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Structural Limitations on the Bifunctional Intercalation of Diacridines into DNA†

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ABSTRACT: An homologous series of diacridines containing two 9-aminoacridine chromophores linked via a simple methylene chain has been studied in order to investigate the minimum interchromophore separation required to permit bifunctional intercalation. Viscometric, sedimentation, and electric dichroism experiments show that compounds having one to four methylene groups in the linker are restricted to monofunctional intercalation, whereas the interaction becomes bifunctional when the chain length is increased to six carbons or more. The results indicate that bifunctional reaction occurs with an interchromophore distance not exceeding 8.8 Å, implying that intercalation by these compounds is not subject to neighbor exclusion if the mode of binding is of the classical intercalation type.

The present study stems from a convergence of several recent advances in our understanding of drug–nucleic acid interaction: the characterization of quinoxaline antibiotics as bifunctional DNA-intercalating agents (Waring & Wakelin, 1974; Wakelin & Waring, 1976), the synthesis of diacridines as a possible route to develop compounds having enhanced binding affinity as well as possible additional specificity (Canellakis et al., 1976a–c), and the use of electric dichroism as a probe for geometrical relationships between ligands bound to DNA and the DNA base pairs (Houssier & Fredericq, 1966; Houssier et al., 1974). The possibility of using diacridines having potentially intercalative chromophores linked by a simple, flexible methylene chain of varying length to investigate the minimum separation between chromophores needed to permit bifunctional interaction has already been suggested (Canellakis et al., 1976a). Here we report an investigation into the interaction between such a series of diacridines and DNA, employing (1) sedimentation and viscometric measurements on closed circular duplex DNA from bacteriophage PM2 to study relative helix-unwinding angles, (2) viscometric observations on sonicated rodlike fragments of DNA to determine helix extension parameters, and (3) electric dichroism measurements designed to yield information concerning the relative orientations of the acridine chromophores with respect to the DNA base pairs. The compounds studied constitute an homologous series in which two 9-aminoacridine rings are joined via their amino groups with a simple methylene chain. For purposes of comparison, some experiments were also performed using the simple intercalating agents 9-aminoacridine and 9-methylaminoacridine.

Materials and Methods

Structural formulas of the acridine derivatives, together with their respective maximum 9-amino nitrogen/9′-amino nitrogen distances and their visible and ultraviolet extinction coefficients, are given in Table I. Also included are the hitherto

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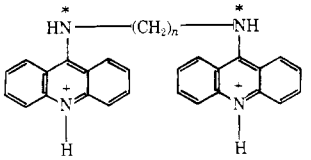
TABLE I: Structure and Properties of Diacridines.[a]



carbon no.	extinction coeff	fully extended N*-N* distance (Å)
2	ϵ_{410} 1.40 × 10^4; ϵ_{258} 8.40 × 10^4	3.9
3	ϵ_{407} 1.60 × 10^4; ϵ_{257} 8.85 × 10^4	5.0
4	ϵ_{409} 1.40 × 10^4; ϵ_{256} 9.00 × 10^4	6.3
5	ϵ_{410} 1.83 × 10^4; ϵ_{256} 1.31 × 10^5	7.5
6	ϵ_{411} 1.96 × 10^4; ϵ_{256} 1.16 × 10^5	8.8
7	ϵ_{410} 1.86 × 10^4; ϵ_{257} 8.96 × 10^4	10.0
8	ϵ_{411} 1.90 × 10^4; ϵ_{257} 7.39 × 10^4	11.3
10	ϵ_{413} 1.60 × 10^4	13.8
12	ϵ_{413} 1.74 × 10^4; ϵ_{262} 7.60 × 10^4	16.3

[a] All visible molar extinction coefficients were determined in buffer of approximate ionic strength 0.02 (pH 7.0) at diacridine concentrations of 5–10 μM with the exception of that for C-10 (DECDAC) in which the buffer also contained 10 μM dimethyl sulfoxide. The ultraviolet extinction coefficients were measured in 0.01 SHE buffer in the same concentration range as above using the visible absorption peak to monitor the exact diacridine concentration. Optical titration measurements show the diacridines to be fully protonated under these conditions such that they carry two positive charges; for example, the pK_a of C-5 is determined to be 9.8. The maximum interchromophore distance (N*-N* distance) is calculated for a fully extended methylene chain in the staggered conformation assuming the carbon–nitrogen and carbon–carbon bond lengths to be both equal to 1.53 Å with all bond angles tetrahedral. The 9-amino nitrogens are constrained to lie in the plane of the acridine rings and are assumed to be sp^3 hybridized. The error introduced by overestimating the carbon–nitrogen bond length is minimal, and the calculations are consistent with measurements made with CPK molecular models. The melting points for derivatives C-5 and C-7 are 190–191.5 and 171–178 °C, respectively (Chen et al., 1978). The visible molar extinction coefficients for 9-aminoacridine and 9-methylaminoacridine in 0.01 SHE buffer determined at concentrations of 5–10 μM are ϵ_{398} 1.01 × 10^4 and ϵ_{404} 1.10 × 10^4, respectively.

unpublished melting points for the C-5 and -7 derivatives. In the interests of clarity, all compounds are referred to by the pseudonym C*n*, where *n* is the number of carbon atoms in the connecting methylene chain. Syntheses and purification were as described by Canellakis et al. (1976a) and Chen et al. (1978). Stock solutions were prepared by dissolving samples in 0.1 M lactic acid at a concentration of 1–2 mg/mL and then titrating to pH 4.0–7.0 with NaOH. Solutions were kept in the dark and stored at −20 °C. Except for the electric dichroism experiments, the buffer used throughout (designated 0.01 SHE) contained 2 mM Hepes,[1] 10 μM EDTA, and 9.4 mM NaCl dissolved in reagent-grade water from a Millipore Milli-Q2 system. It was adjusted with NaOH to pH 7.0 at 20 °C; the resultant ionic strength was 0.01 mol/L. For most experiments, drug solutions were prepared by dilution of the stock with 0.01 SHE, and concentrations were determined by optical absorption measurements; 9-aminoacridine and 9-methylaminoacridine were dissolved directly in 0.01 SHE, the concentration being determined by direct weighing. The pH of the diluted diacridine solutions was measured to be 6.9–7.0, indistinguishable from that of the 0.01 SHE buffer. Solutions were manipulated throughout using siliconized glassware in order to minimize adsorption of drug to glass surfaces.

Bacteriophage PM2 DNA, consisting of over 95% closed circular duplex molecules, was prepared by the method of Espejo et al. (1969) using strains of virus and host bacteria kindly provided by Dr. R. T. Espejo. Samples containing approximately 30% nicked circular molecules (for analytical ultracentrifugation experiments) were generated by repeated freezing and thawing. Calf thymus DNA was purchased from Worthington Biochemical Corp. and also from Sigma Chemical Co. as the highly polymerized sodium salt. DNA concentrations were based on an assumed value for $\epsilon(p)_{260}$ of 6600, where the molar extinction coefficient is expressed with respect to nucleotides.

Analytical Ultracentrifugation. Sedimentation coefficients were measured by boundary sedimentation at 20 °C and 34 000 rpm in a Beckman Model E ultracentrifuge equipped with ultraviolet optics. Details of the procedure and computation of s_{20} values were as previously described (Waring, 1970). Drug–DNA complexes were prepared by method 2 of Waring (1970) in which successive increments of a drug solution in buffer (110 μM) are added directly to an ultracentrifuge cell containing 0.600 mL of PM2 DNA in buffer having an absorbance at 260 nm of 0.600 (i.e., 91 μM in nucleotides). In all cases, the drug and DNA were in contact for at least 30 min before commencement of sedimentation, which was assumed to be adequate for attainment of equilibrium. Occasionally, experiments were repeated several times in order to verify that no effects attributable to slow kinetics were discernible. As an additional check, the spectral change on binding of C-6 to calf thymus DNA was scrutinized (data not shown); it appeared to be practically instantaneous. Sedimentation coefficients are presented as directly determined; they were not corrected for viscosity, buoyancy, or DNA concentration.

Viscometry. Measurements using closed circular PM2 DNA were performed essentially according to the method of Revet et al. (1971) as described by Waring and Henley (1975) using a simple viscometer having a 10-cm capillary of 0.4 mm bore and a bulb of volume 0.7 mL, thermostated at 20 ± 0.01 °C. The flow time for water was 104.0 s. For routine experiments the viscometer contained 1.0 mL of a 303 μM solution of PM2 DNA (OD_{260} = 2.00; flow time 111.7 s). Drugs were added in increments of 1–20 μL from a Burkard precision micrometer syringe of 1-mL volume via a fine plastic tube inserted down the ascending limb of the viscometer; after each addition, complete mixing was effected by bubbling a gentle current of air down the descending limb. Preliminary measurements revealed that the diacridines adsorbed quite strongly to both the glass syringe and the plastic tubing. To counter this problem, they were soaked in drug solution overnight to reach equilibrium saturation, flushed out, and refilled with fresh solution immediately before use. Careful measurements showed that this procedure eliminated further adsorption of the drug from solution. However, the actual concentration of the drug solution being delivered through the plastic tube was always measured before and after each experiment by ultraviolet and visible absorption measurements in order to verify that there were no losses. Solutions were freed of particulate material by centrifuging for 15 min at 4000 rpm before use. Flow times were measured in triplicate to an accuracy of 0.1 s; the average deviation of a set of measurements was 0.1–0.2 s, i.e., approximately 0.1%. No kinetic effects attributable to the rate of complex formation were detected: there was never any consistent trend toward longer or shorter flow times during a

[1] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

set of replicate measurements, even at drug/phosphate ratios where the viscosity was changing rapidly with each increment in drug concentration. Thus, equilibrium must have been attained within a substantially shorter time than the 2–3 min required to make a first measurement. Reduced viscosities η_{red} were calculated by established methods, taking into account the small dilution caused by the addition of drug solutions (Revet et al., 1971; Waring & Henley, 1975).

For experiments designed to measure the helix extension produced by binding of diacridines, calf thymus DNA (Sigma) was sonicated to fragments of molecular weight 5×10^5 (Crothers & Zimm, 1965) as previously described (Wakelin & Waring, 1976). Viscometric measurements on this DNA were performed essentially by the method of Cohen and Eisenberg (1966, 1969) using the same apparatus described above. In this case, however, because of the smaller reduced viscosity of the sonicated fragments, the DNA concentration was increased to 606 μM (OD_{260} = 4.0), which gave a flow time of 110.7 s in the absence of drugs. The data were transformed directly from flow times to values for the relative contour length using the expression

$$L/L_0 = \left[\frac{t_C - t_0(V)}{t_D - t_0(V)}\right]^{1/3}$$

where L is the contour length in the presence of drug, L_0 is the contour length of free DNA, t_C is the flow time for the complex, t_D is the flow time for pure DNA, and $t_0(V)$ is the flow time for buffer at a given total volume, V, in the viscometer. This expression derives directly from the theory of Cohen and Eisenberg (1966, 1969) with the added assumption that the intrinsic viscosity approximates to the reduced viscosity for the complexes, known to be the case for at least one other bifunctional intercalating agent (Wakelin & Waring, 1976).

Electric Dichroism. Calf thymus DNA (Worthington) was dissolved in 0.1 M phosphate buffer (pH 6.8) by gentle shaking overnight at 2 °C and then sonicated as described by Müller and Crothers (1968). The molecular weight of the DNA after this treatment was estimated to be $1.25–1.5 \times 10^5$ by hydrodynamic measurements. DNA–diacridine complexes having drug/nucleotide ratios in the range 0.05–0.06 were prepared by diluting stock diacridine solutions with 0.2 mM NaCl and mixing 2.5 mL with 2.5 mL of stock DNA solution. Electric dichroism measurements were performed as described by Yamaoka & Charney (1972, 1973). The samples were equilibrated in a cell thermostated at 10 °C and were changed every four pulses.

Fractionation of the DNA on a calcium phosphate gel column (Bernardi, 1971) indicated that when the DNA was sonicated in low salt (Müller & Crothers, 1968) it yielded a high proportion of single-stranded DNA. Predominantly double-stranded DNA was obtained when the sonication was performed in 0.1 M phosphate buffer (pH 6.8). This stock DNA solution was dialyzed against 0.2 M NaCl prior to use. The stock diacridine solutions were prepared by dissolving solid diacridine in water and neutralizing to pH 7.0 with 0.1 M lactic acid.

Results

Interaction with Closed Circular DNA. All the diacridines remove and reverse the supercoiling of covalently closed circular DNA in the fashion characteristic of intercalating agents (Waring & Wakelin, 1974; Wakelin & Waring, 1976; Revet et al., 1971; Waring 1970, 1972). Figure 1 shows typical effects of two derivatives on the sedimentation coefficient of PM2 DNA; for C-3 and C-8 the equivalence points occur at drug/nucleotide input ratios of 0.100 ± 0.020 and 0.051 ± 0.008,

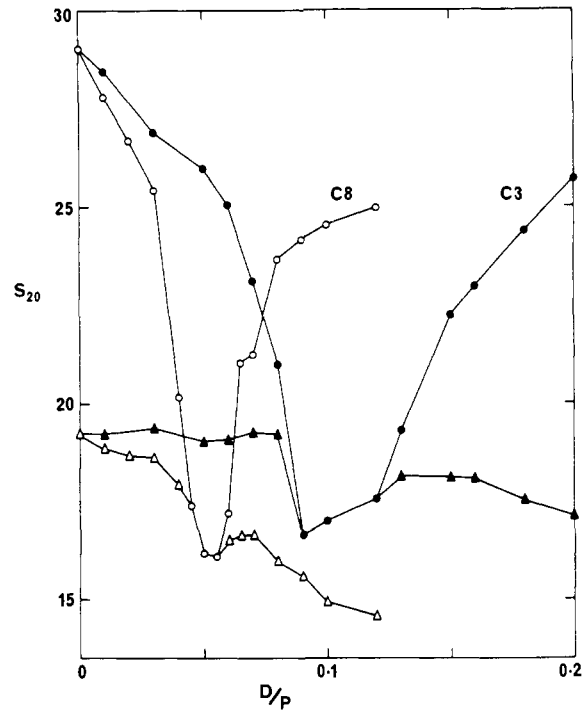


FIGURE 1: Effects of C-8 (open symbols) and C-3 (filled symbols) on the sedimentation coefficient of PM2 DNA. The DNA preparation contained 70% closed circular duplex molecules whose s_{20} is represented by circles; that of the nicked circular molecules is represented by triangles; and when both components cosedimented as a single unresolved boundary only the circular symbol was plotted, representing the weight-average sedimentation coefficient for both species together. The abscissa shows the *input ratio*, i.e., the molar ratio of added drug to DNA nucleotides.

respectively. In addition, both compounds cause the expected decrease in s_{20} of the nicked circular DNA associated with intercalation (Waring, 1970, 1972). It is obvious that the two derivatives differ markedly in the magnitude of their helix-unwinding angles such that the effectiveness of C-8 is almost exactly double that of C-3. Furthermore, C-8 causes a more pronounced lowering of the sedimentation coefficient of the nicked circular DNA than does C-3 at comparable levels of drug binding. Figure 2 shows equivalence input ratios for the complete series of compounds determined by sedimentation measurements (filled circles) as a function of chain length n. It appears that the equivalence ratio rises slightly as n increases from 0 to 3 and then falls precipitously to less than half of the peak value at n = 7, after which it again rises marginally. Thus, with one exception, the compounds fall into two distinct groups with C-6, -7, -8 and -12 having approximately twice the unwinding angle of C-2, -3, -4 and 9-methylaminoacridine. On this basis, we identify the members of the first group as being capable of bifunctional intercalation, whereas those in the latter group are limited to monofunctional reaction. C-5 has an unwinding effect that places it intermediate between the two groups.

These conclusions are subject to one assumption, namely, that all, or nearly all, of the added drug is bound to the DNA at equivalence. In order to determine if any corrections need be applied to unwinding angles estimated by the sedimentation experiments to correct for incomplete binding, and also to provide independent corroboration of the results, viscosity titrations using PM2 DNA were undertaken. Results for the effects of three derivatives, C-4 to C-6 on the viscosity of PM2 DNA are shown in Figure 3. The responses to all three compounds are qualitatively similar and comparable to that seen with ethidium as regards the shapes of the curves and the

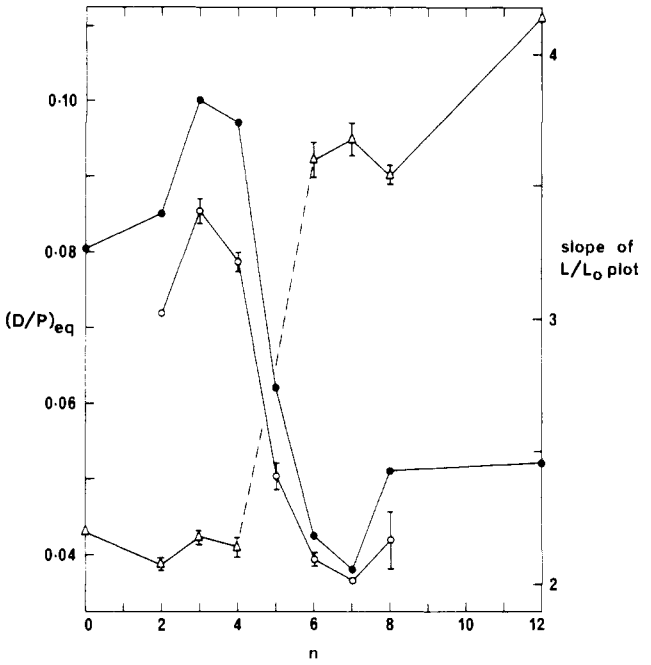


FIGURE 2: Variation of the helix-unwinding angle and the helix extension with length of the connecting methylene chain $-(CH_2)_n-$ for simple dimeric 9-aminoacridine compounds. Circular symbols represent the equivalence points (left-hand ordinate) derived from the closed circular DNA studies. Filled circles show values from sedimentation experiments where the equivalence points are not corrected for binding; open circles represent the results from viscometric measurements and are true equivalence binding ratios whose standard deviations are indicated by the error bars. For C-2 and C-7, the errors were smaller than the symbol plotted. Triangular symbols represent the results of viscometric measurements on sonicated DNA (right-hand ordinate) and are expressed as the slope of the L/L_0 plot (see Figure 5) with standard deviation as indicated. The results shown for $n = 0$ were obtained with 9-methylaminoacridine.

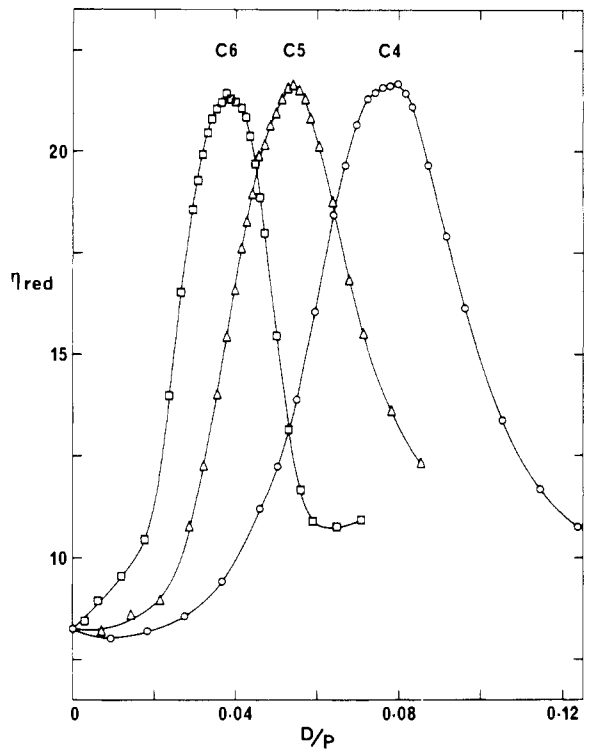


FIGURE 3: Effects of diacridines on the viscosity of closed circular duplex PM2 DNA. The initial DNA concentration was 295 μM in nucleotides for compounds C-4 and C-5 and 267 μM for C-6. Diacridines were added as 200–400 μM solutions in 0.01 SHE buffer. The ordinate represents the reduced viscosity in dL/g; the abscissa shows the molar ratio of *added* drug to DNA nucleotides. The effects of compounds C-4 to C-6 are represented by the symbols ○, △, and □, respectively.

values of the reduced viscosities of the relaxed circular DNA complexes (Waring & Henley, 1965). However, again it appears that C-4 has only approximately half the unwinding ability of C-6, while C-5 remains intermediate in character. To determine true equivalence binding ratios, the positions of equivalence peaks were determined for compounds C-2 to C-8 at a range of DNA concentrations. In Figure 4 the concentration of each drug required to relax the supercoiling of the circles is plotted as a function of the DNA concentration. For a simple mass-action interaction, such a plot should take the form of a straight line with slope equal to the equivalence binding ratio and intercept on the ordinate equal to the free drug concentration in equilibrium with the relaxed circular DNA complex (Revet et al., 1971; Waring & Henley, 1975). The data in Figure 4 reveal that this condition is satisfactorily met, and the resulting equivalence binding ratios are plotted in Figure 2 (open circles). The intercepts shown in Figure 4 are not statistically significantly different from zero; thus, no free drug is detectable at equivalence within the limits of sensitivity of this method. Although the viscometrically determined values of equivalence points for C-2 to C-8 are systematically lower than those determined by sedimentation (Figure 2), they always fall within the range of the single cosedimenting boundary observed in the latter experiments and, judged by the results of either method, there is a clear transition from monofunctional to bifunctional reaction on lengthening the methylene chain from four carbons to six.

Interaction with Sonicated DNA. The extension of the DNA helix associated with binding of the diacridines was investigated by viscometric titration of sonicated rodlike fragments of calf thymus DNA. Figure 5 shows representative results for three derivatives: the relative increase in contour length, L/L_0, in the presence of the ligand is plotted as a function of the drug/nucleotide input ratio. In the left-hand panel it can be seen that the data for C-2 lie on the straight line expected for monofunctional interaction $[L/L_0 = 1 + 2(D/P)]$, whereas those for C-7 lie close to the line expected for bifunctional intercalation $[L/L_0 = 1 + 4(D/P)]$ (Waring & Wakelin, 1974; Wakelin & Waring, 1976). Once more the result for C-5 is enigmatic; data from two separate experiments are presented in the right-hand panel of Figure 5 where it is obvious that the compound fails to yield a simple straight-line response. At low levels of binding the data lie on the line describing a monofunctional reaction, but at input ratios above approximately 0.06 the points deviate upwards to reach a slope of 3.3 which is nearer to that characteristic of the bifunctional diacridines. The slopes of least-squares fitted lines from L/L_0 vs. D/P plots like those shown in Figure 5 for the diacridine series are included in Figure 2 (with the exception of C-5) for comparison with the helix-unwinding measurements. Again the data (triangular symbols: right-hand ordinate) fall into two distinct classes, with the shorter chain derivatives C-2 to C-4 and 9-methylaminoacridine characterized by slopes in the range 2.1–2.2 as expected for monofunctional intercalation, and the longer chain homologues C-6 to C-8 and C-12 with slopes in the region of 3.5–4.1 indicative of bifunctional reaction. In no case did the experimental points deviate downwards toward the axis of D/P at higher input ratios; evidently, therefore, at the high DNA concentration and moderate input ratios (up to 0.12) used in these experiments essentially all of the added drug is bound.

Electric Dichroism. Having thus defined the region of

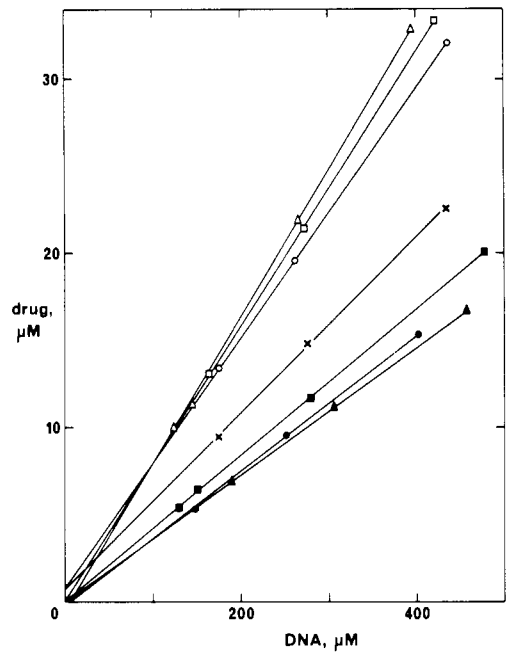


FIGURE 4: Titration of the supercoiling of PM2 DNA by diacridines. The ordinate and abscissa show, respectively, the absolute concentrations of total diacridine and DNA in the viscometer at the maxima of reduced viscosity plots like those presented in Figure 3. For the different experiments, the starting DNA concentration was varied between 150 and 500 μM in nucleotides. The straight lines were fitted by computer using a least-squares program. Identification of symbols: (○) C-2; (△) C-3; (□) C-4; (×) C-5; (●) C-6; (▲) C-7; (■) C-8.

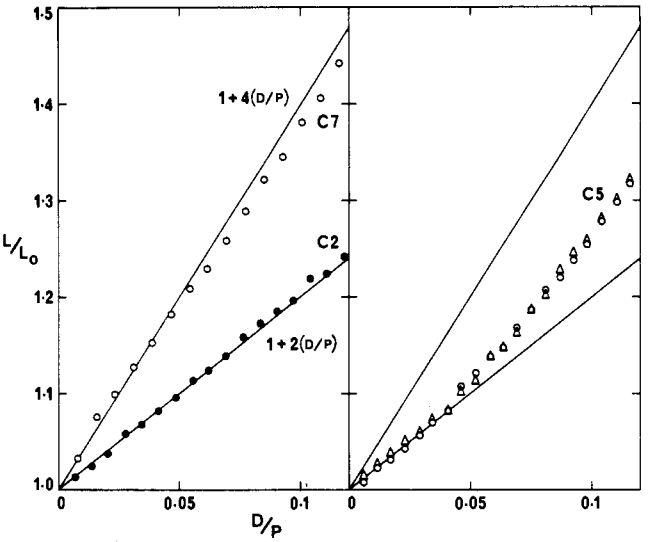


FIGURE 5: Effects of C-2, C-5, and C-7 on the relative contour length of sonicated calf thymus DNA fragments. The ordinate represents the calculated ratio of contour lengths in the presence (L) or absence (L_0) of the diacridines. The abscissa represents the molar ratio of *added* drug to DNA nucleotides. Each panel contains reference lines corresponding to the relations $L/L_0 = 1 + 4\,(D/P)$ and $L/L_0 = 1 + 2(D/P)$. The data for C-2 and C-7 (left-hand panel) are represented by ● and ○, respectively; those for C-5 are shown in the right-hand panel where results from two independent experiments are presented.

transition from mono- to bifunctional reaction to lie between compounds C-4, C-5, and C-6, we sought information regarding the geometrical disposition of the chromophores in the DNA complexes formed with these compounds using electric dichroism measurements. Since for 9-aminoacridine the longest wavelength intense absorption is due to a short-axis polarized transition (Zanker & Schiefele, 1958), such measurements yield values for the twist angle between the acridine planes and the helix axis. Values for the average twist angle per bound drug molecule were derived by treating the reduced dichroic ratios of the complexes according to eq 30 of Yamaoka and Charney (1972, 1973)

$$\left(\frac{\Delta\epsilon}{\epsilon}\right)_A = \tfrac{3}{2}(3\cos^2\theta_A - 1)\,\Phi_A$$

$$\left(\frac{\Delta\epsilon}{\epsilon}\right)_B = \tfrac{3}{2}(3\cos^2\theta_B - 1)\,\Phi_B$$

where $\Delta\epsilon/\epsilon$ is the reduced dichroism, θ_A is the angle that the base transition moment makes with respect to the electric-field direction, θ_B is the angle that the drug transition moment makes with respect to the electric-field direction, and Φ is the orientation function. Average twist angles, derived assuming that $75° \leq \theta_A \leq 90°$ for the compounds C-2 to C-12, as well as that for 9-aminoacridine, are presented in Table II. It is apparent that for derivatives with methylene chains containing seven carbons or more the acridine chromophores are approximately parallel to the base planes as, indeed, is the case for 9-aminoacridine. For compounds C-3 and C-4, the average twist angle is 16–17°, which indicates that, since these derivatives react monofunctionally (see above), the nonintercalated chromophore must be inclined at an angle of some 32–34° with respect to the base planes. A similar argument for C-2 shows that the nonintercalated chromophore lies, in this case, approximately parallel to the intercalated acridine ring and the base planes. The average twist angles determined for C-5 and C-6 are intermediate between those obtained for C-3 or C-4 and those obtained for chain lengths of seven carbons or longer. For the bifunctional intercalation of C-6, this may be interpreted as implying that both chromophores are marginally twisted with respect to the base planes. However, for C-5 any interpretation must of necessity be equivocal, since for this compound the mode of intercalation (whether mono- or bifunctional) is uncertain. If the binding of C-5 is really bifunctional then the same conclusion drawn for C-6 is applicable, whereas if the interaction is merely monofunctional at this level of binding ($D/P \approx 0.05$–0.06) then the nonintercalated ring appears inclined at an angle of about 22° with respect to the base planes.

TABLE II: Electric Dichroism Data for Diacridine–DNA Complexes.[a]

compd	drug/phosphate ratio	reduced dichroism	av twist angle (deg)
DNA	0	−0.54	
9-aminoacridine	0.096	−0.60	0
C-2	0.060	−0.57	0
C-3	0.058	−0.42	17
C-4	0.054	−0.44	16
C-5	0.047	−0.48	11
C-6	0.053	−0.49	10
C-7	0.051	−0.55	0
C-8	0.054	−0.61	0
C-10	0.058	−0.57	0
C-12	0.057	−0.60	0

[a] The average twist angle is defined as the approximate average angle of the acridine chromophores with respect to the DNA base pairs per added drug molecule. It was evaluated from the measured reduced dichroism as described in the text.

In addition to the electric dichroism experiments, we also measured circular dichroism (CD) and magnetic circular dichroism (MCD) spectra for 9-aminoacridine and diacridine

complexes with DNA. Since no magnetic circular dichroism signals were obtainable (i.e., there is essentially no difference between CD and MCD measurements made on the same sample), we conclude that these compounds show no significant amount of external attachment to the helix in complexes formed under these experimental conditions.

Discussion

All three types of hydrodynamic experiments agree that within the present series of diacridines the change from monofunctional to bifunctional intercalation occurs on lengthening the linker from one of four methylene groups to one of six. They also concur in assigning a distinctly intermediate character to the C-5 derivative. Simple geometrical considerations (see Table I) reveal that maximum extension of the methylene chain of C-6 leads to an internuclear separation of the acridine chromophores of 8.8 Å, which is thus the minimum distance unambiguously permitting bifunctional intercalation. Similarly, the maximum internuclear chromophore separation of the C-4 compound, 6.3 Å, sets the unequivocal upper limit to the chromophore separation in those compounds restricted to monofunctional reaction. Thus, the transition in functionality must lie between interchromophore distances of 6.3 and 8.8 Å. Unfortunately, the results obtained with C-5 are so equivocal as to preclude their use to define more precisely the transition region. With the exception of C-5, none of the compounds produced effects indicative of peculiar or nonclassical intercalative behavior; in particular, the results of the helix-extension measurements are not consistent with the formation of highly kinked diacridine–DNA complexes. Consequently, we favor interpreting our results in terms of a classical Fuller–Waring (1964) type of intercalation model in which the distortion of the DNA structure accompanying drug binding is minimized. Based upon this model, the "transition window" of 6.3–8.8 Å implies that there is a single base pair sandwiched between the two intercalated chromophores of C-6, since to accommodate two base pairs would require a separation of 10.2 Å which is clearly unattainable with this derivative. This conclusion is immediately at variance with the generally favored "neighbor-exclusion" model for the interaction of acridines with DNA (Waring, 1972; Crothers, 1968) which predicts at least a "two-base-pair sandwich" as the minimum unit permitting bifunctional intercalation. Further information relevant to this point could be gained from a careful measurement of the stoichiometry of binding. The "one base-pair sandwich" model predicts a saturation binding level (m) of 0.168 drug molecule per nucleotide (neglecting end effects) if there is neighbor exclusion between bound drug molecules or a value of $m = 0.250$ in the absence of such a restriction. On the other hand, the "two-base-pair sandwich" model with neighbor exclusion between bound diacridines would yield saturation binding levels of $m = 0.125$. Experiments to investigate the values of m for representative diacridines are in progress.

If the Fuller–Waring (1964) requirement for strictly parallel stacking of base pairs and chromophores is adhered to, the "one base-pair sandwich" model must also apply in the case of C-7, whereas for compounds with linker chains equivalent in length to those of C-8 or C-9, either model may be applicable. For the higher homologues such as C-10 and C-12, up to three nucleotide pairs may be accommodated in the sandwich.

It is pertinent to query whether there are any indications of exceptional helix distortion in the complexes formed between C-6 or C-7 and DNA. The unwinding angles (33.1° and 35.5°, respectively, based on an ethidium unwinding angle of 26° [Wang, 1974; Keller, 1975]) are not exceptional for bifunctional intercalating agents: cf. the values reported for echinomycin under various experimental conditions (Waring & Wakelin, 1974; Wakelin & Waring, 1976) or 38° for the bifunctional bis(9-aminoacridine) derivative described by Le Pecq et al. (1975) (see below). Since for both the latter compounds the preferred binding model is of the "two base-pair sandwich" type (Wakelin & Waring, 1976; Le Pecq et al., 1975; Ughetto & Waring, 1977; Lee, 1977), the implication is that the degree of helix unwinding associated with the one base-pair sandwich is neither unusually large nor small. Furthermore, the helix extension which accompanies binding of C-6 and C-7 to DNA is indistinguishable from that associated with binding of echinomycin at the same ionic strength (Waring & Wakelin, 1974; Wakelin & Waring, 1976). Thus, it appears that none of the measured properties of the C-6– and C-7–DNA complexes imply exceptional perturbation of the DNA structure, and, moreover, the apparent twisting of the chromophores in the C-6 complex by 10° is seen to be readily reduced to zero on lengthening the methylene chain by one carbon. Consequently, although it may be possible to construct highly kinked CPK molecular models of a "two base-pair sandwich" for C-6– and C-7–DNA complexes of the type described by Sobell et al. (1977), we see no evidence of the substantial helix distortions which such models would presumably demand.

The ambivalent behavior of C-5 is perplexing. Given that the maximum interchromophore separation is 7.5 Å, the data are not inconsistent with a bifunctional reaction which is characterized by an unusually small unwinding angle (26°) accompanied by severe kinking of the duplex backbone which, in turn, is reflected in moderately twisted chromophores (11°) and an apparently small helix extension at low levels of binding. It has been shown that simple treatment (Cohen & Eisenberg, 1966, 1969) of viscosity data derived from measurements on sonicated DNA fragments can lead to erroneous estimates of helix extension if there is severe kinking of the DNA associated with drug binding (Balcarova et al., 1978; Reinert, 1972). When this occurs, it is necessary to assess separately the contribution of stiffening or bending of the DNA to the viscosity enhancement, e.g., by measuring the effects of drug binding on high-molecular-weight DNA (Balcarova et al., 1978; Reinert, 1972). On the other hand, the experimental data for C-5 might equally well be interpreted in terms of monofunctional intercalation characterized by an unusually large unwinding angle associated with an increased viscosity enhancement above a critical level of binding (perhaps due to some form of helix stiffening). Finally, of course, the actual mode of interaction might involve some combination of a mono- and bifunctional reaction, though in this case the L/L_0 plot (Figure 5) would imply that monofunctional intercalation dominates at low levels of binding but gradually gives way to bifunctional reaction as D/P increases—the reverse of what one might naively have expected.

Lastly, it is of interest to compare the results of this study of simple aliphatic 9-aminoacridine dimers with those of a similar study by Le Pecq et al. (1975) of polyamine-linked diacridines derived from 2-methoxy-6-chloro-9-aminoacridine. It was reported that when the linking chain consisted of $-(CH_2)_3NH(CH_2)_3-$ the resulting compound was monofunctional notwithstanding its maximum interchromophore distance of 9.9 Å which would easily permit formation of a one-base-pair bifunctional sandwich (Le Pecq et al., 1975). This result contrasts sharply with our results for the simple C-6 compound whose interchromophore link is shorter by one NH unit and which appears unambiguously bifunctional. Only on lengthening the polyamine chain by a further $-CH_2-$ or by

$-CH_2NH(CH_2)_3-$ to yield the spermidine and spermine derivatives, respectively, did Le Pecq et al. observe a bifunctional reaction, the characteristics of which were reported to be indistinguishable from one another (Le Pecq et al., 1975). The latter compounds may be extended sufficiently to accommodate two nucleotide pairs between the intercalated acridine rings, which prompted the above authors to propose a two base-pair sandwich model. This interesting difference between the results for the two diacridine series may be explained if the nature of the bridging chain and/or the substituents on the acridine ring has a profound effect on the ability of diacridine compounds to act as bifunctional intercalating agents.

With this in mind, we performed some preliminary experiments with spermine and spermidine diacridines derived from 9-aminoacridine, the results of which do indeed implicate the connecting chain as a determinant in the character of the DNA–diacridine complex. Helix-unwinding experiments revealed a very peculiar behavior in that, while an equivalence point of 0.036 ± 0.003 drug molecule bound per nucleotide was observed for the spermidine derivative (indicative of bifunctional reaction), the dip in the s_{20} vs. D/P plot was anomalously sharp. Furthermore, for the spermine derivative the usual two species of PM2 DNA apparent in the ultracentrifuge photographs (covalently closed circles and nicked circles) split into three components which did not coalesce into a single boundary at any ligand concentration. Neither of these strange effects was ever observed with the simple aliphatic diacridines and it seems likely that they may be related to cooperative binding or to inter- or intrastrand cross-linking by diacridines of this type. Such behavior might be facilitated by additional interactions between the charged polyamine linkages and phosphate groups of the DNA backbone or hydrogen bonding groups of the nucleotides themselves.

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