The Interaction between Acridine Dyes and Deoxyribonucleic Acid[1]

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Abstract: The interaction of acridine orange and proflavine with DNA at pH 6.5 has been studied at several ionic strengths by equilibrium dialysis, absorption spectrophotometry, and low-shear viscosimetry. The results are consistent with an intercalation binding model incorporating the following novel features: (1) the total number of possible intercalation sites is fixed *a priori* to include every slot between successive DNA base pairs; (2) an intercalated dye cation inhibits intercalation at the two slots immediately adjoining the occupied one; (3) an intercalated dye cation may associate with a nonintercalated dye cation to produce a spectroscopically distinct bound dimer. At degrees of binding between 0.2 and 0.3 mol of dye/mol of DNA phosphate, at least one additional bound dye species, presumably of a higher aggregate type, begins to make its appearance. This species is difficult to characterize by the methods employed here because of incipient precipitation of the dye–DNA complex.

The interactions between cationic acridine dyes and DNA have attracted wide attention. The acridine dyes possess mutagenic activity[5-8] and their polycyclic aromatic structure resembles that of various potent carcinogens. In addition, dye–nucleic acid systems hold excellent promise for elucidating the general nature of interactions between small ions and polyelectrolytes.

Numerous studies have been devoted to this topic, which is the subject of a recent review article by Blake and Peacocke.[9] The picture which has emerged so far may be summarized as follows: two modes of interaction between dye molecules and DNA are distinguished; a "strong" binding mode involving about 20–25% of the DNA phosphate groups and a "weak" binding mode involving the remaining phosphate groups.[10] The "strongly" bound dye molecules are understood to be intercalated between successive base pairs of the double helix, a process accommodated by a lengthening and a slight local untwisting of the helix.[11,12] The "weakly" bound dye molecules are conjectured to be attached to the helix exterior by means of electrostatic interaction with the phosphate groups. The evidence for intercalation based on the observed increase in molecular dimensions of the DNA helix[11-16] is convincing from a qualitative point of view; however, a clear quantitative understanding of this binding mode and of its effect on the DNA conformation is lacking. Even less is known about the weak binding process. The customary procedure in treating these phenomena has been to express the experimental binding curves in terms of a sum of Langmuir isotherms with arbitrary parameters.[9] This "curve fitting" leads to concepts difficult to rationalize. For instance, the number of "strong" binding sites emerging from such treatments is usually less than half that expected from the molecular structure of the DNA, and, moreover, apparently varies with the ionic strength.

In an attempt to improve our understanding of these phenomena we have employed a combination of three types of physical measurement: equilibrium dialysis, absorption spectroscopy, and low-shear viscosimetry. The binding of the two dyes, acridine orange and proflavine, has been studied in aqueous solutions of tetramethylammonium cacodylate at pH 6.5 and at ionic strengths ranging from 0.002 to 0.2. As will be seen, we are able to propose a simple binding model consistent with all our experimental results.

Experimental Section

Materials. Salmon sperm DNA (Worthington Biochemical Corporation; Lot 6HA) was precipitated twice in 95% ethanol from dilute NaCl solution. A 0.2% stock solution of the purified product prepared in 0.01 *M* NaCl was filtered through a 0.45-μ Millipore filter. This primary stock solution was stored frozen. When needed, thawed portions were dialyzed against the desired buffer solution and then maintained in a frozen state until just prior to use. Phosphate analysis[17] and spectrophotometry gave an extinction coefficient at 259 nm of 6550 ± 30/g-atom of phosphorus. The intrinsic viscosity of the sample in 0.2 *M* NaCl solution at 25° was 985 l./equiv of DNA–phosphorus corresponding[18-20] to an average molecular weight of about 4 million calculated as Na–DNA. Concentrations of DNA solutions were determined spectrophotometrically at 259 nm.

Acridine orange (AO) hydrochloride was obtained as the $ZnCl_2$ double salt (J. T. Baker Co.). Zinc and other divalent metal ions were removed according to the following procedure: the free base

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was precipitated from a well-stirred, ice-cold 10% aqueous ethanol solution by slow addition of 0.35 *M* NaOH. An excess of sodium ethylenediaminetetraacetate (Na_4EDTA) was added and the free base was extracted into a small volume of chloroform; the extract was then washed repeatedly with 0.1 *M* Na_2EDTA solution. After removal of the chloroform by vacuum evaporation, the free base was dissolved in ethanol, neutralized with an equivalent amount of standard HCl, and the solid AO–HCl precipitated by dropwise addition of the ethanolic solution into 10 vol of ethyl ether. The acid chloride was then recrystallized several times by dropwise addition of methanolic solutions into a large excess of ether. An X-ray spectrographic analysis of the crystalline product indicated virtually complete removal of metallic impurities. Both potentiometric titration for chloride ion and spectrophotometric assay based on the empirical equation of Stone and Bradley[21] gave a molecular weight within 2% of the theoretical value of 301.5. Concentrations of AO–HCl in solution were determined spectrophotometrically using the experimentally obtained value of 4.20×10^4 for the molar extinction coefficient at the isosbestic point of 475 nm.

Proflavine (PF) monohydrochloride, purified by means of an extraction procedure similar to that described above for AO, was supplied by Dr. E. J. Gabbay. Concentrations of PF in solution were calculated on the basis of the value of the molar extinction coefficient of 4.1×10^4 at the 444 nm maximum.[22]

Cacodylic acid (CP grade, Mann Research Laboratories) was purified by passing a 95% ethanolic solution through Dowex 50 ion-exchange resin in the H form. The filtered effluent was evaporated to incipient precipitation and the acid precipitated with ethyl ether. The product was washed with ether, assayed by potentiometric titration with standard base, and was found to have a purity of better than 99%.

Tetramethylammonium hydroxide (TMAOH) pentahydrate (RSA Corporation of Ardsley, N. Y.) solutions were assayed by potentiometric titration with reagent grade potassium acid phthalate. All other chemicals used were reagent grade.

Solutions. Buffer solutions at pH 6.50 ± 0.05 (TMACac buffers) were prepared by mixing appropriate volumes of standardized solutions of cacodylic acid and TMAOH followed by filtration through 0.22-μ Millipore filters.

Stock solutions of the pure dyes in buffer were prepared by weight and stored at 4° in foil-wrapped Teflon bottles. Under these conditions the stock solutions were spectrophotometrically stable for periods of several months.

Solutions of the dye–DNA complexes were prepared by weight in Teflon bottles. To prevent spontaneous precipitation of the complexes the stock DNA weighed into the bottle was diluted with buffer and the dye was added slowly with rapid stirring in very dilute solution. Previous findings[23] that it is impossible to prepare precipitate-free solutions of AO–DNA complexes containing more than about 0.3 mol of AO/mol of DNA phosphorus were confirmed. Studies concerned with this precipitation phenomenon will be presented elsewhere.[24] Those solutions of dye–DNA complexes that could not be examined immediately after preparation were protected from photolytic degradation.[25,26] However, the time between preparation and measurement never exceeded 4 hr.

Methods. All equilibrium dialysis experiments were carried out using cellulose casings (Union Carbide Corp., Food Products Div.) cleaned by five consecutive 30-min boilings in fresh portions of distilled water and stored in distilled water at 4°. A typical dialysis experiment was performed by placing two different but known concentrations of DNA in separate casings which had been previously rinsed with buffer and with the appropriate filling solution. Pure buffer was placed in a third casing in order to provide a "blank" for each experiment. The three sealed casings were placed inside a wide-mouth polyethylene bottle containing dye solution of the desired concentration. The bottle was tumbled for about 60 hr in a darkened room in which the temperature was controlled at 22°. On several occasions, dye–DNA complexes were placed inside the cellulose casings and allowed to dissociate into an external solution which was initially pure buffer. The equilibria obtained were found to be completely independent of the manner in which dye had been distributed initially.

Because of extensive adsorption of dye on the cellulose casings, it was necessary to analyze both internal and external solutions for equilibrium dye concentration. External solutions (and internal "blanks") were assayed directly by determining the solution optical density at appropriate wavelengths. Internal solutions were analyzed spectrophotometrically after dissociation of the dye–DNA complexes with an equal volume of 0.2 *M* LiCl in pure methanol. Upon addition of this solvent, it was found that the visible absorption spectra of both dyes became independent of both dye and DNA concentrations, and furthermore, that the uv absorbances of dye and DNA became directly additive. Thus, the concentrations of both dye and DNA could be calculated from the solution absorbance measured at the visible absorption maximum of the dye and at 259 nm. The visible maximum molar extinction coefficients of the dyes in 0.1 *M* LiCl, which is 50% by volume methanol, were found to be: acridine orange, ϵ_{max} 6.80×10^4 at λ 493 nm; proflavine, ϵ_{max} 4.95×10^4 at λ 453 nm. In practice, the DNA analysis is precise only within 5% because the nucleic acid absorbance is typically only a small fraction of the total absorbance. Since the DNA concentration determined by this method always agreed with the known concentration of DNA placed in the casing initially, it was generally assumed that changes in the concentration of polymer during the equilibration period were negligible.

All spectra were recorded on a Cary Model 14 recording spectrophotometer which was located in a room maintained at 22°. Relatively new, matched high-quality silica cuvettes (Suprasil, Precision Cells, Inc.) were employed; soft glass and older silica cells were found to absorb dye excessively. Maximum spectral reproducibility was obtained by placing dye solutions directly in unrinsed cuvettes previously washed with water and ethanol and dried with filtered air. Spectra were recorded as soon as possible after filling the cuvettes. Corrections for sample fluorescence were completely negligible.

Viscosities were measured at 22.00 ± 0.03° by means of a Zimm-Crothers viscosimeter (Beckman Instruments, Spinco Division) equipped with a rotor producing a shear stress calculated to be 0.008 dyn/cm².[27] For DNA solutions in the molecular weight range considered in this study, the viscosities determined at such a low value of the shear stress may be taken to correspond to the limit of zero shear stress.[28] A persistent source of difficulty associated with the use of this type of viscosimeter in aqueous solutions is the formation of surface films.[27,29] This film formation, which manifests itself as a progressive decrease in the turning rate of the rotor, was avoided by adding routinely about 2 μl. of a 5% solution of sodium dodecylsulfate in 50% aqueous ethanol to the solution meniscus in the viscosimeter. In this way, satisfactory reproducibility was achieved. From visual observation, based on changes in dye fluorescence due to the presence of ethanol in the detergent solution, it was ascertained that the depth of penetration of the detergent solution never exceeded 1–2 mm. An additional drop or two of the detergent solution was found to give no additional change in the rate of revolution of the rotor.

Results

Equilibrium Dialysis. All dialysis experiments were carried out in the presence of large excess of simple salt under conditions such that Donnan effects could be neglected. Thus the concentration of dye bound to DNA, C_B, was taken to be the difference between the total concentration of the dye inside the casing and that outside, denoted by C_T.[30] Experimental data for the AO–DNA and the PF–DNA systems at several ionic strengths, I, are summarized in Table I. The quantity β is defined as the ratio of C_B to C_D where the latter is the

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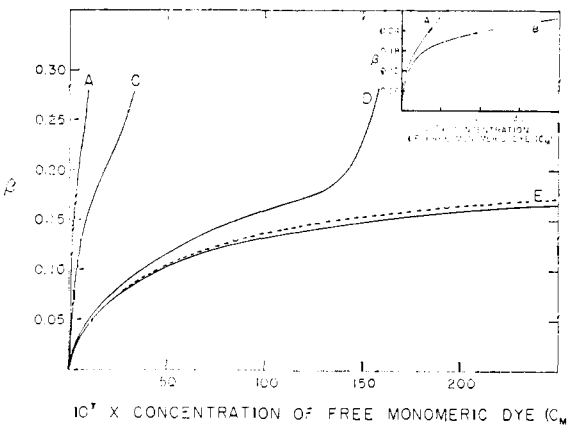

Figure 1. Experimental binding isotherms for AO–DNA and PF–DNA complexes at 22° and pH 6.5 in TMACac buffer. Curve A, AO–DNA at $I = 0.002$; curve B, PF–DNA at $I = 0.002$; curve C, AO–DNA at $I = 0.020$; curve D, AO–DNA at $I = 0.200$; curve E, PF–DNA at $I = 0.200$. Dashed curve is theoretical isotherm calculated from eq 3 with $K_1 = 1.0 \times 10^5$ l. mol^{-1}.

concentration of DNA expressed in units of moles of DNA phosphorus per liter. At a given value of C_T, β was found to be independent of the DNA concentration.[31]

Table I. Equilibrium Dialysis Data for the Interaction of Acridine Orange and Proflavine with DNA at pH 6.5 and 22°

$I = 0.002$		$I = 0.020$		$I = 0.200$	
$C_T \times 10^7$	β	$C_T \times 10^7$	β	$C_T \times 10^7$	β
		Acridine Orange–DNA			
0.6	0.055	1.4	0.062	2.7	0.019
1.2	0.092	2.3	0.087	3.7	0.027
1.5	0.109	4.5	0.118	6.6	0.036
1.4	0.122	7.1	0.141	21.0	0.070
1.9	0.131	9.6	0.152	43.0	0.102
1.9	0.135	11.8	0.168	55.3	0.115
2.8	0.168	14.2	0.181	64.0	0.122
3.9	0.193	22.0	0.210	83.8	0.135
4.8	0.197	26.2	0.230	127	0.157
5.0	0.212	32.8	0.265	173	0.170
6.4	0.224	38.0	0.285	218	0.192
6.3	0.233			235	0.218
7.1	0.245			250	0.254
9.2	0.285			252	0.255
10.8	0.312			274	0.360
		Proflavine–DNA			
0.7	0.068			7.4	0.042
1.6	0.108			30.0	0.087
2.4	0.138			50.3	0.102
4.3	0.166			68.0	0.117
5.2	0.177			107	0.128
8.4	0.204			154	0.149
11.8	0.220			225	0.161
19.0	0.237			265	0.168
23.7	0.246			387	0.186
32.2	0.274			567	0.194
38.7	0.284			491	0.195
48.8	0.300			719	0.198
72.9	0.335			632	0.200
92.7	0.398			798	0.216
102	0.425				
145	0.500				

Both AO and PF are known to aggregate in aqueous solutions,[22,32–34] and for the construction of binding

(31) It should be noted that the quantity β is frequently denoted by r in publications describing dye-DNA systems.

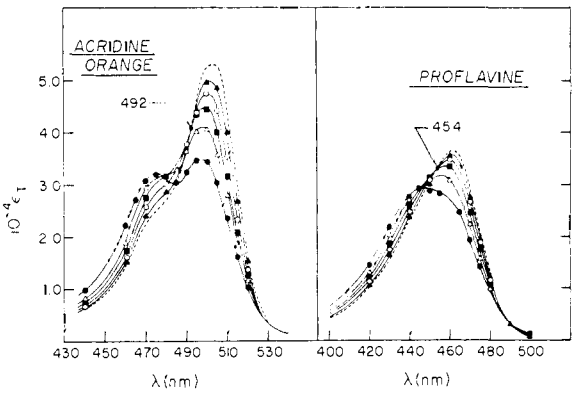

Figure 2. Absorption spectra at $I = 0.002$ for acridine orange and proflavine bound to native DNA corresponding to various values of β: ▲, $\beta = 0.06$; ○, $\beta = 0.12$; ■, $\beta = 0.18$; △, $\beta = 0.24$; ●, $\beta = 0.32$. Dashed curves are spectra extrapolated to $\beta = 0$, *i.e.*, bound monomer spectra. Arrows show isosbestic points for spectra with $\beta < 0.2$.

isotherms the concentration of the free monomeric dye, C_M, rather than that of the total dye, C_T, is required. On the basis of the low value of the dimerization constant for PF[22] the differences between C_T and C_M were found to be negligible even at the highest concentrations of free PF encountered. In contrast, the corrections for the AO data based on recently determined dimerization constants[34] are significant, amounting to as much as 40% of C_T at the highest ionic strength studied. The resulting isotherms are shown in Figure 1. Experimental points have been omitted for the sake of greater clarity. In agreement with previous reports,[10,35–38] the binding of the cationic acridines was found to diminish significantly with increasing ionic strength.

Absorption Spectra. The visible absorption spectra of dye–DNA complexes at low ionic strength ($I = 0.002$) are shown for both AO and PF in Figure 2, where the molar extinction coefficient of bound dye, ϵ_T, is given as a function of the wavelength, λ, for several selected values of β. More extensive tabulations of bound dye extinction coefficients will be presented elsewhere.[39] The spectra shown in Figure 2 have been calculated by correcting the optical densities of the dye–DNA solutions for the contribution of free dye, a correction determined from the known binding isotherms and the spectra of the pure dyes at the appropriate concentrations.

Inspection of the curves shown in Figure 2 reveals that the spectra of both bound dyes display isosbestic points provided that β does not exceed about 0.2. On this basis one may conclude that up to a dye loading of $\beta = 0.2$ there are two, and only two, spectroscopically unique bound dye species. These two spectroscopic varieties of bound dye will be referred to as bound

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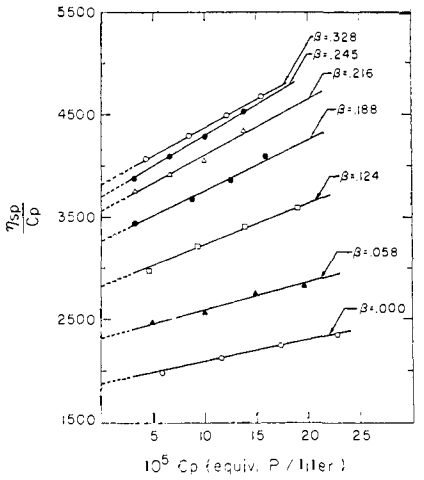

Figure 3. A representative set of reduced viscosity curves measured at various values of β, as shown. Data apply to AO–DNA complexes at $I = 0.002$.

monomer and bound dimer, these designations to be justified below. At dye loadings in excess of $\beta = 0.2$ the trends in the bound spectra indicate the existence of one or more additional bound spectroscopic species. These will be referred to as bound higher aggregates. Of the various spectroscopic bound dye species the spectra of bound monomers may be obtained unambiguously by extrapolating the bound spectra determined at finite values of β to $\beta = 0$. The bound monomer spectra found in this way are shown in Figure 2 by the dashed curves. Relative to the corresponding free monomer maxima for AO and PF the absorption maxima of the bound monomers are red shifted by 445 cm^{-1} and 825 cm^{-1}, respectively.

Viscosimetry. Intrinsic viscosities pertaining to dye–DNA complexes in various states β were obtained by linear extrapolation of the reduced viscosity measured at four DNA concentrations.[40] Dilution was carried out at constant β by using a dye solution known to be in dialysis equilibrium with the complex in state β. As a typical set of reduced viscosity data, the results of AO–DNA complexes at the ionic strength of $I = 0.002$ are shown in Figure 3. The viscosity effects shown are qualitatively consistent with previous reports;[11,15] binding of the acridine dyes significantly increases the intrinsic viscosity of DNA. The intrinsic viscosities of all the systems studied are collected in Figure 4. For convenience of presentation, in this figure the intrinsic viscosity of each dye–DNA complex has been divided by the intrinsic viscosity of DNA alone, measured at the corresponding ionic strength. The latter values were found to be 1090, 1250, and 1870 l./equiv of DNA phosphorus at ionic strengths of 0.2, 0.02, and 0.002, respectively; these values increase linearly with $I^{-1/2}$, a behavior which has also been reported for much higher molecular weight T-2 phage DNA.[41] Huggins constants corresponding to the intrinsic viscosities given in Figure 4 are presented graphically in Figure 5.

Three general observations contained in Figure 4 need emphasis. First, the viscosity enhancement

(40) Reduced viscosities, η_{sp}/C_p, and intrinsic viscosities, $[\eta]$, are expressed in units of l./equiv of DNA phosphorus. It should be noted that, according to this convention, bound dye is not included in the DNA concentration in the denominator of the reduced viscosity.

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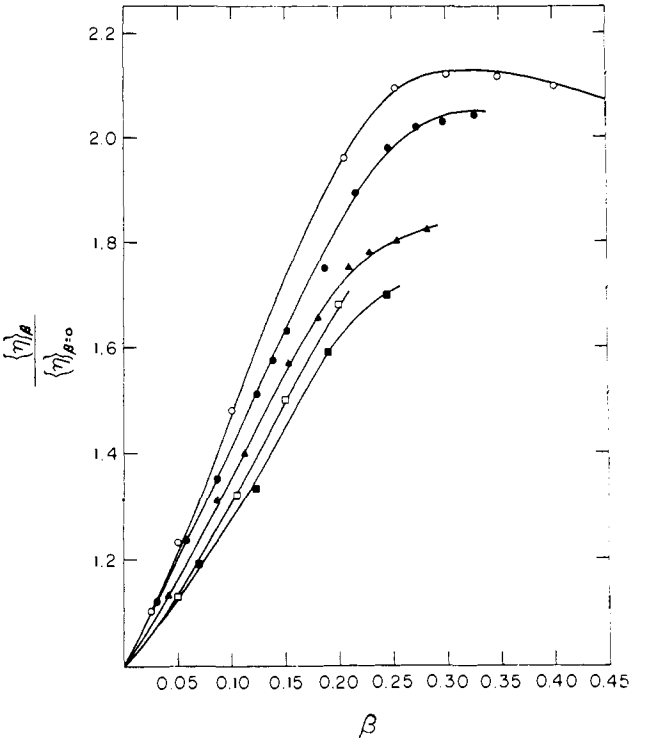

Figure 4. Intrinsic viscosity of dye–DNA complexes relative to intrinsic viscosity of DNA alone at the same ionic strength: ○, $I = 0.002$; △, $I = 0.020$; □, $I = 0.200$. Filled points apply to AO–DNA complexes, open points to PF–DNA complexes.

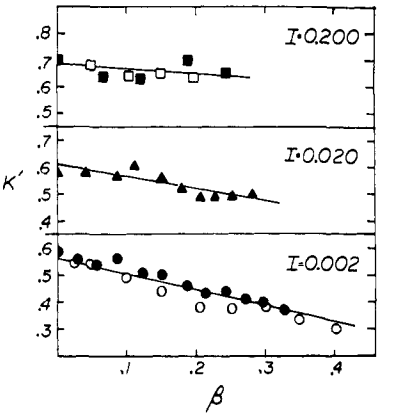

Figure 5. Huggins constant for dye–DNA complexes as function of β. Designation of points is the same as in Figure 4.

phenomenon is most pronounced in the range of β of interest here ($0 < \beta < 0.2$) and begins to level off above this range. The results obtained at ionic strength 0.002, where high enough values of β are experimentally attainable, show that the intrinsic viscosity reaches an upper limit at about $\beta = 0.3$. Second, at corresponding values of β the intrinsic viscosity enhancement diminishes with increasing ionic strength for each dye–DNA complex. Finally, under identical conditions of β and ionic strength, the intrinsic viscosity is enhanced somewhat more by PF than by AO; this difference is most pronounced at high values of β and appears to vanish as β becomes sufficiently small. This last observation bears a strong resemblance to the relative behavior of the binding isotherms for the two dyes at the same ionic strength illustrated in Figure 1.

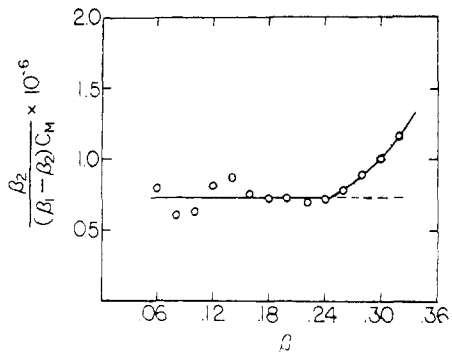

Figure 6. Test plot for eq 5 for AO–DNA complexes at I = 0.002.

Drummond, *et al.*,[15] have previously reported the specific effect of the binding of PF on the viscosity of DNA. Although the general trends of their results are similar to those presented here, the actual magnitudes of their reported viscosity increases are considerably larger than those shown in Figure 4. Since Drummond, *et al.*, do not mention any specific technique used to suppress surface effects referred to above, surface film formation is thought to be a possible contributing factor to their relatively high viscosity values.

Discussion

Description of Model. As shown by our spectroscopy results in the range of $0 < \beta < 0.2$ only two spectroscopically distinct species of bound dye exist. We shall restrict our discussion here to these two species. The additional species appearing at higher values of β cannot be characterized adequately from the present data because incipient precipitation prevents obtaining sufficient quantities in homogeneous solution. We propose that the two presently characterizable species are governed by the following two postulates. (1) *Every slot between two successive base pairs of the DNA helix constitutes a possible binding site for one intercalated dye molecule. Intercalated dye is distributed randomly over all possible binding sites with one restriction—intercalation does not occur at sites immediately adjacent to one already occupied.* (2) *Every intercalated dye molecule is a possible binding site for an additional, nonintercalated dye molecule. The pair of bound dye molecules so formed constitutes a spectroscopically distinct dimer species. This binding of nonintercalated dye molecules proceeds in a random fashion and does not affect the contour length of the DNA.*

Postulate 1 extends previous intercalation concepts in two ways. First, it specifies *a priori* the total number of binding sites in a simple way; second, it denies intercalation at adjacent sites. This prohibition limits intercalation to a theoretical maximum of one dye molecule per two base pairs or four DNA phosphate groups, in accord with all previously reported observations. Alternate proposals of accounting for this limitation by assuming the dye binding to be base pair specific appear to be ruled out by recent findings indicating that the binding of amino acridines[10,42] and related molecules[43] to DNA is insensitive to major changes in the base composition. The assignment of equal *a priori* weights to all binding sites implicit in postulate 1 is consistent with these findings.

Quantitative supporting evidence for postulates 1 and 2, based on our own experimental data, is discussed in the following sections.

Binding Isotherms. The theoretical mass action law for intercalation according to postulate 1 is derived in the Appendix, and may be written in the form

$$K_1 = \frac{2(\beta_1 - \beta_2)(1 - 2\beta_1)}{C_M(1 - 4\beta_1)^2} \quad (1)$$

where β_1 is the number of intercalated dye molecules per DNA nucleotide, β_2 is the number of dye molecules per DNA nucleotide bound according to postulate 2, C_M is the molar concentration of free dye monomer, and K_1 is the apparent binding constant for intercalation. The assumption implicit in eq 1 that the electrostatic potential of the DNA is unaffected by the dye binding over the small range of β treated here will be examined below. The value of K_1 may be determined from the experimental binding data by the relation

$$K_1 = \lim_{\beta = 0} (2\beta/C_M) \quad (2)$$

since the ratio β_1/β approaches unity in this limit.

It is well known—and also consistent with our own spectroscopic data—that at high ionic strength the interaction of proflavine and DNA may be described in terms of a single, spectroscopically unique, bound dye species.[9,10] For such systems $\beta_2 = 0$, $\beta_1 = \beta$, and eq 1 simplifies to

$$K_1 = \frac{2\beta(1 - 2\beta)}{C_M(1 - 4\beta)^2} \quad (3)$$

The dashed curve in Figure 1, calculated from this equation with an appropriate choice of K_1, is seen to nearly coincide with the isotherm E in Figure 1, representing the experimental proflavine binding data at ionic strength 0.2. We consider the close fit as compelling evidence that the intercalation process is governed by postulate 1.

The spectroscopic data for proflavine at ionic strength 0.002 as well as those for acridine orange at all ionic strengths indicate the existence of two bound species. In agreement with this indication, the binding isotherms for these systems cannot be fitted to eq 3. However, eq 1, together with eq 2 and the relation

$$\beta_1 + \beta_2 = \beta \quad (4)$$

now permits a unique determination of β_1 and β_2 from the experimental dialysis equilibrium data. The values so obtained will be used in this and the following sections in further tests of the applicability of our binding hypotheses.

The mass action law corresponding to postulate 2 is given by the expression

$$K_2 = \frac{\beta_2}{C_M(\beta_1 - \beta_2)} \quad (5)$$

where K_2 is the apparent binding constant for nonintercalated bound dye. The assumption concerning the constancy of the electrostatic potential pertaining to eq 1 also applies to eq 5. A test for eq 5 is shown in Figure 6 for acridine orange–DNA complexes at ionic

(42) L. M. Chan and Q. van Winkle, *J. Mol. Biol.*, 40, 491 (1969).
(43) M. J. Waring, *ibid.*, 13, 269 (1965).

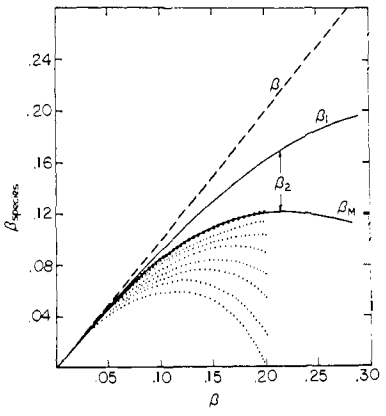

Figure 7. Calculated distributions of bound species obtained by different methods for AO–DNA complexes at $I = 0.002$. Dotted curves represent family of hypothetical curves for β_M, derivable from spectra of bound dye alone. The full curves are derived from dialysis equilibrium data by means of eq 1, 4, and 7 for $\beta \leq 0.2$ and by means of eq 1, 5, and 7 for $\beta > 0.2$.

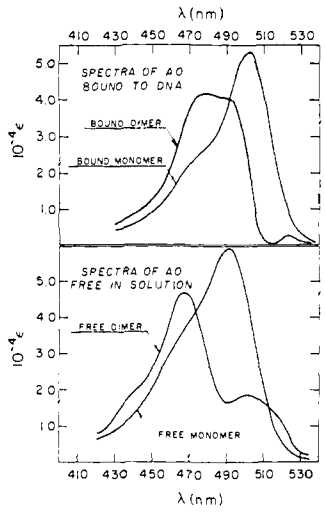

Figure 8. Spectra for AO monomer and dimer bound to DNA compared with spectra of AO monomer and dimer free in solution.

strength 0.002. The equation is clearly applicable over the range of β for which the spectroscopic data show only two bound species, in support of the validity of postulate 2. The rise of the ordinate at higher values of β is consistent with the spectroscopic evidence for additional bound species, presumably higher dye aggregates.

The values of K_1 and K_2 determined from our data by means of eq 1, 2, and 5 are collected in Table II.

Table II. Binding Constants for Dye–DNA Complexes at 22° and pH 6.5

	$10^{-5} K_1$, l. mol^{-1}		$10^{-5} K_2$, l. mol^{-1}	
I	AO–DNA	PF–DNA	AO–DNA	PF–DNA
0.002	30 ± 5	30 ± 5	7.3 ± 1	1.5 ± 0.5
0.020	12 ± 3		1.0 ± 0.3	
0.200	1.3 ± 0.3	1.0 ± 0.3	0.08 ± 0.03	Negligible

Within the experimental uncertainty the monomer binding constants, K_1, are the same for the two dyes at constant ionic strength. This result lends support to a partial intercalation process in which a significant portion of an intercalated dye molecule, probably including its amino group substituents, is not buried between DNA base pairs. Complete intercalation should result in different values of K_1 for AO and PF because of the disparity in the steric and hydrophobic nature of the ring substituents of these dyes. In contrast, at constant ionic strength the secondary binding constant, K_2, is generally larger for AO than for PF complexes. This result is consistent with the known differences in the tendency of the two dyes to dimerize in free solution.[22,32–34]

Bound Dye Spectra. The existence of only two bound dye species up to $\beta \approx 0.2$ permits the molar extinction coefficient of bound dye, ϵ_T, to be expressed at each wavelength in terms of molar extinction coefficients of bound monomer, ϵ_M, and of bound dimer, ϵ_D, by the relation

$$\epsilon_T = (\epsilon_M \beta_M + \epsilon_D \beta_D)/\beta \quad (6)$$

where β_M and β_D are defined by the equations

$$\beta_M = \beta_1 - \beta_2 \quad (7)$$

$$\beta_D = 2\beta_2 \quad (8)$$

and represent the number of bound dye molecules per DNA nucleotide existing as monomer and dimer, respectively. It is noteworthy that while the spectroscopic data alone do not permit a unique determination of either ϵ_D or β_M and β_D, knowledge of a single value of any of these quantities in a single experimental system would be sufficient for the complete determination of these quantities over the whole range of experimental conditions under consideration here. Thus, by a selection of arbitrary values of one of these quantities, a family of curves relating β_M to β may be constructed from the spectroscopic data. A representative sample of this family for the AO–DNA complexes at ionic strength 0.002 is shown in Figure 7 by the dotted curves. It is clear that the correct mass action law must be one of the members of this family. It is therefore meaningful that the solid β_M curve obtained independently from the dialysis equilibrium data by the application of eq 1, 2, 4, and 7 indeed coincides with one of the spectroscopic curves. This coincidence provides additional evidence for the validity of postulates 1 and 2.

Once the distribution of spectroscopic species has been determined in this way the absorption spectrum of the bound dimer becomes uniquely specified. The bound dimer spectrum for AO is shown in Figure 8. For comparison, the figure includes the spectra of bound monomer as well as those of the free monomer and dimer species.[34] The free dimer spectrum has been interpreted[34] using a theory proposed by McRae[44] as a "sandwich" dimer with a coupling energy corresponding to 3200 cm^{-1}. In the absence of dichroic ratios, the analysis of the bound dimer spectrum is more speculative. With the tentative assumption that the polarization states of the vibronic bands of the bound dimer are the same as those found for the free dimer, the use of the energy level and transition-intensity diagrams developed for the free dimer would yield a coupling energy for the bound dimer corresponding to about 2150 cm^{-1}. This value would be compatible with a modified "sandwich" model for the bound dimer

(44) E. G. McRae, *Aust. J. Chem.*, 14, 329, 344 (1961).

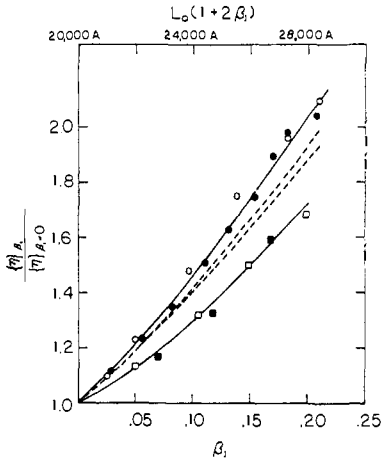

Figure 9. Viscosity enhancement for dye–DNA complexes as a function of the number of dye molecules intercalated per nucleotide. Designation of points is the same as in Figure 4. Dashed curves have been calculated for pure DNA with corresponding contour length; see text.

in which the overlap of the dye molecules would be incomplete to allow for partial intercalation of one of the partners.

Viscosity of the Complexes. If the viscosity data of Figure 4 are replotted against β_1 rather than against β, all dye-specific effects disappear. This finding, illustrated in Figure 9, is consistent with the interpretation that only those bound dye molecules which are intercalated contribute to the intrinsic viscosity, and, furthermore, that intercalated PF and AO molecules produce identical effects. Since the computation of β_1 values is based on postulates 1 and 2, these results furnish further support for the validity of these postulates.

The viscosity results also furnish direct evidence against the hypothesis that dimers are bound externally, *i.e.* that neither partner of a bound dimer is intercalated. With this hypothesis, only monomers would be intercalated, and their number would be proportional to $\beta_1 - \beta_2$, *i.e.*, β_M, rather than β_1. However, in Figure 7 it is seen that for AO at $I = 0.002$, the quantity β_M passes through a maximum at $\beta = 0.22$, while β_1 does not. The maximum in β_M should produce a corresponding maximum in the intrinsic viscosity. However, at this value of β none is observed.

It is reasonable to assume that each intercalated dye molecule makes a contribution of 3.4 Å to the DNA contour length, the equivalent of a DNA base pair. According to this assumption, intercalation of β_1 dye molecules per nucleotide would increase the contour length by a factor of $(1 + 2\beta_1)$. This consideration suggests a comparison of the viscosity results obtained for the DNA–dye complexes with those to be expected for pure DNA of corresponding contour length.

The appropriate values for the intrinsic viscosity of pure DNA may be obtained essentially by an indirect interpolation of experimental literature data following a method recently described by Ullman.[45] In this method the DNA is represented by a wormlike chain model which is characterized by four parameters, its contour length, L, its persistence length, a ($a = 1/2\lambda$ in Ullman's notation), its diameter, d, and a hydrodynamic parameter, α. Customarily, values of d and α are assigned *a priori;* we found that the results of our calculations were insensitive to the particular values chosen for these parameters. In line with previous findings we used $d = 25$ Å and $\alpha = 10$ throughout. The contour length of our experimental DNA sample corresponds to $L_0 = 20{,}000$ Å. Values for a were 115 Å and 625 Å at ionic strengths 0.002 and 0.2, respectively. These values were taken to be independent of the contour length of the pure DNA, an assumption valid over the range of L considered here where the influence of excluded volume effects may be neglected.

The dashed curves in Figure 9 represent the intrinsic viscosity increases to be expected from these calculations if the chain length of our DNA were increased by a factor of $(1 + 2\beta_1)$ through the addition of further base pairs.[46] A comparison between the dashed and full curves then indicates that the intrinsic viscosity of the dye-free DNA is much less sensitive to changes in the ionic strength than that of the dye-containing DNA. The bound cationic dye molecules clearly cannot bring about an increase in the net electric charge of the DNA. Therefore we interpret the observed ionic strength behavior as indicating that the intercalation of dye renders the DNA helix more flexible. This conclusion is in agreement with the results of combined sedimentation–viscosity studies on the PF–DNA system.[47]

Physical Basis of Proposed Model. In the preceding paragraphs it has been shown that the proposed binding model provides a self-consistent rationale for the thermodynamic, spectroscopic, and hydrodynamic properties of the dye–DNA complexes studied. We have considered a large number of alternate binding hypotheses none of which were consistent with all of the experimental data. Specifically, a model based on the assumption that only a fraction p of the slots between base pairs are binding sites was irreconcilable with the experimental results for any single value of p. It remains to examine some of the molecular aspects of our findings.

The fit between the experimental binding data and the mass action laws represented by eq 1 and 5 appears to justify the implicit assumption that the electrical potential is insensitive to the binding of dye. Further evidence for this unusual phenomenon is provided by experiments, carried out in this laboratory, which show that at ionic strength 0.002 the electrophoretic mobility of DNA is not affected by bound acridine orange at least up to $\beta = 0.2$.[48] It is noteworthy that a recent rigorous theoretical treatment predicts that, for the limiting case of zero ionic strength, the effective charge of polyelectrolytes maintains itself at a critical constant value by means of condensation of counterions as long as the formal charge is higher than the critical value.[49] If we assume that at least the general concept of this theory is applicable at the finite ionic strength values used in our experiments, we may ascribe the observed

(45) R. Ullman, *J. Chem. Phys.*, **49**, 5486 (1968).

(46) In conformity with our definition of the intrinsic viscosity for the DNA–dye complexes, these additional base pairs must not be counted toward the value of the DNA concentration.[39] Accordingly, the calculated intrinsic viscosity values were multiplied by a correction factor of $(1 + 2\beta_1)$.

(47) P. H. Lloyd, R. N. Prutton, and A. R. Peacocke, *Biochem. J.*, **107**, 353 (1968).

(48) U. P. Strauss and C. Papon, unpublished observations.

(49) G. S. Manning, *J. Chem. Phys.*, **51**, 924 (1969).

invariance of the potential to a release of condensed tetramethylammonium ions upon the binding of the dye ions.

Evidence that the potential varies with ionic strength is provided by the large observed decrease in the values of K_1 and K_2 with increasing ionic strength. The observed changes are of the order of magnitude expected if both apparent binding constants were proportional to the usual Boltzmann factor containing the potential and if the latter varied with the ionic strength according to the predictions of current polyelectrolyte theories.[50] However, since the same Boltzmann factors should apply to both K_1 and K_2, these parameters should show the same ionic strength dependence. In fact, the variation is somewhat greater for K_2 than for K_1. This finding suggests that additional factors are involved, perhaps related to a specific involvement of the tetramethylammonium ion.[51,52] Comparative studies of the effects of changes in the nature of the supporting electrolyte on the DNA–dye complexes are needed to elucidate this point.

The prohibition of intercalation in adjacent sites has not been predicted on theoretical grounds and is based entirely on experimental evidence. One possible explanation is that the intercalation of one dye molecule produces subtle but critical local distortions in the helix sufficient to cause steric hindrance toward intercalation in the nearest neighboring sites. A second possibility is that the prohibition results from a modification of the water structure in the immediate vicinity of an intercalated dye molecule. The critical role played by water in the intercalation process is indicated by X-ray diffraction studies on dye–DNA films showing that the small change in relative humidity from 100 to 93% induces complete transfer of intercalated dye to external binding sites.[14]

Acknowledgment. This work benefited greatly from a previous exploratory investigation conducted by Mr. Lawrence Muschiatti in this laboratory. Expert and efficient technical assistance provided by Sylvia Taylor is also gratefully acknowledged.

Appendix

Derivation of Eq 1 and 5. We consider the DNA to be immersed in a solution containing both dye monomers, M, and dye dimers, D. The following equilibria are considered where 0 denotes an empty slot between

$$M + 0 = A \qquad \text{(A-1)}$$

$$D + 0 = B \qquad \text{(A-2)}$$

base pairs, A a slot in which a dye monomer is intercalated, and B a slot in which a dye dimer is intercalated. We shall use the letter a with the appropriate subscripts to denote the activities of these species and groups. We have then

$$a_A/a_M a_0 = K_M \qquad \text{(A-3)}$$

$$a_B/a_D a_0 = K_D \qquad \text{(A-4)}$$

where K_M and K_D may be considered as intrinsic binding constants for the intercalation of monomers and dimers, respectively. The activities a_M and a_D pertain in the immediate neighborhood of the DNA which is assumed to be at an electrostatic potential ψ with respect to a DNA-free solution in dialysis equilibrium with the DNA solution. If the corresponding activities in this DNA-free solution are denoted with a prime, we have

$$a_M = a_M' \exp(-e\psi/kT) \qquad \text{(A-5)}$$

$$a_D = a_D' \exp(-2e\psi/kT) \qquad \text{(A-6)}$$

Since the matrix method has been thoroughly treated elsewhere,[53] we shall give only the barest outline of the derivation here. The matrix from which the partition function is derived is given by the expression

$$W = \begin{pmatrix} a_0 & a_0 & a_0 \\ a_A & 0 & 0 \\ a_B & 0 & 0 \end{pmatrix} \qquad \text{(A-7)}$$

This matrix contains the prohibition of occupancy of adjacent slots. Following the customary procedure, we find

$$\theta_A = 2K_M a_M/[q(q+1)] \qquad \text{(A-8)}$$

$$\theta_B = 2K_D a_D/[q(q+1)] \qquad \text{(A-9)}$$

where θ_A and θ_B are the fractions of slots occupied by monomer and dimer, respectively, and q is given by

$$q = [1 + 4(K_M a_M + K_D a_D)]^{1/2} \qquad \text{(A-10)}$$

The binding parameters used in the text, β_1 and β_2, are related to θ_A and θ_B by the relations

$$\beta_1 = (\theta_A + \theta_B)/2 \qquad \text{(A-11)}$$

$$\beta_2 = \theta_B/2 \qquad \text{(A-12)}$$

Combining eq A-8–A-12, we obtain

$$K_M a_M = \frac{2(\beta_1 - \beta_2)(1 - 2\beta_1)}{(1 - 4\beta_1)^2} \qquad \text{(A-13)}$$

$$K_D a_D = K_M a_M \frac{\beta_2}{\beta_1 - \beta_2} \qquad \text{(A-14)}$$

Denoting the dimerization constant of dye in DNA-free solutions by K_d, we have

$$K_d = a_D'/a_M'^2 \qquad \text{(A-15)}$$

Equations A-14, A-15, A-5, and A-6 then yield

$$K_2' a_M = \beta_2/(\beta_1 - \beta_2) \qquad \text{(A-16)}$$

where K_2' is defined by

$$K_2' = K_D K_d/K_M \qquad \text{(A-17)}$$

The apparent binding constants K_1 and K_2 are defined by the relations

$$K_1 = K_M a_M/C_M \qquad \text{(A-18)}$$

$$K_2 = K_2' a_M/C_M \qquad \text{(A-19)}$$

which, applied to eq A-13 and A-16, immediately leads to eq 1 and 5. It should be noted that by use of eq A-5 and A-6, eq A-18 and A-19 can also be written in the form

$$K_1 = K_M \gamma_M' \exp(-e\psi/kT) \qquad \text{(A-20)}$$

$$K_2 = K_2' \gamma_M' \exp(-e\psi/kT) \qquad \text{(A-21)}$$

respectively, if γ_M' is the activity coefficient of the monomer dye cation in the DNA-free solutions. These expressions are useful for the discussion of the dependence of K_1 and K_2 on β and ionic strength.

(50) See for example, L. M. Gross and U. P. Strauss in "Chemical Physics of Ionic Solutions," B. E. Conway and R. G. Barradas, Ed., John Wiley & Sons, Inc., New York, N. Y., 1966, p 347.

(51) M. Leng and G. Felsenfeld, *Proc. Nat. Acad. Sci. U. S.*, **56**, 1325 (1966).

(52) U. P. Strauss, C. Helfgott, and H. Pink, *J. Phys. Chem.*, **71**, 2550 (1967).

(53) S. Lifson, *J. Chem. Phys.*, **26**, 727 (1957).