Summary

The experimental results presented here may be recapitulated as follows. For uroporphyrin I interacting with quinine, one to one stoichiometry and noncooperative binding is found. The ring current shifts indicate a $\pi-\pi$ type dimer is formed in solution with a structure like that shown in Figure 8A.

For urohemin I interacting with quinine, the minimum stoichiometry is two urohemins bound to one quinine. Consistent with the general carbon NMR shift pattern of the uroporphyrin–quinine complex and the NMR line broadening, a π type sandwich structure is postulated. However, this system exhibits cooperativity when quinine binds to urohemin I, indicating two nonequivalent interacting sites for quinine binding on a given urohemin molecule. One of these sites must be characterized by the aromatic ring–ring interaction that contributes to the sandwich structure postulated here and to the protohemin IX–quinine complex previously identified.[4] The other site is most likely axial iron ion coordination by the quinine via its 9-hydroxyl group to one of the urohemins, as has been shown to exist in other systems.[5] Our data give no evidence for deciding whether the coordination is carried out by an alkoxide ($-O^-$) or a hydroxyl (–OH) moiety. Note that cooperativity is absent in quinine binding to uroporphyrin I where axial coordination is an impossibility. Again, the line broadening of the 9- and 8-position quinine carbons is consistent with this postulate, and our results indicate no other simple possibilities that can account for all of the data presented here. The minimal unit structure for this complex that fits our data is shown in Figure 8B.

(36) Constantinidis, I.; Satterlee, J. D. *J. Am. Chem. Soc.*, in press.

Additional support for this concept comes from completed work[36] with the urohemin I–chloroquine complex. Chloroquine has a structure similar to quinine in that both possess the quinoline type aromatic rings. However, the aliphatic substituents at position 4′ are different, and in the case of chloroquine, the 9-position carbon (C–OH in quinine) is replaced by a nitrogen, rendering axial ligation impossible. Our results show that chloroquine binding to urohemin is noncooperative.[36]

In view of these results the proton NMR characterization of quinine interacting with aqueous protohemin IX and the conclusion reached that iron ion coordination was not indicated require additional scrutiny.[4] It seems obvious to us that the reported strong perturbation of the quinine 9-, 2-, 6-, and 8-position proton resonances upon addition of increasing amounts of protohemin IX[4] can easily be interpreted as indicating 9-OH coordination to heme iron. It is clear from model building studies (and noted by Behere and Goff[5]) that conformations of quinine exist in which the quinuclidine 2-, 6-, and 8-position proton resonances would be perturbed owing to 9-OH coordination to the heme iron ion.

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Registry No. Quinine, 130-95-0; urohemin I chloride, 92284-96-3; uroporphyrin I, 607-14-7.

Interactions between DNA and Mono-, Bis-, Tris-, Tetrakis-, and Hexakis(aminoacridines). A Linear and Circular Dichroism, Electric Orientation Relaxation, Viscometry, and Equilibrium Study

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Abstract: The interaction between DNA and a series of mono-, bis-, tris-, tetrakis-, and hexakis-intercalating 9-aminoacridines has been studied with flow linear dichroism (LD), circular dichroism (CD), electric orientation relaxation (EOR) techniques, and with viscometry and equilibrium analyses. The orientation of the 9-aminoacridine ligand relative to the average orientation of the DNA bases, measured by LD, shows that with both 9-aminoacridine and the bis(acridines) the in-plane short axes of the acridine ligands are oriented perfectly parallel to the planes of the DNA bases, as expected for classical intercalation, whereas the long axes are found to be significantly tilted. This is supported by the DNA lengthening measured by EOR, which for 9-aminoacridine is 1.5 base-pair units, compared with 1.0 for ethidium bromide. Also in case of the tris(acridines) LD, CD, viscometry, and equilibrium data indicate that all acridine ligands are intercalated. The binding analysis shows an increasing degree of cooperativity in the sequence 9-aminoacridine < bis(acridines) < tris(acridines), and the corresponding binding densities, 4, 8, and 11–14, respectively, are in good agreement with those expected from the nearest-neighbor exclusion principle. The LD and CD measurements show that the tetrakis- and hexakis(acridines), despite long and flexible links, bind to DNA with only three of the acridine ligands intercalated.

The DNA intercalation of polycyclic aromatic compounds has been the subject of much interest due to their potential as cytostatic agents and as probes in molecular biology.[1-5]

Binding to DNA by intercalation was originally explained by Lerman[6] as a process whereby the intercalator was sandwiched

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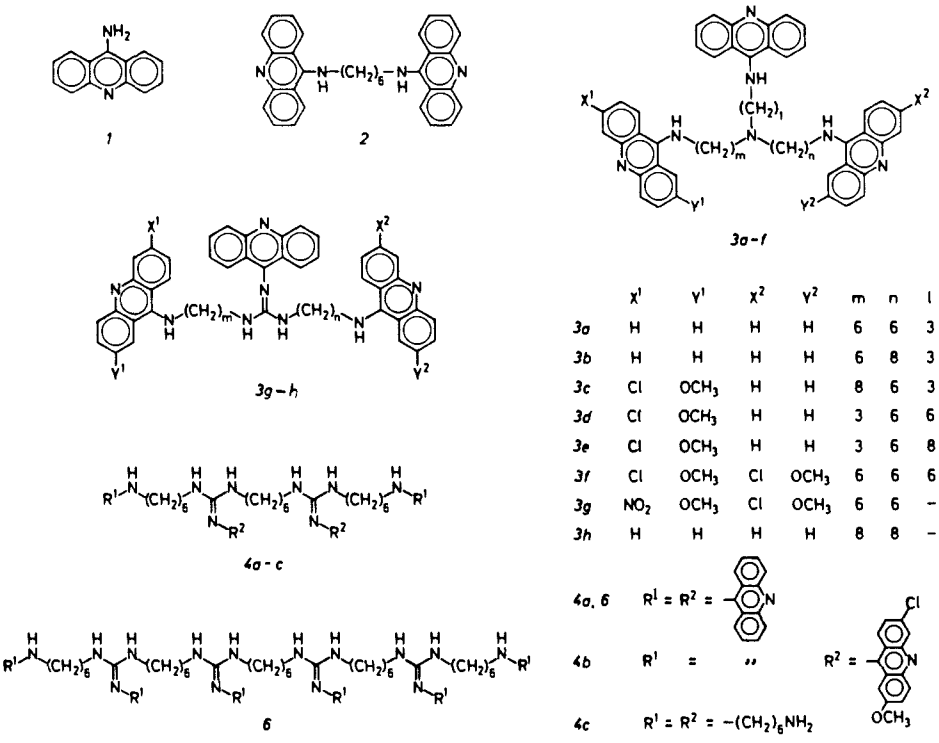
(1) Capelle, N.; Barbet, J.; Dessen, P.; Blanquet, S.; Roques, B. P.; Le Pecq, J. B. *Biochemistry* **1979**, *15*, 3354-3360.
(2) Shafer, R. H.; Waring, M. J. *Biopolymers* **1980**, *19*, 431-443.

0002-7863/88/1510-0932$01.50/0

Chart I



	X¹	Y¹	X²	Y²	m	n	l
3a	H	H	H	H	6	6	3
3b	H	H	H	H	6	8	3
3c	Cl	OCH_3	H	H	8	6	3
3d	Cl	OCH_3	H	H	3	6	6
3e	Cl	OCH_3	H	H	3	6	8
3f	Cl	OCH_3	Cl	OCH_3	6	6	6
3g	NO_2	OCH_3	Cl	OCH_3	6	6	-
3h	H	H	H	H	8	8	-

between two adjacent base pairs. According to the model, the intercalator is coplanar with the base pairs and lengthens the helix by 3.4 Å, corresponding to the van der Waals thickness of an aromatic intercalator, while simultaneously resulting in an unwinding of the DNA helix.

The binding is in part due to hydrophobic and dispersive forces between the aromatic moieties of the bases and the intercalator. However, in most cases electrostatic interactions between the phosphates of the DNA backbone and cationic groups of the intercalator also play an important role in the binding.

The static interaction between DNA and several intercalators has been studied by X-ray diffraction of di- and oligonucleotide complexes,[7,8] which has confirmed the model. Furthermore, the dynamic interactions between DNA and intercalators have been examined by absorption and emission spectroscopy,[9] hydrodynamic techniques,[10] and NMR spectroscopy.[11] The results from these studies also support the general intercalation model but show that the individual intercalators interact differently with the DNA on the molecular level. By introducing more than one potentially intercalating ligand in the same molecule it has been shown that bis-[12] and trisintercalation[9,10] can occur.

We have synthesized and examined a large number of mono and potential bis and higher intercalators in order to examine their cytostatic activity, as well as their application as molecular photoprobes. An integral part of this project involves in vitro investigations of the molecular binding properties to DNA. In the present study we applied a number of spectroscopic techniques amended in our laboratory to selected oligoaminoacridines for the purpose of studying DNA binding geometry: specifically, phase-modulation detected flow linear dichroism (LD), DNA-induced circular dichroism (CD), and electric orientation relaxation (EOR) have been studied at very low reagent/DNA binding ratios. In addition, the DNA affinity of these reagents has been studied by using a two-phase partition equilibrium technique; viscometry has been employed in the range of intermediate to high reagent/DNA ratios.

Materials and Methods

Chemical Reagents. Acridines. All of the acridine compounds were stored as hydrochlorides, and the pH 7 used in all measurements assures that they are positively charged when examined for intercalation. 9-Aminoacridine hydrochloride was obtained from Ega Chemie and used without further purification. The following acridines were prepared as previously described or analogously.[13-15] These compounds do not have well-defined melting points. The structural formulas are presented in Chart I. **2**·2HCl·2H_2O,[13] **3a**·4HCl,[14] **3g**·3HCl·3H_2O, and **3h**·3HCl·3H_2O[15] were samples from the previous publications.

3b·4HCl·4H_2O.[14] Anal. Found (Calcd) for $C_{56}H_{61}N_7$·4HCl·4H_2O: C, 64.72 (64.05); H, 7.42 (7.01); N, 8.62 (9.34); Cl, 12.69 (13.50).

3c·3HCl·4H_2O.[14] Anal. Found (Calcd) for $C_{56}H_{63}N_7Cl_2O_2$·3HCl·4H_2O: C, 60.68 (60.97); H, 7.00 (6.52); N, 7.68 (8.58); Cl, 16.44 (15.52).

3d·4HCl·4H_2O.[14] Anal. Found (Calcd) for $C_{55}H_{58}N_7ClO$·4HCl·4H_2O: C, 60.72 (60.80); H, 7.01 (6.49); N, 8.38 (9.03); Cl, 16.89 (16.32).

3e·3HCl·4H_2O.[14] Anal. Found (Calcd) for $C_{57}H_{62}N_7ClO$·3HCl·4H_2O: C, 64.11 (63.50); H, 7.14 (6.83); N, 8.34 (9.10); Cl, 14.73 (13.16).

3f·4HCl·4H_2O.[14] Anal. Found (Calcd) for $C_{56}H_{59}N_7Cl_2O_2$·4HCl·4H_2O: C, 59.69 (58.44); H, 7.07 (6.62); N, 8.88 (8.52); Cl, 17.35 (18.48).

4a·5HCl·8H_2O.[15] Anal. Found (Calcd) for $C_{72}H_{74}N_{12}$·5HCl·8H_2O: C, 60.67 (60.31); H, 6.44 (6.68); N, 12.57 (11.72); Cl 13.63 (12.36).

4b·5HCl·8H_2O.[15] Anal. Found (Calcd) for $C_{74}H_{76}N_{12}Cl_2O_2$·5HCl·8$H_2O$: C, 56.58 (56.87); H, 6.70 (6.25); N, 12.51 (10.24); Cl, 16.24 (15.88).

Compound **6** was prepared from the corresponding **4c**, which in turn was prepared from *N*-9-acridinyl-*N'*-[6-[(*tert*-butoxycarbonyl)amino]-

(3) Waring, M. J. *Annu. Rev. Biochem.* **1981**, *50*, 159-192.
(4) Topal, M. D. *Biochemistry* **1984**, *23*, 2367-2372.
(5) Wakelin, L. P. G. *Med. Res. Rev.* **1986**, *6*, 275-340.
(6) Lerman, L. S. *J. Mol. Biol.* **1961**, *3*, 18-30.
(7) Wang, A. H. J.; Ughetto, G.; Quigley, G. J.; Hakoshima, T.; van der Marel, G. A.; van Boom, J. H.; Rich, A. *Science (Washington, D.C.)* **1984**, *225*, 1115-1121.
(8) Ughetto, G.; Wang, A. H. J.; Quigley, G. J.; van der Marel, G. A.; van Boom, J. H.; Rich, A. *Nucleic Acids Res.* **1985**, *13*, 2305-2323.
(9) Atwell, G. J.; Leupin, W.; Twigden, S. J.; Denny, W. F. *J. Am. Chem. Soc.* **1983**, *105*, 1913-1914.
(10) Hansen, J. B.; Koch, T.; Buchardt, O.; Nielsen, P. E.; Wirth, M.; Nordén, B. *Biochemistry* **1983**, *22*, 4878-4886.
(11) Delbarre, A.; Shafer, R. H.; James, T. L. *Biopolymers* **1983**, *22*, 2497-2500.
(12) Shafer, R.; Waring, M. J. *Biopolymers* **1982**, *21*, 2279-2290.
(13) Hansen, J. B.; Langvad, E.; Frandsen, F.; Buchardt, O. *J. Med. Chem.* **1983**, *26*, 1510-1514.
(14) Hansen, J. B.; Buchardt, O. *J. Chem. Soc., Chem. Commun.* **1983**, 162-163.
(15) Hansen, J. B.; Thomsen, T.; Buchardt, O. *J. Chem. Soc., Chem. Commun.* **1983**, 1015-1016.

hexyl]thiourea (**5**) and 1,6-bis[9-acridinyl(thiocarbaminoyl)amino]hexane (**7**), which again was prepared from 9-isothiocyanatoacridine and 1,6-hexanediamine.[15]

7: mp 192–193 °C. Anal. Found (Calcd) for $C_{34}H_{32}N_6S_2$: C, 67.98 (69.35); H, 5.65 (5.49); N, 14.20 (14.28); S, 10.76 (10.86).

4c·9HCl·18H_2O. Anal. Found (Calcd) for $C_{86}H_{104}N_{18}$·9HCl·18H_2O: C, 49.92 (50.57); H, 6.18 (7.35); N, 12.18 (12.35); Cl, 15.46 (15.63).

5: mp 174–175 °C. Anal. Found (Calcd) for $C_{25}H_{32}N_4O_2S$: C, 66.27 (66.34); H, 7.13 (7.02); N, 12.41 (12.38); S, 7.20 (7.08).

6·7HCl·10H_2O. Anal. Found (Calcd) for $C_{112}H_{118}N_{20}$·7HCl·10H_2O: C, 61.55 (61.71); H, 5.77 (6.70); N, 11.86 (12.85); Cl, 11.10 (11.34).

The ^1H NMR spectra of all of the acridines were in correspondence with the assigned structures. Calf thymus DNA was of commercial quality (Sigma Chemical Co, calf thymus DNA type I), and the remaining unknown salt in the commerical DNA was of negligible importance. The A_{260}/A_{280} ratio indicated that the protein content was less than 2%. Control experiments with further purified DNA (phenol and ethanol treatment) indicated that remaining impurities could not have any significance for the present results. Other reagents were of p.a. grade. Dextran (MW 500 000) was purchased from Pharmacia Co, Uppsala, Sweden, and poly(ethylene glycol) (MW 6000) from Union Carbide Co. Poly(dGdC) with an average of 500 base pairs was a product from Pharmacia Co.

Stock solutions of DNA were prepared in distilled and Millipore-deionized water containing NaCl (p.a.) and Na_2EDTA (p.a.). The DNA concentration was, when not otherwise stated, 0.7 mM nucleotide in 1 mM NaCl and 1 mM Na_2EDTA. Stock solutions of the ligands were prepared by dissolving the compounds in distilled water and adding NaCl and Na_2EDTA to obtain solutions approximately 0.1 mM ligand in 1 mM NaCl and 1 mM Na_2EDTA. The final ligand concentration was determined spectrophotometrically on a Cary 219 spectrophotometer.

Short DNA for electric orientation relaxation and viscometry was obtained by sonicating calf thymus DNA (sonicator equipment: Sonifier B-12, Branson Sonic Power Co.) and thereafter fractionating the product on a column of Sephacryl S500 (Pharmacia) to obtain a homogeneous fraction of DNA with an average length of 210 base pairs. The effect of enzymatic removal of "blunt ends" was tested. In fact, for the purpose of determining the relative length increase of DNA upon interaction with the ligands, the EOR curve obtained from sonicated DNA, without further treatment, was found to be fully sufficient.

Linear Dichroism. Linear dichroism (LD) is defined (eq 1) as the differential absorption of orthogonal forms of linearly polarized light, where $A_\parallel$ denotes the absorbance of the sample measured with the electric vector of light polarized parallel to the macroscopic orientation direction (equal to the direction of flow), and $A_\perp$ is the corresponding perpendicular absorbance. By dividing LD with the absorbance of the unoriented

$$\mathrm{LD} = A_\parallel - A_\perp \tag{1}$$

sample at rest, A_{iso}, the "reduced" linear dichroism, $\mathrm{LD_r} = \mathrm{LD}/A_{iso}$, is defined, which may be related to the orientation (S) of DNA and the angle (α) between the respective light-absorbing transition moment and the DNA helix axis according to eq 2.[16] Flow orientation was produced

$$\mathrm{LD_r} = S \times {}^1/_2(3\cos^2\alpha - 1) \tag{2}$$

in a Couette cell (with either the inner or the outer cylinder rotating). As a consequence of a non-uniaxial orientation distribution, the function S will become a rather complex average over two angular coordinates.[17] It will therefore be determined in an empirical way from the $\mathrm{LD_r}$ value for the intrinsic DNA absorption and the assumption that $\alpha = 90°$ for the DNA bases. This is a simplification, the justification of which will be considered later. Then eq 2 can be used to provide a "characteristic angle" for the ligand orientation (eq 3). It should be noted that this

$$\alpha_L = \arccos\,[{}^1/_3 - (\mathrm{LD_r})_L/3(\mathrm{LD_r})_{DNA}]^{1/2} \tag{3}$$

application assumes that (1) the ligand is not heterogeneously distributed over DNA regions with different "local" orientations (or it is assumed that S of eq 2 is the same for all bases) and (2) the DNA structure can be represented with a fixed orientation ($\alpha = 90°$) of the bases (the Watson–Crick structure). This interpretation serves the purpose of providing a sensitive measure of the *ligand orientation relative to the DNA bases*. For example, if an apparent $\alpha_L = 90°$ is obtained for a certain ligand absorption band, this indicates that the corresponding ligand transition is most probably parallel to the DNA base plane, whereas a value significantly smaller than 90° indicates a corresponding deviation from coplanarity. It should be emphasized that our model does not exclude the possibility of isotropic deviations (either static or dynamic) of the DNA base planes from the perpendicular orientation, but any such deviation will be included in S instead of in α. Thus, a higher $|\mathrm{LD_r}|$ amplitude than for the DNA bases has in a few cases been observed for some nonintercalating ligands by Nordén et al.,[17] indicating a tilt of the bases. As to the question of whether the bases are tilted with respect to a preferred inclination axis, see the Discussion. Against this background and to avoid confusion, we will use the angle $\pi/2 - \alpha$ to denote the deviation of the ligand from coplanarity with the bases.

The flow cell was of a design according to Wada and Kozawa,[18] and the light was propagated through the quartz cylinder, perpendicular to the rotation axis. LD was measured with a phase-modulation technique on a Jasco J-500 spectropolarimeter modified with a quarter-wave device as described previously.[19,20] DNA was oriented at a spinning rate corresponding to the flow gradient $G = 1200 \pm 2\ s^{-1}$.

Circular Dichroism. Circular dichroism (CD) of the DNA adduct systems provides information on two levels. First, the (average) local conformation of DNA is represented through the CD of the intrinsic DNA absorption centered at 260 nm. Second, independent information about the ligand orientation relative to the bases can be obtained from any CD induced in the ligand absorption bands as has been shown by Nordén and Tjerneld.[21] This induced CD can be interpreted in terms of a nondegenerate perturbation mechanism, at sufficiently low ligand/DNA binding ratios (to exclude ligand–ligand interactions),[22] mainly through pair-wise couplings between electric dipole allowed transitions of the ligand and in the adjacent DNA bases. The rotational strength in the ligand absorption region (R_L) can be expressed by eq 4,

$$R_L = f|\mu|^2(1 - 2\cos^2\gamma) \tag{4}$$

where f is a constant factor dependent on the DNA structure (negative for B form DNA),[21,23] μ is the ligand transition moment, and γ is an "azimuthal angle" (in a plane perpendicular to the helix axis) defining the orientation of the ligand transition moment. The induced CD according to this model will be negative for dye transitions polarized parallel to a direction bisecting the helical angle between the lines connecting the glycosyl bonds of adjacent base pairs ($\gamma = 90°$), while the CD will be positive for transitions polarized parallel to the dyad axis of the DNA helix ($\gamma = 0$). This essentially corresponds to orientations parallel to the long axis and short axis, respectively, of the base pairs surrounding the intercalator. At higher ligand/DNA occupancy, in addition to the above-mentioned "monomeric" CD, one can also expect exciton CD due to the interaction from proximate ligands. The CD spectra were recorded on a Jasco J-500 spectropolarimeter.

Equilibrium Analysis. Binding constants were determined from free-ligand measurements at low ionic strength by the dextran/poly(ethylene glycol) partition method.[17,24] Since the Scatchard plots in no case clearly indicated more than one type of strong complex (Figure 3), only a single binding constant (K) and corresponding binding site density, i.e., number of ligand sites/DNA base ($1/n$), have been considered.[17] The free reagent concentration and the DNA concentration were determined spectrophotometrically in the top and the bottom phase. For all compounds it was determined that the binding data did not depend significantly on the DNA concentration in the region $(1–5) \times 10^{-4}$ mol dm^{-3} DNA phosphate. The binding data were analyzed in terms of nonlinear least-squares fits to McGhee–von Hippel binding isotherms.[25]

For the more strongly binding DNA adducts generally a large element of error was observed in the limit of low binding ratios. A systematic error owing to traces of DNA in the PEG phase, particularly at low salt concentrations, could be corrected for after quantifying the DNA with a fluorescent probe. However, the strong dependence of the intercept at zero binding still makes the binding constants obtained via the McGhee–von Hippel method relatively uncertain for the multiintercalators, whereas the binding site density ($1/n$) and the cooperativity number (ω) seem to come out more reliably.

In order to obtain a better measure of affinity, for the purpose of comparison, we shall define a "relative binding constant" K', referring to a standard binding ratio that is chosen sufficiently high to guarantee accurate equilibrium analysis (eq 5). Here r is the binding ratio ex-

(16) Nordén, B. *Appl. Spectrosc. Rev.* **1978**, *14*, 157–248.
(17) Nordén, B.; Tjerneld, F.; Palm, E. *Biophys. Chem.* **1978**, *8*, 1–15.
(18) Wada, A.; Kozawa, S. *J. Polym., Sci. Part A* **1964**, *2*, 853–864.
(19) Davidsson, Å.; Nordén, B. *Chem. Scr.* **1976**, *9*, 49–53.
(20) Nordén, B.; Seth, S. *Appl. Spectrosc.* **1985**, *39*, 647–655.
(21) Nordén, B.; Tjerneld, F. *Biopolymers* **1982**, *21*, 1713–1734.
(22) Schipper, P. E.; Nordén, B. *Chem. Phys. Lett.* **1979**, *67*, 99–102.
(23) Schipper, P. E.; Nordén, B.; Tjerneld, F. *Chem. Phys. Lett.* **1980**, *70*, 17–21.
(24) Albertsson, P. Å. *Partition of Cell Particles and Macromolecules*; Wiley: New York, 1971.
(25) McGhee, J. D.; von Hippel, P. H. *J. Mol. Biol.* **1974**, *86*, 469–489.

$$K' = r[\mathrm{L}]^{-1}\left(\frac{1}{n} - r\right)^{-1} \quad (5)$$

pressed in acridyl units/DNA phosphate, $1/n$ is the binding site density (also with respect to acridyl units), and [L] is the free ligand concentration. The values of n will be adopted from the binding isotherm, e.g., $n = 4$ for 9-aminoacridine, etc.

The relative binding constants were evaluated for a binding ratio corresponding to 0.15 acridine unit/DNA phosphate, which for the trifunctional ligands was found to give a sufficiently reduced error in $r/[\mathrm{L}]$. Equivalent ratios with respect to acridine moieties were employed for the di and mono compounds.

Electric Orientation Relaxation. A considerably improved signal-to-noise ratio when using a powerful laser source for measuring electric birefringence, together with appropriately designed data aquisition, is shown to make electric orientation relaxation a sensitive method for determining the relative lengthening of the DNA helix as a result of intercalative binding.

The linear birefringence due to the application of an electric field orthogonal to the light path as a rectangular pulse (response times 50 ns, duration 1–1000 μs) was measured on the DNA solutions at low ionic strength (1 mM NaCl) by use of a Kerr cell with a 1-cm optical path length and 2-mm gap between the parallel, polished electrodes of stainless steel in a block of Teflon. The rear and front windows were of birefringence-free fused silica (Suprasil), and the tightening was designed to minimize strain birefringence. The experimental setup consists of a 5-W argon ion laser (Spectra-Physics) used in the 488-nm mode, the measuring cell between two crossed, high-performance Glan polarizers, a Jobin-Yvon grating monochromator (H.10 C UV) followed by an end-on photomultiplier (Hamamatsu R928) with a preamplifier connected online to a Iwatsu TS-8123 storage oscilloscope.

The reorientation relaxation curve in the absence of an electric field was fitted by a multiexponential function with fast and slow components adjusted to the time characteristics of the instrument electronics. The DNA sample was assumed to have an "effective length" obtained from the rotational diffusion constant of a rigid rod,[26,27] according to the Broersma–Perrin relation[28,29] (eq 6), where L is the length and b the

$$\theta = \frac{3kT}{\pi\eta_0 L^3}\,[\ln L/b - 1.57 + 7[1/\ln(L/b) - 0.28]] \quad (6)$$

width of DNA, T the absolute temperature, η_0 the viscosity of the solvent, and θ the rotational diffusion constant, calculated as $\theta = 1/6\tau_i$ with τ_i the relaxation time. The rod thickness b was taken to be 13 Å (not a critical choice).

The length of DNA, $L(r)$, obtained in the presence of a ligand at a given binding ratio r was determined correspondingly and the lengthening expressed as the apparent number of additional base pairs N per added bound ligand molecule. We shall define N in the limit of zero binding as shown in eq 7. For a classical intercalator one would expect $N = 1.0$, which corresponds to a lengthening of DNA by 3.4 Å per adduct molecule; for a bisintercalator $N = 2.0$ is expected, and so on.

$$N = \lim_{r\to 0}\,[L(r) - L(0)]/2L(0)r \quad (7)$$

Viscometry. Viscosity was measured with a modified Ubbe-Lohde type viscometer with a 100-mm-long × 0.4-mm-wide capillary and a 1-mL bulb thermostated to 20 °C. The sonicated DNA in a buffer of 10 mM Tris-HCl and 10 M EDTA, pH 7, was filtered through a 0.45-μ Sartorius membrane. Viscosity was measured at DNA concentrations of $\sim 5 \times 10^{-4}$ M and reagent/base pair ratios r less than 0.15. The viscosity index α_v is obtained from the flow times at varying reagent/base pair ratios according to[30] eq 8, where t_r, t_{DNA}, and t_0 denote the flow times

$$L/L_0 = [(t_r - t_0)/(t_{DNA} - t_0)]^{1/3} = 1 + \alpha_v r \quad (8)$$

of a given fixed volume of DNA complex (at reagent/phosphate ratio r), free DNA, and buffer, respectively. As indicated by the left-hand side equality, viscometry is assumed to directly provide the relative DNA lengthening (L/L_0).

Unwinding. An agarose gel migration–retardation assay was used to measure the unwinding of super-coiled plasmid DNA induced by the acridines. This assay is based on the observation that high-affinity DNA ligands stay bound to DNA upon gel electrophoresis (Nielsen et al., unpublished results) combined with the influence of superhelical density on the migration of circular DNA. The unwinding angles of the acridines were determined relative to that of compound **2** (34°)[5] from plots of reagent/base–pair ratio versus DNA migration. The ratios causing 50% retardation were used for the calculation.

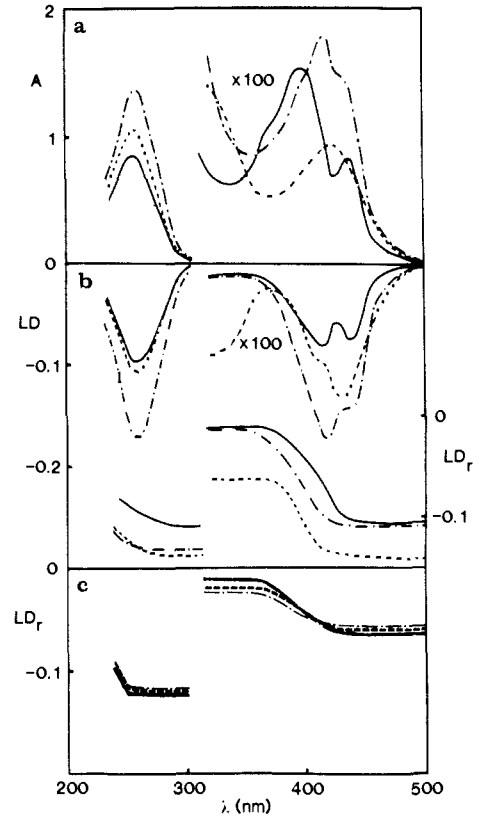


Figure 1. Key: a, absorbance (A); b, flow linear dichroism (LD) and reduced linear dichroism (LD$_r$) spectra of **3a** (—, [DNA] = 0.17 mM phosphate), **3g** (–·–, [DNA] = 0.26 mM), **3h** (---, [DNA] = 0.21 mM); c, LD$_r$ spectra ([DNA] = 0.31 mM) of **4a** (—), **4b** (---), **6** (–·–). All spectra are normalized to an optical path length of 1 cm. The LD spectra were run at a flow gradient of 1200 s^{-1}. The solutions were prepared to have a reagent/DNA phosphate ratio of 0.005. The ionic medium was 1 mM NaCl and 0.15 mM Na_2EDTA.

Results

Linear and circular dichroism spectra were measured at low reagent/DNA binding ratios in order to minimize effects of interactions between the ligands and perturbation of the global conformation of DNA. Representative absorbance (A), linear dichroism (LD), and reduced linear dichroism (LD$_r$) spectra are shown in Figure 1. The LD corresponding to the main, long-wavelength absorption band of the acridinyl chromophore has for all the compounds a negative sign and a shape roughly that of the absorption curve. The LD$_r$ amplitudes observed for the long-wavelength bands of 9-aminoacridine and the bis(acridinyl) compound correlate well with that of the 260-nm DNA base absorption, as expected for intercalation.[16,31] The 400-nm absorption in 9-aminoacridine is polarized parallel to the short axis in the molecular plane,[32,33] and the LD result indicates that this axis is oriented essentially parallel to the DNA bases. The absorption at 350 nm is essentially long-axis polarized,[32,33] and for all compounds the LD$_r$ amplitude of this band is significantly reduced compared with the 260-nm band of DNA, indicating a tilt of the long axis relative to the average orientation of the DNA bases. The apparent tilts $\pi/2 - \alpha$, calculated from eq 3, obtained for the various DNA complexes in the limit of low binding, are given in Table I for the in-plane short axis of acridine relative to the DNA bases.

(26) Elias, J. G.; Eden, D. *Macromolecules* **1981**, *14*, 410–419.
(27) Hagerman, P. J. *Biopolymers* **1981**, *20*, 1503–1535.
(28) Broersma, S. *J. Chem. Phys.* **1960**, *32*, 1626–1631 and 1632–1635.
(29) Perrin, F. *J. Phys. Radium* **1934**, *7*, 497–511.
(30) Cohen, G.; Eisenberg, H. *Biopolymers* **1969**, *8*, 45–52.
(31) Hansen, J. B.; Koch, T.; Buchardt, O.; Nielsen, P. E.; Nordén, B.; Wirth, M. *J. Chem. Soc., Chem. Commun.* **1984**, 509–511.
(32) Matsuoka, Y.; Nordén, B. *Chem. Phys. Lett.* **1982**, *85*, 302–306.
(33) Fornasiero, D.; Kurucsev, T. *Chem. Phys. Lett.* **1985**, *117*, 176–180.

Table I. Linear Dichroism, Electric Orientation Relaxation, Viscosity, and Unwinding Data for the Studied DNA Complexes

compd	$LD_{r,L}/LD_{r,DNA}$[a]	app tilt of short axis ($\pi/2 - \alpha$),[a] deg		lengthening,[b] N	viscosity index,[c] α_v	unwinding angle, deg
		av value	heterogeneous alternative			
1	1.00	0		1.5	1.5	
2	1.00	0		2.1	1.8	34
3a	1.06	0		2.4	4.0	44
3g	0.95	5			3.1	
3h	0.85	15	two at 0, one at 23		3.0	27
4a	0.60	20	three at 0, one at 30	2.4	3.0	41
4b	0.50	25	three at 0, one at 50		5.2	
6	0.49	25	three at 0, three random	2.8	3.6	50

[a] Reduced linear dichroism values of short-axis-polarized bands in the 9-aminoacridine chromophore at low reagent/DNA binding ratios (r = 0.005). Apparent tilts $\pi/2 - \alpha$ of the 9-aminoacridine short axis with respect to the average base plane. [b] DNA lengthening according to eq 7, obtained with EOR on sonicated calf thymus DNA at very low binding ratios ($r < 0.03$). [c] Viscosity index values, α_v, according to eq 8, obtained in the region of intermediate binding ratios ($r < 0.15$).

Table II. Binding Data According to the Single-Site-Binding Isotherm of McGhee and von Hippel

reagent	1.00 × 10^{-3} mol dm^{-3} NaCl			0.100 mol dm^{-3} NaCl		
	K/mol^{-1} dm^3	n	ω	K/mol^{-1} dm^3	n	ω
1	4.0 × 10^5	4	1	2.1 × 10^4	4	1
2	(2.2 × 10^5)	9	20	4.8 × 10^4	8	35
3a	(1.3 × 10^6)	17	20	4.0 × 10^4	13	80
3g	(4.0 × 10^6)	13	23	5.0 × 10^4	14	115
3h	(1.3 × 10^7)	13		(4.0 × 10^5)	11	

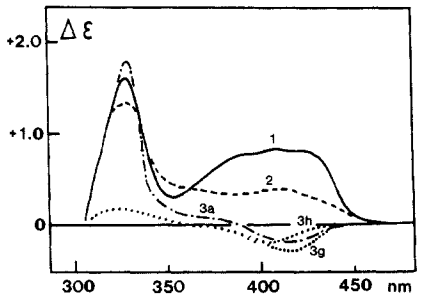


Figure 2. CD spectra of **1**, **2**, **3a**, **3g**, and **3h** in 1 mM NaCl and 0.15 mM Na_2EDTA. The CD spectra of **4a**, **4b**, and **6** coincide with the spectrum of **3h**.

The circular dichroism, of the DNA–reagent complex in the DNA absorption region, (not shown) is not significantly altered compared with that of pure DNA (B form), demonstrating that the average DNA conformation is not measurably perturbed by the interaction, at least at low binding ratios. As shown in Figure 2, the 400-nm absorption system of the adduct, when bound to DNA, displays a relatively weak induced circular dichroism with a sign and magnitude that varies between the compounds. With 9-Aminoacridine (**1**) the strongest induced CD is observed, with a positive sign for the 400-nm band; whereas the bis(acridine) **2** has a considerably weaker CD (per acridyl unit) and the tri compounds **3a**,**g**,**h**, like the tetrakis (**4a**,**b**) and hexakis(**6**) compounds, only show very weak negative signals. The 300-nm absorption band of the acridyl chromophore displays a relatively strong positive induced CD for **1**, **2**, and **3a**, but practically no induced CD for the other compounds.

The results of the equilibrium studies, and fitted binding isotherms, (exemplified in Figure 3) are summarized in Table II. Owing to experimental uncertainty and systematic errors, a decreasing fidelity is expected in the limit of low binding ratios. For this reason quantitative conclusions regarding particularly the larger binding constants are difficult. As expected, the binding of 9-aminoacridine to DNA shows no cooperativity (ω = 1), whereas a significant degree of positive cooperativity ($\omega > 1$) is observed for most of the oligoacridines.[34] The n value obtained

(34) A lack of cooperativity reported for compound **2** by Wakelin et al.[35] could be due to the different ionic conditions (0.5 M NH_4F) used in their study.

(35) Wakelin, L. P. G.; Creasy, T. S.; Waring, M. J. *FEBS Lett.* **1979**, *104*, 261–265.

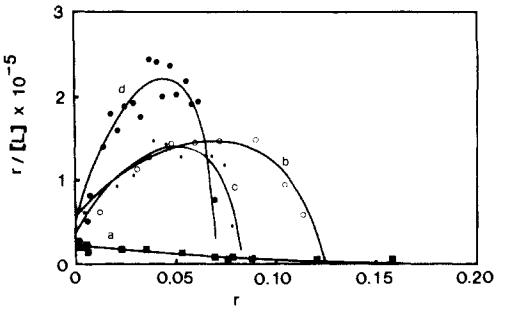


Figure 3. Binding isotherms of **1** (a), **2** (b), **3a** (c), and **3h** (d). The ionic medium was 100 mM NaCl and 0.15 mM Na_2EDTA. Solid lines represent a nonlinear least-squares fit to the model of McGhee and von Hippel with three parameters, K, n, and ω.

Table III. Relative Binding Constants, $K' = r[L]^{-1}(1/n - r)^{-1}$, for the Following r Values: 0.05 for Tris-, 0.075 for Bis-, 0.15 for Monoacridine[a]

reagent	K'/mol^{-1} dm^3	
	1.00 × 10^{-3} mol dm^{-3} NaCl	0.100 mol dm^{-3} NaCl
1	4.0 × 10^5	2.5 × 10^4
2	(2 × 10^7)	3.8 × 10^6
3a	(3 × 10^7)	4.6 × 10^6
3g	(5 × 10^7)	7.1 × 10^6
3h	(3 × 10^8)	1.4 × 10^7

[a] The following n values were adopted based on the intercepts of the binding isotherms: n = 4 (mono), 8 (bis), 12 (tris).

at high ionic strength (0.1 M) is in agreement with 9-aminoacridine being a monointercalator obeying the nearest-neighbor exclusion principle (n = 4) and compound **2** being a bisintercalator (n = 8). Reagents **3a**,**g**,**h** behave essentially as trisintercalators (n = 12 would be expected with all nearest neighbors excluded).

The relative binding constants (Table III) as defined by eq 5 are thought to reflect more reliably the binding affinities of the multiintercalators than are the constants obtained from the fitted isotherms. Whereas the apparent binding constants of Table II do not vary significantly when increasing the number of potentially intercalating groups, the relative binding constants do: $K_1 < K_2 < K_3$ (Table III).

The increase in viscosity of sonicated calf thymus DNA was measured as a function of reagent base-pair ratio (Table I). The monoacridine (**1**) gave an α_v value of 1.5, the diacridine (**2**) gave an α_v value of 1.8, whereas the tris-, tetrakis-, and hexakis-(acridines) showed a more complex behavior giving values from 3 to 5, depending on the reagent/base-pair ratio. For tris and higher acridine derivatives the viscosity increased measurably during the first 15–60 min, analogously with the increase in LD (Table IV).[31] Viscometry measurements on the aminoacridines do not agree with the simple model for intercalation, according to which α_v should increase linearly as a function of the number of intercalating ligands, i.e., α_v = 2 for mono-, 4 for bis-, 6 for trisintercalators, etc. However, qualitatively the results are compatible with the conclusion that the mono-, bis-, and tris-

Table IV. Time Needed To Reach 90% of Maximum LD (LD_r) Value When Mixing Equal Volumes of DNA and Reagent[a]

reagent	t, min: 1.00×10^{-3} mol dm^{-3} NaCl	t, min: 0.100 mol dm^{-3} NaCl
1	0[b]	0[b]
2	0[b]	0[b]
3a	10[c]	5–10
3g	15	5–10
3h	15	5–10
4a	30	10–20
4b	40	10–20
6	60	20–30

[a] [DNA phosphate] = 0.30 mM, $r = 0.005$, $G = 1800 \pm 2\ s^{-1}$. [b] LD signal develops immediately (within 0.1 s). [c] From ref 31.

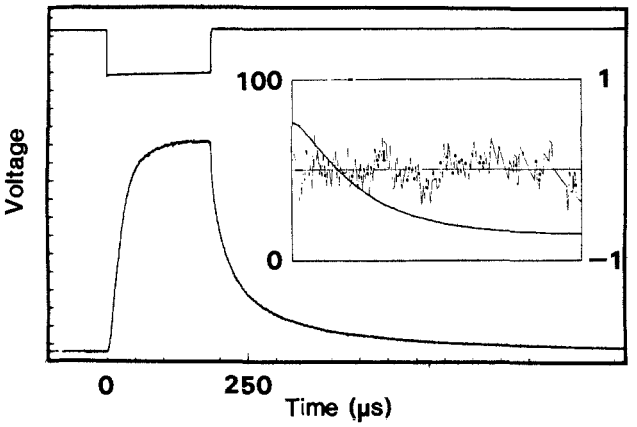


Figure 4. Representative response from an electric orientation relaxation measurement demonstrating the favorable signal/noise ratio. Lower curve: photomultiplier response. Upper curve: the corresponding orienting electric pulse (amplitude of field strength, 7.5 kV cm^{-1}). Inset: the corresponding numerical fit to the relaxation part of the response curve (arbitrary scale 0–100) and the corresponding residual (−1 to +1).

(acridines) bind to DNA by mono-, bis-, and trisintercalation, whereas the results indicate that **4a,b,c** only bind by trisintercalation.

In Figure 4 a typical response curve, and the corresponding exponential fit, from an electric orientation relaxation measurement is shown. Figure 5 provides the results in terms of a lengthening of short, sonicated DNA molecules expressed as the "number of apparent additional base pairs" per intercalated ligand molecule (eq 6, 7). The bis(acridine) compound lengthens DNA by approximately two base pairs per total ligand unit, as expected for bisintercalation. However, 9-aminoacridine gives a lengthening of 1.5 compared with 1.0 measured for ethidium bromide.

Discussion

Linear Dichroism. The observation that the short, in-plane symmetry axis of the acridine unit is oriented parallel to the DNA base pairs is significant. Such an orientation of planar DNA ligands is often taken as evidence for true intercalation. The very similar orientations observed for all acridine groups in the bis- and tris(acridine) compounds, strongly indicate that these are indeed bis- and trisintercalators. It is interesting that Denny et al.[36] recently reported that the unwinding angles of a series of tris(acridines) with amide-containing linkers indicated that these were only bisintercalators. Our preliminary results with a new gelelectrophoresis may suggest that the unwinding angles of the tris(acridines) **3a,g** are close to the one of reagent **2**, in agreement with the above results. It is thus conceivable that the tris(acridines) reported[36] are in fact trisintercalating, analogous to compounds **3a,g**, but that the difference in DNA binding geometry of the tris(acridines) versus the bis(acridines) (cf. the discussion of CD

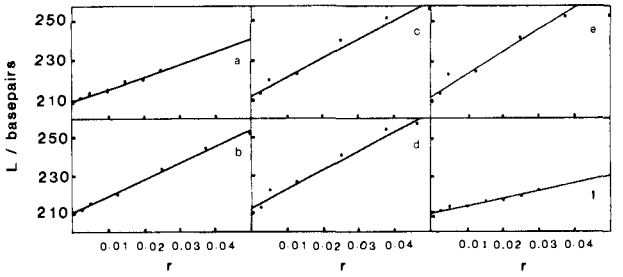


Figure 5. Results from electric orientation relaxation measurements. Relative lengthening, N, (see eq 7) versus [L]/[DNA phosphate]. Key: a, **1**; b, **2**; c, **3a**; d, **4a**; e, **6**; f, ethidium bromide. The DNA concentration was 0.15 mM phosphate. The ionic medium was 1 mM NaCl, 0.15 mM Na_2EDTA. The values obtained for the number of base pairs per bound reagent (eq 7) are 1.5, 2.1, 2.4, 2.4, 2.8, and 1.0, respectively.

results) also results in different unwinding angles per ligand.

The significant apparent tilt of the short axes of the tetrakis- and hexakis(acridines) correspondingly demonstrates that not all of the acridine groups are parallel to the average orientation of the DNA bases. A straightforward explanation of this finding is that at most three of the acridine groups are bound to DNA by intercalation. If, however, the binding induces a local DNA bending, the results might still be compatible with intercalative binding of more than three acridine groups.

A somewhat unexpected observation is the indication of a considerable tilt (at least 20°) of the acridine long axis of the monoacridine as well as the other compounds. This conclusion is based on polarization determinations in 9-aminoacridine from stretched film measurements.[32] The intensity of the absorption in the 300–350-nm region, where the second $\pi \rightarrow \pi^*$ transition (with a polarization parallel to the long axis) is situated,[32] is relatively weak, and the influence from other transitions (such as $n \rightarrow \pi^*$) therefore cannot be excluded. On the other hand, in a recent reinvestigation of the purity of these transitions, Fornasiero and Kurucsev[33] found evidence for a mixture of long-axis polarization at the short-wavelength side of the 400-nm band system. The observed variation of LD_r at the short-wavelength side of the first absorption band for all of the studied ligands upon binding to DNA (Figure 1) is thus also consistent with an inclination of the long axis of the acridine group by approximately 20° relative to the base planes.

As will be inferred from the circular dichroism results, the long axis of intercalated 9-aminoacridine is likely to be oriented parallel to the longest dimension of the intercalation pocket. It is therefore relevant to question whether the evidenced tilt of the long axis of the acridine group might be reflecting a corresponding "natural" inclination of the base pairs. Johnson et al.[37–39] found that shorter wavelength bands for DNA give a less negative reduced flow dichroism than does the 260-nm absorption, indicating base tilts as large as 20–30° relative to a preferred inclination axis. Base tilts have also been evidenced from electric dichroism measurements,[40,41] including intercalated 9-aminoacridine (though with confused long- and short-axes transitions),[42] however, as pointed out by Edmondson and Johnson,[38] the results must be interpreted with caution as they refer to single wavelengths and very high fields where distorsion of DNA may occur.

Our assumption of taking $\alpha = 90°$ for the DNA bases has been based on fiber B-form data (which has, due to overlapping absorptions, an "effective" α at 260 nm equal to 86°).[43] Perpen-

(36) Denny, W. A.; Atwell, G. J.; Willmott, C. A.; Wakelin, L. P. G. *Biophys. Chem.* **1985**, *22*, 17–26.
(37) Causley, G. C.; Johnson, W. C., Jr. *Biopolymers* **1982**, *21*, 1763–1780.
(38) Edmondson, S. P.; and Johnson, W. C., Jr. *Biopolymers* **1985**, *24*, 825–841.
(39) Edmondson, S. P.; Johnson, W. C., Jr. *Biopolymers* **1986**, *25*, 2335–2348.
(40) Wu, H. M.; Dattagupta, N.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6808–6811.
(41) Charney, E.; Chen, H. H.; Henry, E. R.; Rau, D. C. *Biopolymers* **1986**, *25*, 885–904.
(42) Hogan, M.; Dattagupta, N.; Crothers, D. M. *Biochemistry* **1979**, *18*, 280–288.
(43) Matsuoka, Y.; Nordén, B. *Biopolymers* **1982**, *21*, 2433–2452.

dicularity was supported by the value of the limiting LD_r of sonciated DNA in gel.[44] In the solution structure, however, it can be anticipated that the bases deviate significantly from perpendicular orientation owing to dynamics. Still, it can be justified to use $\alpha = 90°$ if the base tilting is uniform (no preferred inclination axis), since the tilt may then be represented by a corresponding reduction of the orientation factor S.

The present results do not allow any conclusion as to whether the 9-aminoacridine tilt is due to a natural base tilt (with a preferred inclination axis) or not. One DNA drug, methylene blue, which at low salt is intercalated similarly to 9-aminoacridine according to induced CD results,[21] gives for its long-axis polarized transition practically the same LD_r as the 260-nm DNA band does. This is strong evidence that, at least for that compound, the long axis of the base pairs is not more tilted than the short axis is.

Circular Dichroism. The order of magnitude and the shape of the induced 400-nm CD of 9-aminoacridine bound to DNA are consistent with the CD mechanism for an electric dipole allowed nondegenerate transition of an intercalator.[21,23] The positive sign indicates that $\gamma < 45°$ (eq 4), i.e., the short axis of 9-aminoacridine is more perpendicular than parallel to a direction defined by the glycosyl bonds of the adjacent base pairs, or in other words, that the projection of the long axis tends to be parallel to the longest dimension of the base-pair pocket. This is also the orientation expected from comparison with other intercalators.[17,23]

The induced 400-nm CD, calculated per acridine unit, falls off in the sequence mono- > bis- > tris-, tetrakis-, hexakis(acridines). A very similar induced CD would have been expected if all the 9-aminoacridine groups had had the same orientation. The observed variation can be due to acridine chromophores interacting with each other or to the strain and charge distribution of the linkers forcing the acridines somewhat away from their monointercalator orientation. For several reasons we believe the latter explanation. The positive CD band at about 320 nm may arise from an $n \rightarrow \pi^*$ transition of the acridine chromophore, which could explain a virtual insensitivity to the γ-orientation of the intercalator.

Except for one of the tris(acridines) (**3h**) the induced long-wavelength CD of all of the ligands has the expected absorption band shape without any indication of exciton CD. The absence of exciton CD excludes self-stacking of the acridines, as well as nearest-neighbor intercalation. If 9-aminoacridine was intercalated perfectly along the base-pair pocket ($\gamma = 0°$ for short axis, eq 4), the CD variation from mono- to bis(acridine) would correspond to a twist of the acridine groups around the helix axis by some 30° upon intercalation. The sign change for the tris(acridines) further indicates that one or more of the acridine groups has been turned more than 45°. It should be noticed that decreasing CD amplitudes are generally expected with an increasing number of differently oriented intercalators. As seen from eq 4 the rotational strength averages to zero for a random γ-distribution.

Furthermore, the analogous behavior of the tris(acridines) (except for **3h**) and the tetrakis- and hexakis(acridines) supports the proposal that they bind to DNA by trisintercalation. (We have at present no explanation for the departing behavior of **3h** in CD and LD).

Electric Orientation Relaxation. The exploitation of the classical method of electric birefringence for measuring the rotational diffusion of DNA,[26,27] in order to determine DNA lengthening by intercalation at low binding ratios seems to be attempted for the first time. The elongation of sonicated DNA fragments, of an average length of approximately 200 base pairs, is readily detectable even at a binding ratio as low as 0.0025, i.e., one ligand molecule per DNA molecule (Figure 5).

The results for the present 9-aminoacridines indicate that the intercalation is not described by the classical lengthening of 3.4 Å for each intercalated ligand. First, the monointercalator 9-aminoacridine exhibits an expansion as large as 1.5 (compared with 1.0 for ethidium bromide), which suggests a more pronounced distortion of the DNA structure upon binding than that found for ethidium bromide. This conclusion is in agreement with the observed tilt of the acridine long axis relative to the average orientation of the DNA base pairs, which of course would require more space than the untilted stacking. Second, the degree of expansion appears not to be additive in the sequence mono-, bis-, and trisintercalators, as judged from the lengthening numbers $1.5 < 2.1 < 2.4$. At the present stage of development of this method, however, it is difficult to interpret these results in detail.

From electric birefringence relaxation of sonicated calf thymus DNA Matsuda and Yamaoka[47] found that 9-aminoacridine ($r = 0.1$) makes the weight-average length of DNA increase, compared with that in 1 mM NaCl, but also that the field-free relaxation time (and thus the apparent length) decreases with increasing field of the pulse. An earlier reported length increase by less than 3.4 Å per bound 9-aminoacridine, measured from electric dichroism during the pulse,[42] may be due to field perturbation or too high drug concentration.

Equilibrium Analysis. The relative binding constants and the apparent values of n (site density), shown in Table II, provide clear evidence that progression from mono- to bis- to tris(aminoacridine) is accompanied by substantially enhanced DNA affinities. The binding site densities are consistent with nearest-neighbor exclusion. However, in agreement with results by others on both di- and triacridines,[35,45,46] we do not find the degree of enhancement that would be expected if the free energy of binding of the acridinyl units were additive: with similar entropy terms, one would expect the binding constants of a bis- and trisintercalator to be approximately equal to, respectively, the square and the cube of that of the mono compound. The considerably lower experimental values can thus indicate a substantial entropy difference (for example, due to solvent rearrangement) or an unfavorable extra enthalpy cost associated with the distortion of DNA upon bis- and trisintercalation. As mentioned before, the CD results suggest that strain from the linkers leads to different (less favorable) orientations of the intercalating groups compared with the monofunctional compound. On the other hand, the linkers of the multifunctional compounds also contain basic groups, which contribute significantly to the binding energy by electrostatic interaction with the acidic deoxyribophosphate backbone.

The increasing cooperativity parameter ω in the progression mono- < bis- < trisintercalator is significant and deserves a comment. A positive cooperativity has earlier been reported for the DNA interaction with several diacridines.[12,35] However, this effect has not yet been satisfactorily explained. All evidence in the present study indicates that the bis- and tris(acridines) are indeed bis- and trisintercalators, respectively. A positive cooperativity is therefore not simply an effect of an increased self-association tendency. As pointed out by Wakelin et al.,[35] self-association of the diacridines only enhances the cooperativity very moderately and only reduces the binding site density slightly. We propose that cooperativity is caused by changes in the DNA structure upon binding, facilitating the introduction of additional intercalating groups. Indeed, preliminary results with probes covalently bound to DNA indicate that perturbation of the DNA structure is generally not localized to a single pair of bases but is transmitted to surrounding base pairs.

Unwinding. The unwinding results support our conclusions regarding the differences in DNA binding of compounds **2**, **3a**, **3h**, **4a**, and **6**. If all three acridines were intercalated with identical binding geometry, one would expect an unwinding angle of ~50° for compound **3a**, which by LD measurements appears to be trisintercalating. We find a smaller value, which we interpret as evidence of a different binding geometry resulting in a smaller DNA unwinding of at least one acridinyl ligand, in agreement with the observation that the induced CD spectra of **2** and **3a** are different. In this connection it is noteworthy that unwinding angles

(44) Matsuoka, Y.; Nordén, B. *Biopolymers* **1982**, *22*, 1731–1746.
(45) Laugaa, P.; Markovits, J.; Delbarre, A.; LePecq, J.-B.; Roques, B. P. *Biochemistry* **1985**, *24*, 5567–5575.
(46) Assa-Munt, N.; Denny, W. A.; Leupin, W.; Kearns, D. R. *Biochemistry* **1985**, *24*, 1441–1449.
(47) Matsuda, K.; Yamaoka, K. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 1727–1733.

of ~30° have previously been reported for several tris(acridines).[36,48] The rather small unwinding angle of compound **3h** corresponds well with the finding by LD that this tris(acridine) does not bind to DNA by trisintercalation. Similarly, the unwinding angles of compounds **4a** and **6** support the LD results, suggesting that these tetrakis- and hexakis(acridines) are only trisintercalating when binding to DNA.

Concluding Remarks. A number of significant conclusions can be made on the basis of the results presented in this paper. The combined evidence from the LD_r, binding site density, viscometry, unwinding, and EOR results leave little doubt that the tris(acridines) **3a,g** bind to DNA with all three acridine moieties intercalated in the DNA helix while obeying the neighbor exclusion principle. Regarding the tetrakis- and hexakis(acridines) **4a,b** and **6**, our LD_r results suggest that these are at the most trisintercalators. In agreement with previous reports[5,31,48] we also find that bis- and trisintercalation only contribute moderately to an increase in the affinity for DNA. However, the binding is characterized by a large cooperativity factor, ω.

It has previously been shown that certain 9-aminoacridines bind to DNA with some sequence specificity.[5] In particular, it has been inferred that bis(acridines) show increased affinity for poly(dAdT) as compared with poly(dGdC). It would be expected that sequence specificity was more pronounced with DNA intercalating compounds, which contain more than one intercalating group. The present results have not given evidence of any sequence specific DNA binding for any of the studied multiacridines. We have also been unable to detect such an effect using the DNase I footprinting technique. We did find a distinct difference between 9-aminoacridine, which did not affect the DNase, whereas a significant inhibition of the DNase I action was exhibited by bis-, tris-, and tetrakis(acridines) (unpublished results). We ascribe this effect to the linker between the intercalating groups, which is believed to occupy the minor groove where the DNase is presumed to bind and cleave the DNA strand.[49]

Furthermore, our results allow for a comparison between the LD, CD, viscometry, and EOR techniques for studying intercalative binding of reagents to DNA. The technique of EOR, developed here for the determination of the DNA lengthening upon the binding of ligand molecules, must be considered as at least equal and possibly superior to the use of viscometry for this purpose. An advantage of the EOR method as compared with viscosity measurements is that much smaller binding ratios, at which virtually all reagent molecules are bound to DNA at a distance large enough to eliminate ligand–ligand interactions, can be employed without loss of sensitivity. It is clear that neither EOR nor viscometry measurements can unequivocally determine whether an oligoacridine binds to DNA with all of its acridine groups; it is also clear that these two techniques do not appear to yield exactly the same information although in principle they both rely on the DNA helix extension.

Results obtained by LD are much more easily interpreted, since this technique yields information about the binding geometry of the DNA-associated ligand more directly. The combined CD and LD results for 9-aminoacridine can be interpreted in terms of a binding geometry where the ligand molecule intercalates with its short axis parallel to the DNA bases but with the long axis tilted at some 60–70° from the helix axis, and not 90° as would be expected for a perfect intercalator. This tilted binding geometry is accompanied by an unusually large value of the specific lengthening detected with EOR.

The DNA-induced CD of 9-aminoacridine could be rationalized in terms of nondegenerate coupled oscillator theory, with the long axis of acridine being preferentially parallel to the longest dimension of the intercalation pocket. Upon introducing more acridinyl units in the reagents the induced CD gradually decreases, which is ascribed as an effect of a rotation or a changed angular distribution of the different acridine ligands in their intercalation pockets relative to the surrounding DNA bases.

The time dependence of the binding of oligoacridines with DNA, detected both in LD and EOR measurements when using calf thymus DNA, and not observable when using synthetic poly(dGdC), is probably the result of the attempt of the reagents to find the binding geometry with the lowest energy.

Abbreviations

LD, linear dichroism; CD, circular dichroism; EOR, electric orientation relaxation.

Registry No. 1, 90-45-9; **2**, 58903-52-9; **3a**, 91790-14-6; **3b**, 111794-83-3; **3c**, 111794-84-4; **3d**, 111794-85-5; **3e**, 111794-86-6; **3f**, 111794-87-7; **3g**, 111794-88-8; **3h**, 111794-89-9; **4a**, 111794-90-2; **4b**, 111822-67-4; **4c**, 111794-91-3; **6**, 111794-92-4.

(48) Denny, W. A.; Atwell, G. J.; Baguley, B. C.; Wakelin, L. P. G. *J. Med. Chem.* **1985**, *28*, 1568–1579.
(49) Drew, H. R. *J. Mol. Biol.* **1984**, *76*, 535–557.