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Effects of Ring Substituents and Linker Chains on the Bifunctional Intercalation of Diacridines into Deoxyribonucleic Acid†

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ABSTRACT: Sedimentation experiments with closed circular duplex PM2 deoxyribonucleic acid (DNA) and viscosity measurements with sonicated rodlike DNA fragments have been performed to investigate unwinding and extension of the DNA helix associated with binding of homologous series of diacridines. When the acridine nuclei are linked via 9-amino substituents with chains permitting interchromophore separation in the critical region between 8.8 and 11.3 Å, it is found that substituents at positions 2, 3, and 6 restrict bifunctional intercalation to compounds capable of sandwiching at least two base pairs in the intercalated complex. Unsubstituted diacridines, and derivatives bearing an ethyl group at position 4, can bis-intercalate with a significantly shorter linking chain. The presence of amino groups in the linker does not affect the ability to bis-intercalate but increases the helix unwinding angle by 30–40% and decreases the helix extension per bound ligand molecule by 11–18%. Bifunctional reaction is observed with a diacridine linked via the 4–4′ positions showing that 9-aminoacridine can intercalate in at least two major orientations with the 9 or 10 position directed toward the helix axis. Both orientations can be accommodated simultaneously within a single diacridine linked via the 9–4′ positions with a bridge sufficiently long to encompass two base pairs. An acridone substituted at positions 4 and 5 with positively charged side chains intercalates with a helix unwinding angle indistinguishable from that of ethidium whereas an uncharged bisacridone failed to yield a detectable complex with DNA. Tilorone dihydrochloride, a structurally similar antiviral drug, was found to intercalate with an unwinding angle approximately half that of ethidium. The potentially bifunctional bisquinoline drug dequalinium causes relaxation of the supercoiling of PM2 DNA but probably not by simultaneous intercalation of both its chromophores.

Efforts to develop potential chemotherapeutic DNA-binding drugs endowed with enhanced selective toxicity center largely on studies of polyfunctional intercalating agents (Waring & Wakelin, 1974; Le Pecq et al., 1975; Canellakis et al., 1976; Cain et al., 1978; Gaugain et al., 1978; Kuhlmann et al., 1978; Wakelin et al., 1978). The underlying principle is that bis-intercalation affords the opportunity for improving both nucleotide sequence selectivity and specificity for DNA per se as a result of the larger binding constants expected for drugs of this type. Recently, it has been demonstrated that the transition from mono- to bifunctional reaction in an homologous series of diacridines is indeed accompanied by a substantial increase in the binding constant (Wakelin et al., 1979).

LePecq et al. (1975) studied a series of diacridines derived from 6-chloro-2-methoxyacridine linked through the 9 position via various polyamines. They concluded that bifunctional reaction only occurs when the linkage is sufficiently long to permit two base pairs to be accommodated between the chromophores. Similarly, it is likely that the rigid cyclic peptide which separates the quinoxaline chromophores in echinomycin by 10.2 Å restricts this antibiotic to binding via a "two-base-pair sandwich" model (Waring & Wakelin, 1974; Wakelin & Waring, 1976; Ughetto & Waring, 1977; Cheung et al., 1978). In contrast to these two examples, Wakelin et al. (1976, 1978) showed that in a series of unsubstituted diacridines linked via a simple methylene chain bis-intercalation is first observed when the linkage is long enough to span only one base pair, assuming a Fuller–Waring-type geometry for the intercalated complex (Fuller & Waring, 1964). Kuhlmann et al. (1978) reported bifunctional reaction for a dimer of ethidium linked through the para position of the 6-phenyl ring, whereas Gaugain et al. (1978) failed to observe bis-intercalation with an ethidium–acridine dimer and a simple ethidium dimer, both of which were linked through the quaternary ring nitrogen.

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The principal objective of the present work was to investigate the reason(s) for the apparently different lengths of linker chain needed to permit bis-intercalation of diacridines in the studies of LePecq et al. (1975) and Wakelin et al. (1976, 1978). Helix unwinding angles derived from sedimentation measurements on closed circular duplex DNA from bacteriophage PM2 and measurements of helix extension obtained from viscometric experiments with sonicated rodlike fragments of DNA were employed to assess the capacity of ligands to bis-intercalate, as in the previous studies. A priori one might imagine that the different results could arise from the presence of substituents on the acridine rings or from the inclusion of charged amino groups in the linker chains, or perhaps both. We find that the influence of substituents on the chromophores is most important. In addition, we use novel diacridines to establish that the acridine chromophore can become intercalated in at least two major orientations with respect to the base pairs of the DNA helix. We also investigate the capacity for intercalation of related substances including acridones, tilorone, and dequalinium in an effort to widen our understanding of structural constraints which affect the process.

Experimental Procedures

Materials

Buffers. Buffer solutions were prepared in reagent-grade water from a Millipore Milli-Q2 system. Buffer A contained 2 mM Hepes,[1] 10 μM EDTA, and 9.4 mM NaCl adjusted with NaOH to pH 7.0 at 20 °C. Buffer B consisted of 2 mM Mes, 10 μM EDTA, and 9.25 mM NaCl brought to pH 6.0 with NaOH. Buffer C contained 10 mM Tris, 10 μM EDTA, and 20 mM NaCl adjusted with HCl to pH 7.5. Buffers D and E were respectively 10 mM and 200 mM sodium acetate solutions at pH 5.0 containing 10 μM EDTA. Buffer A was used routinely except where stated otherwise in the text.

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

Drugs. Structural formulas, together with the molar extinction coefficients of the drugs in the experimental buffer used, are given in Table I. Tilorone dihydrochloride was a gift from Dr. K. Jacobsen; a pharmaceutically pure sample of dequalinium dihydrochloride was provided by Dr. D. Jack of Allen and Hanburys Ltd., United Kingdom; quinacrine dihydrochloride was purchased from Sigma Chemical Co. These compounds were used as supplied without further purification. Diacridines **2**, **3**, and **8** and 9-(methylamino)-acridinium chloride were obtained from Dr. E. S. Canellakis of Yale University. Their synthesis and purification are described by Canellakis et al. (1976). All remaining acridines were purified by using standard techniques and yielded satisfactory elemental analyses. Their synthesis will be described elsewhere (R. M. Acheson, E. Constable, J. P. Gibbs, and R. G. McR. Wright, unpublished experiments). In the dry state drugs were stored in a desiccator in the dark at 0–4 °C; solutions were freshly prepared whenever possible and maintained in the dark at 0–4 °C or stored frozen at −22 °C. Extinction coefficients of compounds with limited aqueous solubility were determined by dissolving the solid in dimethyl sulfoxide or 0.025 M lactic acid and diluting copiously with buffer to reduce the concentration of the original solvent to an insignificant level (<0.25% v/v). In these instances absorbance measurements were made in 100 mm light path quartz cuvettes.

Methods

Analytical Ultracentrifugation. Sedimentation coefficients were measured by boundary sedimentation at 20 °C and 34000 rpm in a Beckman Model E ultracentrifuge equipped with ultraviolet optics. Details of procedures 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 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, a period assumed adequate for attainment of equilibrium. Sedimentation coefficients are presented as directly determined; they were not corrected for viscosity, buoyancy, or DNA concentration.

Viscometry. Measurements were made essentially by the method of Cohen & Eisenberg (1966, 1969) using a simple viscometer having a 10-cm capillary of 0.4-mm bore and a bulb of volume 1.5 mL, thermostated at 20 ± 0.01 °C. The calf thymus DNA used was sonicated to fragments of molecular weight 3×10^5–5×10^5 (Crothers & Zimm, 1965) as previously described (Wakelin & Waring, 1976). For routine experiments the viscometer contained 1.8 mL of a 606 μM solution of sonicated DNA. Drugs were added in increments of 1–20 μL to a maximum of 500 μL from a Burkard precision micrometer syringe via a fine plastic tube. The syringe and tube were thoroughly soaked in drug solution prior to use to minimize losses of material by adsorption to glass and plastic surfaces. Even so, the actual concentration of the drug solution being delivered from the tip of the plastic tube was always determined before and after each experiment by visible absorption measurements. Solutions were freed of particulate material by centrifuging for 15 min at 4000 rpm before use. The data were transformed directly from flow times to values for the relative contour length as described previously (Wakelin et al., 1978) by using the equation

$$\frac{L}{L_0} = \left(\frac{t_c - t_0(v)}{t_d - t_0(v)} \right)^{1/3}$$

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.

Equilibrium Dialysis. Binding curves were measured by equilibrium dialysis using a Measuring and Scientific Equipment Dianorm apparatus as previously described (Wakelin et al., 1979). Dialysis cells were separated by a Spectrapor 2 regenerated cellulose membrane and loaded with 4 mL of 454 μM calf thymus DNA in one chamber and the same volume of the appropriate drug solution in the other. The cells were rotated to establish equilibrium in a water bath at 20 °C for 20 h, after which the drug concentration in each chamber was determined spectrophotometrically. For the free drug side the

[1] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Mes, 4-morpholineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Me_2SO, dimethyl sulfoxide.

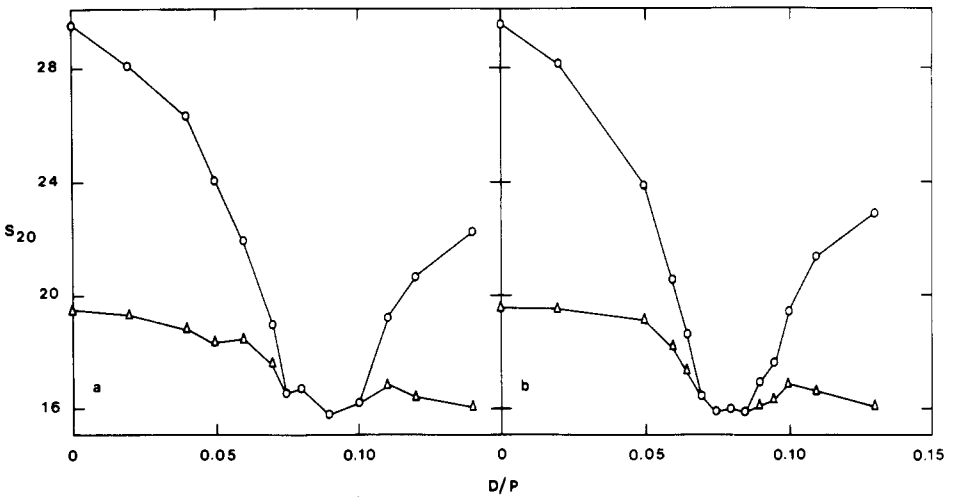


FIGURE 1: Effects of 9-aminoacridine and 9-(methylamino)acridine on the sedimentation coefficient of PM2 DNA. The DNA preparations contained 70% closed circular duplex molecules whose s_{20} is represented by circles; that of the nicked circular molecules is shown as triangles; when both components cosedimented 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. (a) 9-aminoacridine; (b) 9-(methylamino)acridine.

extinction coefficients listed in Table I were used; for the other side the complex was dissociated by addition of an equal volume of dimethyl sulfoxide, and the total concentration was estimated by using the following molar extinction coefficients determined for 50% (v/v) buffer–Me_2SO mixtures: 9-(methylamino)acridine, 1.14×10^4 (411 nm); 10-methylacridine, 3.54×10^3 (419 nm). The intrinsic association constant for an isolated potential binding site, $K(0)$, and the number of nucleotides occluded by a single bound drug molecule, n, were estimated by an iterative procedure designed to satisfy eq 10 of McGhee & Von Hippel (1974)

$$\frac{r}{c} = K(0)(1 - nr)\left[\frac{1 - nr}{1 - (n-1)r}\right]^{n-1}$$

given the experimentally determined values of the binding ratio, r, and the free drug concentration, c, together with an initial guess of n [see Wakelin & Waring (1976)].

Results

Before investigation of potentially bifunctional diacridines, the properties of relevant simple mononuclear acridines were examined. 9-Aminoacridine removed and reversed the supercoiling of PM2 DNA with an equivalence input ratio (i.e., molar ratio of total added drug to DNA nucleotides which causes complete relaxation of supercoiling) of 0.087 ± 0.015 (Figure 1a). The equivalence input ratio was corrected to a binding ratio of 0.076 ± 0.012 by using equilibrium parameters determined for calf thymus DNA in buffer of identical ionic strength [$K(0) = 1.54 \times 10^5$ M^{-1} and $n = 3.73$; W. Wilson, B. C. Baguley, L. P. G. Wakelin, and M. J. W. Waring, unpublished experiments]. This correction assumes that contributions from the free energy of supercoiling to the effective association constant vanish for fully relaxed circles and that the intrinsic binding parameters are the same for these two DNAs of equal base composition. The experiment yields a helix unwinding angle of 17.4°, taking that of ethidium to be 26° (Wang, 1974; Keller, 1975), which is indistinguishable from the 17.2° measured for proflavin [Müller et al. (1973); corrected to the same standard of 26° for ethidium).

9-Aminoacridine caused a linear increase in the relative contour length of sonicated calf thymus DNA up to the highest binding ratio studied ($r = 0.175$). The data lie on a line of slope 1.79, falling slightly below the theoretical value of 2.0 expected for idealized monofunctional intercalation. Again, the result is very similar to that found for proflavin [slope = 1.67; Cohen & Eisenberg (1969)] indicating a close correspondence between the parameters of intercalation for these two drugs.

The effects of methylation of the 9-amino group on these parameters are shown in Figure 1b and Table I. The equivalence input ratio of 0.080 ± 0.010 drug molecules per nucleotide when corrected for binding [$K(0) = 2.25 \times 10^5$ M^{-1} and $n = 4.20$; equivalence binding ratio = 0.073 ± 0.008] indicates an unwinding angle of 18.2° (Figure 1b). The helix extension measurements, similarly corrected, yield a value for the slope of 2.21 (Table I). Thus, the presence of the methyl group has little effect on the unwinding angle but appears to cause a longer extension of the helix per intercalated molecule. The position of methylation is clearly important, however, since quaternization of acridine at position 10 yields a compound whose equivalence input ratio occurs at 0.135 ± 0.020 drug molecules per nucleotide. Correction of this value for binding shows the unwinding angle to be 18.4° and reveals that the binding constant is markedly lower for 10-methylacridine [$K(0) = 2.33 \times 10^4$ M^{-1} and $n = 3.89$; equivalence binding ratio = 0.072 ± 0.009].

Diacridines Lacking Ring Substituents Connected by Various Linker Chains. Linking two 9-aminoacridine chromophores via $-(CH_2)_3NH(CH_2)_3-$ produces a diacridine (compound **1**, Table I) whose equivalence input ratio is 0.031 ± 0.006 (Figure 2a), less than half the value of the monomer. Figure 3a shows that the helix extension produced by binding of this derivative is well described by a straight line of slope 3.20, a value nearly twice that found with 9-aminoacridine. Taken together, these findings identify this compound as a bis-intercalator. A compound similar to **1** which differs only by the possession of methoxy and chloro substituents in positions 2 and 6, respectively (designated III in LePecq et al., 1975), was shown to behave monofunctionally in buffer containing 0.2 M sodium acetate at pH 5.0 (LePecq et al., 1975). To check that the different results for these two compounds do not arise simply from the disparity in solvent conditions, we also measured the equivalence input ratio for compound **1** in 0.2 M sodium acetate at pH 5.0 and found it to be 0.037 ± 0.005 (Figure 2b, Table I), again typical of bifunctional reaction (LePecq et al., 1975; Wakelin et al., 1976, 1978).

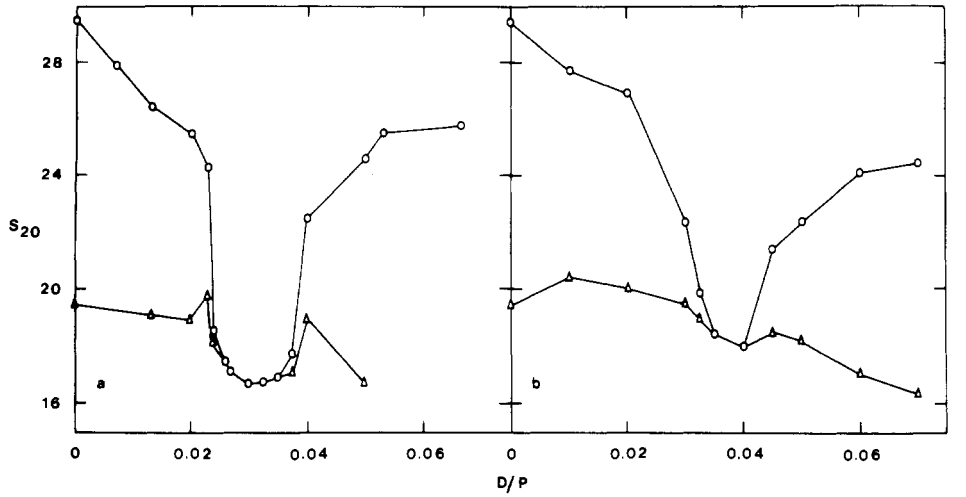


FIGURE 2: Effects of compound **1** on the sedimentation coefficient of PM2 DNA. For details see legend to Figure 1. (a) Measurements made in buffer A; (b) measurements made in buffer B.

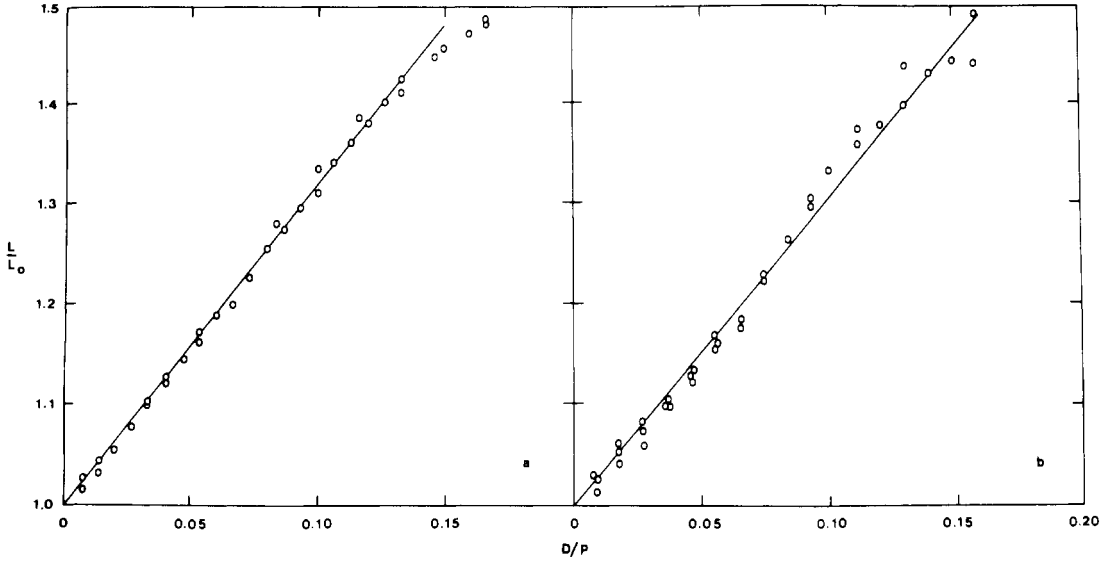


FIGURE 3: Effects of compounds **1** and **3** on the relative contour length of sonicated calf thymus DNA. The ordinate represents the calculated ratio of contour lengths in the presence (L) or absence (L_0) of the drug. The abscissa shows the input ratio, i.e., the molar ratio of added drug to DNA nucleotides. (a) Compound **1**; (b) compound **3**.

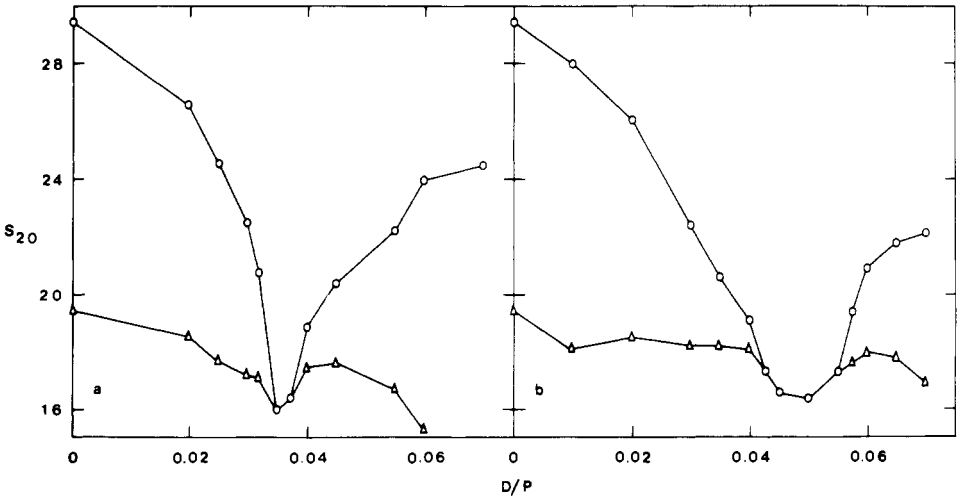


FIGURE 4: Effects of compounds **2** and **5** on the sedimentation coefficient of PM2 DNA. For details see legend to Figure 1. (a) Compound 2; (b) compound 5.

Hence it appears that the chloro and methoxy ring substituents must be responsible for preventing bis-intercalation by the diacridine III described by LePecq et al. (1975). Extending the linker chain of compound **1** by one–CH_2–unit (compound 2, Table I) has little effect on the helix unwinding angle (equivalence input ratio = 0.036 ± 0.003, Figure 4a) and helix

extension parameter (slope = 3.00, Table I). However, the appearance of the equivalence region is unusually sharp (Figure 4a), and the slope of the L/L_0 plot is noticeably less than seen with diacridines constructed with simple aliphatic chains (Wakelin et al., 1976, 1978). These peculiarities reappeared in an exaggerated form with compound **3** (Table I) which has a connecting link derived from spermine. Here the sedimenting species of PM2 DNA seen in the ultracentrifuge photographs appeared as three components which did not coalesce into a single boundary at any ligand concentration. Furthermore, the helix extension plot was again characterized by a low gradient (Figure 3b), and the measurements revealed clear evidence of slow reaction kinetics. The flow time for the drug–DNA complex was found to rise steadily for ~10 min subsequent to addition of an aliquot of this diacridine. However, stopped-flow studies with an analogue of compound **3** substituted at positions 2 and 6 with methoxy and chloro groups, respectively, revealed fast association kinetics accompanied by very slow dissociation processes (Capelle et al., 1979). Thus, it is possible that the slow kinetic effect observed here is related to redistribution of ligand between DNA molecules during mixing of concentrated solutions of reactants.

Compound **4**, having two 9-aminoacridine chromophores linked via $-(CH_2)_2O(CH_2)_2-$ (Table I), provides information on a substitution which has the practical advantage of considerably increasing the aqueous solubility of the diacridine without adding to its overall positive charge. Compound **4** is analogous to the 9-aminoacridine dimer (designated C5) studied by Wakelin et al. (1978), whose linkage comprises a five-membered methylene chain. C5 was found to behave enigmatically, yielding an unwinding angle approaching that of its bifunctional homologues but producing helix extension typical of monofunctional reaction at low levels of binding (Wakelin et al., 1978). These characteristics are accentuated in the results for compound **4** which has an equivalence input ratio of 0.036 ± 0.006 (Table I), a value very typical of bifunctional reaction [cf. results for compounds **1** and **2** and the bifunctional diacridines described by LePecq et al. (1975) and Wakelin et al. (1978)], together with a helix extension plot of slope 1.75, almost identical with the value found for 9-aminoacridine. Thus, substitution of a carbon by an oxygen atom produces little effect on the mode of binding of a diacridine whose chain length lies in the critical region between mono- and bifunctional reaction. One difference between these two analogues, however, is that the helix extension per bound drug molecule is not enhanced at elevated binding ratios for compound **4** unlike the results reported for the C5 diacridine (Wakelin et al., 1978).

Diacridines Bearing Nuclear Substituents and Simple Methylene Bridges. Since the 6-chloro and 2-methoxy substituents of LePecqs' diacridines (LePecq et al., 1975) seem to be the features which prevent bifunctional reaction for the dimer linked via $-(CH_2)_3NH(CH_2)_3-$, we were prompted to investigate the effects of ring substituents on the behavior of diacridines containing simple methylene bridges of similar length. Measurements were first made with quinacrine (Table I) to assess the effects of chloro and methoxy groups on a single intercalating chromophore. The equivalence input ratio was found to be 0.060 ± 0.008 (Table I), corresponding to an unwinding angle of not <22.1°. This may be compared with a value for the equivalence ratio of 0.070 measured in buffer of ionic strength 0.05 and pH 5.0 (Saucier et al., 1971).

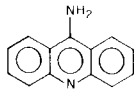
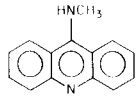
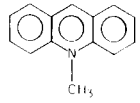
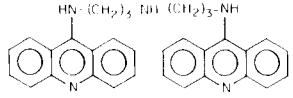
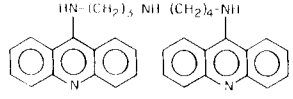
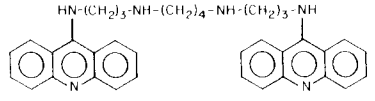
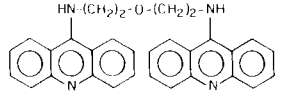
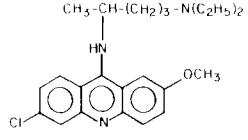
Compound **5** (Table I) has two 6-chloro-2-methoxy-9-aminoacridine chromophores joined via a six-membered methylene bridge, and its equivalence input ratio was measured to be 0.050 ± 0.007 (Figure 4b). Thus the helix unwinding angle of **5** appears somewhat greater than that of quinacrine but less than that which characterizes the bifunctional analogue lacking the ring substituents [designed C6, equivalence input ratio = 0.043 ± 0.010; Wakelin et al. (1976, 1978)]. This ambiquity in the mode of binding of **5** does not extend to its effect on the contour length of DNA. Its limited solubility at pH 7.0 required that helix extension measurements be made at pH 6.0 where it was found that the L/L_0 plot has a slope of 2.28, typical of monofunctional reaction for dimeric acridines (Wakelin et al., 1976, 1978).

Removal of the 2-methoxy group from compound **5** and addition of a further chlorine atom at position 3 (compound **6**) illustrates more clearly the ability of ring substituents to influence the potential for bis-intercalation of diacridines which otherwise possess linker chains sufficiently long to permit bifunctional reaction. Thus, at pH 6.0, compound **6** yields an equivalence input ratio of 0.055 ± 0.010 drug molecules per nucleotide, a value barely distinguishable from that of quinacrine and a helix extension plot of slope 1.75, a value almost identical with that of 9-aminoacridine. However, extending the linker chain by two methylene units (compound **7**) now produces a diacridine with apparently conflicting parameters of intercalation. The equivalence input ratio rises to 0.071 ± 0.006 (Table I) at pH 6.0, a value indicative of monofunctional reaction, whereas the helix extension measurements (conducted at pH 5.0 because of poor solubility in less acid buffers) yield a plot of slope 3.40 which is practically double that found for the shorter chain analogue (compound **6**) and strongly suggestive of bifunctional reaction.

Evidently the position and/or type of substitution of the acridine nucleus is an important factor affecting its ability to intercalate. Compound **8**, in which two 4-ethyl-9-aminoacridine chromophores are again linked via a six-membered methylene bridge, is clearly bifunctional. The equivalence input ratio of 0.031 ± 0.003 and helix extension parameter of 3.71 (Table I) unequivocally demonstrate that addition of an ethyl group at position 4 does not prevent simultaneous intercalation of both acridine rings. However, it was noted that the increment in contour length for complexes with drug/nucleotide ratios of <0.02 was consistently lower than seen at higher levels of binding, suggesting that a proportion of isolated bound molecules may intercalate monofunctionally.

Diacridines Linked via Position 4. Bifunctional bis(9-aminoacridines) linked via the exocyclic amino group are obliged to bind to DNA with the ring nitrogen at position 10 directed toward the intercalation cavity. However, a study of diacridines having two 9-aminoacridine chromophores joined via the 4 position would indicate whether the acridine ring is also capable of intercalation in the reverse orientation, i.e., with the 9-amino group leading the insertion into the binding site. Compound **9** is such a dimer constructed with a long, hydrophilic linker and whose equivalence input ratio of 0.029 ± 0.005 (Figure 5a) clearly places it amongst the bifunctional group of intercalating agents (Waring & Wakelin, 1974; LePecq et al., 1975; Wakelin et al., 1976, 1978). The helix extension plot for compound **9** is well described by a straight line of slope 3.99 up to a drug/nucleotide ratio of 0.025, corroborating bifunctional reaction; above this, however, the slope diminishes to a value of 2.25 pointing to monofunctional reaction as the DNA lattice becomes more fully occupied. It is not likely, under the experimental conditions used, that the binding constant for this diacridine is so small that the bend in the L/L_0 plot results from a significant proportion of added drug remaining free in solution at input ratios as low as 0.03.

Table I

compound	M_r	no./name	buffer	extinction coeff at λ_{max}	equivalence input ratio	slope of L/L_0 plot
 HCl, H_2O	249	9-aminoacridine	A	$E_{398} = 1.01 \times 10^4$	0.076 ± 0.012^a	1.79^a
 HCl	245	9-(methylamino)acridine	A	$E_{404} = 1.10 \times 10^4$	0.073 ± 0.009^a	2.21^a
 Cl^-	250	10-methylacridine	A	$E_{414} = 3.59 \times 10^3$	0.072 ± 0.008^a	
 3HCl, $2H_2O$	631	1	A E	$E_{410} = 1.96 \times 10^4$	0.031 ± 0.006 0.037 ± 0.005	3.20
 3HCl, $3H_2O$	664	2	A	$E_{412} = 1.80 \times 10^4$	0.036 ± 0.003	3.00
 4HCl, $4H_2O$	775	3	A	$E_{412} = 1.81 \times 10^4$		3.05
 2HCl. H_2O	550	4	A	$E_{410} = 1.82 \times 10^4$	0.036 ± 0.006	1.75
 2HCl, $2H_2O$	518	quinacrine	A		0.060 ± 0.008	

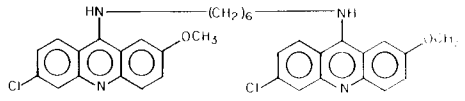
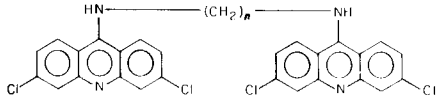
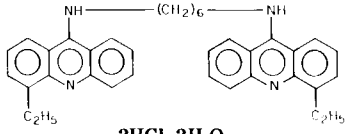
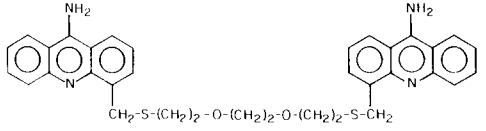
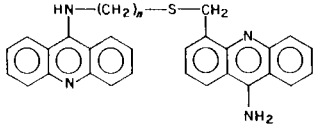
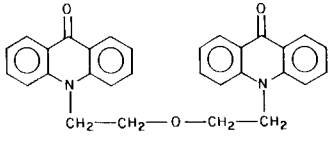
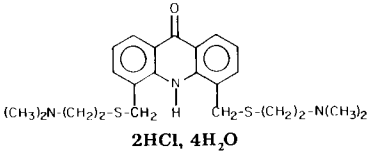
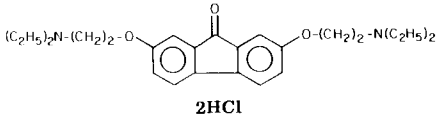
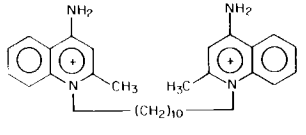
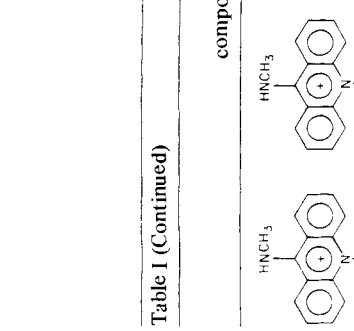
 **2HCl**		672	5	A B	$E_{420} = 1.62 \times 10^4$	0.050 ± 0.007	2.28
 **free base**	$n = 6$ $n = 8$	608 636	6 7	B B D	$E_{405} = 1.71 \times 10^4$ $E_{407} = 1.31 \times 10^4$ $E_{407} = 1.42 \times 10^4$	0.055 ± 0.010 0.071 ± 0.006	1.75 3.40
 **2HCl, 2H$_2$O**		635	8	A	$E_{412} = 2.46 \times 10^4$	0.031 ± 0.003	3.71
 **2HCl, 2H$_2$O**		703	9	A	$E_{405} = 1.30 \times 10^4$	0.029 ± 0.005	3.39 at $D/P < 0.025$ 2.25 at $D/P > 0.030$
 **2HCl, x H$_2$O**	$n = 2$ ($x = 1^1/_4$) $n = 6$ ($x = 3$) $n = 8$ ($x = 1^1/_4$)	556 644 640	10 11 12	A A A	$E_{405} = 1.58 \times 10^4$ $E_{405} = 1.70 \times 10^4$ $E_{407} = 1.51 \times 10^4$	0.058 ± 0.010 0.055 ± 0.010 0.034 ± 0.008	2.13 3.49 at $D/P < 0.05$ 2.03 at $D/P > 0.06$ 3.25 at $D/P < 0.05$ 2.23 at $D/P > 0.06$
		461	13	A B		no detectable binding no detectable binding	
 **2HCl, 4H$_2$O**		574	14	A	$E_{403} = 1.04 \times 10^5$	0.048 ± 0.008	2.22
 **2HCl**		484	tilorone	C		0.128 ± 0.022	
 **2Cl$^-$**		528	dequalinium	A	$E_{328} = 2.75 \times 10^4$	0.11 ± 0.03	

Table I (Continued)

compound	M_r	no./name	buffer	extinction coeff at λ_{max}	equivalence input ratio	slope of L/L_0 plot
 $2Cl^-$, HCl, $^1/_2CH_3OH$	624	15	A	$E_{415} = 2.22 \times 10^4$	0.075 ± 0.010	2.01
HCl	387	16	A		not <0.5	

[a] These values are corrected for binding. See text for details.

Furthermore, the slope remains constant over the remainder of the accessible range (up to $D/P = 0.10$) and does not show increasing deviations from linearity at higher input ratios.

The conclusion that intercalation can occur with 9-aminoacridine bound in either of two distinct orientations is confirmed by the results for a series of diacridines in which the bridge extends from the 9-amino group of one acridine nucleus to the 4 position of the other. Evidence of bifunctionality of longer chain homologues in such a series (compounds 10, 11, and 12, see Table I) necessarily requires that, within the same molecule, one chromophore be bound with the 9-amino group directed toward the helix axis while the other acridine ring must have the N10 nitrogen oriented into the intercalation site. Compound **10** (Table I) has a short linkage and does not have the facility for bifunctional reaction; it therefore stands as a definitive example of a monofunctional member of this class of diacridine. Figure 6a shows that it has an equivalence input ratio of 0.058 ± 0.010; its helix extension plot is a straight line of slope 2.13 with no deviation up to the highest input ratio studied (0.125, see Table I). Lengthening the chain by four methylene groups (compound **11**) has no significant effect on the unwinding of the helix which is characterized by an equivalence input ratio of 0.055 ± 0.010. Judged on this criterion alone compound **11** appears to be monofunctional. However, the viscosity measurements revealed two unusual features. First, the flow time of the drug–DNA complex continued to rise for 5–10 min after mixing the components, attributable to slow reaction kinetics (a similar effect was noted for compound **3**). Second, as found for compound 9, the initial slope of the L/L_0 plot (3.49, see Table I) was typical of bifunctional reaction up to an input ratio of 0.05, whereas at higher levels of binding (D/P(max) = 0.14) the slope fell to 2.03. Thus, the viscosity results for compound **11** are suggestive of bifunctional reaction at low drug/nucleotide ratios with monofunctional intercalation taking over as the degree of binding increases.

Addition of a further two methylene groups to the linking chain (compound **12**) produces a diacridine whose behavior is less equivocal. Figure 6b shows that the equivalence input ratio (0.034 ± 0.008 drug molecules per nucleotide) falls in the range typical of bifunctional reaction: its helix unwinding angle therefore appears a factor of 1.71 larger than that found for the monofunctional analogue **10**. Bifunctional intercalation is also supported by the results of the helix extension measurements up to an input ratio of 0.05 (slope = 3.25), but at higher binding levels the gradient decreases to 2.23. Again, slow reaction kinetics were observed. Hence compound **12** can be identified as an intrinsically bifunctional agent which engages in mixed chromophore orientations when forming a bis-intercalated complex with DNA.

Acridones and Tilorone. The ability of naturally occurring uncharged antibiotics such as the actinomycins, quinomyins, and triostins to intercalate into DNA (Müller & Crothers, 1968; Waring, 1970; Waring & Wakelin, 1974; Lee & Waring, 1978), as well as the example of daunomycin in which a positive charge is located elsewhere than on the anthracycline chromophore, prompted us to study the intercalative potential of acridones. These derivatives of acridine offer the opportunity to investigate the importance of net charge and its distribution, which may prove relevant to future efforts to modify the pharmacological properties of drugs based on polymeric acridines.

Compound **13** (Table I) is a bisacridone linked through the ring nitrogens. It is uncharged in neutral solution and extremely insoluble, in this regard reminiscent of the bifunctional

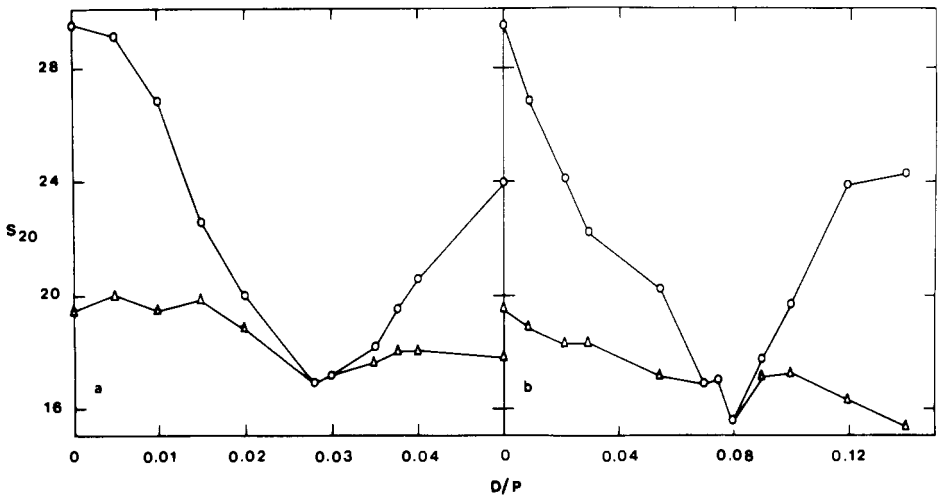


FIGURE 5: Effects of compounds **9** and **15** on the sedimentation coefficient of PM2 DNA. For details see legend to Figure 1. (a) Compound 9; (b) compound 15.

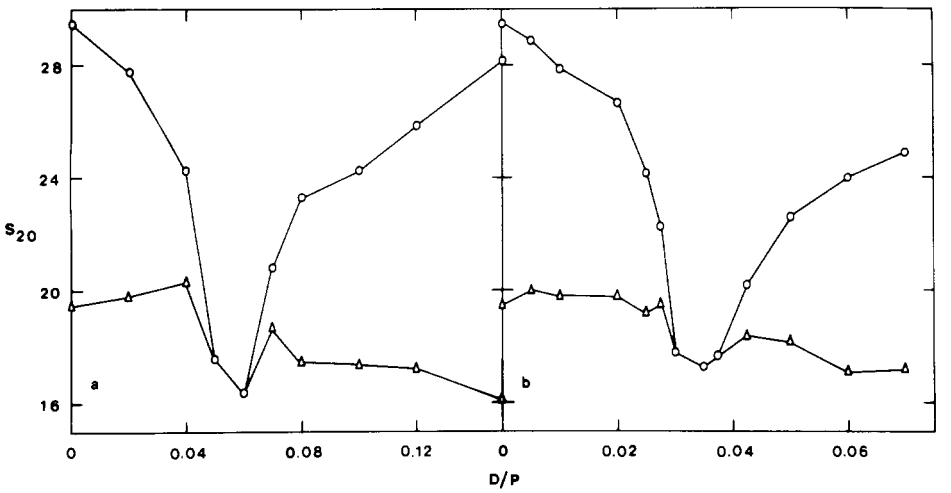


FIGURE 6: Effects of compounds **10** and **12** on the sedimentation coefficient of PM2 DNA. For details see legend to Figure 1. (a) Compound 10; (b) compound 12.

intercalating antibiotic echinomycin (Waring & Wakelin, 1974). However, unlike echinomycin, it was not possible to detect the formation of a complex with DNA at all, even by shaking the solid material with moderately concentrated DNA solutions at pH 6 or 7 for several hours. Therefore, either this bisacridone has no affinity for DNA or its aqueous solubility is so low as to prevent the attainment of significant (detectable) levels of binding.

A soluble mononuclear acridone was synthesized by substituting basic functions at positions 4 and 5 (compound **14**); it bears two positive charges centered on the amino nitrogens which are separated from the chromophore by four intervening atoms. This acridone was found to remove and reverse the supercoiling of PM2 DNA in the fashion characteristic of intercalating agents with an equivalence input ratio of 0.048 ± 0.008, a value indistinguishable from that of ethidium (Wakelin & Waring, 1974). Like established intercalating agents, it also decreased the sedimentation coefficient of nicked circular PM2 DNA, and its visible absorption peak displayed bathochromic and hypochromic shifts on binding. Furthermore, it caused a linear increase in contour length up to the highest drug/nucleotide ratio studied (0.12), the slope of the L/L_0 plot being 2.22. These findings establish that a charged acridone in which the charge is centered some distance from the aromatic ring (which classically would be considered electrically neutral) is capable of binding to DNA by an intercalation process, the parameters of which are typical of other intercalating ligands.

The antiviral agent tilorone bears an interesting structural resemblence to the mononuclear acridone (Table I) which prompted a comparison of their helix unwinding capacities. Again, tilorone affords an example where the positive charge of a potential intercalating drug molecule is not located on the aromatic chromophore. Figure 7a shows that titrating PM2 DNA with tilorone in buffer of ionic strength 0.027 elicits the typical intercalation response with an equivalence input ratio of 0.128 ± 0.022. Chandra & Woltersdorf (1976) showed that the apparent association constant for tilorone binding to calf thymus DNA at ionic strength 0.1 was very similar to that reported for ethidium under comparable conditions (LePecq & Paoletti, 1967), and Chandra et al. (1972) demonstrated that, like ethidium, the affinity of tilorone for DNA increases at lower salt concentrations. Hence, by analogy with the binding behavior of ethidium the equivalence binding ratio for tilorone may be estimated as ~0.10 drug molecules per nucleotide, corresponding to a helix unwinding angle about half that of ethidium. The substantial difference between the helix unwinding behavior of tilorone and the acridone may reflect differences in the structure of their intercalation complexes. For example, the acridone has the facility for binding with the chromophore fully inserted into the intercalation site and the side chains projecting toward phosphate groups in the same

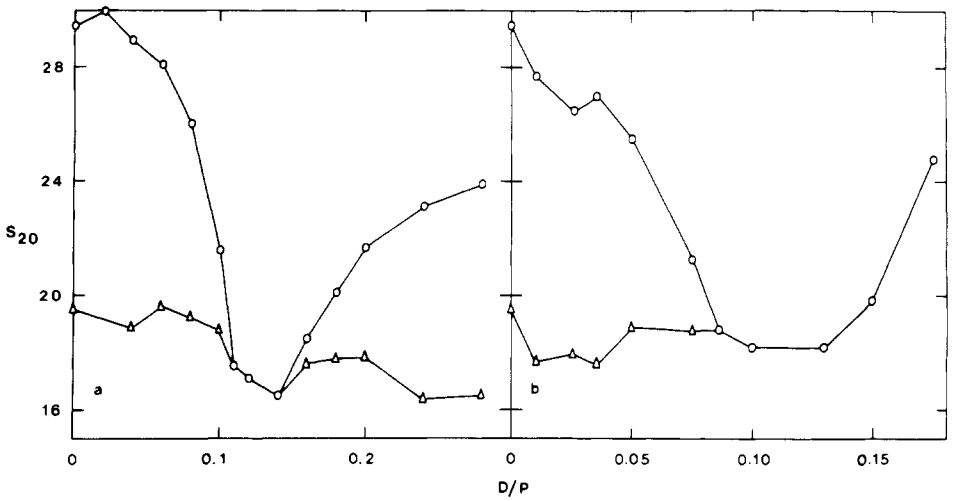


FIGURE 7: Effects of tilorone and dequalinium on the sedimentation coefficient of PM2 DNA. For details see legend to Figure 1. (a) Tilorone dihydrochloride; (b) dequalinium dihydrochloride.

DNA groove. However, this configuration for tilorone would yield markedly poorer stacking interaction between its chromophore and the DNA bases because of the position of attachment of the side chains to the fluorenone ring. Hence, tilorone might increase its hydrophobic binding energy by intercalating "sideways" thereby projecting one side chain into each groove in a fashion similar to that suggested for a dicarbethoxyamino derivative of ethidium whose unwinding effect on PM2 DNA is experimentally indistinguishable from that found for tilorone (Wakelin & Waring, 1974).

Bisquaternary Compounds. Two compounds were examined to investigate the effects of bridging chromophores via their ring nitrogen atoms. Dequalinium (Table I) is a clinically useful antibacterial bisquinoline in which two 2-methyl-4-aminoquinoline rings are linked via a 10-membered methylene chain. It adsorbs strongly to glassware from aqueous solution; accordingly, all glass surfaces were siliconized and saturated with drug prior to use. Figure 7b reveals that dequalinium removes and reverses the supercoiling of PM2 DNA with an equivalence input ratio of 0.11 ± 0.03. At drug/nucleotide ratios of 0.15 and 0.175, the nicked circular DNA appeared to be selectively precipitated from solution; at higher drug concentrations, both nicked and closed circular species were precipitated, presumably due to the formation of an electrostatically neutral complex. The apparent helix unwinding angle of dequalinium is calculated to be not <12.1°, which probably assigns it to the monofunctional group of intercalating agents, although it is not possible rigorously to rule out bifunctional reaction in the absence of DNA binding data. However, as much as 60–70% of the drug added at equivalence would have to be free if dequalinium intercalates bifunctionally (assuming normal unwinding angles) which would indicate an exceptionally poor affinity constant. It is of interest that Cain et al. (1978) synthesized a comparable bisquinoline compound which also showed only monofunctional reaction. These findings suggest that simple bisquinoline compounds linked via flexible bridges may be unable to establish bis-intercalated complexes. By contrast, the relatively rigid peptide linkage in quinoxaline antibiotics seems actively to promote bifunctional reaction as observed for the bisquinoline analogue of echinomycin (Gauvreau et al., 1980).

Compound **15** is a quaternary dimer of 9-(methylamino)-acridine linked via a six-membered methylene chain, and Figure 5b shows that it yields an equivalence input ratio of 0.075 ± 0.010. Its helix extension plot is well described by a straight line of slope 2.01 up to the highest input ratio studied (0.08). These parameters are extremely similar to those of the 9-(methylamino)acridine monomer (see Table I) and clearly identify **15** as a monofunctional intercalator. Thus the length of the $-(CH_2)_6-$ chain is insufficient to permit bifunctional reaction when attached via the ring nitrogen as opposed to the other locations around the acridine ring system.

A tert-Butyl-9-aminoacridine. Müller et al. (1973) demonstrated that 2,7-di-*tert*-butylproflavin binds to DNA by a nonintercalative mechanism which does not involve detectable unwinding of the DNA helix. Experiments with a derivative of 2,7-di-*tert*-butyl-9-aminoacridine (compound **16**) showed that this compound relaxed the supercoiling of PM2 DNA at an input ratio of 0.5 but failed to reverse it at the highest accessible ligand concentration. While this result cannot be taken as definitive evidence of normal intercalation, it does reveal a significant difference between the modes of binding of 2,7-di-*tert*-butyl derivatives of 9-aminoacridine and proflavin. Compound **16** evidently affects the winding of the DNA helix, perhaps as a result of partial intercalation of the chromophore at, for example, a kinked binding site (Sobell et al., 1977).

Discussion

Effects of Ring and Linker Substituents on Bis-Intercalation. The major conclusions from this work bear on the structural features which dictate the length of linker chain required to permit a potentially bifunctional diacridine to bind to DNA bis-intercalatively. The results for compound **1** clearly demonstrate that removal of the chloro and methoxy groups from the monofunctional diacridine III of LePecq et al. (1975) permits bis-intercalation. LePecq et al. (1975) calculated the distance between the 9-amino group nitrogen atoms in the fully extended chain of diacridine III to be 9.9 Å and surmised that this is insufficiently long to accommodate two base pairs. If this is so, it follows that the bis-intercalated complex observed on removal of the ring substituents must involve a one base pair sandwich structure, assuming a Fuller-Waring (Fuller & Waring, 1964) type of intercalation model [see Wakelin et al. (1978)].

The dominant effect of ring substituents is substantiated by the finding that addition of chloro and methoxy groups to the acridine nuclei of the simple *n*-hexane-bridged diacridine of Wakelin et al. (1978) (designated C6), whose linkage was found to be the shortest compatible with bifunctional intercalation, restricts the ligand to monofunctional reaction (compound **5**). Similarly, substitution of C6 with 3,6-dichloro

residues prevents bifunctional reaction (compound **6**). Lengthening the methylene chain of the latter diacridine to one of eight carbon atoms (compound **7**) produces a derivative whose maximum interchromophore distance is 11.3 Å [see Wakelin et al. (1978)] and whose helix extension speaks for bifunctional reaction.

The ability of compound **8**, the ethyl derivative of the C6 diacridine, to intercalate bifunctionally shows that simple alkyl groups in position 4 are able to penetrate through the DNA helix and do not prevent insertion of either of the acridine chromophores. A similar observation has been made for analogues of actinomycin in which the methyl groups in positions 4 and 6 of the phenoxazone chromophore have been replaced by ethyl and other alkyl residues (Meienhofer & Atherton, 1977). The capacity of acridine positions 4 and 5 to bear bulky substituents without inhibition of biological activity has also been noted for antitumor drugs in the acridinylmethanesulfonanilide series (Cain et al., 1977). All these results can be rationalized by postulating that intercalation is relatively insensitive to substituents which are positioned near the minor axis of the acridine nucleus and that it is the dimension of the long axis of the acridine chromophore which determines the critical length of linker chain needed to permit bifunctional reaction.

Effects of Linker Amino Groups on Parameters of Intercalation. In comparing the results for simple methylene-bridged diacridines reported by Wakelin et al. (1978) with those for equivalent molecules having nitrogen atoms in the linker chain, two significant differences are noticeable. First, the slopes of the L/L_0 plots for the nitrogen-substituted compounds spread from 3.0 to 3.2 whereas those for their pure methylene homologues are grouped about the value 3.6. Second, the equivalence input ratios for the simple alkane derivatives are substantially larger than those found here for compounds **1** and **2** under identical ionic conditions. Thus incorporation of charged amino groups into the linkage causes an apparent reduction in helix extension by approximately 11–18% with a concomitant increase in unwinding angle of approximately 30–40%. It is plausible that these changes are a consequence of hydrogen and/or ionic bonding interactions of the amino groups with the base carbonyl and/or sugar phosphate oxygen atoms of the DNA, which induce additional unwinding of the helix and possibly introduce a degree of kinking or bending into the polymer. The anomalous behavior of compound **3** in the ultracentrifuge experiments, which are to some extent presaged by the unusually sharp equivalence region found for compound **2**, and the exceptionally slow reaction kinetics of this diacridine suggest that the mechanism of binding becomes more complex with increasing numbers of amino group substituents in the linking chain. It is possible that these irregular phenomena are related to the ability to form inter- or intrastrand cross-links, which may or may not lead to direct transfer between binding sites (Bresloff & Crothers, 1975; Capelle et al., 1979; Wakelin & Waring, 1980) with diacridines of this type.

Bis-Intercalated Complexes with Different Chromophore Orientations. The results for compounds **9**, **10**, **11**, and **12** reveal that the acridine ring can intercalate with at least two different orientations in the DNA binding site. Moreover, intercalation via both of these configurations can be accommodated in a single diacridine (compound **12**) with an internuclear separation that permits the formation of a two base pair sandwich. Thus, neither form of the intercalated complex causes sufficiently extensive structural changes in the polynucleotide backbone or linker chain to inhibit binding at nearby intercalation sites. It is interesting that the helix extension measurements for compounds **9** and **12** show the first clear evidence of conversion from bifunctional to monofunctional reaction as the level of binding increases. This distinguishes them from the simple bifunctional methylene-bridged and polyamine-linked dimers and suggests that bis-intercalation may be inherently less stable for asymmetrically connected diacridines and those whose bifunctionally intercalated complexes involve mixed chromophore orientations.

Acridones. In addition to reacting with nucleic acids, positively charged acridines have a marked affinity for proteins (Nicholson, 1973) and undoubtedly exhibit a general tendency to bind to negatively charged macromolecules in vivo, which may partly explain their poor selective toxicity when used as chemotherapeutic agents. The construction of polymeric acridines having numerous positive charges might well exaggerate these undesirable features. The present finding that acridone serves as a perfectly acceptable intercalating chromophore suggests that it may be better suited to the development of polyfunctional agents since the net molecular charge, and its distribution, can be controlled and is not dictated by the use of quaternized or protonated aminoacridine rings.

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Carcinogen Aflatoxin B_1 Is Located Preferentially in Internucleosomal Deoxyribonucleic Acid following Exposure in Vivo in Rainbow Trout†

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ABSTRACT: The purpose of this work was to investigate the distribution in chromatin of deoxyribonucleic acid (DNA) adducts of aflatoxin B_1, following exposure in vivo. Rainbow trout were injected intraperitoneally with radiolabeled aflatoxin B_1, a potent procarcinogen known to readily induce hepatocellular carcinomas in these fish. After maximum incorporation, liver nuclei were prepared and digested with micrococcal nuclease. Mono-, di-, and trinucleosomal fractions were purified from several stages of nuclease digestion, and the lengths and specific activities of their DNA were determined. The results indicate that aflatoxin B_1 is ~5 times as likely on a per nucleotide basis to localize on internucleosomal (linker) DNA as on nucleosomal core DNA in this system.

Although chemical carcinogens are believed to initiate their action through mutagenic interaction with the genome (Miller & Miller, 1971; Ames, 1979), our knowledge of the actual sites of carcinogen attack in higher organisms is at present very limited. One initial approach to this problem involves examining the influence of those chromatin structural features which one can clearly define on the accessibility of carcinogens to DNA in vivo. The genome of all eukaryotes is now recognized to be organized into a repeating subunit structure [for reviews, see Kornberg (1977); Garret (1979)]. This structure, called the nucleosome, consists of about 165-245 base pairs of DNA[1] together with all of the histones. The nucleosome can in turn be subdivided into a ubiquitous "core particle" containing 146 base pairs of DNA wound one and three-fourths turns around a core of eight histone molecules (two each of histones H_2A, H_2B, H_3, and H_4) and a variable region of 20-70 base pairs of "linker" DNA. The linker provides additional binding sites for histones H1 and other nonhistone proteins of suspected regulatory or structural function (Noll & Kornberg, 1977; Lohr et al., 1977a; Levy-Wilson et al., 1979; Weisbrod et al., 1980). These elements, perhaps in combination with local variations in histone sequence (Spinelli et al., 1979) or modification (Davie & Candido, 1978) are believed to control chromatin configuration in some manner which reflects, and perhaps mediates, gene expression and differentiation patterns. For example, regions of the genome enriched in transcribing sequences appear to be preferentially digested by brief treatment of chromatin with pancreatic DNase I (Weintraub & Groudine, 1976; Garel & Axel, 1976; Weisbrod et al., 1980) though the structural basis for this is incompletely defined at present.

Several groups have recently begun to investigate the chromosomal distribution of carcinogen-DNA adducts formed in vivo in animal systems by using DNase I or micrococcal nuclease (MNase), both of which can selectively degrade linker DNA under appropriate conditions, as probes of chromatin structure. Adducts of the bulky procarcinogen *N*-hydroxy-2-acetylaminofluorene were reported to be preferentially distributed in the acid-soluble fractions upon exhaustic digestion of rat liver nuclei with MNase (Metzger et al., 1977), suggesting a preferred linker distribution for this carcinogen. However, the same adducts were also found to be enriched in the fractions resistant to exhaustive DNase I digestion (Ramanathan et al., 1976a; Metzger et al., 1977) which implies a preferred core DNA distribution. By comparison,

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[1] Abbreviations used: DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; dpm, disintegrations per minute; $NaDodSO_4$, sodium dodecyl sulfate.

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