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Studies on the Structure of Deoxyribonucleoproteins. Spectroscopic Characterization of the Ethidium Bromide Binding Sites†

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ABSTRACT: In order to understand the chemical nature and the specificity of the interactions between ethidium bromide and its binding sites in deoxyribonucleoproteins, we have measured the quantum yield, fluorescence lifetime, and polarization of fluorescence of bound dye as a function of the DNA-phosphate/dye ratio (P/D). These studies have enabled us to interpret more accurately the basis for the heterogeneity of ethidium bromide binding sites in deoxyribonucleoproteins (DNP), which we have found to be even greater than previously reported (Angerer, L. M., and Moudrianakis, E. N. (1972), *J. Mol. Biol. 63*, 505). Although no differences in the nature of the intercalation sites between DNP and DNA are apparent from measurements of the quantum yield and fluorescence lifetime of bound dye, Scatchard plots of titration data indicate that there are at least two classes of highly fluorescent intercalation sites in DNP compared to only one class in DNA. In addition, polarization studies show that the rotational relaxation time for dye bound to DNP is about 470 nsec compared to only about 200 nsec for dye bound to DNA, suggesting that the internal structure of the DNP fiber is more rigid than that of DNA. Changes in polarization values as a function of the P/D ratio also raise the possibility that the sites of intercalation are more clustered in DNP than those in DNA. By preparing partial DNP's deproteinized by various procedures which control the type and order of removal of different classes of histones, we have demonstrated that the restriction of the dye-binding capacity of DNP is dependent upon the kind as well as the amount of its complexed protein. On a weight basis, the lysine-rich histones appear to be the most effective inhibitors of dye binding in DNP.

A number of physical and biochemical studies have recently been made on deoxyribonucleoproteins[1] (DNP) to probe the organization of proteins along the DNA. These studies have examined the sensitivity of whole and partially deproteinized DNP's to various nucleases and the capacity of these DNP preparations to bind various small molecules and synthetic polypeptides (Mirsky, 1971; Mirsky and Silverman, 1972; Clark and Felsenfeld, 1971; Itzhaki, 1971; Kleiman and Huang, 1971; Minyat *et al.*, 1970) or to serve as templates for RNA and DNA synthesis (Murray, 1969; Georgiev *et al.*, 1966; Spelsberg and Hnilica, 1971; Smart and Bonner, 1971; Schwimmer and Bonner, 1965). In general, it has been found that a portion of the DNA in DNP is masked through its association with bound protein, although the amount of DNA estimated as masked depends on the probe used and the conditions of the measurement. Recently, the interaction of the intercalative drug, ethidium bromide (EtBr), with whole and partial DNP's and DNA has been characterized by both absorption and fluorescence spectroscopy in order to determine the relative effectiveness of various chromosomal proteins in altering the dye-binding capacity of DNP and to compare the nature of the dye-binding sites in DNP and DNA (Angerer and Moudrianakis, 1972; Lurquin and Seligy, 1972; Williams *et al.*, 1972). These studies have shown that the optical properties of EtBr bound to either DNP or to DNA are identical and that both primary (highly fluorescent) and secondary binding sites are present in both DNP and DNA. However, the number of

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[1] Abbreviations used are: DNP, deoxyribonucleoproteins; EtBr, ethidium bromide.

available binding sites is reduced in DNP, and in one study (Angerer and Moudrianakis, 1972) the affinity of the highly fluorescent sites of intercalation of the dye was found to be lower in DNP than in DNA. Furthermore, studies of the binding of the dye to selectively deproteinized calf thymus DNP's suggested that the earliest extractable proteins, comprising primarily histone f1, had the greatest inhibiting effect on the dye-binding capacity of whole DNP. The interpretation of this result, however, is not unambiguous since it is possible that the maintenance of specific structures with reduced dye-binding capacity depends on interactions between more than one class of chromosomal proteins. Thus, it cannot be decided from these studies alone whether the presence of histone f1 specifically or the presence of a certain quantity of protein is more important in excluding EtBr from certain regions of DNP.

In the experiments reported here, we have extended these dye binding studies to determine whether the reduced dye-binding capacity of DNP depends upon the presence of either a *specific kind* or a *critical amount* of bound protein by *initially* extracting from DNP a class of histone *other* than histone f1. In addition, we present a complete analysis of the variation of the quantum yield and fluorescence lifetime as a function of the ratio of the concentrations of DNA–phosphate and dye (P/D) present in the reaction mixture. Both of these measurements are critical for a better understanding of the molecular basis of the apparent heterogeneity of the highly fluorescent binding sites present in DNP, but absent in DNA. The nature of the sites of intercalation has been further investigated by comparing the polarization of fluorescence of dye bound to DNP and to DNA at various P/D ratios. Evidence will be presented to show that (1) there is a specific class of proteins which, on a weight basis, is more effective in restricting EtBr binding than other proteins in DNP, and this class is the lysine-rich histones, (2) there are some DNA-like higher affinity sites of intercalation in DNP as well as the intercalation sites of lower affinity for the dye that were described previously, and (3) on the average the rigidity of the intercalation sites is substantially greater in DNP than in DNA. The polarization studies also suggest that the sites of intercalation of EtBr might be more clustered in DNP than those in DNA.

Materials and Methods

Reagents. Ethidium bromide was obtained from Calbiochem. It was judged to be pure since (1) it gave a single spot upon thin layer chromatography in a solvent of butanol–water–acetic acid in a ratio of 8:2:2 (Le Pecq, 1972), (2) its absorption and fluorescence emission spectra agreed with previously published spectra (Waring, 1965; Bittman, 1969), (3) its quantum yield in a variety of solvents was independent of exciting wavelength, and (4) an elemental analysis of its carbon, hydrogen, and nitrogen content (Galbreath Laboratories, Inc.) showed that its chemical composition was, within experimental error, equal to its expected composition (*Anal.* Calcd: C, 64; H, 5.1; N, 10.7. Found: C, 61.5; H, 5.2; N, 10.1). Concentrations of the dye were determined spectrophotometrically by assuming an extinction coefficient of 4.8×10^3 mol^{-1} cm^{-1} at 460 nm (Waring, 1965). All other chemicals were reagent or spectral grade.

Preparation of DNP, Partial DNP's, and DNA. Calf thymus DNP, salt-extracted DNP's, and DNA were prepared and assayed for chemical composition as described previously (Angerer and Moudrianakis, 1972). The extraction of slightly lysine-rich histones, f2b and f2a2, from DNP was accomplished according to the method of Kleiman and Huang (1972) with the following modifications. A solution containing 8 M urea, 0.4 M NaCl, and 0.1 M $NaHSO_3$ (pH 7.8) was added to an equal volume of a solution of DNP at a concentration of 200 μg/ml. The partial DNP was purified from the dissociated proteins by sedimenting it in the SW 25.1 rotor at 50,000*g* for 24 hr through a step gradient consisting of a 3-ml cushion of 40% sucrose and two 2-ml steps of 15 and 5% sucrose. Each sucrose solution also contained 4 M urea, 0.2 M NaCl, and 0.05 M $NaHSO_3$. The pelleted material was resuspended by several strokes in a glass–glass homogenizer and dialyzed against 10^{-3} M sodium cacodylate (pH 7.0). This procedure allows a recovery of greater than 85% of this partial DNP and removes almost completely and exclusively histones f2b and f2a2, which constitute about 55–60% of the total protein.

Titrations. For the fluorometric titrations, the signal from the phototube amplifier (Keithley Model 141) was fed to a Keithley digital multimeter Model 160 through a low-pass RC filter of 5-sec time-constant which resulted in a substantial improvement of the signal-to-noise ratio.

For dilute solutions of dye with absorbance less than or equal to 0.05, the following equations are valid

$$I_b = 2.3 I_{ex} \epsilon_b c_b q_b d \quad (1)$$

$$I_f = 2.3 I_{ex} \epsilon_f c_f q_f d \quad (2)$$

$$I_r = 2.3 I_{ex} \epsilon_f c_r q_f d \quad (3)$$

where I refers to fluorescence intensity, ϵ to extinction coefficient, c to concentration, and q to quantum yield. Subscripts b and f designate bound dye and free dye, respectively. I_{ex} is the intensity of exciting light, and in eq 3 I_r is the fluorescence intensity of a reference free dye solution of concentration c_r. The optical length of the cuvet in centimeters is given by d.

If, in addition, I is the total fluorescence intensity, *i.e.*, $I = I_b + I_f$, and c_0 is the total dye concentration, *i.e.*, $c_0 = c_b + c_f$, then from eq 1, 2, and 3 we obtain the following relation

$$c_b = \frac{\epsilon_f[(I/I_r)(c_r - c_0)]}{(\epsilon_b q_b/q_f) - \epsilon_f} \quad (4)$$

The ratio R of the fluorescence intensity of a dilute dye solution of high DNA–phosphate/dye (P/D) ratio, for which virtually all of the dye is bound, to the fluorescence intensity of the same concentration of a free dye solution is given by

$$R = \frac{\epsilon_b q_b}{\epsilon_f q_f} \quad (5)$$

Equations 4 and 5 yield

$$c_b = \frac{(I/I_r)(c_r - c_0)}{R - 1} \quad (6)$$

Relation 6 was used for the calculations of fluorometric titrations. Instrumental sensitivity corrections were applied to the ratio (I/I_r) as explained in the section on quantum yield determinations below. For the measurements the exciting wavelength was 520 nm and the emission was monitored at 600 nm. Aliquots (5 μl) of dye (10^{-3} M) were added to solutions of DNP or DNA at concentrations of approximately 50 μg/ml.

Spectrophotometric titrations were carried out as described previously (Angerer and Moudrianakis, 1972) by measuring the absorbance at 460 nm in 10-cm cuvets in a Cary 14 recording spectrophotometer at room temperature. The titrations were extended to lower r values (higher concentrations of

bound dye) by increasing the DNA–P concentration from 6–8 $\times 10^{-5}$ to 1.2–1.4 $\times 10^{-4}$ M.

Quantum Yield Determinations. Quantum yield determinations as a function of the exciting wavelength were made by correcting the excitation spectra for the absorbance of the sample and for the intensity of the exciting light. The latter was determined by employing a 3 g/l. solution of rhodamine B in ethylene glycol as a quantum counter. Excitation spectra were recorded on a spectrofluorometer constructed in this laboratory and previously described (Angerer and Moudrianakis, 1972). Absorption spectra were recorded on a Cary 14 recording spectrophotometer.

The correction curve for the emission monochromator–photomultiplier combination was determined by comparison of spectra taken with this spectrofluorometer and a calibrated one in Dr. H. H. Seliger's laboratory in this department. Since the correction factor was too high for wavelengths longer than 700 nm, the complete corrected spectra could not be determined. However, it was possible to obtain the corrected emission maxima which, for quantum yield measurements relative to free dye in water, were assumed proportional to the areas under the fluorescence spectra. This assumption is true, of course, if the shapes of the spectra are the same for the solution under study and for the free dye. Since there is a small difference in the width of the fluorescence spectra of the dye bound to DNP and DNA compared to free dye, relative quantum yield determinations should be considered only *approximate*. All the measurements were carried out in nondegassed dilute solutions of 10^{-5} M EtBr. A 3-66 Corning filter was used in the emission side to cut off any contribution to the signal from the exciting light.

Fluorescence Lifetime Measurements. Fluorescence lifetimes were measured with an instrument constructed in this laboratory. The instrument employs a discharge lamp driven by a blocking oscillator, at a frequency of 5 kHz, through an HY-2 hydrogen thyratron (Ware, 1971). The half-width of the light pulses in air, at a pressure of 1 atm, is about 3.5 nsec. Air-equilibrated solutions of DNP and DNA containing concentrations of EtBr ranging from 10^{-5} to 5 $\times 10^{-5}$ M were excited through a 7-54 Corning filter and the fluorescence was detected through a 3-67 filter. The fluorescence signal was detected by a TVP Amperex photomultiplier, which has a 2-nsec rise time, and fed to a Tektronix 1S1 sampling unit. The output of the sampling unit was scanned by a slow ramp, passed through a low-pass RC filter of 0.15-sec time-constant, and plotted on a Honeywell X–Y recorder Model 520.

From the theory of averaging through an RC circuit (Rolfe and Moore, 1970; Bendat and Piersol, 1971) it can be easily shown that an abrupt change in the decay curve can be faithfully reproduced by a low-pass filter with a distortion of less than 5% and with a time resolution T_1 given by

$$T_1 = 3RCT_2/T_s \tag{7}$$

For the circuit employed here, the scanning time $T_x \simeq 180$ sec, the scanned time interval (corresponding to 10 cm of the oscilloscope trace at a sensitivity of 5 nsec/cm) $T_2 \simeq 50$ nsec, and the filter time constant $RC = 0.15$ sec. From eq 7 we then obtain $T_1 \simeq 0.12$ nsec. Thus, the low-pass filter allows the resolution of a fast component of the decay curve in the subnanosecond region with an error of less than 5%. The distortion can be further reduced to less than 2% by employing a scanning time of 4 min. Averaging of the fluorescence signal by the RC filter resulted in a substantial improvement of the signal-to-noise ratio.

The effect of the duration of the exciting light pulse on the fluorescence decay becomes important for short lifetimes and a computer program was employed to convolute the data. This is accomplished by synthesizing the experimental decay curve from decay curves corresponding to trial values of the lifetime and the instrumental response function using the convolution theorem. This procedure is discussed by Ware (1971). That value of the lifetime which best fits the experimental curve is taken as the true lifetime.

Fluorescence Polarization Studies. Fluorescence polarization studies were carried out by employing a Polacoat polarized plastic film GS 10 as polarizer and a Telesar polarizing filter as analyzer. The degree of polarization, p, was calculated from the relation

$$p = \frac{I_{VV} - I_{VH}(I_{HV}/I_{HH})}{I_{VV} + I_{VH}(I_{HV}/I_{HH})} \tag{8}$$

where the first subscript refers to the position of the polarizer and the second subscript to that of the analyzer, and V and H stand for vertical and horizontal, respectively. The ratio I_{HV}/I_{HH} is the transmittance, which was found to be about 0.75 and only slightly dependent on wavelength.

The average cosine square of the angle ω swept by the emission oscillator between the time of absorption and emission of light is related to the degree of polarization p and the limiting degree of polarization p_0 by

$$\cos^2 \omega = \frac{p(2 - p_0) + p_0}{p_0(3 - p)} \tag{9}$$

(Perrin, 1929; Weber, 1952).

The rotational relaxation time ρ is given by

$$\rho = \frac{\tau p(3 - p_0)}{p_0 - p} \tag{10}$$

where τ is the fluorescence lifetime (Perrin, 1929; Weber, 1952).

Medium of Assays. All photometric measurements in this study were made with samples of DNP's or DNA in a buffer of 10^{-3} M cacodylate (pH 7.0) unless otherwise specified. When glycerol or sucrose was added to the assay mixture for quantum yield and lifetime measurements, their presence in the medium (within the concentration range reported here) did not cause any change in the absorption or emission spectra nor the fluorescence lifetime of the dye.

Results

Titrations. The comparison of Scatchard (1949) plots of previous spectrophotometric titrations of whole and partial DNP's from calf thymus (Angerer and Moudrianakis, 1972) indicated that the removal of the *first histone*, *i.e.*, histone f1, resulted in a greater increase in the dye-binding capacity of DNP than the *subsequent* removal of other classes of histones. In the experiments presented here, we have attempted to answer the question of whether the presence of histone f1 specifically or the presence of a certain quantity of protein is more important in reducing the dye-binding capacity of DNP by initially extracting from DNP a class of histone other than f1. The binding curves are shown in Figure 1. Removal of 60% of the total protein, including all of histone f1 and a smaller proportion of histones f2b and f2a2 (curve D), results in a greater increase in the dye-binding capacity than removal of a *different group* of proteins, also comprising 60% of the total protein, which includes primarily histones f2b and f2a2 (curve C). Furthermore, DNP, treated to remove 25% of its total

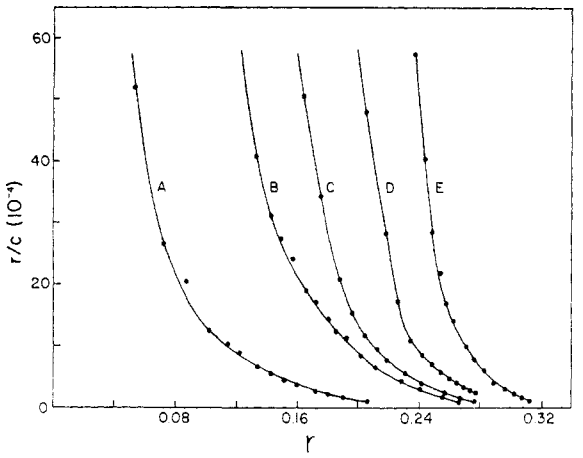

FIGURE 1: Scatchard plots of *spectrophotometric* titrations: (A) whole DNP; (B) 0.6 M NaCl extracted DNP (histone f1 removed); (C) 4 M urea and 0.2 M NaCl extracted DNP (histones f2b and f2a2 removed); (D) 9.0 M NaCl extracted DNP (histone f1 and most of histones f2b and f2a2 removed); (E) DNA. Extraction procedures are described in Materials and Methods. It should be noted that all of the curves are shifted to slightly lower values of r and the average slope of the lower part of the curves is slightly higher compared to the curves published previously (Angerer and Moudrianakis, 1972). This is attributed to a small error (*ca.* 4%) in the determination of the stock dye concentration in the previous experiments. However, with the exception of detecting the sites of highest affinity for the dye, the relationship of the binding curves to each other is essentially the same in these experiments and those already published. Please note that Figure 2 of the earlier publication corresponds approximately to the lower third of Figure 1 here. In addition, the scale of the abscissa here has been expanded relative to that of Figure 2 of the earlier publication (Angerer and Moudrianakis, 1972).

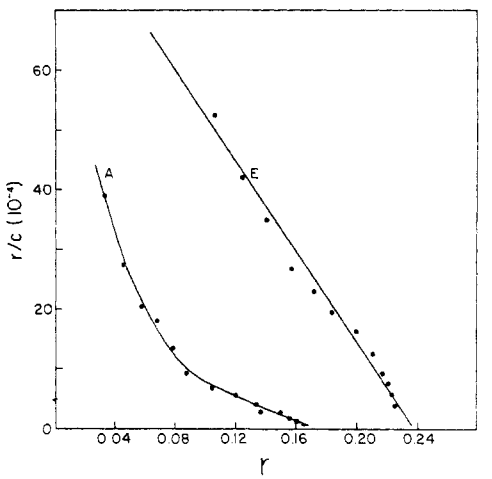

FIGURE 2: Scatchard plots of *fluorometric* titrations: (A) whole DNP; (E) DNA. The difference between curve E of Figure 1 and curve E of Figure 2 suggests that DNA in solutions of *low ionic strength* possesses an additional class of high affinity binding sites in which bound dye molecules do not fluoresce appreciably. A more detailed explanation is offered in the Discussion.

protein, comprising mostly histone f1 (curve B), has a somewhat greater increase in dye-binding capacity in proportion to the amount of protein removed than does DNP depleted of histones f2b and f2a2 (curve C). These results demonstrate that the increase in dye-binding capacity depends on the kind as well as the amount of protein removed. On a weight basis, histone f1 specifically is more effective than other histones in limiting the dye-binding capacity of DNP.

As can be seen by comparing these curves to those reported previously (Angerer and Moudrianakis, 1972), we have extended these titrations to lower values of r by improving the sensitivity with which small amounts of free dye can be detected (see Materials and Methods, under Titrations). It must be noted that as r decreases, these binding curves, and in particular the DNP curve (curve A), show considerably more curvature than previously observed, which results from the presence of some binding sites of higher affinity for the dye. Although it is known (Waring, 1965) that the extinction coefficient of bound dye at 460 nm does not vary with r at high P/D ratios, it is possible that some of the curvature may be attributed to dye bound in some lower affinity sites at low P/D which might have slightly different extinction coefficients.

While some sites of very high affinity have been detected in DNP in the present titrations, it can be seen that the increase in the slope as r decreases is more gradual for the DNP curve (curve A) than for the DNA curve (curve E), suggesting that some of the binding sites in DNP may have reduced affinities for the dye. In this respect it is important that fluorometric titrations presented below also indicate that there is a greater heterogeneity of highly fluorescent intercalation sites in DNP compared to DNA. However, because the Scatchard plots of EtBr binding to DNP are not linear, accurate binding stoichiometry cannot be quantitatively determined. Even in multisite systems where the number of different independent classes of binding sites is known, the determination of the number of available sites and the magnitude of the association constants for each class of site is quite complicated (Klotz and Hunston, 1971). Thus, in this system, the best description of binding is a *qualitative* one since the dye-binding capacity of DNP probably includes a variety of different binding sites.

In spectrofluorometric titrations, there is some uncertainty in those points of the binding curve which define the highest affinity sites, since the accuracy of the measurement depends on the accuracy of the determination of the concentration of free dye whose quantum yield is much lower than that of bound dye. Nevertheless, distinct curvature in Scatchard plots has been consistently found for the titrations of DNP but not for the titrations of DNA. Typical binding curves are presented in Figure 2. The curvature in the plot of the DNP titration cannot be caused either by the quenching of fluorescence of already bound dye or by the existence of dye bound in secondary sites whose quantum yield is not enhanced (Le Pecq and Paoletti, 1965), because the reduction in the slope of the curve results from fluorescence intensities higher than those predicted for a straight line. Thus, in DNP, *at least two* classes of highly fluorescent dye-binding sites exist. Again a quantitative evaluation of these sites is complicated. It should be noted that at the P/D ratios where the break occurs, the lifetime of the excited state of bound dye begins to decrease slightly although the decay is still a single exponential function of time. Hence the experimental curve shown here does not represent the true binding curve, which would exhibit a greater change in slope as r increases if the quantum yield q were constant throughout the entire range of the titration. The decreased lifetime at low P/D for both DNP and DNA affects significantly only the lowest few points on these binding curves.

As stated above, in spectrofluorometric studies there is some inaccuracy in determining the position of the points defining that part of the curve representing the binding sites of highest affinity for the dye. However, if this uncertainty is taken into account in the case of DNA, the slope of the curve representing these sites is somewhat less than that obtained by spectrophotometric titrations. These results may be taken to imply that the *strength* of association between a *dye-binding*

site in DNA and the dye *bound to that site* may be dependent on the electronic state of the bound dye, *i.e.*, on whether the dye molecule is in the ground or the excited electronic state. One way by which such differences could be detected is through the use of time-resolved fluorescence spectroscopy. Spectroscopic investigations in our laboratory on the origin of the spectral shifts upon the intercalation of EtBr have suggested that the association of the dye with DNA may in fact be stronger when the dye is in the ground state (S. Georghiou and E. N. Moudrianakis, manuscript in preparation).

The Scatchard plots of fluorometric titrations of DNP and DNA are displaced to slightly lower r values compared to the corresponding plots of spectrophotometric titrations (see Figures 1 and 2). The difference between these curves may be attributed to the presence of a small percentage of bound dye whose absorption changes are readily detectable but whose quantum yield is low. It can be shown from the analysis of binding curves in two-site systems given by Klotz and Hunston (1971) that for the case in which K_1 (primary type binding) is much greater than K_2 (secondary type binding), secondary binding would contribute only slightly to this difference. From spectrophotometric titrations of EtBr binding to DNA, K_1 and K_2 have been estimated to be 19.3 and 0.45 $\times$ 10^6 l./mol, respectively. Thus, it appears that at least part of the difference between spectrophotometric and fluorometric binding curves is due to the existence of a few dye molecules which are bound in high affinity sites and whose quantum yield is low. These extra dye molecules, which cause a shift of the spectrophotometric binding curve to values of r greater than 0.24, are probably able to bind to DNA as a result of an opening or loosening of its secondary structure due to the low ionic strength of the assay medium.

Quantum Yields and Fluorescence Lifetimes. In order to evaluate the binding data obtained by fluorometric titrations more clearly, we have examined the variation in the quantum yield of dye bound to DNP and to DNA as a function of both the P/D ratio and the exciting wavelength. The data for dye bound to DNP are presented in Figure 3, and similar results have been obtained for dye bound to DNA. When appropriate corrections are made, *i.e.*, for the differences in the corrected emission spectra for bound and free dye at 600 nm and for the intensity of exciting light as a function of wavelength (see Materials and Methods), the relative quantum yield of EtBr bound either to DNP or to DNA at high P/D ratios is ten times higher than that of the free dye in water, and it is independent of exciting wavelength. As the P/D ratio decreases, the relative quantum yield decreases and becomes increasingly dependent on the exciting wavelength. At shorter wavelengths, the relative quantum yield is lower than that measured at longer wavelengths. These effects can at least partially be explained by summing the separate contributions of free and bound dye to the total quantum yield at each wavelength. The equation used for such a procedure is

$$q = \frac{\epsilon_b c_b q_b + \epsilon_f c_f q_f}{\epsilon_b c_b + \epsilon_f c_f} \qquad (11)$$

where ϵ, c, and q are the extinction coefficient, concentration, and quantum yield, respectively, and subscripts b and f refer to bound and free dye, respectively. Our measurements show that q_b/q_f is about 10, which, for the purpose of the following discussion, is the important parameter for establishing the magnitude of the difference between calculated and experimental values. For the calculations, we have taken the value 0.19 as the absolute quantum yield of bound EtBr. This was derived using the reference rhodamine B in ethanol, whose

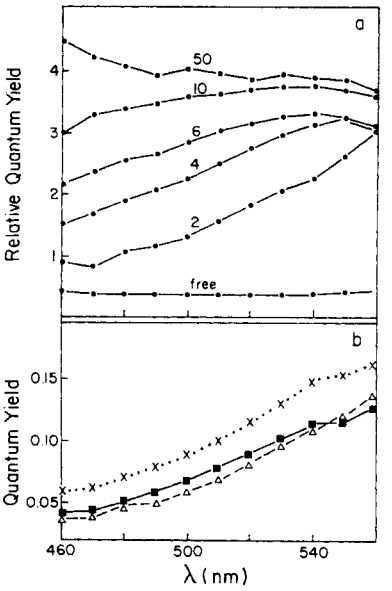

FIGURE 3: (a) Relative quantum yield as a function of exciting wavelength for dye bound to DNP at P/D = 2, 4, 6, 10, and 50. (b) EtBr bound to DNP at P/D = 2: (X···X) calculated from spectrophotometric determination of the concentration of bound dye; (△–△) calculated from fluorometric determination of the concentration of bound dye; (■–■) experimental curve.

quantum yield is 0.97 (Weber and Teale, 1957). If instead of 0.97, 0.69 is used as the quantum yield of rhodamine B in ethanol, as given by Parker and Rees (1960), a value of 0.14 is obtained for the quantum yield of EtBr bound to DNA, which agrees with the value published by Le Pecq and Paoletti (1971). The question of which of these two values for the quantum yield of EtBr bound to DNA is closer to being correct should not have any effect at all on the curves presented in Figure 3a (relative quantum yields) and would cause a small, near-parallel displacement of the curves in Figure 3b downward if the value of Parker and Rees were used in the calculations instead. The latter, however, would be of insignificant consequence to the conclusions drawn in this paper since we are only interested in the *change* of the quantum yield as a function of the exciting wavelength rather than in the *absolute* value of the quantum yield itself, which remains to be resolved. From eq 11, it can be seen that the magnitude of the calculated quantum yield depends on the value taken for the concentration of bound dye. As indicated in the titrations, the estimated value for the concentration of bound dye at a given P/D ratio is higher when determined from absorption measurements than from fluorescence measurements. It can be seen in Figure 3 that the experimental and calculated values derived from a fluorometric determination of c_b are in good agreement, and, thus, with respect to highly fluorescent dye binding, *and only that kind of binding*, the variation of quantum yield with exciting wavelength can be approximated in terms of two states of dye, *i.e.*, bound and free. The calculated quantum yields derived from spectrophotometric determinations of c_b are 25–30% higher than the experimental values. The higher values of c_b obtained from absorption studies could result from dye bound in either primary and/or secondary binding sites whose quantum yield is very low.

We have also measured the lifetime of fluorescence of dye bound to DNP and to DNA at various P/D ratios in order to better understand the reason for the variation of the quantum yield of the bound dye as a function of P/D ratio. As can be seen in Table I, the lifetime of the excited state decreases as the

TABLE I: Variation of Fluorescence Lifetime and Degree of Polarization of Dye Bound to DNP and DNA as a Function of P/D Ratio.[a]

	P/D	Lifetime (nsec)	Polarization
DNP	1	16.5	0.15
	2	18.0	
	10	25.0	
	20	25.0	0.27
	20 + 40% sucrose		0.29
	20 + 80% glycerol		0.32
	130		0.36
	130 + 40% sucrose		0.38
DNA	1	14.3	0.14
	2	17.5	
	4	24.0	
	20		0.27
	20 + 40% sucrose		0.31
	20 + 80% glycerol		0.33
	50	24.8	
	130		0.31
	130 + 40% sucrose		0.37
DNA	2 (10^{-1} M NaCl)	20.0	
	2 (1 M NaCl)	24.7	

[a] Absorption spectra, fluorescence emission spectra, and lifetime measurements were found to be unaffected by the addition of sucrose or glycerol in the medium within the range tested.

P/D ratio decreases. Similar results have been obtained by Burns (1969) for EtBr–DNA complexes. Since the decay appears to be a single exponential function of time at all the P/D ratios examined, the fluorescence observed probably reflects dye bound in either one kind of site or in several sites whose lifetimes are very similar. There are several possible ways to account for the observed decrease in the fluorescence lifetime with decreasing P/D ratio. Excitation energy could migrate from dye bound in intercalation sites to nearby dye molecules whose lifetime is comparatively short. The fluorescence lifetime might also be sensitive to changes in the microenvironment of the binding sites possibly induced by increasing numbers of bound dye molecules. As more dye is bound, the binding sites would gradually become populated with dye whose fluorescence lifetime, on the average, is progressively shorter. There is, however, strong evidence which suggests that migration of energy can occur from primary to secondary sites at low P/D ratios. If the salt concentration is increased to 1 M to reduce secondary electrostatic binding of the dye, the fluorescence lifetime of dye bound to DNA becomes identical with that observed for dye bound at high P/D ratio and under conditions of low ionic strength (see Table I). At low P/D, the decay curve contains a fast component which, after convolution of the data (see Materials and Methods), can be attributed to dye with a fluorescence lifetime of about 1 nsec. This fast component undoubtedly arises from free dye.

Polarization Studies. The nature of the EtBr binding sites in DNP has been further compared to those in DNA by measuring the degree of polarization of fluorescence of EtBr bound to DNP and to DNA as a function of P/D ratio (see Table I). The dye concentration in each determination was 10^{-5} M, and excitation and emission wavelengths were 520 and 600 nm, respectively. The degree of polarization of fluorescence of EtBr bound to DNA at P/D = 130 (0.31) is significantly less than the maximum observable degree of polarization of EtBr in glycerol (0.41). Such a depolarization has been interpreted to reflect an internal local oscillation of intercalated dye (Wahl *et al.*, 1970). At an equivalent P/D ratio of dye bound to DNP there is less depolarization (p = 0.36), which by the same argument would suggest that the intercalated dye in DNP has less freedom to oscillate. The addition of 40% sucrose decreases the oscillation of the intercalated dye in both the DNP and DNA binding sites as shown by the greater degree of polarization of fluorescence of the dye.

When the P/D ratio is decreased to 20, where more than 96% of the dye is still bound in highly fluorescent intercalation sites, the degree of polarization of dye bound to DNP decreases from 0.36 to 0.27 while that of dye bound to DNA shows a smaller decrease from 0.31 to 0.27. It seems likely that the larger decrease in p for dye bound to DNP compared to that for dye bound to DNA is due to energy migration between bound dye molecules whose sites are more clustered in DNP than in DNA. Although it might be possible that the intercalation of increasing amounts of dye in DNP alters the structure of the DNA helix and thereby permits more internal motion of the chromophore, one would expect this effect to be greater for DNA than for DNP. Furthermore, the energy migration hypothesis is supported by the fact that the increase in the degree of polarization of fluorescence at P/D = 20 upon addition of 40% sucrose or 80% glycerol is relatively small. Such a result would not be expected if the low degree of polarization at P/D = 20 were due *solely* to a decrease in the rigidity of the binding sites at this lower P/D ratio.

Discussion

Deoxyribonucleoproteins have a lower capacity to bind EtBr than does DNA. However, because DNP contains a heterogeneity of highly fluorescent binding sites, *i.e.*, those containing intercalated dye, it is difficult to quantitate exactly how many base pairs in DNP are masked either totally or partially with respect to EtBr intercalation. In a qualitative sense our results agree with other studies of binding of various other cationic dyes to DNP which have suggested that approximately one-third to one-half of the DNA is available for binding these dyes (Itzhaki, 1971; Klein and Szirmai, 1963; Borisova and Minyat, 1970; Chambrom *et al.*, 1966). The remaining DNA in DNP is excluded from dye binding either partially or completely by the presence of bound chromosomal proteins.

The EtBr binding capacity of various partial DNP's, deproteinized by two different extraction procedures which vary the *order of removal* of different classes of histones, has been investigated. These studies indicate that the inhibition of dye binding to DNA by chromosomal proteins is dependent upon the class as well as the amount of protein remaining bound to DNA. On a weight basis, the proteins extracted by 0.6 M NaCl (primarily the lysine-rich histone, f1) are the strongest inhibitors of dye binding. Since the net positive charge per mole of protein is greatest for these histones and least for the slightly lysine-rich histones (f2b and f2a2), one plausible explanation is that a given weight of lysine-rich histones could interact electrostatically with a proportionately greater number of negatively charged phosphate groups on the DNA. Thus, this class of histones could more effectively block EtBr

intercalation, which is undoubtedly preceded and facilitated by electrostatic forces (Bittman, 1969). In addition, any or all of the chromosomal proteins could inhibit intercalation by inducing conformation changes in DNP which make the binding sites unavailable to the dye or by altering the structure of the binding site such that the absorption and fluorescence spectra of the dye are not altered upon binding.

Lurquin and Seligy (1972) have recently reported a spectrophotometric study of the binding of EtBr to whole and partially deproteinized chick blood DNP's and DNA. Their preparations have a considerably lower dye-binding capacity than we have observed for calf thymus DNP and DNA, and, in general, they detected no difference in the affinity of DNP and DNA for the dye. The difference between our results and those of Lurquin and Seligy is probably due to the fact that the corresponding preparations of DNP were isolated from different tissues, by different methods, and were assayed under different ionic conditions. In a future publication we will deal with the problem of tissue specificity and the effects of ions (*in toto* and specific) on the physicochemical properties of isolated chromatin. Nevertheless, it is interesting that the Lurquin and Seligy studies show that histone V, which is specific to nucleated erythrocytes and is the most basic of the histones in that system, has the greatest inhibiting effect on the binding of EtBr to DNP, as does histone f1 in the thymus DNP studied here. The possibility that the structural roles of these two histones in DNP may be similar has also been suggested from X-ray diffraction studies (Murray *et al.*, 1970).

In the experiments reported here, we have extended the titrations over a wider range of r by improving the accuracy with which small amounts of free dye can be detected in both spectrophotometric and fluorometric titrations. The results reveal an even greater heterogeneity of binding sites in DNP than previously detected, since the slope of the binding curve increases rapidly at low r values, indicating that some higher affinity sites are present. Furthermore, fluorometric titrations indicate that there is at least one additional class of highly fluorescent binding site which is present in DNP but absent in DNA. Analysis of the variation of quantum yield and fluorescence lifetime with the P/D ratios used throughout the titrations indicates that the curvature observed as r increases in the Scatchard plot of the fluorometric titration of DNP cannot be attributed to quenching or to the presence of dyes bound in sites with a lower quantum yield, and, therefore, must represent dye molecules bound in highly fluorescent sites having a lower affinity for the dye.

There are several possible explanations for the existence of lower affinity, but highly fluorescent, binding sites in DNP. For example, there may be regions in DNP in which parts of proteins or whole proteins are not bound tightly enough to completely restrict intercalation, or the presence of such proteins could merely provide sufficient positive charge in the area of the available intercalation site to inhibit partially the first step in the intercalation process. Alternatively, there could be several distinct types of DNA–protein structures in DNP whose affinity for the dye depends upon the conformation of the DNA or the geometry of the intercalation site.

Although there is a greater diversity of binding affinities in DNP compared to DNA, the nature of the binding sites is very similar since no significant difference in the quantum yield, fluorescence lifetime, emission spectrum, or absorption spectrum of dye bound either to DNP or to DNA can be detected. However, we have observed a greater depolarization of fluorescence of dye bound to DNA compared to that of dye bound to DNP at very high P/D ratios (130), which suggests that the binding site is much more rigid in DNP than in DNA. We believe that this depolarization of fluorescence of intercalated dye bound at very high P/D ratios results from some degree of internal flexibility of the macromolecule rather than from migration of the excitation energy between binding sites since on addition of 40% sucrose to these solutions, the fluorescence polarization approaches the value of 0.41, which is the value obtained for EtBr in glycerol which approximates the limiting polarization. Wahl *et al.* (1970) have also concluded that the depolarization of EtBr bound to DNA must be attributed to some form of oscillation or deformation of the base pairs in the nanosecond scale, since the rotation of the macromolecule during the lifetime of the excited state is insignificant (Callis and Davidson, 1969), and the time taken for the dye to dissociate is long (Li and Crothers, 1969). An estimate of the mobility of the intercalated dye can be obtained by applying eq 9 and 10 with $p_0 = 0.41$. Such a calculation shows that the angle swept by the emission oscillator between the times of absorption and emission of light is about 18° for EtBr bound to DNP and 25° for EtBr bound to DNA. This corresponds to rotational relaxation times of approximately 470 and 200 nsec for EtBr bound to DNP and DNA, respectively. Although these results are only approximate since the true value of p_0 is not accurately known, nevertheless they indicate that the extent of internal motion of intercalated dye allowed in DNP is much less than that permitted in DNA. In this regard, it is noteworthy that Burns (1971) has reported that addition of bovine serum albumin to ribosomal RNA containing intercalated EtBr in its base-paired regions increases the polarization of fluorescence of the dye.

It is also interesting that the polarization studies suggest that intercalated EtBr molecules are more clustered in DNP than in DNA, which undoubtedly is a manifestation of the clustering along the DNA of specific proteins or of specific higher order nucleoprotein structures which inhibit dye binding. We are currently extending our investigations into the structure of these regions of DNP.

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Action of Micrococcal Nuclease on Chemically Modified Deoxyribonucleic Acid†

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ABSTRACT: The exposure of alkali-denatured DNA to the reagent *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide (Cmc) *p*-toluenesulfonate results in the substantial blocking of the thymidine and deoxyguanosine moieties of the polynucleotide chains with Cmc groups. The hydrolysis of this modified DNA with micrococcal nuclease followed by the removal of the blocking groups produces a series of oligonucleotides, whereas, under the same enzymatic conditions, unmodified DNA is reduced to mono- and dinucleotides. The oligonucleotides in various size groups have been analyzed by degradation with alkaline phosphatase together with snake venom phosphodiesterase or spleen phosphodiesterase to determine their chain lengths and the identity of their 5'- and 3'-terminal nucleotides. In addition, the tetranucleotides, pdA-dT-dA-dT and pdT-dA-dT-dA, and their di-Cmc derivatives have been digested with the nuclease and the resulting products have been identified. From these results a specific pattern for the action of the nuclease on the modified DNA can be derived: (i) a preferential endonucleolytic cleavage takes place at the -Np-dA- bonds (where N is a modified or unmodified deoxyribonucleoside); (ii) each oligonucleotide, so formed, can then undergo the normal exonucleolytic degradation at its 3' terminal depending on whether it contains a modified or unmodified nucleotide at its 3' terminal; (iii) those oligonucleotides that contain a blocked dT or dG at their 3' terminals tend to be resistant to this exonucleolytic action while the rest of the oligonucleotide species are subject to the stepwise removal of unblocked nucleotides until a blocked dT or dG is located at their 3' terminals. This restricted activity of micrococcal nuclease constitutes a method for the specific cleavage of polydeoxyribonucleotides that is expected to be of some value in future studies on the sequence analysis of DNA.

In comparison with the sequence analysis of RNA molecules the direct determination of the primary structure of DNA chains has presented a somewhat more difficult problem. While a number of sequences of some small sections of DNA molecules have already been assigned it has become apparent that one of the main difficulties in this work arises from the relative lack of enzymatic methods for the cleavage of polydeoxyribonucleotides into specific smaller fragments for subsequent analysis. In the case of the work on RNA, the relative ease with which sequence information can be obtained depends, in large part, on the availability of base-specific endonucleases such as the ribonucleases A, T_1, and U_2. In addition, the reversible chemical modification of certain nucleotides within an RNA chain can be used to induce even greater specificity on the action of these ribonucleases. There are no known counterparts of these ribonucleases that

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