Reichman, M., Karlan, D., and Penman, S. (1973), *Biochim. Biophys. Acta 299,* 173.
Reichman, M., and Penman, S. (1973), *Biochim. Biophys. Acta 324,* 282.
Robertson, H. D., Webster, R. E., and Zinder, N. D. (1968), *J. Biol Chem. 243,* 82.
Ro-Choi, T. S., Choi, Y. C., Savage, H. E., and Bush, H. (1973), *Methods Cancer Res. 9,* 71.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 851.
Shutt, R. H., and Krueger, R. G. (1972), *J. Immunol. 108,* 819.
Snyder, A. L., Kann, H. E., and Kohn, K. W. (1971), *J. Mol. Biol. 58,* 555.
Soeiro, R., and Basile, C. (1973), *J. Mol. Biol. 79,* 507.
Soeiro, R., Vaughan, M., and Darnell, J. E. (1968), *J. Cell. Biol. 36,* 91.
Spirin, A. S., Belitsina, N. V., and Lerman, M. I. (1965), *J. Mol. Biol. 14,* 611.
Staynov, D. Z., Pinder, J. C., and Gratzer, W. B. (1972), *Nature (London), New Biol. 235,* 108.
Tavitian, A., Uretsky, S. C., and Acs, G. (1968), *Biochim. Biophys. Acta 157,* 33.
Vaughan, M. H., Soeiro, R., Warner, J. R., and Darnell, J. E. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 1527.
Vesco, C., and Penman, S. (1968), *Biochim. Biophys. Acta 169,* 188.
Wagner, E. K., Penman, S., and Ingram, V. M. (1967), *J. Mol. Biol. 29,* 371.
Warner, J. R., Girard, M., Latham, H., and Darnell, J. E. (1966), *J. Mol. Biol. 19,* 373.
Warner, J. R., and Soeiro, R. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 1894.
Weinberg, R. A., Loening, U., Willems, M., and Penman, S. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 1088.
Weinberg, R. A., and Penman, S. (1970), *J. Mol. Biol. 47,* 169.
Weiss, J. W., and Pitot, H. C. (1974a), *Arch. Biochem. Biophys. 160,* 119.
Weiss, J. W., and Pitot, H. C. (1974b), *Cancer Res. 34,* 581.
Wellauer, P. K., and Dawid, I. B. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2827.
Wilkinson, D. S., Cihak, A., and Pitot, H. C. (1971), *J. Biol. Chem. 246,* 6418.
Wilkinson, D. S., and Pitot, H. C. (1972), *J. Biol. Chem. 248,* 63.
Wilkinson, D. S., and Tlsty, T. D. (1974), *Proc. Amer. Assoc. Cancer Res. 15,* 47.
Willems, M., Penman, M., and Penman, S. (1969), *J. Cell Biol. 41,* 177.
Zadrazil, S., Fucik, V., Bartl, P., Sormova, Z., and Sorm, F. (1965), *Biochim. Biophys. Acta 108,* 701.
Zain, B. S., Adams, R. L. P., and Imrie, R. C. (1973), *Cancer Res. 33,* 40.
Zubay, G., Wilkins, M. H. F. (1960), *J. Mol. Biol. 2,* 105.

Thermal Denaturation of the DNA–Ethidium Complex. Redistribution of the Intercalated Dye during Melting†

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ABSTRACT: The temperature dependence of the circular dichroism of the DNA–ethidium bromide complex at elevated temperatures provides evidence that the optical activity of the complex near 307 nm originates from interactions between intercalated dye molecules while the optical activity near 515 nm results from singly intercalated ethidium bromide molecules. The behavior of the circular dichroism of the complex at elevated temperatures also explains the higher ellipticities near 307 nm which characterize complexes formed between ethidium bromide and denatured DNA. Finally the circular dichroism data indicate that the melting of the complex takes place in a stepwise manner with some DNA regions, probably AT-rich regions, dissociating first. The implications of these findings regarding the inhibiting effect of ethidium bromide on the function of DNA polymerase are examined.

Ethidium bromide is an antitrypanosomal drug which is known to inhibit nucleic acid synthesis both *in vivo* (Tomchick and Mandel, 1964) and in cell-free systems (Elliott, 1963; Waring, 1964). It has been suggested (Waring, 1964) that the pharmacological properties of ethidium are at least partly due to inhibition of polymerases involved in nucleic acid synthesis. This inhibition may be a direct consequence of the physicochemical interaction between DNA templates and the planar phenanthridinium ring structure of the drug (Waring, 1965a).

The interaction between DNA and ethidium bromide results in pronounced changes in the physical properties of both components such as an increase in the fluorescence quantum efficiency of ethidium (LePecq and Paoletti, 1967; Wahl *et al.*, 1970) and an increase in the viscosity of DNA (Cohen and Eisenberg, 1969). In terms of a physical model, the interaction may be described as an insertion of the planar phenanthridinium ring between adjacent nucleotide pairs in DNA. The binding depends primarily on hydrophobic interactions occurring between ethidium bromide and

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the surrounding bases (Fuller and Waring, 1964) as well as the electrostatic forces operating between phosphate residues and the positively charged nitrogens of the phenanthridinium ring (Gilbert and Claverie, 1968). This type of interaction is referred to as intercalation and it occurs almost exclusively at low ethidium bromide to DNA phosphate ratios (approximately 0.25 and below), *i.e.*, under conditions associated with strong binding of the dye. At higher ethidium to DNA phosphate ratios, secondary binding resulting from electrostatic attraction between phosphate residues and dye molecules attached to the exterior of the helix also takes place (Waring, 1965b; Aktipis and Kindelis, 1973).

The intercalation complex formed between DNA and ethidium bromide is characterized by major circular dichroism bands between 300 and 360 nm and a band of much lower ellipticity centered near 515 nm (Aktipis and Kindelis, 1973). In the present study we have examined the dependency of the circular dichroism of the complex at elevated temperatures and we have obtained information regarding the origin of the circular dichroism bands induced by ethidium bromide. The changes noted in the circular dichroism with temperature have also provided information on the probable sequence of events which accompany the thermal denaturation of the DNA-ethidium bromide complex.

Experimental Section

Preparation of Solutions. Calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J.) was dissolved (4 mg/ml in Tris-HCl buffer (0.04 M, pH 7.9) and dialyzed twice *vs.* the same buffer. From this stock solution working solutions were prepared by diluting with buffer. The concentration of these solutions was determined using an extinction coefficient of 6600 at 260 nm (Mahler *et al.*, 1964). Denatured DNA was prepared by immersing a stoppered flask containing a 2×10^{-3} M solution of DNA phosphate in boiling water for 15 min and subsequently cooling the hot solution in ice-water. The residual hyperchromicity of the denatured DNA was 13%. By comparison the native DNA solution exhibited a hyperchromicity of about 39%.

Ethidium bromide (Lot No. 100301, Calbiochem, Los Angeles, Calif.) solutions were prepared in the same buffer shortly before each experiment. Concentrations were determined using a molar extinction coefficient of 5600 at 480 nm (Waring, 1965a).

Solutions of the DNA-ethidium bromide complex for various molar ratios of ethidium bromide to DNA phosphate were prepared by mixing appropriate volumes of the working solutions and diluting with buffer to a standard volume (usually 20 ml).

In most instances, as indicated in the corresponding figures, a constant amount of dye was used and the amount of DNA was varied. Occasionally the reverse procedure was used.

Circular Dichroism Measurements. Circular dichroism spectra were recorded on a modified Jasco ORD/UV5 spectropolarimeter (SS-10 modification; sensitivity 2×10^3 deg/cm). Measurements were carried out in cells with optical path lengths ranging from 0.1 to 10.0 cm so that optical densities in each instance did not exceed 2.0.

The difference in extinction coefficient between left and right circularly polarized light $\epsilon_l - \epsilon_r$ was calculated from

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

where degrees of ellipticity were obtained directly from the recorder chart, the concentration c was expressed in moles/liter, and l, the path length of the cell, was expressed in centimeters. For the calculation of $\epsilon_l - \epsilon_r$ the concentration of either DNA phosphate or DNA-bound ethidium bromide was used as indicated in the corresponding figures.

Measurements at Elevated Temperatures. Optical density *vs.* temperature measurements were carried out using a Beckman T_m analyzer connected to a Moseley X-Y recorder (Hewlett Packard Corp., Model No. 7035B). Circular dichroism-temperature profiles were obtained using a modified T_m analyzer which permits automatic recording of CD *vs.* temperature (Martz and Aktipis, 1971). The maximum optical path length accommodated by the cell holder of this instrument is 1.0 cm. Alternatively for the measurement of low ellipticities it was necessary to use a jacketed 10-cm cell. This cell was connected to a Lauda K-2/R constant temperature circulator (Brinkman Instruments, Westbury, N.Y.) and the temperature was increased at 5° increments at a rate of approximately 1°/min. All other heating rates, with one exception noted in the legend of the corresponding figure, were adjusted to 3°/min.

Binding Studies. The binding of ethidium to DNA at elevated temperatures was determined spectrophotometrically by monitoring absorbance at 460 nm as described previously (Aktipis and Kindelis, 1973). The calculation of molar circular dichroism for each temperature is feasible from ethidium bromide binding data. These data are easily obtained because the absorption of DNA-bound ethidium at 460 nm is apparently independent of temperature as indicated by the constancy of the apparent absorption of complex formed between DNA (6×10^{-5} M) and ethidium (3×10^{-5} M) in the 25-70° region. (An increase in the apparent absorbance noted between 70 and 100° can be accounted for by the release of bound ethidium molecules resulting from the dissociation of the DNA-ethidium bromide complex.) Also the effect of temperature on the absorption spectrum of free ethidium bromide is limited to a rather small shift in the absorption maximum from 480 nm at 25° to 490 nm at 90° and a concommitant linear decrease in the apparent extinction coefficient at 460 nm from 4850 to about 4250.

Results

Circular Dichroism of Thermally Denatured DNA-Ethidium Complex. Addition of ethidium bromide to thermally denatured DNA induces circular dichroism similar to that reported for the DNA-ethidium complex (Aktipis and Martz, 1970; Aktipis and Kindelis, 1973). The complex formed with denatured DNA (Figure 1) at ratios of added ethidium to DNA phosphate of 0.10 and 0.20 exhibits at 307 nm $\epsilon_l - \epsilon_r$ values, based on the concentration of DNA, of 1.3 and 3.5, respectively. By comparison the corresponding $\epsilon_l - \epsilon_r$ magnitudes for the native DNA-ethidium bromide complex for identical ratios are 0.8 and 2.8.

Similar results, shown in Figure 2, are obtained by plotting the molar circular dichroism at 307 nm of ethidium bromide complexes formed with native and with denatured DNA *vs.* the molar ratio of bound ethidium to DNA phosphate (r). Ellipticities are consistently higher for all the complexes formed with denatured DNA and an apparent maximum $\epsilon_l - \epsilon_r$ value of about 25 is achieved at a lower ratio for these complexes.

The increase in $\epsilon_l - \epsilon_r$ noted for the denatured DNA-ethidium bromide complex may, at first, be a little surprising.

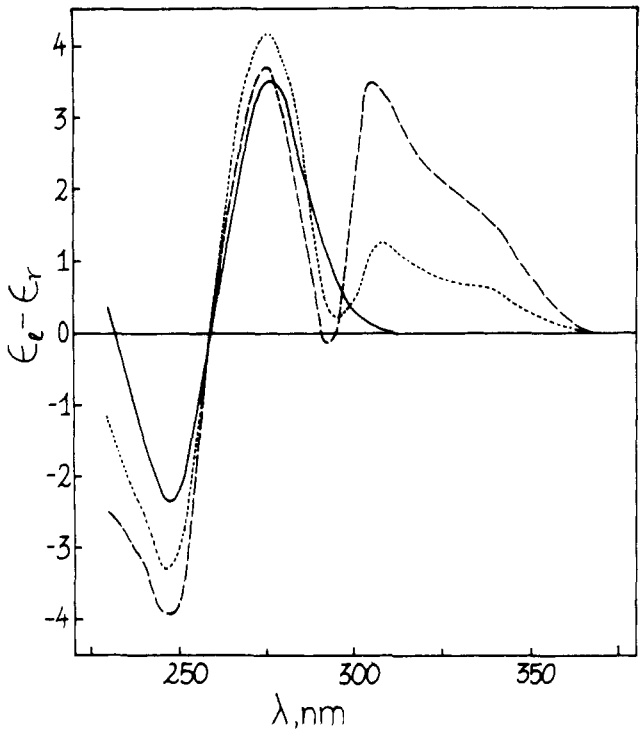

FIGURE 1: Circular dichroism of heat denatured DNA (—) and the denatured DNA-ethidium complex for added ethidium/DNA ratios of (···) 0.1 and (- - -) 0.2. Since ellipticities are calculated on the basis of DNA concentration the shape of the spectra is identical with that directly recorded on the chart of the spectropolarimeter.

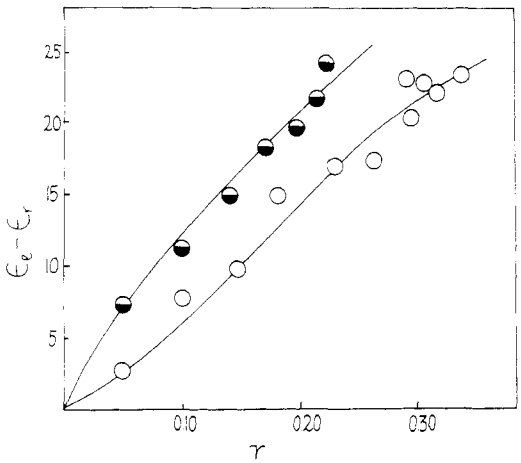

FIGURE 2: Dependency of ellipticity at 307 nm of the DNA-ethidium complex on the bound dye/DNA ratio. Ellipticities are calculated on the basis of the concentration of DNA-bound ethidium. DNA phosphate, 2×10^{-4} M; (○) native DNA; (◒) heat denatured DNA.

Optical activity in the 300–350-nm region originates from ethidium bromide molecules intercalated in double-stranded helical sites (Aktipis and Kindelis, 1973) and denatured DNA certainly has fewer regions containing such sites. On this basis, and in view of the fact that ethidium binds almost exclusively to the double-stranded regions of denatured DNA (LePecq and Paoletti, 1967), one might have expected the complex formed with denatured DNA to exhibit lower rather than higher ellipticities. In fact, under a similar set of conditions the maximum fluorescence enhancement of ethidium bromide bound to DNA, which also originates from dye molecules associated with primary binding sites, decreases by more than 50% for the denatured DNA-ethidium bromide complex (LePecq and Paoletti, 1967).

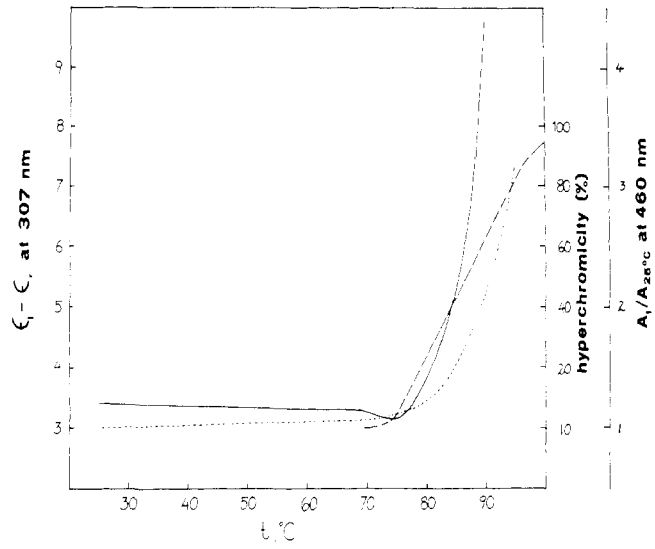

FIGURE 3: Dependency of the ellipticity at 307 nm (left ordinate) (—) and absorbancies at 260 nm (- - -) and 460 nm (···) (right ordinate) on temperature for the DNA-ethidium complex at an added ethidium/DNA of 0.07; [EB], 2×10^{-5} M. The optical density-temperature profile at 260 nm was obtained using a 0.2-cm cell and a heating rate of 0.3°/min. Ellipticities are calculated on the basis of the concentration of DNA-bound ethidium.

This decrease undoubtedly results from the reduction in the content of double-stranded regions available for ethidium bromide intercalation in denatured DNA.

The increase in the circular dichroism noted for the denatured DNA–ethidium bromide complex is, however, consistent with the notion that the optical activity in the 300–350-nm region does not simply originate from the intercalation of single ligands and their interaction with the surrounding nucleotides, as the case is with fluorescence, but rather results from the interactions between intercalated ligands within an intact double-stranded polynucleotide region (Aktipis and Kindelis, 1973). As it will become apparent from the subsequent discussion, the possibility of such interactions is substantially increased with denatured DNA.

Circular Dichroism of DNA–Ethidium Complex at Elevated Temperatures. Additional information regarding the increase in the interactions between ethidium bromide molecules intercalated in denatured DNA is obtained by examining the behavior of the native DNA–ethidium bromide complex at elevated temperatures.

The dependence on temperature of the circular dichroism of this complex at 307 nm, is shown in Figure 3. At a ratio of 0.07 the circular dichroism decreases slightly with increasing temperatures up to 75°. This is followed by a strong increase in the circular dichroism, in the 75–90° range.

At this low ratio practically all ethidium present is initially bound to DNA and, as suggested by the constancy of the absorption of the complex at 460 nm, the binding changes very little for temperatures up to about 70°. By contrast the sharp increase in the circular dichroism of the complex above 75° is associated with a pronounced decrease in ethidium binding. The increase in the circular dichroism also appears to be concomitant with the elimination of double-stranded regions in DNA which serve as binding sites for ethidium bromide. More precisely, the increase in the optical density of the complex at 260 nm just precedes and accompanies the increase in molar circular dichroism. The increase in ellipticity of the complex near 307 nm thus

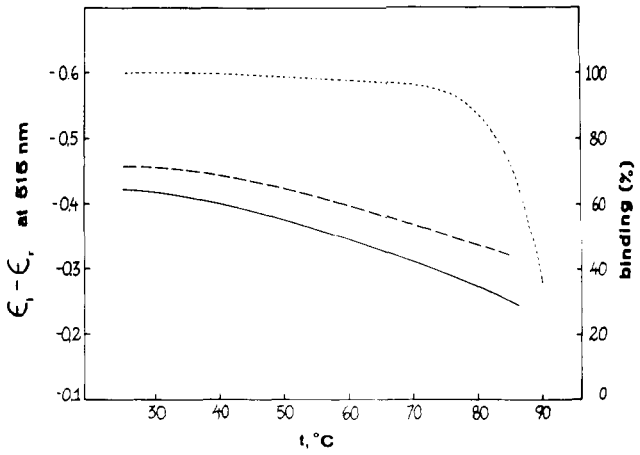

FIGURE 4: Dependence of the binding of ethidium to DNA at the ethidium/DNA ratio of 0.07 (right ordinate) (• • •) and of the ellipticities for the DNA-ethidium complex at 515 nm (left ordinate) on temperature at ethidium/DNA ratios of (—) 0.07 and (- - -) 0.10. Ellipticities are calculated on the basis of concentration of DNA-bound ethidium. [EB], 3×10^{-5} M.

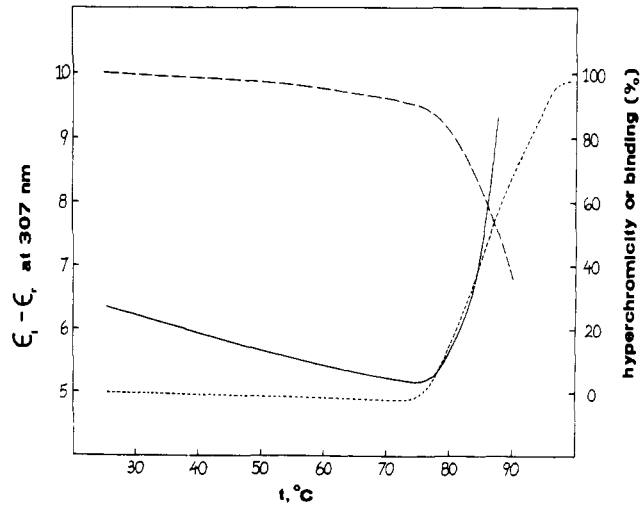

FIGURE 5: Dependence of the binding of ethidium to DNA (right ordinate) (- - -), the ellipticities at 307 nm (—) (left ordinate), and the absorbancies at 260 nm (• • •) (right ordinate) on temperature for an added ethidium/DNA ratio of 0.10. Ellipticities are calculated on the basis of the concentration of DNA-bound ethidium. [EB], 3×10^{-5} M.

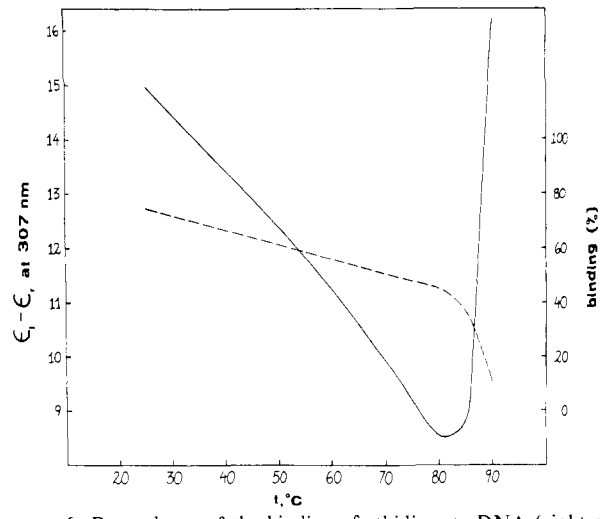

FIGURE 6: Dependence of the binding of ethidium to DNA (right ordinate) (- - -) and the ellipticities at 307 nm (left ordinate) (—) on temperature for an added ethidium/DNA ratio of 0.30. Ellipticities are calculated on the basis of the concentration of DNA-bound ethidium. [EB], 3×10^{-5} M.

appears to occur concomitantly with the decrease in the double-stranded content of DNA. As the case is apparently with denatured DNA, such decreases in the available binding sites results in an increase in the probability for interaction between drug molecules intercalated in the remaining sites, since clearly substantial amounts of drug remain bound to DNA for temperatures as high as 90°.

For temperatures below 70°, the double-stranded structure of DNA is maintained intact and the amount of intercalated dye remains nearly constant. It is also quite clear that no significant change in the factors which influence the circular dichroism of the complex near 307 nm takes place until thermal dissociation of the DNA strands is initiated near 75°.

Circular Dichroism of DNA-Ethidium Complex near 515 nm. A totally different dependency of the circular dichroism of the 0.07 complex on temperature is noted near 515 nm (Figure 4). The molar circular dichroism of the complex appears to decrease constantly and evenly over the entire range of temperatures used, though almost all the decrease in the binding of ethidium occurs sharply over the 75–90° region. The decrease in ellipticity near 515 nm, which apparently results from thermally induced changes in the conformation of the complex, is entirely consistent with the postulated origin of the circular dichroism in this region, namely the interaction between individual ethidium molecules with the surrounding nucleotide base pairs (Aktipis and Kindelis, 1973). Any redistribution of the intercalated molecules occurring during melting, which alters the probability that two dye molecules bind to neighboring sites and produces the increase in circular dichroism noted near 307 nm, would not be expected to appreciably influence ellipticities at 515 nm.

The appearance of optical activity near 515 nm requires, of course, that bound dye molecules are held in a relatively rigid manner, which confers a definite asymmetric character to the binding site. Therefore, it is reasonable to assume that the decrease of the circular dichroism at this wavelength with increasing temperatures relates to changes in the conformation of the complex. However, because of the intrinsically low circular dichroism of the complex near 515 nm as well as the decrease in the effective concentration of the complex at temperatures associated with dissociation of DNA strands, it is difficult to obtain accurate quantitative data for this wavelength at melting temperatures. It is clear though that an increase parallel to that noted for the circular dichroism of the complex at 307 nm above 75° is definitely not occurring at 515 nm.

Circular Dichroism of DNA-Ethidium Complex at Higher Ethidium to DNA Ratios. Results generally analogous to those obtained at an r of 0.07 are also observed at slightly higher ratios. At 0.10, for instance, the dependence of the circular dichroism at both 515 nm (Figure 4) and 307 nm (Figure 5) on temperature is similar to that obtained at 0.07. The deviation in the behavior of the two complexes is limited to the expected difference, namely, that the dissociation of the complex for the 0.10 ratio begins at somewhat lower temperatures as it can be seen from comparison of the temperature optical density profile at 460 nm for the 0.07 ratio with that of the 0.1 ratio (not shown here).

At a ratio of 0.30 (Figure 6), however, significant differ-

ences in the behavior of the circular dichroism at 307 nm are noted. Although increases in ellipticities do not level off at the same temperature for all complexes, some useful comparison between the ellipticities of complexes with different ratios can be made below 90°. Specifically, the increase in the ellipticity of the 0.30 complex within the melting range is smaller, *i.e.*, twofold, between 80 and 90°, than it is for the 0.07 ratio for which the ellipticity increases by a factor of 3 between 75 and 90°. For the latter complex, as the melting of the double-stranded complex begins, apparently considerable redistribution of the intercalated ethidium molecules is feasible, leading to increased binding of the dye on the remaining double-stranded regions of the polynucleotide.

The ellipticity at 307 nm is strongly dependent on the molar ratio of ethidium bromide bound to DNA over phosphate at room temperature (r) (Aktipis and Martz, 1970; Dalgleish *et al.*, 1971) or, to phrase it differently, the ellipticity at this wavelength is highly sensitive to the density with which a particular polynucleotide region is "packed" with ethidium bromide molecules. This accounts for the low $\epsilon_l - \epsilon_r$ values obtained at room temperature for the DNA–ethidium bromide complex at an r of 0.07 and the higher ellipticities obtained at higher ratios of bound drug. Furthermore during melting of the complex at low ratios the redistribution of a constant number of ethidium bromide molecules over a decreasing number of sites suitable for intercalation leads to an increase in the density of "packing" of the DNA helix with dye molecules and, therefore, results in increased ellipticities. In contrast, at the higher ratios the sites of DNA available for ethidium bromide intercalation are nearly saturated at room temperature. Therefore, any redistribution of ethidium bromide molecules during melting would not change the density of intercalated molecules to a degree sufficient to produce changes in circular dichroism as extensive as those noted for the lower ratios.

Nevertheless, the twofold increase noted in the ellipticity of the 0.30 complex between 80 and 90° is indicative of the fact that considerable dye redistribution also takes place for this complex during melting. Such redistribution is possible because, although at this ratio DNA is about 80% saturated with ethidium bromide molecules at 25°, as the temperature is increased to 80°, these molecules dissociate leading to a complex of an actual ratio of approximately 0.14 (Figure 6). Thus, redistribution of the dye becomes again possible over the melting range of 80–90°.

A more striking difference in the optical properties of the 0.07 and the 0.30 complexes is, however, noted if the change in ellipticities for these two complexes is compared at 25 and 90°. Between these two temperatures the ellipticity of the 0.07 complex is increased by a factor of 3 while the overall increase in the ellipticity of the 0.30 complex appears to be negligible. This more pronounced difference in the properties of these two complexes is partly obscured if consideration is given to the changes which occur over the melting temperature region only. This treatment, however, introduces some distortion because of the decrease in ellipticity of the 0.30 complex that occurs between 25 and 80°. This decrease, though, is unrelated to the rearrangement which occurs during melting and simply results from the thermally induced dissociation of ethidium bromide prior to melting (Figure 6). Such dissociation produces an increase in the average distance between bound ethidium bromide molecules which results in a decrease in interactions between intercalated molecules and therefore a concomitant decrease in the circular dichroism of the complex (Aktipis and Kindelis, 1973).

In an attempt to provide a more clear-cut and elegant demonstration of the small effects of temperature on the circular dichroism of DNA–ethidium complexes for which reintercalation of the dye is not feasible the circular dichroism properties of complexes with ratios (r) higher than 0.3 were also examined. However, for the ethidium bromide concentrations used in these series of experiments, complete binding of the dye to DNA cnnnot be maintained at elevated temperatures even at ratios considerably higher than 0.3. If, in order to achieve constant binding, substancially larger concentrations of DNA are used, *i.e.*, 2×10^{-4} M, extremely high optical densities are obtained especially at the higher ratios (r), making the determination of temperature–optical density profiles at 260 nm awkward. In view of these considerations the results obtained with the 0.3 complex appear to be, experimentally at least, the most desirable compromise.

Discussion

The circular dichroism properties of the DNA–ethidium complex at elevated temperatures provide additional support for the notion that the circular dichroism of the complex near 307 nm originates from interactions between intercalated ethidium bromide molecules in neighboring sites as suggested previously (Aktipis and Martz, 1970; Dalgleish *et al.*, 1971; Aktipis and Kindelis, 1973). The properties of the circular dichroism near 515 nm are also consistent with and support the suggestion that circular dichroism in this region results from interactions between individual ethidium bromide molecules with the surrounding nucleotide pairs (Aktipis and Kindelis, 1973). Thus, it appears that while the induced optical activity at 307 nm depends upon the spatial relationship that exists among intercalated dye molecules, the circular dichroism near 515 nm is a property of individually bound ethidium bromide molecules.

The variation noted in the circular dichroism of the DNA–ethidium bromide complex near 307 nm provides also some insight into the mechanism of the melting process for this complex. Free DNA can be viewed as consisting of "regions" of nucleotide sequences of certain overall base composition which are surrounded by other "regions" of different composition and, therefore, different thermal stability. The melting temperature of each "region," although influenced by the surrounding sequences, is primarily dependent on the base composition of the region. Thus, upon heating, AT-rich "regions" would be expected to melt first and GC-rich "regions" later. The stepwise nature of the melting process has been inferred by denaturation mapping in which the DNA molecule is induced to melt at room temperature by suitable adjustment of the pH (Inman and Schnös, 1970). The same conclusion has also been reached by preventing the reannealing of partially melted DNA by reaction with formaldehyde (Inman, 1966) or by quenching at low ionic strength (Fuke *et al.*, 1970).

The changes in the circular dichroism of the DNA–ethidium bromide complexes of various composition suggest that these complexes also melt in blocks depending on local nucleotide composition of the parent DNA. The presence of the ligand, however, is expected to influence the process of melting. Specifically, the phase transition boundary of each "region" is expected to shift to higher temperatures in the presence of ethidium bromide since dyes are known to preferentially stabilize the helix form over the random coil form

of DNA (Gersch and Jordan, 1965). The shift in the melting boundary for every region, though, may be the same since ethidium bromide does not exhibit any base specificity with respect to nucleotide binding (Waring, 1965b).

Thus, the melting of the DNA–ethidium bromide complex appears to take place in a manner generally similar to that of free DNA in the sense that AT-rich regions of the complex dissociate first while GC-rich "regions" are still maintaining a double-stranded conformation. The dye molecules dissociated from the AT-rich region of DNA during the initial stages of melting are apparently reintercalated within GC-rich regions which are still intact producing the increase in ellipticities which accompanies the melting of the complex. This notion is also supported by the observation that the fluorescence of intercalated proflavine undergoes a sharp increase at elevated temperatures. This increase has been interpreted as indicative of the interaction between dye molecules, released from melted regions, with sites in the remaining DNA regions which maintain a double-stranded structure (Daune and Chambron, 1968).

The behavior of the circular dichroism of the DNA–ethidium bromide complex at elevated temperatures also satisfactorily explains the higher ellipticities noted near 307 nm for complexes formed between the dye and thermally denaturated DNA. An analogous increase in the magnitude of the Cotton effect was previously observed for the denatured DNA–proflavine complex and was reported without comment (Blake and Peacocke, 1966). The increase in the circular dichroism of the denatured DNA–ethidium bromide complex, as well as, in retrospect, the increase in the circular dichroism of the corresponding proflavine complex are easily explained if the conformational characteristics of denatured DNA are taken into account. Denatured DNA consists of regions of double-stranded helix separated by other less organized regions which are described as random coil structures. For this reason, the denatured DNA–ethidium bromide complex contains, for each and every ethidium bromide to DNA ratio examined, a decreased number of primary binding sites as compared to native DNA. This decrease in the overall number of binding sites, however, enhances the probability that dye molecules will be "packed" more densely within the remaining intact helical regions. The resulting increase in the probability for interaction between intercalated molecules produces the enhanced optical activity noted over the 300–360-nm region for the denatured DNA–ethidium complex.

The inhibitory effect of ethidium bromide on the function of DNA polymerase may be, at least partly, due to the stabilization of the DNA double helix. The thermal dissociation of the helix in the presence of ethidium bromide appears to take place in a manner analogous to that of free DNA, *i.e.*, the melting process is characterized by a temperature region in which helix and coil regions coexist at finite equilibrium concentrations (Poland and Scheraga, 1966). The presence of ethidium bromide apparently shifts the melting region to higher temperatures without affecting the differences in the stabilities of double-stranded regions of different nucleotide composition. Thus, ethidium could be viewed as a component which, when added to replicating DNA, interferes with the dissociation of the double helix without changing the sequence by which various regions of the helix undergo dissociation prior to replication.

References

Aktipis, S., and Kindelis, A. (1973), *Biochemistry 12,* 1213.

Aktipis, S., and Martz, W. W. (1970), *Biochem. Biophys. Res. Commun. 39,* 307.

Alberts, B. M., and Frey, L. (1970), *Nature (London) 227,* 1313.

Blake, A., and Peacocke, A. R. (1966), *Biopolymers 4,* 1091.

Cohen, G., and Eisenberg, H. (1969), *Biopolymers 8,* 45.

Dalgleish, D. G., Peacocke, A. R., Fey, G., and Harvey, C. (1971), *Biopolymers 10,* 1853.

Daune, M., and Chambron, J. (1968), *J. Chim. Phys. Physicochim. Biol. 65,* 72.

Elliott, W. H. (1963), *Biochem. J. 86,* 562.

Fuke, M., Wada, A., and Tomizawa, J. (1970), *J. Mol. Biol. 51,* 255.

Fuller, W., and Waring, M. J. (1964), *Ber. Bunsenges. Phys. Chem. 68,* 805.

Gersch, N. F., and Jordan, D. O. (1965), *J. Mol. Biol. 13,* 138.

Gilbert, M., and Claverie, P. (1968), *J. Theor. Biol. 18,* 330.

Inman, R. B. (1966), *J. Mol. Biol. 18,* 464.

Inman, R. B., and Schnös, M. (1970), *J. Mol. Biol. 49,* 93.

LePecq, J. B., and Paoletti, C. (1967), *J. Mol. Biol. 27,* 87.

Mahler, H. R., Kline, B., and Mehrotra, B. D. (1964), *J. Mol. Biol. 9,* 801.

Martz, W. W., and Aktipis, S. (1971), *Anal. Biochem. 39,* 327.

Poland, D., and Scheraga, H. A. (1966), *J. Chem. Phys. 45,* 1464.

Tomchick, R., and Mandel, H. G. (1964), *J. Gen. Microbiol. 36,* 225.

Wahl, Ph., Paoletti, J., and LePecq, J. B. (1970), *Proc. Nat. Acad. Sci. U. S. 65,* 417.

Waring, M. J. (1964), *Biochim. Biophys. Acta 87,* 358.

Waring, M. J. (1965a), *Mol. Pharmacol. 1,* 1.

Waring, M. J. (1965b), *J. Mol. Biol. 13,* 269.