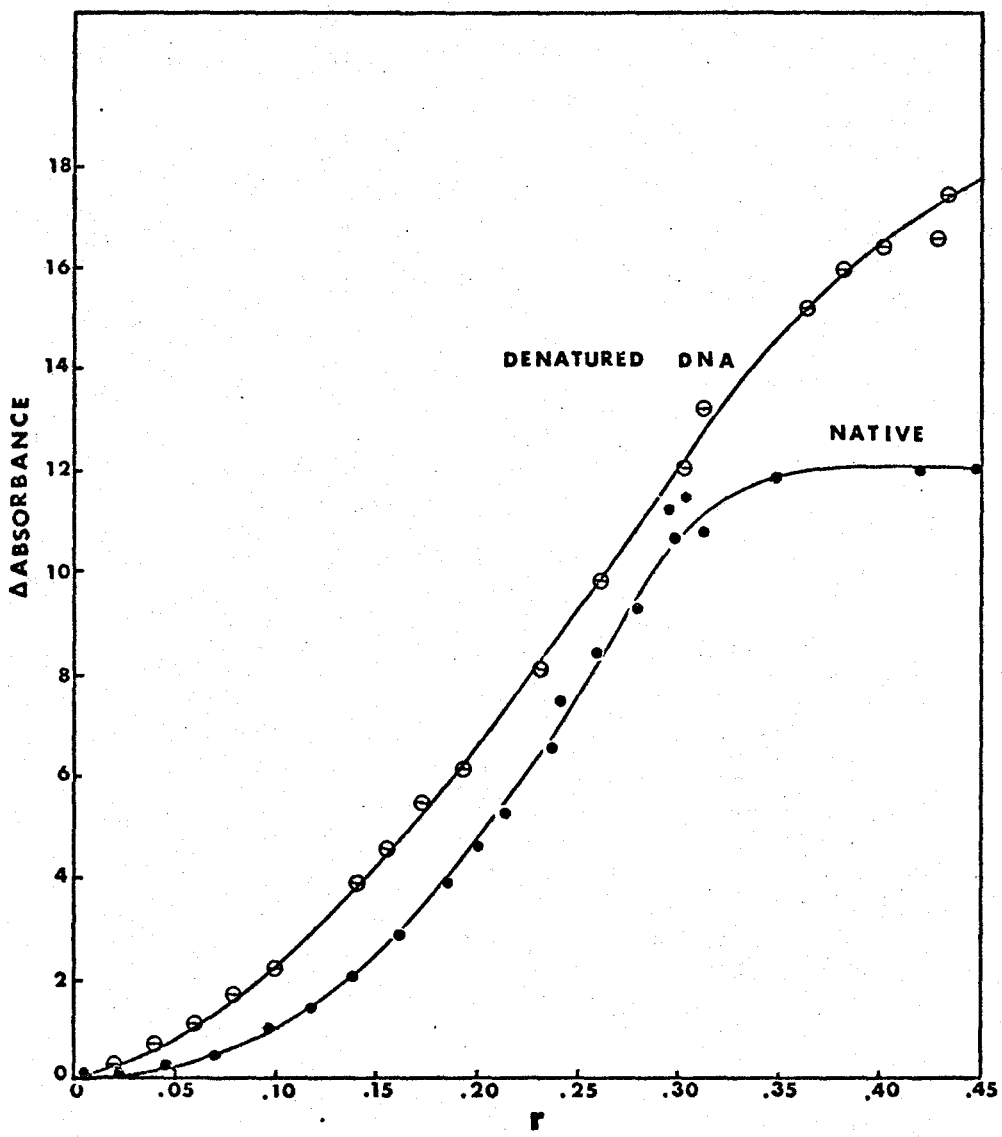


Fig. 33. Dependence of Δ Absorbance at 307 nm on the ratio of bound EB to DNA phosphate for EB-denatured DNA. [(DNA) = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.]

TABLE 15

Dependence of Δ Absorbance at 307 nm on Bound Dye to Nucleotide Ratios for Ethidium Bromide-Denatured DNA

r bound	Δ Absorbance 307 nm
0.020	0.28
0.040	0.64
0.060	1.07
0.080	1.63
0.100	2.11
0.120	2.91
0.140	3.76
0.156	4.43
0.173	5.40
0.193	6.03
0.238	8.06
0.261	9.79
0.303	11.98
0.325	13.18
0.364	15.12
0.383	15.88
0.406	16.32
0.431	16.48
0.436	17.30

In Tris-HCl (0.04 M) buffer, pH 7.9.

absorption of left and right circular polarized light, at 307 nm, ΔA, is plotted as a function of r for EB-DNA and EB-denatured DNA complexes. As previously mentioned, ΔA for the EB-native DNA complex increases from zero at an r of 0.005 to a constant maximum value of 11.8 at an r of 0.30.

The situation is somewhat different in the case of the EB-denatured DNA complex. The values of ΔA are higher than those for the EB-"native" DNA complex at every value of r and throughout the range of r examined reaching a value of 17.3 at an r of 0.44. The failure of ΔA to attain a constant value corresponding to the saturation of primary binding sites may be partially caused by the overlapping of the CD bands at 290 and 307 nm. The behavior of the Δ absorbance and molar circular dichroism (Fig. 32) at r greater than 0.30 may be explained on a similar basis.

Thus, the spectra of EB complexes with native and denatured DNA between 300 and 360 nm are similar with regard to shape and location of the CD bands. However, differences in the dependence of the molar circular dichroism and ΔA on r indicate a different behavior of ethidium toward native and denatured DNA.

I. TEMPERATURE-ABSORBANCE PROFILES OF ETHIDIUM BROMIDE-DNA COMPLEXES AT 260 nm

The temperature-absorbance profiles at 260 nm (Tm profiles) of DNA to which increasing amounts of EB have been

added are shown in Fig. 34. At a ratio of added EB per phosphate of 0.40 the temperature at which the initial rise in absorbance occurs (Ti) increases from 64° for DNA, to about 91° for EB-DNA.

Complete temperature absorbance transitions are not realized at any of the EB/P ratios examined with the exception of the 0.05 ratio for which the maximum attained hyperchromicity is 38.5%. This value is comparable to the 39.8% hyperchromicity obtained with DNA under the same conditions. Maximum hyperchromicity at 96° C decreases from 39.8% for DNA to about 14.0% for EB/P at 0.40. At EB/P of 1.0 and 1.5 a transition is not observed. These results are in agreement with previous observations (6,56) indicating that EB stabilizes DNA toward thermal denaturation.

The two-step temperature-absorbance profile noted for the 0.05 ratio suggests that DNA denaturation occurs first in regions free from EB. The temperature at which the initial rise in absorbance occurs (64°) is the same as for DNA itself. A second step of the Tm profile may be distinguished at temperatures above 80° C. At these temperatures and under similar conditions DNA undergoes nearly a complete transition. This indicates that ethidium stabilizes DNA against thermal denaturation up to temperatures at which free DNA would have been completely denatured.

The circular dichroism results (Chapter III, E2) may suggest that EB interaction with DNA occurs in a manner

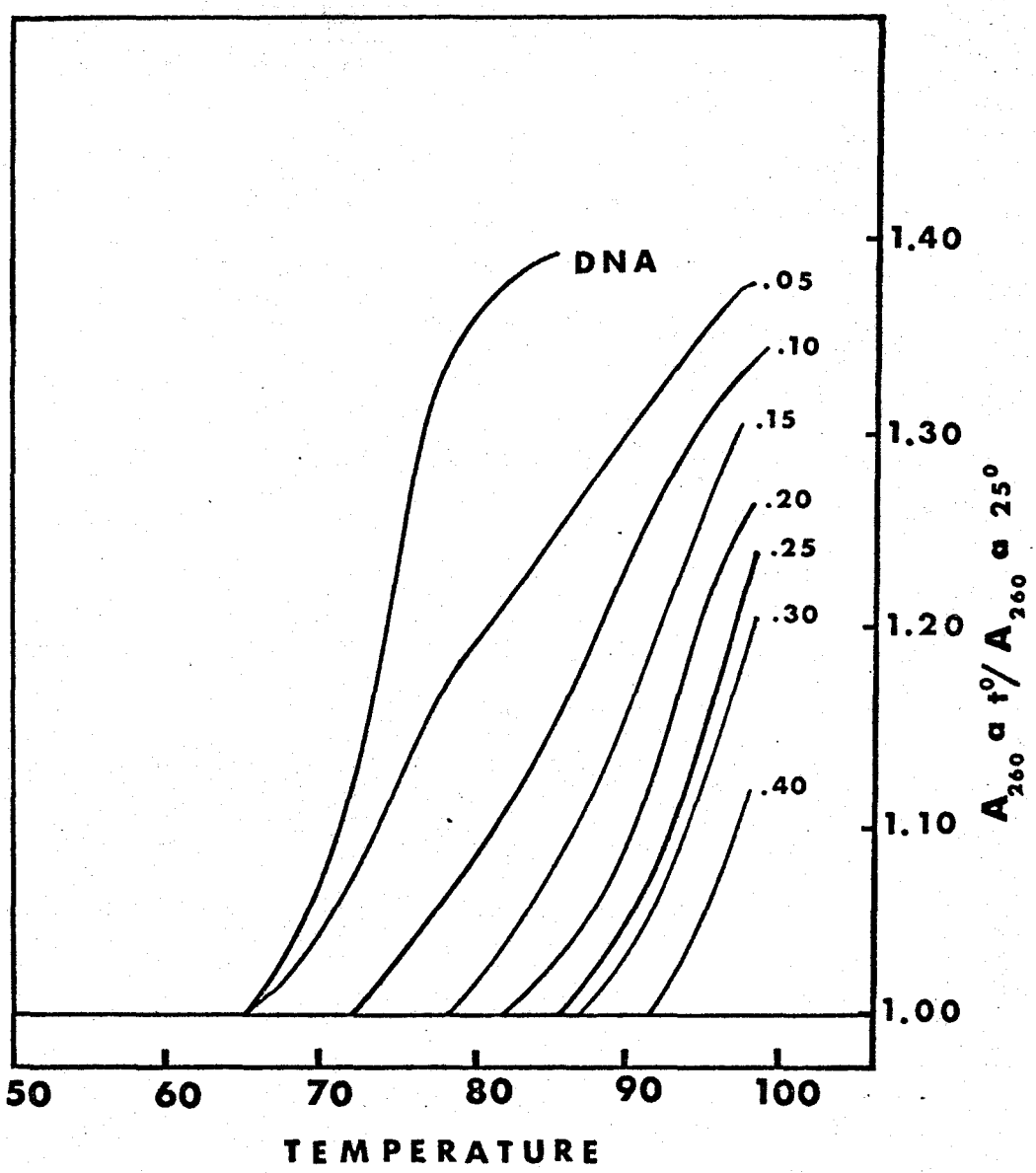


Fig. 34. Temperature-absorbance profiles at 260 nm of EB-DNA at several ratios of added EB to DNA phosphate. ([DNA] = 0.60 μM/ml in 0104 M Tris-HCl, pH 7.9. Path length = 0.20 mm.)

which favors the intercalation of dye molecules between adjacent base pairs rather than equal distribution throughout the DNA helix. The observed two-step temperature-absorbance profile at an EB/P ratio equal to 0.05 is consistent with this view. The one step profile noted at higher EB/P ratios indicate that the concentration of bound EB is sufficient for stabilization of the entire DNA helix. The temperature-absorbance profiles at EB/P ratios above 0.05 indicate that at these ratios the complex does not reach the midpoint transition until the temperature exceeds that at which the inflection occurs for the 0.05 ratio (80°). The correlation between the temperature at which this inflection occurs and the Ti at the 0.10 ratio suggests that this temperature corresponds to the temperature at which dissociation of the EB-DNA complex occurs.

J. TEMPERATURE EFFECTS ON THE CIRCULAR DICHROISM OF THE EB-DNA COMPLEX

1. Effect on CD Spectra from 300 to 360 nm

The effect of elevated temperature on the circular dichroism of the ethidium bromide-DNA complex at an added EB/P of 0.30 is shown in Fig. 35. At 25° a CD maximum is noted at 307 and a shoulder at 335 nm. The E_L-E_R, on the basis of bound EB, at 307 nm is 19.3. A temperature increase to 93.5° results in a decrease in the observed ΔA over the entire spectrum. However, the molar circular dichroism at 307 nm calculated on the basis of bound EB is decreased only to 16.0.

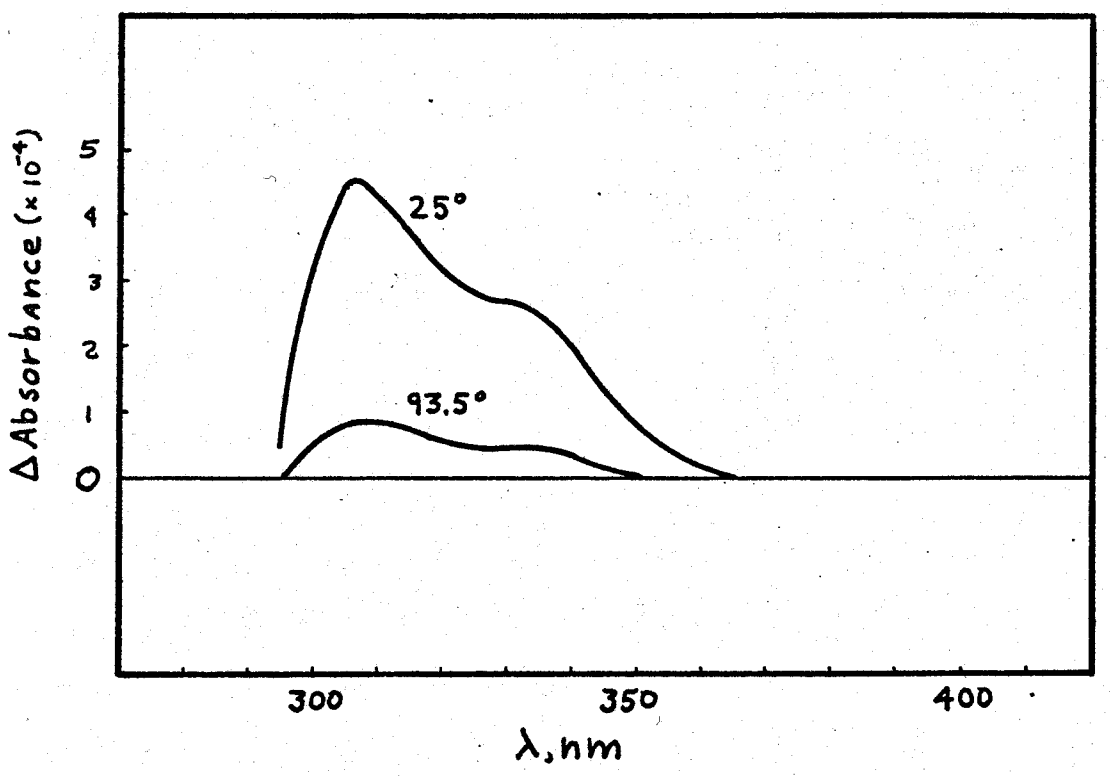


Fig. 35. Effect of temperature on the circular dichroism of EB-DNA at an added EB to phosphate ratio of 0.30. ([DNA] = 0.20 μM/ml in Tris-HCl (0.04 M), pH 7.9.) The circular dichroism is expressed in terms of Δ absorbance rather than E_L-E_R because the concentration of bound EB is different for each spectrum.

Somewhat different results are obtained at lower EB/P ratios of 0.10. At 25° E_L-E_R at 307 nm is 5.0 (Fig. 36). An increase in temperature to 96° results in enhancement rather than decrease of the circular dichroism throughout the spectrum and E_L-E_R is increased to 28.5 at 307 nm.

2. Effect on CD Spectra from 400 to 600 nm

The effect of elevated temperatures on circular dichroism in the spectral region from 400-600 nm is shown in Fig. 37. At 25° (a) maxima are exhibited at 500 and 540 nm with E_L-E_R of -0.36 and +0.30 respectively. An increase in temperature to 97° results in a considerable decrease of the magnitude of the negative circular dichroism. At this temperature the spectrum resembles that of free EB (Fig. 22c). The presence of residual induced CD at elevated temperature, observed in both the 300-360 nm and 450-540 nm regions, indicates that EB continued to interact with DNA even at temperatures at which DNA strands are at least partially dissociated (Fig. 34).

K. INFLUENCE OF TEMPERATURE ON THE MOLAR CIRCULAR DICHROISM OF THE ETHIDIUM BROMIDE-DNA COMPLEX AT 307 nm

The results discussed in the following section indicate that, at high temperatures, EB bound to DNA may dissociate from the complex. Prior to the onset of strand dissociation (Ti) E_L-E_R values at 307 nm either remain constant (EB/P = 0.05 - 0.10), decrease (0.15 - 0.40) or increase (1.00 - 1.50) depending on the particular EB/P ratio involved.

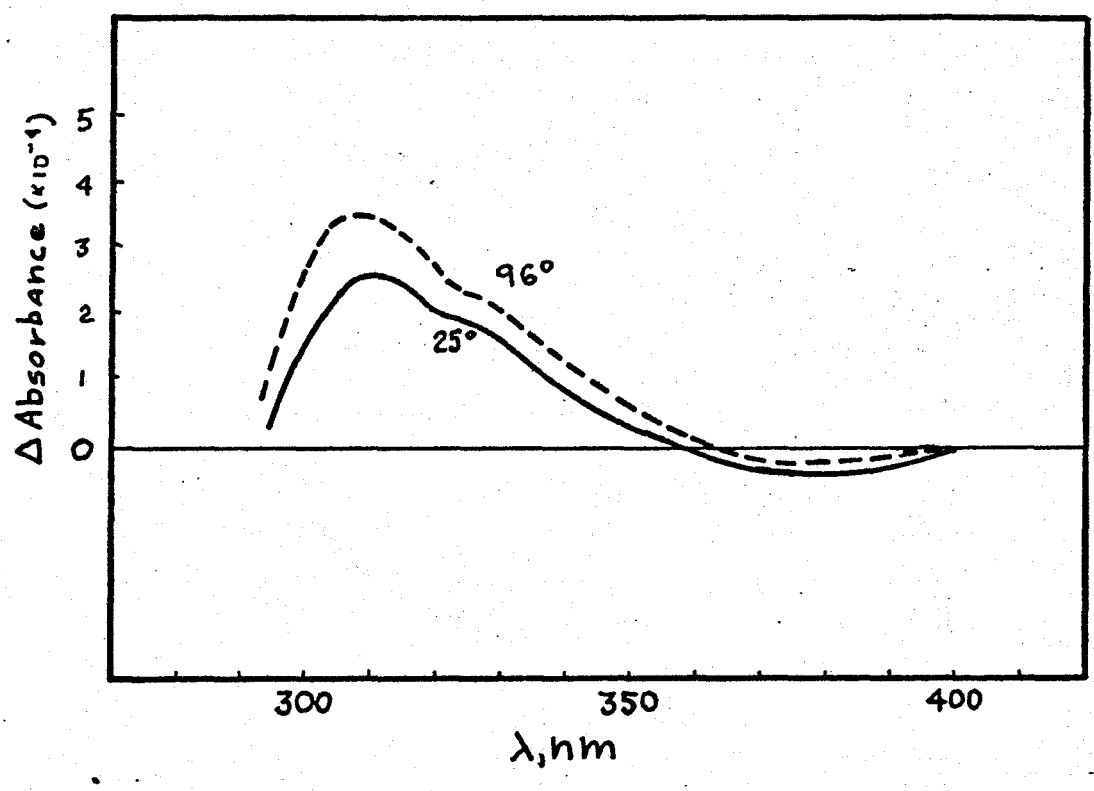


Fig. 36. Effect of temperature on the circular dichroism of EB-DNA at an added EB to phosphate ratio of 0.10. ([DNA] = 0.60 μM/ml in Tris-HCl (0.04 M), pH 7.9). The circular dichroism is expressed in terms of Δ absorbance rather than E_L-E_R because the concentration of bound EB is different for each spectrum.

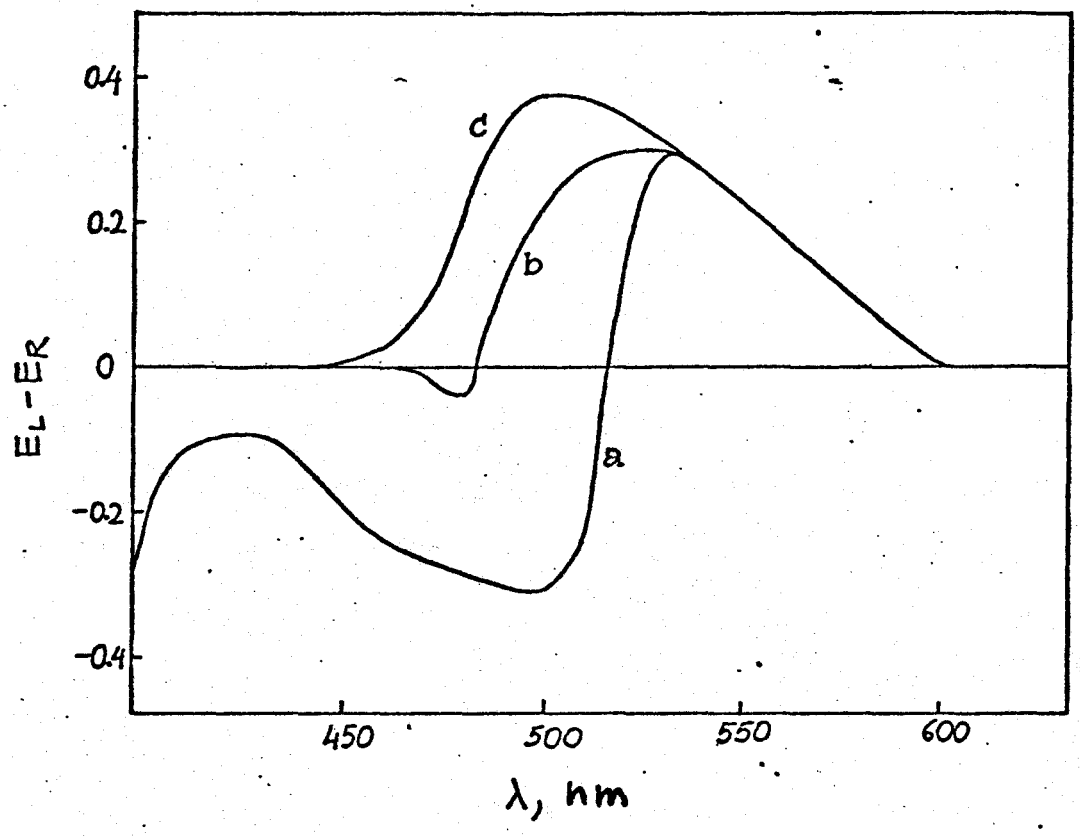


Fig. 37. Circular Dichroism of EB-DNA in 0.04 M Tris-HCl, pH 7.9 obtained in a 10 cm cell (a) EB-DNA (DNA = 0.60 μM/ml, EB = 0.09 μM/ml) at 25°C (b) Same as in a at 97°C (c) EB (0.09 μM/ml).

Within the temperature regions at which strand separation is occurring, E_L-E_R rises sharply, for every ratio, to a maximum value which subsequently decreases as higher temperatures are attained.

1. Circular Dichroism at 307 nm Preceding Strand Dissociation

(a) Low Ethidium Bromide - DNA Ratios

As indicated in Chapter III, A3 (Fig. 23) at low EB/P ratios (0.05-0.10) and at a DNA concentration of 0.20 μM/ml all added EB is bound to DNA. At these ratios only primary binding sites are occupied (111). At an added EB/P of 0.05 (Fig. 38) r is not affected by increasing temperatures up to 77°C and E_L-E_R also remains constant at 2.0 until the temperature exceeds this limit. Similar results are obtained at EB/P = 0.10 (Fig. 39). The molar circular dichroism remains constant at 4.8 for temperatures up to 74° and r does not change until the temperature exceeds 65°.

The constancy in r values indicates that at these low added EB/P ratios, the equilibrium between free and bound EB strongly favors the formation of the EB-DNA complex even at elevated temperatures.

(b) Intermediate Ethidium Bromide - DNA Ratios

A different behavior for r and E_L-E_R is observed at intermediate ratios of added EB between 0.15 and 0.40. At these ratios the increase in temperature results in partial

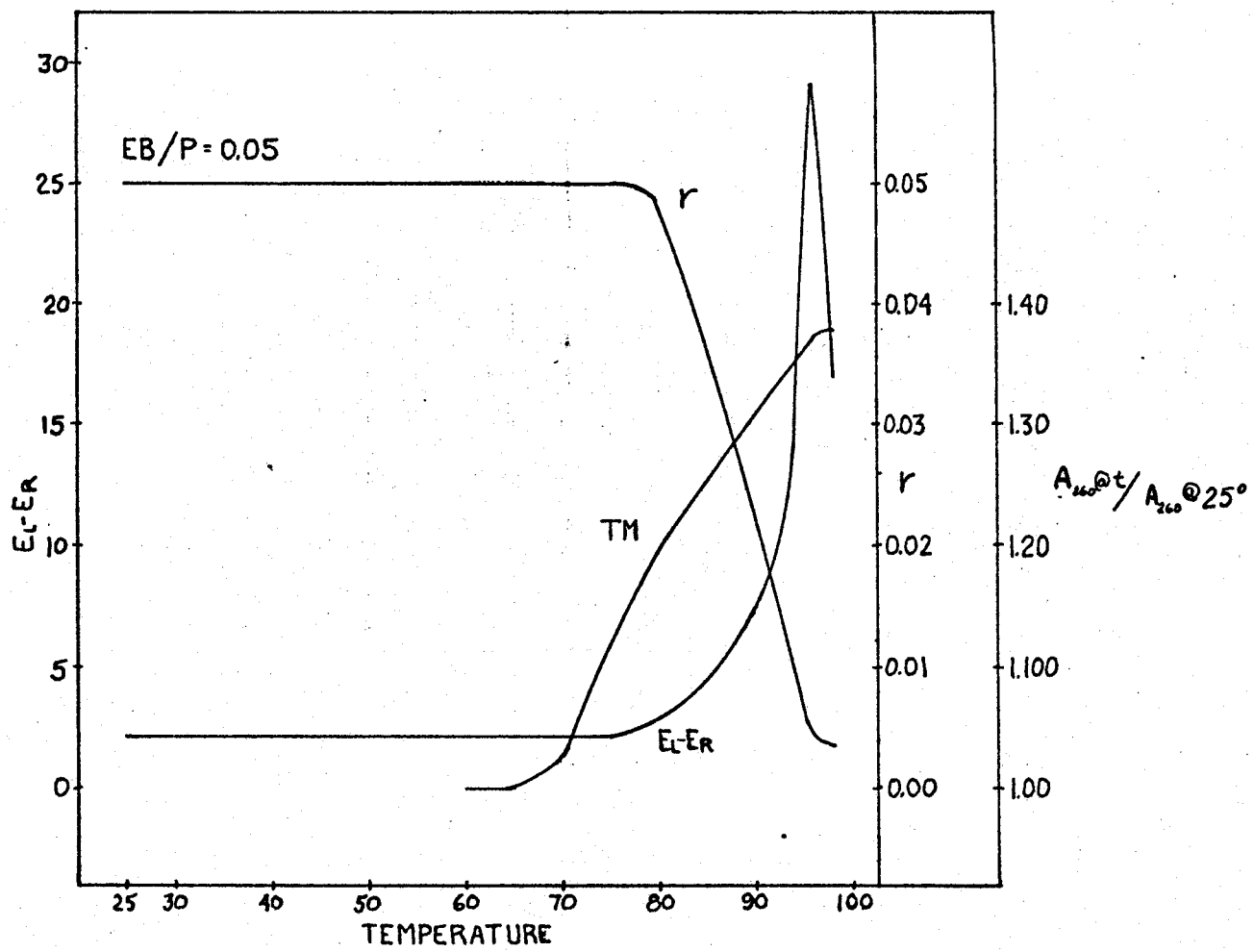


Fig. 38. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. Also shown is the temperature-absorbance profile at 260 nm. [DNA] = 0.60 μM/ml, [EB] = 0.03 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio equal to 0.05.

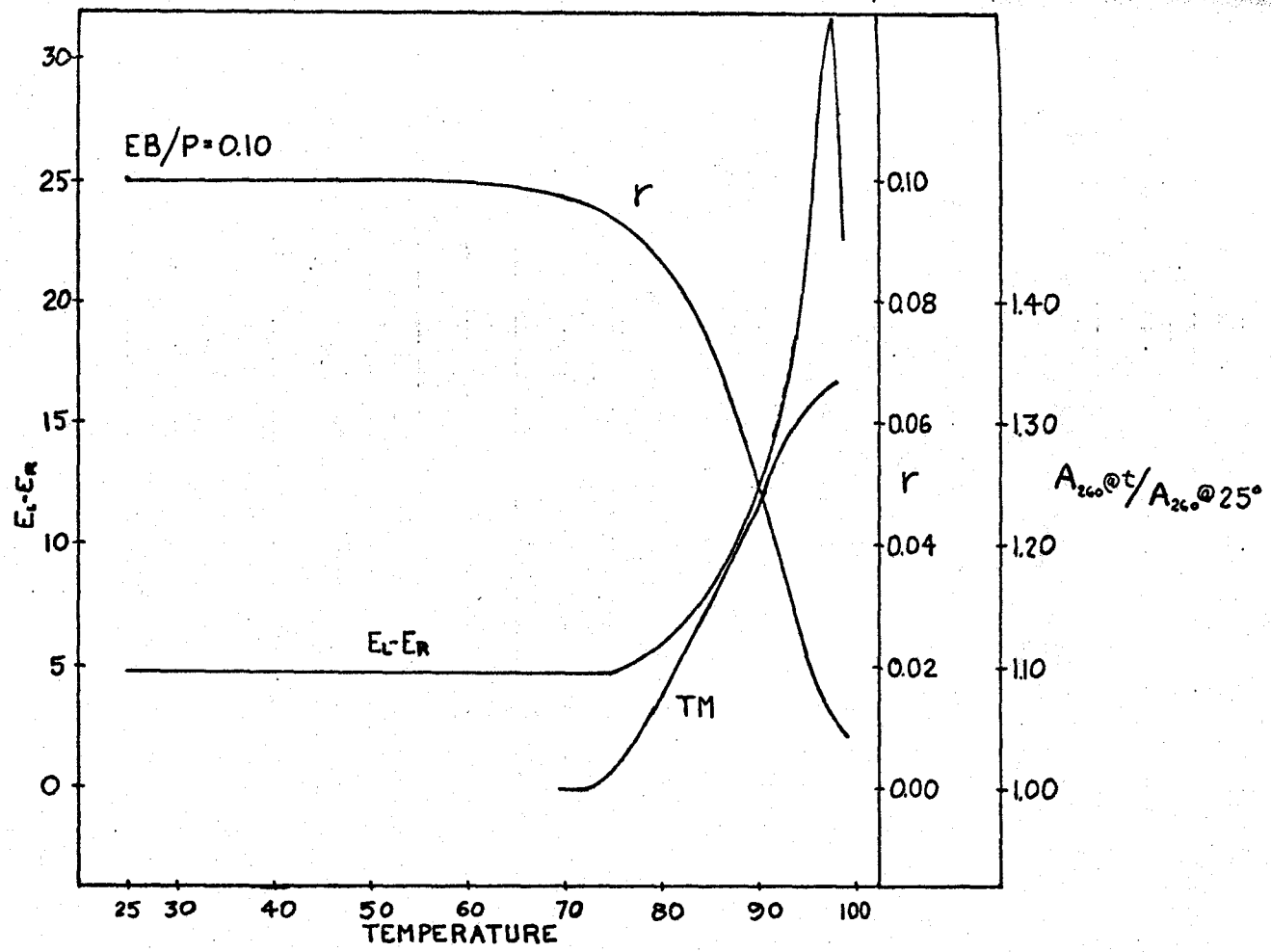


Fig. 39. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. Also shown is the temperature-absorbance profile at 260 nm. [DNA] = 0.60 μM/ml, [EB] = 0.06 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio equal to 0.10

dissociation of the EB-DNA complex. The effect of the resulting decrease in r on E_L-E_R depends on the specific EB/P ratio involved. At an added EB/P of 0.30, r is 0.26 at 25° and E_L-E_R is 20.5. An increase in temperature to 87° results in decreases in both r and E_L-E_R to 0.19 and 14.0 respectively.

Similar behavior is also observed at increased temperatures for EB/P ratios in the 0.15-0.40 range (Figs. 40-44). For each of these ratios, increased temperatures result in decreases in both the binding of EB to DNA and the molar circular dichroism up to temperatures at which the temperature-absorbance profile indicates the beginning of DNA strand separation.

(c) High Ethidium Bromide - DNA Ratios

A somewhat different behavior is observed at the higher added EB/P ratios of 1.00 and 1.50 (Figs. 45 and 46). The initial r values for these ratios are 0.40 and 0.52 respectively. Elevated temperatures up to 96° do not cause any increase in the absorbance at 260 nm. Changes occur, however, in both r and E_L-E_R. At EB/P of 1.00 a change in temperature from 25° to 96° results in a decrease in r from 0.40 to 0.27. For EB/P of 1.50 r is decreased over the same temperature range from 0.52 to 0.23.

This decrease in r parallels that observed at the lower EB/P ratios. However, in contrast to the results obtained for EB/P ratios between 0.15 and 0.40, the temperature promoted decrease in the amount of bound EB at these

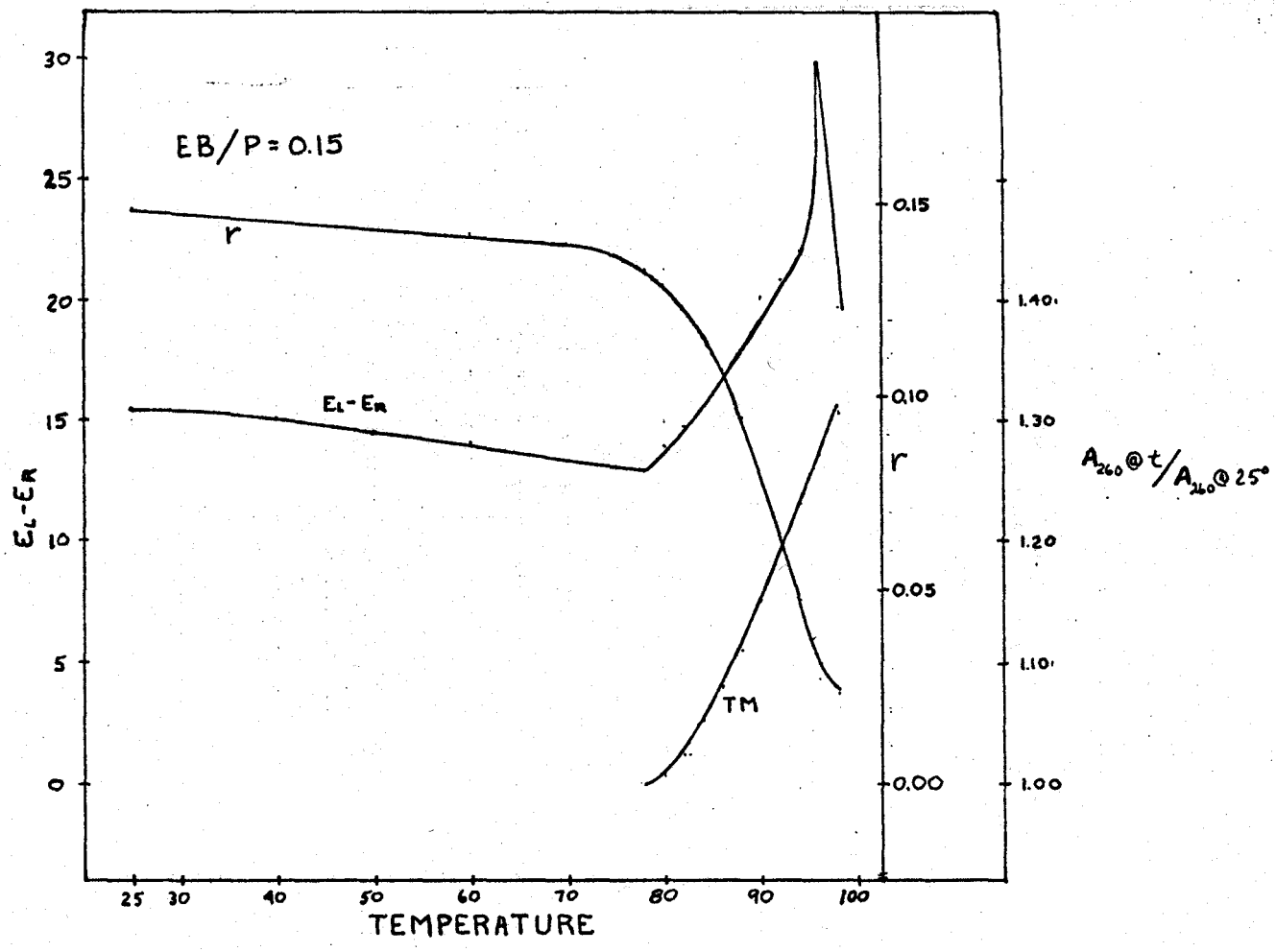


Fig. 40. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. Also shown is the temperature-absorbance profile at 260 nm. (DNA) = 0.60 μM/ml, (EB) = 0.09 μM/ml in 0.04 M Tris HCl, pH 7.9. Added EB/P ratio equal to 0.15.

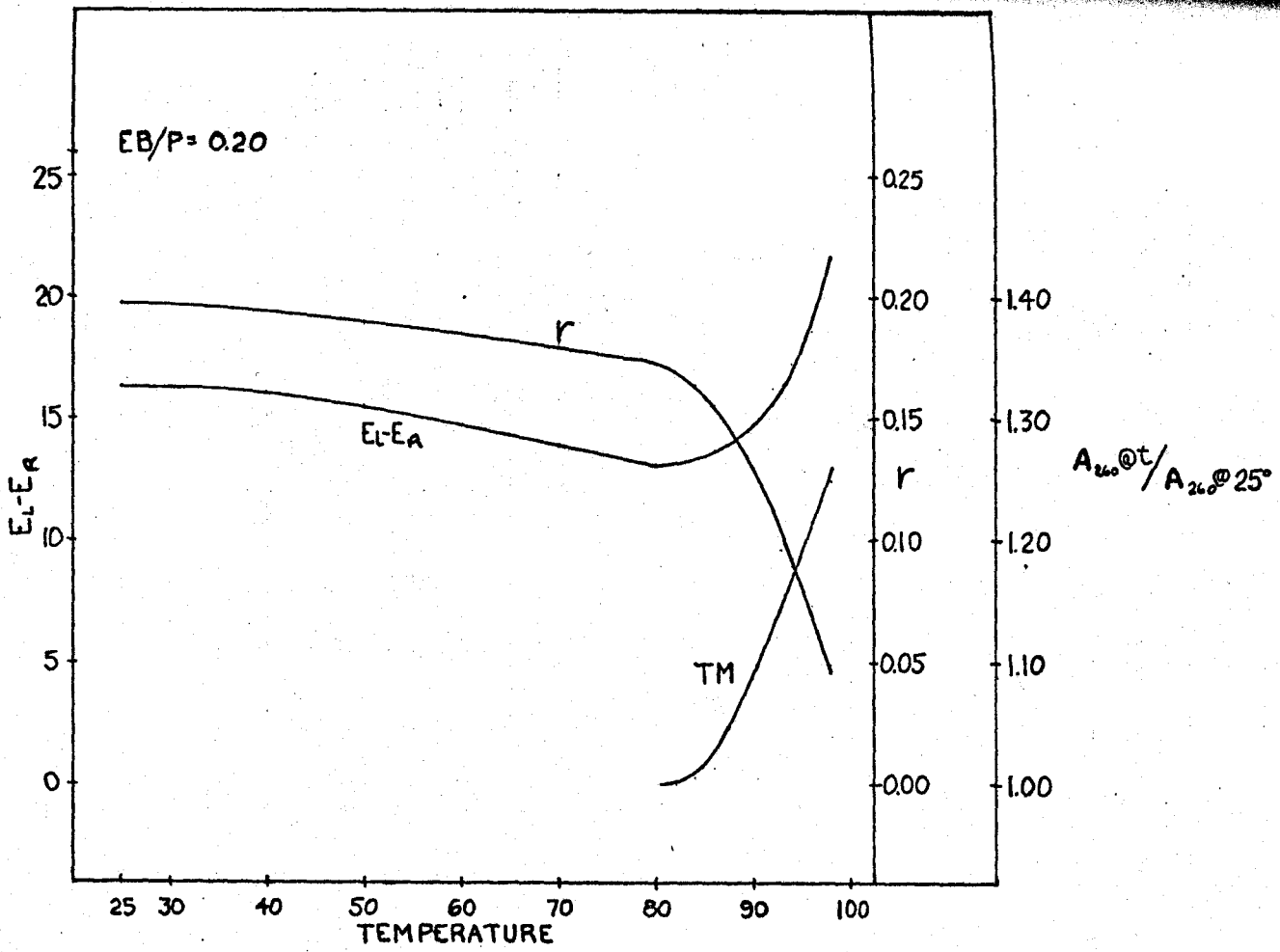


Fig. 41. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. Also shown is the temperature-absorbance profile at 260 nm. [DNA] = 0.60 μM/ml, [EB] = 0.12 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio equal to 0.20.

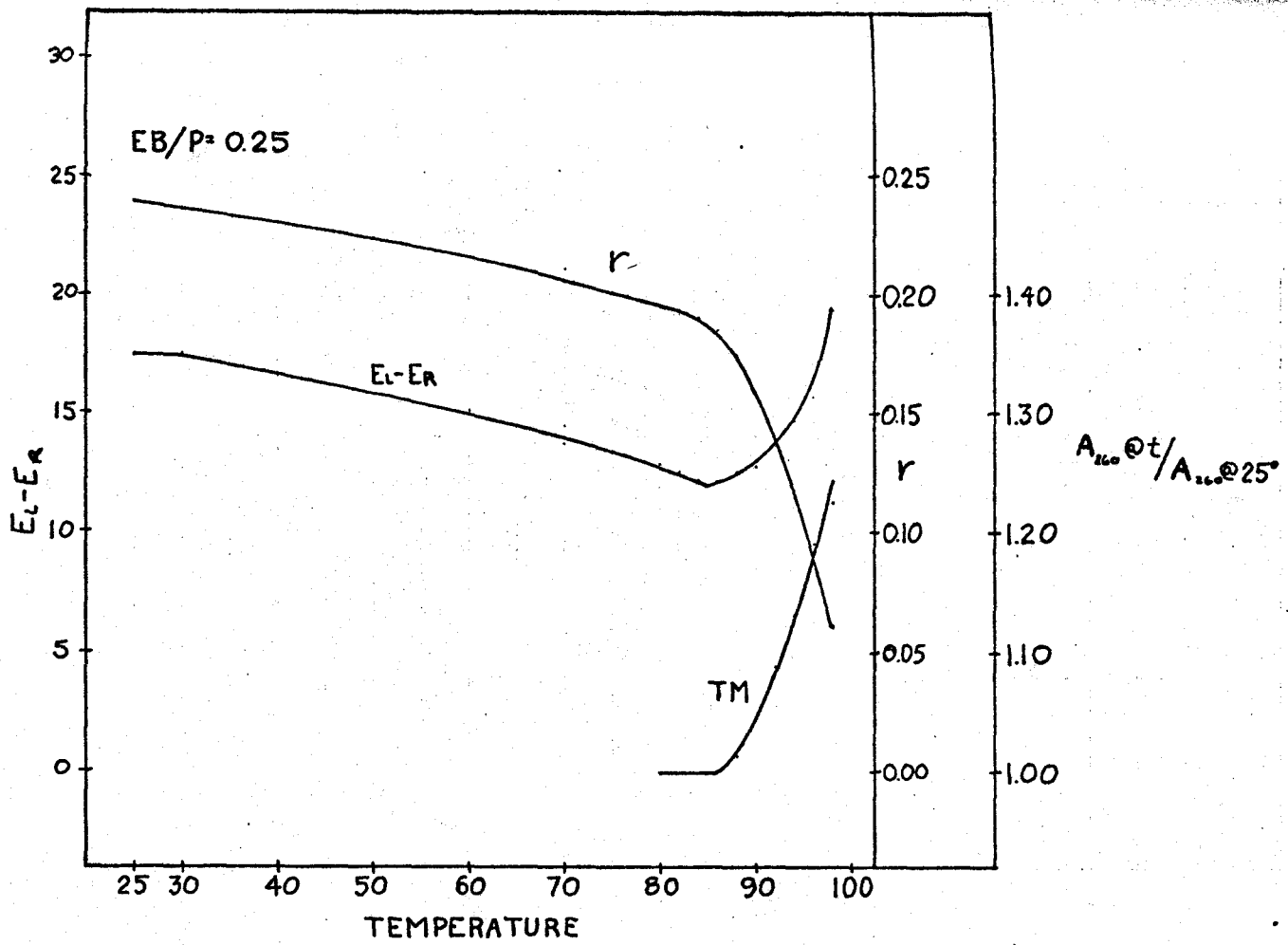


Fig. 42. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. Also shown is the temperature-absorbance profile at 260 nm. [DNA] = 0.60 μM/ml, [EB] = 0.15 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio equal to 0.25.

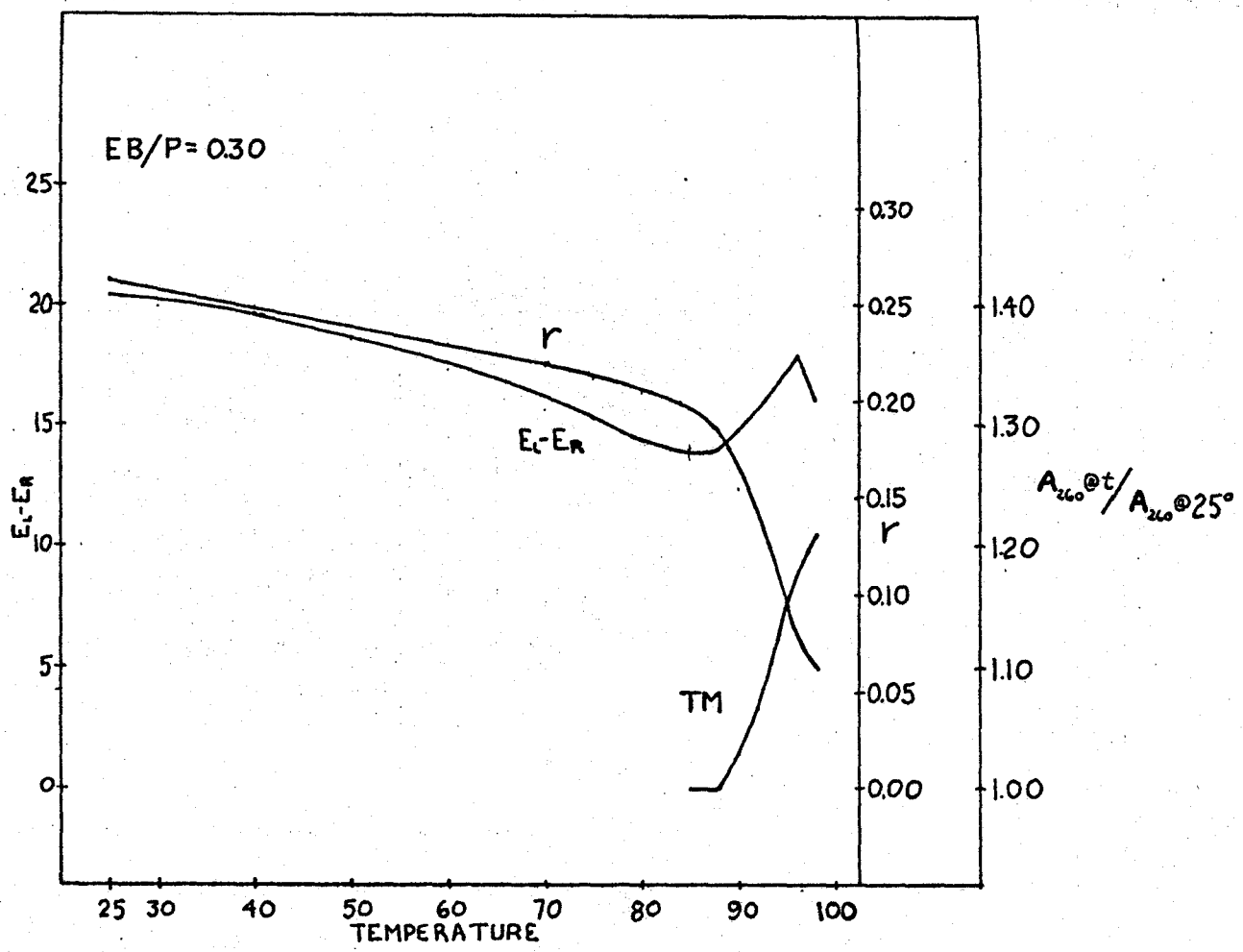


Fig. 43. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. Also shown is the temperature-absorbance profile at 260 nm. [DNA] = 0.60 μM/ml, [EB] = 0.18 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio equal to 0.30.

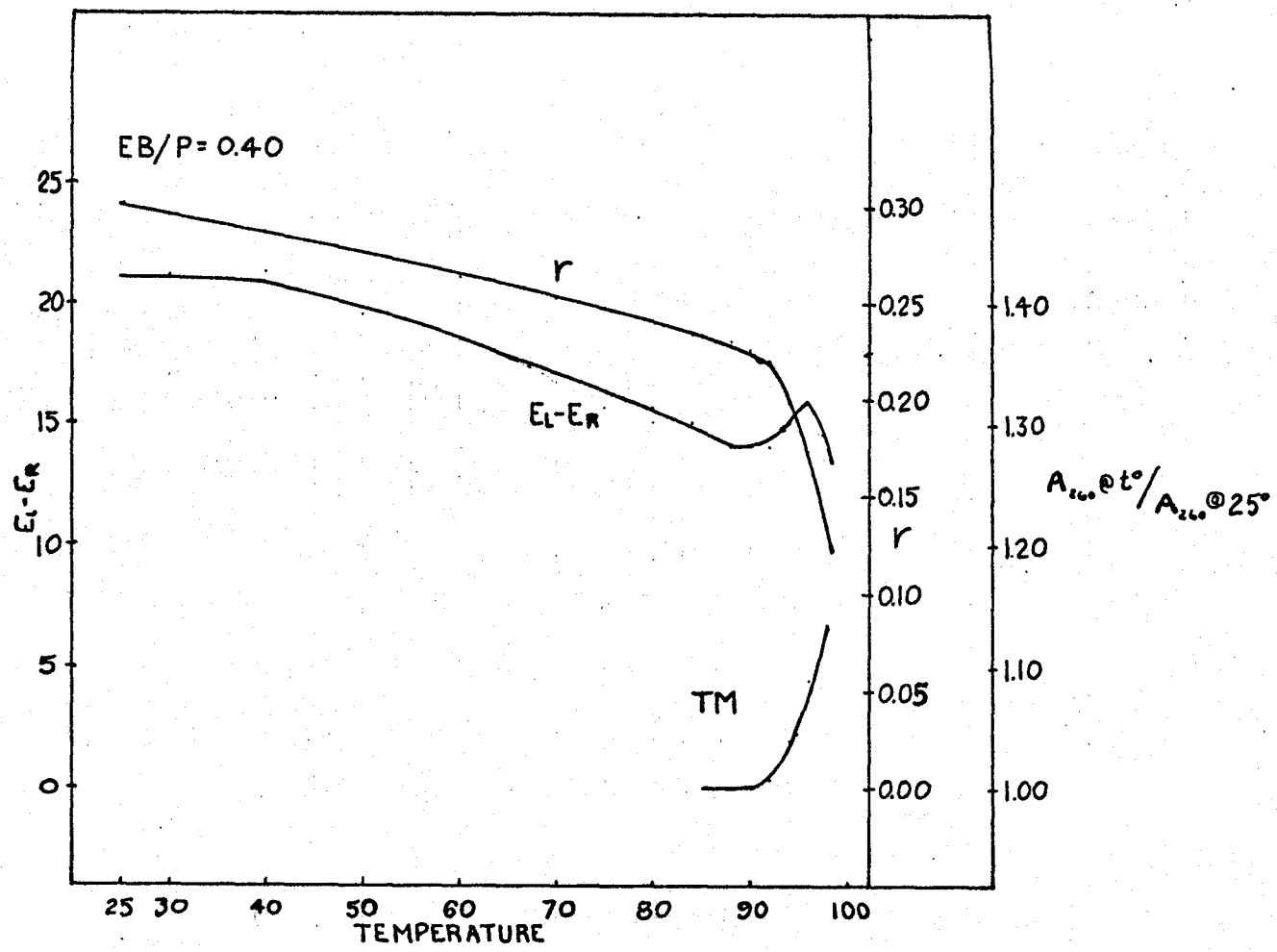


Fig. 44. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. Also shown is the temperature-absorbance profile at 260 nm. [DNA] = 0.60 μM/ml, [EB] = 0.24 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio equal to 0.40.

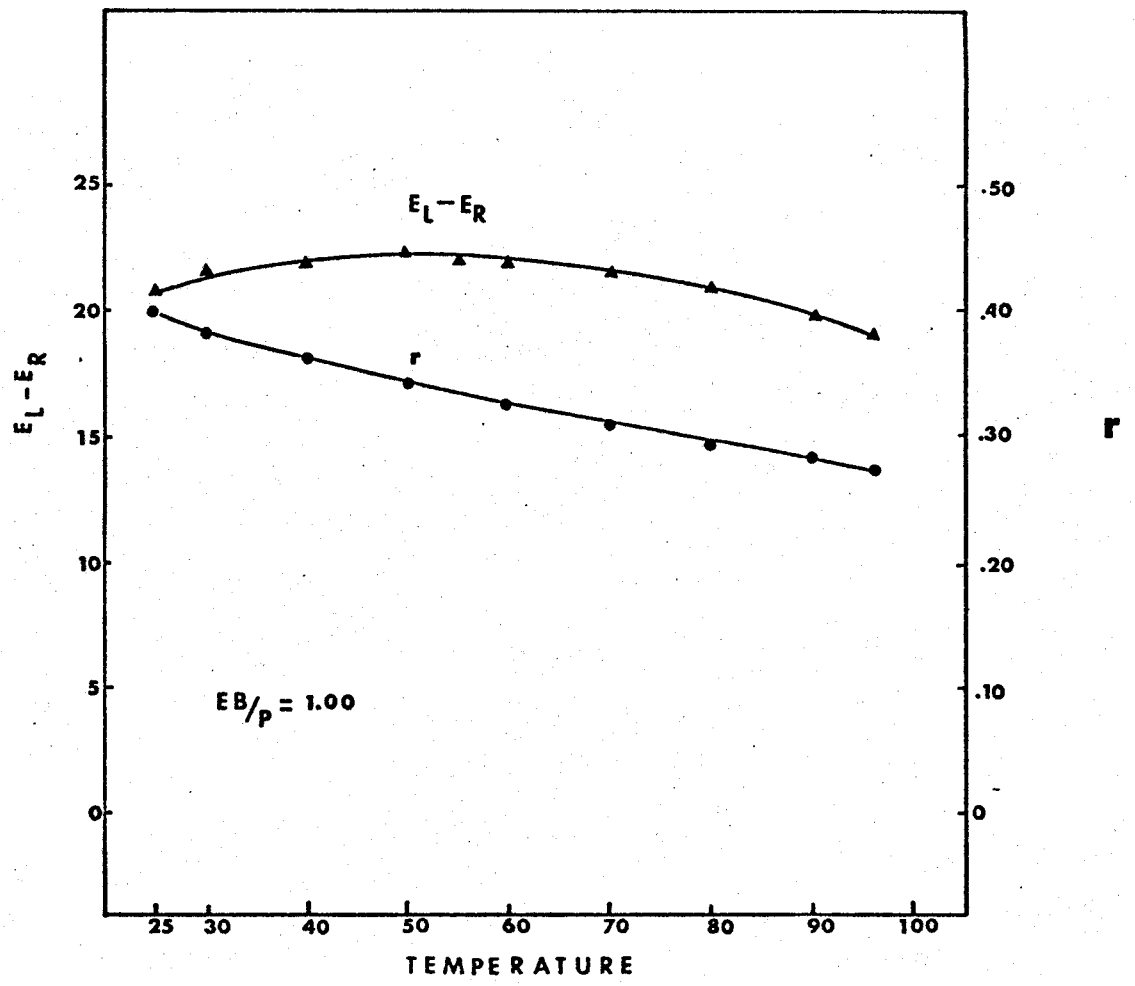


Fig. 45. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. ([DNA] = 0.60 μM/ml, [EB] = 0.60 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio equal to 1.00.)

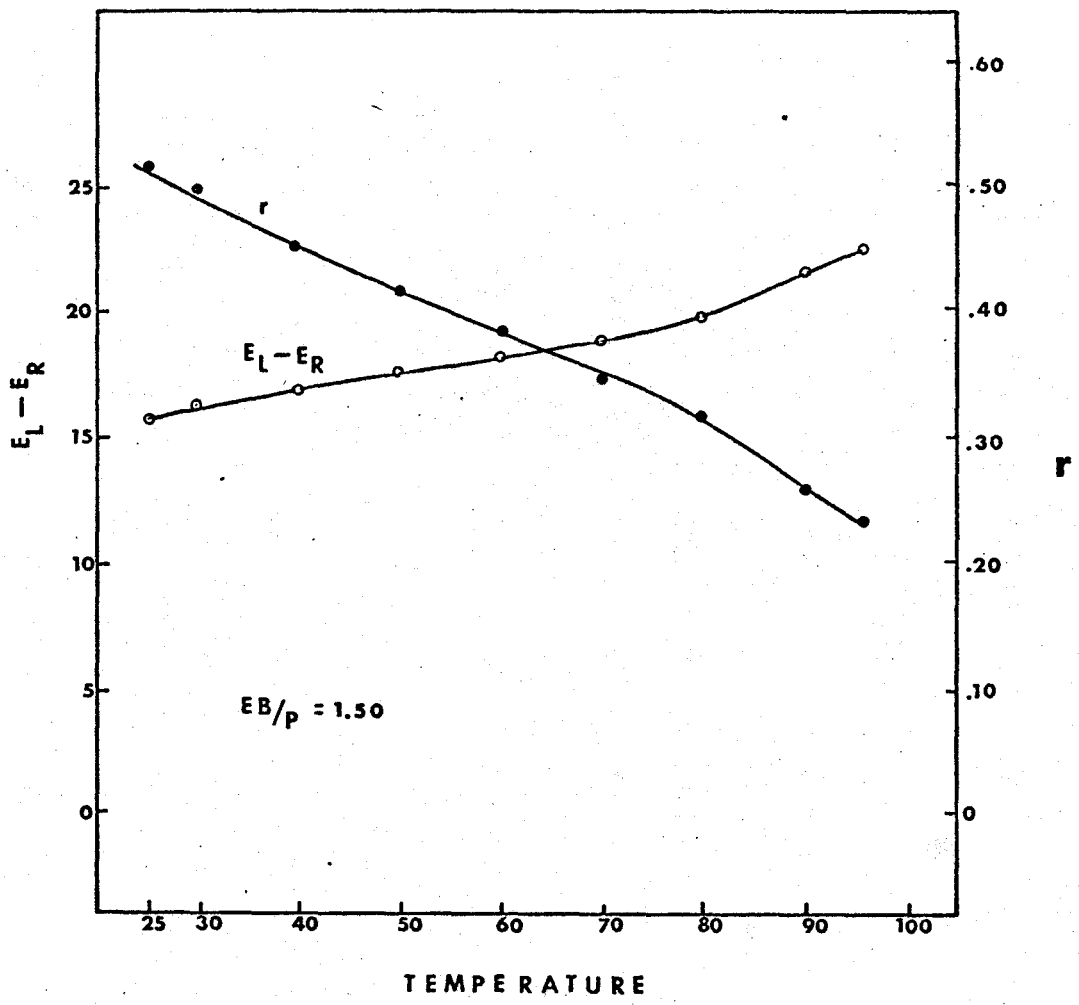


Fig. 46. Dependence of molar circular dichroism at 307 nm and bound EB ratio on temperature. ([DNA] = 0.60 μM/ml, [EB] = 0.90 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio equal to 1.50.)

higher ratios is accompanied by an increase in E_L-E_R. For an EB/P of 1.00 (Fig. 45), E_L-E_R is 20.5 at 25°C and increases to 22.0 at 50°C. At temperatures exceeding 50° E_L-E_R subsequently decreases to 19.0 at 96°. For an added EB/P ratio of 1.50 (Fig. 46), E_L-E_R at 25° is 15.8 and increases continuously to a final value 22.4 at 96°.

Some explanation for the difference in the behavior of E_L-E_R at 307 nm for the various EB/P ratios may be offered if we assume that the band responsible for the circular dichroism at 307 nm originates from nearest-neighbor interactions between two or more EB molecules bound to adjacent primary binding sites. The appearance of the E_L-E_R maximum at an r of 0.30 is consistent with the expectation that at this ratio the interaction between EB and DNA would result in saturation of all available primary binding sites. A more detailed explanation of these data is offered in the next section.

(d) <u>The Relationship Between Molar Circular Dichroism and r at Elevated Temperatures</u>

As previously indicated, at an EB/P of 0.05 and 0.10 neither EB binding nor E_L-E_R are affected until the temperature exceeds that at which DNA strand separation begins to occur (Ti). At r values between 0.15 and 0.30, however, elevated temperatures result in a decrease in both binding and E_L-E_R. For an EB/P of 0.30, for example, the initial r at 25° is 0.26 and E_L-E_R is 25.0. As the temperature

increases to 80° r decreases to 0.19 and E_L-E_R to 14.0. The E_L-E_R values of 21.0 and 14.0 agree well with values of 22.0 and 15.0 obtained at room temperature (Fig. 47). A similar correlation is also observed for EB/P ratios between 0.15 and 0.40 for which the initial r values at 25° are higher than 0.30.

As previously indicated, at high ratios (EB/P = 1.00 and 1.50) elevated temperatures cause a decrease in r values similar to that observed at the lower EB/P ratios. However, an apparent inconsistency arises at ratios above 0.30;a decrease in r brings about an increase in E_L-E_R.

A comparison between the dependence of the molar circular dichroism on r at room temperature and at continuously increasing temperatures is shown in Fig. 47. The magnitudes of E_L-E_R and r in curve B are obtained from Fig. 45 and are replotted as E_L-E_R versus r. Curve B represents values obtained over a temperature range from 25° to 98°C at total added EB/P ratio of 1.00 and an initial bound EB ratio of 0.40 at 25°C.

Comparison of these curves indicates that at EB/P ratios sufficient to prevent DNA strand separation up to 98°, elevated temperatures cause dissociation of the EB-DNA complex with concomitant effects on nearest-neighbor interactions. In curve B, the dependence of E_L-E_R on r is similar to that noted for the same range of r in curve A. Curves A and B exhibit a maximum E_L-E_R near the same r of about 0.30.

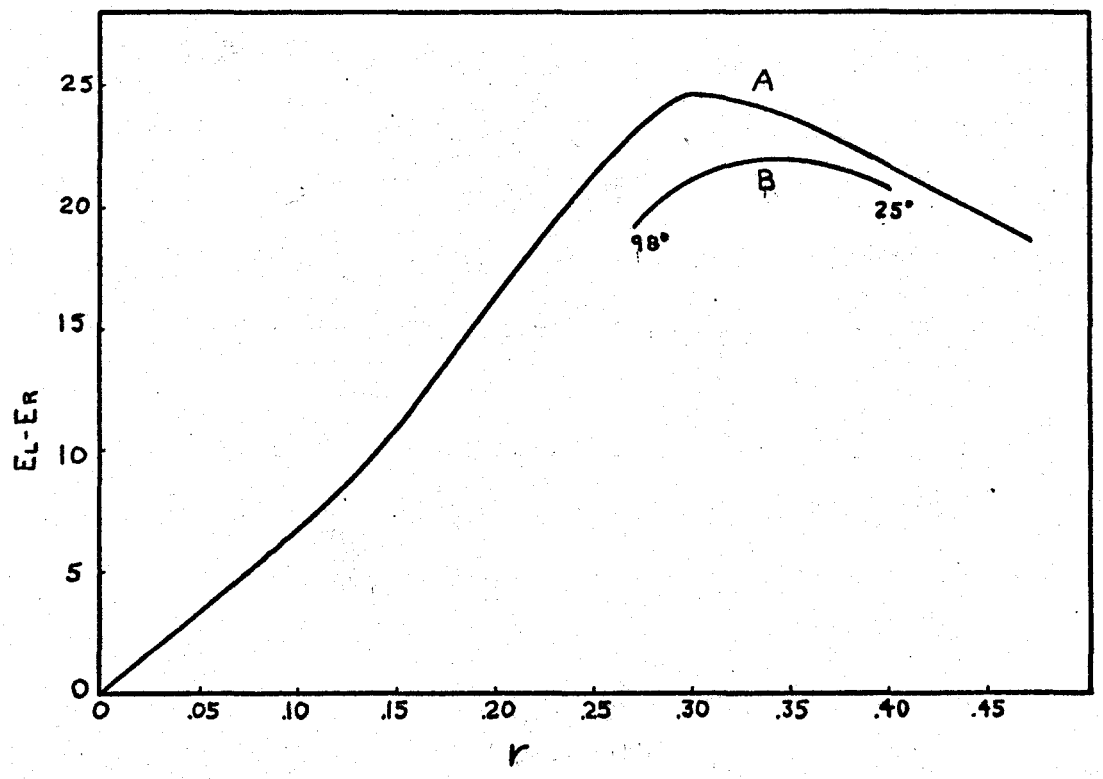


Fig. 47. Comparison of the dependence of molar circular dichroism at 307 nm on r obtained at two widely different experimental conditions; A, represents values obtained at room temperature and B, values obtained from Fig. 45 over temperatures from 25° to 98°. (See Chapter III, Kld for details.)

The molar circular dichroism decreases above and below this ratio. Ellipticities are higher in curve A than B, particularly at values of r below 0.35. These small differences however may result from the influence of elevated temperatures on the conformation of the EB-DNA complex.

It must be remembered that data used to construct curve B is obtained by determining E_L-E_R values for a single sample at continuously increasing temperatures from 25° to 98°. Since the temperature absorbance profile does not exhibit a transition at temperatures as high as 98°, strand dissociation has not occurred. Furthermore, a considerable portion of the EB is still complexed at 98° since r equals 0.26 at this temperature.

As noted previously, the induced CD at 307 nm may be the result of nearest-neighbor interactions between two or more EB molecules bound to adjacent primary sites (Chapter III, E2). This interaction may require that EB molecules are held firmly in adjacent sites in a specific geometric relation to each other. The lower E_L-E_R values noted for curve B may thus be the result of a temperature induced alteration in the relative geometry between mutually interacting DNA-bound EB molecules. Since E_L-E_R values at r below 0.32 in curve B are obtained at temperatures (Fig. 45) above the Ti (64°) for DNA, the occurrence of induced conformational changes is likely at these temperatures.

2. Circular Dichroism at 307 nm during Strand Separation

Ethidium bromide-DNA complexes at EB/P ratios between 0.05 and 0.40 exhibit sharp increases in E_L-E_R at 307 nm at temperatures exceeding their respective Ti's. This increase occurs at all EB/P ratios for which temperature-absorbance profiles indicate strand separation below 98°. At an r of 0.148 at 25°, for example, which decreases to 0.135 at a temperature equal to Ti (78°) for this complex (Fig. 40), E_L-E_R, over the same temperature range, decreases from 15.4 to 13.0. Once Ti is exceeded, r decreases rapidly reaching 0.025 at 98°. At the same time E_L-E_R rises sharply, reaching a peak value of 30.0 at 96°. Beyond this temperature, E_L-E_R decreases to 20.5 at 98°.

Similar abrupt changes occur in the binding and molar circular dichroism as the Ti for other complexes in the region of r from 0.05-0.40 is exceeded. The binding decreases rapidly and E_L-E_R undergoes a sharp increase up to a temperature of 96°, above which E_L-E_R begins again to decrease.

The temperature absorbance profile of DNA gives an indication of the temperature at which bases in the DNA helix are becoming unstacked. As base unstacking proceeds, strands are expected to begin separating (22). Since a base-paired double stranded structure is required for primary binding (111), the sharp decrease noted in r at

elevated temperatures appears to be the result of a temperature induced decrease in the number of primary binding sites.

The increase in E_L-E_R at temperatures exceeding Ti occurs for every EB/P ratio between 0.05 and 0.40 although the magnitude of the increase depends on the specific ratio. The temperature induced increase in E_L-E_R is greatest for the lower EB/P ratios. For example, at an added EB/P of 0.05 (Fig. 38), E_L-E_R increases from 2.0 at Ti to a maximum value of 30.0 at 96°; whereas at an added EB/P ratio of 0.30 (Fig. 43) E_L-E_R increases from 13.5 to 18.0 between Ti and 96°.

The magnitude of the increase in E_L-E_R at temperatures exceeding Ti may be a reflection of the degree of primary site saturation at each EB/P ratio. Since nearest-neighbor interactions are already nearly maximal at intermediate EB/P ratios, only small increases in E_L-E_R are expected for those ratios at temperatures exceeding Ti. However, at low EB/P ratios nearest-neighbor interactions responsible for the 307 nm circular dichroism are less extensive. Temperature induced increase in the relative amount of nearest-neighbor interaction, at these ratios, may be reflected in a comparatively large increase noted in the molar circular dichroism at 307 nm.

L. INFLUENCE OF TEMPERATURE ON THE MOLAR CIRCULAR DICHROISM AT 500 nm

The effect of temperature on the magnitude of the molar circular dichroism at 500 nm at an EB/P ratio of 0.15

is shown in Fig. 48. At 25°C, r is 0.148 (Fig. 40) and E_L-E_R is equal to -0.72. With increasing temperatures, the molar circular dichroism is slightly decreased to -0.60 at 80°C and at 95°C it is further decreased to -0.50.

Clearly, the dependence of the 307 nm and 500 nm molar circular dichroism on temperature at this EB/P ratio are distinctly different. The CD at 307 nm shows a slight decrease from 15.4 at 25° to 13.0 at 77° followed by a substantial increase to 30.0 as the temperature approaches 96°C. Thus, the temperature dependence of the 307 nm and 500 nm circular dichroism bands is similar to the dependence of the respective bands on r. A temperature induced decrease in EB binding has pronounced effects on the molar circular dichroism at 307 nm.

In contrast, the magnitude of the molar circular dichroism at 500 nm is independent of r. EB bound to either primary or secondary binding sites appears to contribute equally to the circular dichroism at 500 nm. The decrease in E_L-E_R, at 500 nm, from -0.72 to -0.50 between 25° and 95° may be the result of temperature effects on the geometry of the EB chromophore within the environment of the DNA polymer.

M. EFFECT OF pH ON THE MOLAR CIRCULAR DICHROISM OF THE ETHIDIUM BROMIDE-DNA COMPLEX AT 307 nm

1. The Dependence of the Molar Circular Dichroism on pH

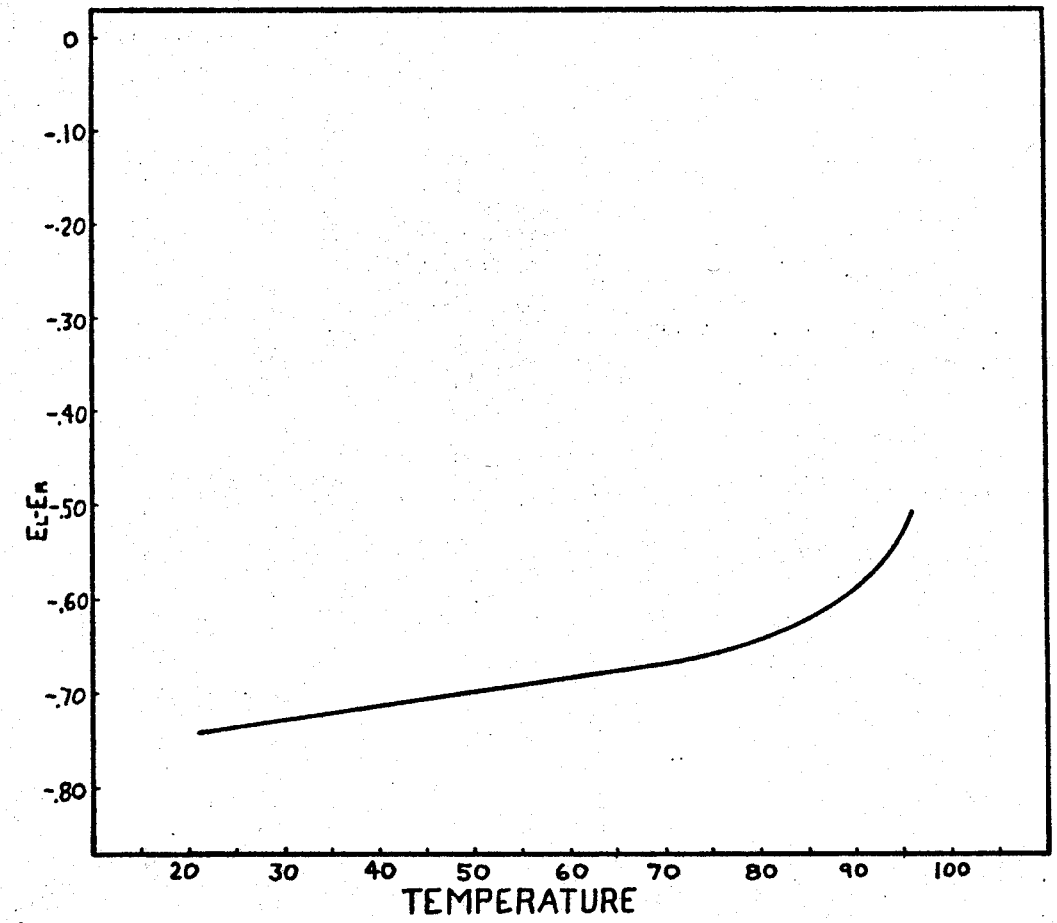


Fig. 48. Dependence of molar circular dichroism at 500 nm on temperature. [DNA] = 0.60 μM/ml, [EB] = 0.09 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB/P ratio 0.15.

The molar circular dichroism at 307 nm and r for an EB/P ratio of 0.15 are shown as functions of pH in Fig. 49, which is constructed from the data listed in Table 16. In the pH range between 5.0 and 10.5, the magnitudes of r and E_L-E_R decrease slightly with decreasing pH. At pH 5.0 and 10.5 r is 0.123 and 0.131 respectively and E_L-E_R is 10.0 and 11.0. At pH values above and below these limits r decreases rapidly, reaching zero at pH 3.0 and 12.0.

The dependence of E_L-E_R on pH below 5.0 is quite different from that observed above pH 10.5. Below pH 5.0 both r and E_L-E_R decrease rapidly in a roughly parallel manner. Between pH 11.3 and 11.7 E_L-E_R increases sharply to a maximum of nearly 26 at pH 11.7. Above this pH, E_L-E_R decreases, reaching zero near pH 12.0. In the pH range between 11.3 and 12.0 at which E_L-E_R reaches a maximum, r decreases from 0.136 to zero.

These results indicate that at decreasing pH between 11.0 and 5.0 there is a decrease in the ability of DNA to bind EB. A similar dependence of EB binding to DNA has been reported by LePecq _et al_. (56) who noted a 20 percent decrease in the fluorescence intensity of EB-DNA complexes with decreasing pH between 11.0 and 5.0.

Interpretation of these results requires an understanding of DNA secondary structure at these pH values. Spectral (123) and other physical methods (101) have shown

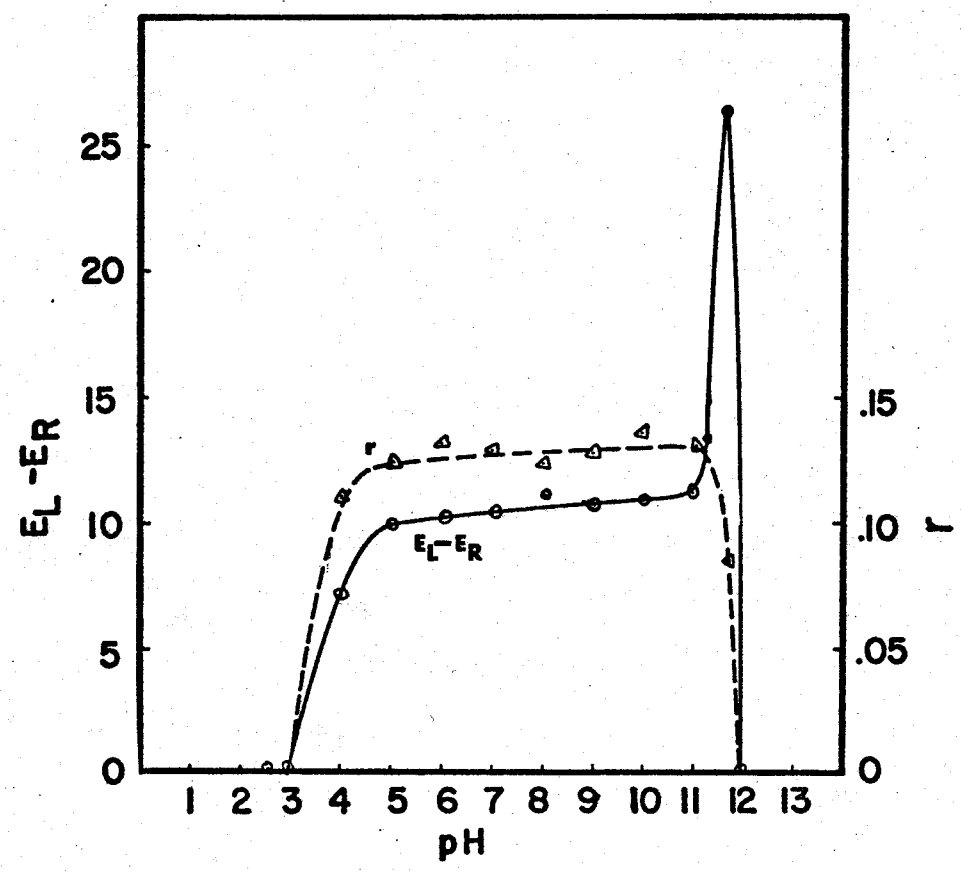


Fig. 49. Relationship between molar circular dichroism at 307 nm and EB binding on pH. [DNA] = 0.20 μM/ml, [EB] = 0.03 μM/ml. Added EB/P ratio = 0.15.

TABLE 16

Dependence of EB Binding and Molar Circular Dichroism on pH

pH	Bound EB per DNA Nucleotide	Molar Circular Dichroism 307 nm
2.5	0.000	0.00
3.0	0.000	0.00
4.0	0.110	7.15
5.0	0.123	9.94
6.0	0.134	10.03
7.0	0.129	10.42
8.0	0.123	11.08
9.0	0.128	10.64
10.0	0.139	10.90
11.0	0.133	11.20
11.3	0.136	12.18
11.7	0.093	26.29
12.0	0.000	0.00

DNA concentration 0.20 μM/ml. (EB) = 0.03 μM/ml. Buffers for the various pH ranges were: Acetate (0.04 M), pH 2.5-6.0; Tris-HCl (0.04 M), pH 6.0-9.0; Diethanolamine (0.04), pH 9.0-12.0.

that in the pH region between 3.5 and 8.0 DNA undergoes reversible conformational changes. Zimmer and Triebel (123) have reported that these conformational changes, which become more significant near pH 5.0, are more pronounced in DNA of high G + C content. Further information obtained by titration of synthetic polynucleotides indicates that acid-induced conformational changes begin with protonation of cytosine (18). This protonation may result in tautomeric changes which alter the hydrogen bonding characteristics of the cytosine moiety (72). These changes are present at pH values above those at which strand dissociation occurs and may be responsible for the decrease in binding and the reduced molar circular dichroism at 307 nm.

The double stranded requirement for EB primary binding has been emphasized in previous reports (27,34). The rapid decrease in both r and E_L-E_R below pH 5.0 may thus be the result of two phenomena, (i) acid-induced modifications in the DNA secondary structure prior to strand separation at pH 4.0 and (ii) the onset of the helix-coil transition. Primary binding sites in double helical DNA are modified by acid promoted tautomeric changes of cytosine residues. The beginning of the helix-coil transition, on the other hand, will bring about a decrease in the number of primary binding sites.

2. <u>The Relationship Between Molar Circular Dichroism and Bound Ethidium Bromide to Nucleotide Ratio at pH 11.7</u>

The dependence of E_L-E_R at 307 nm as a function of r for pH 11.3 and 11.7 is shown in Table 17 and illustrated in Fig. 50. The sharp increase in E_L-E_R above pH 11.3 was observed previously (Fig. 49). At pH 11.3, E_L-E_R increases with increasing r reaching a maximum of 24.2 at an r of 0.30 (Fig. 50). The increasing molar circular dichroism with increasing r at pH 11.3 is similar to that noted at pH 7.9 (Fig. 46). E_L-E_R values at pH 11.7 are higher for every ratio than those obtained at either pH 7.9 or 11.3. This difference is particularly pronounced in the range between r of 0.05 and 0.20. Specifically, at r equal to 0.05, E_L-E_R is 13.3 at pH 11.7 and 4.20 at pH 11.3. At r equal to 0.20, E_L-E_R is 26.6 and 17.0 respectively. Thus, the large increase in E_L-E_R at pH 11.7 noted in Fig. 49 for the added EB/P ratio of 0.15 is not peculiar for this ratio but occurs for all r's between zero and 0.50.

The increase in E_L-E_R between pH 11.3 and 11.7 is similar to the increase for the EB-DNA complex at temperatures at which strand dissociation occurs. In both cases, the sharp increase in E_L-E_R takes place as the DNA strands are partially dissociated.

Recently reported absorption (2) and proton release (3) data indicate that alkali-induced DNA strand separation

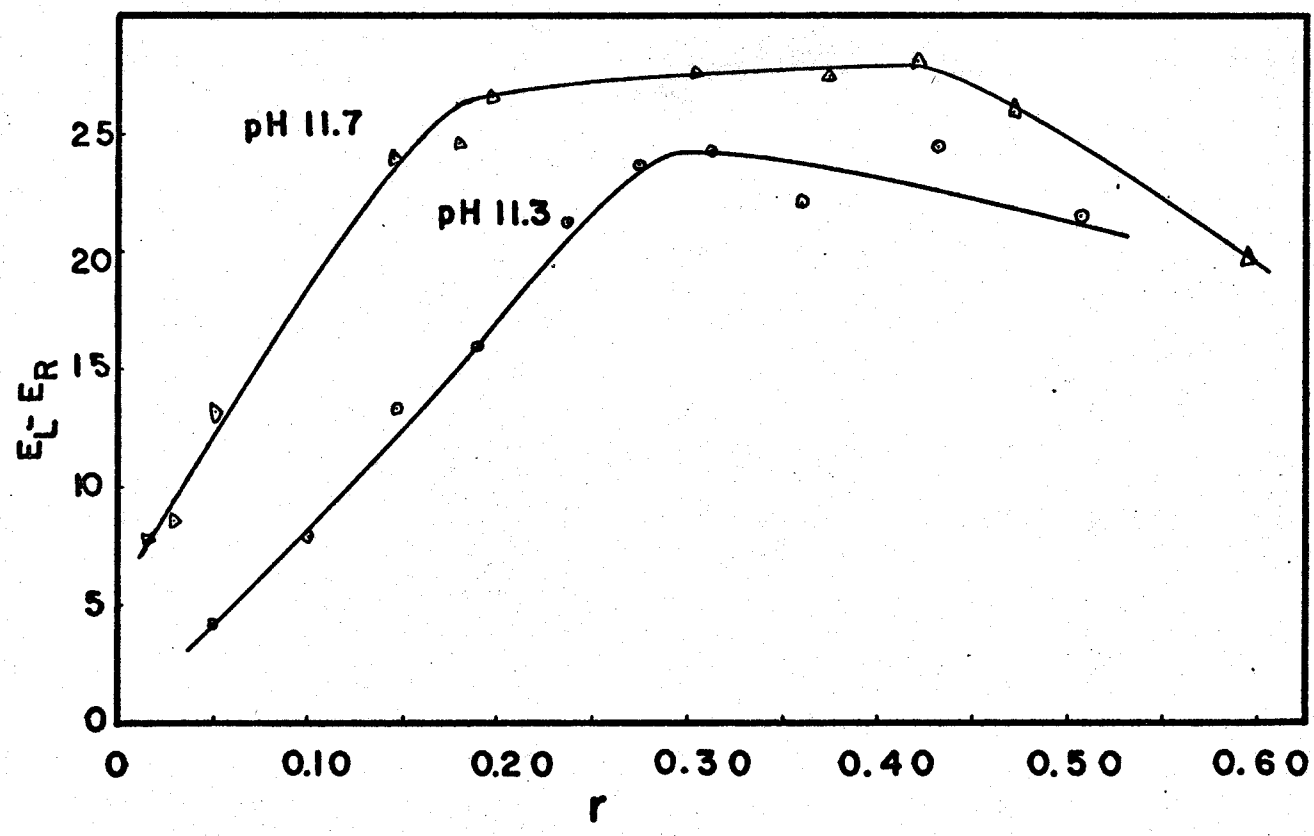


Fig. 50. Dependence of molar circular dichroism at 307 nm on r at pH 11.3 and 11.7. (DNA) = 0.20 μM/ml. EB concentration as shown in Table 7.

TABLE 17

Dependence of Molar Circular Dichroism at 307 nm on Bound Ethidium Bromide to Nucleotide Ratio at pH 11.3 and 11.7

pH 11.3		pH 11.7	
r bound	Molar Circular Dichroism 307 nm	r bound	Molar Circular Dichroism 307 nm
0.050	4.20	0.015	7.73
0.100	7.80	0.030	8.43
0.146	13.35	0.050	13.28
0.192	16.35	0.100	14.50
0.236	21.22	0.093	26.29
0.275	23.74	0.145	23.95
0.311	24.29	0.179	24.55
0.360	22.03	0.197	26.54
0.373	23.85	0.303	27.63
0.431	24.49	0.342	27.97
0.507	21.48	0.385	30.36
		0.421	28.18
		0.473	25.90
		0.596	19.81

DNA concentration 0.20 μM/ml in diethanolamine buffer (0.04 M). EB concentrations as shown in Table 7.

proceeds gradually between pH 11.5 and 12.0. For T2 phage DNA, for example, the alkali promoted helix-coil transition may be reversed if during the forward titration the pH of 11.7 is not exceeded (2). DNA strand separation in aqueous alkali may be initiated by dissociation of A-T regions followed by disruption of hydrogen bonding between guanosine and cytosine bases (2).

For the pH range within which A-T rich DNA regions are dissociated but G-C base-pairs remain intact, DNA clearly possesses a structure consisting of both single and double-stranded regions. The presence of such regions in partially denatured DNA by alkali has been confirmed by electron photomicrography (49).

The data summarized in Figs. 49 and 50 suggests that the development of the 307 nm dichroic band is the result of nearest-neighbor interactions between EB molecules bound to adjacent primary binding sites. Maximum E_L-E_R coincides with saturation of primary binding sites. Binding of EB to these sites permits such nearest-neighbor interactions to occur between two or more DNA-bound EB molecules.

Partially denatured DNA at pH 11.7, which probably is double-stranded only in the vicinity of G-C rich regions, is a structure quite different from DNA at pH 7.9 which is a base-paired double helix. At pH 11.7 EB binds to primary sites which are situated within the double-stranded regions of the partially denatured polynucleotide. Therefore, a

greater probability exists that one EB molecule will bind to a primary binding site adjacent to a previously bound EB. The resulting increase in nearest-neighbor interactions would then give rise to increase molar circular dichroism at 307 nm.

At pH values exceeding 11.7, both r and E_L-E_R decrease to zero. This decrease which accompanies complete alkali denaturation of DNA may be the direct result of elimination of double-stranded primary binding sites.

N. CIRCULAR DICHROISM OF ETHIDIUM BROMIDE COMPLEXES WITH SYNTHETIC POLYNUCLEOTIDES

We have noted that the addition of ethidium bromide to DNA results in the appearance of several induced circular dichroism bands. The band centered near 307 nm apparently reflects only primary binding. The dependence of the molar circular dichroism at 307 nm on the bound EB to nucleotide ratio (Fig. 26) indicates that this band originates from nearest-neighbor interactions between EB molecules bound to adjacent primary binding sites. By examination of the circular dichroism of the ethidium bromide complexes with synthetic polynucleotides of known secondary structure, the polynucleotide structural requirements for primary complex formation may be determined.

In this section the EB to DNA ratios are expressed in terms of add (EB/P) rather than bound (r) EB. The determination of the concentration of EB bound to these

polynucleotides is difficult since the spectra of EB in the bound form, which is different in each case, cannot be obtained for many of these synthetic polymers. The spectra can be compared however since the polynucleotide concentrations are identical (0.20 μM/ml) in each case.

1. Interaction of Ethidium Bromide with Single-Stranded Polynucleotides

The effect of EB on the circular dichroism of single-stranded polynucleotides is shown in Figs. 51-55. Poly A, in the single-stranded conformation (65) exhibits circular dichroism below 300 nm with maxima near 265 and 245 nm (Fig. 51). In the presence of EB a decrease in E_L-E_R at 265 nm is observed from 29.0 for poly A to 23.5 and 18.4 for added EB/P ratios of 0.10 and 0.40 respectively. Under these conditions, the circular dichroism in the 300 to 360 nm region is not influenced. This indicates that interactions between EB and single-stranded poly A, if occurring at all, are different from that which occurs between EB and DNA.

The influence of EB on the CD of other single-stranded ribosyl homopolymers, namely poly C, poly U, poly I and a mixture of the non-interacting homopolymers poly A and poly C, abbreviated as poly (A + C), indicates that in each of these cases the addition of EB results in the appearance of circular dichroism in the 300-360 nm region.

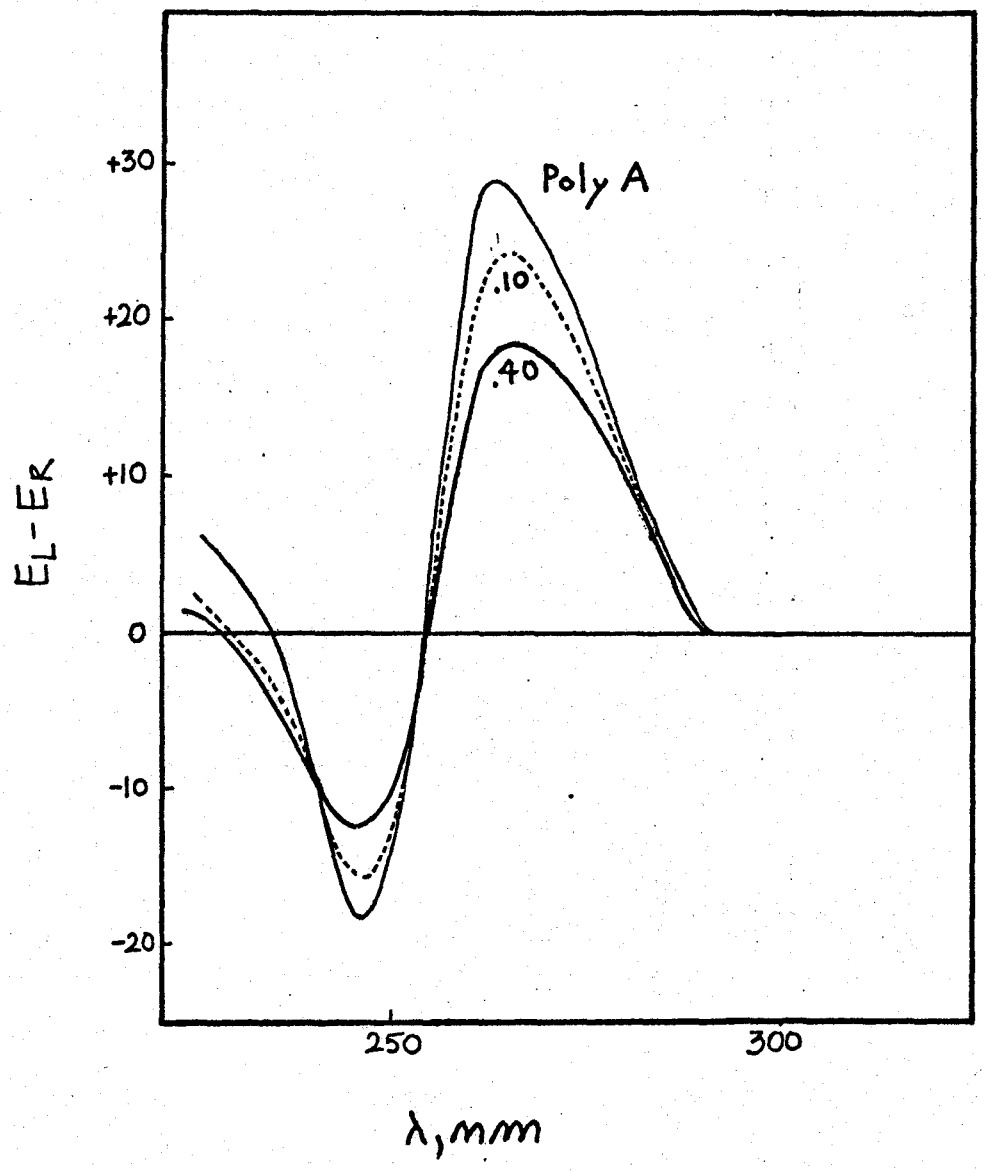


Fig. 51. Circular dichroism of poly A and EB-poly A at added EB to nucleotide ratios of 0.10 and 0.40. [P] = 0.20 μM/ml in 0.04 M Tris-HCl pH 7.9.

Poly C (Fig. 52), which under the conditions of the experiment exists in the single-stranded conformation (71), exhibits a positive CD spectrum below 300 nm with a maximum E_L-E_R of 30.0 near 276 nm. In the presence of EB at EB/P ratios of 0.20 and 0.50 a broad positive circular dichroism at wavelengths below 345 nm is noted. For an added EB/P ratio of 0.50, E_L-E_R is relatively constant at approximately 14.0 from 305 to 265 nm. No discernible maxima are noted.

The CD of poly U (Fig. 53) exhibits a positive maximum with E_L-E_R near 270 nm of 4.50. Addition of EB at an EB/P ratio of 0.20 produces a decrease in E_L-E_R to 1.10 but no shift in the 270 nm maximum. At an EB/P ratio of 0.50, circular dichroism is observed below 370 nm. The E_L-E_R increases gradually to +3.95 near 300 nm and remains nearly constant at +3.90 below 300 nm.

Poly I (Fig. 54) exhibits circular dichroism at wavelengths below 280 nm with an E_L-E_R maximum of +3.10 at 245 nm. At an EB/P ratio of 0.20, positive maxima are noted near 250 and 285 nm with approximately equal E_L-E_R of 3.10. At an EB/P ratio of 0.50, circular dichroism is observed at wavelengths below 355 nm. E_L-E_R increases gradually from zero at 355 nm to 4.00 near 300 nm and remains nearly constant below this wavelength.

The mixture of the non-interacting polynucleotides poly A and poly C exhibits the CD shown in Fig. 55 which is

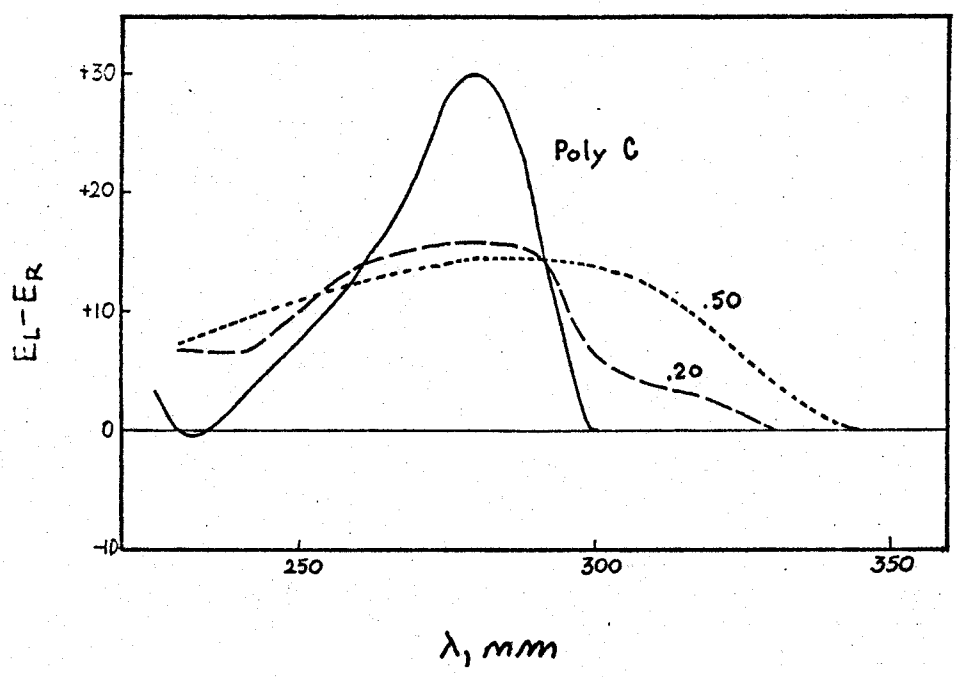


Fig. 52. Circular dichroism of poly C and EB-poly C at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl pH 7.9.

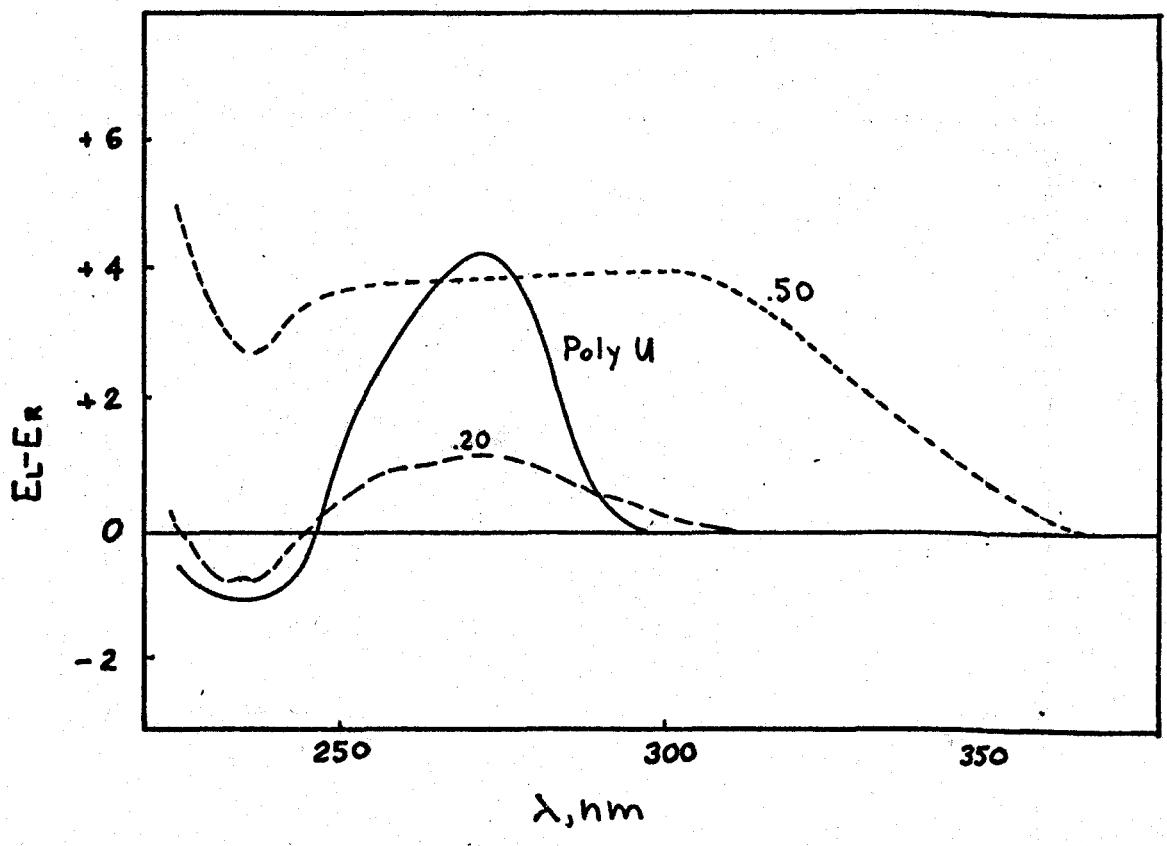


Fig. 53. Circular dichroism of poly U and EB-poly U at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl pH 7.9.

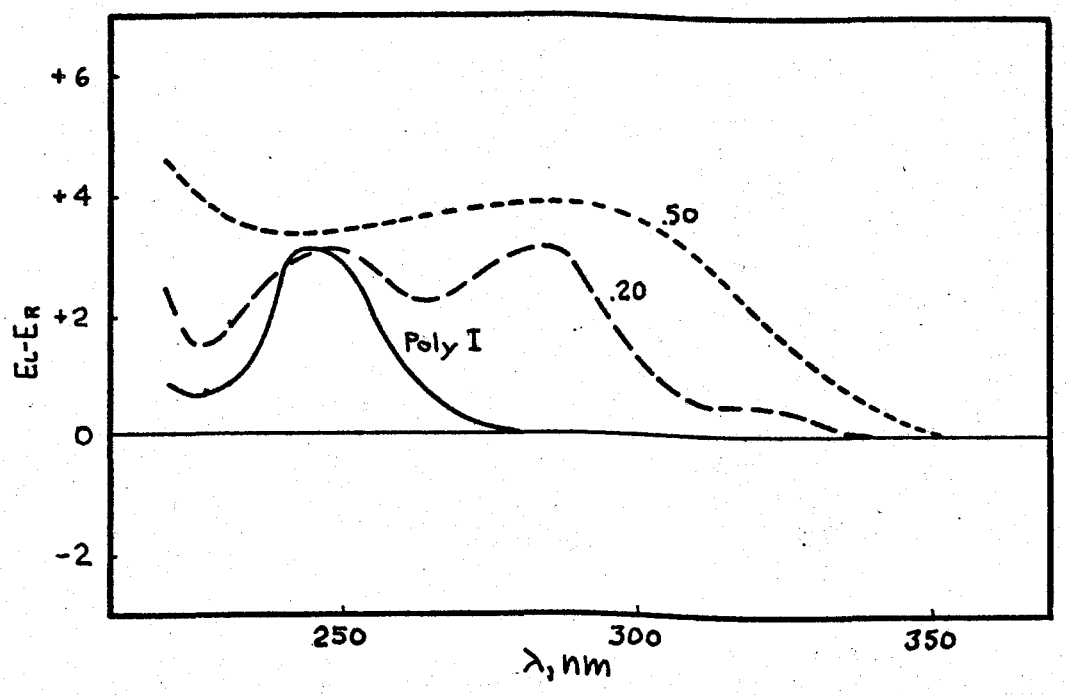


Fig. 54. Circular dichroism of poly I and EB-poly I at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

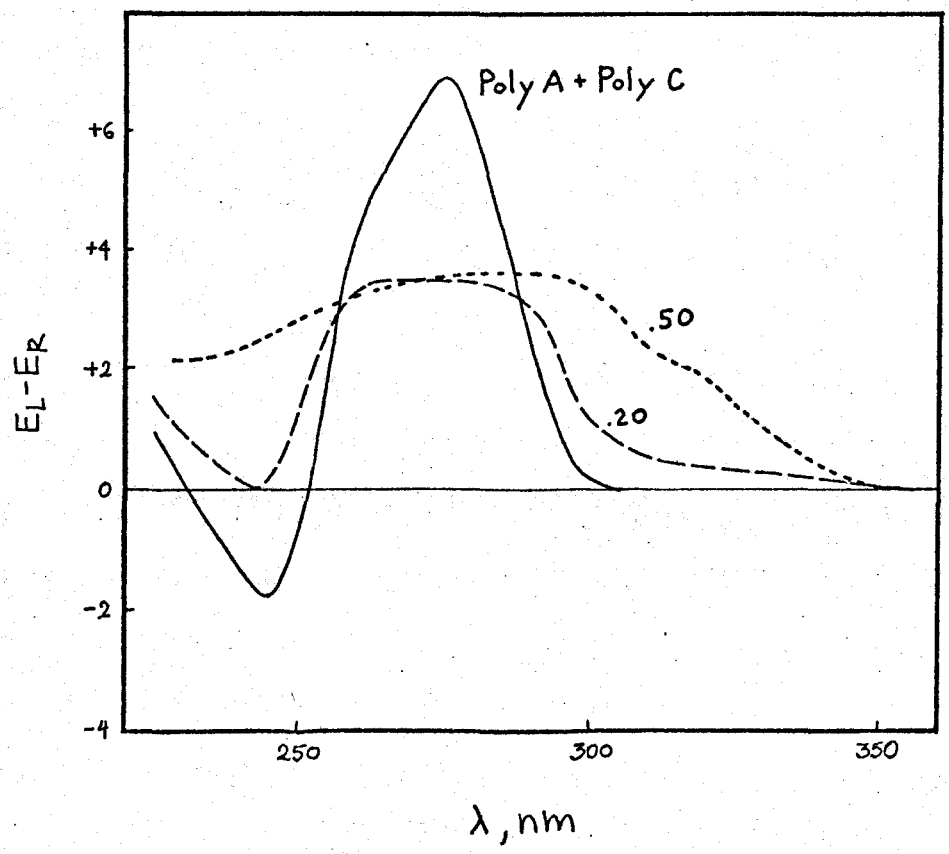


Fig. 55. Circular dichroism of an equimolar mixture of poly A and poly C in the presence of added EB to nucleotide ratios of 0.20 and 0.50. Total [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

the algebraic sum of the circular dichroism of the free polynucleotides. A maximum is noted near 275 nm with an E_L-E_R of approximately 26.60. Ethidium bromide, present at an EB/P ratio of 0.20, reduces the positive circular dichroism at wavelengths below 330 nm. In the 300 to 330 nm region E_L-E_R values are small but increase sharply from 300 to 290 nm. Between 255 and 290 nm, E_L-E_R is constant at 13.60 but decreases to nearly zero at 242 nm. At an EB/P ratio of 0.50, circular dichroism is observed at wavelengths below 350 nm. E_L-E_R increases steadily from zero at 350 nm to 13.00 at 295 nm.

Thus, the circular dichroism spectra of ethidium bromide complexes of the single-stranded polynucleotides, poly C, poly U, poly I and the poly A-poly C mixture exhibit, in general, many similarities. The distinct maximum noted for the EB-DNA complex (Fig. 20) is not apparent in these complexes. Rather, a broad positive circular dichroism is observed below 350 nm.

These findings indicate that an interaction between ethidium bromide and single-stranded polynucleotides may occur. The absence of distinct maximum at 307 nm, however, is an indication that this interaction is distinct from primary binding. Ethidium has been reported (55,112) to bind to single-stranded polynucleotides only at EB/P ratios considerably higher than those required for DNA. Absorption techniques have shown that under these conditions EB forms easily dissociated complexes with poly A.

Interactions have also been reported to occur between EB and polyvinylsulfate (Chapter I, F5) (111). Clearly however, primary binding involving intercalation of EB is not possible for this polymer. Thus, it is not surprising that EB interaction with polyvinylsulfate does not result in induced CD between 300 and 350 nm as is the case with single-stranded polynucleotides. The origin of the induced CD observed for EB complexes with poly C, poly U, poly I and a mixture of poly A and poly C may, therefore, be different from either primary binding by intercalation or secondary ionic binding. This is apparently the case since primary binding does not occur between EB and these polynucleotides (40) and secondary interactions apparently do not result in induced CD of similar characteristics.

2. The Effect of Magnesium (II) on the Ethidium Bromide-Single-Stranded Polynucleotide Interaction

The effect of ethidium bromide on the circular dichroism of poly C in the presence of 4×10^{-3} M $MgCl_2$ is shown in Fig. 56. At EB/P ratios of 0.20 and 0.50, a slight decrease in the ellipticity of the 276 nm maximum is noted. E_L-E_R decreases from 26.0 for poly C to 24.5 for an EB/P ratio of 0.50.

The effect of addition of EB to poly (A + C) in the presence of 4×10^{-3} M $MgCl_2$ may be examined in Fig. 57. At EB/P ratios of 0.20 and 0.40, a decrease in the magnitude of the 270 nm band and a concomitant red shift of the

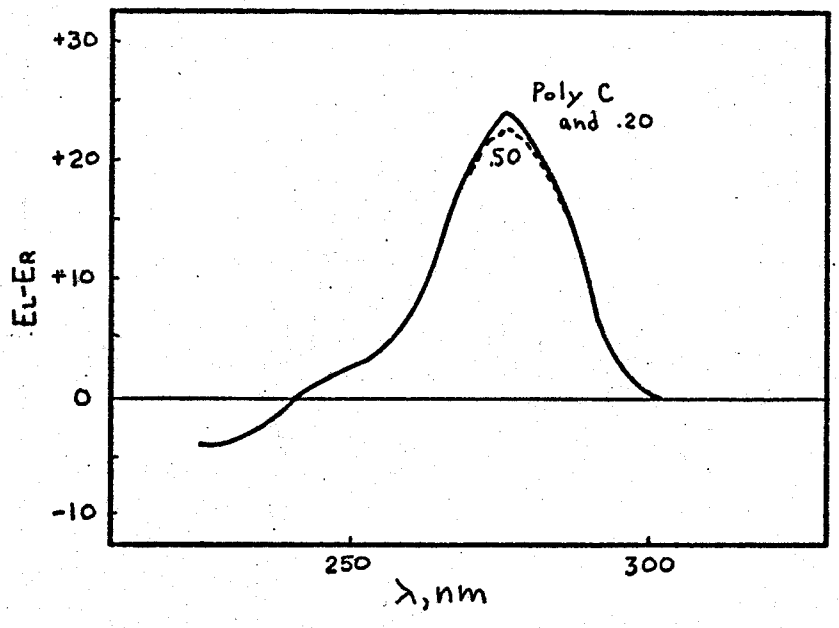


Fig. 56. Circular dichroism of poly C and EB-poly C in the presence of 4 x 10^{-3} M magnesium chloride at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

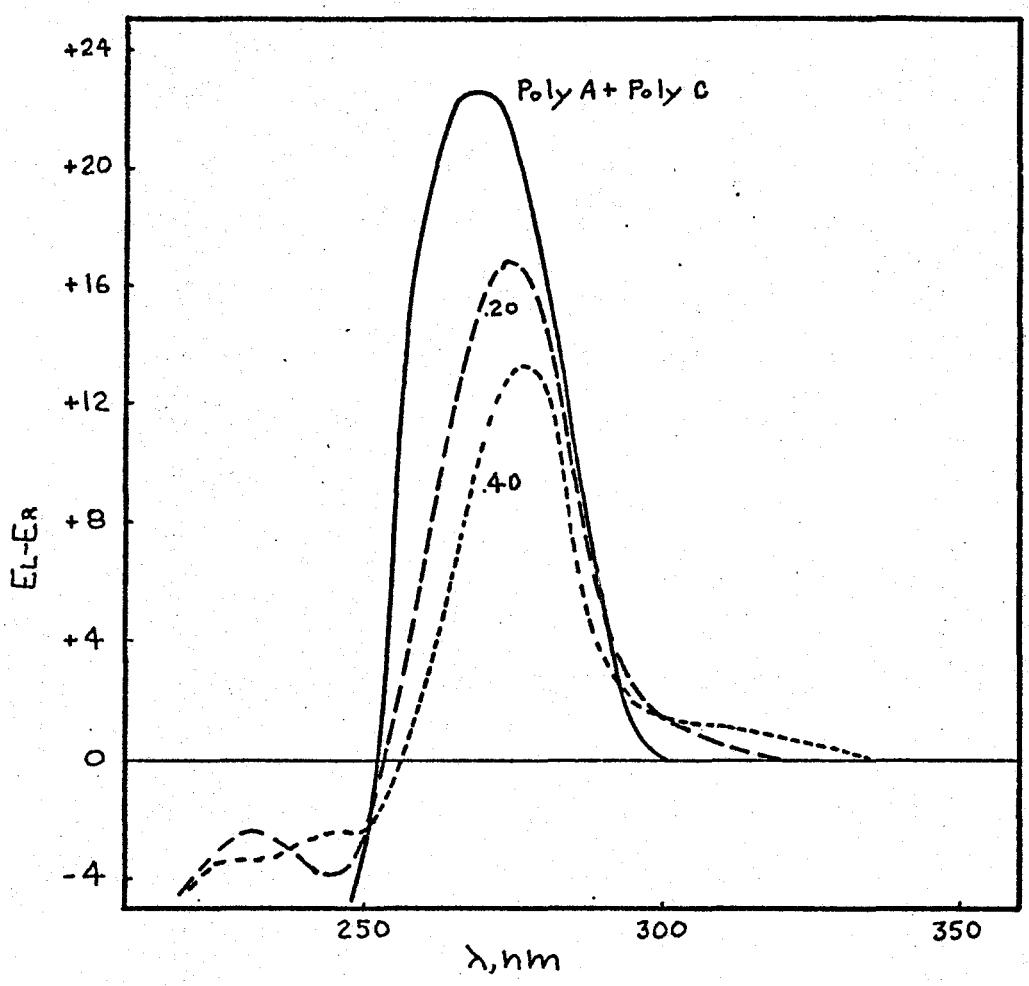


Fig. 57. Circular dichroism of an equimolar mixture of poly A and poly C in the presence of 4 x 10^{-3} M magnesium chloride at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

maximum are noted. At 0.40, E_L-E_R decreases from 23.0 at 270 nm for poly (A + C) to 13.5 near 277 nm.

The effect of EB on the circular dichroism of poly U in the presence of 4×10^{-3} M $MgCl_2$ is shown in Fig. 58. Under these conditions, poly U exhibits dichroism only below 295 nm with a maximum E_L-E_R of 5.00 near 270 nm. EB/P ratios of 0.20 and 0.50 result in the appearance of circular dichroism from 300 to 350 nm. The ellipticity at 270 nm, however, is not significantly affected for the 0.20 ratio. For the 0.50 ratio, E_L-E_R at 300 nm is approximately 2.00, and at 270 nm is reduced to 3.80.

In the absence of magnesium, each of the single-stranded polynucleotides, poly C, poly U and poly I and poly (A + C), exhibit induced circular dichroism between 300 and 350 nm when EB is present. Thus, magnesium (II) ions alter the CD of the complexes between ethidium bromide and most of these polynucleotides.

In the case of poly C and poly (A + C), magnesium apparently interferes with the interaction responsible for the induced CD in the 300 to 350 nm region. This, however, is not the case with EB-poly U complexes which suggests either that ethidium bromide exhibits a preference for binding to the uridine moiety or simply that the observed differences in circular dichroism between single-stranded polynucleotides in the presence of EB results from differences in the secondary structure of the polynucleotides.

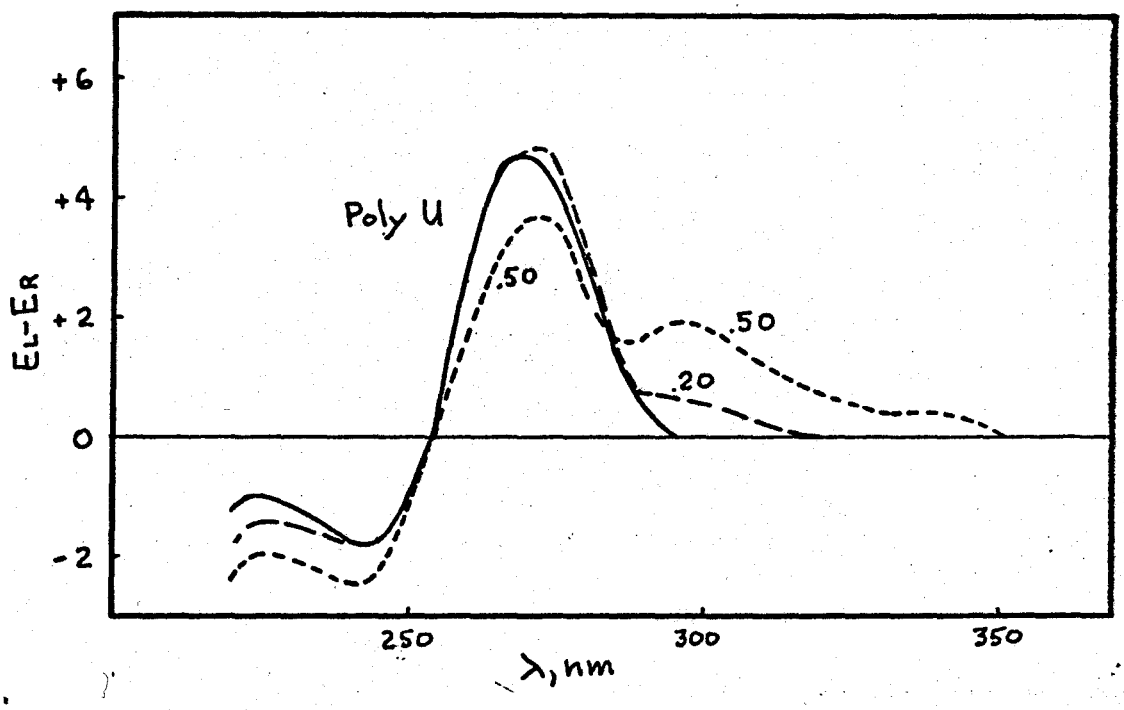


Fig. 58. Circular dichroism of poly U and EB-poly U in the presence of 4 x 10^{-3} M magnesium chloride at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

Studies of the interaction of EB with various mononucleotides indicate that uridine does not exhibit any unusual binding behavior (112). Therefore, differences in secondary polynucleotide structure appear as the more likely cause for the observed differences in the effect of EB on single-stranded polynucleotides. Poly A, at neutral pH, exists as a relatively stiff, rod-like, single-stranded helix (12). Poly C exists as a single-stranded coil with intermittent regions of helicity (71), whereas poly U, at room temperature, may lack any regular cooperative structure (70).

Poly A apparently interacts with EB only by secondary binding and does not exhibit induced CD in the region from 300 to 350 nm. The origin of the induced CD can not, therefore, be the result of simple ionic interaction between dye and single-stranded polynucleotides.

Although strong primary binding may not occur with these polynucleotides as it does between EB and DNA, an ethidium bromide bound to the ribosyl phosphate moiety may also interact with the nitrogen bases of the polynucleotide. Whether interaction with the bases occurs or not probably depends on the secondary structure of each polynucleotide. In a polynucleotide such as poly A, which possesses a rigid helical structure, this type of interaction is not expected to occur because of the inaccessibility of the bases for interaction with ethidium molecules bound on the external surface of the helix. However, in the case of poly C or

poly U, either of which possess varying amounts of random coil secondary structure, folding of the polynucleotide may permit EB bound to the polymer to interact with the bases in different regions of the strand.

3. Interaction of Ethidium Bromide With Double-Stranded Polynucleotides

(a) Poly dAT

The effect of ethidium bromide on the circular dichroism of base-paired double-stranded ribo- and deoxyribopolynucleotides is shown in Figs. 59-66. Poly dAT, a deoxyribopolynucleotide consisting of alternating adenine and thymine nucleotide residues (49), exhibits a CD (Fig. 59) with maxima near 245 and 263 nm. E_L-E_R is -4.00 at 245 nm and +4.40 at 263 nm.

Addition of EB to dAT results in modification of the circular dichroism of poly dAT below 300 nm and the appearance of induced CD between 300 and 360 nm with a predominant band near 307 nm and a shoulder near 335 nm. A similar CD band at 307 and shoulder at 335 nm are also observed for the EB-DNA complex (Fig. 20). The ellipticity of the band at 307 nm increases as the EB/P ratio increases from 0.10 to 0.50. At added EB/P ratios, 0.10, 0.30 and 0.50, E_L-E_R is respectively 2.2, 6.3 and 7.6. For these ratios a continued increase in the magnitude of the positive maximum at 263 nm and a red shift in this maximum reaching 278 nm at an EB/P of 0.50 are noted.

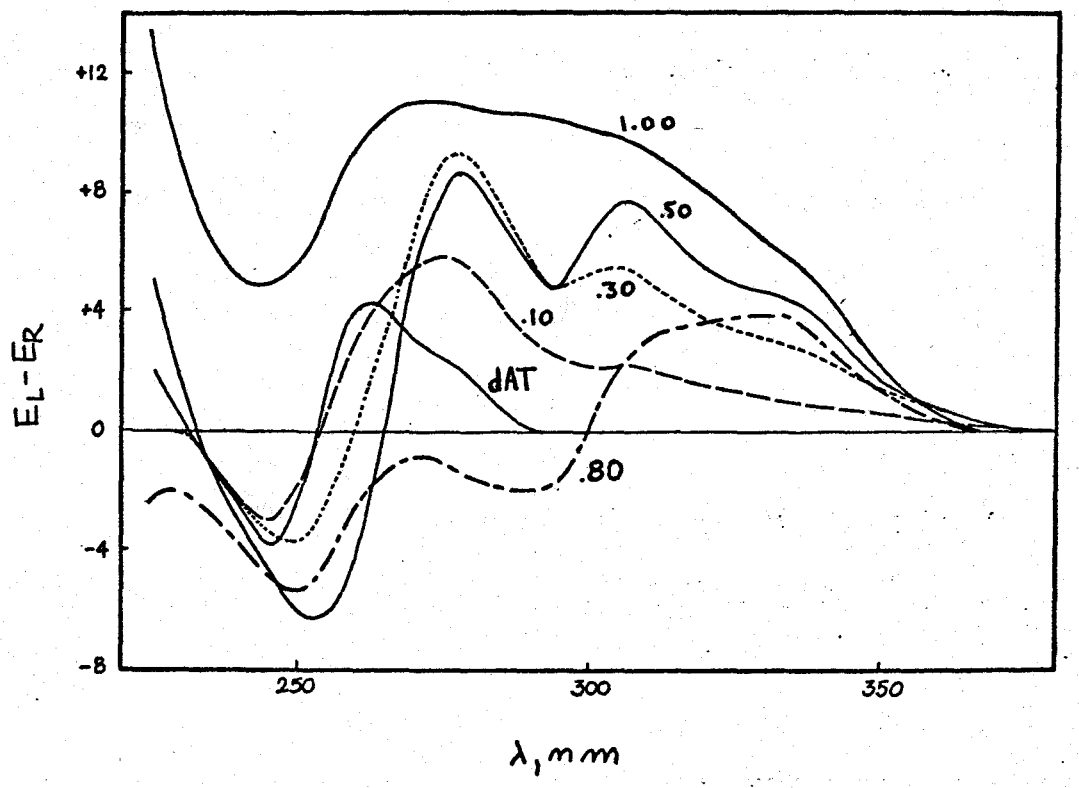


Fig. 59. Circular dichroism of poly dAT and EB-poly dAT at added EB to nucleotide ratios of 0.10, 0.30, 0.50, 0.80 and 1.00. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

The circular dichroism of the EB poly dAT complex undergoes additional more pronounced changes as the EB/P ratio increases from 0.50 to 1.00. Both the 307 and 278 nm bands decrease up to an EB/P of 0.80, while the magnitude of the 335 nm shoulder remains substantially unchanged at EB/P ratios of 0.50, 0.80 and 1.00. The CD at an EB/P of 1.00 is reminiscent of that observed upon addition of EB to single-stranded homopolymers in the absence of magnesium and quite different from that observed for the EB-DNA complex for the same EB/P ratio. In contrast, the shape of the EB-DNA complex remains the same between 300 and 350 nm at all EB/P ratios; only the magnitude of E_L-E_R changes.

The similarity between the CD of EB-DNA and EB-poly dAT at low EB/P ratios from 300 to 360 nm suggests that at these ratios the primary binding between EB and poly dAT may be similar to that occurring between EB and DNA.

The circular dichroism of the EB-poly dAT complex in the presence of 4×10^{-3} M $MgCl_2$ is shown in Fig. 60. EB induces strong CD in the 290 to 360 nm region with a distinct CD maximum near 307 nm. A red shift in the CD maximum of poly dAT from 263 to 277 nm is also noted. In general the spectra are similar to those in the absence of magnesium especially at low EB/P ratios. However at an EB/P ratio of 1.00, the 307 maximum is still distinct as

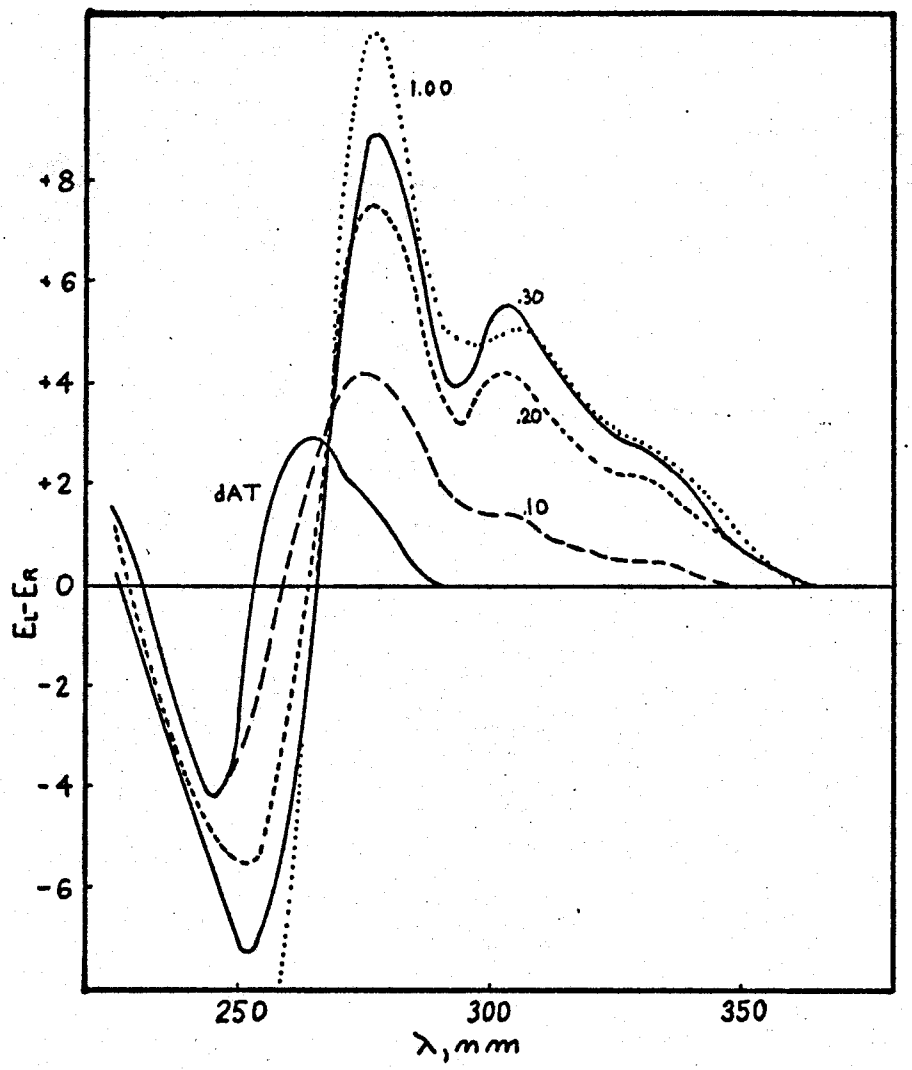


Fig. 60. Circular dichroism of poly dAT and EB-poly dAT in the presence of magnesium chloride (4×10^{-3} M) at added EB to nucleotide ratios of 0.10, 0.30, 0.50 and 1.00. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

compared to the broad spectrum noted for EB-poly dAT in the absence of magnesium. This may indicate that magnesium stabilizes the poly dAT helix against disruption by EB at high EB/P ratios.

(b) Poly (A + U)

The effect of EB on the circular dichroism of other double-stranded polynucleotides provides further evidence that a base-paired polynucleotide is required for primary binding to take place.

The CD of poly (A + U) (Fig. 61), a double-stranded polyribonucleotide consisting of one strand of poly A and one strand of poly U, exhibits maxima near 243 and 265 nm with E_L-E_R of -4.00 and +8.90 respectively. Red shifts in the maxima to 257 and 274 nm and a decrease in ellipticity at 274 nm are noted with increasing EB/P ratios. The red shift is similar to that observed for the double-stranded deoxyribosylcopolymer poly dAT. Furthermore, the addition of increasing concentrations of EB to poly (A + U) results in the development of a strong induced CD from 300 to 360 nm similar to that observed for DNA, denatured DNA and poly dAT.

The appearance of a strong induced CD at 307 nm may be indicative of EB binding to primary sites within the poly (A + U) double helix. These results are in agreement with those obtained by absorption (112) and fluorescence (55) methods indicating that EB forms primary complexes with poly (A + U) similar to those formed with DNA.

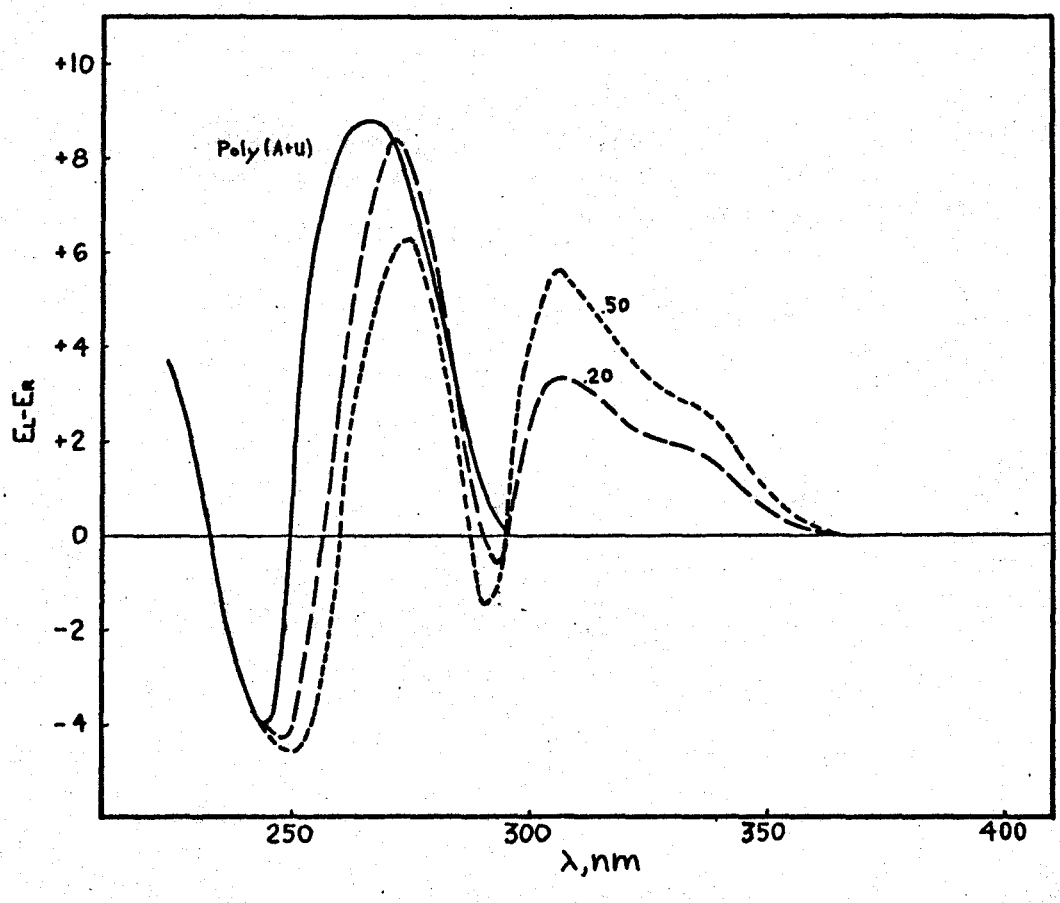


Fig. 61. Circular dichroism of poly (A + U) and EB-poly (A + U) at added EB to nucleotide ratios of 0.10, 0.20 and 0.30. [P] = 0.20 μM/ml in 0.04 M Tris HCl, pH 7.9.

The effect of magnesium ions on the circular dichroism of the EB-poly (A + U) complex is shown in Fig. 62. The spectra are similar in the presence and absence of magnesium. Increasing EB/P ratios result in a progressive red shift in the positive CD maximum from 262 to 273 nm and the appearance of increased CD from 300 to 360 nm. It is interesting to note that the negative CD band near 290 nm is considerably more pronounced in the case of EB-poly (A + U) in the absence of magnesium than it is for EB-DNA or EB-poly dAT.

(c) Yeast RNA

The effect of addition of EB to yeast RNA is shown in Fig. 63. At EB/P ratios of 0.20 and 0.50, a complex CD band of low intensity is observed from 300 to 360 nm. For an EB/P ratio of 0.50, E_L-E_R at 307 nm is equal to 1.8 as compared with 5.40 for DNA and 5.80 for poly (A + U). Increasing EB/P ratios result in a red shift of the positive CD maximum from 266 to 272 nm. A small reduction in the E_L-E_R at 307 nm is noted in the presence of 4×10^{-3} M $MgCl_2$ (Fig. 64). At an EB/P ratio of 0.50, E_L-E_R is 1.80 in the absence and 1.10 in the presence of magnesium.

(d) Poly dG:dC

Poly dG:dC is a double-stranded deoxyribosyl polymer with one strand consisting entirely of polydeoxyguanosine and the other of polydeoxycytosine (90). The polynucleotide exhibits circular dichroism below 300 nm

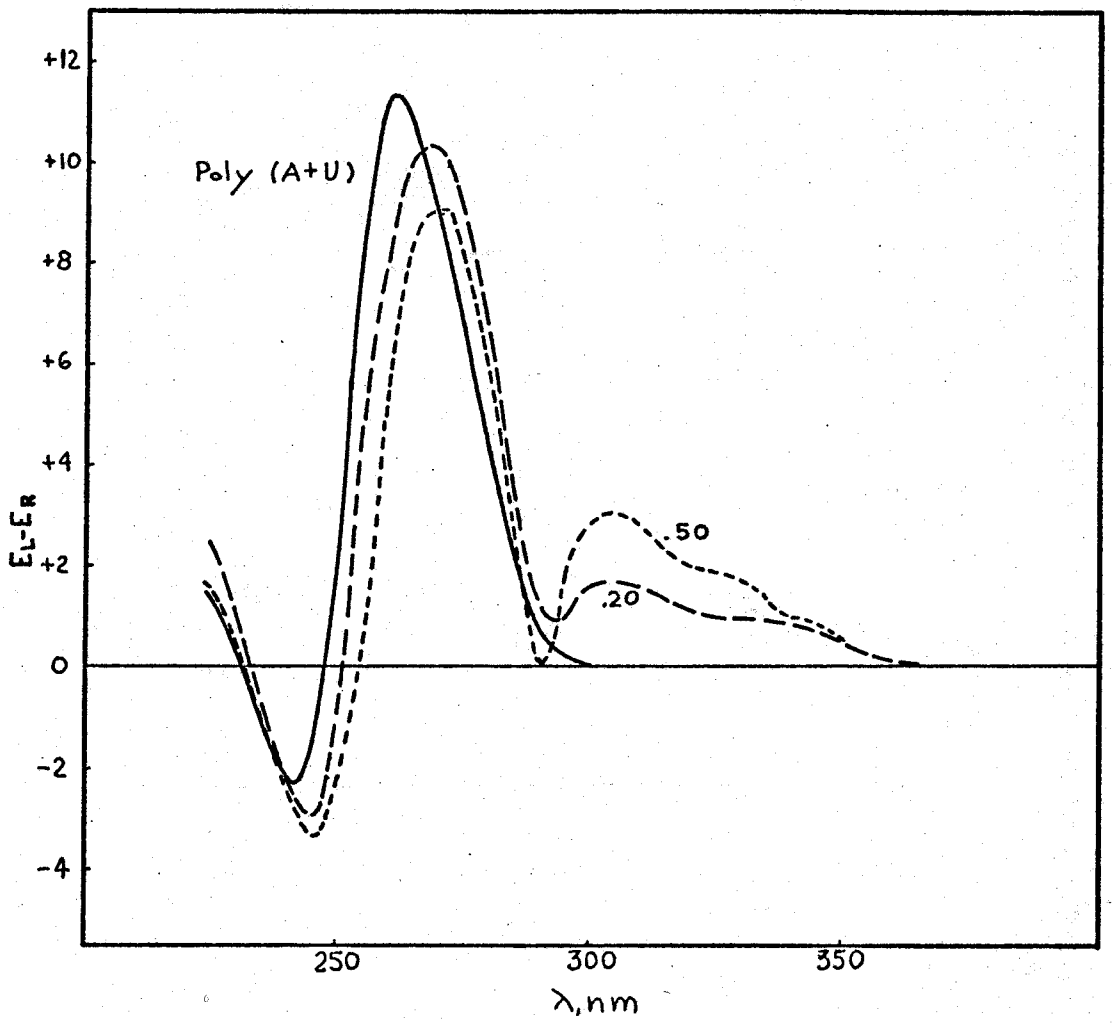


Fig. 62. Circular dichroism of poly (A + U) and EB-poly (A + U) in the presence of magnesium chloride (4×10^{-3} M) at added EB to nucleotide ratios of 0.10, 0.20 and 0.30. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

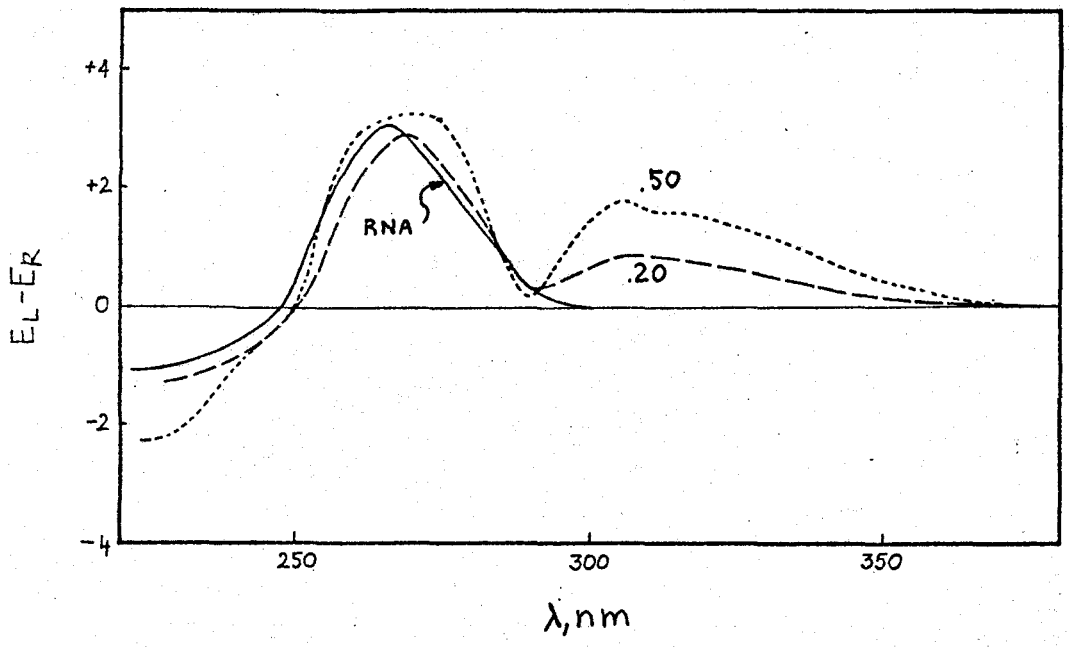


Fig. 63. Circular dichroism of yeast RNA and EB-yeast RNA at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

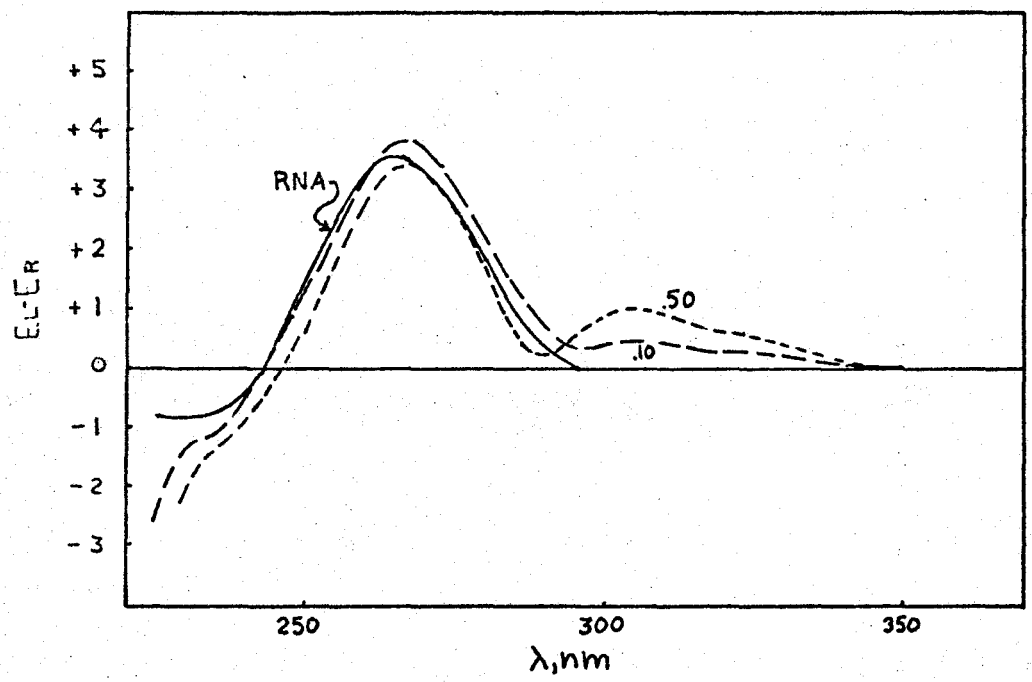


Fig. 64. Circular dichroism of yeast RNA and EB-yeast RNA in the presence of magnesium chloride (4×10^{-3} M) at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

with a positive maximum E_L-E_R near 263 nm of 5.3 (Fig. 65). Addition of EB to the poly dG:dC at an EB/P ratio of 0.20 results in the appearance of CD bands with apparent maxima near 307 and 335 nm and a decrease in E_L-E_R at 263 nm from 5.3 to 3.9. At an EB/P of 0.50 the spectrum exhibits increased ellipticity at both 307 and 335 nm. E_L-E_R at 307 nm has increases to 3.90 as compared with 2.60 at EB/P of 0.20. The spectra for each of these ratios exhibit apparent maxima at 307 and 335 nm. Some minor differences between these two ratios are noted, however, below 300 nm. In this region the CD may result from contributions of EB, EB-poly dG:dC complex and poly dG:dC.

The influence of magnesium ions on the interaction of EB with poly dG:dC is shown in Fig. 66. The CD at EB/P ratios of 0.20 and 0.50 exhibit distinct maxima near 307 and 335 nm. Differences in the EB-poly dG:dC spectra in the presence and absence of magnesium are apparent. The appearance of the induced CD from 300 to 360 nm is indicative of formation of a primary complex between ethidium bromide and poly dG:dC. At EB/P ratios of 0.20 and 0.50, maxima are noted at 307 nm. However, in the absence of magnesium, E_L-E_R is about twice as large as in the presence of magnesium. A similar decrease in E_L-E_R at 307 nm was previously observed for DNA (Fig. 29).

More pronounced differences in the EB-poly dG:dC are found in the region below 300 nm for these two systems.

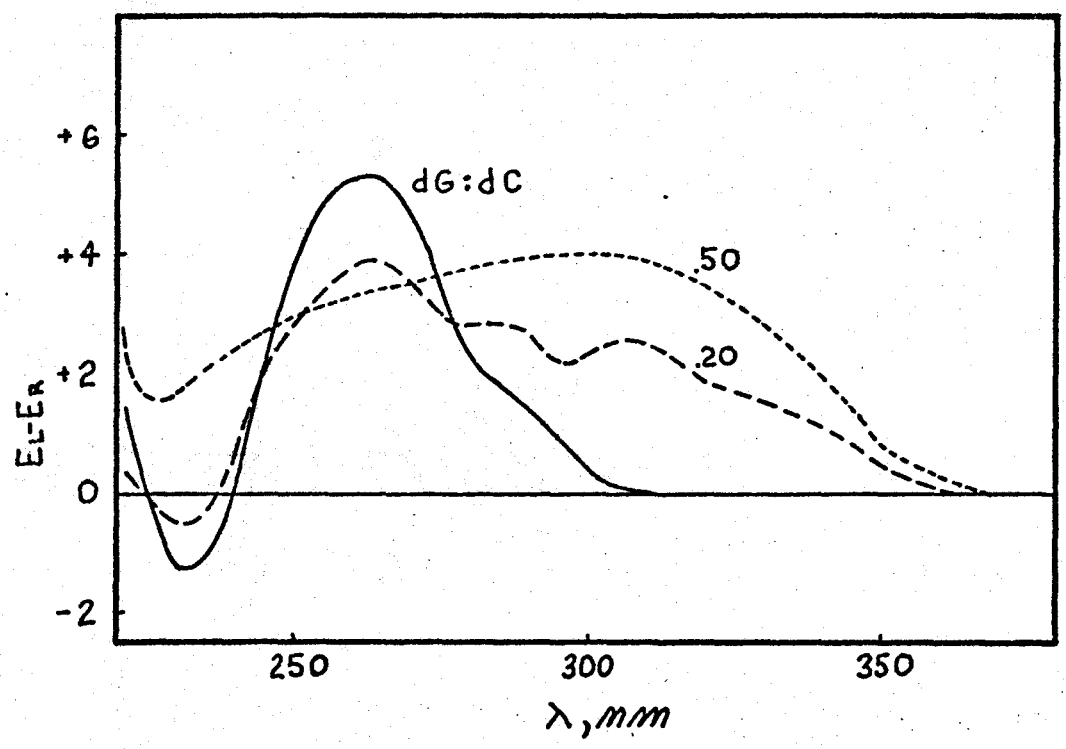


Fig. 65. Circular dichroism of poly dG:dC and EB-poly dG:dC at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

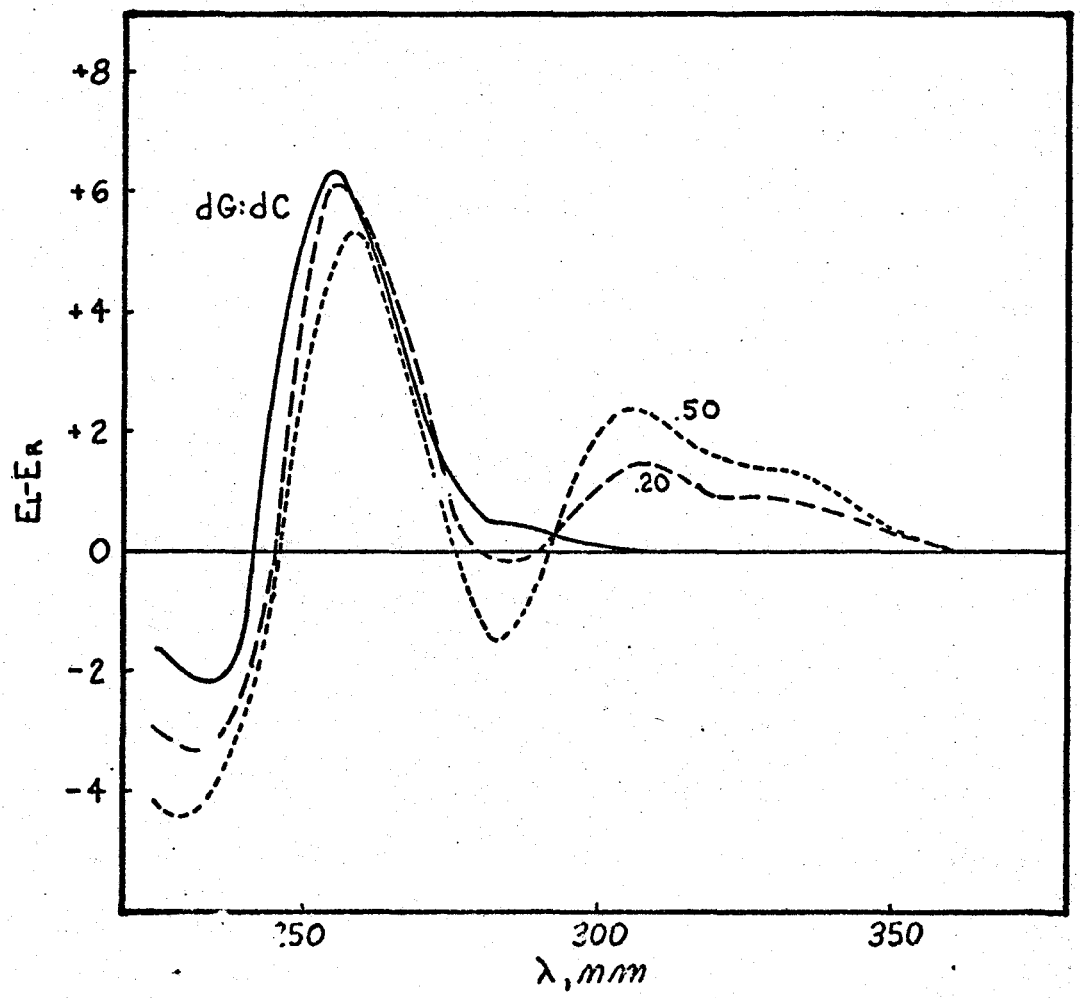


Fig. 66. Circular dichroism of poly dG:dC and EB-poly dG:dC in the presence of magnesium chloride (4×10^{-3} M) at added EB to nucleotide ratios of 0.20 and 0.50. [P] = 0.20 μM/ml.

In this region, in the absence of magnesium, the integrity of the positive maximum of poly dG:dC is lost at an added EB/P ratio of 0.50. Similar observations can be made with poly dAT (Figs. 59 and 60). The presence of magnesium (II) ions apparently stabilizes the helical structures of poly dG:dC and poly dAT toward disruption which may result from the primary binding of ethidium bromide. The distinct band at 290 nm for EB-poly dG:dC at a ratio of 0.50 is similar to that noted for EB-poly (A + U) also in the presence of magnesium ion.

4. Interaction of Ethidium Bromide with Unusual Double-Stranded Polynucleotides

(a) Poly (A + I)

The investigation of the interaction of ethidium bromide with double-stranded polynucleotides possessing base-pairing not usually found in DNA or RNA is expected to provide further information about structural features of polynucleotides which are essential for the development of nearest-neighbor interaction between bound ethidium bromide molecules.

Poly (A + I), which consists of a strand of poly A hydrogen-bonded to a strand of poly I (85), exhibits CD below 300 nm with a positive maximum with E_L-E_R of 4.50 noted near 270 nm (Fig. 67). At an EB/P ratio of 0.20, a very weak CD centered near 307 nm may be noted. E_L-E_R at this wavelength is only 0.50 compared with 2.80 for DNA at

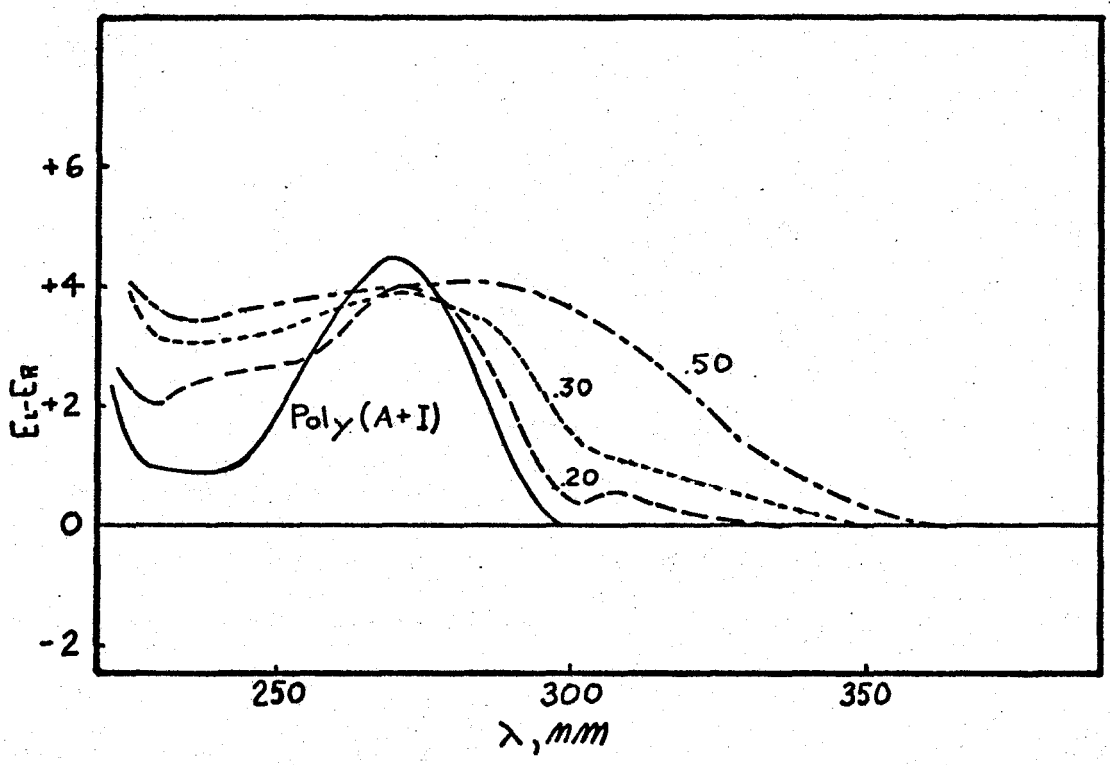


Fig. 67. Circular dichroism of poly (A + I) and EB-poly (A + I) at added EB to nucleotide ratios of 0.20, 0.30 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

the same ratio. At EB/P ratios of 0.30 and 0.50 E_L-E_R increases to 1.00 and 3.80 respectively. However, the CD for the 0.30 and 0.50 ratios does not exhibit a distinct maximum near 307 nm.

The appearance of the 307 nm band at EB/P ratios of 0.20 may be an indication that at low ratios some nearest-neighbor interaction takes place within the poly (A + I) structure. The CD for poly (A + I) at EB/P ratios of 0.30 and 0.50 however, is similar to that noted for the single-stranded polynucleotides, poly C and poly U, under the same conditions.

(b) Poly (I + C)

Another double-stranded structure possessing bas-pairing not usually found in DNA or RNA is poly (I + C). The effect of ethidium bromide on the circular dichroism of this polynucleotide is shown in Fig. 68. Poly (I + C) exhibits CD below 300 nm with a maximum E_L-E_R of +3.8 near 275 nm. Addition of EB at EB/P ratios of 0.20 and 0.50 results in induced CD below 360 nm. However, distinct positive maxima are not observed either at 307 nm or at 335 nm. The spectra are, in general, similar to those observed with poly (A + I) or with single-stranded poly-nucleotides such as poly C and poly U at the same EB/P ratios.

Formation of complexes with geometry similar to that described for EB-DNA primary complexes, thus, is not

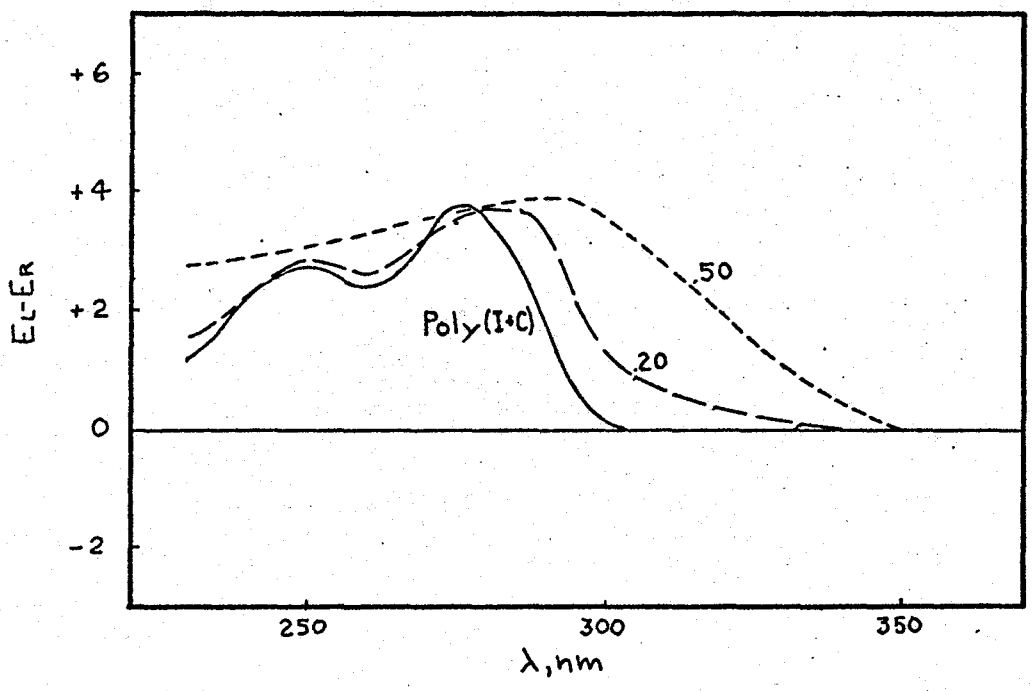


Fig. 68. Circular dichroism of poly (I + C) and EB-poly (I + C) at added EB to nucleotide ratios of 0.20, 0.30 and 0.50. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

likely with poly (A + I) and poly (I + C). The CD of the EB complexes of these double-stranded polynucleotides rather indicates the formation of a complex similar to that obtained between EB and poly C or poly U.

(c) Poly A at pH 5.6

A particularly unusual double-stranded polynucleotide is poly A at pH 5.6. At this pH poly A assumes a protonated double-stranded structure (86). The adenine-adenine hydrogen bonding occurs between the N_{10} and the N_7 of adenine and N_{10} and O_6 of the phosphate on the opposite strand (1, 86). Hydrogen bonding of this type can occur only if adenine rings are tilted with respect to each other in a propeller-like manner (Fig. 69). Therefore, in this polynucleotide, bases of each pair are not coplanar.

The effect of ethidium bromide on the CD of poly A at pH 5.6 is shown in Fig. 70. At this pH, poly A exhibits circular dichroism below 300 nm with a maximum E_L-E_R of 26.0 near 262 nm. Addition of EB at EB/P ratios of 0.20 and 0.40 results in a decrease in E_L-E_R to 16.60 and 3.20 respectively. Induced circular dichroism above 300 nm is not observed for either ratio. The results with double-stranded poly A at pH 5.6 are similar to those observed for single-stranded poly A (Fig. 50).

The failure to observe circular dichroism with a maximum at 307 nm to appear upon EB addition to double-stranded poly A indicates that double-strandedness alone is

Fig. 69. CPK space-filling model of hydrogen bonded adenine residues in poly A at pH 5.6. Hydrogen bonding is postulated to occur between the N_{10} and the N_7 of adenine and the N_{10} and O_6 of the phosphate of the opposite strand.

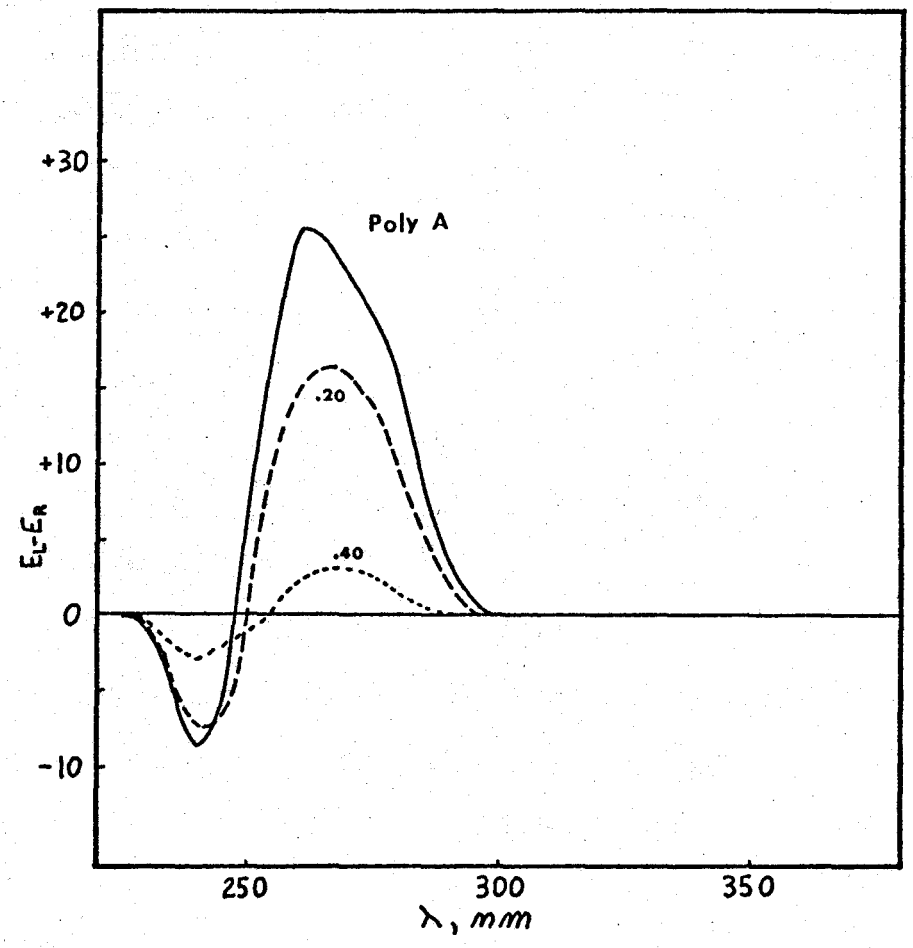


Fig. 70. Circular dichroism of double-stranded poly A and EB-poly A at pH 5.6 and added EB to nucleotide ratios of 0.20 and 0.40. [P] = 0.20 μM/ml in 0.04 M acetate, pH 5.6.

not sufficient for the intercalation of EB. The geometry of the bases at the potential binding site is obviously a crucial factor in determining whether intercalation can take place.

5. Dependence of the Molar Circular Dichroism of the EB-poly (A + U) Complex on the Bound Ethidium Bromide to Nucleotide Ratio

The relationship between the molar circular dichroism at 307 nm and the ratio (r) of bound ethidium bromide per nucleotide of poly (A + U) is shown in Fig. 71 which is constructed from the data listed in Table 18. E_L-E_R increases with increasing r up to an E_L-E_R of 21.0 at an r of 0.24. Above this ratio some precipitation of poly (A + U) was observed.

E_L-E_R values for the EB-poly (A + U) complex corresponds closely to those obtained for the EB-DNA complex for every ratio. The similarity between the CD and the dependence of E_L-E_R on r for EB-DNA and EB-poly (A + U) indicates that the structure of the complex may also be similar. The binding parameters for poly (A + U) of n equal to 0.21 and k, 4×10^{-7}, obtained from Scatchard plots from the shift in the absorption spectrum near 500 nm (112), differ appreciably from those of n equal to 0.15 and k, 15×10^{-7} obtained from similar plots by fluorescence spectroscopy (55). These differences are substantial particularly with respect to the values of n. It should be noted,

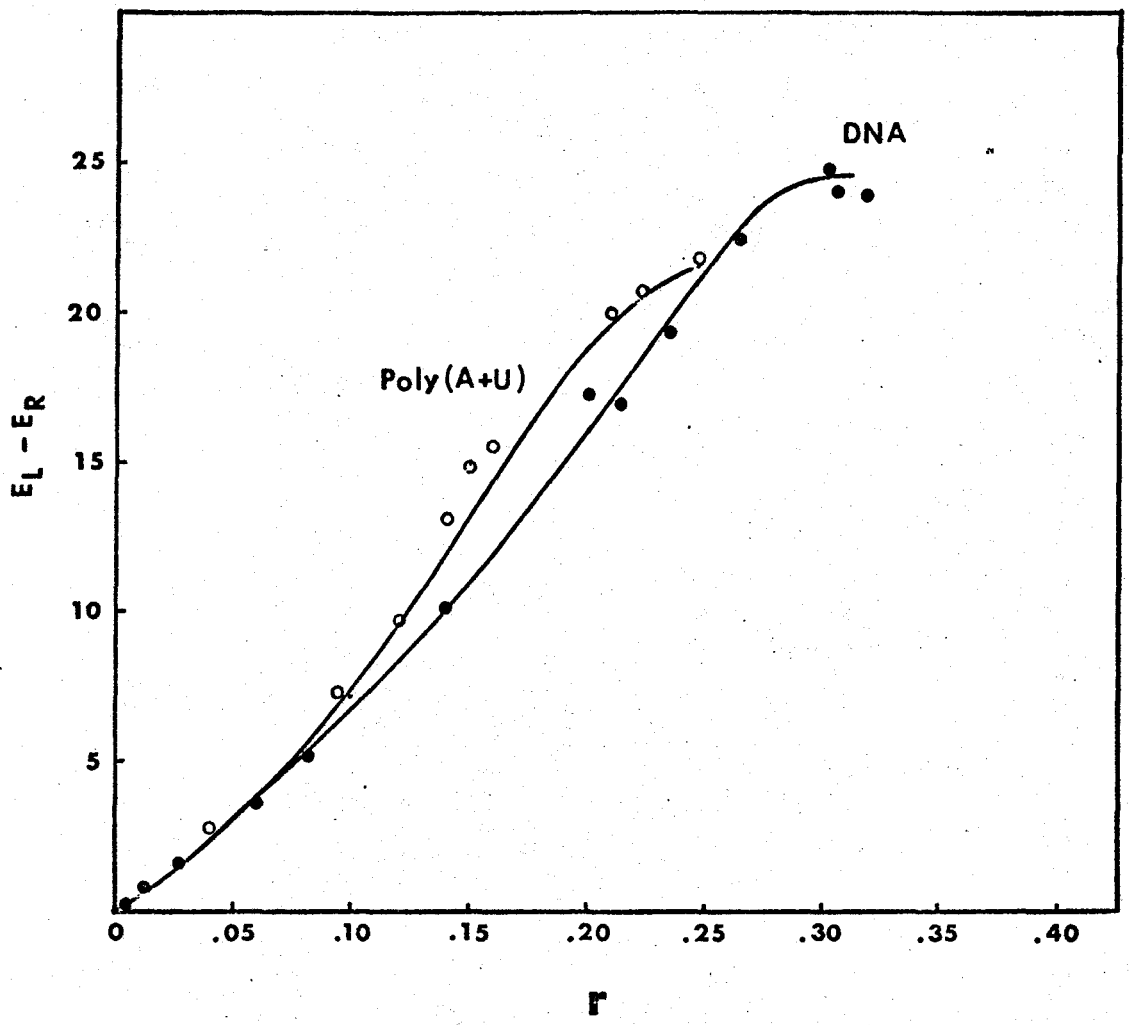


Fig. 71. Dependence of molar circular dichroism of EB-poly (A + U) at 307 nm on the bound EB to nucleotide ratio. [P] = 0.20 μM/ml in 0.04 M Tris HCl, pH 7.9. EB concentrations vary from 0.008 to 0.100 μM/ml.

TABLE 18

Dependence of Molar Circular Dichroism at 307 nm on Bound Ethidium Bromide to Nucleotide Ratio for EB-poly (A + U)

r bound	Molar Circular Dichroism 307 nm
0.04	2.75
0.06	3.58
0.08	5.15
0.10	7.02
0.12	9.67
0.14	13.21
0.16	15.46
0.18	17.40
0.20	19.35
0.22	20.21
0.24	21.00

[P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9.

however, that the absorption results were obtained at 0.04 M Tris-HCl while fluorescence measurements were carried out in 0.20 M NaCl - 0.20 M Tris-HCl and the values of k are known to vary with ionic strength (56). On the other hand, the two methods agree closely on the number of binding sites for DNA at n equal to 0.20. This may indicate that differences in the values of n obtained by the two methods for poly (A + U) are due to effects of ionic strength on the secondary structure of poly (A + U). Such changes may not occur with DNA.

6. Effect of Ethidium Bromide on the Temperature-Absorbance Profiles of Synthetic Polynucleotides

(a) Double-Stranded Polynucleotides

The effect of ethidium bromide on the temperature-absorbance profiles of several double-stranded polynucleotides provides further evidence for the formation of primary complexes between EB and these polymers. We noted previously (Chapter III, I) that EB stabilizes DNA toward thermal denaturation. The temperature-absorbance profiles of poly (A + U) and poly dAT in 0.04 M Tris buffer (Fig. 72) indicate a similar behavior towards these polynucleotides.

Poly (A + U) exhibits a temperature-absorbance transition in the 55°-65° temperature range with a Tm of 60°. At an EB/P of 0.20, the temperature at which the transition occurs increases to 75°-85° and the Tm to 82°. The Tm of poly dAT is 64° and increases to 92° at an EB/P ratio of 0.50.

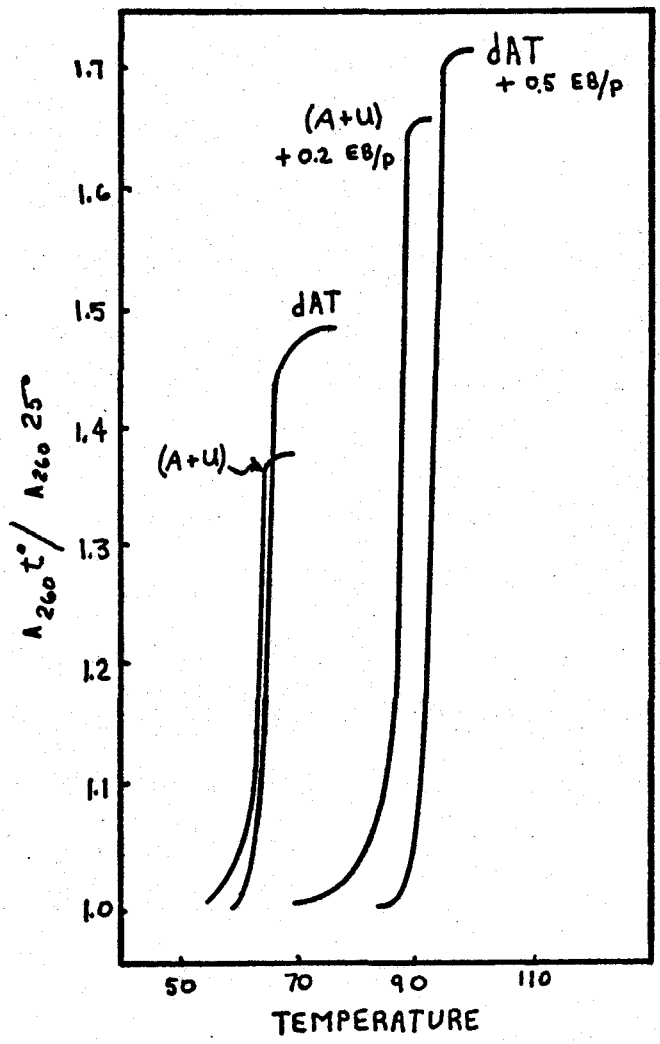


Fig. 72. Effect of ethidium bromide on the temperature-absorbance profiles at 260 nm of poly (A + U) and poly dAT. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB to nucleotide ratios are as indicated.

Yeast RNA which is a heterogeneous mixture of various RNA's, is also stabilized toward thermal denaturation by EB. Yeast RNA (Fig. 73) exhibits a broad transition from 30° to 75°. Almost no transition is observed at an added EB/P of 0.50.

Poly (A +I) (Fig. 73) exhibits a temperature-absorbance transition with a Tm of 41°. This transition which occurs over a narrow temperature range indicates that poly A and poly I form a base-paired stacked structure (85). The presence of EB at an EB/P ratio of 0.20 does not stabilize poly (A +I) toward thermal denaturation indicating that complexes similar to those formed with DNA are not formed with poly (A + I).

(b) Single-Stranded Polynucleotides

The effect of EB on the temperature-absorbance profiles of poly A and poly U is shown in Fig. 74. The temperature-absorbance profiles of poly A and EB-poly A coincide, as do those of poly U and EB-poly U. Thus, EB does not stabilize single-stranded polynucleotides toward thermal denaturation as it does in the case of DNA and other double-stranded polynucleotides including poly dAT and poly (A + U). This may be regarded as further evidence that EB complexes with single-stranded polynucleotides are different than those formed with DNA

The temperature-absorbance profiles and circular dichroism studies indicate that, of the polynucleotides

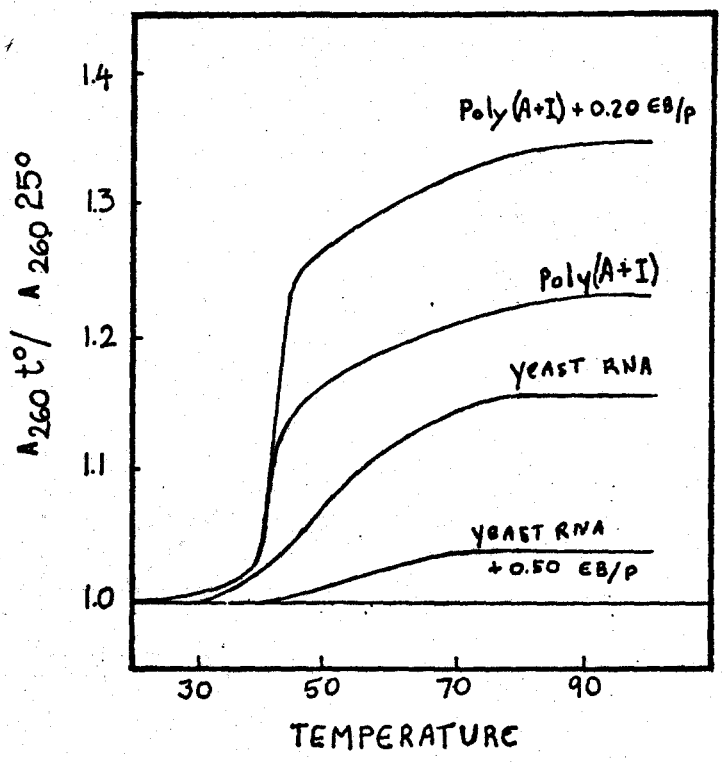


Fig. 73. Effect of ethidium bromide on the temperature-absorbance profiles at 260 nm of poly (A + I) and yeast RNA. [P] = 0.20 μM/ml in 0.04 M Tris-HCl, pH 7.9. Added EB to nucleotide ratios are as indicated.

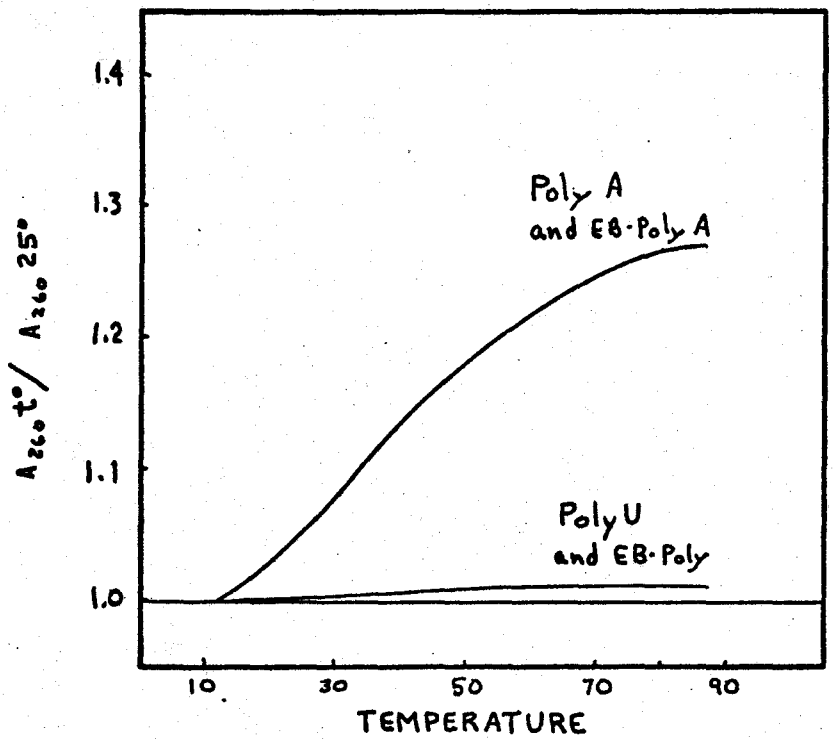


Fig. 74. Effect of ethidium bromide on the temperature-absorbance profiles at 260 nm of poly A and poly U. [P] = 0.20 μM/ml in 0.04 M Tris HCl, pH 7.9. Added EB to nucleotide ratios are as indicated.

examined, poly (A + U), poly dAT, poly dG:dC and yeast RNA may form primary complexes with ethidium bromide similar to that formed with DNA. Double-stranded polynucleotides possessing base-pairing not usually found in naturally occurring DNA or RNA, such as poly (A + I) and poly (I + C) apparently do not form similar complexes.

CHAPTER IV: DISCUSSION

A. THE CIRCULAR DICHROIC PROPERTIES OF THE EB-DNA COMPLEX

The induced bands centered near 307 and 500 nm apparently arise from different sources. The circular dichroism is dependent on r at 307 nm and independent of this ratio at 500 nm.

1. The Origin of the 500 nm Band

At 500 nm, the molar circular dichroism is constant up to about twice the amount of ethidium bromide required to saturate primary binding sites (Chapter III, E1). Since primary sites are saturated at a ratio of about 0.25 (56, 111), additional EB binding above this ratio can only occur at secondary sites. Therefore, EB bound to primary as well as secondary sites contributes equally to the negative circular dichroism of the EB-DNA complex at 500 nm.

Induced circular dichroism in the 500 nm region is also observed for EB interacting with polyvinylsulfate Chapter III, D). In view of the fact that the interaction between EB and polyvinylsulfate is in the nature of secondary binding, the appearance of circular dichroism, in this case, is consistent with the assumption that secondary interactions contribute to the circular dichroism of the EB-DNA complex at 500 nm. The relative independence of the

circular dichroism at 500 nm on temperature under conditions at which strand separation occurs, also supports this conclusion.

The appearance of induced optical activity at low dye to nucleotide ratios and the insensitivity of the molar circular dichroism to this ratio suggests that the induced optical activity in the 450 to 540 nm region, results from asymmetry induced upon the binding of individual EB molecules within the asymmetric environment of the macromolecule. Yamaoka and Resnik (120) reported similar behavior for the DNA-acridine orange complex at low ratios (r = 0.001-0.002). In addition, it has been reported that one of the bands induced upon the interaction of an analog of proflavine with DNA is independent from dye to nucleotide ratios (60).

2. <u>The Origin of the 307 nm Band</u>

The behavior of the circular dichroic band at 307 nm differs from that at 500 nm. Circular dichroism at 307 nm increases with increasing ratios of EB bound to nucleotide. Maximum ellipticity is noted at a ratio of 0.30 which corresponds with the saturation of primary binding sites in DNA (Chapter III, E3).

This value of r is slightly higher than the generally noted value of 0.20-0.25. However, as previously noted (Chapter III, E2), the proportion of bound EB is measured by absorption spectroscopy and therefore contributions resulting from secondary binding are included in

this value of r. Consequently, the molar circular dichroism reaches a maximum value at a total bound EB to nucleotide ratio higher than that corresponding to the saturation of primary binding sites alone.

A similar dependence of the induced optical activity on bound dye to nucleotide ratio has been reported for the DNA-proflavine (8, 23) and the DNA-acridine orange (122) systems (Chapter I, H). In each of these systems, the DNA-dye interaction results in the induction of a predominant circular dichroic band. The molar circular dichroism of the respective bands increases with increasing amounts of bound dye and reaches a maximum value at a bound dye to nucleotide ratio of about 0.25. This ratio corresponds well with the saturation of the primary binding sites of DNA in these systems.

At low dye to nucleotide ratios, EB is bound only to primary binding sites (Chapter I, C3a) (56,111). The negligible amount of optical activity in the DNA-EB complex at 307 nm at very low EB to nucleotide ratios ($r < 0.005$) indicates that isolated EB molecules bound to primary binding sites do not contribute to the induced circular dichroism at this wavelength. Under these conditions, the probability that one EB molecule would be intercalated in a position adjacent to another is low. This probability, however, increases with increasing ratios of bound EB.

The continuous increase in ellipticity with increasing EB to nucleotide ratios which we have noted, suggests that the development of the 307 nm circular dichroism is the result of nearest-neighbor interactions between two or more EB molecules bound to adjacent primary binding sites.

3. The Dependence of Circular Dichroism Upon Formation of Dye Clusters

The binding of small molecules to adjacent binding sites on a polymer in groups of two or more, may be treated on a statistical basis (19). From this type of treatment, it is possible to determine the probability of two or more dye molecules binding to adjacent primary sites. It is also possible in principle, on the basis of the observed relation between bound dye to DNA ratios and the observed circular dichroism, to determine the number of interacting dye molecules in groups of a specific size.

The fraction of dye molecules that are bound at n adjacent sites may be calculated as follows: Let N equal the total number of binding sites and γ be the probability of any site being occupied. The probability that n sites will be occupied in sequence with adjacent sites empty is $\gamma^n (1-\gamma)^2$ and the number of molecules in such groups is then $nN\gamma^n (1-\gamma)^2$. The total number of molecules in runs or clusters of n or more is:

$$\sum_{n=n}^{n=\gamma} nN\gamma^n (1-\gamma)^2 = nN\gamma^n + N(1-n)\gamma^{n+1}$$

and the fraction of molecules which are bound in such runs is:

$$\frac{nN\gamma^n + N(1-n)\,\gamma^{n+1}}{\text{Total Number of bound molecules}}$$

Since the ratio ($\frac{\text{total number of bound molecules}}{N}$) is equal to γ , the fraction, Θ_n, of EB molecules bound in groups or clusters of n or more may be expressed as:

$$\Theta_n = n\,\gamma^{n-1} + (1-n)\,\gamma^n$$

The fraction, Θ_n, of molecules bound in groups of n, equal to 2, 3 and 4, is plotted as a function of γ in Fig. 75. The values for Θ_n are calculated assuming that the bound ligand, in this case EB, will be distributed randomly throughout the polymer. Binding parameters, i.e. the dissociation constant and the number of primary binding sites, for EB complexes calculated on the basis of Scatchard plots, have been shown not to vary with DNA's of varying guanosine + cytosine content (56,111). Thus, since EB does not exhibit any preference for specific binding sites in DNA, random distribution of the ligand is a reasonable assumption in this system.

Clearly, the dependence of ellipticities on bound EB to nucleotide ratio which we have noted, does not correspond exactly to any of the calculated probability functions shown in Fig. 75. This apparent discrepancy may be the result of either one of two reasons. First, it is

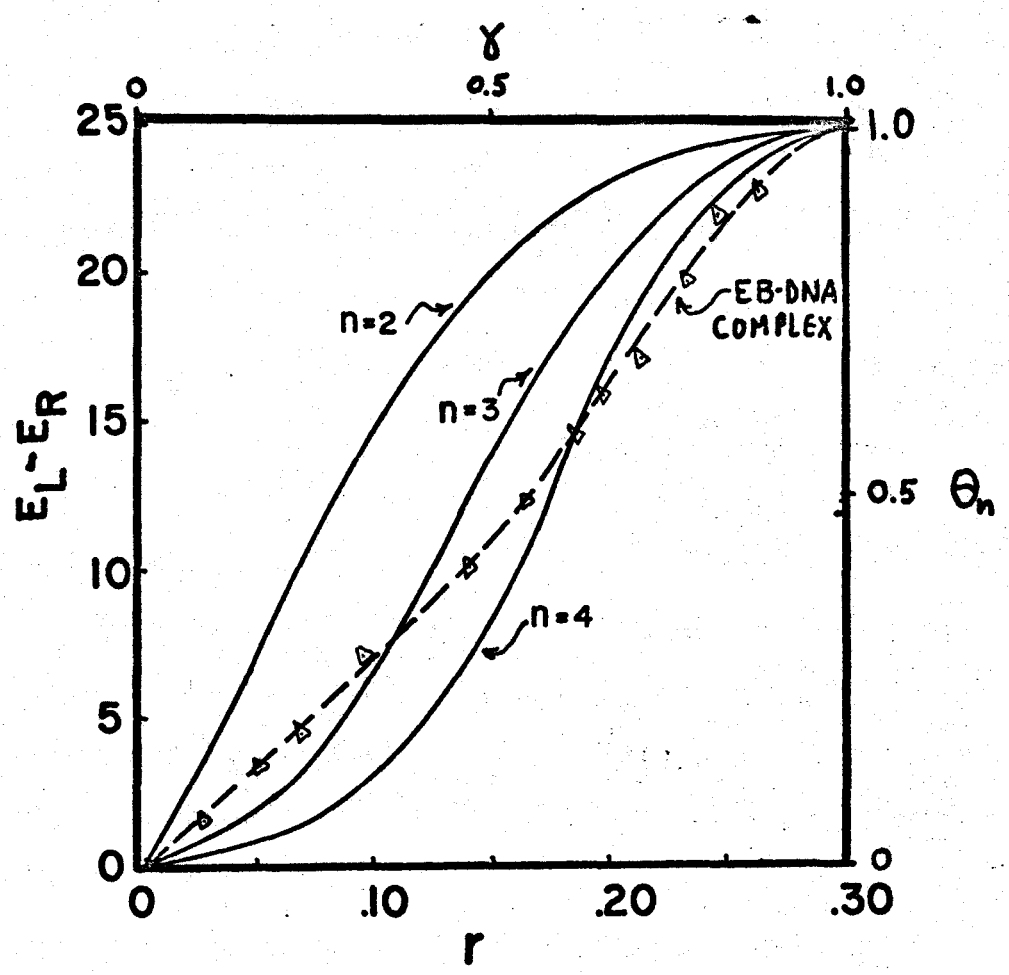


Fig. 75. Probability functions for the formation of "clusters" of dye molecules bound to a polynucleotide. The values of n equal to 2, 3 and 4 refer to the probability that n dye molecules bind to adjacent primary sites. The data for the EB-DNA complexes are reproduced from Fig. 26.

possible that "clusters" of three and four EB molecules coexist and the contribution of clusters consisting of different numbers of EB molecules, varies with bound-EB to nucleotide ratios. A second reason for this behavior, may be the fact that in the case of EB, the probability functions (Θ_n) are calculated with the assumption that primary binding site saturation occurs at a dye to nucleotide ratio of 0.30. Since this assumption is not completely correct, calculations made on this basis would be expected to distort the shape of the probability functions.

At first examination, the circular dichroism results in the presence of magnesium (Fig. 30) would appear to be better suited for comparison with the probability functions. The bound EB to nucleotide ratio at which the molar circular dichroism reaches a maximum, decreases from 0.30 in the absence of magnesium to 0.25 in the presence of magnesium. The ratio of 0.25 corresponds exactly with the saturation of primary binding sites calculated by other methods.

However, the maximum molar circular dichroism attained is decreased from 25.0 to 22.5 respectively in the absence and presence of magnesium ions. This suggests that magnesium alters the nature of the EB-polynucleotide interaction in a manner that may limit maximum nearest-neighbor interaction. A direct effect on nearest-neighbor interactions is not likely, since the dependence of the molar circular dichroism on bound EB ratio is not effected by the

presence of magnesium up to a ratio of 0.25. However, magnesium ions may decrease the number of primary binding sites in DNA. It should be noted that increased ionic strength or temperature have been reported to decrease the number of primary-DNA binding sites (29), and alter the circular dichroism of the proflavine-DNA complex (60).

Since the structure of dye-DNA primary complexes appears to be sensitive to environmental conditions, and these conditions can affect the circular dichroism of the complex, the lack of a strict correlation between the observed ellipticities and any one of the derived Θ_n functions is not surprising.

4. Additional Evidence for the Formation of EB "Clusters"

The behavior of the circular dichroism of the EB-DNA complex at elevated temperatures and extremes of pH gives further support to the concept of nearest-neighbor interactions between bound EB molecules.

At added EB to nucleotide ratios between 0.05 and 0.40 and temperatures exceeding those at which DNA strands begin to separate, a rapid decrease in the concentration of bound EB and concomitant increase in the molar circular dichroism are observed (Figs. 38-44). This may be interpreted by assuming that clusters of bound EB molecules are more resistant to thermal disruption than regions interacting only with isolated EB molecules. The increase in molar

circular dichroism at temperatures exceeding that at which the DNA strands begin to separate could then be attributed to dissociation of isolated EB molecules from the EB-DNA complex. Clearly, the dissociation of such EB molecules would increase the relative content of EB in the form of clusters.

The effect of this change on the molar circular dichroism of the complex may be more clearly discussed by referring to Fig. 76. This figure presents a hypothetical distribution of EB in the EB-DNA complex at bound EB to nucleotide ratio of 0.20. The actual distribution of EB molecules throughout the helix, of course, is not known. In addition to isolated EB's, EB molecules occupying from two to four adjacent binding sites are shown.

At elevated temperatures, strand separation may first occur around regions accommodating isolated EB molecules. DNA regions containing "clusters" may remain intact at elevated temperatures resulting in a considerably higher amount of EB in the form of clusters than that present prior to heating. Thus, the observed increase in the molar circular dichroism at 307 nm, at 95°C, may simply reflect the relative increase of EB clusters and the concomitant increase in nearest-neighbor interactions.

The increase in circular dichroism between pH 11.0 and 11.7 (Fig. 49) may also be viewed as a result of a relative increase in the EB present in the form of clusters.

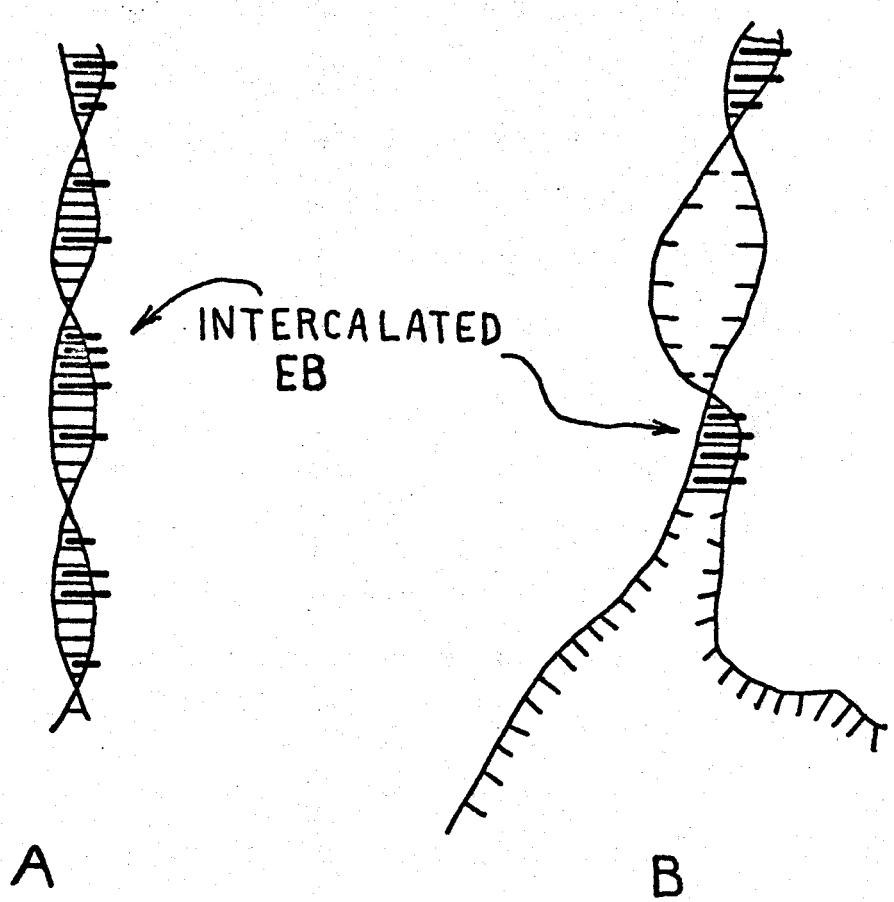


Fig. 76. A scheme proposed to explain the effect of temperature on the circular dichroism properties of EB-DNA primary complex. (A) a hypothetical distribution of bound EB at a ratio of 0.20 and (B) the same complex at elevated temperatures.

Addition of EB to partially denatured DNA at pH 11.7, favors the formation of clusters since only those regions containing a large proportion of hydrogen bonded guanosine-cytosine base pairs remain intact at this pH (50).

The bound dye to nucleotide ratio at which the molar circular dichroism reaches a maximum value corresponds to the saturation of primary binding sites. This correlation is clearly shown in Fig. 50, in which the ratio at which maximum circular dichroism is observed, decreases from 0.30 at pH 11.3 to 0.16 at pH 11.7.

Partial alkaline denaturation of DNA occurs in the adenine-thymine rich regions (2), which make up 58 percent of the total bases in calf thymus DNA. Therefore, a decrease of about 50 percent in the amount of double-stranded regions is expected in partially alkaline denatured DNA. This decrease in double-stranded regions will result in a concomitant decrease in the number of primary DNA binding sites since primary binding occurs only with double-stranded polynucleotides. At pH 11.3, just below the pH required for alkaline denaturation of A-T regions in DNA, the number of primary binding sites corresponds to an r of 0.30. However, at pH 11.7, approximately 50% of these binding sites are denatured and the number of primary binding sites decrease to about 0.16.

The decrease in EB binding at acidic pH may be the result of the effect of hydrogen ion concentration on either

the ethidium molecule, the DNA, or both. The pK of primary amine substituents of aromatic compounds as present in EB is in the 4-5 range (97). Thus, the decrease in the bound EB to nucleotide ratio at increasing hydrogen ion concentration (Fig. 49) is probably not directly due to protonation of the amino groups of EB. Furthermore, since neither the absorption nor the circular dichroism spectra of ethidium bromide are appreciably modified in the pH range between 3.0 and 12.0, the effect of pH on the EB molecule may be ruled out as a factor contributing to the decrease in the ability of EB to bind to DNA between pH range 4 to 5.

At pH below 5.0, protonation of the EB amino groups at the 3 and 8 positions of the phenanthridinium ring takes place. This would be expected to enhance EB binding. Induction, by resonance, of partial positive charges on the amino groups of aminoacridine dyes enhances the ability of the dyes to form primary complexes with DNA (58). A similar structure would be expected in the case of ethidium bromide. The +1 charge on each amino group resulting from EB protonation is somewhat different than the $\delta+$ due to resonance. Both phenomena, however, may be expected to have similar effects on EB primary binding.

These observations do not rule out the possibility that the circular dichroism below pH 5.0 is due to the effect of pH on the electronic properties of EB. However, it seems more likely that the decrease in both bound EB and

molar circular dichroism between pH 5.0 and the onset of the helix-coil transition near pH 3.5 are the result of changes in DNA secondary structure induced by protonation of the bases.

5. Nearest-Neighbor Interactions: Two Alternative Interpretations

We have interpreted the dependence of the molar circular dichroism at 307 nm on the bound EB to nucleotide ratio as indicative of the existence of nearest-neighbor interactions. Based on DNA-proflavine studies, two possibilities have been proposed to explain the dependence of circular dichroism on the amount of bound dye (7-9). (i) Interactions occurring between DNA-bound dye molecules may increase as the number of molecules within a group increases. (ii) The progressive binding of dye molecules in the vicinity of molecules already bound, may continuously alter the conformation of the DNA polymer. In this latter case, induced optical activity would be the result of interaction of dye molecules with DNA base pairs rather than with other dye molecules. In either case, the environment of a particular bound ligand is obviously determined by the number of other bound ligands in the same vicinity.

Unfortunately, the statistical approach presented in a previous section, although it gives an indication of the magnitude of the circular dichroism which may be expected from clusters of a given size, cannot distinguish between these two possibilities.

Direct interaction between EB molecules bound in clusters, is somewhat difficult to visualize since neighboring dye molecules are separated by hydrogen bonded pairs. In fact, fluorescence studies indicate that energy transfer does not occur between bound EB molecules (56). The possibility of course, exists that interaction takes place between the phenyl groups of neighboring EB molecules. Indeed, examination of the CPK space-filling model (Fig. 77) of the EB-DNA primary complex does not rule out this possibility. However, proflavine which does not contain a similar substituent also gives rise to nearest-neighbor interactions upon binding to DNA (7-9, 59). Although investigations with ethidium analogs which do not contain a 6-phenyl substituent would resolve this question, interactions between phenyl groups do not appear to be an essential part of the interaction between DNA-bound EB molecules.

Thus, if we assume that EB molecules are situated in relation to the helix, in a way which would prevent the interaction between adjacent phenyl groups, it would be difficult to understand how EB chromophores interact. It is improbable that electronic interactions are transmitted either through or around the intervening base-pairs (103). In this sense, the alternative interpretation as in (ii) might be preferable. In this interpretation, the addition of a second EB molecule alters the electronic environment of an EB molecule already bound to an adjacent primary

Fig. 77. CPK space-filling model depicting the stereo-chemical relationship between two EB molecules intercalated at adjacent binding sites.

binding site and the addition of a third EB molecule alters the environment of the second EB molecule, but probably does not affect the first (23).

This explanation appears attractive in view of the report that factors which influence the electronic environment of the proflavine-DNA complex, such as increased ionic strength and temperature, also affect its circular dichroism (60). These factors are believed to influence the circular dichroism by changing the degree to which the helix unwinds around the intercalated dye molecule and thus altering the environmental asymmetry around the intercalation site. These changes in asymmetry may alter the relative electronic relationships between the base-pairs and the intercalated dye molecule (60).

6. The Possibility of Existence of a "Third" Type of Binding

Nearest-neighbor interactions appear enhanced for the EB-denatured DNA primary complex. This enhancement may be due to an increase in the formation of clusters resulting from the lower relative content of double-stranded regions in denatured DNA.

The circular dichroism results obtained with DNA indicate that the ethidium complexes with native and denatured DNA may be similar. Both the shape of the induced bands, as well as the wavelength of the maximum at 307 nm and shoulder at 335 nm, are similar for the two complexes.

However, some indications for a different behavior between native and denatured DNA toward EB also exist. The dependence of circular dichroism, for example, expressed either as E_L-E_R (Fig. 32) or Δ absorbance (Fig. 33) on the bound EB to nucleotide ratio are different for the EB-DNA and EB-denatured DNA complexes.

The results obtained by spectrophotometric techniques (111) also indicate a different behavior of ethidium bromide toward native and denatured DNA. The Scatchard plot for denatured DNA deviates from linearity at ratios of bound EB to nucleotide as low as 0.10 (111). This suggests that at these low ratios, either primary binding at one site in denatured DNA may affect the binding at a nearby site or that binding occurs at more than one type of binding site. Furthermore, fluorescence measurements indicate that the maximum fluorescence enhancement observed for ethidium bromide bound to denatured DNA is only about one-half that noted for native DNA (56). This is not surprising since fluorescence enhancement apparently depends only on EB bound to primary sites. Denatured DNA, of course, possesses considerably fewer hydrogen-bonded regions of the type required for primary binding.

The increase in the ratio of bound EB to nucleotide, at which maximum dichroism is observed, from 0.30 in native DNA to 0.36 in denatured DNA, supports the notion that an additional type of binding, distinct from primary or secondary binding, may be present in the denatured polynucleotide.

Assuming that primary binding occurs only at double-stranded regions, the number of primary binding sites in denatured DNA would be expected to decrease. For native DNA, the ratio at which the maximum E_L-E_R occurs corresponds approximately to the saturation of primary binding sites. In the case of the EB-denatured DNA complex, however, the expected decrease in the number of primary sites is not reflected by a corresponding decrease in the r at which maximum E_L-E_R is observed.

These results suggest that induced circular dichroism in the spectral region from 300 to 350 nm may occur upon the interaction of EB with regions in denatured DNA which are not double-stranded. Since secondary interactions do not result in the appearance of induced circular dichroism in this spectral region, the possibility of an EB-polynucleotide interaction different from that resulting from the postulated primary or secondary binding is suggested. This possibility is discussed in more detail in the following sections dealing with the interaction of ethidium bromide with synthetic polynucleotides.

B. POLYNUCLEOTIDE STRUCTURAL REQUIREMENTS FOR COMPLEX FORMATION

The preceding discussion has dealt primarily with the circular dichroism of the EB-DNA primary complex with respect to the origin of the 307 nm and 500 nm circular dichroic bands and the nature of nearest-neighbor

interactions between DNA-bound EB molecules. Examination of the circular dichroism of ethidium bromide complexes with synthetic single and double-stranded polynucleotides, gives information on polynucleotide structural requirements for complex formation.

1. Double-Stranded Polynucleotides

The circular dichroism of ethidium bromide complexes with double-stranded polynucleotides possessing base pairing similar to that found in DNA and RNA, such as poly dAT (Figs. 59,60), poly (A + U) (Figs. 61,62) and poly dG:dC (Figs. 65,66), is similar to that of EB-DNA complexes. The magnitude of the circular dichroism at 307 nm increases in each instance with increasing added ethidium bromide to DNA ratios up to 0.50. This behavior may be an indication that the primary complexes formed between EB and poly dAT, poly (A + U) or poly dG:dC are structurally similar to the primary complex formed with DNA. The close agreement in ellipticities for EB-DNA and EB-poly (A + U) complexes at each ratio of bound EB to nucleotides up to 0.25, is a further indication of this structural similarity. Blake and Peacocke, on the basis of ORD studies, have reached a similar conclusion regarding the proflavine-DNA and proflavine-poly (A + U) complexes (9).

The nearest-neighbor interactions responsible for the development of induced circular dichroism in the 300 to 360 nm region in the EB-DNA complex would also be expected

to occur with EB-poly dAT, EB-poly (A + U) and EB-poly dG:dC complexes. The circular dichroism above 300 nm of the poly dAT and poly dG:dC complexes indicate that the EB complexes with double-stranded polynucleotides are similar for both the ribo- and the deoxyribo-structures. The similarities in the excitation spectra (6, 56) and Scatchard plots (6, 111) for DNA and transfer RNA support the idea that EB-DNA and EB-RNA primary complexes are also similar.

2. Interaction of Ethidium Bromide with Polynucleotides Possessing Unusual Base Pairing

The situation is different, however, for polynucleotides possessing unusual base-paired structures. The circular dichroism indicates that complexes formed between ethidium bromide and poly (A + I) (Fig. 67), poly (I + C) (Fig. 68) or double-stranded poly A at pH 5.6 (Fig. 70) are different from the complexes formed with DNA.

The circular dichroism of EB-poly (A + I) and EB-poly (I + C) indicate the existence of interactions between ethidium and these polynucleotides. This is confirmed by the results of spectrophotometric studies (112). However, the circular dichroism between 300 and 360 nm is not characteristic of an EB-DNA primary complex since a distinct maximum such as that noted for EB-poly (A + U), EB-poly dG:dC and EB-DNA is not present. Instead, a closer similarity to the circular dichroism of EB complexes with

single-stranded polynucleotide poly C, poly I, and poly U is noted.

It should be noted that the absence of primary EB binding to poly (A + I) or poly (I + C) similar to that which occurs for EB-DNA is not likely to be due to preferential binding of EB to sites comprised of specific base pairs such as adenine-thymine or guanosine-cytosine. The results of both absorption (111) and fluorescence (56) studies indicate that ethidium bromide binds equally well to DNA's with guanosine + cytosine content that varies from 35 to 72%. The results obtained with poly dAT, poly (A + U) and poly dG:dC also indicate that ethidium forms primary complexes with these polynucleotides. Since no indication of base specificity was observed with either these polynucleotides or the DNA's, it is unlikely that preferential binding is the reason for the absence of nearest-neighbor interactions in the EB-poly (A + I) and EB-poly (I + C) complexes.

A more plausible explanation for this behavior between ethidium and poly (A + I) or poly (I + C) is that the geometry of the hydrogen-bonded base-pair in these polynucleotides is such that it does not permit intercalation of EB in a manner which promotes nearest-neighbor interactions.

3. Hydrogen Bonding in Intercalated Ethidium Bromide

In this connection, it might be useful to examine the geometry of primary binding sites in the model of the EB-DNA complex proposed by Fuller and Waring (Chapter I, E2) (35). The intramolecular distance between the C'-1 carbons of the deoxyribose moieties of the opposite DNA strands is 11 Å (Fig. 78) (116). A similar distance separate opposite polynucleotide strands between adenine and thymine and adenine and uracil in poly dAT and poly (A + U). The distance between the amino nitrogens of ethidium, 11 Å, is of the same magnitude.

As previously indicated (Chapter I, D6) both hydrogen bonding and hydrophobic forces participate in the stabilization of the primary complex between ethidium bromide and DNA. Furthermore, hydrogen bonding between the ethidium amino groups and the phosphate groups of DNA may orient the ethidium ring system within the binding site in a manner most favorable for stabilization of the complex. The overall distance between the phosphate oxygens in the EB-DNA complex (14.8 Å) (35) is nearly optimal for the occurence of hydrogen bonding interaction with the amino groups of the ethidium moiety. Examination of space filling models indicates that hydrogen-bonding may orient the phenanthridinium ring in such a manner as to become almost completely immersed in the interior of the double helix.

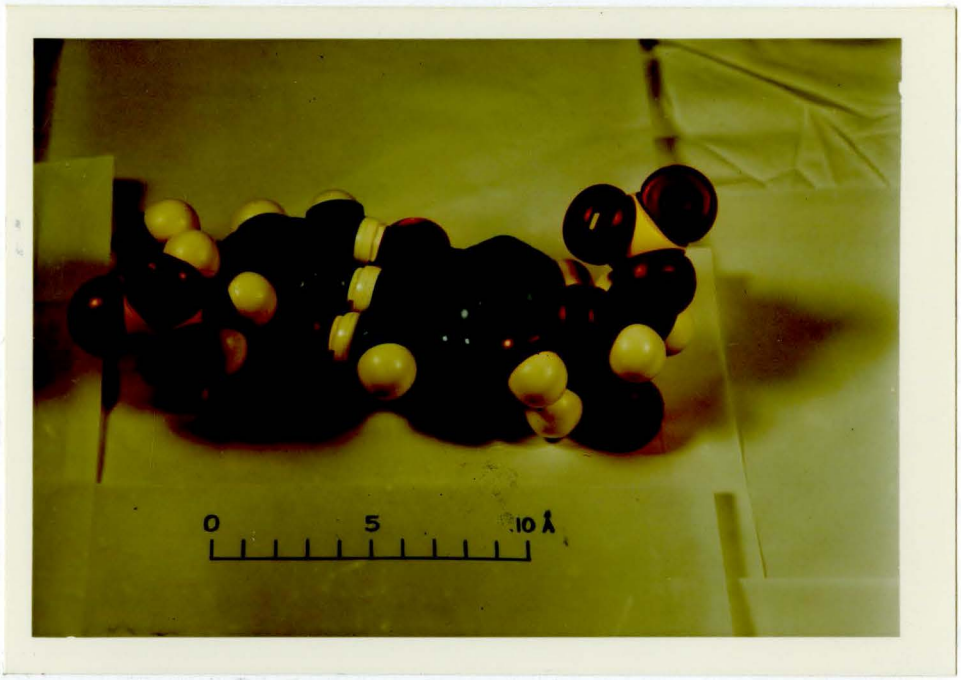


Fig. 78. Top view of a guanosine-cytosine hydrogen bonded base pair as it occurs in DNA.

Studies of the binding of proflavine and proflavine analogs to DNA (Chapter I, C) confirm the importance of such hydrogen bonding for the stabilization of the primary complex (9, 62). In general, proflavine analogs with structures that interfere with hydrogen bond formation dissociate more easily from DNA than proflavine and do not promote nearest-neighbor interactions (29,62).

Hydrogen bonding between the amino groups in ethidium and DNA phosphate oxygens on opposite polynucleotide strands cannot take place in poly (A + I). Hydrogen bonding in this nucleotide occurs between purines. This results in an intramolecular distance between phosphate oxygen on opposite strands of about 18 Å (Fig. 79) which is 3.5 Å greater than that between guanosine and cytosine or adenosine and thymidine in poly dG:dC and poly dAT respectively.

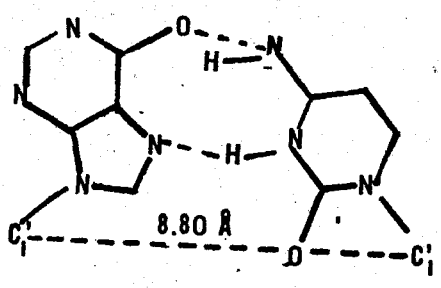
Although ethidium is not expected to form an EB-poly (A + I) primary complex identical to the EB-DNA or EB-poly (A + U) complexes, intercalation may still occur. The forces which contribute to the stabilization of the primary complex double-stranded polynucleotides may also be operative in the EB-poly (A + I) complex. However, hydrogen bonding between both ethidium amino groups and the phosphate of opposite polynucleotide strands, which may occur with DNA, is not possible in the case of EB-poly (A + I). The intramolecular distance between the two polynucleotide

Fig. 79. The interaction between ethidium bromide and poly (A + I). (A), view of an adenine-inosine hydrogen-bonded base pair as it may occur in poly (A + I); (B), a possible spatial relationship between the ethidium bromide amino groups and the phosphate moieties in opposite poly (A + I) strands.

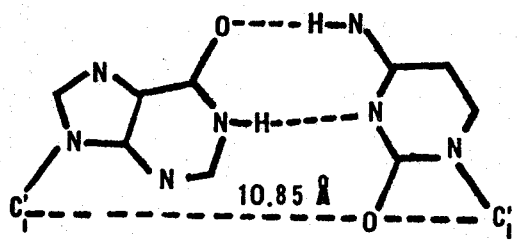
strands is too large to permit both amino groups in the ethidium ring to simultaneously participate in hydrogen bonding.

Waring (112) and Le Pecq (55) have reported that relatively strong binding occurs between EB and poly (A + I). However, the absence of a strong induced circular dichroism upon formation of the EB-poly (A + I) complex suggests that intercalation alone may not be sufficient for formation of a primary complex with a geometry similar to that of the complex formed between EB and DNA. Hydrogen bonding may be important in the orientation the ethidium molecule within the primary binding site in a manner which promotes nearest-neighbor interactions.

Similarly, poly (I + C) apparently does not form primary complexes with either ethidium bromide or proflavine (102) similar to the EB-DNA complex. The circular dichroism results that we have obtained, as well as spectrophotometric measurements (112), confirm this conclusion. If hydrogen bonding between poly I and poly C were of the Watson and Crick type (116,118) i.e. if it occurred between inosine and cytosine at the same sites as those involved between the guanosine-cytosine pair (Fig. 8OB), the intermolecular distance between poly I and poly C should be the same as that between guanosine and cytosine and adenine and thymidine in poly dG:dC and poly dAT. Base pairing of this type should therefore, permit intercalation of the ethidium ring system. However, this apparently is not the case.



(A)



(B)

Fig. 80. Two possible alternatives for hydrogen bonding in poly (I + C). (A) Hoogsteen type (B) Watson-Crick type.

It is possible that hydrogen bonding between poly I and poly C occurs as proposed by Hoogsteen (Fig. 80A) (45, 118) with bonds extending between the N_7 and O_6 of inosine and the N_1 and N_7 of cytosine. Under these conditions, it is unlikely that EB intercalation could take place since phosphate oxygen on opposite strands would be spearated by 11 Å which is about 4 Å less than the corresponding distance of 14.8 Å in DNA. Examination of these structures indicates that hydrogen bonding between EB amino groups and phosphate oxygen groups in poly (I + C) cannot occur.

The circular dichroism results indicate that very little interaction may take place between ethidium bromide and double-stranded poly A at pH 5.6. In this system, intercalation of ethidium may be prevented by the lack of flexibility of the poly A double helix (86). At this pH, the opposite strands are parallel and bases are tilted with respect to one another (Chapter III, N4c). These factors may prevent the insertion of the phenanthridinium ring between base pairs.

Furthermore, even if intercalation would take place, proper hydrogen bonding between EB and the phosphate residues would not be possible. The intercalation of EB requires the backrotation, i.e. local unwinding, of the phosphate-sugar backbone (Fig. 7, 10) in order to provide space for the phenanthridinium ring system. In double-stranded poly A at pH 5.6, the strands are held together

by hydrogen bonds occurring between N_{10} and N_7 on one hand, and oxygen on the phosphate on the other hand. Hydrogen bonding of this nature prevents the separation of adjacent base pairs necessary for accommodating EB.

Clearly then, some insight on the nature of the polynucleotide structural requirements for primary complex formation may be obtained from studies of the interaction of ethidium with synthetic polynucleotides. Polynucleotides possessing base-pairing similar to that in DNA, such as poly dAT and poly dG:dC, or RNA, such as poly (A + U), form primary complexes with ethidium bromide that may be similar to the EB-DNA complex. These complexes exhibit induced circular dichroism between 300 and 360 nm with a distinct maximum at 307 nm. The dependence of the molar circular dichroism of the EB-poly (A + U) complex at 307 nm or the bound EB to nucleotide ratio is similar to that noted for the EB-DNA complex which indicates that nearest-neighbor interactions between intercalated ethidium molecules may also be involved in this system.

Thus, the results of the circular dichroism obtained with synthetic double-stranded polynucleotides are in good agreement with the model for primary binding of ethidium to DNA proposed by Fuller (35), which postulates complete intercalation of the phenanthridinium ring. If primary binding of ethidium involved only partial rather than complete intercalation, then EB would be expected to form

primary complexes with polynucleotides such as poly (A + I) and poly (I + C) similar to the EB-DNA complex. The results of the circular dichroism studies with these polynucleotides, as well as with double-stranded polynucleotides that have interstrand distances which allow hydrogen bonding between both ethidium amino groups and DNA phosphate, indicate that only the latter may participate in primary binding by intercalation in which the geometry of the complex promotes nearest-neighbor interactions.

4. The Interaction of Ethidium Bromide with Single-Stranded Polynucleotides

Both absorption (112) and fluorescence (55) studies indicate that primary binding of ethidium bromide to single-stranded polynucleotides does not occur. However, induced circular dichroism is observed in the region between 300 and 360 nm. EB though, in the presence of poly C, poly U, or poly I does not exhibit the distinct circular dichroism maximum at 307 nm noted in the EB-DNA complex. The appearance of circular dichroism is not surprising since the absorption results (112) have indicated that some interaction occurs between ethidium and single-stranded polynucleotides.

Since primary binding is not possible in these systems, it may be argued that secondary binding is responsible for the noted induced circular dichroism in the spectral region above 300 nm. However, based on the

observation that upon interaction of EB with either polyvinylsulfate or poly A, no induced circular dichroism in the 300 to 350 nm region appears, it is reasonable to assume that the development of circular dichroism in this region is not the direct result of secondary binding either. In the case of poly A, spectrophotometric studies have indicated that weak interaction with EB does occur, but the lack of any induced circular dichroism or fluorescence enhancement (56) indicates that this interaction cannot be described as primary binding. Furthermore, only secondary interactions may occur between EB and a polyanion such as polyvinylsulfate.

The appearance of induced circular dichroism also indicates that ethidium bromide interacts with cytosine, uridine and inosine homopolymers. Primary binding cannot take place in these systems either, and secondary binding, at least such as that occurring with polymeric structures of the polyvinylsulfate type, does not induce circular dichroism in the spectral region between 300 and 350 nm. It is therefore, likely that the interaction between EB and these polynucleotides may be of different character.

5. Modified Intercalation between Ethidium Bromide and Single-Stranded Polynucleotides

While induced circular dichroism may not be the direct result of secondary interactions between EB and the polynucleotide phosphate groups, electrostatic forces may

place the ethidium molecule in the vicinity of the polynucleotide phosphate in a geometrical arrangement similar to that proposed by Peacock (Chapter I, D7) for acridine intercalation in DNA (Fig. 13) (82).

The stereochemical relationship between EB and poly U (Fig. 81) may depend upon both electrostatic interactions between the quarternary nitrogen on the ethidium bromide and the phosphate groups of poly U and the partial intercalation of the phenanthridinium ring between adjacent uracil bases. The forces which stabilize such a complex would be considerably weaker than those maintaining the EB-DNA complex. Partially intercalated ethidium is under the influence of both hydrophobic and ionic forces. In this position, the ethidium ring may only interact with the ring systems of individual uracil bases. This is in sharp contrast to the situation present in the EB-DNA complex in which the ethidium ring is completely intercalated between the DNA base pairs.

It should be noted that the available evidence does not permit the selection of one among various geometric configurations possible for this model. The proposed arrangement simply describes one stereochemically acceptable possibility.

The interaction of ethidium bromide with poly (A + I) and poly (I + C) may take place in a manner similar to that postulated for poly U. The similarities in the

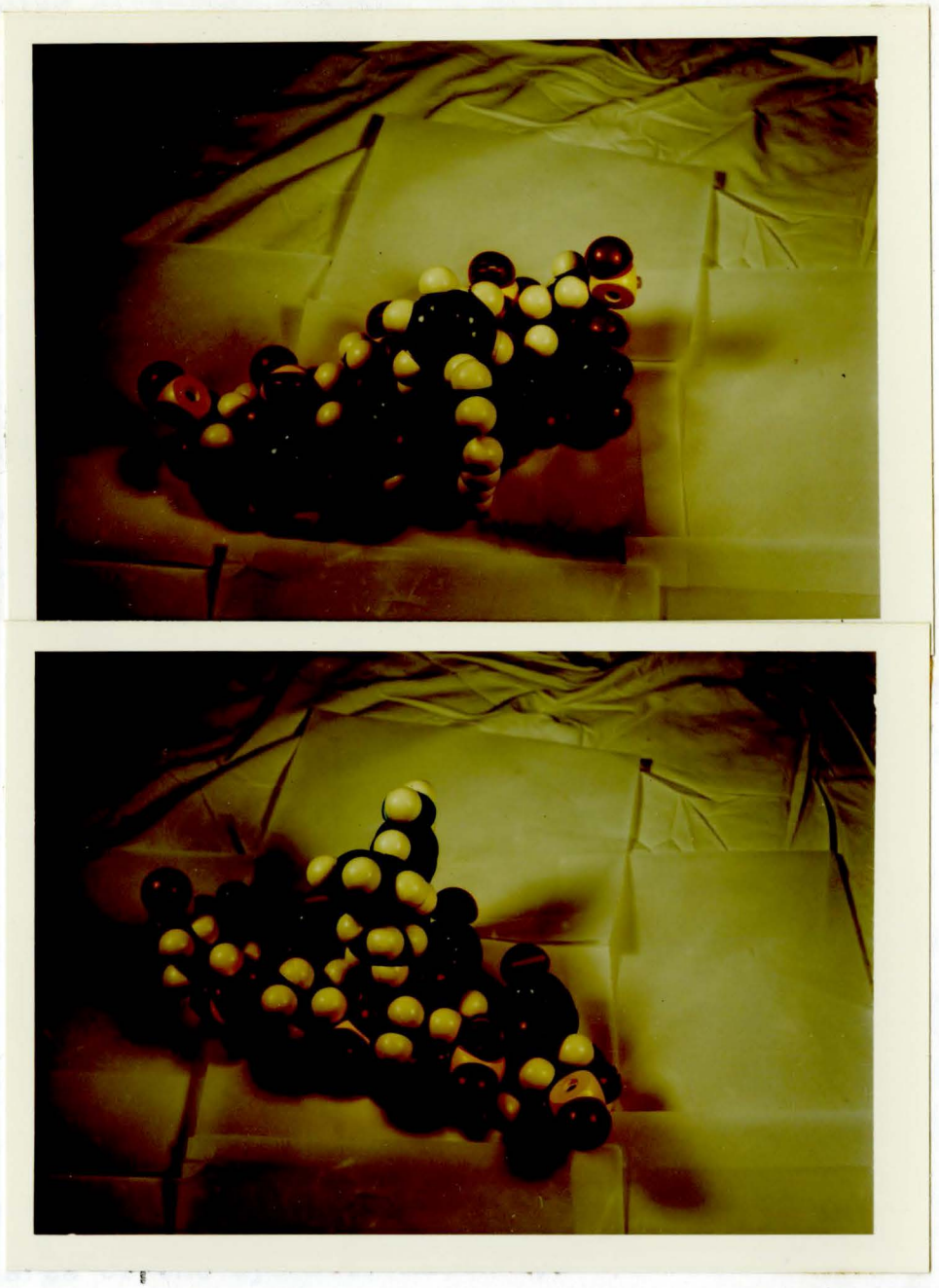


Fig. 81. Two views of a possible stereochemical relationship between ethidium bromide and poly U. The phenanthridinium ring of EB is partially intercalated between adjacent uracil residues in poly U.

circular dichroism spectra of EB-poly U and EB-poly (A + I) suggest that these complexes may be similar to each other.

As previously noted, the interstrand distance in poly (A + I) is too large to permit hydrogen bonding between the amino groups of EB and phosphate groups of opposite polynucleotide strands. The available evidence does not permit a clear distinction between structures in which EB is partially intercalated and completely intercalated between adjacent base pairs in poly (A + I). The second possibility, however, receives support from the observation that higher ethidium bromide concentrations are required, than is the case with DNA, to achieve a given ratio of bound EB per DNA nucleotide (110). The dissociation constants, from Scatchard plots, are 5.5×10^{-7} and 40×10^{-7} for EB-DNA and EB-poly (A + I) respectively. This indicates that while binding of EB to poly (A + I) is not as strong as that occurring between EB and DNA, it is considerably stronger than that noted for single-stranded polynucleotides. Furthermore, hydrophobic forces in general favor complete insertion of the phenanthridinum ring since this maximizes the area of the dye over which hydrophobic forces are manifested (35).

In conclusion, the binding of ethidium bromide to DNA and other double-stranded polynucleotides possessing similar base pairing, such as poly (A + U), poly dAT and poly dG:dC, results in the induction of a strong dichroic

band centered near 307 nm. The strong dependence of the molar circular dichroism at 307 nm on the bound EB to nucleotide ratio for the EB-DNA and EB-poly (A + U) complexes indicates that this band originates from nearest-neighbor interactions resulting from the binding of EB molecules to adjacent primary sites. The large increase in the molar circular dichroism at a given bound EB ratio, under conditions of temperature and pH which decrease the relative amount of double-stranded regions in DNA, is regarded as further evidence that the nearest-neighbor interactions are responsible for the 307 nm band.

Increased circular dichroism at 307 nm is also noted for the EB complex with ice quenched heat denatured DNA. However, an apparent increase occurs in the number of primary binding sites compared to native DNA. This observation as well as the circular dichroism results of EB complexes with poly C and poly U suggest that a partial intercalation of EB may occur with single-stranded polynucleotide regions.

GLOSSARY

Δ Absorbance: Difference in absorbance between left and right circular polarized light.

EB/P: Molar ratio of total added ethidium bromide to nucleotide phosphate.

E_L-E_R: Molar circular dichroism; the difference in molar extinction coefficients between left and right circular polarized light. In this thesis, molar circular dichroism is calculated on the basis of the concentration of either nucleotide phosphate or bound ethidium bromide as specified.

Hyperchromicity: The absorbance of a polynucleotide at 260 nm at temperature t divided by the absorbance at 25°C.

Intercalation: Interaction of a dye with a double-stranded polynucleotide occurring by insertion of the dye ring-system between adjacent base pairs of the polynucleotide.

r: The molar ratio of polynucleotide-bound ethidium bromide to nucleotide phosphate.

Tm profile: The normalized dependence of the absorbance of a polynucleotide at 260 nm on temperature.

Ti: The temperature, in a Tm profile, at which the initial increase in absorbance is noted.

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APPROVAL SHEET

The dissertation submitted by William Warren Martz has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

March 31, 1971
Date

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