THE INTERACTION OF ACRIDINE DYES WITH THE DENSELY PACKED DNA OF BACTERIOPHAGE

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ABSTRACT The interactions of acridine dyes with intact phage DNA differ from those with extracted DNA in the following respects. Strong binding (intercalation) is greatly reduced in intact phage but probably not eliminated. The cooperative, weak binding is stronger and the stacking tendency is increased. In gels of DNA the stacking tendency is seen to increase with decreasing hydration. These influences of the dense packing of DNA must be taken into account when using basic dyes to study chromosome structure.

INTRODUCTION

Acridine dyes participate in a variety of interesting and useful interactions with nucleic acids. (For a general discussion see Albert, 1966.) Among the in vivo effects of these interactions are the production of insertion-deletion type mutations, the elimination of episomes, the suppression of genetic recombination, and the sensitization of DNA to photodynamic action. In addition many of these compounds are of great value in studies of nucleic acids in vitro. For instance acridine orange (AO), 3,6-bis-dimethylaminoacridine, has long been used in cytology as a fluorochrome to differentiate DNA and RNA and more recently to determine the orientation of DNA in certain biological structures (MacInnes and Uretz, 1966, 1968).

As a result of the interest in these properties many physicochemical studies of the interactions of acridines with isolated nucleic acids have been reported. Nearly all of these studies, however, were performed in relatively dilute solutions, while nucleic acids in vivo and in cytological preparations are frequently in a densely packed form. The principal exception is the study of Neville and Davies (1966) who analyzed acridine-stained gels of DNA and found changes in the interaction depending on the degree of hydration. As a result of the above considerations and inasmuch as we are using fluorescence emission from acridine dyes in the analysis of chromosome structure, we have undertaken a more detailed investigation of the effect of dense packing on the interaction of DNA with these dyes.

We have chosen for study bacteriophage $T4o_1$ as a convenient and relatively well-defined model system with densely packed DNA. Two methods of analyzing the interaction have been employed. The first was to measure the binding of the dye over a wide range of concentrations by means of equilibrium dialysis. This method has previously been employed in the study of the interaction of proflavine, 3,6-diaminoacridine, with free DNA (Peacocke and Skerrett, 1956). Two modes of binding were found, which they termed "strong" and "weak" binding. The strong binding saturates at about 0.2 proflavine molecules per phosphate and the weak binding at one dye per phosphate. It is now generally accepted that the strong binding results from intercalation of the acridine molecule between adjacent base pairs of the DNA (Lerman, 1961, 1963, 1964), and that the weak interaction results from binding of the dye on the outside of the DNA helix (Li and Crothers, 1969).

The second method was to determine the stacking tendency of densely packed DNA. As the concentration of AO in a solution is increased the fluorescence color shifts from green to red as a result of the aggregation of AO molecules (Zanker, 1952). Similarly as the amount of AO bound to a nucleic acid increases, the absorption and fluorescence spectra shift as a result of the "stacking" of the dye molecules with one another (Bradley and Wolf, 1959). The "stacking tendency" of a polymer is then defined as some measure of the amount of dye that must be bound to result in a certain degree of stacking as evidenced by the spectral shift. It has been found that the stacking tendency is a sensitive function of the secondary structure of a nucleic acid (Bradley and Wolf, 1959; Bradley and Felsenfeld, 1959) and is insensitive to many other possible variables (Stone and Bradley, 1961). The effects of dense packing and dehydration, however, have not to our knowledge been studied in detail.

MATERIALS AND METHODS

Experiments were carried out with phage suspended in either phage buffer (ϕB), containing per liter of distilled water 7.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 5.0 g NaCl, 1.0×10^{-3} M $MgSO_4$, 1.0×10^{-4} M $CaCl_2$, and 0.01 g gelatin, or in a 10-fold dilution of this buffer (DϕB).

Phage T4Bo: was grown in nutrient broth on *Escherichia coli* B/r. The purification process involved a low speed centrifugation, filtration through Hyflo Super-Cel (Fisher Scientific Co., Pittsburgh, Pa.), a Millipore filtration (Millipore Corporation, Bedford, Mass.), a high speed centrifugation, and overnight resuspension of the phage in a small volume of ϕB. The optical absorption at 260 nm per plaque-forming unit was 3.0×10^{-12} cm^2 (corrected for light scattering assuming λ^{-4} dependence). This indicates that nearly all the UV-absorbing material was viable phage (Luria et al., 1951).

Phage DNA was prepared from mature phage by phenol extraction. Salmon sperm DNA and yeast RNA were obtained from Calbiochem (Los Angeles, Calif., catalogue No. 2620 and 55712, respectively). Depurinated DNA was made from salmon sperm DNA by the method of Tamm et al. (1952). DNA concentration was determined assuming an ϵ_{260} (P) of 6500.

Proflavine hydrochloride, "purified by acetylation procedure" by one N. H. Smith, was free of other dyes as determined by paper chromatography in two solvents (Albert, 1966, p. 150). AO was purified by the method of Freifelder and Uretz (1966). The concentration of

proflavine stock solutions was checked using ϵ_{440} of 3.5×10^4 (Weill and Calvin, 1963) and ϵ_{444} of 3.9×10^4 (Albert, 1966), which agreed within 5%.

Fluorescence was measured on a Turner model 110 fluorometer (G. K. Turner Associates, Inc., Palo Alto, Calif.) equipped with a blue fluorescent excitation lamp, a Corning 5–58 (blue) primary filter (Corning Glass Works, Laboratory Products Dept., Corning, N. Y.), and a secondary filter of either Corning 3–69 (yellow) for proflavine, 3–68 (yellow) for AO, or 3-66 (orange) for determination of the color of AO fluorescence. The color scale was defined as the ratio of intensity measured with the 3–66 filter to the intensity with the 3-68 filter. Optically dense samples were measured in a capillary tube cuvette.

In order to measure dye binding to the intact phage DNA we took advantage of the large activation energy required for dye to pass through the protein coat of the phage (Cramer and Uretz, 1966). At 45°C it takes about 50 min for AO binding to reach 90% of saturation, while at 0°C the rate is nearly 1000-fold slower. The method used was to incubate phage in dye at 45°C for 2 hr, chill on ice, and then separate the phage from dye on a column 1.0 cm I.D. containing 3.0 g of coarse Sephadex G-25 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden). Fractions of 4.0 ml were collected in tubes which could be used directly as cuvettes in the fluorometer. Fluorescence was measured shortly after separation (for fluorescence intensity and color of bound dye) and after heating the fractions a half-hour at 70°C (to release dye and eliminate quenching in order to determine the amount of dye bound). The column and fluorometer were in a cold room at about 5°C. It was assumed that there were 3.3×10^5 phosphates in the DNA of each phage particle. The phage concentration was always sufficiently low that less than 0.1% of the dye was bound, which prevented any significant heterogeneous binding due to cooperative interactions of bound dye molecules.

Proflavine binding to extracted DNA employed a dialysis chamber (The Chemical Rubber Co., Cleveland, Ohio) with approximately 1 ml capacity on each side of the membrane with an air bubble inside each chamber to provide stirring. The dialysis was carried out inside an incubator at 45°C. The dye concentration on each side was determined daily by taking 2- or 10-μl samples which were diluted into 4.0 ml of 50% (volume) aqueous ethanol, in order to eliminate quenching, and measured for fluorescence. After equilibrium had been reached (usually 1 day was sufficient), more dye was added for the determination of another point. The entire binding curve was determined on a single sample of DNA.

The color of fluorescence from gels of DNA in equilibrium with air of various water contents was determined by supporting the gels on a coil of tinned copper wire which was suspended over saturated solutions of various salts in a stoppered tube which could be used as a cuvette in the fluorometer. The saturated salt solutions maintained the humidity at fixed levels. The fluorescence color was determined daily until a steady state was reached.

RESULTS

Binding

Data on the binding of proflavine to intact phage and extracted DNA are presented in Fig. 1. It may be seen that at low dye concentrations the intact phage DNA binds only about one-seventh as much dye as the extracted DNA. At higher concentrations a cooperative binding apparently takes place since the slope of the curve exceeds unity. Furthermore this cooperative binding is sufficiently extensive that over a certain range the intact phage DNA actually binds more dye than the extracted DNA. Finally the binding saturates at about 0.6 dye molecules per phosphate.

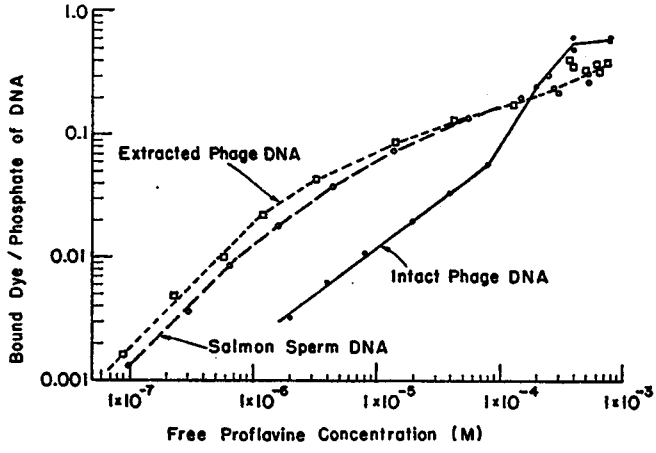

FIGURE 1 Binding of proflavine to intact phage DNA and to extracted DNAs in ϕB at 45°C.

In order to analyze the binding more thoroughly we have employed the following theory, which describes the binding of adsorbate to linear polymers (Steiner and Beers, 1961). For dilute solutions,

$$k_0 m = \frac{\beta - 1 + 2\theta}{\beta + 1 - 2\theta} e^{W/kT},$$

where $\beta \equiv [1 - 4\theta(1 - \theta)(1 - e^{-W/kT})]^{1/2}$, k_0 is the intrinsic association constant for one molecule of adsorbate with a single binding site, m is the molar concentration of free adsorbate, θ is the fraction of occupied sites, W is the free energy of interaction of a nearest neighbor pair of bound adsorbates, k is the Boltzmann constant, and T is temperature in degrees Kelvin. For later use we also define B as the number of binding sites per DNA phosphate.

Two types of binding curves predicted by this theory are plotted in Fig. 2. With no interaction between bound dye molecules ($W = 0$) one obtains the Langmuir adsorption isotherm. For this case, on a log-log plot, the slope of the curve is unity at low free dye concentrations and steadily decreases to zero as binding is saturated. When there are attractive forces between bound dye molecules ($W < 0$) one obtains a cooperative binding curve. In this situation the slope is also unity at low free dye concentrations but *increases* to some value greater than one and then decreases to zero as saturation is approached.

With these facts in mind we may examine Fig. 1 again. The binding to free DNA resembles the Langmuir isotherm except at high dye concentrations where some excess binding occurs. The data at dye concentrations below 2×10^{-5} M fit the theory fairly well with the following parameters: for salmon sperm DNA $k_0 = 1.0 \times 10^5$ and $B = 0.12$; for phage DNA $k_0 = 1.3 \times 10^5$ and $B = 0.14$. We estimate that the uncertainty in these parameters is about 20%.

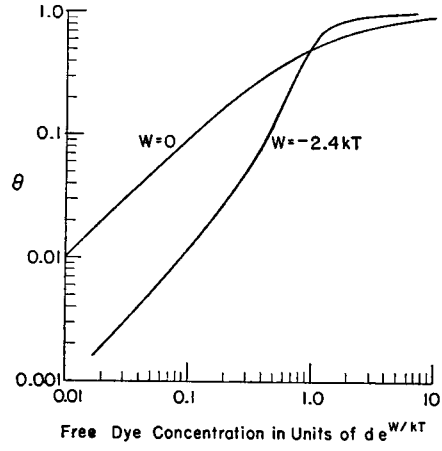

FIGURE 2 Two types of binding curves predicted by theory.

The binding curve of intact T4o$_1$ may be separated into three regions. At low dye concentrations the slope is less than one. In the intermediate region the slope is greater than one, and at high concentrations the slope is near zero. The simplest interpretation of this curve is that there is a cooperative binding process which predominates at high and intermediate concentrations but that the low concentration region is dominated by a Langmuir adsorption with a large association constant and a small number of sites. It might be anticipated that this latter mode of binding is by a mechanism similar to that which occurs with free DNA at low dye concentrations, but that there are a restricted number of sites for this type of binding in the intact phage.

That such a model is sufficient to explain the binding to intact T4o$_1$ is clear from Fig. 3. Here data on the binding of proflavine to phage T4o$_1$ in ϕB and its 10-fold dilution, DϕB, are compared with a model having the following parameters: for the strong binding mode $k_0 = 1.0 \times 10^5$ (ϕB) or 6.7×10^5 (DϕB), $B = 0.02$, and $W = 0$; for the cooperative binding mode $k_0 = 4.0 \times 10^2$ (ϕB) or 2.7×10^3 (DϕB), $B = 0.60$, and $W = -1.5$ kcal/mole. The fit of the data to the theory is good, with an estimated uncertainty of about 20% for each parameter.

Two aspects of these data are worth noting. First the 10-fold decrease in ionic strength of the buffer changed both association constants by a factor of about seven, but the stacking interaction (W) was apparently not changed. Approximately the same dependence on ionic strength of proflavine strong binding to extracted DNA was observed by Peacocke and Skerrett (1956). Thus, in this respect, densely packed DNA does not differ appreciably from extracted DNA. These data support the general idea that binding to DNA is largely ionic but stacking of dye molecules is not. Secondly, since intercalation usually leads to strong binding, the low number of strong binding sites in the intact phage indicates that intercalation is greatly sup-

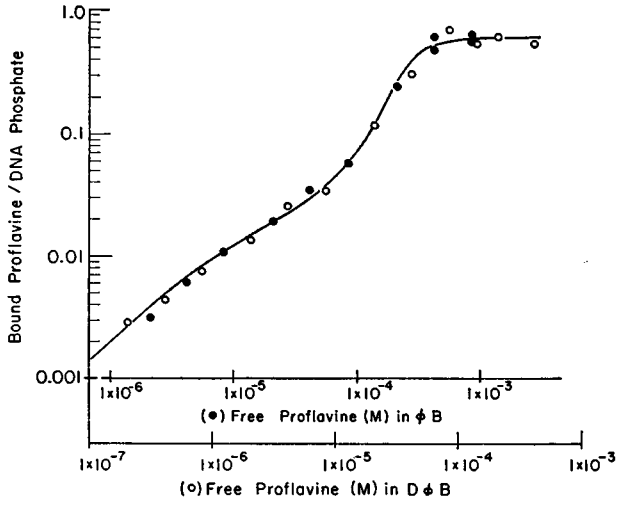

FIGURE 3 Comparison of proflavine binding to intact phage T4o$_1$ DNA with theory. Theoretical parameters: for the strong binding mode $k_0 = 1.0 \times 10^5$ (ϕB) or 6.7×10^5 (DϕB), $B = 0.02$, and $W = 0$; for the cooperative binding mode $k_0 = 4.0 \times 10^3$ (ϕB) or 2.7×10^3 (DϕB), $B = 0.60$, and $W = -1.5$ kcal/mole. Temperature, 45°C.

pressed; however, the fact that what strong binding there is in the phage has nearly the same association constant as strong binding in free DNA indicates that the remaining strong binding may be due to a limited amount of intercalation.

Controls were run to insure that the phage were not disrupted by incubation in the dyes. This was done by sedimenting phage with radioisotope-labeled DNA through sucrose gradients. Since intact phage have a sedimentation coefficient more than 10 times that of released DNA, this is a sensitive technique. No detectable proportion of phage incubated in proflavine was disrupted. On the other hand incubation in AO at concentrations above about 1×10^{-5} M resulted in disruption of a substantial proportion of the phage. At lower concentrations the binding of AO to phage could be measured and was found to be nearly identical with the binding of proflavine. The two dyes also bound to free DNA to the same extent. Thus our results are not unique to a particular dye.

Fluorescence Color

In Fig. 4 *a* data are presented concerning the color of fluorescence of various amounts of AO bound to DNA, RNA, and depurinated DNA. As expected the stacking tendency decreases with base pairing. In addition it should be noted that in the limit of low amounts of dye bound to DNA or RNA the color of fluorescence is less red than for free AO in solution which has a redness of 0.15. This shift is probably caused by interactions between the dye and bases of the nucleic acid and is expected on the basis of the studies of Weill and Calvin (1963). It is interesting to note

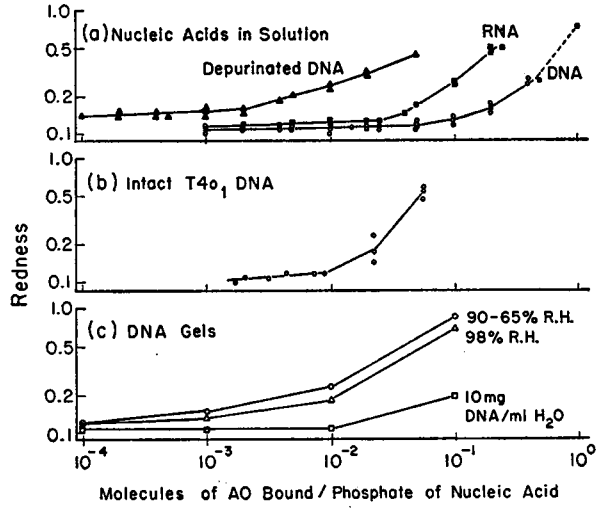

FIGURE 4 The color of fluorescence from AO bound to nucleic acids under various conditions. On this color scale values of 0.1–0.2 are green and 0.5–0.8 are red. R.H., relative humidity.

that depurination greatly reduces the number of such interactions. This observation is consistent with previous reports which indicate that acridine dyes form complexes with purine-containing nucleic acid derivatives which are stronger and lead to more dramatic spectral changes than complexes with pyrimidine-containing derivatives (Peacocke and Skerrett, 1956; Hruska and Danyluk, 1968; Yamabe, 1969).

In Fig. 4 *b* analogous data are presented for AO bound to intact phage DNA. It may be seen that the intact phage exhibits a stacking tendency between that of RNA and depurinated DNA and certainly much higher than for free DNA. In addition, at the limit of low amounts of bound dye the fluorescence color is the same as for free DNA. This suggests that small amounts of dye do intercalate intact phage DNA.

For the sake of comparison we also present similar data in Fig. 4 *c* for gels of DNA at different degrees of hydration. It is clear that decreasing the hydration of DNA increases its stacking tendency quite substantially, as Neville and Davies observed (1966).

DISCUSSION

Method

The interpretation of the data rest on the assumption that, under the conditions used, virtually all of the dye bound to the phage is bound to its DNA. A method of measuring the binding to phage which was previously used (Cramer and Uretz, 1966) was to centrifuge the phage out of the dye solution and measure the decrease in fluorescence of the solution. This method suffers from the possibility that dye may

bind to the protein of the phage as well as to its DNA. Indeed we found that with only brief exposure to dye substantial binding took place when measured in this fashion. This indicates that the protein on the outside can in fact bind the dye since binding to the DNA of the phage requires extended incubation in dye because of the permeability barrier (Cramer and Uretz, 1966).

We believe that the method used in the present study eliminates this problem. It seems clear that virtually all the dye bound outside the permeability barrier of the phage is washed free on the column since no detectable amount of dye elutes with the phage if they have not been incubated with dye. It seems equally certain that negligible amounts of dye bound inside the permeability barrier are lost on the column since no trailing of dye from the phage peak is observed and from data on the rate of photodynamic sensitization of the phage by AO (Cramer and Uretz, 1966) it is expected that less than 0.1% of dye inside the barrier would be lost under these conditions.

Furthermore there is good evidence that in addition to simply being inside the permeability barrier most of the dye is actually bound to the DNA, at least at high dye concentrations. This evidence is based on the fact that acridines are not known to bind to proteins cooperatively or with stacking interactions. In contrast our results with the phage demonstrate cooperative binding of proflavine and stacking of AO. These are the types of interactions one expects for nucleic acids. In view of the above considerations we feel confident that in our experiments nearly all the dye bound to phage at high dye concentrations is bound to its DNA.

The situation at low dye concentrations is not so clear. It is possible that the strong binding observed in intact phage is due to binding to protein inside the permeability barrier. The observed fluorescence color, binding constant, and influence of ionic strength on binding are, however, all consistent with the hypothesis that small amounts of proflavine can intercalate the DNA of intact phage and this seems to be the most reasonable explanation of strong binding in the phage. In any case this point is not crucial to the principal conclusions.

Binding

The parameters describing proflavine binding are summarized in Table I. As mentioned before, strong binding is greatly reduced in the intact phage. The fraction of strong binding retained in intact phage may be calculated as 0.15 (0.02 sites per phosphate of intact phage DNA/0.13 sites per phosphate of extracted DNA) if it is assumed that all of the DNA of the phage is accessible to dye or as 0.25 (0.02/0.60 sites per phosphate of accessible phage DNA/0.13 sites per phosphate of extracted DNA) if, on the basis of 0.6 dye molecules per phosphate at saturation, one assumes that only 60% of the DNA is accessible. Since intercalation in free DNA leads to strong binding, these numbers probably represent an upper limit on the amount of intercalation in the intact phage.

TABLE I

PARAMETERS DESCRIBING THE BINDING OF PROFLAVINE TO DNA

DNA	Buffer	Strong binding			Cooperative binding		
		k_0	B	W	k_0	B	W
							kcal/mole
Salmon sperm	ϕB	1.0×10^5	0.12	0	—	—	—
Extracted phage	ϕB	1.3×10^5	0.14	0	—	—	—
Intact phage	ϕB	1.0×10^5	0.02	0	4.0×10^2	0.60	−1.5
Intact phage	DϕB	6.7×10^5	0.02	0	2.7×10^3	0.60	−1.5

The cooperative binding in the intact phage is very pronounced. It was particularly surprising to find that the intact phage actually bound more at certain dye concentrations than the free DNA, which indicates that for some reason this cooperative binding is more favored in the intact phage than in free DNA. This point will be elaborated in the next section.

Stacking Tendency

It has previously been shown that in solution extracted phage T4 DNA has the same stacking tendency as DNA from many other sources (Stone and Bradley, 1961). A comparison of Figs. 4 *a* and *b*, however, clearly indicates that the intact phage DNA has a higher stacking tendency than free DNA. The question now arises as to whether this is further evidence that some of the DNA of intact phage is denatured as has been suggested (Tikchonenko et al., 1966, 1967; Gorin et al., 1967). Rigler (1966) has interpreted the red shift of fluorescence which he observed for AO-stained phage T2 in this way. On the other hand Maestre and Tinoco (1967) attribute the peculiarities of intact phage DNA which they have observed to dehydration.

A test of the effect of dehydration on stacking tendency is presented in Fig. 4 *c* which clearly indicates that dehydration does result in a higher stacking tendency. This is in agreement with the studies of Neville and Davies (1966) who found that low hydrations resulted in less intercalation of acridine dyes and increased stacking in the case of AO, without disrupting the DNA structure. X-ray diffraction studies (North and Rich, 1961) on phage T2 (which is nearly identical with T4) indicate that the lateral spacing of DNA in completely hydrated phage corresponds to that of free DNA at approximately 90% relative humidity. From a comparison of Figs. 4 *b* and *c* it may be seen that a dehydration corresponding to 90% relative humidity is in a rough way sufficient to account for the higher stacking tendency of the intact phage DNA. Thus we conclude that the higher stacking tendency of intact phage DNA cannot be taken as evidence for denaturation and may be the direct result of dense packing of the DNA.

Conclusions

From the evidence presented it seems clear that when aminoacridines bind to densely packed DNA the amount of intercalation is reduced and the strength of the stacking interaction is increased over that found for free DNA in solution. Each of these changes separately works to increase the "stacking tendency" (as defined by Bradley) of densely packed DNA. The role of these effects arising from the dense packing of DNA must be kept in mind when using basic dyes to study chromosome structure in more complex systems as well as in viruses.

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