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Viscometric analysis of the interaction of bisphenanthridinium compounds with closed circular supercoiled and linear DNA

W.David Wilson*, Rebecca A.Keel, Robert L.Jones and Carol W.Mosher+

Department of Chemistry and Laboratory for Microbial and Biological Sciences, Georgia State University, Atlanta, GA 30303, and +Bio-Organic Chemistry, Life Sciences Division, SRI International, Menlo Park, CA 94025, USA

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ABSTRACT

The interaction with closed circular supercoiled and linear DNA of bisphenanthridinium compounds substituted through both the meta and para positions of the 6-phenyl group, along with appropriate monomer intercalators as controls, has been investigated by viscometric titration. When CPK models for the phenanthridinium rings of the three bis-compounds are oriented in a parallel manner as a model for intercalation, their ring plane to ring plane distances are approximately 7 to 8 Å (SR 2430), 11 Å (SR 2193), and 15 Å (SR 2166). In SR 2430 the two phenanthridines are linked through the para positions of the 6-phenyl group; this chain allows intercalation of the two rings at adjacent binding sites in DNA, but is not long enough to accommodate an excluded site between bound rings. The other two compounds, SR 2193 and 2166, joined through the meta positions of the 6-phenyl, can double intercalate with an excluded site. The viscometric titrations with both superhelical and linear DNA clearly indicate that SR 2430 gives results close to those of the monomer control compounds while SR 2193 and SR 2166 have approximately twice the unwinding angle and DNA length increase on binding to DNA as the monomer compounds. These phenanthridinium compounds, therefore, are capable of bisintercalation only if their linking groups are of sufficient length to allow an excluded binding site between base pairs. This conclusion is supported by DNA thermal denaturation experiments in the presence of these compounds.

INTRODUCTION

It is now well established that a large variety of planar aromatic molecules can bind to DNA through an intercalation process (1-4). Many of these intercalating molecules have significant medicinal activity (1, 4). One of the most interesting aspects of intercalation binding is the apparent "neighbor exclusion" of binding sites which limits saturation of the double helix to one bound intercalator for every two base pairs (1-5). Several series of potential bisintercalating systems have been synthesized with the goals of (i) increasing the medicinal activity of intercalating drugs and (ii) using these molecules as probes for analysis of binding site exclusion (for example, 6-12). Early results with bisacridine derivatives suggested that bisintercalation could occur only when the linking chain was long enough to allow the two

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rings to skip one potential binding site in agreement with neighbor exclusion (6). More detailed investigations with a variety of bisacridines, however, have shown, for example, that bisacridines substituted only through a 9-amino group can bind at neighboring sites in apparent violation of neighbor exclusion (8, 9). Although they are the only compounds which have been shown to bind to DNA without neighbor exclusion, their existence raises questions about the molecular basis of intercalation site exclusion. Bisintercalation with and without neighbor exclusion as well as potential modes of monointercalation of bis compounds are schematically illustrated for reference in Figure 1.

Because of the importance of understanding the origin of neighbor exclusion, we have investigated the interaction of several bisphenanthridinium derivatives (Figure 2) with DNA. The linking groups on these derivatives have been constructed with size and geometry to allow binding only at adjacent sites (SR 2430), with one excluded site (SR 2193), or with potentially more than one excluded site (SR 2166). This series then effectively covers the ring separation range that was shown with the 9-aminoacridine derivatives to bind without site exclusion. It should be mentioned that the usual method of calculating ring to ring distance with the acridines, simply measuring the length of the fully extended connector chain, will not work with the derivatives of Figure 2. The linking chains in these compounds contain the rigid phenyl groups which are fixed roughly perpendicular to the phenanthridinium ring and it is the position and length of the other substituents that is critical for the maximum separation of the phenanthridinium rings. As has been done with other bisintercalators (6-9), we have specified a conformation with the intercalating rings oriented to allow both rings to intercalate. To simply specify maximum separation distance is meaningless with these compounds since, for example, the rings of SR 2430 can be separated farther than its structural isomer SR 2193 but in an intercalation alignment the rings of SR 2430 are much closer. To measure distances, we have constructed CPK models, oriented the two planar ring systems in a parallel alignment, extended the linking groups to the fullest extent permissible through normal bond rotation, and then measured the center plane to center plane separation distance for the two ring systems. These separation distances are approximately 7 to 8Å for SR 2430, 11Å for 2193, and 15Å for SR 2166. Because of steric interactions of the two para substituted phenyl rings in SR 2430, the conformation of this compound with parallel phenanthridinium rings is quite rigid. Single bond rotations in the two meta substituted compounds give these derivatives a much more flexible structure when the two phenanthridinium rings are parallel for inter-

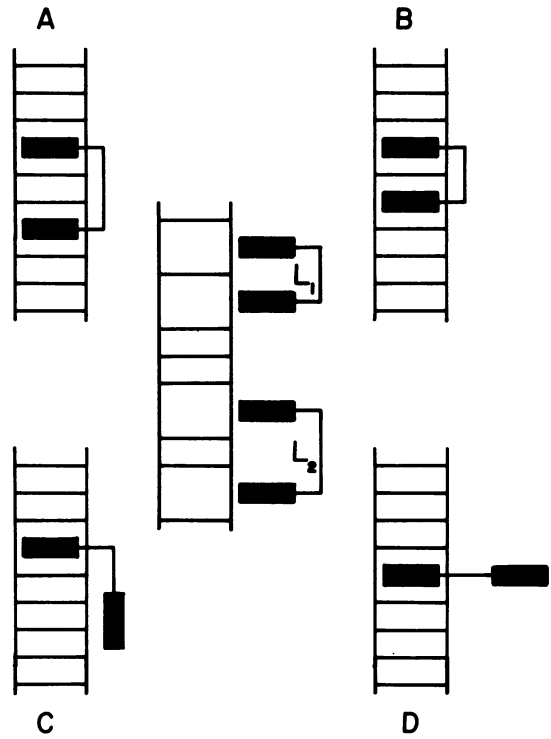
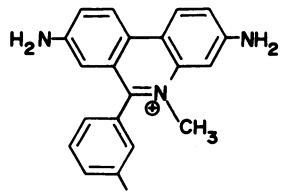
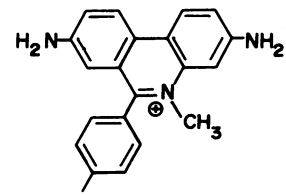


Figure 1. Possible interaction mechanisms of potential bisintercalators with double helical DNA are schematically illustrated. The center drawing illustrates base pair separation distances for binding of a bisintercalator with a short connector (L_1) and for binding with a longer connector (L_2) in a complex allowing exclusion of a center binding site between the intercalated rings. The L_2 compound can bind by bisintercalation in either a complex of type A or B while the L_1 compound can only form a type B structure. Compounds which can not bind by bisintercalation, for example the L_1 compound if it can not form a complex of type B, can still bind to the double helix through monointercalation complexes as in the C and D examples. C is characteristic of a range of monointercalation structures with the nonintercalated ring system bound into either of the grooves of the double helix. The circular dichroism spectrum of either B or C might be quite different than the spectrum for A or for monointercalators. The DNA length increase and unwinding angle for C will be similar to a monointercalator and approximately one half that for structures A or B. This suggests that when evaluating potential nonclassical bisintercalator binding modes using model compound results, some type of hydrodynamic experiment is needed.

calation interactions. Calculation of the phenanthridinium ring to ring distances using standard bond lengths and angles is reasonably straight for-



R



R'

COMPOUND	RING TO RING DISTANCES (Å)	STRUCTURE
dimidium	---	RH
SR 2183	---	$RCONHC_2H_5$
SR 2193	11	$RCONH(CH_2)_2NHCOR$
SR 2166	15	$RCONH(CH_2)_5NHCOR$
SR 2430	7-8	$R'CONH(CH_2)_2NHCOR'$

Figure 2. Structures for the phenanthridinium derivatives are shown. Ethidium is identical to dimidium except that the nitrogen quaternizing alkyl group is ethyl instead of methyl. Ring plane to ring plane distance were determined as described in the text.

ward with the meta derivatives SR 2193 and SR 2166. With the phenyl rings perpendicular to the phenanthridinium rings and the methylene groups in a staggered arrangement, we calculate center plane separation distances of 10.4 Å for SR 2193 and 14.2 Å for SR 2166. The agreement between calculated and CPK measured distances is in good agreement for a specific conformation as has been found for acridine bisintercalators (8). It should be emphasized that these are maximum separation distances and that both SR 2193 and SR 2166 can adopt conformations with the phenanthridinium rings closer together or in non-parallel alignments. Other conformational factors, such as giving a slight helical rotation to one phenanthridinium ring with respect to the other ring in the same molecule, can have slight effects on ring to ring distances and are no doubt necessary to optimize intercalator-DNA interactions in a complex.

Previous analysis of the interaction of SR 2166 and SR 2193 with DNA by circular dichroism suggested that both of these compounds bind by bisintercal-

ation (11) in a type A structure (Figure 1). CD results for SR 2430 are different than for either monomer phenanthridinium control compounds (Figure 2) or for the two meta substituted derivatives (11). Since the ring separation distance in SR 2430 is similar to that which gives bisintercalation in acridine derivatives without site exclusion, it seems possible that a similar nonstandard binding interaction (B in Figure 1) might be occurring with the para substituted bisphenanthridinium and that this could account for the anomalous CD results. Monointercalation interactions of SR 2430 of type C or some combination of types C and D from Figure 1 can also lead to circular dichroism results which are different from classical mono or bisintercalation with neighbor exclusion. To determine which of these possibilities is correct, we report here a quantitative viscometric analysis of the interaction of the compounds of Figure 1 with closed circular superhelical DNA and sonicated DNA for evaluation of unwinding angles and length increases. We have also measured DNA melting temperatures for these derivatives to determine their relative strengths of binding to DNA.

MATERIALS AND METHODS

The derivatives shown in Figure 2 were prepared at SRI as previously described (10-12). Ethidium bromide, DNA preparations, and viscometric methods were as previously described (13). All viscometric experiments were conducted in PIPES 10 buffer: 0.01M 1,4 piperazinediethane sulfonic acid, 10^{-3}M EDTA, 0.1M NaCl adjusted to pH 7.0 with NaOH. We found it very difficult to quantitatively dissolve the compounds of Figure 2 as stock solutions in PIPES 10. Compounds SR 2193, SR 2166, and SR 2430 were available as chloride salts and could be dissolved at low concentration in water. Compound SR 2183 as the bromide salt was quite difficult to dissolve in water even at low concentration. All compounds dissolved readily in dimethylsulfoxide (DMSO). Extinction coefficients were determined by dissolving weighed amounts of the derivatives in water (with prolonged warming) or in DMSO with subsequent dilution with water to give a final DMSO concentration of 1%. The two methods gave excellent agreement and averaged long wavelength extinction coefficients are 9550 $M^{-1}cm^{-1}$ (498 nm) for SR 2166, 10,000 $M^{-1}cm^{-1}$ (499 nm) for SR 2193, 9,860 $M^{-1}cm^{-1}$ (495 nm) for SR 2430, and 6,130 $M^{-1}cm^{-1}$ (487 nm) for SR 2183. Viscometric titrations were also conducted by two methods. In Method A phenanthridinium stock solutions at concentrations near 5×10^{-4} M were prepared in DMSO and diluted by a factor of ten with PIPES 10 for the titration. Flow time readings were corrected for the effect of DMSO by conducting a blank

titration of DNA using a ten fold dilution of pure DMSO with PIPES 10. In Method B stock solutions of the more soluble compounds were prepared in water at concentrations also near 5 X 10^{-4} M. To determine whether these solutions decreased in concentration due to surface absorption or precipitation, concentrations were analyzed spectrophotometrically before viscometric titrations. For some reason SR 2193 seems particularly inclined to surface absorption. Titration solutions were prepared by making ten fold dilutions of the aqueous stock solutions into PIPES 10 buffer. Since the volume of drug solution used in a titration is much less than the volume of DNA solution in the viscometer, no corrections for ionic strength changes are necessary. Thermal denaturation studies of DNA in the presence of these compounds were carried out as previously described (10). Denaturation experiments were conducted at 5.2x10^{-5} M DNA-P in 0.01 M phosphate, 10^{-5} M EDTA buffer at pH 7. Compounds were added to the desired ratio as DMSO solutions and all samples were diluted to a final DMSO concentraton of 5% for consistency. Sealed cells were used so that temperatures above 100°C could be attained.

RESULTS

Unwinding of closed circular superhelical DNA has empirically evolved as an identifying characteristic of intercalation binding reactions (1). Typical viscometric titrations of closed circular superhelical Col E1 DNA with the compounds of Figure 2 are shown in Figure 3. All compounds give the viscometric maxima expected for intercalation. All also give smooth curves with similar values of η/η_0, indicating that there are no significant nonstandard conformational equilibria in their interactions with the superhelical DNA. Quantitation of DNA unwinding can be done accurately by conducting viscometric titrations of the type shown in Figure 3 at several different DNA concentrations and plotting the results according to the Vinograd equation:

$$C_T^{\sim} = \upsilon' N_T^{\sim} + C_F^{\sim} \qquad (1)$$

where $C_T^{\sim}$ = total drug concentration, $\upsilon' = C_B^{\sim}/N_T^{\sim}$, $C_B^{\sim}$ = bound drug concentration, $N_T^{\sim}$ is the total DNA concentration in base pairs, $C_F^{\sim}$ = free drug concentration, and all quantities are those determined at the principal maximum in a viscometric titration (13, 14). Using experimentally determined υ' values from equation (1), unwinding angles, Φ_u, can be calculated from equation (2):

$$\Phi_u = \frac{\Phi_s \cdot \upsilon_s'}{\upsilon_u'} \qquad (2)$$

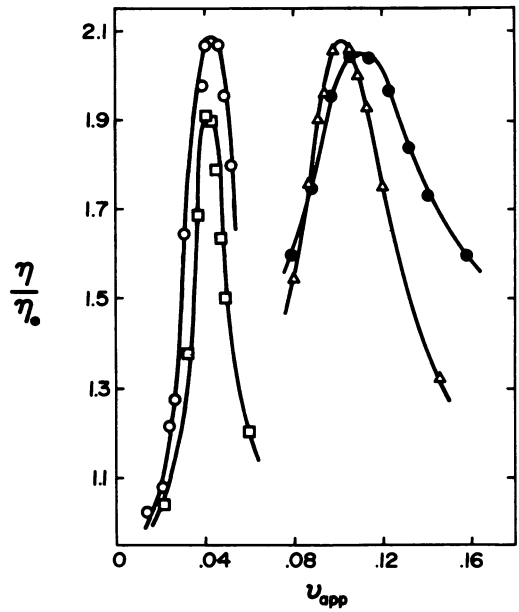


Figure 3. Viscometric titrations by Method A are shown for SR 2193 (O), SR 2166 (□), SR 2183 (Δ), and SR 2430 (●). The titrations were performed at 25°C in PIPES 10 buffer at a DNA concentration of 3.91 X 10^{-5} M base pair equivalents. The reduced specific viscosity ratio (η/η_o), where η is the reduced specific viscosity of a DNA-phenanthridinium solution and η_o is the same quantity for DNA alone, is plotted versus the molar ratio (υ_{app}) of phenanthridinium compound added per mole of DNA base pairs.

where ϕ_s is the known unwinding angle for a standard compound such as ethidium bromide, and υ'_u and υ'_s are determined from a plot according to equation (1) for the unknown and standard respectively. The υ'_u and υ'_s values can also be determined independently through a single viscometric titration and appropriate thermodynamic characterization of the ligand-DNA interaction (9). The Vinograd method does not require assumptions about binding models for fitting binding data and allows a direct linear plot for minimization of the error in determining υ' values. Plots according to equation 1 for the compounds of Figure 2 are shown in Figure 4. All plots are linear within experimental error and the slopes (equivalence binding ratios, υ' from equation (1)) are collected in Table 1. Using 26° as the unwinding angle for ethidium bromide, unwinding angles have been calculated using equation (2) and are also collected in Table 1. To determine what effect DMSO might have on these results, viscometric titrations were done without DMSO for ethidium bromide and the

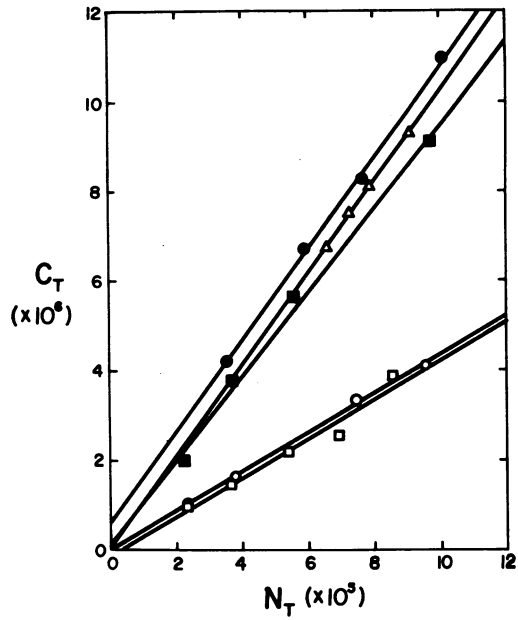


Figure 4. Total phenanthridinium derivatives and total DNA base pair molarities, determined from the maxima in viscometric titrations such as those shown in Figure 3, are plotted for SR 2193 (○), SR 2166 (□), SR 2183 (■), SR 2430 (●) and ethidium (△). All titrations in this Figure were performed by Method A and solid lines plotted in the Figure were calculated using a linear least squares regression computer program.

bisintercalators SR 2166 and SR 2193. Results for these titrations are also included in Table 1. Within experimental error, no difference in the υ' values for ethidium bromide is found by the two methods. The results for SR 2166 are also quite close by the two methods while those for SR 2193 (60 and 68°) are somewhat outside of experimental error. Because of particular difficulties of working with aqueous solutions of SR 2193 (Methods Section), we do not feel this difference is significant. The primary point is that all of the phenanthridinium compounds fall into one of two classes based on unwinding angles. Ethidium, SR 2183, and the bis compound SR 2430 have unwinding angles near 26° while the other bis-derivatives SR 2166 and SR 2193 have similar unwinding angles which are approximately twice this 26° value.

Another characteristic that distinguishes intercalation from nonintercalation binding modes is the length increase of sonicated DNA that results from intercalation (1, 15). Viscometric titrations of DNA with monointercalators

can be evaluated using the following equation:

$$\frac{L}{L_0} = \left(\frac{\eta}{\eta_0}\right)^{1/3} = 1 + \upsilon \qquad (3)$$

where L is the length of the double helix in the presence of the small molecule and L_0 is the length of the free DNA molecule. The right hand side of the equation is $1 + 2\upsilon$ for bisintercalators. Viscometric titrations of ethidium bromide and SR 2166 are shown in Figure 5A. The results for SR 2193 are quite similar to those for SR 2166 while those for SR 2183 and SR 2430 are quite similar to those for ethidium. Plots of L/L_0 were also constructed from these results according to equation (3) and are shown in Figure 5B. To obtain accurate slopes in the L/L_0 plots the length increase must be plotted versus the amount of bound ligand. Calculations of υ were conducted using the neighbor exclusion equation with an equilibrium constant under these conditions for ethidium of 10^5 on a molar scale. If only υ values below 0.2 are used, the corrections are small. Since the lower C_F values for the bisinter-

Table 1
SUMMARY OF VISCOMETRIC RESULTS

Compound	Titration Method[a]	υ'[b]	ϕ[c]	Slope[d]
SR 2166	A	.0439	60	1.5-2.0[e]
SR 2166	B	.0431	61	--
SR 2193	A	.0440	60	1.5-2.0[e]
SR 2193	B	.0376	68	--
SR 2430	A	.1022	26	.75
SR 2183	A	.0937	28	.85
ethidium	A	.1018	26	--
ethidium	B	.1008	26	.63

a) Titration methods are described in the Materials and Methods Section.
b) Slopes for plots such as those shown in Figure 3.
c) Based on the 26° standard value for ethidium (1, 18, 19).
d) Slopes of L/L_0 plots such as those shown in Figure 4B.
e) These plots are not linear and slopes in the range 1.5 to 2.0 are obtained depending on the area of the curve analyzed.

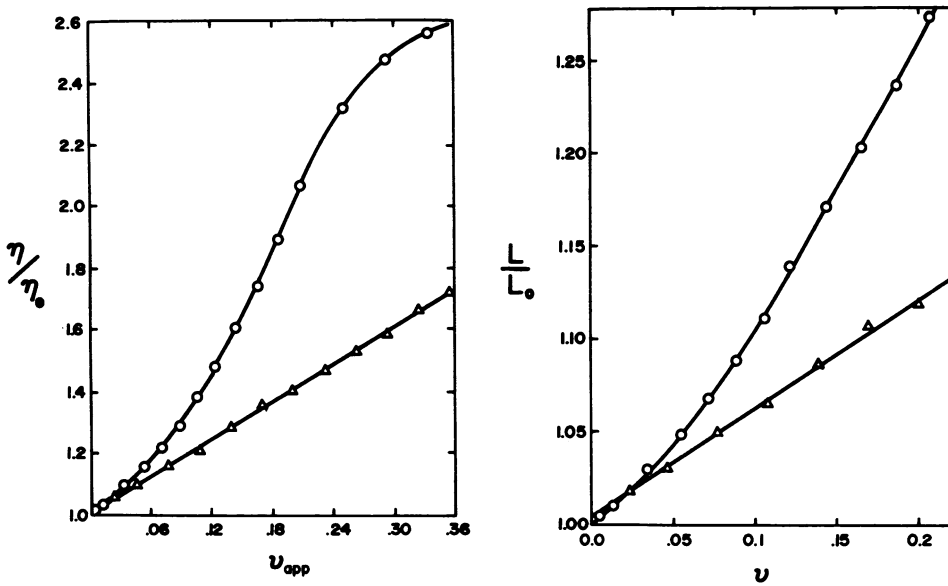


Figure 5A. Example viscometric titrations, using Method B, plotted as in Figure 3, of sonicated calf thymus DNA are shown for SR 2166 (O) and for ethidium (Δ).

Figure 5B. The results of Figure 5A are replotted as L/L_0 versus the amount of compound bound per DNA base pair equivalent, υ. Symbols are as in Figure 5A.

calators suggest that their binding constants are, as expected, greater than for ethidium, no corrections were made for these molecules in this region of binding. The L/L_0 plots for ethidium, SR 2183, and SR 2430 were linear with slopes in the range 0.6 to 0.8. Although a slope of 1.0 is expected for an ideal monointercalator, experimental slopes are usually below this ideal value. As can be seen from Figure 5, the viscometric titration of SR 2166 is not linear and the L/L_0 plots are also somewhat curved. Depending on the area chosen, slopes of from 1.5 to 2.0 are obtained for SR 2166 and SR 2193. A slope of two is expected for ideal bisintercalators. No matter what the exact values for the slopes are, the compounds once again fall into one of two groups with SR 2166 and SR 2193 having length increases approximately twice as large as those for ethidium, SR 2183 and SR 2430.

Thermal denaturation studies of DNA in the presence of these compounds are shown in Figure 6 and also support the idea of a stronger interaction of SR 2166 and SR 2193 with DNA than for the other compounds. The Tm curves for the biscompounds are all biphasic with the two phases having individual Tm

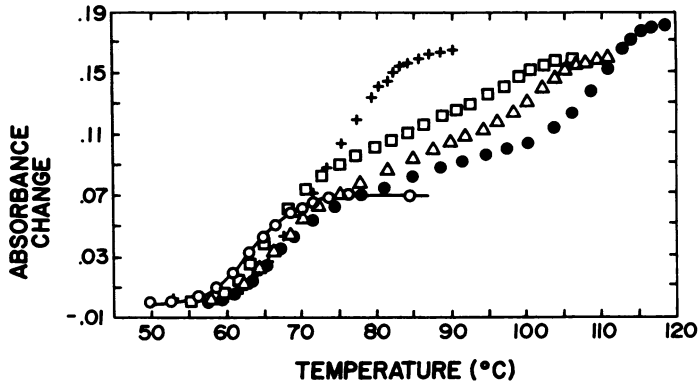


Figure 6. Thermal denaturation curves for DNA and its complexes with the derivatives of Figure 2 are shown: DNA (Tm=67°), O ; SR 2183, + ; SR 2430, □ ; SR 2193, Δ ; and SR 2166, ● . The monomer compound, SR 2183, was analyzed at a ratio of 0.1 moles per mole of DNA-P and all melting curves with the bisphenanthridinium compounds are reported at a ratio of 0.05. The buffer used in these experiments was 0.01 M phosphate, 10^{-5} M EDTA at pH 7 and containing 5% DMSO.

values which can differ by over 40° (SR 2166). The Tm of the low melting phase is only slightly stabilized relative to DNA alone (ΔTm<5°C) suggesting that this region of the DNA is not greatly perturbed by bisintercalator binding. The upper phase represents regions of DNA which are stabilized by interaction with bisintercalators. As can be seen from Figure 6, the stabilization of these high melting regions is greatest for SR 2166 followed by SR 2193 and SR 2430. The monomer, SR 2183, gives monophasic melting even at a 0.1 ratio with a Tm of 76° (ΔTm=9°). An increase in ΔTm for all of the bis versus the mono compounds would be expected due to the fact that the bis compounds are dications. The increase in ΔTm for SR 2430 relative to the monomer compounds is in the range expected in going from a monocation to a dication at this ionic strength. The additional increase in ΔTm for SR 2193 and especially SR 2166 indicates a much more favorable interaction of these compounds with DNA than for SR 2430 or the monomers. Bisintercalation of SR 2193 essentially eliminates any flexibility from the linking chain of this derivative. SR 2166 still has considerable flexibility in a bisintercalation complex which may allow for an optimization of binding interactions and could account for the larger ΔTm for this derivative relative to SR 2193.

DISCUSSION

The viscometric results for all of the phenanthridinium derivatives of Figure 2 fall into one of two well defined groups. Ethidium, SR 2183, and SR 2430 give unwinding angles and length increases expected for monointercalators. SR 2166 and SR 2193, in agreement with CD studies (11), give results characteristic of bisintercalation. The anomalous results obtained in the CD experiments with SR 2430 are not observed in the viscometric titrations of closed circular superhelical and sonicated DNA with this compound. The viscosity and Tm experiments with this derivative clearly support monointercalation. The different CD results obtained with this compound, relative to both mono and bisintercalators could be due to asymmetrical orientation of the nonintercalated ring system in one of the grooves of DNA. It is known from the work of Dervan and Becker (16) that longer para-substituted bisphenanthridinium compounds can bind to DNA by bisintercalation. This indicates that para substitution alone of SR 2430 cannot account for its lack of bisintercalation. It has been suggested that the ability of acridines to bind to DNA by bisintercalation depends strongly on the substituents on the acridine ring (9). Several bisacridines substituted only through the 9-amino linking group, and one 9 amino-4-ethyl compound were able to bind to DNA by bisintercalation in apparent violation of neighboring binding site exclusion (8, 9). Acridine rings with 6-chloro-2-methoxy or with 3,6-dichloro substituents could bind by bisintercalation only at alternate base pairs in agreement with the site exclusion binding model. The phenanthridinium derivatives of Figure 2 also can apparently bind by bisintercalation only at alternate binding sites. The para substituted derivative, SR 2430, has the phenanthridinium rings separated far enough for bisintercalation at neighboring sites but not separated far enough for site exclusion without significant distortion of the double helix. The meta substituted structural isomer of SR 2430, SR 2166, has the phenanthridinium rings separated far enough to bisintercalate with an excluded site and this compound, as well as SR 2193, binds to DNA by bisintercalation. The bisphenanthridinium compounds then apparently have the same structural constraints on bisintercalation as the 6-chloro-2-methoxy substituted bisacridine compounds.

At this time only bis-9-aminoacridine derivatives which are unsubstituted or which have limited substitution on the acridine short axis have been shown to bind to DNA without binding site exclusion. Acridines with substituents on the long axis can bind only with neighbor exclusion. In the same manner the phenanthridinium compounds of Figure 2 have the amino substituents on the long

axis of the chromophore and can only bind with site exclusion. Analyzing a CPK space filling model of the DNA double helix with both the phenanthridinium and acridine compounds has not revealed any large structural difference in their potential for binding to DNA. It should also be mentioned, however, that model building with monointercalators has also failed to explain binding site exclusion. Sobell and co-workers have proposed a conformational change at alternating sugar residues as an explanation for neighbor exclusion of binding sites (17) but the apparent violation of neighbor exclusion by the bis-9-aminoacridine derivatives brings this explanation into question. It will be of interest to study a broad range of potential bisintercalating ring systems with connectors of varying length to determine what type structures are required to bind to DNA without neighbor exclusion.

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*To whom correspondence should be addressed.

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