Optical Properties of the Deoxyribonucleic Acid–Ethidium Bromide Complex. Effect of Salt†

Stelios Aktipis* and Antonis Kindelis

ABSTRACT: The circular dichroism (CD) of the DNA–ethidium bromide complex is characterized by optical activity in the 290- to 600-nm range. Major bands are noted between 300 and 360 nm. A band of much lower ellipticity is centered near 515 nm. The complex between DNA and 3,8-diamino-5,6-dimethylphenanthridinium bromide, a structural analog of ethidium bromide in which the 6-phenyl group has been replaced by a methyl group, also exhibits similar CD properties. The independence noted in the ellipticities at 515 nm on the concentration of either of the above complexes indicates that optical activity in this region arises from asymmetry in the double helix induced in the vicinity of each intercalated dye molecule. In spite of a change in the association constant (K) between DNA and ethidium bromide from 2×10^6 l./mol in 0.04 M buffer to 2.8×10^5 l./mol at 5.0 M sodium chloride, the conformation of the complex appears substantially unaltered at high salt as indicated by the constancy of the CD for concentrations of sodium chloride up to 5.0 M. The CD of the complex in the 290- and 360-nm region is characterized, especially at high salt, by both positive and negative components. The dependence of the ellipticities at the maxima of these bands on the ratio of DNA-bound ethidium bromide to DNA phosphate may, of course, be explained on the assumption that the binding of increasing numbers of ethidium bromide molecules in the vicinity of molecules already bound alters progressively the conformation of the complex. In view, however, of the typical exciton splitting shape noted for the CD in the 290- to 360-nm region it is preferable to attribute the CD of the complex to manifestations of direct interactions between ethidium bromide molecules intercalated in neighboring binding sites. A better knowledge of the properties of the DNA–ethidium bromide complex is expected to contribute to our understanding of the mechanism by which ethidium bromide interferes with the synthesis of nucleic acids.

The antitrypanosomal properties of ethidium bromide appear to depend on the ability of this drug to interfere with nucleic acid synthesis (Newton, 1963; Tomchick and Mandel, 1964) by inhibiting the function of nucleic acid polymerases (Waring, 1964). This inhibition appears related to the formation of a complex between ethidium bromide and nucleic acids (Waring, 1965a).

Partly as a result of this realization the interaction of ethidium bromide with DNA has received in recent years considerable attention. The interaction is accompanied by changes in the physical properties of both the DNA and the dye. Among other changes, increases in the fluorescence quantum efficiency of ethidium bromide (LePecq and Paoletti, 1967; Wahl *et al.*, 1970) and a metachromic shift in the absorption spectrum near 500 nm (Waring, 1965b) take place. An increase in viscosity and a decrease in the sedimentation coefficient of DNA (Cohen and Eisenberg, 1969) are also noted.

At low ratios of ethidium bromide to DNA phosphate the interaction may simply be described as an insertion of the planar phenanthridinium ring system between adjacent base pairs of the double helix (Fuller and Waring, 1964). This process of "intercalation" depends on hydrophobic interactions occurring between the DNA and the dye (Fuller and Waring, 1964) as well as electrostatic forces exerted between DNA phosphate residues and the positively charged nitrogen on the phenanthridinium ring or the amino group substituents (Gilbert and Claverie, 1968).

Examination of the binding parameters associated with the DNA–ethidium bromide interaction has indicated the existence of two types of binding. A strong interaction which apparently is associated primarily with intercalation predominates at ethidium bromide to polynucleotide ratios below 0.25. In addition, secondary binding arising from electrostatic interactions between phosphate residues and ethidium bromide molecules attached to the outside of the helix may take place at the higher ratios (Waring, 1964, 1965b). Recent circular dichroism studies of the DNA–ethidium bromide complex have revealed the presence of induced optical activity in the 290- to 600-nm region (Aktipis and Martz, 1970; Dalgleish *et al.*, 1971). The dependence of the CD near 308 nm on DNA-bound ethidium bromide over phosphate ratios noted in this system has been interpreted as an indication that the CD of an intercalated ethidium bromide depends upon the effect of a second dye molecule also intercalated in the vicinity of the first.

In this report we will present some new findings on the dependence of the CD of complex on the ethidium bromide: DNA ratio, and such factors as ionic strength and temperature and we will examine the possible origins of the CD near 308 and 515 nm.

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, a working solution (1×10^{-3} M) was prepared before each experiment by diluting with buffer. The concentration of the working solution was determined spectrophotometrically using an extinction coefficient of 6600 at 260 nm (Mahler *et al.*, 1964). Ethidium

† From the Department of Biochemistry and Biophysics, Stritch School of Medicine, Loyola University of Chicago, Maywood, Illinois 60153. *Received September 25, 1972.* This investigation was supported by National Institutes of Health Grant No. CA10346.

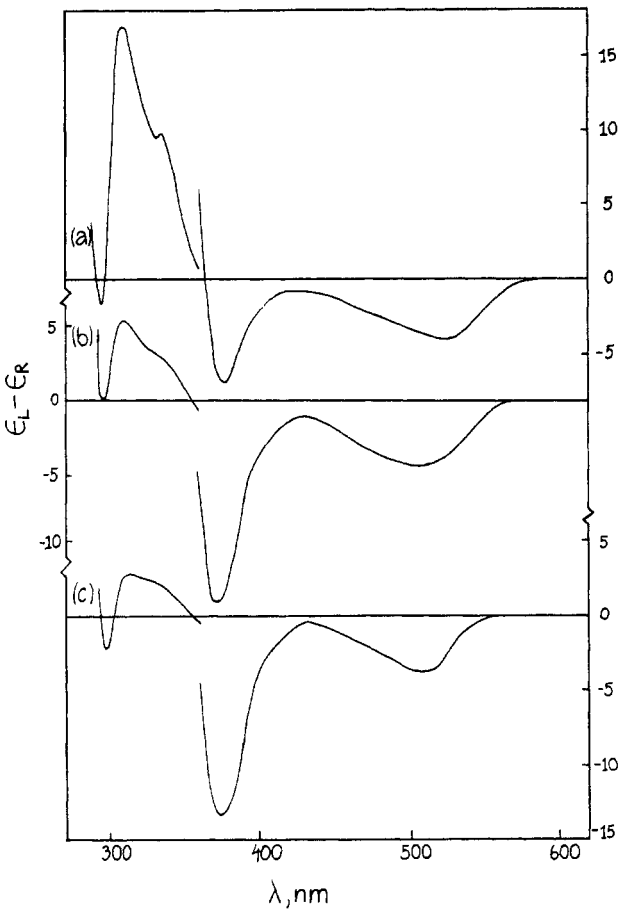

FIGURE 1: The circular dichroism of DNA–ethidium bromide at pH 7.9, DNA(P) 2×10^{-4} M. Molar ratios of ethidium bromide to DNA phosphate are expressed on the basis of added ethidium bromide (ethidium bromide:P) and/or on the basis of bound ethidium bromide (r). (a) DNA–ethidium bromide in 0.04 M Tris-HCl buffer, ethidium bromide:P of 0.35; (b) DNA–ethidium bromide in 0.04 M Tris-HCl buffer, ethidium bromide:P of 0.1; r of 0.1; (c) DNA–ethidium bromide in 5.0 M sodium chloride, ethidium bromide:P of 0.1; r of 0.086. $\epsilon_l - \epsilon_r$ scale for wavelengths below 360 nm as shown. For higher wavelengths $\epsilon_l - \epsilon_r$ values must be multiplied by a factor of 0.1.

bromide (lot no. 100301, Calbiochem, Los Angeles, Calif.) solution was freshly prepared before each experiment in the same buffer and the solution was kept in the dark until it was used. Ethidium bromide 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 added ethidium bromide to DNA phosphate (ethidium bromide:P) were prepared by mixing appropriate volumes of the working solutions and diluting with buffer to a standard volume (usually 10 ml). As a rule, a constant amount of DNA was used (2.0 ml of a 10^{-3} M solution) and the amount of the dye was varied. In some instances however, as indicated in the corresponding figures, the reverse procedure was used. For experiments carried out at higher ionic strengths a series of solutions was prepared by adding sodium chloride to Tris-HCl buffer (0.04 M, pH 7.9) so that final sodium chloride concentrations ranged between 0.025 and 5.0 M. Solutions of the complex containing various concentrations of sodium chloride were prepared by mixing DNA and ethidium bromide in the appropriate sodium chloride solution and adjusting to a constant volume.

3,8-Diamino-5,6-dimethylphenanthridinium bromide solutions were prepared by dissolving 1.6 mg of the dye in 10 ml of buffer. Samples with various dye to phosphate ratios were prepared by mixing aliquots of this solution (2 ml) with varying volumes of DNA solution and adjusting with buffer to 10 ml. A crystalline sample of 3,8-diamino-5,6-dimethylphenanthridinium bromide was kindly provided by Dr. T. I. Watkins (Boots Pure Drug Co., Ltd., Great Britain).

Binding Studies. The concentration of the DNA–ethidium bromide complex and the molar ratio of DNA-bound ethidium bromide to DNA phosphate (r) were calculated from the shift in the absorption maximum of ethidium bromide upon binding to DNA as described previously (Peacocke and Skerett, 1956). The same procedure was used for the determination of binding of 3,8-diamino-5,6-dimethylphenanthridinium bromide (in 0.04 M Tris-HCl buffer) and for the binding of ethidium bromide at 5.0 M sodium chloride. In both instances the absorption spectra provided little, if any, evidence of aggregation for the dye in the concentration range employed in these studies and the spectra of the complexes exhibited satisfactory isosbestic points near 486 and 516 nm, respectively. Binding was calculated from the optical densities at 450 nm for the 3,8-diamino-5,6-dimethylphenanthridinium bromide and at 460 nm for ethidium bromide.

Intrinsic association constants (K) were obtained from the Scatchard relation ($r/c = Kn - Kr$) by plotting r/c *vs.* r; where c is the molar concentration of free ethidium bromide and r the molar ratio of DNA-bound dye to DNA phosphate.

Circular Dichroism Measurements. Circular dichroism spectra were recorded using a Durrum-Jasco ORD/UV-5 spectropolarimeter modified to a maximum sensitivity of 2×10^{-3} deg/cm (Durrum Instruments, Palo Alto, Calif.). Measurements were carried out in cells with optical path lengths ranging from 0.1 to 5.0 cm so that for every measurement 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

$$\epsilon_l - \epsilon_r = \frac{\text{degrees of ellipticity}}{33 \times c \times l}$$

where degrees of ellipticity were obtained directly from the recorder chart, the concentration c was expressed in moles per liter and l, the path length of the cell, was expressed in centimeters. $\epsilon_l - \epsilon_r$ values were calculated on the basis of the concentration of bound rather than "total added" ethidium bromide except as otherwise specified.

Measurements at Elevated Temperatures. The CD of the DNA–ethidium bromide complex at elevated temperatures was examined using a modified Beckman Tm analyzer with a Moseley X–Y recorder (Hewlett Packard Corp., Model No. 7035B) which permits automatic recording of CD *vs.* temperature (Martz and Aktipis, 1971). The calculation of molar circular dichroism for each temperature is feasible from ethidium bromide binding data at elevated temperatures. Such data can be conveniently obtained because 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° with a concomitant decrease in the apparent extinction coefficient from 5600 to about 5470. Furthermore, both the apparent extinction coefficient of free ethidium bromide and the absorption of the DNA–ethidium bromide complex at 460 nm can be automatically monitored by recording optical densities *vs.* temperature using the Beckman Tm analyzer. Thus, the concentrations of bound ethidium bromide can be calculated from

the optical densities at 460 nm. For the complexes examined, the concentration of bound ethidium bromide calculated by this method was in good agreement with the results of equilibrium dialysis obtained at elevated temperatures. For instance, for a complex prepared by addition of ethidium bromide (0.09 μmol/ml) to DNA (0.6 μmol/ml) and dialyzed at 96° for 20 hr the amount of bound ethidium bromide was determined to be 0.017 μmol/ml giving an r of 0.027; the value of r determined spectrophotometrically for the same complex was 0.028.

Results

Circular Dichroism of the DNA–Ethidium Bromide Complex. The effect of ethidium bromide on the CD of DNA is shown in Figure 1. At molar ratios of added dye to DNA phosphate (ethidium bromide:P) of 0.10 and 0.35 a complex positive band is generated in the 300- to 350-nm region with a maximum near 308 nm and a shoulder near 335 nm (Figure 1a,b, respectively). Similar results were reported previously (Aktipis and Martz, 1970) and are in agreement with findings published more recently (Dalgleish *et al.*, 1971). In the region between 350 and 600 nm two distinct negative bands of low intensity are also noted; one centered near 515 nm and the second one centered near 375 nm. By comparison, free ethidium bromide does not exhibit any optical activity over the 300- to 600-nm region.[1]

The positions of the induced CD bands in the DNA–ethidium bromide complex are consistent with the absorption characteristics of the complex. The absorption spectrum of free ethidium bromide exhibits an absorption shoulder near 310 nm and a broad band extending between 400 and 550 nm.

Examination of the CD of the complex reveals that the dependence of ellipticities, expressed as $\epsilon_l - \epsilon_r$ per bound ethidium bromide, on the ethidium bromide:P ratio differs for various wavelengths. The CD of the 308-nm band is highly dependent on ethidium bromide:P. In contrast the 515-nm band appears totally independent of this ratio. The behavior of the 375-nm band is more difficult to determine because of the overlap between this band and the CD between 300 and 350 nm. The inverse relation which exists between the ellipticities of the bands at 308 and 375 nm, however, indicates that these two bands do not originate from the same transition.

Finally, a small band centered near 295 nm can be distinguished for the complex with ethidium bromide:P ratio of 0.35. The presence of this band may have escaped previous detection partly because of the low intensity of the CD generally associated with it and partly because this band is completely absent at the lower ethidium bromide:P ratios.

Dependence of the CD on the Ratio of Ethidium Bromide to DNA. More informative comparisons between the CD of various DNA–ethidium bromide complexes are, of course, possible when these complexes are specified in terms of the amount of bound rather than the amount of total added ethidium bromide. $\epsilon_l - \epsilon_r$ values for the complex remain nearly constant at about −0.5 for r between 0.05 and 0.22. By comparison, the dependence of $\epsilon_l - \epsilon_r$ at 308 nm, on bound ethidium bromide to nucleotide ratios (r), shown in Figure 2, differs sharply from the behavior noted at 515 nm. $\epsilon_l - \epsilon_r$ values near 308 nm have been found to depend on the ratio of bound ethidium bromide (Aktipis and Martz, 1970) and reach a maximum of about 25 at an r between 0.30 and 0.35 (Martz, 1971; Dalgleish *et al.*, 1971), a ratio which corresponds to the saturation region of the DNA–ethidium bromide binding.

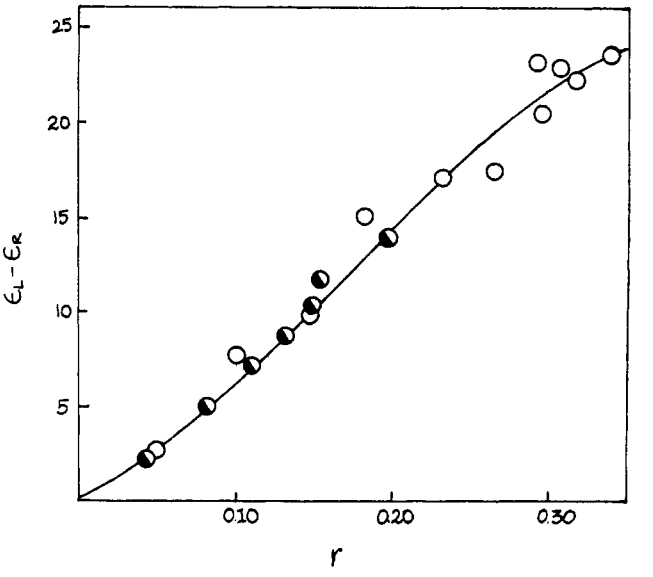

FIGURE 2: The dependence of ellipticities at the band maximum near 308 nm for DNA–ethidium bromide at pH 7.9; DNA(P) 2×10^{-4} M. (○) 0.04 M Tris-HCl buffer; (◐) 5.0 M sodium chloride.

The circular dichroism near 308 nm has previously been attributed to interactions between ethidium bromide molecules intercalated in neighboring binding sites (Aktipis and Martz, 1970; Dalgleish *et al.*, 1971). In contrast the independence of the CD on r for the 515-nm band is an indication that the band is associated with the interaction between individual intercalated ethidium bromide molecules and the surrounding nucleotide bases. The ellipticities characteristic of such interactions would presumably be independent from the extent to which other ethidium bromide molecules are present in the vicinity of the binding site.

Circular Dichroism of the Complex at High Ionic Strength. The general features of the CD of the DNA–ethidium bromide complex are not substantially altered in 5.0 M sodium chloride solution (Figure 1c). Apart from an increase in the ellipticity of the 375-nm band both the ellipticities and the maxima of the other bands located at wavelengths higher than 300 nm remain unchanged. In fact, the results listed in Table I indicate that ellipticities at 308 nm as well as 515 nm remain remarkably constant for salt concentrations which vary between 0.04 and 5.0 M. Furthermore, examination of Figure 1 reveals that the dependence of the ellipticities near 308 nm on bound ethidium bromide to DNA phosphate ratios (r) at 5.0 M sodium chloride exhibits a close similarity to the results obtained at 0.04 M buffer. The only apparent qualitative difference between the spectra at 0.04 M buffer and 5.0 M sodium chloride is that in the latter the negative CD band located near 295 nm is present for all ethidium bromide to DNA ratios examined, while at the lower ionic strength this band is present only for high ethidium bromide to DNA ratios.

These results suggest that contributions from negative CD

[1] We have previously reported (Aktipis and Martz, 1970) some minor optical activity for ethidium bromide, the magnitude of which was apparently within the limits of experimental error of the instrument that was available to us at the time (Durrum-Jasco ORD/UV-5). Optical activity was not observed in subsequent measurement when the same spectropolarimeter modified to operate with a highly improved signal to noise ratio (maximum sensitivity of 2×10^{-3} deg/cm) was employed.

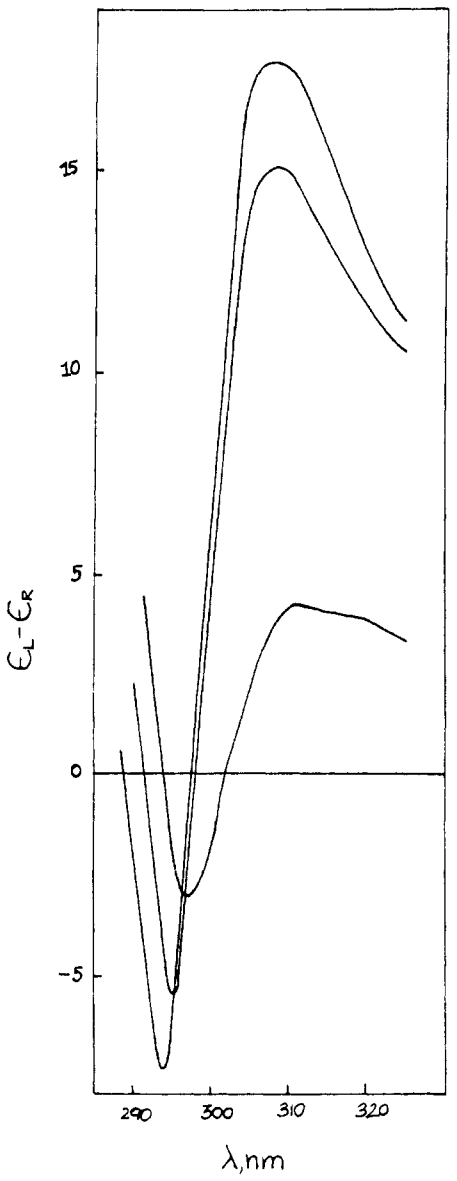

FIGURE 3: The circular dichroism of DNA–ethidium bromide in 5.0 M sodium chloride–buffer (pH 7.9) for various molar ratios of DNA-bound ethidium bromide to DNA phosphate; DNA (P) 2×10^{-4} M; upper curve r 0.086; middle curve r 0.162; lower curve r 0.180.

in the vicinity of this band may in fact be present for low ethidium bromide to DNA ratios as well but a definite band near 295 nm can be distinguished only at higher ionic strengths or at higher ratios.

The increase in the CD and the shift in the apparent maximum of this band from 296 to 292 nm noted with increasing r at constant concentration of ethidium bromide of 0.3×10^{-4} M and changing DNA concentrations (Table II) may simply be interpreted as the result of two distinct trends: the increase in the negative CD originating from transitions characteristic of the interaction between DNA and ethidium bromide and the increase in the overlap between the CD originating from this interaction with the positive CD of DNA present at wavelengths below 300 nm. This overlap would tend to produce the shift in the apparent maximum of the 295-nm band and would limit ellipticities even at the higher ethidium bromide:P ratios.

TABLE I: Circular Dichroism of the DNA–Ethidium Bromide[a] Complex at Various Salt Concentrations.

$[Na^+]$ (mol/l.)	$\epsilon_l - \epsilon_r$ at 308 nm[b]	$\epsilon_l - \epsilon_r$ at 515 nm
None	5.2	−0.53
0.12	6.1	−0.41
0.20	6.0	−0.46
0.50	6.1	−0.48
0.60	5.4	−0.45
0.75	5.5	−0.41
1.0	6.0	−0.50
5.0	5.4	−0.46

[a] Measurements were carried out for samples of added ratios (ethidium bromide:P) between 0.085 and 0.28 with r's ranging between 0.085 and 0.14; DNA concentration maintained constant at 2×10^{-4} M. [b] $\epsilon_l - \epsilon_r$ values are reported for an r of 0.085 and were estimated from plots of $\epsilon_l - \epsilon_r$ *vs.* r.

Much higher ellipticities near 295 nm are noted at 5.0 M sodium chloride (Figure 3). This increase is consistent with the expected decrease in the contribution of the positive CD of DNA below 300 nm. The CD of DNA in 6.0 M lithium chloride is characterized by reduced ellipticities in the region between 290 and 260 nm and an especially pronounced reduction in the CD above 290 nm (Studdert *et al.*, 1972). We have obtained similar though less drastic changes in the CD of DNA at 5.0 M sodium chloride which would, of course, tend to strengthen the 295-nm band.

The variations in the ellipticities of the complex near 295 nm with r noted both at 0.04 M buffer and at 5.0 M sodium chloride, when the CD is obtained at constant DNA concentrations (2×10^{-4} M) (Figure 3, Table III) deserves some comment. The only apparent explanation for these results is that the negative CD near 295 nm exhibits an intrinsic dependence on the ethidium bromide:P ratios, increasing as this ratio increases. The behavior of the 295-nm band appears therefore to be similar to that noted for the positive CD band near 308 nm and distinct from that of the band centered at 515 nm. This indicates that the 295- and the 308-nm bands may have similar origin. In fact, the relationship between these two bands in terms of both their neighboring positions and the dependence of their ellipticities on r strongly suggests that these bands are manifestations of the positive and the negative CD components of the same conservative transition (Tinoco, 1964).

The generally lower ellipticities noted for the negative component and the somewhat assymmetric shape of the doublet are, of course, consistent with the overlap between this band and the CD of DNA below 300 nm.

Interaction between Ethidium Bromide and DNA in 5.0 M Sodium Chloride. The remarkable similarities noted in the CD of the DNA–ethidium bromide complex at 0.04 M buffer and at 5.0 M sodium chloride raise the question whether these similarities are consistent with the changes in the binding interactions between DNA and ethidium bromide in the presence of high concentrations of salt. The association constant for the DNA–ethidium bromide interaction is sensitive to salt. Specifically the Scatchard plot for this interaction (Figure 4) in 0.04 M buffer permits the calculation of an intrinsic

TABLE II: Dependence of the Circular Dichroism[a] near 295 nm on the Molar Ratio of DNA-Bound Ethidium Bromide over DNA Phosphate (r).

Added Ratio EB:P	Bound Ratio r	λ_{max} (nm)	$\epsilon_l - \epsilon_r$ at λ_{max}
0.15	0.15	296	−0.7
0.25	0.21	295	−1.7
1.00	0.24	292	−2.0

[a] Obtained in 0.04 M Tris-HCl buffer; ethidium bromide (EB) concentration maintained constant at 0.3×10^{-4} M.

TABLE III: Dependence of the Circular Dichroism[a] near 295 nm for the DNA–Ethidium Bromide Complex on the Molar Ratio of DNA-Bound Ethidium Bromide over DNA Phosphage (r).

Added Ratio EB:P	Bound Ratio r	λ_{max} (nm)	$\epsilon_l - \epsilon_r$ at λ_{max}
0.10	0.085	297	−3.2
0.20	0.133	296	−3.6
0.40	0.162	295	−5.4
0.50	0.164	295	−5.7
0.65	0.164	295	−6.3
0.80	0.165	294	−6.4
1.00	0.180	294	−7.2

[a] Obtained in 5.0 M sodium chloride; DNA concentration maintained constant at 2×10^{-4} M.

association constant K of 2×10^6 l./mol. Values for this constant of 1.4×10^6 l./mol (Waring, 1965b) and 2.7×10^6 l./mol (Angerer and Moudrianakis, 1972) can be calculated from available binding data but such differences are, of course, within the limits of experimental error. A substantial change in K to 2.8×10^5 l./mol is noted, however, at 5.0 M sodium chloride. The number of apparent binding sites (n) is also decreased from approximately 0.27 at 0.04 M buffer to 0.19 at 5.0 M sodium chloride.

The change noted in the association constant at the higher salt concentration is not surprising since, at neutral pH, electrostatic interactions between positively charged dye molecules and the phosphate residues in DNA appear to be the main contributors to the binding force (Gilbert and Claverie, 1968). In fact, the interaction may essentially be described as the distribution of charges from a small region with the dimensions of the ethidium bromide molecule to the much larger volume occupied by the DNA macromolecule. Although other factors may also participate in the stabilization of the complex (Gersch and Jordan, 1965), the dispersion of charges which accompanies intercalation is clearly a process strongly dependent on ionic strength. A similar 10-fold decrease in the association constant between T2 DNA and ethidium bromide has been reported to occur with increasing ionic strength in the presence of 4×10^{-3} M magnesium chloride (Waring, 1965b).

For each specific set of conditions the maximum amount of dye intercalated in DNA appears to depend, among other factors, on the exact geometry of the helix. Although conflicting views have been advanced on the precise effect of intercalation on the geometry of the complex (Fuller and Waring, 1964; Bauer and Vinograd, 1970; Paoletti and LePecq, 1971), such interactions undoubtedly bring about changes in rotation and translation along the helix. These changes are expected to influence the subsequent binding of additional molecules of ethidium bromide. It has been suggested that, at high sodium chloride concentrations, the sodium ions tend to hinder this dye-promoted alteration of the geometry of the helix resulting in a decrease in the number of binding sites (Angerer and Moudrianakis, 1972).

In spite of the changes noted in the association constant and the number of binding sites for the DNA–ethidium bromide complex, there is no reason to assume that the basic nature of the interactions between ethidium bromide molecules bound to adjacent primary sites on DNA has been substantially altered at high salt. As previously noted, the overall shape of the CD as well as the ellipticities at both 308 and 515 nm remain unchanged over a wide range of sodium chloride concentrations. This is an indication that, aside from the change in the association constant detected from the Scatchard plots, the nature of the DNA–ethidium bromide complex is not drastically modified.

Furthermore, changes in ionic strength do not appear to affect the interactions between intercalated neighboring ethidium bromide molecules. In fact, at 5.0 M sodium chloride the ellipticity of the DNA–ethidium bromide complex near

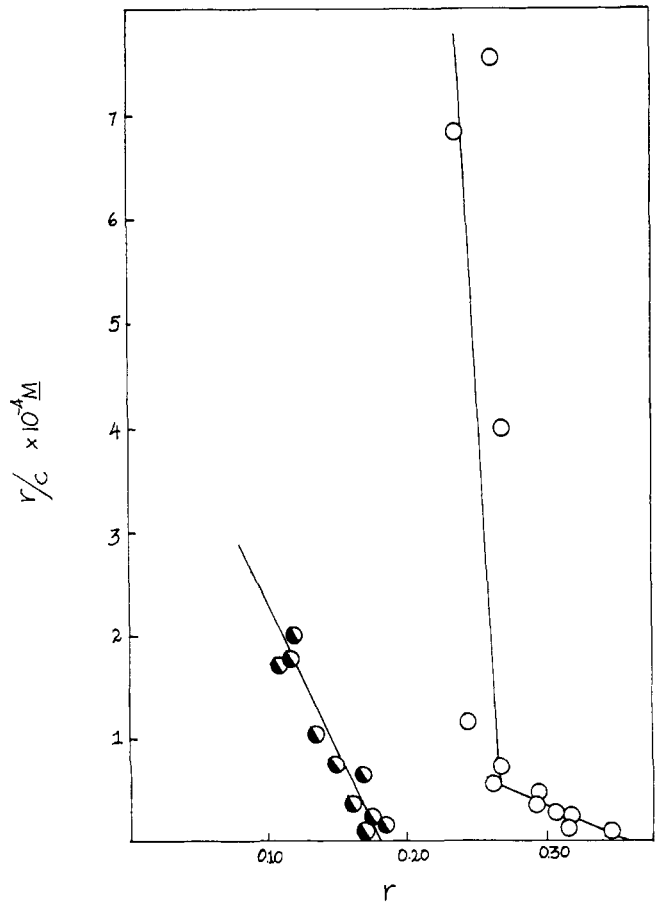

FIGURE 4: Scatchard plots for the interaction between DNA (2×10^{-4} M) and ethidium bromide at pH 7.9. (○) 0.04 M Tris-HCl buffer; (◐) 5.0 M sodium chloride.

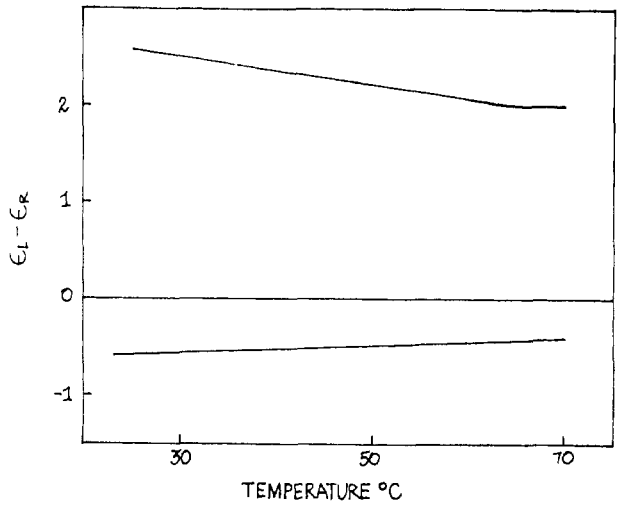

FIGURE 5: Effect of temperature on the circular dichroism of DNA–ethidium bromide complex in 0.04 M Tris-HCl (pH 7.9); DNA(P) 6×10^{-4} M. Upper curve CD at 307 nm; lower curve CD at 515 nm. The molar ratio of DNA-bound ethidium bromide to DNA phosphate (r) remains constant between 25 and 70° at 0.05.

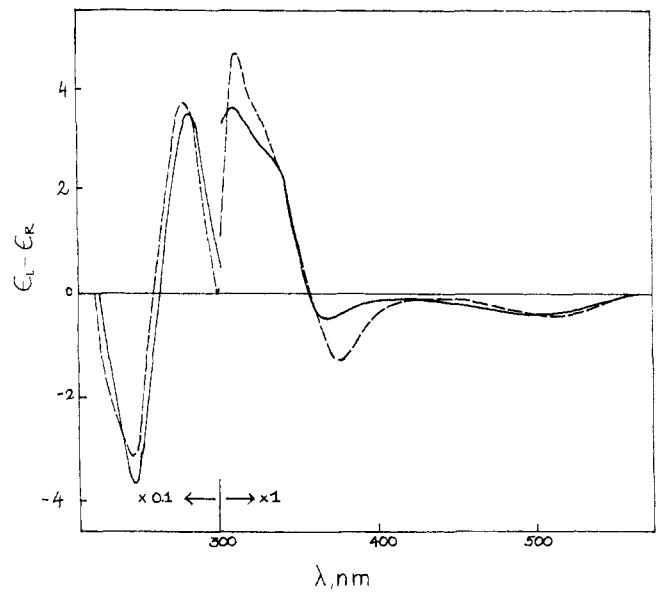

FIGURE 6: The circular dichroism of DNA–3,8-diamino-5,6-dimethylphenanthridinium bromide complex in 0.04 M Tris-HCl buffer (pH 7.9) at a molar ratio of DNA-bound dye to DNA phosphate (r) of 0.10; DNA (P) 1×10^{-4} M. The CD of DNA–ethidium bromide under the same conditions is indicated by the dotted line. $\epsilon_l - \epsilon_r$ scale for wavelengths above 300 nm as shown. For below 300 nm, scale reading must be multiplied by a factor of 10.

308 nm increases with r in a manner which closely parallels that observed at 0.04 M buffer (Figure 2). Clearly then, both the CD of the complex and the dependence of the ellipticities on r indicate that similar interactions occur between intercalated ethidium bromide molecules at both the lower and the higher concentrations of salt. This conclusion is in agreement with the results obtained with the DNA–proflavine complex for which it was also concluded that the CD effects induced by dye–dye interaction are independent of salt concentration (Li and Crothers, 1969; Blake and Peacocke, 1966).

Effect of Temperature on the CD of the DNA–Ethidium Bromide Complex. Although at elevated temperatures a sharp decrease in the binding between ethidium bromide and DNA is expected to accompany the separation of DNA strands (Aktipis and Martz, 1970), the binding of ethidium bromide is rather insensitive to temperature variations in the 25–70° range.

In spite of this constancy in the binding below 70° the observed molar CD near 515 nm appears to decrease with temperature (Figure 5). Since the CD in this region may be the result of electronic interactions between single intercalated ethidium bromide molecules and the surrounding DNA bases the observed small change is not surprising. Optical properties are clearly sensitive to the exact three-dimensional structure of the complex and conformational changes occurring at the sites of ethidium bromide intercalation are apparently sufficient to modify the interaction between ethidium bromide and the surrounding base pairs. The decrease noted in the molar CD near 308 nm with increasing temperatures below 70° is more pronounced and it can be explained on similar terms since the interactions between ethidium bromide molecules bound to adjacent sites in DNA would again be expected to depend on the conformation of the complex.

CD of the DNA–3,8-Diamino-5,6-dimethylphenanthridinium Bromide Complex. In an attempt to assess whether the phenyl substituent present on the phenanthridinium ring of the dye is involved in the interaction between intercalated molecules, the CD of a complex prepared from DNA and a structural analog of ethidium bromide, 3,8-diamino-5,6-dimethylphenanthridinium bromide in which the 6-phenyl group has been replaced by a 6-methyl substituent, was obtained and compared with the CD of the DNA–ethidium bromide complex.

The spectra shown in Figure 6 exhibit striking similarities throughout the 220- to 600-nm region which is an indication that the three-dimensional structures of the two complexes have a number of common characteristics. The similarity in the shape of the CD bands in the 300- to 360-nm region, the comparable ellipticities noted for the two complexes over this region and, most significantly, the dependence of the CD of the DNA–3,8-diamino-5,6-dimethylphenanthridinium complex on the ratio of the bound dye both near 308 nm (Figure 7) and at 515 nm (Table IV) are additional evidence that the nearest-neighbor interactions giving rise to the induced CD in this region as well as the interactions between nucleotide residues and dye molecules are similar for the two complexes.

Discussion

It is generally recognized that the effects which accompany the binding of a dye to a polymer depend, as a rule, upon both the exact nature of the polymer and such factors as concentration and dye to polymer molar ratios. Optical activity in the dye–polymer complexes at very low dye to polymer ratios is attributed to asymmetry induced by a monomeric chromophore bound within the asymmetric environment of the polymer (Yamaoka and Resnik, 1966). At higher ratios, however, the possibility exists that coupling between chromophores of the bound dye will contribute increasingly to the optical activity of the complex (Gardner and Mason, 1967).

The optical properties of the DNA–ethidium bromide complex appear to originate from both models of interaction cited above. The CD properties of the complex over the 290- to 360-nm region are radically different from those noted over

TABLE IV: Circular Dichroism[a] of the DNA–3,8-Diamino-5,6-dimethylphenanthridinium Bromide Complex on DNA-Bound Dye over DNA Phosphate (r).

Added Ratio	Bound Ratio r	$\epsilon_l - \epsilon_r$ at 505 nm
0.05	0.050	−0.36
0.10	0.100	−0.36
0.25	0.217	−0.31
0.50	0.237	−0.41

[a] Obtained in 0.04 M Tris-HCl buffer; dye concentration maintained constant at 1×10^{-5} M.

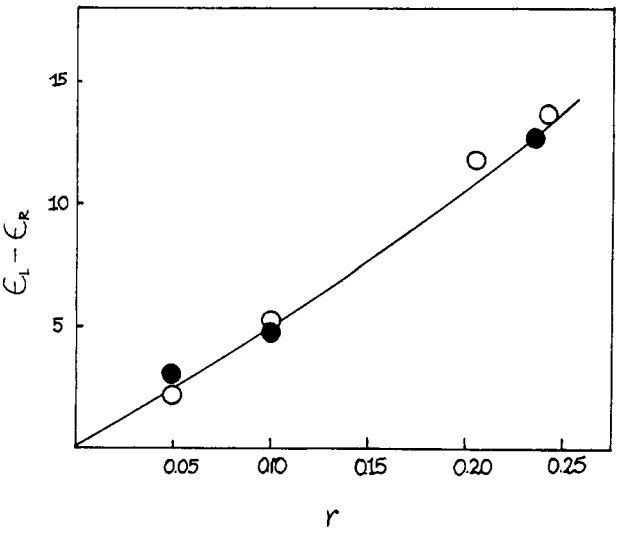

FIGURE 7: The dependence of ellipticities at the band maxima near 307 nm for the DNA–dye complex in 0.04 M Tris-HCl buffer (pH 7.9). Dye concentration is maintained constant at 10^{-5} M. (●) 3,8-Diamino-5,6-dimethylphenanthridinium bromide; (○) ethidium bromide.

the 450- to 550-nm region. Consequently the bands with the 308-nm and 515-nm maxima must be attributed to different sources of asymmetry.

Specifically, the characteristics of the long wavelength band, *i.e.*, appearance of optical activity at low ethidium bromide to nucleotide ratios and independence of the molar circular dichroism from this ratio, suggest that the optical activity in this region results from asymmetry induced upon the binding of individual ethidium bromide molecules within the environment of the macromolecule.

The binding of ethidium bromide to DNA at the lower ethidium bromide to nucleotide ratios is, of course, predominantly of the "primary" type (Waring, 1965). Therefore, the induced CD at 515 nm may be viewed as a direct result of the interaction between intercalated ethidium bromide molecules with the surrounding DNA base pairs.

In contrast, the CD band near 308 nm exhibits a behavior which indicates a distinctly different origin. The molar circular dichroism of this band increases with increasing ratios of bound ethidium bromide to nucleotide reaching a maximum value at a ratio which corresponds to the saturation of the primary binding sites of DNA (Figure 4). It may be noted that this dependence is analogous to that reported for the proflavine–DNA complex (Li and Crothers, 1969; Dalgleish *et al.*, 1969).

The negligible molar CD of the DNA–ethidium bromide complex near 308 nm at very low ethidium bromide to nucleotide ratios ($r < 0.05$) indicates that isolated ethidium bromide molecules bound to primary binding sites contribute very little, if at all, to the induced CD at this wavelength. Under these conditions, the probability that one ethidium bromide molecule is intercalated in a position adjacent to another is very low but this probability, of course, increases at the higher ethidium bromide to DNA ratios.

The increased ellipticities observed at higher ratios suggest that the development of induced CD near 308 nm may be the result of direct interaction between two or more regularly ordered ethidium bromide molecules bound to adjacent sites in DNA. In this discussion we will be referring to this mode of interactions as model I. Alternatively, it is possible that the progressive binding of increasing numbers of ethidium bromide molecules in the vicinity of molecules already bound alters continuously the conformation of the complex (Dalgleish *et al.*, 1969). According to this scheme (model II), the increased CD near 308 nm is a reflection of this conformational change. In either of these two possibilities the exact environment of every bound ethidium bromide molecule clearly depends upon the presence of other ethidium bromide ligands in the vicinity of the binding site.

All currently available evidence is consistent with either of the above models. In fact a clear-cut choice between the two may not be possible because evidence which would exclude one in favor of the other is difficult to come by. Model II is perhaps the most elusive with respect to direct testing because definite theoretical predictions regarding the characteristics of the CD associated with it are not available. It is, therefore, not possible at this time to eliminate this model at least as a potential contributor to the optical activity of the complex in the vicinity of the 308-nm band. More definite statements, however, can be made regarding the character of the CD of model I which is expected to exhibit a conservative doublet characteristic of exciton splitting (Tinoco, 1964).

In fact, the contribution of direct interactions mediated by the intervening base pairs to the circular dichroism for the DNA–ethidium bromide complex has been questioned previously (Dalgleish *et al.*, 1971) precisely because of the absence of a characteristic conservative doublet. The elusive nature of this doublet, however, appears to be simply the result of an overlap between the negative component of the doublet near 295 nm and the strong positive band centered at 275 nm. This latter band, which is present in DNA and becomes intensified in the complex, apparently masks the negative component of the doublet to the extent that, under the normally employed conditions of low ionic strength, this component is either completely absent or drastically decreased.

Clearly, however, the CD of the complex, at least under conditions of high salt, behaves as predicted from classical models for stacked chromophores. This supports the notion that the interactions between intercalated ethidium bromide molecules described by model I are important contributors to the CD of the complex at high salt. Furthermore, this conclusion may be extended and applied to lower ionic strengths if it is assumed that under these conditions the conformation of the complex is not substantially altered. There are indeed indications that, aside from a minor change in the average angle of rotation of the helix, the conformation of the DNA is not particularly sensitive to salt (Wang, 1969). The similarities in the CD of the complex at 0.04 M buffer and 5.0 M

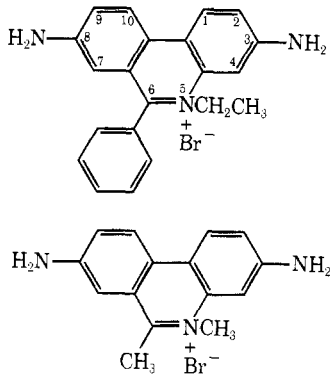

FIGURE 8: The structures of ethidium bromide (above) and 3,8-diamino-5,6-dimethylphenanthridinium bromide (below). The IUPAC instituted numbering system is indicated.

sodium chloride indicate that a similar condition may apply to the DNA–ethidium bromide complex. Interactions between stacked dye chromophores are also expected to occur in the proflavine–DNA complex. It is of interest to note that circular dichroism of a conservative type is also apparently associated with this system (Li and Crothers, 1969).

One question which may be raised regarding the origin of the interactions between dye molecules intercalated in adjacent sites is whether such interactions are transmitted through the nucleotide base pairs which intervene between dye molecules or occur in some other more direct manner. The intercalation of the phenanthridinium ring places the phenyl and ethyl substituents within the large groove of DNA (Fuller and Waring, 1964). Furthermore, if it is assumed that the prevailing view, that the 3- and 8-amino groups of the ring are in contact with the phosphates in opposite strands, is correct, it becomes apparent that no part of the phenanthridinium ring is accessible for direct interactions between intercalated molecules. In this configuration, however, such interactions could conceivably occur *via* the phenyl rings which are not shielded by the bases surrounding the intercalated ring. The behavior of the CD for a complex between DNA and 3,8-diamino-5,6-dimethylphenanthridinium bromide, which is a phenanthridinium derivative differing from ethidium bromide in the sense that it does not carry a phenyl substituent (Figure 8), indicates, however, that if interactions between phenyl groups of intercalated ethidium bromide molecules occur to any significant degree, such interactions do not contribute appreciably to the CD of the complex near 308 nm.

It appears, therefore, that interactions between intercalated ethidium bromide molecules are exerted from one ethidium bromide molecule to the next *via* the intervening bases.

The dependence of the CD on temperature at both 308 and 515 nm indicates that elevated temperatures bring about a conformational reorganization of the complex. This reorganization would, of course, be reflected by the CD near 308 nm regardless of whether the source of optical activity is best described by model I or II. The latter model, however, may be a better candidate for pronounced changes in the CD at elevated temperatures since changes in conformation would probably affect both the distances as well as the relative position between neighboring ethidium bromide molecules. This model would, therefore, be expected to be particularly susceptible to changes which affect the CD originating from direct interactions between intercalated molecules.

Since such interactions would depend on both distance and relative position of the chromophores (Tinoco, 1962), these results indicate that a statement generally made about macromolecules, namely, that changes in conformation whether achieved by elevated temperatures or other means are frequently reflected by the CD, is clearly applicable to the DNA–ethidium bromide complex. This reinforces the conclusion that the stability of the CD of the DNA–ethidium bromide complex at high concentrations of salt is an excellent indication that the conformation of the complex is not appreciably altered in the presence of salt.

In summary, it appears that the applicability of classical models for mutually interacting stacked chromophores can be tested in this system. The conservative CD noted for the complex at high ionic strength is consistent with model I. Furthermore, there are indications that contributions from a conservative doublet are also present in the CD at lower ionic strengths.

The possibility that the alternative model for DNA–ethidium bromide interactions (model II) contributes to the CD of the complex cannot, of course, be excluded. It must be noted, though, that convincing evidence, which supports this alternative in preference to the stacked chromophore model, which depends upon direct interactions among intercalated ethidium bromide molecules, remains to be advanced. Therefore, it may be stated that the first model not only describes the interactions occurring between DNA and ethidium bromide in a completely satisfactory manner but it is also the most compatible with all the currently available experimental evidence.

A detailed description of the physicochemical nature of the interaction between intercalated ethidium bromide and double-stranded DNA is, of course, essential for eventually arriving at a better understanding of the relationship between the structure of the DNA–ethidium bromide complex and the known biological properties of ethidium bromide.

Acknowledgment

We are indebted to Dr. William W. Martz for carrying out a number of preliminary experiments and to Dr. T. I. Watkins for generously providing us with a sample of 3,8-diamino-5,-6-dimethylphenanthridinium bromide.

References

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

Angerer, L., and Moudrianakis, E. N. (1972), *J. Mol. Biol. 63*, 505.

Bauer, W., and Vinograd, J. (1970), *J. Mol. Biol. 47*, 419.

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

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

Dalgleish, D. G., Fujita, H., and Peacocke, A. R. (1969), *Biopolymers 8*, 633.

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

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

Gardner, B. J., and Mason, S. F. (1967), *Biopolymers 5*, 79.

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

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

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

Li, H. J., and Crothers, D. M. (1969), *Biopolymers 8*, 217.

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

Biol. 9, 801.
Martz, W. W. (1971), Ph.D. Dissertation, Loyola University, Chicago, Ill.
Martz, W. W., and Aktipis, S. (1971), *Anal. Biochem. 39*, 327.
Newton, B. A. (1963), *Metab. Inhibitors 2*, 285.
Paoletti, J., and LePecq, J. B. (1971), *J. Mol. Biol. 59*, 43.
Peacocke, A. R., and Skerett, J. N. H. (1956), *Trans. Faraday Soc. 52*, 261.
Studdert, D. S., Patroni, M., and Davis, R. C. (1972), *Biopolymers 11*, 761.
Tinoco, Jr., I. (1962), *Advan. Chem. Phys. 4*, 113.
Tinoco, Jr., I. (1964), *J. Amer. Chem. Soc. 86*, 297.
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.
Wang, J. C. (1969), *J. Mol. Biol. 43*, 25.
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.
Yamaoka, K., and Resnik, R. A. (1966), *J. Phys. Chem. 70*, 4051.

Glycoproteins Associated with Nuclei of Cells before and after Transformation by a Ribonucleic Acid Virus†

Albert A. Keshgegian and Mary Catherine Glick*

ABSTRACT: A comparison was made by gel filtration of Pronase-digested glycopeptides derived from nuclei of baby hamster kidney fibroblasts (BHK_{21}/C_{13}) and the same clone transformed by the Bryan strain of Rous sarcoma virus (C_{13}/B_4). The comparison showed the presence of glycopeptides associated with the nuclei from the transformed cells labeled with radioactive D-glucosamine or L-fucose which were not seen to the same extent in the nuclei from nontransformed cells. Extraction of the nuclei with Triton X-100 from both cell lines removed most of the fucose- and glucosamine-containing glycoproteins. The nuclei appeared intact after this procedure suggesting that the glycoproteins are associated with the outer nuclear membrane. The ribosomal fractions from both cell lines showed a small amount of a heterogeneous population of fucose-containing glycopeptides. The glucosamine-containing glycopeptides of the nuclei differed somewhat in their patterns of elution from those derived from the surface membranes of the corresponding cells. However both the nucleus and surface membrane-derived glycoproteins from the transformed cells always showed the presence of glycopeptides which were not seen to the same extent in these cell fractions from the nontransformed fibroblasts.

The glycoprotein composition of surface membranes has been observed to change after transformation by RNA (Buck *et al.*, 1970) or DNA viruses (Buck *et al.*, 1971). These changes were demonstrated by gel filtration of surface membrane digests labeled with radioactive D-glucosamine or L-fucose. Further fractionation and chemical analyses showed that the glycoproteins which were more apparent after virus transformation actually contained in addition to fucose more sialic acid, mannose, and galactose suggesting a lengthening of a portion of the carbohydrate moiety (Glick, 1971). The appearance of these glycoproteins on the cell surface has been followed in hamster fibroblasts after viral infection and shown to correlate with the tumorgenicity of the cell population (Glick *et al.*, 1972).

Others when comparing 3T3 and SV40 transformed 3T3 mouse fibroblasts, have observed small (Wu *et al.*, 1969) or no (Sakiyama and Burge, 1972) differences in the membrane glycoproteins. The reason for this discrepancy is not apparent. Hamster embryo cells, transformed by SV40 virus, show the appearance of specific glycoproteins characteristic of the other transformed cells examined (M. C. Glick, unpublished observations).

In order to see if the glycoproteins expressed on the cell surface following virus transformation are present also in nuclei, a comparison was made of the glycopeptides from nuclei of cells before and after virus transformation. The results show that changes in the glycopeptides associated with the nucleus also accompany virus transformation.

Materials and Methods

Cell Culture. Baby hamster kidney fibroblasts (BHK_{21}/C_{13}) and the same clone transformed by the Bryan strain of Rous sarcoma virus (C_{13}/B_4) were grown as described previously (Buck *et al.*, 1970). The cells were of early passage stocks and in no case were used beyond the twelfth passage in our laboratory. Examination of the cultures at routine intervals for *Mycoplasma* showed them to be negative.

The cells were cultured for 72 hr in the presence of L-[1-^{14}C]fucose (50.8 Ci/mol), L-[G-^{3}H]fucose (4.3 Ci/mmol), D-[μ-^{14}C]glucosamine (10.7 Ci/mol), or D-[6-^{3}H]glucosamine (1.3 Ci/mmol) obtained from New England Nuclear Corp., Boston, Mass. These procedures as well as those used for

† From the Departments of Therapeutic Research and Biochemistry (A. A. K.) School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received September 22, 1972.* This work was supported by American Cancer Society Grant PRA-68 and U. S. Public Health Service Grants 5PO1 A107005-06 and 5TO5 GMO2046.

* Address correspondence to this author at the Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia, Philadelphia, Pa. 19146.