# Circular Dichroism and Temperature–Optical Density Studies on the Conformation of Polynucleotide–Ethidium Bromide Complexes†

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ABSTRACT: Circular dichroism and temperature–optical density studies indicate that the nature of the complexes formed between ethidium bromide and polynucleotides is strongly dependent on the structure and conformation of the polynucleotide component. DNA-like or RNA-like double-stranded polynucleotides such as poly(dA-dT)/(dA-dT) and poly(A-U) form complexes with three-dimensional structures similar to that of the DNA–ethidium bromide complex. In the latter, the phenanthridinium ring of the dye is completely intercalated between DNA base pairs in a manner favoring the formation of hydrogen bonds between the amino groups of the ring and the phosphate groups of the polynucleotide backbone. In more unusual double-stranded polynucleotide structures, such as poly(A-I) and poly(I-C), the phenanthridinium ring may also be inserted between stacked base pairs but with an orientation which permits interaction between the polynucleotide backbone and the ring nitrogen. Single-stranded polynucleotides with relatively flexible structures, such as poly(C) and poly(U), may form complexes similar to those indicated for poly(A-I) and poly(I-C). By contrast, the rather inflexible polynucleotide structure represented by poly(A) apparently interacts with ethidium bromide in a rather nonspecific fashion.

Ethidium bromide is a phenanthridinium derivative which interferes with nucleic acid synthesis both *in vivo* (Tomchick and Mandel, 1964) and in cell-free systems (Elliott, 1963; Waring, 1964). These properties have been attributed, at least partially, to the inhibition of enzymes involved in the synthesis of nucleic acids (Waring, 1964). The observed inhibition of the DNA and the RNA polymerases appears to be a direct consequence of the physical interaction between template DNA and the phenanthridinium ring (Waring, 1965a).

The interaction between ethidium bromide and DNA, especially at high molar ethidium bromide to nucleotide phosphate ratios, includes a nonspecific component originating from electrostatic interactions between the two species. At lower ratios, however, this component is proportionally decreased and a rather specific "primary" complex predominates (Waring, 1965b; Lee *et al.*, 1973). This primary complex may be described as a structure in which the ethidium bromide ring system is inserted between DNA base pairs in a manner apparently favoring the formation of hydrogen bonds between the amino group substituents of the ring and phosphate oxygen (Fuller and Waring, 1964).

The formation of such a specific complex apparently depends on the structure and conformation of the polynucleotide binding site, in the sense that DNA can provide optimum dimensions for the formation of hydrogen bonds between ethidium bromide and phosphate groups on the opposite polynucleotide strands. In many instances, the capacity of dyes to interact with nucleic acids appears to depend primarily on the conformation of the specific polynucleotide structures involved rather than the chemical structures of the constituent nucleotides. For example, the preferential binding of actinomycin to DNA of high guanine plus cytosine content may be attributed to a specific conformation characteristic of this type of DNA rather than on any binding specificity of the dye toward either guanine or the guanine–cytosine pair (Wells, 1971).

Studies of the factors influencing the interactions between dyes and nucleic acids are of interest not only on their own right but also because they may yield data which can illuminate the nature of the interactions between DNA and other molecules of biological significance.

In this report we are presenting the results of circular dichroism and temperature–optical density dependence experiments on the interactions between ethidium bromide and various polynucleotides. Previous studies on this topic have revealed a correlation between the ability of polynucleotides to provide sites for strong primary interaction with the dye and the presence of base-paired helical structures (Waring, 1966). These studies, however, which were based on the changes occurring in the absorption spectrum of ethidium bromide, mostly reflect the differences in the excited electronic states of dye which accompany the binding of ethidium bromide and as such provide little information on the precise nature of the interactions. A re-examination of the factors involved, based on methods more sensitive toward conformational change, may thus be of value in providing a more detailed description of the nature of the polynucleotide–ethidium bromide interactions.

## Experimental Section

*Preparation of Solutions.* Ethidium bromide (Calbiochem, Los Angeles, Calif.) solutions were freshly prepared before each experiment in Tris-HCl buffer (0.04 M, pH 7.9) and kept in the dark until used. Concentrations were determined using a molar extinction coefficient of 5600 at 480 nm (Waring, 1965a). Polyriboadenylate (poly(A), type I, Lot 1990), polyribocytidylate (poly(C), type I, Lot 119B-0280), and polyriboinosinate (poly(I), type I, Lot 10C-0390) were purchased

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from Sigma Chemical Co., St. Louis, Mo. These polynucleotides and polyribouridylate (poly(U), P-L Biochemicals, Inc., Milwaukee, Wis.) were received as lyophilized powders and were used for the preparation of stock solutions (4 mg/ml) in the same buffer. Poly(deoxyadenylate-deoxyribothymidylate) [poly(dA-dT)/(dA-dT), Lot 6-4317, Miles Laboratories, Kankakee, Ill.] powder (10 absorbance units) was dissolved in 4 ml of buffer and the solution was used without further dilution.

The exact concentration of each polynucleotide solution was determined using the following extinction coefficients: poly(A) 9180 at 259 nm (Rich, 1958), poly(C) 6300 at 269 nm (Warner, 1957), poly(I) 10,300 at 250 nm (Rich, 1958), poly(U) 9330 at 260 nm (Michelson and Monny, 1966), and poly(dA-dT)/(dA-dT) 5650 at 260 nm (Mahler *et al.*, 1964).

Intermolecular complexes [poly(A-I), poly(I-C), and poly(A-U)] were prepared by mixing the component polynucleotides in equimolar proportions, with respect to nucleotides, and storing the solutions at room temperature for several hours before use (Steiner and Beers, 1961). The hypochromic effect associated with these solutions indicated that the double-stranded structures were indeed formed.

For the preparation of the ethidium bromide–polynucleotide complexes, polynucleotide solutions (0.40 $\mu$mol/ml) and ethidium bromide solutions (2.0 $\mu$mol/ml), prepared by appropriate dilution of the corresponding stocks in Tris-HCl buffer, were mixed so that samples with different molar ratios of ethidium bromide to polynucleotide phosphate (usually 0.20 and 0.50) would result.

For the preparation of the ethidium bromide–poly(vinyl sulfate) complex, poly(vinyl sulfate) (Lot 88254, General Biochemical Co., 5 ml of 10 $\mu$mol/ml) and ethidium bromide solutions (0.20 ml, 8 $\mu$mol/ml) were diluted with 10.0 ml of buffer. Final concentrations were [$SO_4^{2-}$] 5.0 $\mu$mol/ml and [ethidium bromide] 0.16 $\mu$mol/ml, giving an ethidium bromide to sulfate ratio of 0.032.

*Determination of the Ethidium Bromide–Polynucleotide Binding.* The concentrations of the polynucleotide–ethidium bromide complexes and the molar ratio of polynucleotide-bound ethidium bromide to DNA–phosphate ($r$) were calculated in the case of DNA and poly(A-U) from the shift in the absorption maximum of the ethidium bromide upon binding to the polynucleotide as described previously (Peacocke and Skerett, 1956). The absorption spectra provided no evidence of dye aggregation in the concentration range used in these studies and the spectra of the complexes exhibited single isosbestic points. Binding was calculated from the optical densities at 460 nm.

*Circular Dichroism Measurements.* Circular dichroism spectra were recorded using a Durrum-Jasco ORD/UV5 spectropolarimeter. Measurements were carried out in strain-free quartz cells with appropriate optical path lengths so that in every instance optical densities remained below 2.0. The difference in extinction coefficient between left and right circularly polarized light, $\epsilon_l - \epsilon_r$, was calculated from

$$\epsilon_l - \epsilon_r = \text{degrees of ellipticity}/33cl$$

where degrees of ellipticity were obtained directly from the recorder chart, the concentration ($c$) was expressed in moles per liter, and $l$ (path length of the cell) was expressed in centimeters. $\epsilon_l - \epsilon_r$ values were normally based on polynucleotide phosphate concentrations. In some instances, however, as indicated in the corresponding figures, the concentration of the complex calculated from the extent of ethidium bromide binding was used instead.

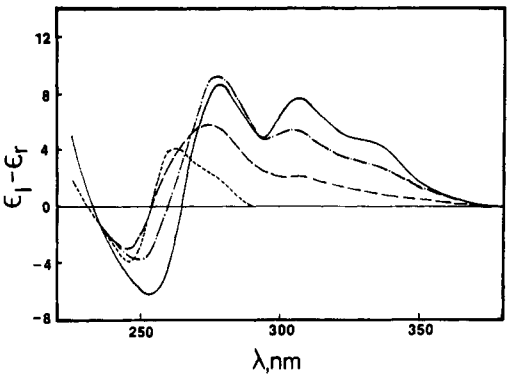


FIGURE 1: The circular dichroism of the poly(dA-dT)/(dA-dT)–ethidium bromide complex in 0.20 M Tris-HCl, pH 7.9; [polynucleotide phosphate], 0.20 $\mu$mol/ml. The added ethidium bromide to polynucleotide phosphate ratios are: (–) 0.50; (–·–) 0.30; (— —) 0.10. The circular dichroism of the free polynucleotide is indicated by the dashed line (- - -).

*Temperature–Optical Density Measurements.* Temperature–optical density profiles were obtained using a modified $T_m$ analyzer (Beckman Instruments, Inc.) attached to a Cary 15 spectrophotometer. Blanks containing samples identical with those being heated were employed and optical densities were automatically plotted by an X–Y recorder. The results were then re-plotted as hyperchromicity *vs.* temperature.

## Results

*Interaction between Ethidium Bromide and DNA-Like or RNA-Like Polynucleotides.* The effect of ethidium bromide on the circular dichroism (CD) of poly(dA-dT)/(dA-dT) is shown in Figure 1. The spectrum below 300 nm is modified and a new complex band with a maximum near 307 nm appears. Ellipticities generally increase as the ratio of added ethidium bromide to polynucleotide phosphate increases from 0.10 to 0.50.

Similar results, shown in Figure 2a, are obtained with a second double-stranded polynucleotide, poly(A-U). An apparent distinguishing characteristic in the CD of this complex is the presence of a small negative band in the 250–300-nm region. However, a similar negative band is present in the DNA–ethidium bromide complex at high ethidium bromide to DNA ratios and especially under conditions of high ionic strength. This band appears to be part of a conservative transition centered near 300 nm and it is not exclusive with poly(A-U) (Aktipis and Kindelis, 1973a).

Results obtained previously by absorption (Waring, 1966) and fluorescence methods (LePecq and Paoletti, 1965) have indicated that ethidium bromide forms a strong primary complex with poly(A-U). The binding parameters for this complex are, in fact, comparable to those determined for the DNA–ethidium bromide interaction.

The similarity between the CD of the ethidium bromide complexes of poly(dA-dT)/(dA-dT) and poly(A-U) and that of DNA (Figure 2b) over the 300–350-nm region suggests that the geometry of the primary binding between ethidium bromide and these polynucleotides (group I) may be analogous to that characteristic of the DNA–ethidium bromide complex.

The macromolecular structures of the typical double-stranded polynucleotides poly(dA-dT)/(dA-dT) and poly-

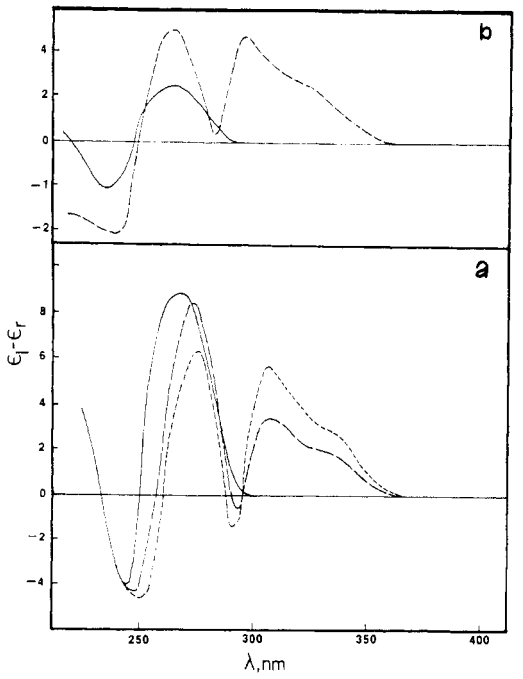


FIGURE 2: (a) The circular dichroism of the poly(A-U)–ethidium bromide complex at ethidium to polynucleotide phosphate ratios of: (- - -) 0.50 and (– –) 0.20. (b) The circular dichroism of the DNA–ethidium bromide complex at a ratio of 0.30. Complexes were prepared in 0.20 M Tris-HCl (pH 7.9); [polynucleotide phosphate], 0.20 μmol/ml. The circular dichroism of DNA is indicated by the solid line.

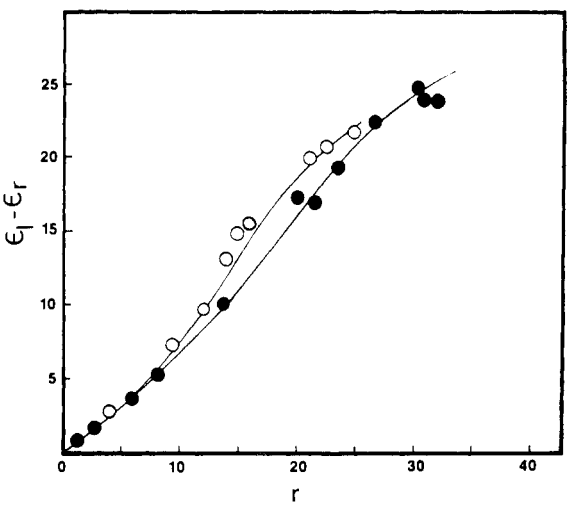


FIGURE 3: The dependence of molar circular dichroism of polynucleotide–ethidium bromide complexes at 307 nm on the bound ethidium to polynucleotide phosphate ratio (*r*). Complexes were prepared in 0.04 M Tris-HCl (pH 7.9); [polynucleotide phosphate], 0.20 μmol/ml. Ethidium bromide concentrations varied from 0.008 to 0.100 μmol/ml: (●) DNA–ethidium bromide; (○) poly(A-U)–ethidium bromide.

(A-U) are of course in general terms similar to that of DNA. Specifically, the distances between the opposite strands of DNA of these polynucleotides are comparable. A model analogous to that proposed for the DNA–ethidium bromide complex in which the intercalated ethidium bromide is aligned in a manner permitting the formation of hydrogen bonds between the amino groups of the ring and charged oxygen of the phosphate groups of both polynucleotide strands (Fuller and Waring, 1964) may, therefore, adequately describe the ethidium bromide complexes of poly(dA-dT)/(dA-dT) and poly(A-U).

The suggestion of structural similarities between these three complexes is further supported by the dependence noted between the molar circular dichroism at 307 nm and the ratio of bound ethidium bromide to polynucleotide (*r*) for the poly(A-U)–ethidium bromide complex shown in Figure 3. $\epsilon_l - \epsilon_r$ increases with increasing *r* up to about 22 at an *r* of 0.25 and for every ratio examined the ellipticities noted correspond closely to those obtained for the DNA–ethidium bromide complex. It may be noted, parenthetically, that for the latter some additional increase in ellipticities occurs for *r* up to approximately 0.32 although strong binding appears to be saturated at an *r* of about 0.28 (Aktipis and Kindelis, 1973a). It is, therefore, conceivable that some intercalation sites with decreased affinity for ethidium bromide are still available at *r* values higher than 0.28.

The dependence between molar circular dichroism and *r* in the DNA–ethidium bromide complex has been interpreted as indicative of interactions between intercalated ethidium bromide molecules (Aktipis and Martz, 1970; Dalgleish *et al.*, 1971; Aktipis and Kindelis, 1973a). Restrictions in the positioning of ethidium molecules, imposed by the intercalation into the double-stranded helix, apparently force a molecular geometry necessary for the establishment of these interactions. The similarity in the circular dichroism properties of the DNA–ethidium bromide and poly(A-U)–ethidium bromide complexes, therefore, provides additional evidence of a close analogy in the geometry of these two complexes.

Similarities in the structures between DNA–dye and poly(A-U)–dye complexes have also been previously discussed in connection with the mechanism of proflavine intercalation (Blake and Peacocke, 1967).

*Interaction between Ethidium Bromide and Some Unusual Double-Stranded Polynucleotides.* The circular dichroism properties of ethidium bromide complexes formed with two polynucleotides possessing base pairing of a type not commonly associated with naturally occurring nucleic acids, *i.e.* poly(I-C) and poly(A–U), are quite distinct from those noted for polynucleotides in group I.

The poly(I-C)–ethidium bromide complex (Figure 4b) at an ethidium bromide/polynucleotide phosphate ratio of 0.20 exhibits only a minor increase in ellipticities below 300 nm and a small circular dichroism tail above 300 nm. At higher ratios a broad band over the 200–350-nm region develops. This band lacks the 307-nm maximum associated with complexes of polynucleotides in group I.

Similar results are obtained with the poly(A-I) complex (Figure 4a). At ethidium bromide/polynucleotide phosphate ratios up to 0.50 induced circular dichroism develops above 300 nm. Again the distinct properties noted for the DNA–ethidium bromide, poly(dA-dT)/(dA–dT)–ethidium bromide, and poly(A-U)–ethidium bromide complexes are absent.

In spite of these differences in circular dichroism, the binding curves describing the interaction of ethidium bromide with poly(I-C) and poly(A-I) especially are of the same nature as those obtained for DNA and poly(A-U) and are, therefore, characteristic of the occurrence of primary binding (Waring, 1966). The strong binding noted with these complexes, though not a proof, is a good indication that ethidium bromide interacts with these polynucleotides also by intercalation. If this is, indeed, the case, the differences noted in the circular dichroism properties between ethidium bromide complexes formed with poly(A-I) or poly(I-C) (group II) and ethidium bromide complexes formed with typical double-stranded

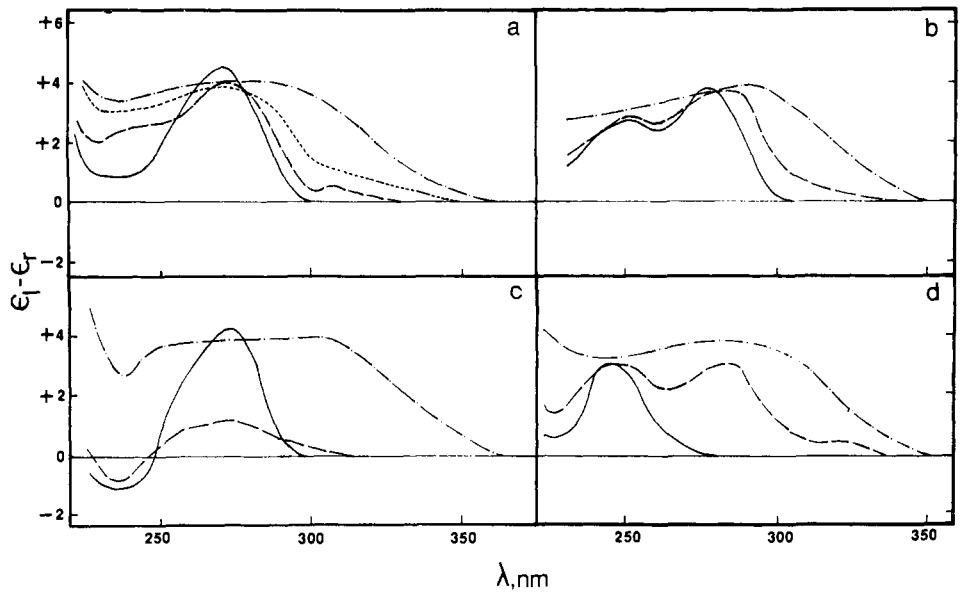


FIGURE 4: The circular dichroism of complexes of ethidium bromide with single- and unusual double-stranded polynucleotides ([polynucleotide phosphate], 0.20 μmol/ml) at various ethidium bromide to polynucleotide phosphate ratios in 0.20 M Tris-HCl (pH 7.9): (a) poly(A-I) at a ratio of: (–·–) 0.50; (- - -) 0.30; (– –) 0.20; (b) poly(I-C) at a ratio of: (–·–) 0.50; (– –) 0.20; (c) poly(U) at a ratio of: (–·–) 0.50; (– –) 0.20; (d) poly(I) at a ratio of: (–·–) 0.50; (– –) 0.20. The circular dichroism of the free polynucleotides is indicated by the solid lines.

polynucleotides may simply reflect differences in the specific conformation of these complexes at the vicinity of the binding sites rather than gross differences in overall structures. One of the major differences in the conformation of these complexes may, in the case of poly(A-I), originate from the intermolecular distance between phosphate oxygen atoms on the opposite strands. This distance turns out to be, based on inspection of Corey–Pauling–Koltun (CPK) molecular models, too large to permit the intercalated dye to participate in hydrogen bonds with both polynucleotide strands simultaneously.

The complexes formed between ethidium or proflavine and poly(I-C) appear to also have distinct characteristics from those of DNA and the other polynucleotides in group I. If hydrogen bonding between the poly(I) and the poly(C) strands in this polynucleotide occurs as proposed by Hoogsteen (1959) then the binding site might turn out to be too narrow to permit simultaneous hydrogen bonding of the intercalated ethidium ring with both polynucleotide strands.

Clearly then, although the interactions between ethidium and poly(I-C) or poly(A-I) may involve intercalation, the precise positioning of the phenanthridinium ring in these systems appears to be different from that occurring with DNA. This difference may simply relate to the inability of these polynucleotides to provide sites within which ethidium bromide can form hydrogen bonds bridging the two polynucleotide strands. Evidence obtained from complexes of phenanthridinium derivatives of various structures carrying either one or two amino group substituents also indicates that hydrogen bonding may be primarily responsible for the exact orientation of the phenanthridinium ring within the primary binding site (Aktipis and Kindelis, 1973b).

*Interaction between Ethidium Bromide and Some Single- and Triple-Stranded Polynucleotides.* Characteristic circular dichroism patterns are also obtained for complexes formed between ethidium bromide and such polynucleotides as poly(U) (Figure 4c), poly(I) (Figure 4d), and poly(C) (Figure 5) (group III). Both absorption (Waring, 1966) and fluorescence (LePecq and Paoletti, 1965) studies have indicated that single-stranded polynucleotides do not participate in binding comparable to the strong interaction occurring between DNA and ethidium bromide. Nevertheless, the circular dichroism of polynucleotides in this group is substantially modified in the presence of the dye.

The effects noted for poly(C), poly(U), and poly(I) at ethidium bromide/polynucleotide phosphate ratios of up to 0.50, *i.e.* the development of a broad circular dichroism band lacking a maximum near 307 nm, are very similar to those occurring with poly(A-I) or poly(I-C) and distinct from the properties of the double-stranded polynucleotide complexes in group I. It thus appears that the complexes formed with these single-stranded polynuclotides may, in fact, have conformations analogous to those of the poly(A-I)–ethidium bromide and the poly(I-C)–ethidium bromide complexes.

Further differences in the behavior of various polynucleotides toward ethidium bromide are indicated by the circular dichroism properties of the poly(A)–ethidium bromide complex (Figure 6). The dye interacts with this polynucleotide as indicated by the shift in the absorption maximum in the ethidium bromide spectrum (Waring, 1966). However, in this case the interaction fails to produce either the fluorescence enhancement (LePecq and Paoletti, 1965) or the characteristic circular dichroism above 300 nm which accompanies the

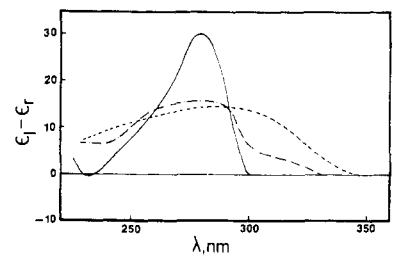


FIGURE 5: The circular dichroism of poly(C) and the poly(C)–ethidium bromide complex in 0.20 M Tris-HCl (pH 7.9); [polynucleotide phosphate], 0.20 μmol/ml. Ethidium bromide to polynucleotide phosphate ratios are: (– –) 0.50; and (–·–) 0.20. The circular dichroism of the free polynucleotide is indicated by the solid line.

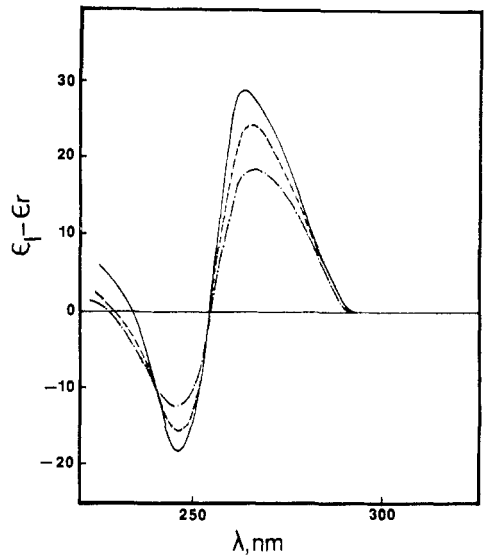


FIGURE 6: The circular dichroism of poly(A) and the poly(A)–ethidium bromide complex in 0.04 M Tris-HCl (pH 7.9). The ethidium to polynucleotide phosphate ratios are (- - -) 0.10 and (–·–) 0.40. The circular dichroism of free poly(A) is indicated by the solid line; [polynucleotide phosphate], 0.20 μmol/ml.

intercalation of ethidium bromide to DNA (Aktipis and Martz, 1970; LePecq and Paoletti, 1967). Rather this absence of fluorescence and circular dichroism effects is reminiscent of the properties of polyionic structures unrelated to nucleic acids, such as poly(vinyl sulfate), which bind ethidium bromide principly *via* electrostatic forces (LePecq and Paoletti, 1967).

The interaction between ethidium bromide and poly(A) may, therefore, also be of electrostatic nature, involving the partial positive charges on the amino group of the dye and the

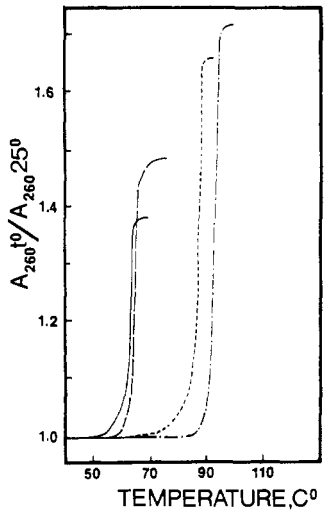


FIGURE 7: The effect of ethidium bromide on the temperature–optical density profiles of poly(dA-dT)/(dA-dT) and poly(A-U) in 0.04 M Tris-HCl (pH 7.9); [polynucleotide phosphate], 0.20 μmol/ml; (- -) poly(dA-dT)/(dA-dT); (–·–) poly(dA-dT)/(dA-dT)–ethidium bromide complex at an ethidium to polynucleotide phosphate ratio of 0.50; (—) poly(A-U); (- - -) poly(A-U)–ethidium bromide complex at a ratio of 0.20. The different hyperchromicities noted between free polynucleotides and the corresponding ethidium bromide complexes result mostly from changes in the absorption of the complexes at elevated temperatures as ethidium bromide is released during strand dissociation.

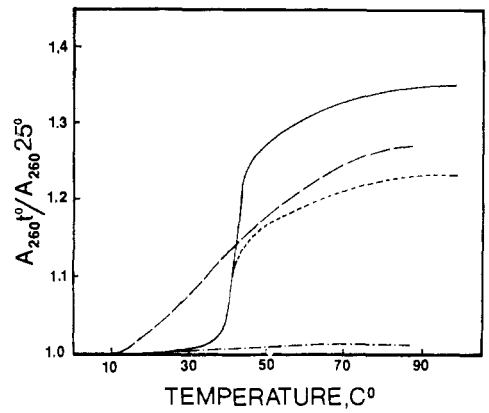


FIGURE 8: The effect of ethidium bromide on the temperature–optical density profiles of poly(A), poly(U), and poly(A-I) in 0.04 M Tris-HCl (pH 7.9) at [polynucleotide phosphate], 0.20 μmol/ml: (- -) poly(A) and the poly(A)–ethidium bromide complex at an ethidium to polynucleotide ratio of 0.20; (–·–) poly(U) and the poly(U)–ethidium bromide complex at a ratio of 0.20; (- - -) poly(A-I); (—) the poly(A-I)–ethidium bromide complex at a ratio of 0.20.

negative charges on the polynucleotide phosphates. Apparently such interaction is not a sufficient condition for the inducement of circular dichroism over the 300–350-nm region. For the development of optical activity, some specific positioning of the phenanthridinium ring with respect to the polymeric structure, and perhaps more specifically intercalation of the ring, would be required. However, since at neutral pH poly(A) may be, in comparison to poly(C) or poly(U), a rather stiff rod-like structure, insertion of the phenanthridinium ring between stacked adenine residues may be difficult to achieve.

*Temperature–Optical Density Profiles of the Polynucleotide–Ethidium Bromide Complexes.* The temperature–optical density profiles of polynucleotide–ethidium bromide complexes provide additional evidence for differences in conformation among these various complexes. Polynucleotides in group I are effectively stabilized by ethidium bromide. Poly(A-U) in 0.04 M Tris-HCl buffer, pH 7.9, exhibits a transition with a midpoint ($T_m$) of about 60°. By comparison, the $T_m$ of the poly(A-U)–ethidium bromide complex at a ratio of ethidium bromide/polynucleotide phosphate of 0.20 is 82° (Figure 7). Similarly, the $T_m$ of poly(dA-dT)/(dA-dT) increases from 64 to 92° at an ethidium bromide/polynucleotide phosphate ratio of 0.50. These results indicate that ethidium bromide is approximately as effective in stabilizing the structures of poly(A-U) and poly(dA-dT)/(dA-dT) as it is in protecting DNA from thermal denaturation.

By contrast, ethidium bromide does not stabilize poly(A-I) which exhibits a $T_m$ of approximately 42° in either the presence or the absence of ethidium bromide (Figure 8). Similarly, the temperature–optical density profiles of poly(A) and poly(A)–ethidium bromide coincide as do those of poly(U) and poly(U)–ethidium bromide. Furthermore, in the latter case the lack of a sharp transition is typical of highly flexible polynucleotides and is expected on the basis of the known properties of poly(U) (Michelson and Monny, 1966). Clearly then, although DNA and generally polynucleotides in group I are stabilized by ethidium bromide, the stability of the other polynucleotides examined is not appreciably influenced by interaction with the dye.

The stabilization of polynucleotides in the presence of ethidium bromide is caused by the stronger and therefore

preferential binding of the dye to double-stranded regions over single-stranded regions which are gradually produced during the process of thermal denaturation (Gersch and Jordan, 1965). The lack of any ethidium bromide effect on the thermal denaturation of single-stranded polynucleotides such as poly(A) and poly(U) and the stabilization noted with double-stranded polynucleotides such as DNA poly(A-U) and poly(dA-dT)/(dA-dT) are, therefore, quite understandable.

The behavior of poly(A-I) is consistent with differences in conformation between the complexes formed with this polynucleotide and those of complexes of the typical double-stranded polynucleotides in group I. Apparently as a result of these differences, ethidium bromide binding to polynucleotides in group II is weaker than it is to polynucleotides in group I. In fact, it appears that the affinity of ethidium bromide for poly(A-I) is not much higher for this complex than it is for the polynucleotide components of this structure. Such binding properties would explain the ineffectiveness of ethidium bromide in providing protection against the thermal denaturation of poly(A-I).

## Discussion

The circular dichroism properties and the temperature–optical density profiles of the complexes formed between ethidium bromide and various polynucleotides indicate that the conformation of these complexes depends on the overall structural characteristics of the corresponding polynucleotides rather than a specific polynucleotide base content or sequence.

Hydrogen bonding apparently plays an important role in determining the conformation of these complexes. Examination of CPK space-filling models indicates that in the DNA–ethidium bromide complex the dye may easily intercalate in a manner which places the amino substituents of the ring in the vicinity of the phosphate groups of the polynucleotide backbone. In this position the ring is completely inserted in the interior of the double helix and hydrogen bonds can be formed between phosphate groups and the ring. Similar observations can also be made regarding the ethidium bromide complexes of poly(A-U) and poly(dA-dT)/(dA-dT). It may be noted that circular dichroism and binding studies of proflavine and other acridines to DNA have also implicated hydrogen bonding as an important factor in the structure of the corresponding dye–polynucleotide complexes (Dalgleish *et al.*, 1969).

Intercalation of ethidium bromide in a conformation similar to that specified for polynucleotides in group I [*i.e.* DNA, poly(A-U), and poly(dA-dT)/(dA-dT)] is obviously impossible for single-stranded polynucleotides and highly unlikely for such double-stranded polynucleotides as poly(A-I) or poly(I-C).

In general, the exact location of a dye bound to a polynucleotide depends on the specific structures of both components. Specifically, in the case of acridines, the 3,6-diaminoacridine (proflavine) binds to DNA in the classical configuration in which both amino groups are oriented toward the phosphate backbone while the configuration of other acridines is, to a large degree, determined by the ring nitrogen. Such differences in configuration are apparently related to differences in charge distribution on the ring, since in acridine the positive charge is primarily located at the ring nitrogen while in the amino derivatives the charge is also distributed over the amino groups (Löber and Achtert, 1969).

Since the classical configuration characteristic of the DNA–ethidium bromide complex appears structurally impossible for the complexes of this dye with poly(A-I) and poly(I-C), it is reasonable to assume that in these complexes the orientation of the phenanthridinium ring is also to a degree determined by the ring nitrogen. In fact, although at neutral pH charges on ethidium bromide are almost equally distributed between the amino group nitrogen and the ring nitrogen, the latter may be slightly more positive than the amino groups (Giacomoni and LeBret, 1973). Thus, it is quite conceivable that the phenanthridinium ring is located within the binding site in a manner similar to that proposed for acridine in the DNA–acridine complex (Pritchard *et al.*, 1966). In this configuration the ethidium ring would not be hydrogen bonded to the two complementary polynucleotide strands, but rather it may interact with two successive bases on the same polynucleotide chain in a way permitting the negatively charged oxygen of the phosphate backbone to be placed between the charged heterocyclic nitrogen and the amino group on $C_3$ of the dye.

A similar conformation may characterize the complexes formed between ethidium bromide and the single-stranded polynucleotide poly(C), which is a relatively flexible structure with intermittent regions of ellipticity (Fasman *et al.*, 1964), and poly(U), which is a highly flexible polynucleotide (Michelson and Monny, 1966). Poly(I), which may exist as a triple-stranded structure (Rich, 1958), appears to be included in this category also.

The similarities noted in the circular dichroism of the ethidium bromide complexes formed with polynucleotides in groups II and III and the identical behavior exhibited by ethidium bromide, with respect to the stabilization of these polynucleotides against thermal denaturation, are consistent with similarities between the conformations of these complexes. The positioning of the dye between base pairs in these complexes may be favored over an alternative arrangement simply because it reduces the area of the dye over which hydrophobic forces can be exercised.

The rather unusual circular dichroism properties exhibited by poly(A) have indicated that the interaction between ethidium bromide and this polynucleotide may be of purely electrostatic nature. Apparently, because of a certain degree of rigidity associated with the stacked helical structure of poly(A) (Holcomb and Tinoco, 1965), interaction with the bases in the manner described for the other single-stranded polynucleotides is not favored. Instead ethidium bromide apparently binds to the exterior of the poly(A) helix.

In conclusion, the present studies have indicated that the interaction of ethidium bromide with polynucleotides as determined by circular dichroism is a sensitive indicator of polynucleotide conformation. The precise configuration of polynucleotide–ethidium bromide complexes is clearly determined by the geometry of the polynucleotide binding site. This geometry appears to depend not only on whether single- or double-stranded polynucleotides are involved but also, among double-stranded polynucleotides, on the exact dimensions of the binding site. The configuration of the complex is apparently also influenced decisively by the degree of flexibility of the polynucleotide structure.

The involvement of such factors in the interaction between dyes and polynucleotides and the observation that DNA can react with small molecules such as ethidium bromide in a very specific way are consistent with the notion that gene expression can be regulated on the basis of the structural and conformational characteristics of the DNA. According to this notion the geometry of certain polynucleotide regions

within the gene could conceivably determine the degree of interaction with other molecules in a manner which influences the activity of specific regions of the chromosome.

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# Distribution of Pyrimidine Oligonucleotides in Complementary Strand Fractions of *Escherichia coli* Deoxyribonucleic Acid†

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ABSTRACT: The distribution of pyrimidine oligonucleotide clusters (isostichs) in complementary fractions L and H of *Escherichia coli* DNA separated by MAK chromatography has been determined. Preparations of native and single-stranded DNA were degraded with diphenylamine in formic acid, and the released isostichs under the general formula of $Py_nP_{n+1}$ were separated on DEAE-cellulose according to chain length. Eleven isostichs were recovered from strand fractions L and H in unequal proportions. Each isostich fraction was subfractionated according to base composition on DEAE-cellulose at pH 3.0. Fifty-nine different nonisomeric pyrimidine oligonucleotides were separated from both strand fractions. The findings show an asymmetric distribution of pyrimidine clusters between the L and H strand fractions, with a predominance of oligonucleotides of length 6–11 in the H fraction. The distribution bias between the fractions involves both the cytosine-rich and thymine-rich oligonucleotides to the same extent. Unlike *Bacillus subtilis* DNA where the asymmetry between the strands is extensive and follows certain regularities, in *E. coli* the bias is more limited and less regular. The findings can be correlated with the extent of asymmetric transcription in these two bacterial species.

Alkali-denatured DNA preparations from *Bacillus subtilis* (Rudner *et al.*, 1968a) and from several microbial species (Rudner *et al.*, 1969) can be separated by a technique of *intermittent gradient* elution from an MAK column into two distinct components designated by their buoyant densities, as light (L) and heavy (H). On the basis of several criteria such as transforming activity, temperature–absorbance behavior, nucleotide composition, and hybridization to RNA, it was previously concluded that the two MAK fractions isolated from denatured *B. subtilis* DNA represent two families of strand fragments, each derived from one of the original chains (Rudner *et al.*, 1968a,b, 1969; Karkas *et al.*, 1968, 1970;

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