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# THE BINDING OF 9-AMINOACRIDINE TO CALF THYMUS DNA IN AQUEOUS SOLUTION

## ELECTRONIC SPECTRAL STUDIES

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Absorption, circular dichroism and steady-state fluorescence spectra were determined of 9-aminoacridine solutions in the presence of DNA at an ionic strength of 0.001 mol $dm^{-3}$. Up to a dye/DNA phosphorus ratio of about 0.2 the results are fully consistent with the requirements and predictions of a binding model already shown to apply to the binding of other aminoacridines to DNA. The apparently anomalous spectroscopic behaviour of the 9-aminoacridine/DNA system compared with proflavine/DNA, for example, can be satisfactorily explained from a consideration of the magnitudes of exciton interactions between dyes bound to DNA.

## 1. Introduction

The antibacterial, mutagenic and antitumour activity of aminoacridines [1] and their extensive use in biological staining techniques [2] explain the interest in the study of the binding of these compounds to nucleic acids in solution. When the molar ratio of dye to DNA base-pair is very small, aminoacridines that do not carry bulky side groups are bound to DNA by intercalation between adjacent base-pairs [3]. As this ratio increases binding of the dye molecules to DNA will take place externally, without insertion into the helix, as well. Specifically, it has been proposed that the initial external binding sites are in the immediate vicinity of already intercalated dye molecules so that, initially, external binding produces 'dimeric' bound dye species consisting of an intercalated and an externally bound dye molecule [4]. This model, which will be referred to as the AKS model, has been shown to be successful in interpreting the absorption and circular dichroism (CD) spectra of DNA solutions in the presence of the aminoacridines acridine orange and proflavine [5] which are the most widely studied DNA/dye systems.

Earlier studies have shown that other aminoacridines, 9-aminoacridine (9AA) specifically, exhibit CD spectra when complexed to DNA which differ significantly from the corresponding spectra shown by, for example, DNA/proflavine solutions under identical circumstances [6,7]. These observations have led to speculations that the difference in the CD spectra may reflect differences in the geometry of the dye/DNA intercalation complexes. However, in terms of the AKS model, the major contribution to the CD of DNA/proflavine complexes arises from the exciton interactions between dye molecules in the bound dimer species. Were this also applicable to the CD of DNA/9AA solutions then the previous inference concerned with the geometry of intercalation complexes may not be valid. It was for this important reason that the work to be described was undertaken.

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We have set out to find the answer first to the question of whether the AKS model applied to the binding of 9AA to DNA. As will be seen the three independent spectroscopic methods, absorption, CD and steady-state fluorescence affirm the validity of the AKS model for this system. The answer to the subsequent second question of why the CD spectrum of 9AA bound to DNA appears anomalous is then found by considering the inherent electronic spectral properties of aminoacridines.

## 2. Materials and methods

9-Aminoacridine hydrochloride was obtained from A.G. Fluka; it was recrystallized twice from ethanol followed by drying in vacuo. Calf thymus DNA from Sigma Chemical Co. was used; concentrated stock solutions of DNA in $10^{-3}$ mol $dm^{-3}$ NaCl were prepared in the cold (0–4°C) with repeated stirring over a 7 day period followed by centrifugation to remove undissolved particulate matter. The dye/DNA solutions were prepared in spectroscopic silica cells by weight and kept overnight at 4° in a refrigerator to ensure complete equilibration. The pH of the solutions were between about 6.3 and 6.7.

Ultraviolet absorption measurements were made on a Zeiss DMR 10 double-beam recording spectrophotometer and the digitised data were transferred to a CDC Cyber 173 computer for treatment. CD measurements were made on a Jasco J-40CS recording spectropolarimeter equipped with an external microprocessor control unit allowing the averaging of data to improve signal-to-noise ratios. The digitised data were again transferred for further treatment to the Cyber 173. Fluorescence spectra were obtained on a Perkin Elmer model 3000 spectrofluorimeter observing the usual precautions associated with such measurements [8,9].

All data refer to room temperature measurements near 21°C.

## 3. Results and interpretation

### 3.1. The AKS binding model

This model has been applied to binding data including the dyes proflavine and acridine orange and both DNA [4,5,10] and RNA [11]. We denote the concentrations of intercalated and externally bound dye by $C_1$ and $C_2$, respectively. The model now asserts that at relatively low values of $C_2$ each externally bound dye is closely associated with an intercalated dye. Thus, the total bound dye (of concentration $C_B = C_1 + C_2$) may be subdivided into 'monomeric' intercalated species of concentration $C_1 - C_2$ and 'dimeric' species of concentration $C_2$ where the latter consists of one intercalated dye and one externally bound dye.

The binding equilibria are summarised as follows

$$A_F + DNA \overset{K_1}{\rightleftharpoons} A_1$$

$$A_1 + A_F \overset{K_2}{\rightleftharpoons} A_1A_2$$

where $A_F$, $A_1$ and $A_2$ refer to dye molecules which are, respectively, free and non-associated in solution, bound to DNA in intercalated form and bound externally. The symbol $A_1A_2$ refers to the bound dimer which consists of an intercalated ($A_1$) and a non-intercalated ($A_2$) bound molecule. A summary of the dye species postulated and the corresponding symbols to be used here are given in table 1.

### 3.2. Absorption spectra

Representative spectra are shown in fig. 1; the ionic strength of the solutions was kept low ($I = 0.001$) in order to ensure a relatively large extent of dye binding [5]. In recording these spectra the total dye concentration, $C_T$, was held approximately constant and the DNA concentration, $C_{DNA}$, expressed as DNA phosphorus per $dm^3$, was varied; the numbers refer to the ratio $\beta = C_T/C_{DNA}$.

In terms of the AKS model each element of the molar absorptivity matrix, $\epsilon^{\beta\lambda}$, is generated by the presence of three species and we may write

$$\epsilon^{\beta\lambda} = \alpha_F^\beta \epsilon_F^\lambda + \alpha_M^\beta \epsilon_M^\lambda + \alpha_D^\beta 2\epsilon_D^\lambda \quad (1)$$

Referring to fig. 1 one expects that the spectrum corresponding to the high value of $\beta$ would incorporate a much larger proportion of bound dimer than that for the low $\beta$ value. Yet, the shapes of

Table 1

Dye species according to the AKS model

| Species | Concentration | Fraction | Absorptivity | Molar CD | Fluorescence |
|---|---|---|---|---|---|
| Free dye ($A_F$) | $C_F$ | $\alpha_F$ | $\epsilon_F^\lambda$ | – | $F_F^\lambda$ |
| Intercalated dye ($A_1$) | $C_1$ | $\alpha_1$ | – | $\Delta\epsilon_1^\lambda$ | – |
| Externally bound dye ($A_2$) | $C_2$ | $\alpha_2$ | – | $\Delta\epsilon_2^\lambda$ | – |
| Monomer | $C_1 - C_2$ | $\alpha_M$ | $\epsilon_M^\lambda$ | – | $F_M^\lambda$ |
| Dimer | $C_2$ | $\alpha_D \equiv \alpha_2$ | $2\epsilon_D^\lambda$ | $\Delta\epsilon_D^\lambda$ | – |

the two spectra are not very different which implies that $\epsilon_D^\lambda$ is approximately proportional to $\epsilon_M^\lambda$. We write, therefore,

$$\epsilon_D^\lambda = p\epsilon_M^\lambda + \delta^\lambda \tag{2}$$

where $p \leqslant 1$ is near unity and $\delta^\lambda$ is a relatively small correction term. If now we define the following quantities

$$\chi^\beta = \alpha_M^\beta + 2p\alpha_D^\beta \quad \text{and} \quad \Delta^{\beta\lambda} = 2\alpha_D^\beta\delta^\lambda$$

then eq. 1 may be transformed into

$$\epsilon^{\beta\lambda} = \alpha_F^\beta\epsilon_F^\lambda + \chi^\beta\epsilon_M^\lambda + \Delta^{\beta\lambda} \tag{3}$$

In this equation the vectors $\epsilon_F$ and $\epsilon_M$ are known; the former corresponds to the free dye spectrum and the latter to the extrapolated dye spectrum in the limit of infinite DNA concentration. The magnitudes of the first two terms are about 4 orders larger than $\Delta^{\beta\lambda}$ so that we may fit each spectrum to eq. 3 using as variables $\epsilon_F^\lambda$ and $\epsilon_M^\lambda$ to obtain, as fitting parameters, the pair $\alpha_F^\beta$ and $\chi^\beta$ for each solution and simultaneously the residuals which correspond to $\Delta^{\beta\lambda}$. As an indication of whether the above procedure is justified, one expects that the shapes of the residual spectra should be independent of $\beta$; this indeed is shown to be the case in fig. 2 where the values of the residuals are plotted normalised arbitrarily to the set for $\beta = 0.168$.

Taking account of the postulated equilibria between dye species in solution we can express the relation between $\chi^\beta$ and $\alpha_F^\beta$ by

$$\chi^\beta = \left(1 - \alpha_F^\beta\right)\left(1 + 2pK_2\alpha_F^\beta c_T^\beta\right)/\left(1 + 2K_2\alpha_F^\beta C_T^\beta\right) \tag{4}$$

and thus obtain from the fit of these data the unknown parameters $K_2$ and $p$. However, we found that a satisfactory fit of the $\chi^\beta$, $\alpha_F^\beta$ set to eq. 4 could be obtained only for the region below about $\beta = 0.2$. This value of $\beta$ represents then the upper limit for the validity of the AKS model. The values of $K_2$ and $p$ so derived are shown in table 2.

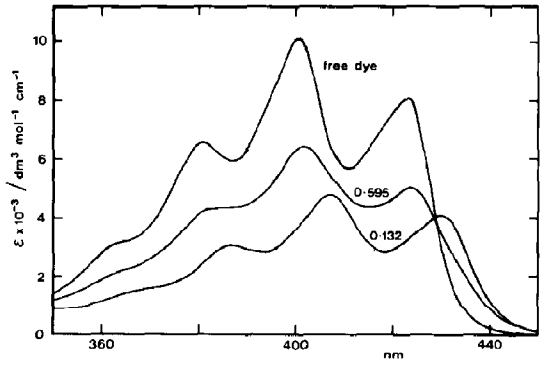


Fig. 1. Absorption spectrum of 9AA in 0.001 M NaCl solution in the presence of DNA at the values of $\beta$ indicated.

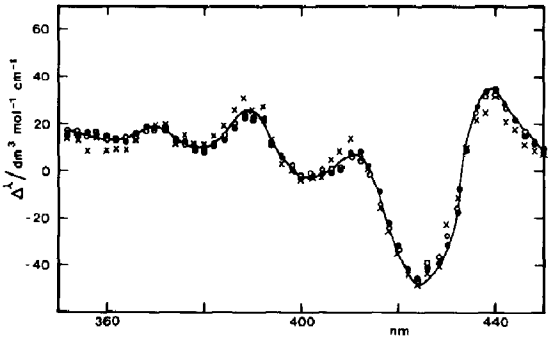


Fig. 2. Residuals arising from fit of absorption data to eq. 3 normalised to those of $\beta = 0.168$. (———) Averaged smoothed result. (○) $\beta = 0.595$, (●) $\beta = 0.278$, (×) $\beta = 0.186$.

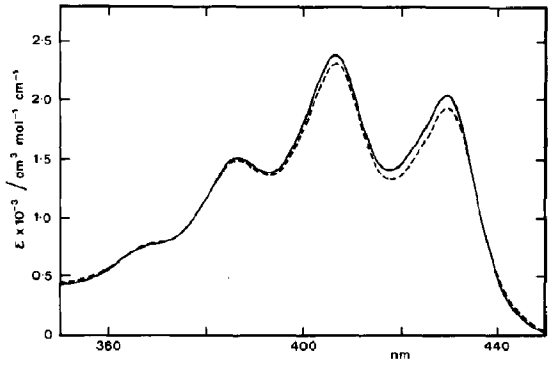


Fig. 3. Spectra of the monomeric (———) and dimeric (------) species of 9AA bound to DNA.

We can also relate $\Delta^{\beta\lambda}$ to $\alpha_F^\beta$

$$\Delta^{\beta\lambda} = \delta^\lambda(1 - \alpha_F^\beta)2K_2\alpha_F^\beta C_T^\beta/(1 + 2K_2\alpha_F^\beta C_T^\beta) \quad (5)$$

and we obtained satisfactory fits for a series of wavelengths, provided again that $\beta \leqslant 0.2$, with fitting parameters $K_2$ and the set $\delta^\lambda$. The average $K_2$ obtained from these fits is also included in table 2.

From a knowledge of the constant $p$ and the $\delta^\lambda$ terms we may construct the bound dimer spectrum, $\epsilon_D$, which is shown together with the bound monomer spectrum, $\epsilon_M$, in fig. 3.

### 3.3. Circular dichroism

Representative spectra of dye/DNA solution of ionic strength 0.001 mol dm$^{-3}$ are shown in fig. 4. In terms of the AKS model the observed CD is given by [5]

$$\Delta\epsilon^{\beta\lambda} = \alpha_1^\beta\Delta\epsilon_1^\lambda + \alpha_2^\beta\Delta\epsilon_2^\lambda + 2\alpha_D^\beta\Delta\epsilon_D^\lambda \quad (6)$$

If we allow for the fact that $\Delta\epsilon_2^\lambda$ has the same shape as $\Delta\epsilon_1^\lambda$ [11] we may put $\Delta\epsilon_2^\lambda = q\Delta\epsilon_1^\lambda$ where $q$ is a proportionality constant and we obtain the working equation:

$$\Delta\epsilon^{\beta\lambda} = \Delta\epsilon_1^\lambda(\alpha_1^\beta + q\alpha_2^\beta) + 2\alpha_D^\beta\Delta\epsilon_D^\lambda \quad (7)$$

where the vector $\Delta\epsilon_1$ is obtained by extrapolation of the CD data to $\beta = 0$.

The visible-near ultraviolet spectrum of 9AA has been assigned to two ($\pi^*$, $\pi$) electronic transitions [13,14], both polarised in the plane of the acridine ring. More specifically, the lowest energy transition which shows well-defined vibronic bands is polarised along the short axis and the much less intense transition, which peaks near 330 nm, along the long axis of the molecule [15,16]. We shall consider the CD spectra separately for these two regions.

Table 2

Parameters derived using the AKS model

| Equation | $K_2(\times 10^{-5})$ | $p$ | $q$ |
|---|---|---|---|
| 4 | 3.8±0.3 | 0.969 | |
| 5 | 3.9±0.6 | | |
| 8 | 4.3±0.2 | | 1.90 |
| 9 | 4.2±1.1 | | |

The CD spectrum that corresponds to the long-axis transition near 330 nm does not appear to change shape as $\beta$ is varied. We conclude that in this region exciton interactions between dye molecules in the species $A_1A_2$, which dominate in the case of proflavine, for example, are negligible in this system so that for the region corresponding to this transition we may put $\Delta\epsilon_D^\lambda \approx 0$. This finding is not surprising, since the oscillator strength of the long-axis transition of 9AA is only about 1/40th of that of proflavine. With neglect of the last term in eq. 7 and using the equilibrium expression for the formation of the dimers we derive

$$\Delta\epsilon^{\beta\lambda} = \Delta\epsilon_1^\lambda(1 - \alpha_F^\beta)\left[1 + K_2\alpha_F^\beta C_T^\beta(1 + q)\right]/ \left(1 + 2K_2\alpha_F^\beta C_T^\beta\right) \quad (8)$$

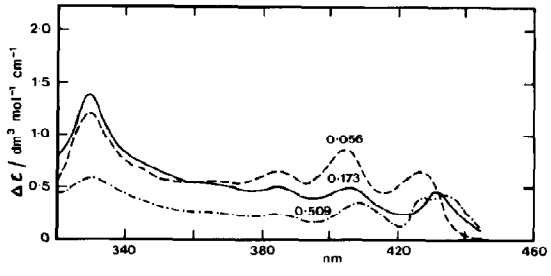


Fig. 4. CD of 9AA in 0.001 M NaCl solution in the presence of DNA at the values of $\beta$ indicated.

Using $\alpha_F^\beta$ interpolated from the absorption spectral results we fitted the data corresponding to the maximum in this region and found the fit satisfactory provided the data set was restricted to below $\beta = 0.2$; the resulting parameters, $K_2$ and $q$, are shown in table 2.

In the region that corresponds to the short-axis transition one observes from fig. 4 that there is some change in the shape of the spectrum as one varies $\beta$; thus $\Delta\epsilon_D^\lambda$ although small is not negligible and one would need to use eq. 7 to interpret the data. In our previous work which involved proflavine and acridine orange [5] we could proceed through the calculation of exciton spectra using vibronic exciton theory with various exciton coupling strengths until we could reproduce satisfactorily the experimental spectra. A necessary condition for such calculations is that the vibronic transition may be taken to consist of a single progression to a good approximation; as we have shown elsewhere [16] this is not so for 9AA and for this reason no further analysis of this region of the CD spectrum was carried out.

### 3.4. Fluorescence spectra

Representative spectra are shown in fig. 5. We write for the observed fluorescence intensity at any wavelength and value of $\beta$ an equation similar in form to eq. 3

$$F^{\beta\lambda} = \alpha_F^\beta F_F^\lambda + \alpha_M^\beta F_M^\lambda + R^{\beta\lambda} \qquad (9)$$

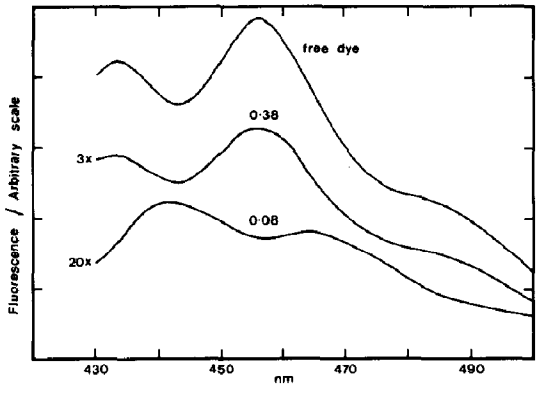


Fig. 5. Fluorescence of 9AA in 0.001 M NaCl solution in the presence of DNA at the values of $\beta$ indicated.

where the vectors $\boldsymbol{F}_F$ and $\boldsymbol{F}_M$ are known; the former is the fluorescence of the free dye and the latter the fluorescence extrapolated to $\beta = 0$ and $\boldsymbol{R}^{\beta\lambda}$ is the matrix of residuals. The first term in eq. 9 corresponds to the contribution of the unbound dye to the observed fluorescence. In fig. 6a it may be seen that the $\alpha_F^\beta$ values derived from the fluorescence data agree well with those obtained from the absorption spectra.

Turning to the fluorescence of the bound dyes it has been shown [9] that the fluorescence of a large fraction of 9AA bound to DNA is quenched and that those dye molecules which emit fall into at least three different classes distinguished by differing quantum yields but not by different spectra; at the same time the intercalative binding equilibrium itself does not appear to depend on the emitter class. Thus, if there is no site specificity of binding only that of quantum yield, we may take $\boldsymbol{F}_M$ to describe the spectrum of the intercalated dyes whose magnitude is an average of the quantum yields of the various classes of bound dyes. Since the fluorescence of dimeric bound dye is likely to be quenched the second term in eq. 9 is the contribution to the observed fluorescence of

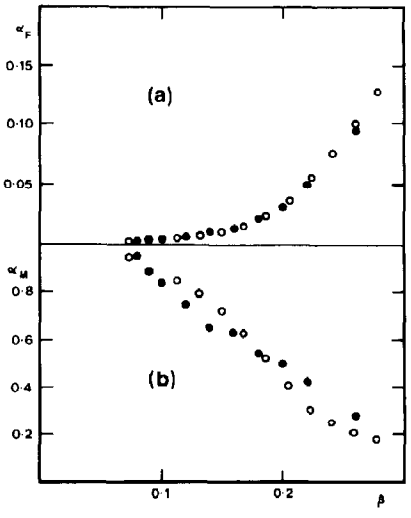


Fig. 6. Comparison of the fractions of free dye (a) and of monomeric bound dye (b) derived from absorption spectra with those obtained from fluorescence. (○) Absorption and (●) fluorescence measurements.

monomeric, intercalated molecules and this interpretation is fully supported by the good agreement between $\alpha_M^\beta$ obtained from eq. 9 and those derived from the absorption data as shown in fig. 6b. Furthermore, the value of $K_2$ calculated from the pairs $\alpha_F^\beta$, $\alpha_M^\beta$ at $\beta \leqslant 0.2$ and $C_T$ shown in table 2 is also consistent with those obtained from the absorption and CD data.

Finally, the residuals, $R^{\beta\lambda}$, in eq. 9 describe a shape that is approximately independent of $\beta$ which implies that there may be some fluorescence contribution from bound dimeric dyes as well. As seen in fig. 6, compared with the results derived from the absorption spectra, the $\alpha_M^\beta$ values at higher $\beta$ are overestimated by eq. 9 which supports the above conclusion. However, lack of precision of the $R^{\beta\lambda}$ matrix has prevented us from deriving quantitatively a dimeric fluorescence spectrum.

## 4. Discussion

Except for the appearance of their visible-near ultraviolet spectra 9AA and proflavine have similar solution properties. Thus their dimerisation constants in aqueous solution are very nearly identical [17–19], about $10^3$ mol$^{-1}$. Similarly, the shapes of the binding isotherms to DNA of these two dyes, the apparent binding constants and the resulting viscosity increase of the DNA solutions are also much the same [20–24]. It would be expected, therefore, that the same overall model of binding to DNA should be valid for both dyes. The first major conclusion arising from this work is that the spectroscopic data are not in contradiction with this expectation, as has been thought before [6]. For both dyes the AKS binding model is valid at not too high dye/DNA ratios and the absorption, CD and fluorescence data yield a consistent value for the formation constant of the bound dimers which is of the same order as that found previously for proflavine [4].

The second major conclusion is that the significant differences in spectroscopic behaviour between the two dyes upon binding to DNA reside in the strength of exciton coupling in the bound dye dimers species and not in possibly distinct intercalative geometries which is not excluded by these results. The magnitude of exciton interactions in a proflavine dimer bound to DNA [5] is of the order of 200 cm$^{-1}$ which is sufficiently large to produce measurable dimer spectra, particularly in CD. Exciton coupling may be taken to be the interaction between transition dipoles and accordingly its magnitude will be proportional to the intensity of the transition; this is about an order of magnitude less for 9-aminoacridine than for proflavine. Thus, even identical geometric relations between the molecules in the dimeric species would result in very weak exciton coupling between the transition moments of 9AA molecules and correspondingly rather small differences between monomeric and dimeric spectra (see fig. 3) compared with the situation in proflavine binding to DNA where the contribution of exciton spectra is particularly important in the observed CD. We thus conclude that the spectra observed are consistent with both dyes forming similar species of bound dimers to DNA.

We noted that the validity of the AKS model is limited to regions below about $\beta \approx 0.2$. At higher values of $\beta$ we believe that external binding produces external dimers and 'stacks' as has been postulated many years ago by Bradley and Wolf [25]. However, precisely because of the weak exciton interactions between the dye molecules, even the spectra of such stacks are not very different from the bound monomer spectrum and virtually indistinguishable from the $A_1A_2$ dimer spectrum as implied by the fact that the shape of the deviation spectrum shown in fig. 2 does not change significantly at high values of $\beta$.

We emphasise that the results of the spectroscopic measurements presented here do not exclude the possibility of different intercalation geometries for the various dyes for which evidence does exist [5,26,27]. Indirect evidence for differences between the intercalation complexes of 9AA and those of proflavine and acridine orange is provided here as well by the fitting of the results to a site exclusion intercalative binding model [28] which in a form relevant here may be written as

$$\frac{r_1}{C_F} = \frac{K_1}{2} \cdot \frac{(1 - 2nr_1)^n}{[1 - 2(n-1)r_1]^{(n-1)}} \qquad (10)$$

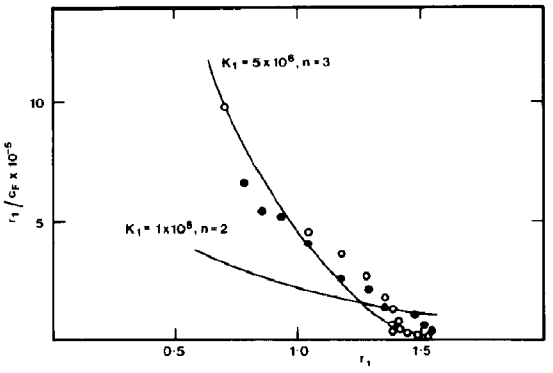


Fig. 7. The ratio $r_1/C_F$ as a function of $r_1$. (○) Absorption and (●) fluorescence measurements. Full lines correspond to eq. 10 with the two separate sets of parameters indicated.

where $r_1$ is the ratio $C_1/C_{DNA}$, $K_1$ the equilibrium constant and $(n-1)$ the number of neighbour sites excluded. As shown in fig. 7, our results appear to obey the model with $n = 3$ in contrast to just nearest-neighbour exclusion ($n = 2$) established for proflavine and acridine orange in 0.001 mol $dm^{-3}$ NaCl solution [4]. At the same time the binding constants $K_1$ are approximately of the same magnitude for all three dye/DNA systems at this low ionic strength. A least-squares fit of the data resulted in the following values: $K_1 = (5.06 \pm 0.33) \times 10^6$ and $n = 2.88 \pm 0.05$.

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