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# DNA Bifunctional Intercalators. 2. Fluorescence Properties and DNA Binding Interaction of an Ethidium Homodimer and an Acridine Ethidium Heterodimer†

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## Appendix: Numerical Solution of McGhee and von Hippel Equations for Competing Ligands

Marc Le Bret#

ABSTRACT: An ethidium homodimer and an acridine ethidium heterodimer have been synthesized (Gaugain, B., Barbet, J., Oberlin, R., Roques, B. P., & Le Pecq, J. B. (1978) *Biochemistry 17* (preceding paper in this issue)). The binding of these molecules to DNA has been studied. We show that these dimers intercalate only one of their chromophores in DNA. At high salt concentration ($Na^+ > 1$ M) only a single type of DNA-binding site exists. Binding affinity constants can then be measured directly using the Mc Ghee & Von Hippel treatment (Mc Ghee, J. D., & Von Hippel, P. H. (1974) *J. Mol. Biol. 86,* 469). In these conditions the dimers cover four base pairs when bound to DNA. Binding affinities have been deduced from competition experiments in 0.2 M $Na^+$ and are in agreement with the extrapolated values determined from direct DNA-binding measurements at high ionic strength. As expected, the intrinsic binding constant of these dimers is considerably larger than the affinity of the monomer (ethidium dimer $K = 2 \times 10^8$ $M^{-1}$; ethidium bromide $K = 1.5 \times 10^5$ $M^{-1}$ in 0.2 M $Na^+$). The fluorescence properties of these molecules have also been studied. The efficiency of the energy transfer from the acridine to the phenanthridinium chromophore, in the acridine ethidium heterodimer when bound to DNA, depends on the square of the AT base pair content. The large increase of fluorescence on binding to DNA combined with a high affinity constant for nucleic acids makes these molecules extremely useful as nucleic acid fluorescent probes. In particular, such molecules can be used in competition experiments to determine the DNA binding constant of ligands of high binding affinity such as bifunctional intercalators.

The biological properties and the antitumoral activity of bifunctional intercalators (Fico et al., 1977; Sinha et al., 1977; unpublished results from our laboratory) are thought to be related to their high DNA binding affinity (Le Pecq et al., 1975). It is therefore of interest to characterize and understand in detail the DNA binding of such molecules. Their interaction with DNA is complex. Several different types of binding are involved and in some cases bisintercalation occurs (Le Pecq et al., 1975; Wakelin et al., 1976).

Because the DNA binding affinity of such molecules is very high, a direct measurement of this binding affinity is partic-

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ularly difficult. Consequently, a detailed study of the DNA interaction of these bifunctional molecules has not yet been done. To permit such a study we have been led to synthesize bifunctional intercalators of particular intercalating moieties. Ethidium and acridine were chosen for their well-known fluorescence properties (Le Pecq, 1976, review). In the preceding paper (Gaugain et al., 1978), the synthesis and the conformational properties of an ethidium homodimer and of an acridine ethidium heterodimer are reported. In this paper we show that these two compounds exhibit a complex DNA binding behavior. Their DNA binding constants have been measured and are found to be high despite their DNA monointercalation properties. The fluorescence properties of these dimers are also described.

## Experimental Section

### *Materials*

Acridine derivatives AcMo,[1] AcDi II, and AcDi III (respectively identical with compounds I, IIB, and IIA in Le Pecq et al., 1975) as well as the phenanthridinium monomer (identical with compound XIV in Gaugain et al., 1978) have been synthesized as described earlier (Barbet et al., 1975; Watkins, 1952). 2,6-Dimethylellipticinium acetate was a gift from Dr. Nguyen-Dat-Xuong.

Calf thymus DNA was purified according to Aubin et al. (1963). λ phage DNA was prepared from the purified phage solution by three phenol extractions. DNAs of yeast mitochondria (82.7% AT, Bernardi et al., 1972) was a gift from Dr. Guerineau. DNAs from *Clostridium perfringens* and *Micrococcus luteus* were purchased from Sigma and DNA from *Proteus vulgaris* was purchased from Miles. Rat DNA was a gift from Dr. Giacomoni. These DNAs were purified by phenol extraction. PM2 DNA, a covalently closed circular DNA, was prepared as described (Le Pecq, 1971). Poly(dA-dT), poly(dA-BrdU), and poly(dG-dC) were obtained from Boehringer. Calf thymus sonicated DNA was prepared as described (Saucier et al., 1971; Le Pecq et al., 1975). The base composition of the DNAs is taken from Fasman (1976).

### *Methods*

Fluorescence measurements were made with a photon counting spectrofluorometer built in the Laboratory of Molecular Pharmacology (Paoletti, 1972).

Fluorescence decay times have been measured by the time correlated single photon counting technique (Yguerabide, 1972) with an instrument built in this laboratory. In both instruments the temperature is regulated at 25 °C.

*Fluorescence Titration and Scatchard Determination.* The fluorescence increment, IF, is the difference of fluorescence intensity between the solution containing the dye and the DNA and the solution containing the dye alone at the same concentration and measured under identical conditions. It is expressed as the number of impulses counted per 10 s in the photon counting instrument and is the average of three determinations. The fluorescence increment IF is plotted as a function of the $r_f$ of the dye ($r_f$ = ratio of the total molar concentration of dye to the molar DNA base pairs concentration).

When the binding of the dye to the DNA is not complete, the concentration of bound dye is computed according to Le Pecq & Paoletti (1967).

*Competition Experiments.* Solutions of ethidium dimer at identical concentration containing various concentrations of competing ligand are added to a DNA solution. The mixed solutions are stored 24 h in the dark at 25 °C to reach equilibrium before fluorescence measurements.

Viscometric measurements were performed at 25 °C in a semimicro dilution capillary viscometer with suspended level (Cannon Instrument Co., State College, Pa.) mounted in a high precision temperature regulated water bath. Flow times were measured to ±0.1 ms by the combined use of photoelectric sensors and of an electronic timer (Revet et al., 1971).

The unwinding angle of the DNA helix caused by the binding of the different derivatives was measured with covalently closed circular DNA from PM2 phage using viscometry (Revet et al., 1971).

To measure the length increase of short DNA segments, the intrinsic viscosity of sonicated DNA in the presence of increasing concentration of the various derivatives was measured; log $(\eta/\eta_0)$ was plotted as a function of log $(1 + r)$ where $\eta$ and $\eta_0$ are the intrinsic viscosity of sonicated DNA in presence and in the absence of bound dye, respectively, and $r$ is the ratio of the molar concentration of bound dye to the molar concentration of DNA base pairs (Saucier et al., 1971). In such experiments the slope of this plot is expected to be between 2 and 3 for a monointercalating agent and between 4 and 6 for a bisintercalating agent.

TABLE I: Unwinding and Lengthening of DNA by Ethidium Dimer and Acridine Ethidium Dimer as Compared to Ethidium and Acridine Monomers.[a]

| | EB | AcMo | ED | AcEtD |
|---|---|---|---|---|
| $\phi$ (deg) | 26 | 17 | 26 | 26 |
| slope log $\eta/\eta_0$ vs. log $(1 + r)$ | 2.3 | 2.1 | 3.6 | 2.3 |

[a] Unwinding angle $\phi$ is derived from viscometric measurement on closed circular DNA as described previously (Revet et al., 1971), using 26° as for the unwinding angle of ethidium (Wang, 1974). The lengthening of the DNA helix is proportional to the slope of the function log $(\eta/\eta_0)$ vs. log $(1 + r)$ (Saucier et al., 1971), where $\eta$ and $\eta_0$ are respectively the intrinsic viscosity of sonicated DNA in presence and in the absence of dye, and $r$ is the ratio of the molar concentration of bound dye to the molar concentration of DNA base pairs. This slope is expected to be between 2 and 3 for a monointercalating agent. Experiments with the ethidium dimer were performed in 0.2 M Tris-HCl buffer (pH 7.4) and in 0.2 M sodium acetate buffer (pH 5.0) for acridine ethidium dimer.

## Results

I. *DNA Binding of Ethidium and Acridine Ethidium Dimers.* In order to determine whether these two molecules are able to bisintercalate in DNA as previously described for acridine dimers (Le Pecq et al., 1975; Canellakis et al., 1976), we have measured the unwinding of the DNA helix caused by these two molecules using closed circular DNA. The lengthening of the DNA helix was also derived from viscosimetric measurements on sonicated DNA (Le Pecq et al., 1975). If bisintercalation occurs, the unwinding and the lengthening of the DNA helix must be twice that observed with the monointercalating agent, ethidium bromide, used as reference. Results are shown in Table I. It is clear that the values of the unwinding angle for the two dimers are close to the value obtained for ethidium. The lengthening of the DNA helix caused by the dimers is also of the same order of magnitude as for the monomer. Therefore, it can be concluded that these two dimers

[1] Abbreviations used: EtDi, ethidium dimer; AcEtDi, acridine ethidium dimer; AcMo, acridine monomer; AcDi II, acridine dimer II; AcDi III, acridine dimer III; EB, ethidium bromide; PhMo, phenanthridinium monomer.

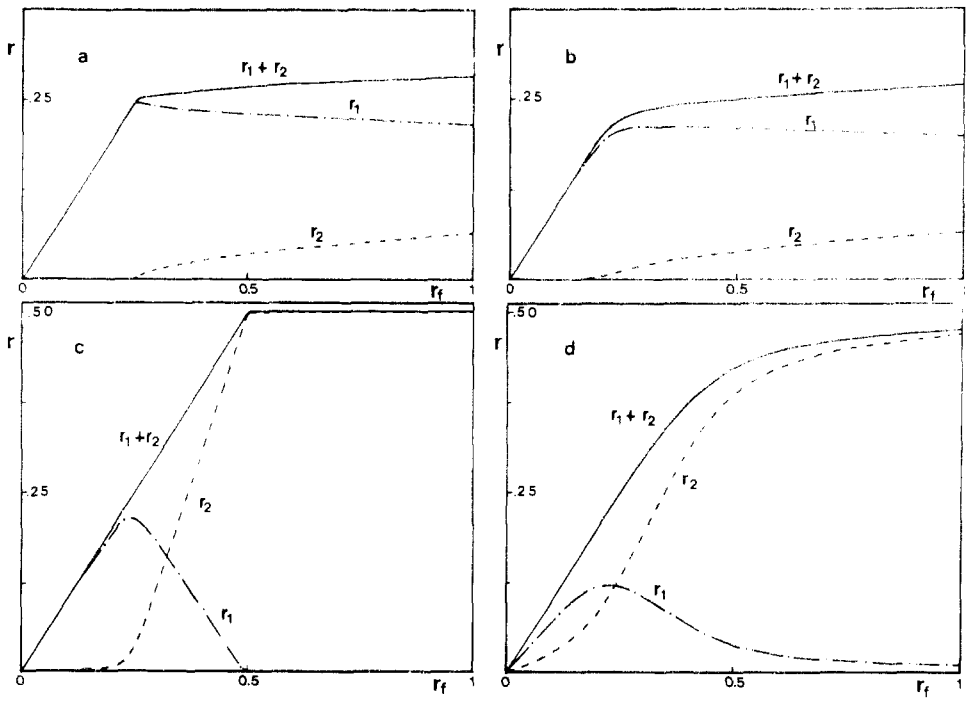


FIGURE 1: Simulation of the binding to DNA of a molecule which can occupy two kinds of sites with $n_1 = 4$ and $n_2 = 2$, referring to primary and secondary sites, respectively. The doted-dashed lines correspond to the binding to the primary sites ($r_1$), while the dashed lines show the binding to the secondary sites ($r_2$). The solid lines correspond to the sum of the bound molecules on the two types of sites ($r_1 + r_2$). (a) Molar DNA concentration $B = 10^{-2}$ M. Binding constant for the primary sites $K_1 = 10^{11}$ $M^{-1}$. Binding constant for the secondary sites $K_2 = 10^6$ $M^{-1}$. (b) Molar DNA concentration $B = 10^{-6}$ M. Binding constant for the primary sites $K_1 = 10^9$ $M^{-1}$. Binding constant for the secondary sites $K_2 = 10^7$ $M^{-1}$. (c) Molar DNA concentration $B = 10^{-2}$ M. Binding constant for the primary sites $K_1 = 10^{11}$ $M^{-1}$. Binding constant for the secondary sites $K_2 = 10^9$ $M^{-1}$. (d) Molar DNA concentration $B = 10^{-6}$ M. Binding constant for the primary sites $K_1 = 10^9$ $M^{-1}$. Binding constant for the secondary sites $K_2 = 3 \times 10^8$ $M^{-1}$. DNA concentration is expressed in base pairs. The high DNA concentration represents approximatively the situation inside bacteria and the low value the usual in vitro situation.

bind to DNA by intercalating only one of their two chromophores.

To evaluate the DNA binding affinities of the dimers, fluorescence titrations have been performed taking advantage of the large increase of fluorescence quantum yield upon binding of these molecules to DNA. However, as shown by McGhee & Von Hippel (1974), the binding affinity constant of oligomers or polymers for DNA cannot be simply deduced from binding measurements using traditional Scatchard plots. For example, if a ligand covers $n$ base pairs the binding equation is:

$$\frac{r}{c} = K\frac{(1-nr)^n}{[1-(n-1)r]^{n-1}} \qquad (1)$$

where $r$ is the ratio of the concentration of bound ligand to the concentration of DNA base pairs, $c$ the free concentration of the ligand, and $K$ the intrinsic binding affinity constant. If $B$ is the molar concentration of DNA base pairs, and $r_f$ is the ratio of the total molar concentration of ligand to the molar concentration of DNA base pairs (B), eq 1 is rewritten:

$$r = BK(r_f - r)\frac{(1-nr)^n}{[1-(n-1)r]^{n-1}} \qquad (2)$$

This equation applies only when there is one type of binding site. In our previous studies on diacridines, it was shown that probably two types of binding sites coexist: one corresponding to bisintercalation, with the highest binding constant which saturates for $r = 0.25$ ($n = 4$) and another, corresponding to monointercalation, with a lower binding constant which saturates for $r = 0.5$ ($n = 2$). Under these conditions and in the absence of cooperative binding, the following equations apply:

$$r_1 = BK_1\frac{(1-n_1r_1-n_2r_2)^{n_1}}{[1-(n_1-1)r_1-(n_2-1)r_2]^{n_1-1}}(r_f - r_1 - r_2) \qquad (3)$$

$$r_2 = BK_2\frac{(1-n_1r_1-n_2r_2)^{n_2}}{[1-(n_1-1)r_1-(n_2-1)r_2]^{n_2-1}}(r_f - r_1 - r_2) \qquad (4)$$

Subscripts 1 and 2 refer to first and second types of binding sites. These equations can be solved numerically (Appendix).

As observed previously in ethidium bromide-DNA studies (Le Pecq & Paoletti, 1967) secondary (electrostatic) binding sites exist which have a lower ligand binding affinity than that associated with the primary (intercalation) sites. In some cases these secondary sites can be populated at the expense of the primary sites and they can even predominate at high free concentrations of ligand. This effect is indeed well accounted for by the Mc Ghee-Von Hippel theory: the total gain in free energy could be greater at saturation when the ligand occupies the secondary sites (rather the primary sites) because the lower binding free energy associated with the weaker secondary site binding constant is compensated by a greater density of secondary sites on DNA. As an example of this phenomenon, we show a simulation of the DNA binding of a molecule which can occupy two kinds of sites, ether a primary site with $n_1 = 4$ and binding constant $K_1$ or a secondary site with $n_2 = 2$ and binding constant $K_2$, such that $K_1 > K_2$ (Figure 1). Indeed, it is observed in Figure 1 that the primary sites are occupied first and that the secondary sites are populated later at the expense of the primary sites. At high DNA concentrations or for large binding constants (large values of the products $BK$'s), the

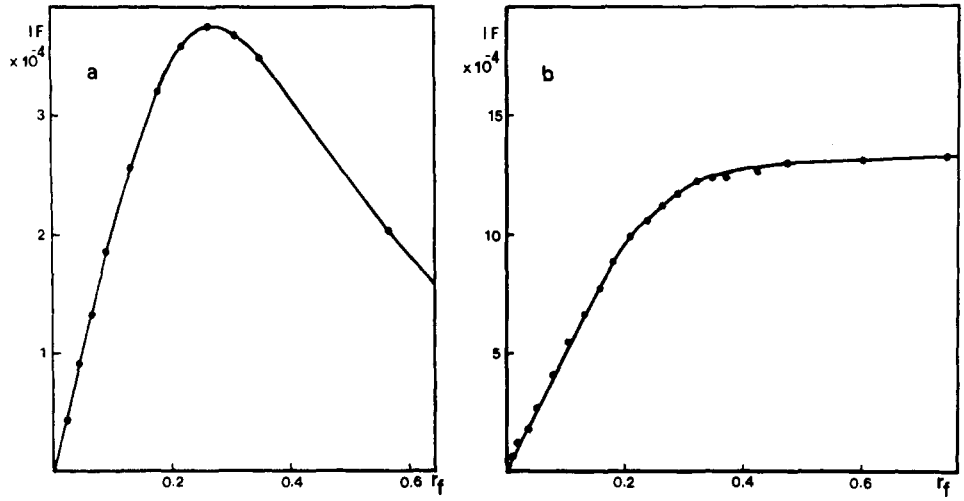


FIGURE 2: Fluorometric titration curves of calf thymus DNA by ethidium dimer in 0.2 M sodium acetate buffer, pH 5.0 (a), and in 0.2 M Tris buffer, pH 7.4 (b) ($\lambda_{exc}$ = 540 nm; $\lambda_{em}$ = 610 nm). DNA base pairs concentration is $0.9 \times 10^{-6}$ and $1.7 \times 10^{-6}$ M in curves a and b, respectively. $r_f$ is the ratio of the total dye concentration to the molar DNA base pair concentration.

transition between the two kinds of sites begins for $r$ values corresponding to the saturation of the primary sites. The displacement is thereafter almost stoichiometric and it is still observed when the ratio $K_1/K_2$ is large ($10^3$). At lower DNA concentrations or for smaller binding constants ($BK$ smaller), this displacement is only observed when $K_2$ is close to $K_1$ and it is no longer complete even for large values of the free ligand concentration. In such cases, it is difficult to deduce $K_1$ and $K_2$ from direct binding measurements. We have therefore measured the binding of the ethidium dimer and the acridine ethidium dimer to DNA as a function of ionic strength, in order to determine the conditions which will minimize secondary site binding ($K_2$, $n$ = 2) and allow direct measurement of primary site binding ($K_1$, $n$ = 4).

Fluorescence titrations of the ethidium dimer with calf thymus DNA at 0.2 M ionic strength are presented in Figure 2, as a function of increasing $r_f$ (added dimer/DNA base pair). In Tris buffer, the fluorescence reaches a plateau at $r_f$ = 0.25 (Figure 2). However, in acetate buffer, the fluorescence levels off for $r_f$ = 0.25 and later decreases for larger values of $r_f$ (Figure 2). In acetate buffer, these results show that the fluorescent binding sites corresponding to $n$ = 4 are first populated. The subsequent decrease in fluorescence shows that secondary nonfluorescent binding sites are populated at the expense of the first sites, as would be expected if the $n$ value for this secondary site is smaller than 4. Quantitative treatment of such results is rendered difficult due to the fluorescent quenching of ethidium dimers which are bound to the first kind of sites by ethidium dimers which are bound to the secondary sites. Even in the case of Tris buffer where the fluorescence reaches a plateau, the curves cannot be correctly fitted, using eq 3 and 4, probably because the secondary sites are sufficiently populated to cause some fluorescence quenching of the primary sites. Thus a direct determination of the dimer binding constant from fluorescent measurements cannot be correctly done under these experimental conditions.

At higher salt concentrations ($Na^+ > 0.5$ M), the binding of the ethidium dimer to DNA is no longer completely stoichiometric (linear) with increasing values of $r_f$. Therefore, the equilibrium concentration of free dimer can be directly measured from the fluorescence titration data. Furthermore, only one type of binding site is observed and the fluorescence titration data can be analyzed according to the Mc Ghee-Von Hippel treatment. The Scatchard plot of the binding of the

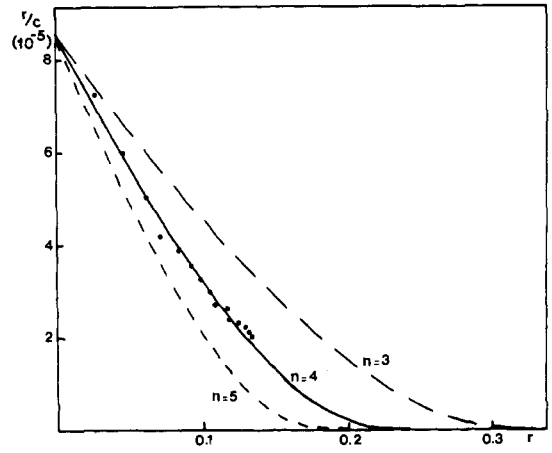


FIGURE 3: Scatchard plot of the binding of ethidium dimer to calf thymus DNA in 1.5 M $Na^+$ and 0.1 M Tris buffer deduced from fluorometric titration ($\lambda_{exc}$ = 540 nm; $\lambda_{em}$ = 610 nm) and fitted according to the Mc Ghee-Von Hippel (1974) treatment. The best fit is observed with the smooth curve where the number of covered base pairs $n$ is chosen equal to four with an intrinsic binding constant for ethidium dimer of $K = 8.5 \times 10^5$ $M^{-1}$. The dashed curves are drawn with the same value of $K$ but with $n$ = 3 and $n$ = 5. DNA base pair concentration is $0.8 \times 10^{-6}$ M.

ethidium dimer to calf-thymus DNA (1.5 M $Na^+$) is shown in Figure 3. These results clearly show that the ethidium dimer binds to DNA covering 4 base pairs, with a binding constant of $8.5 \times 10^5$ $M^{-1}$ in 1.5 M $Na^+$ (Figure 3). The DNA binding constants of the ethidium dimer at various ionic strengths (0.5-4 M $Na^+$) are presented in Figure 4. It is interesting to note that the ethidium dimer binding constants are similar in acetate and Tris buffer, when extrapolated to the same ionic strength (Figure 4).

Binding of the ethidium dimer to DNA was also measured in the presence of various concentrations of other competing ligands, in order to directly evaluate the DNA binding constant of the ethidium dimer at low salt concentrations (below 1 M $Na^+$). Equations 3 and 4 can be applied to the competition between two ligands. In order to simplify, we limit ourself to the case of a ligand which can occupy two kinds of sites with two different values of $n$. $K_1 n_1 r_1$ *and* $K_1' n_1' r_1'$ will refer to primary and secondary sites, respectively. The competing ligand is characterized by $K_2$, $n_2$, and $r_2$.

Under these conditions the following equations apply

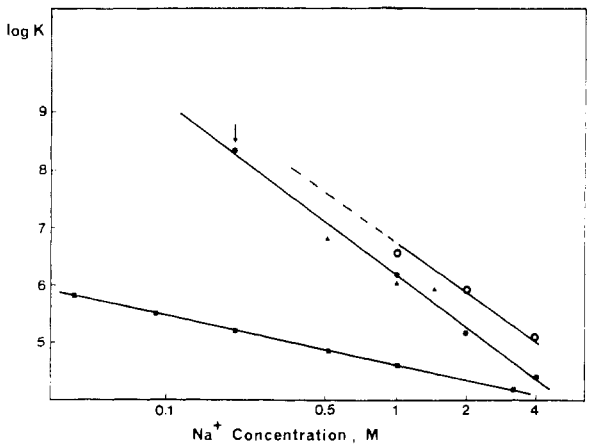


FIGURE 4: Compared effect of the $Na^+$ concentration on the intrinsic DNA binding constant of ethidium bromide, ethidium dimer, and acridine ethidium dimer. Intrinsic binding constants of the ethidium dimer (●, sodium acetate buffer, pH 5.0; ▲, NaCl-Tris buffer pH 7.4) and the acridine-ethidium dimer (○, sodium acetate buffer, pH 5) are measured by fluorometric titration as indicated in the legend of Figure 3. The measurements of Le Pecq & Paoletti (1967) have been reinterpreted according to the Mc Ghee-Von Hippel treatment to obtain the intrinsic association constants of ethidium bromide for DNA at various $Na^+$ concentrations (■). The arrow indicates the logarithm of the binding constant of ethidium dimer in 0.2 M sodium acetate buffer, deduced from the competition experiments shown in Figure 5.

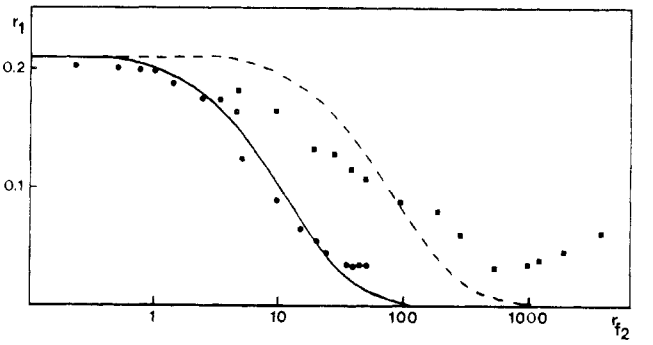


FIGURE 5: Displacement of the ethidium dimer, bound to calf thymus DNA, by competing monointercalating ligand in 0.2 M sodium acetate buffer, pH 5.0. The concentration of bound ethidium dimer per DNA base pair ($r_1$) is deduced from fluorescence measurement ($\lambda_{exc}$ = 540 nm; $\lambda_{em}$ = 610 nm) and $r_{f_2}$ is the ratio of the total molar concentration of the competing ligand to the molar concentration of DNA base pair B ($B$ = $1.5 \times 10^{-6}$ M). Competition by 2,6-dimethylellipticinium: the experimental values of $r_1$ (●) are fitted with eq 7-9 by the solid line ($n_1 = 4$, $K_1 = 2 \times 10^8\ M^{-1}$; $n_2 = 2$, $K_2 = 8 \times 10^5\ M^{-1}$). Competition by acridine derivative AcMo: the experimental values of $r_1$ (■) are fitted with eq 7-9 by the dashed line ($n_1 = 4$, $K_1 = 2 \times 10^8\ M^{-1}$; $n_2 = 2$, $K_2 = 1 \times 10^5\ M^{-1}$). The intrinsic association constant of 2,6-dimethylellipticinium for calf thymus DNA ($K = 8 \times 10^5\ M^{-1}$) has been directly measured by fluorometric titration ($\lambda_{exc}$ = 330 nm; $\lambda_{em}$ = 540 nm) in the same buffer. The intrinsic association constant of the acridine derivative AcMo for calf thymus DNA in the same buffer ($K = 1 \times 10^5\ M^{-1}$) has been deduced from competition experiment with ethidium bromide according to Le Pecq & Paoletti (1967).

with:

$$a = (1 - n_1 r_1 - n_1' r_1' - n_2 r_2) \quad (5)$$

$$b = (1 - (n_1 - 1)r_1 - (n_1' - 1)r_1' - (n_2 - 1)r_2) \quad (6)$$

$$r_1 = BK_1 \frac{a^{n_1}}{b^{n_1 - 1}} (r_{f_1} - r_1 - r_1') \quad (7)$$

$$r_1' = BK_1' \frac{a^{n_1'}}{b^{n_1' - 1}} (r_{f_1} - r_1 - r_1') \quad (8)$$

$$r_2 = BK_2 \frac{a^{n_2}}{b^{n_2 - 1}} (r_{f_2} - r_2) \quad (9)$$

These equations can be solved numerically for $r_1$, $r_1'$, and $r_2$ for different values of $B$, $K_1$, $K_1'$, $K_2$, $r_{f_1}$, and $r_{f_2}$ (Appendix).

Two different intercalating monomers, a fluorescent derivative of ellipticine (Le Pecq & Le Bret, 1976) and a monomeric acridine derivative (AcMo, Le Pecq et al., 1975), were chosen as the competing ligands since their DNA binding parameters ($K$, $n$) have been well established. Experiments were made in 0.2 M $Na^+$ where secondary site dimer binding can be neglected, especially for relatively low values of $r_f$ (Figure 2). Furthermore, only ethidium dimers bound to DNA at the primary sites ($n = 4$) are fluorescent ($\lambda_{em}$ = 610 nm). The competitive binding results are shown in Figure 5. The data can be fitted with eq 7-9, assuming weak secondary site binding of the dimer ($K_1'$ small). With the ellipticine derivative as the competing ligand, the experimental data in Figure 5 can be accurately fitted using a value of $K_1 = 2 \times 10^8\ M^{-1}$. In the case of the acridine derivative as the competing ligand, there is less agreement between the experimental and theoretical competition curves (Figure 5). It can be observed in Figure 5 that the fluorescence of the ethidium dimer begins to *increase* in competitive binding experiments with the acridine derivative $r_{f_2}$ values greater than 800. Control experiments have shown that the fluorescence of the ethidium dimer also increases in the same concentration range of excess acridine monomer in the absence of DNA. This phenomenon suggests that the ethidium dimer can interact with the acridine ligand (at high concentrations of the competing agent) which could account, in part, for the discrepancy between the theoretical and the experimental competition curves with the acridine monomer (Figure 5). Nevertheless, the value of the ethidium dimer bonding constant which fits both the theoretical and experimental binding curves at 50% displacement ($r_1 = 0.1$) is the same as the binding constant used in fitting the ellipticine competition ($K_1 = 2 \times 10^8\ M^{-1}$). It is striking to observe that this is the value expected from extrapolation of direct binding measurements made at high salt concentrations (Figure 4).

The DNA binding constant of the ethidium dimer, which has been determined from the competition binding experiments with monomeric intercalating compounds (Figure 5), can be further used to obtain values of the relative binding affinity of other intercalating dimers. We have measured the displacement of the ethidium dimer bound to DNA as a function of increasing concentration of two competing acridine dimers (AcDi II and AcDi III; Le Pecq et al., 1975). As shown in Figure 6, the competitive displacement of DNA bound ethidium dimer by the acridine dimers can be accurately described according to the Mc Ghee & Von Hippel equations (eq 7-9), assuming both competing acridine dimers occupy one type of binding site with $n = 4$. The validity of the values obtained for the binding constants of the two acridine dimers (AcDi II, $1.5 \times 10^8\ M^{-1}$; AcDi III, $6 \times 10^8\ M^{-1}$) will be discussed later.

Fluorescence titrations of the acridine ethidium dimer with DNA at different ionic strengths indicate a somewhat more complicated interaction between primary and secondary site binding. For example, at low salt concentration ($Na^+ \leq 0.02$ M) the dimer fluorescence increases stoichiometrically (i.e., linearly) up to $r_f = 0.5$ and decreases at larger values of $r_f$ (Figures 10 c,d). At salt concentrations between 0.2 and 0.5 M $Na^+$, the fluorescence increases linearly during the titration up to $r_f = 0.4$-$0.5$ and reaches a plateau at $r_f > 0.5$ as shown in Figure 7. At salt concentrations above 1.0 M $Na^+$, binding of the acridine ethidium dimer to DNA is no longer stoichiometric at low values of $r_f$, which allows a direct measurement

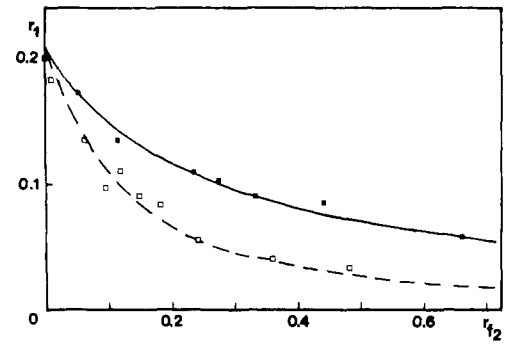


FIGURE 6: Displacement of the ethidium dimer, bound to calf thymus DNA, by competing bisintercalating ligand in 0.2 M sodium acetate buffer, pH 5.0. The concentration of bound ethidium dimer per DNA base pair ($r_1$) is deduced from fluorescence measurement ($\lambda_{exc}$ = 540 nm; $\lambda_{em}$ = 610 nm) and $r_{f_2}$ is the ratio of the total molar concentration of the competing ligand to the molar concentration of DNA base pair B ($B$ = $1.6 \times 10^{-6}$ M). Competition by acridine dimer AcDi II: the experimental values of $r_1$ (■) are fitted with eq 7-9 by the solid line ($n_1 = 4$, $K_1 = 2 \times 10^8$ $M^{-1}$; $n_2 = 4$, $K_2 = 1.5 \times 10^8$ $M^{-1}$). Competition by acridine dimer AcDi III: the experimental values of $r_1$ (□) are fitted with eq 7-9 by the dashed line ($n_1 = 4$, $K_1 = 2 \times 10^8$ $M^{-1}$; $n_2 = 4$, $K_2 = 6 \times 10^8$ $M^{-1}$).

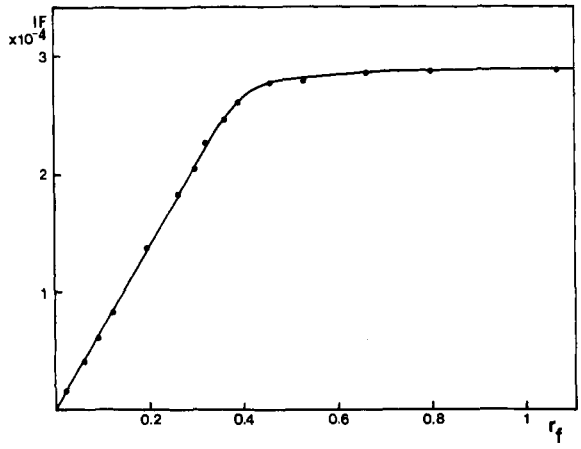


FIGURE 7: Fluorometric titration curve of calf thymus DNA by acridine ethidium dimer in 0.2 M sodium acetate buffer ($\lambda_{exc}$ = 540 nm; $\lambda_{em}$ = 590 nm). DNA base pairs concentration is $7.2 \times 10^{-7}$ M.

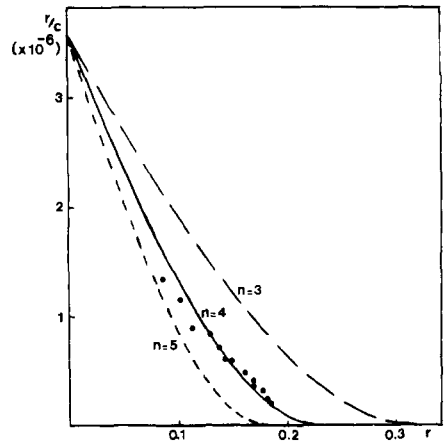


FIGURE 8: Scatchard plot of the binding of acridine ethidium dimer to calf thymus DNA in 1 M sodium acetate buffer deduced from fluorometric titration ($\lambda_{exc}$ = 550 nm; $\lambda_{em}$ = 610 nm) and fitted according to the Mc Ghee-Von Hippel treatment. The best fit is observed with the smooth curve where the number of covered base pairs $n$ is chosen equal to four with an intrinsic binding constant for acridine ethidium dimer of $K = 3.6 \times 10^6$ $M^{-1}$. The dashed curves are drawn with the same value of $K$ but with $n$ = 3 and $n$ = 5. DNA base pair concentration is $1.7 \times 10^{-6}$ M.

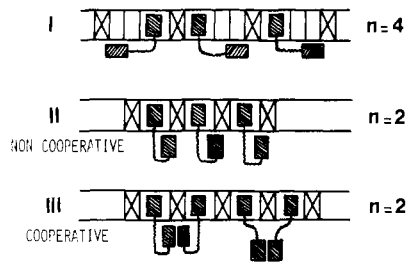


FIGURE 9: Schematic representation of the different possible types of binding of ethidium dimer and acridine ethidium dimer to DNA.

of the binding constant according to the Mc Ghee-Von Hippel treatment. Under these conditions ($Na^+ > 1$ M), the dimer covers 4 base pairs when bound to DNA (Figure 8). The binding constants of the acridine ethidium dimer at different salt concentrations (above 1 M $Na^+$) are reported in Figure 4.

The acridine ethidium dimer fluorescence titration curves with DNA at various salt concentrations suggest that the acridine ethidium dimer can bind to DNA in at least three different modes: (1) a primary binding mode corresponding to $n = 4$, which is the only binding site occupied at high salt concentrations (Figure 9, I); (2) a secondary binding mode corresponding to $n = 2$, which has similar fluorescence characteristics and which is populated at $r_f$ values larger than 0.25 by displacing the primary binding sites (Figure 9, II and III); and (3) a third type of binding mode possibly corresponding to $n = 1$, which can displace both the primary and secondary sites at low ionic strength and at $r_f$ values larger than 0.5. It is surprising to observe that, in 0.5 M $Na^+$, the acridine ethidium dimer binds to DNA almost stoichiometrically up to $r_f$ = 0.5 indicating that both primary ($n = 4$) and secondary ($n$ = 2) binding sites are populated, while in 1 M $Na^+$ only the primary sites ($n = 4$) are occupied (Figure 8). Such a sharp transition between two modes of binding suggests that the secondary sites binding could be cooperative. Mc Ghee & Von Hippel (1974) have shown that, in such cases, a small variation in the binding constant can lead to dissociation of the complex. Furthermore, the fluorescence titration curve of the acridine ethidium dimer in 0.5 M acetate is stoichiometric (linear) up to $r_f = 0.5$ and it can not be fitted to a theoretical binding curve unless the secondary site is assumed cooperative (as schematically illustrated in Figure 9, III).

II. *Fluorescence Properties of Ethidium Dimer and Acridine Ethidium Dimer.* As shown in Table II, the fluorescence quantum yield for emission from the phenanthridinium chromophore ($\lambda_{exc}$ = 540 nm) is greatly enhanced when either the ethidium dimer or the acridine ethidium dimer is bound to their primary sites on DNA (at low values of $r$). The fluorescence emission spectra of the bound dimers are similar to emission spectra of the corresponding N-5 aminoalkyl phenanthridinium derivative PhMo also in the presence of DNA ($\lambda_{exc}$ = 540 nm; $\lambda_{em}^{max}$ = 615 nm). However, these spectra appear to be slightly red shifted in comparison with the emission spectra of bound ethidium bromide ($\lambda_{em}^{max}$ = 605 nm), possibly due to the influence of the positively charged aminoalkyl chain on the emission characteristics of the dimers and the monomeric PhMo derivative.

The fluorescence lifetimes of the ethidium dimer, the acridine ethidium dimer, the phenanthridinium derivative PhMo, and ethidium bromide were measured in the presence of excess DNA and were compared with their respective quantum yield ratios as shown in Table II. In all cases, single exponential fluorescence decays were observed and the ratios of the fluo-

TABLE II: Ratios of Quantum Yield of EtDi to Ethidium Bromide (EB) and of AcEtDi to Monomer PhMo when Bound to DNA as Compared with the Corresponding Ratio of Fluorescence Lifetime, and Ratio between Quantum Yield of EtDi Bound to DNA and Quantum Yield of Free EtDi as Compared with the Same Ratio for AcEtDi.[a]

| | |
|---|---|
| $\frac{\phi_{EtDi(DNA)}}{\phi_{EtDi(free)}} = 40 \pm 4$ | $\frac{\phi_{AcEtDi(DNA)}}{\phi_{AcEtDi(free)}} = 30 \pm 3$ |
| $\frac{\phi_{EtDi(DNA)}}{\phi_{EB(DNA)}} = 0.56 \pm 0.05$ | $\frac{\phi_{AcEtDi(DNA)}}{\phi_{PhMo(DNA)}} = 0.96 \pm 0.05$ |
| $\frac{\tau_{EtDi(DNA)}}{\tau_{EB(DNA)}} = \frac{15 \pm 0.5 \text{ ns}}{24 \pm 0.5 \text{ ns}} = 0.62$ | $\frac{\tau_{AcEtDi(DNA)}}{\tau_{PhMo(DNA)}} = \frac{17.5 \pm 0.5 \text{ ns}}{19 \pm 0.5 \text{ ns}} = 0.92$ |

[a] The ratios of quantum yields were measured as before (Le Pecq & Paoletti, 1967) with a DNA base pair concentration of $2 \times 10^{-5}$ M and a dye concentration of $5 \times 10^{-7}$ M in 0.2 M sodium acetate buffer, pH 5.0. Fluorescence lifetimes were measured with a DNA base pair concentration of $5 \times 10^{-4}$ M and a dye concentration of $1 \times 10^{-5}$ M in the same buffer. Fluorescence excitation was made at $\lambda_{exc} = 520$ nm.

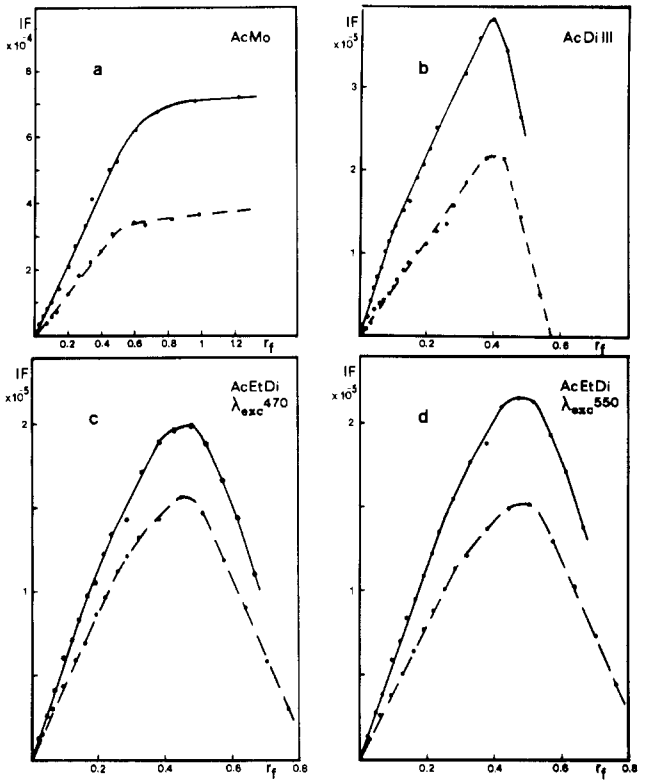


FIGURE 10: Compared fluorometric titration curves of poly(dA-dT) in solid lines and poly(dA-BrdU) in dashed lines in 0.02 M sodium acetate buffer. (a) By acridine monomer AcMo ($\lambda_{exc} = 440$ nm; $\lambda_{em} = 550$ nm); (b) by acridine dimer AcDi III ($\lambda_{exc} = 420$ nm; $\lambda_{em} = 500$ nm); (c) by acridine ethidium dimer AcEtDi ($\lambda_{exc} = 470$ nm; $\lambda_{em} = 600$ nm); (d) by acridine ethidium dimer AcEtDi ($\lambda_{exc} = 550$ nm; $\lambda_{em} = 600$ nm). The concentration of poly(dA-dT) and poly(dA-BrdU) in base pairs is $1.7 \times 10^{-6}$ M. $r_f$ is the ratio of the total molar concentration of dye to the total molar concentration of polynucleotide base pair.

rescence lifetimes are in agreement with the corresponding quantum yield ratios (Table II). These results show that both dimers can be characterized by a single highly fluorescent phenanthridinium species when bound to their primary sites on DNA. It is generally accepted that only DNA intercalated ethidium bromide is highly fluorescent (Le Pecq, 1971, review), which would suggest that the phenanthridinium chromophore of the ethidium and acridine ethidium dimers bind to DNA by intercalation. On the other hand, it is expected that the fluorescence properties of the two chromophores on the ethidium homodimer should be quite different in the presence of DNA since our data clearly indicate that this dimer binds to DNA by intercalation of only one of its phenanthridinium rings (Table I). Instead, we observe that the intercalated and nonintercalated phenanthridinium moieties on the same ethidium dimer appear spectroscopically identical, when the dimer is bound to its primary sites on DNA. These observations suggest that for the ethidium dimer and possibly for the acridine ethidium dimer, the fluorescence emission of the phenanthridinium moiety should not be taken as the only criteria in establishing that the phenanthridinium ring is, in fact, intercalated.

Galley & Purkey (1972) have shown that the bromine atom in poly(dA-BrdU) can serve as a probe for locating the position of fluorescent chromophores on DNA. When a chromophore is located close to the bromine atom its fluorescence is quenched. Fluorescence titrations at low ionic strength of the acridine ethidium dimer and two acridine derivatives with poly(dA-dT) and poly(dA-BrdU) have been compared, in order to explore the possibly different DNA binding modes of these molecules (Figure 10). The results in Figure 10 show the following.

(a) When the acridine monomer AcMo is intercalated in poly(dA-BrdU), its fluorescence is quenched and is only 55% of the fluorescence of the dye bound to poly(dA-dT). As expected the acridine ring is therefore close to the bromine atom in the intercalated state.

(b) In the case of the acridine dimer AcDi III (Barbet et al., 1975), the fluorescence of the dimer bound to poly(dA-BrdU) is also quenched in the region of bisintercalation ($0 < r < 0.2$) and is only 40% of the fluorescence of the dye bound to poly(dA-dT). In the region of monointercalation ($0.25 < r < 0.4$) the quenching appears relatively smaller and the fluorescence of the dye is about 60% of the fluorescence of the dye bound to poly(dA-dT). In a third part of the titration, $r > 0.45$, a very sharp decrease of fluorescence is observed in both cases indicating a third type of binding.

(c) In the case of the acridine ethidium dimer, the phenanthridinium fluorescence has been measured either by excitation in the acridine band (470 nm) or by excitation in the phenanthridinium band (550 nm). The result shows that the fluorescence quenching of the dimer when bound to poly(dA-BrdU) when compared with poly(dA-dT) is about the same, regardless of whether excitation is made directly in the phenanthridinium band ($\lambda_{exc} = 550$ nm) or through the acridine band ($\lambda_{exc} = 470$ nm).

Energy transfer from the acridine to the phenanthridinium chromophores of the acridine ethidium dimer is expected to be very efficient because of the large overlap of the emission fluorescence spectrum of acridine and absorption spectrum of phenanthridinium. The efficiency of energy transfer $E$ from the acridine moiety to the phenanthridinium ring is given by the usual equation (Schiller, 1975):

$$E = \frac{A_A}{A_D}\left(\frac{I_s}{I} - 1\right) \quad (10)$$

where $A_A$ and $A_D$ are respectively the absorption of the acceptor (phenanthridinium) and the donor (acridine) at the wavelength $\lambda$ of excitation. $I_s$ and $I$ are the fluorescence intensities of the acceptor in the presence ($I_s$: sensitized fluorescence) or in the absence ($I$) of transfer from the donor, with excitation at the same wavelength. The ratio $I_s/I$ can be obtained from eq 10:

$$\frac{I_s}{I} = E\frac{A_D}{A_A} + 1 \quad (11)$$

If $I_s/I$ is measured on poly(dA-dT) and on poly(dA-BrdU), we get:

$$\frac{I_{s2}}{I_{s_1}} = \frac{I_2}{I_1}\frac{E_2\left(\frac{A_D}{A_A}\right) + 1}{E_1\left(\frac{A_D}{A_A}\right) + 1} \quad (12)$$

where subscripts 1 and 2 refer to the measured phenanthridinium fluorescence of the dimer when bound at the same $r$ value to poly(dA-dT) and to poly(dA-BrdU), respectively. The ratio $I_2/I_1$ is independent of excitation wavelength and can be obtained directly by measuring the phenanthridinium fluorescence at $\lambda_{exc}$ = 550 nm, where there is no acridine absorption ($A_D = 0$). According to eq 12, the sensitized fluorescence intensity ratio, $I_{s_2}/I_{s_1}$ (which is obtained by excitation into the donor acridine absorption band at 470 nm), can only be equal to $I_2/I_1$ if the efficiency of energy transfer from the acridine moiety to the phenanthridinium ring is the same when the dimer is bound to poly(dA-dT) and poly(dA-BrdU) (i.e., $E_1 = E_2$). The results in Figure 10 (c,d) show that $E_1 = E_2$ for the acridine ethidium dimer when bound to poly(dA-dT) and poly(dA-BrdU), therefore demonstrating that the acridine fluorescence of this dimer is not quenched on poly(dA-BrdU). Since quenching of the acridine moiety would be observed if the acridine ring is intercalated (Figures 10a,b), we conclude that the phenanthridinium ring of the acridine ethidium dimer is intercalated and subsequently quenched due to its proximity to the bromine atom at the intercalation site on poly(dA-BrdU). Furthermore, the acridine ring of the dimer must be located in the minor groove, since exterior binding in the major groove would necessarily lead to contact with the bromine atom and quenching of the acridine fluorescence.

The excitation spectra of the acridine ethidium dimer when bound to DNAs of different AT content are shown in Figure 11, as monitored by the phenanthridinium fluorescence at 610 nm. Previous studies have shown that the acridine chromophore is fluorescent only when bound adjacent to two AT base pairs, which renders its fluorescence dependent on the nucleic acid AT content (Weisblum & de Hasseth, 1972). For a given DNA, with excitation of the bound acridine ethidium dimer in the acridine absorption band at 470 nm, the relation between the fraction of AT base pairs, $f_{AT}$, and $I_s/I$ is given by:

$$\left(\frac{I_s}{I_{470}}\right) = \left[E_1\left(\frac{A_D}{A_{A470}}\right) + 1\right]f_{AT}^2 + \left[E_2\left(\frac{A_D}{A_{A470}}\right) + 1\right]\left[1 - f_{AT}^2\right] \quad (13)$$

where $E_1$ and $E_2$ refer to the transfer efficiency of the acridine chromophore (to the phenanthridinium chromophore), when the acridine ring of the dimer is bound adjacent to two AT base pairs on the DNA ($E_1$) or adjacent to two other base pairs on the DNA ($E_2$). If $I_s/I$ is measured with the dimer bound to

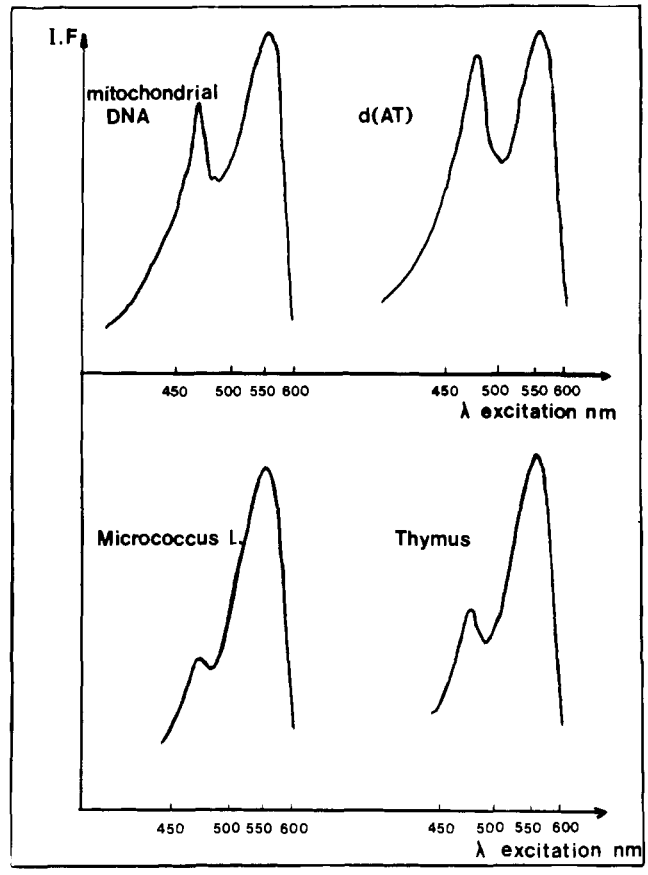


FIGURE 11: Excitation spectra of acridine ethidium dimer for the phenanthridinium fluorescence ($\lambda_{em}$ = 610 nm) in DNA of various AT content. Measurements are made in sodium acetate buffer 0.2 M, pH 5.0. AcEtDi concentration is $5 \times 10^{-7}$ M and DNA base pair concentration is $2 \times 10^{-5}$ M.

poly(dG-dC):

$$\left(\frac{I_2}{I_{470dG\text{-}dC}}\right) = E_2\left(\frac{A_D}{A_{A470}}\right) + 1 \quad (14)$$

and if we assume $E_2$ is small, eq 13 becomes:

$$\left(\frac{I_s}{I_{470}}\right) - \left(\frac{I_s}{I_{470dG\text{-}dC}}\right) = E_1\frac{A_D}{A_A}f_{AT}^2 \quad (15)$$

$I_{470}$ can be computed from the emission intensity measured when excitation is done at $\lambda$ = 550 nm ($I_{550}$) where the acridine does not absorb since:

$$\frac{I_{470}}{I_{550}} = \frac{A_{A470}}{A_{A550}} \quad (16)$$

Therefore:

$$\frac{I_{s470}}{I_{550}} - \frac{I_{s470}}{I_{550}}(dG\text{-}dC) = E_1\left(\frac{A_D}{A_A}\right)f_{AT}^2\frac{A_{A470}}{A_{A550}} \quad (17)$$

Figure 12 clearly shows that a linear relation is effectively obtained for $(I_{s470}/I_{550}) - (I_{s470}/I_{550})$ (dG-dC) as a function of $f_{AT}^2$.

## Discussion

Ethidium bromide has been used as a fluorescent probe of nucleic acids because of its large increase of fluorescence on binding to double stranded DNA or RNA. The ethidium dimer and the acridine ethidium dimer were synthetized in order to take advantage of the fluorescence properties of ethidium. The binding of dimeric compounds to DNA could therefore be

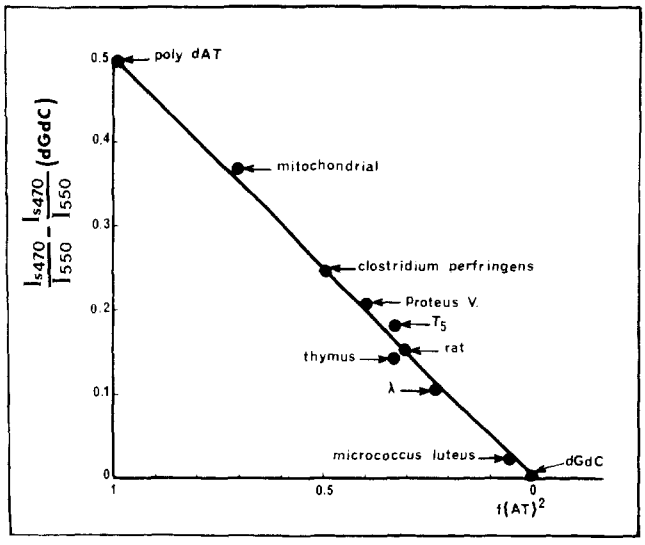


FIGURE 12: Effect of the AT content of DNA on the energy transfer from acridine to phenanthridinium chromophore in DNA-bound acridine ethidium dimer (see explanations in the text). Experimental conditions are the same as in Figure 11.

more easily studied by spectrofluorimetry.

In our previous studies on acridine dimers we observed that, if the chain linking the two rings is shorter than a critical length (10.1 Å), bisintercalation does not occur (Le Pecq et al., 1975). Such a critical length is indeed expected according to the excluded site model which postulates that two base pairs must separate two intercalated rings (Crothers, 1968; Bauer & Vinograd, 1970). Intercalating dimers are therefore expected to cover an average of four base pairs. Wakelin et al. (1976) have studied the binding of different diacridines and have questioned the excluded site model because they observed bisintercalation for dimers with chain lengths smaller than 10 Å. In this study we observed that the acridine ethidium dimer binds to DNA intercalating only one of its rings, although its chain length is long enough to allow bisintercalation according to the excluded site model. In the case of the ethidium dimer, the measurement of unwinding angle suggests monointercalation. The viscometric measurement of DNA lengthening could be interpreted two different ways: the viscometric increase, which is larger than that observed for the monomer, could result from either monointercalation and stiffening of the chain, or from a combination of mono- and bisintercalation. The DNA lengthening measured directly by electron microscopy (Butour et al., 1978), at low concentration of ethidium dimer, clearly shows that monointercalation occurs, but in presence of an excess of ethidium dimer some DNA molecules are extended from 50% up to 80%. Such a behavior is indicative that bisintercalation to adjacent base pairs can occur as suggested by Wakelin et al. (1976). The excluded site restriction, which applies well for an intercalating monomer of relative low DNA affinity, probably represents a thermodynamic limitation which might be bypassed with a dimer of high DNA affinity. The factors which govern mono- or bisintercalation appear more complex than previously thought. Different types of binding can coexist. We have shown that their relative importance is very dependent on environment and on the degree of saturation, especially when cooperative binding phenomenon can take place. The multiplicity of the binding processes which are observed for these dimers probably results from the relative flexibility of their chains. The synthesis of dimers with chains of specific and rigid conformation would be a way to orient these dimers to a more specific type of binding process.

The study of the interaction of the dimers with DNA is generally rendered very difficult because of their high binding affinity and the multiplicity of the binding modes. The binding constant to DNA of these dimers can be more easily measured at high ionic strength using the Mc Ghee-Von Hippel treatment, because only one type of site is then populated and because the binding affinity to this site is then considerably lowered. Binding constants at lower ionic strength can be estimated by extrapolation of the values obtained at high ionic strength. Duane (1972) and Record et al. (1976) have shown that a straight line is obtained when the logarithm of the binding constant is plotted vs. the logarithm of the molar concentration of salt. According to Record et al. (1976) the slope of this line is $m'\psi$, with $m'$ being the number of ion pairs formed in the interaction and $\psi$ a parameter equal to 0.88 for double-stranded DNA. In such a representation (Figure 4), we found $m'\psi = 0.85$ in the case of ethidium bromide which contains only one charge. The $\psi$ value obtained is therefore in very close agreement with the $\psi$ value of Record et al. (1976). In the case of ethidium dimer the slope, which is not known with the same accuracy, is approximately 3 and the apparent number of ion pairs found in the DNA interaction is therefore 3.4, close to the maximum value of 4 corresponding to the number of charges on the dimer. The variation of log $K$ as a function of log ($Na^+$) measured at high ionic strength can therefore serve to estimate by extrapolation the binding affinity at lower ionic strength.

The competition between a dimer and a monomer with a well-known binding constant can, in principle, serve to measure the binding constant of the dimer in lower salt concentration. We limited our study to the case of ethidium dimer because the interference from secondary sites can be neglected. The monomers which are used in such experiments must have absorption spectra such that they do not interfere with the fluorescence measurements of the ethidium dimer. The determination is made easier when the binding affinity of the monomer is large; otherwise the concentration of monomer needed for effective competition becomes so high that other factors could interfere. The constant of self-stacking of monomers varies from 100 $M^{-1}$ for ethidium bromide (Thomas & Roques, 1972) to 1000 $M^{-1}$ for ellipticine derivatives (Delbarre et al., 1976) and can reach $10^4$ $M^{-1}$ for acridine orange (Lamm & Neville, 1965). These values would limit the concentrations of monomer which can be used in competitive binding experiments. As anticipated, better results are obtained in competition experiments between the ethidium dimer and an ellipticine derivative (which has a larger DNA binding affinity) as compared with the results obtained from experiments with an acridine monomer, since at high concentrations the acridine monomer can directly interact with the dimer.

Once the binding affinity of a dimer is known, competition experiments between different dimers can be used to determine their binding constant. Such a method could become the simplest way for the determination of binding constants of dimers or other similar ligands. Nevertheless, as shown in this work, the competition can be quantitatively interpreted only in simple cases where there is only one type of binding site for both the displacing and the displaced ligand. It is also necessary to accurately know the number of base pairs covered by the dyes. A competing ligand would displace a bound ligand more easily if it covers a smaller number of base pairs. The displacement of the bound ethidium dimer by two different acridine dimers can be accurately fitted using the Mc Ghee and Von Hippel treatment (Figure 6), although the results must be considered with caution because interference from secondary binding sites of the acridine dimers cannot be estimated. Nevertheless the

results obtained in such determinations, if considered only as an order of magnitude, can be useful for the study of structure-activity relationships in the design of new biologically active substances. On the other hand, the dimers themselves may have biologically important properties related to their ability to displace a natural ligand on DNA. If this were the case, this ability can be directly measured by similar competition experiments using the binding affinities and the number of base pairs bound by the dimers as important parameters.

Both the ethidium and the acridine ethidium dimers are potentially of interest as probes to nucleic acid structure because, like ethidium bromide, the fluorescence quantum yield of these dimers increases by a large factor when they bind to DNA. Furthermore, they have binding affinities much larger than ethidium bromide, allowing them to be used as probes at very low DNA concentrations. Experiments in progress in our laboratory show that the ethidium dimer can be used to measure DNA concentrations spectrofluorometrically and with much better sensitivity than can be obtained using ethidium bromide. The acridine ethidium dimer could be a useful probe for the study of chromosomes. With conventional acridine dyes, the fluorescent banding pattern observed on chromosomes is difficult to interpret. The fluorescence intensity which is measured in a particular band depends both on the quantum yield of the acridine dye (governed by the AT content of the DNA) and on the quantity of bound dye (Moutchen, 1976). With the acridine ethidium dimer both factors can be separated. For example, the fluorescence of the bound dimer excited in the phenanthridinium absorption band is independent of the AT content of DNA and can serve to measure the total quantity of bound dimer, while the intensity of fluorescence excited in the acridine absorption band will measure the AT content of the DNA.

Acknowledgments

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## Appendix: Numerical Solution of Mc Ghee and Von Hippel Equations for Competing Ligands

We consider $I$ chemically different ligands $L_1, L_2 \ldots, L_i \ldots, L_I$. The total concentration and the free concentration of ligand $L_i$ are respectively $T_i$ and $c_i$. The ligand $L_i$ may bind to the DNA in $J_i$ different modes of intrinsic affinity constant $K_{ij}$ and covering $n_{ij}$ base pairs. All the sites of the DNA molecule are assumed equivalent: $K_{ij}$ does not depend on the base sequence. The number of ligands bound per base pair of the chemical species $L_i$ in the $j$th mode is $r_{ij}$. We want to compute the values of $r_{ij}$ when the DNA concentration in base pairs B and the total concentrations of ligands $L_2 \ldots L_I$ are fixed but when the total concentration of ligand $L_1$ is varied.

Mc Ghee & Von Hippel (1974) showed that:

$$r_{ij} \Big/ \left(1 - \sum_{i=1}^{I} \sum_{j=1}^{J_i} n_{ij} r_{ij}\right) = K_{ij} c_i X^{1-nij} \qquad \text{(A1)}$$

where $X$ is the largest root of the secular equation (Lifson, 1964) or the largest eigenvalue of the matrix (Crothers, 1968) from which the grand partition function of the system may be derived. Here

$$X = 1 + \sum_{i=1}^{I} \sum_{j=1}^{J_i} \left(r_{ij} \Big/ \left(1 - \sum_{i=1}^{I} \sum_{j=1}^{J_i} n_{ij} r_{ij}\right)\right) \qquad \text{(A2)}$$

We set $u_1 = K_{i1} c_i$

$$\sigma = 1 \Big/ \left(1 - \sum_{i=1}^{I} \sum_{j=1}^{J_i} n_{ij} r_{ij}\right)$$

Using eq A1 and the above definitions it may be easily derived

$$\sigma - 1 = \sum_i u_i \left(\sum_{j=1}^{J_1} n_{ij} (K_{ij}/K_{i1}) X^{1-nij}\right) \qquad \text{(A3)}$$

$$X - 1 = \sum_i u_i \left(\sum_{j=1}^{J_1} (K_{ij} K_{i1}) X^{1-nij}\right) \qquad \text{(A4)}$$

$$r_{ij} = u_i (K_{ij}/K_{i1}) X^{1-nij}/\sigma \qquad \text{(A5)}$$

Now the total concentration of the ligand $L_i$ satisfies

$$T_i = c_i + B \left(\sum_{j=1}^{J_i} r_{ij}\right) \qquad \text{(A6)}$$

Multiplying eq A6 by $K_{i1}$ and replacing $r_{ij}$ by its value (A5) we get

$$u_i = T_i \left(K_{i1}\, \sigma \Big/ \left(\sigma + B \sum_{j=1}^{J_i} K_{ij} X^{1-nij}\right)\right) \qquad \text{(A7)}$$

and eq A3 and A4 become:

$$\sigma - 1 = \sigma \sum_{i=1}^{I} T_i \left(\left(\sum_{j=1}^{J_i} n_{ij} K_{ij} X^{1-nij}\right) \Big/ \left(\sigma + B \sum_{j=1}^{J_i} K_{ij} X^{1-nij}\right)\right) \qquad \text{(A8)}$$

$$X - 1 = \sigma \sum_{i=1}^{I} T_i \left(\left(\sum_{j=1}^{J_i} K_{ij} X^{1-nij}\right) \Big/ \left(\sigma + B \sum_{j=1}^{J_i} K_{ij} X^{1-nij}\right)\right) \qquad \text{(A9)}$$

Eliminating $T_1$ (the varying total concentration of ligand $L_1$) between eq A8 and A9 we set:

$$F(\sigma) = (\sigma - 1)\left(\sum_{j=1}^{J_1} K_{1j} X^{1-n}1j\right) - (X - 1)\left(\sum_{j=1}^{J_1} n_{1j} K_{1j} X^{1-n}1j\right) - \sigma \sum_{i=2}^{I} T_i \left[\sum_{j=1}^{J_i} \sum_{j'=1}^{J_1} K_{ij} K_{1j'} (n_{ij} - n_{1j'}) X^{2-nij-n}1j'\right] \Big/ \left[\sigma + B \sum_{j=1}^{J_i} K_{ij} X^{1-nij}\right] \qquad \text{(A10)}$$

Therefore $\sigma$ is a root of $F(\sigma)$.

For physical states, $\sigma$ and $X$ are both larger than 1 and $\sigma$ is larger than $X$. Since $F(0)$ is negative and $F(+\infty)$ is positive, $F(\sigma)$ has necessarily a positive root for a given value of $X$ larger than 1. Now, $F(\sigma)$ has $I - 1$ negative roots respectively less than $-B\, \Sigma_{j=1}{}^{J_i} K_{ij} X^{1-nij}$. As the numerator of $F(\sigma)$ is a polynomial of degree $I$ its positive root is unique. The algorithm is easily explained.

(a) An initial value larger than 1 is chosen for $X$.

(b) The coefficients of $\sigma$ in $F(\sigma)$ are computed (eq A10). The unique positive root of a polynominal of degree $I$ must be found. If $I$ is less or equal to 2, this is not difficult. For larger values of $I$, Newton's algorithm is used setting $\sigma = X$ as the initial value.

(c) The value of $T_1$ is computed through (eq A9). If $T_1$ is negative we jump to point d. Otherwise the values of $u_i$ are computed through eq A7 and those of $r_{ij}$ from eq A5.

(d) The value of $(X - 1)$ is multiplied by a number larger than 1. For this new larger value of $X$, the algorithm is restarted from point b. For a sufficiently large value of $X$, $T_1$ increases and in all cases eventually gets positive.

This algorithm has been run on a small size computer (a 9810 A Hewlett Packard calculator).

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# Magnetic Circular Dichroism of Netropsin and Natural Circular Dichroism of the Netropsin–DNA Complex†

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ABSTRACT: We report the first measurement of the magnetic circular dichroism (MCD) of the basic polypeptide antibiotic netropsin (Nt). The MCD shows that the longest wavelength absorption band of Nt is the sum of more than one component and permits a radically new interpretation of the circular dichroism of the complex which Nt forms with DNA. We conclude that Nt has no major effect on the CD and thus the helical structure of the bases of the DNA to which it is bound. Thus the ability of Nt to inhibit the function of DNA polymerase, RNA polymerase, and the photoreactivating enzyme must be mediated by factors other than a distortion of the helical structure of the bases.

The basic oligopeptide antibiotic netropsin binds to regions of double-stranded DNA which are rich in adenine–thymine base pairs and inhibits both DNA polymerase and RNA polymerase activity (Zimmer, 1975, and references cited therein) and the photoreactivating enzyme (Sutherland, 1978). Netropsin and its complexes with various DNAs and copolymers have been studied extensively by a variety of spectroscopic and hydrodynamic methods (Zimmer et al., 1970, 1971a,b, 1972; Reinert, 1972; Wartell et al., 1974; Luck et al., 1974; Zasedatelev et al., 1974; Zimmer, 1975; and references cited therein).

In this paper we report the first measurement of the mag-

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