A Comparison of the Interaction of an Acridine Dye and a Triphenylmethane Dye with Deoxyribonucleic Acid

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Abstract: The interaction of the acridine dye proflavine with DNA has been compared with that of the triphenylmethane dye pararosaniline under identical conditions of pH (5.0), ionic strength (0.002), and temperature (22°) by means of dialysis, spectroscopic, and viscosimetric measurements. Although the intrinsic strength of binding of pararosaniline is significantly weaker than that of proflavine, both dyes appear to intercalate into the helix up to a degree of dye loading of about 0.1 mol of bound dye per mol of DNA nucleotide. Above the latter degree of binding, little, if any, additional pararosaniline is inserted into the helix even though intercalation of proflavine continues up to a maximum value of about 0.25 mol of intercalated dye per mol of nucleotide. At pH 5.0, the binding of proflavine to DNA is about three times weaker than the value reported previously for the same system at pH 6.5.

The interactions between basic dye cations and native DNA have been under intensive investigation during the past decade. The widespread interest in these systems was largely stimulated by the pioneering work of Lerman who proposed[2,3] that various types of aromatic dye cations have a tendency to intercalate between successive base-pairs inside the double helix, a process accommodated by a local untwisting of the sugar–phosphate backbone and an elongation of the macromolecule. In addition to Lerman's own evidence, an impressive variety of studies[4–8] has provided convincing support for the intercalation model as a general mode of binding in various dye–DNA systems.

Recently a detailed binding model was proposed to account for the thermodynamic, spectroscopic, and hydrodynamic properties of acridine dye–DNA complexes.[8] The essential features of this model are as follows. (1) Up to a total dye loading of about 0.2 mol of bound acridine per mol of nucleotide, the principal dye binding mode is intercalation. (2) Intercalated dye is distributed randomly over the DNA chain with the restriction that dye may not be inserted in any two directly adjacent base-pair "slots." (3) An intercalated dye molecule is incompletely inserted into the helix; the protruding portion may serve as a binding site for a second molecule of dye, a process leading to the formation of a spectroscopically unique bound dimer species. (4) Dimerization becomes the dominant binding mode as the total dye loading exceeds about 0.3 mol of bound dye per mol of nucleotide. At still higher binding ratios, trimerization and higher aggregation processes are encountered. These external aggregation events ultimately lead to precipitation of the dye–DNA complex.

The present investigation was undertaken in part as a test of the applicability of the foregoing binding model to a nonacridine cation–DNA system. The nonacridine dye selected was pararosaniline; at a properly chosen pH, this triphenylmethane dye possesses the same overall unit charge as an acridine, but has a completely different configuration of aromatic rings and a different overall geometry. The general question as to whether triphenylmethane cations intercalate into the helix is somewhat unresolved. In the course of his brief survey of various dye structures, Lerman reported[2,3] that triphenylmethane cations increase the viscosity of DNA to an extent comparable to that found for the acridines, an observation which Lerman regarded as evidence that both types of dye intercalate. Lerman also found[9] that binding to DNA produced marked decreases in the reactivities of the amino substituents on pararosaniline, further evidence that the triphenylmethane cation is at least partially inserted into the double helix. On the other hand, later X-ray diffraction studies by Neville and Davies[5] on pararosaniline–DNA fibers did not reveal the intercalation-induced changes in layer-line spacing as found by the same authors for acridine–DNA complexes. These conflicting reports provided additional impetus for the present comparison of the relative thermodynamic, spectroscopic, and viscosimetric properties of acridine–DNA and triphenylmethane–DNA complexes.

Experimental Section

Materials. Commercial salmon sperm DNA (Worthington Biochemical Corp.; Lot 6HA) was precipitated twice into 95% ethanol from dilute NaCl solution. This sample of DNA is from the same lot as that employed previously;[8] it has a molecular weight of approximately 4×10^6 (as NaDNA) and an extinction coefficient at 259 nm of 6550 l. per mol of nucleotide. A stock solution containing 0.2% DNA was exhaustively dialyzed in the cold against 0.005 *M* NaCl, filtered through a 0.45-μ Millipore filter, and stored frozen.

Proflavine monohydrochloride (Pro) was a purified sample, identical with that used earlier.[8] Concentrations were determined spectrophotometrically using a molar extinction coefficient of 4.1×10^4 at 444 nm.[10]

Pararosaniline monohydrochloride (Para), a pure sample free of *p*-rosaniline and related compounds, was obtained from the Sigma Chemical Corp., Lot P3750. Concentrations were determined

(1) This work is based in part on a thesis submitted by N. M. P. to the Department of Chemistry of Russell Sage College in partial fulfillment of the requirements for the B. A. degree, May 1971.

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(3) L. S. Lerman, *J. Cell. Comp. Physiol., Suppl. 1*, 64, 1 (1964).

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(8) R. W. Armstrong, T. Kurucsev, and U. P. Strauss, *J. Amer. Chem. Soc.*, 92, 3174 (1970).

(9) L. S. Lerman, *J. Mol. Biol.*, 10, 367 (1964).

(10) G. R. Haugen and W. H. Melhuish, *Trans. Faraday Soc.*, 60, 386 (1964).

spectrophotometrically using a molar extinction coefficient of 9.5×10^4 at 542 nm. The latter value was calculated from the absorbance of weighed portions of the dye dissolved in sodium acetate buffer at pH 5.0. All other chemicals employed were reagent grade quality.

Choice of Experimental Conditions. All measurements were performed at 22° on solutions prepared in sodium acetate buffer at an ionic strength of 0.002 *M* and a pH of 5.0. The temperature and ionic strength are identical with those used in a previous study.[8] The pH selection was critical since it was found that the absorbance of pararosaniline solutions decreases very rapidly with increasing pH above about pH 5.5. This decrease in absorbance is related to the progressive conversion of the intensely colored, conjugated dye cation $Para^+$ to the colorless carbinol base ParaOH. On the basis of a spectroscopic study, it was found that the basic dissociation constant for the reaction

$$ParaOH \rightleftharpoons Para^+ + OH^-$$

is equal to 2.0×10^{-7}. Accordingly, at pH 5.0, greater than 99% of the dye is present as the monocation, as desired.

Solutions. Sodium acetate buffers were prepared at an ionic strength of 0.002 *M* and a pH of 5.0 by mixing 25.0 ml each of 0.0800 *M* NaOH and 0.122 *M* $HC_2H_3O_2$, then diluting to a total volume of 1 l. Buffers were prepared fresh daily to minimize bacterial growth. Stock solutions of the dyes were prepared in foil-wrapped Teflon bottles, assayed spectrophotometrically, and then refrigerated. In the absence of DNA, both dyes were found to obey Beer's law over the range of dye concentration employed (10^{-6} to 10^{-4} *M*). Dilutions of the stock dye solutions and preparation of the dye–DNA complexes were done in accordance with procedures described elsewhere.[8]

Methods. Absorption spectra were recorded on either a Perkin-Elmer spectrophotometer, Model 202, or on a Bausch and Lomb spectrophotometer, Model 505. Quartz cells with either 1- or 10-cm path lengths were utilized, as appropriate.

Binding isotherms were established by means of equilibrium dialysis measurements. Equilibration times of 24 ± 1 hr were found to be appropriate in the present study. All equilibrium concentrations were determined in the same manner as that described in detail elsewhere.[8]

All viscosity measurements were carried out at 22.00 ± 0.03° using a Zimm-Crothers viscosimeter (Beckman Instruments, Spinco Division). Reduced viscosities, obtained reproducibly without the need for detergent solution to dispel surface films,[8] were assumed to be shear-independent. Intrinsic viscosities were obtained by extrapolating the trend in reduced viscosity of four DNA solutions to zero concentration. These extrapolations were carried out by means of a linear least-squares analysis. DNA concentrations for viscosity measurements ranged from about 3.5×10^{-4} down to 1.0×10^{-4} mol of nucleotide/liter. Successive dilution of DNA solutions containing bound dye was carried out using a dye solution as a diluent. The concentration of diluent dye corresponded in each case to the free dye concentration which would exist in membrane equilibrium with the particular dye–DNA complex under investigation.

Results and Discussion

Binding Isotherms. The degree of binding of dye to DNA is expressed by the quantity β, defined as the molar concentration of dye bound to DNA (C_B) divided by the DNA concentration expressed in moles of nucleotide per liter. C_B was taken to be the difference between the total equilibrium concentration of dye inside the dialysis casing and that outside the casing, C_F. Experimental plots of β *vs.* C_F for the two dye–DNA systems, both at 22°, pH 5.0, and ionic strength 0.002 *M*, are presented in Figure 1. If one assumes that each binding site on DNA involves two nucleotide units,[11] then the intrinsic strength of binding of a single dye cation to a large excess of DNA binding sites is given by K^0, defined by the relation[8]

$$K^0 = \lim_{\beta \to 0}(2\beta/C_F) \qquad (1)$$

(11) This assumption amounts to an *a priori* specification that each "slot" between DNA base-pairs is a potential binding site for a dye cation.

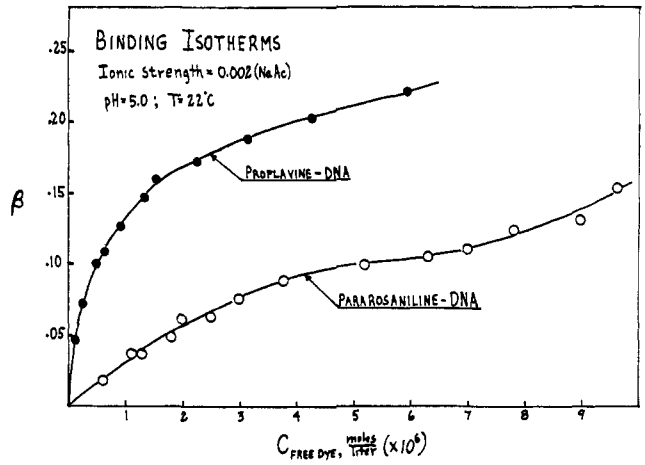

Figure 1.

Application of eq 1 to the proflavine–DNA data in Figure 1 yields a value of K^0 equal to 9.0×10^5 l./mol. This value is smaller by a factor of 3 than the corresponding constant for the Pro–DNA system studied[8] at the same temperature and ionic strength but at pH 6.5 rather than the present value of 5.0. A comparable decrease in binding constant with decreasing pH has been reported[12] for the acriflavine–DNA system. It has been suggested[13] that this reduction in binding constant with decreasing pH may be a consequence of partial protonation of the cytosine bases of DNA, a phenomenon which is probably appreciable at pH 5.0 and low ionic strength. Such a process would clearly diminish the electrostatic potential around the helix, thereby reducing its affinity for dye cations. The reduction in potential would also be expected to promote increased coiling of the macroion. Such an effect is implicit in the observation that under the conditions of ionic strength and temperature employed in the present work, a decrease in pH from 6.5 to 5.0 leads to a 30% decrease in the viscosity of DNA solutions. Despite the lower intrinsic binding constant, the general shape of the proflavine isotherm (Figure 1) is extremely similar to that obtained at pH 5.0. In both cases, β increases smoothly with free dye concentration over the entire range studied with no evidence of an inflection point. An inflection point, as is observed for the pararosaniline system (see below), reflects a rather sudden transition in the mode of binding of the dye.[14]

Turning now to the pararosaniline isotherm in Figure 1, it is apparent that this triphenylmethane dye interacts much more weakly with DNA than does proflavine. This difference in strength of binding is the largest single distinction in the behavior of the two dyes found in the present study. For the Para–DNA system, K^0, as defined by eq 1, was found to be 6.0×10^4 l./mol, a value which is smaller by a factor of 15 than that obtained for the acridine dye under the same conditions. This intrinsically weaker binding of pararosaniline may be due to the fact that its positive charge is exten-

(12) H. Heilweil and Q. Van Winkle, *J. Phys. Chem.*, **59**, 939 (1955).
(13) M. Duane, personal communication.
(14) The absence of an obvious inflection point does not imply that all dye molecules are bound in the same manner. In a prior study[8] it was shown that a proflavine isotherm with a shape very similar to that presented in Figure 1 could reflect two binding modes; a single mode, presumably intercalation, was largely predominant, however.

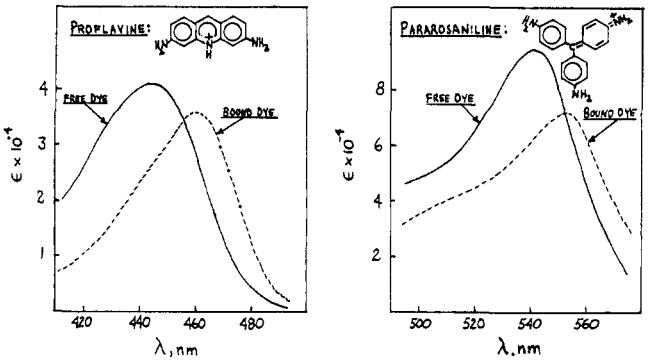

Figure 2.

sively delocalized over the three amino nitrogen atoms. It has been shown repeatedly[8,12,15] for dye–DNA systems that binding constants vary greatly with ionic strength, an observation which clearly implies that these interactions are at least partially a result of electrostatic attraction between the dye cation and the ionized phosphate groups of the nucleic acid. One may speculate that strong binding is favored by the presence of a fairly localized charge on the dye cation. In addition, one could point to differences in the aromatic structures of the two dyes. Perhaps proflavine with its three fused rings can interact more strongly with the organic moieties of DNA than can pararosaniline with its three separate noncoplanar phenyl substituents. This argument would be especially germane if both dyes do in fact intercalate such that their aromatic π systems are in close proximity to the π clouds of the nucleotide bases. Finally, the weaker binding of pararosaniline may be related to the slight nonplanarity of the triphenylmethane molecule. If both dyes are inserted into the helix, surely the planar proflavine could intercalate with less steric hindrance than could the nonplanar molecule.

Examination of the pararosaniline isotherm reveals an inflection point at a value of β of approximately 0.12. This feature implies that the initial mode of binding approaches saturation at a degree of dye loading which is about 50% lower than that found for the Pro–DNA system.[8] The fairly rapid increase in pararosaniline binding above this inflection point may correspond to the attachment of dye to the helix exterior, a binding process which is a widely accepted mode for acridine–DNA systems. In the latter case, however, such "secondary" binding is relatively insignificant below a value of β of about 0.25.[8] It should be noted that great difficulty was encountered in obtaining reproducible data for the Para–DNA system whenever dye loadings exceeded 0.12 bound dye molecule per nucleotide. Higher loadings frequently led to precipitation, a phenomenon which was reported[8] for acridine–DNA systems under conditions of dye loading wherein extensive amounts of bound dye were believed to have been attached to the exterior of the double helix.

The relatively weaker binding of pararosaniline may explain why changes in DNA layer line spacing were not observed for Para–DNA complexes examined in the course of the X-ray diffraction studies of Neville and Davies.[5] In order to ensure significant binding of pararosaniline, a large excess of free dye must be present. In addition, Neville and Davies[5] prepared dye–DNA complexes in the presence of unbuffered 25% ethanol. It is well established[8] that organic solvents, especially alcohols, strongly vitiate cationic dye–DNA interactions. Acridine–DNA interactions, being intrinsically stronger, would be relatively less affected by these conditions.

Spectral Shifts. The absorption spectrum which corresponds to that of a single dye molecule bound to a very large excess of DNA binding sites may be obtained by extrapolating the measured spectra of dye–DNA complexes to zero dye loading. When compared with the spectra exhibited by the corresponding dyes in the absence of nucleic acid, these "bound monomer" spectra reflect the manner in which the energy levels of the dye molecules are perturbed by the interaction with the polyion. The bound monomer spectra for the two dyes studied are shown as the dashed curves in Figure 2; the solid curves pertain to the corresponding dye monomers at the same temperature, pH, and ionic strength, but in the absence of DNA.

It can be seen from Figure 2 that both dye molecules undergo very similar spectral shifts upon binding to DNA. In both cases, the dye absorption maxima are shifted to the red by 10 to 15 nm with comparable slight losses in the intensities of absorption. Although it has been argued[16] that spectral shifts of the type shown in Figure 2 imply an intimate π–π interaction between dye cations and nucleotide bases, and hence, some degree of intercalation, such a conclusion is unwarranted since a very similar spectral shift results when proflavine interacts with the simple synthetic polyanion polystyrenesulfonate. Comparable red shifts have even been reported[17] to arise from the interaction of acridine orange with the polyacrylates, macroions which are totally devoid of aromatic character. Of course, even though spectral shifts of the type obtained for both dye systems may not be taken as direct evidence of intercalation, they surely do not rule out the existence of this type of binding mode either.

Intrinsic Viscosities. The intrinsic viscosity of the DNA sample employed was found to be 1390 ± 30 l. per mol of nucleotide at 22° in $NaC_2H_3O_2$ buffer, pH 5.0, and ionic strength 0.002 *M*. This viscosity value is nearly 30% lower than that reported[8] for the same DNA sample at the same temperature and ionic strength but in the presence of tetramethylammonium cacodylate buffer at pH 6.5. It was first thought that the DNA sample had undergone partial degradation during the interval since the previous report.[8] A viscosity study, carried out at various buffered pH values, revealed, however, a sharp increase in DNA viscosity over the pH range 5.0–7.0. The value obtained at pH 6.5 was virtually identical with that reported earlier.[8] This marked dependence of DNA viscosity at low ionic strength upon pH, even over the range of relatively neutral values, is probably related to increased protonation of cytosine residues with decreasing pH, as discussed earlier.

(15) A. R. Peacocke and J. N. H. Skerrett, *Trans. Faraday Soc.*, **52**, 261 (1956).

(16) H. J. Li and D. M. Crothers, *J. Mol. Biol.*, **39**, 461 (1969).

(17) G. Barone, V. Crescenzi, F. Quadrifoglio, and V. Vitagliano, *Ric. Sci.*, **36**, 939 (1966).

The intrinsic viscosities of DNA with various amounts of bound dye attached are presented in Table I. As

Table I. Effect of Dye Binding on DNA Viscosity

Proflavine–DNA		Pararosaniline–DNA	
β	$[\eta]$, l./mol	β	$[\eta]$, l./mol
0	1390	0	1390
0.051	1700	0.055	1700
0.102	2040	0.103	2200
0.162	2290	0.153	2180
0.175	2720	0.220	2250
0.250	2920		

found in the course of the previous study,[8] proflavine binding produces a monotonic increase in the viscosity of DNA up to a degree of dye loading equal to about 0.20 to 0.25 dye molecules per nucleotide. Above this level, dye binding, which is thought to occur almost exclusively to the helix exterior, produces little or no additional viscosity enhancement. When the ratios of the intrinsic viscosities of the Pro–DNA complexes to the viscosity of DNA alone are plotted *vs.* β, the resulting points (filled circles in Figure 3) are found to fall on virtually the same curve as that reported[8] earlier for pH 6.5. From the absorption spectra of the Pro–DNA complexes with total dye loadings less than about 0.2 mol of bound dye per mol of nucleotide, it was concluded in the earlier study that nearly all[18] bound proflavine is intercalated over this range of β. Since similar viscosity enhancements were obtained for the Pro–DNA system under the present set of conditions, it may be reasonably concluded that the proflavine bound to DNA in the complexes here studied is largely intercalated. The viscosity results obtained for the Pro–DNA system therefore provide a virtual "calibration curve" for the viscosity enhancement to be expected if each bound dye molecule is inserted into the helix.

Table I also contains a compilation of the intrinsic viscosities of Para–DNA complexes; the corresponding viscosity ratios, $[\eta]_\beta/[\eta]_0$, are plotted as open circles in Figure 3. It is readily apparent from these data that pararosaniline binding also promotes an increase in DNA viscosity and, further, that at low dye loadings, both dyes produce very similar viscosity enhancements. Since the intercalation of proflavine is a widely accepted phenomenon, one is led to the conclusion that pararosaniline intercalates also, at least over the range of β wherein the viscosity enhancements coincide. At β values greater than 0.10 to 0.13, additional binding of the triphenylmethane dye produces little additional increase in viscosity. This result is consistent with the binding data which reflected a change in binding mode at precisely the same range of β. Therefore it is probable that intercalation of pararosaniline is restricted to about 0.1 to 0.13 dye molecule per nucleotide, or to about one-half of the binding sites available to the acridine dye. Above this degree of dye loading, it is likely that pararosaniline binds to the helix exterior, a process which would be expected to produce little change in the intrinsic viscosity of a long semirigid macroion like DNA.

(18) The calculated percentages of proflavine molecules intercalated at β values of 0.10, 0.15, and 0.20 were 96, 93, and 89%, respectively.

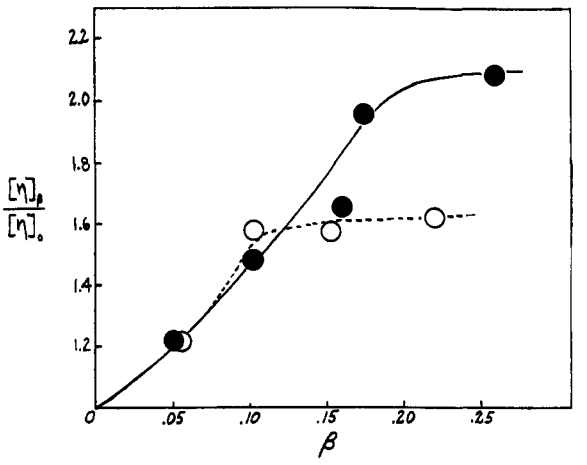

Figure 3.

It has been proposed[8] that proflavine intercalation is a phenomenon that can occur up to the point at which a dye cation is inserted into every alternate internucleotide "slot" along the chain. As a rationale for this model it was suggested that intercalated acridines are only partially inserted into the helix; that the projecting portions of these molecules somehow act to suppress intercalation at their two nearest neighbor "slots." The notion of partial intercalation of dye molecules, as contrasted to total insertion proposed originally by Lerman, has received support from other authors.[5,19,20] Lerman himself concluded that molecules like pararosaniline (though not the acridines) were incompletely inserted. In any case, because of its shape, it is likely that the triphenylmethane dye is less completely intercalated than the acridine. On the basis of the present findings, it is concluded that an intercalated pararosaniline cation acts to suppress similar binding at three neighboring "slots" on either side. This restriction would allow intercalation up to a value of β of 0.125, in agreement with the experimental results.

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