Sequence-Specific Interactions of Methylene Blue with Polynucleotides and DNA: A Spectroscopic Study

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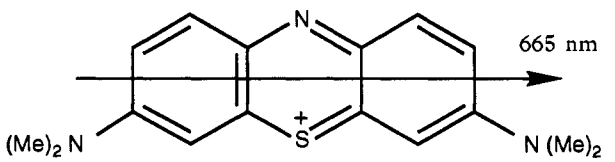
Abstract: The modes of binding of the phenothiazinium dye methylene blue (1) to alternating and nonalternating polynucleotides and to calf thymus (CT) DNA have been characterized using linear dichroism (LD) and circular dichroism (CD) spectroscopy. With the polynucleotide $[poly(dG\text{-}dC)]_2$ the interaction at low binding ratios is shown to be purely intercalative and the binding mode is insensitive to changes in ionic strength. The observed CD spectrum is bisignate, which may be due to intercalation at the different base-pair steps (5'G-C3' and 5'C-G3'), giving rise to CD signals of different sign and shape. By contrast, a single intercalative binding mode with the alternating AT polynucleotide $[poly(dA\text{-}dT)]_2$ is likely only at very low ionic strength; at high ionic strength (200 mM phosphate, pH 6.9), a second binding mode is also manifest which is attributed to groove binding of the dye. The absorption and linear dichroism spectroscopic features of the methylene blue/CT-DNA (42% GC) complex reflect those of complexes of the dye with both $[poly(dA\text{-}dT)]_2$ and $[poly(dG\text{-}dC)]_2$; the circular dichroism spectrum of the methylene blue/CT-DNA complex and its variation with ionic strength reflect the complexity of even this simple system where numerous possible binding sites exist. Comparative binding to the nonalternating polynucleotides poly(dA)·poly(dT) and poly(dG)·poly(dC), which each possess only one base-pair step, was also examined. On the basis of the combined LD and CD evidence, it is proposed that the dye is loosely bound with poly(dA)·poly(dT), probably in the major groove, and intercalated with poly(dG)·poly(dC).

Introduction

The continuing endeavor to design and develop drugs which interact with specific DNA structural motifs or sequences demands that the underlying principles by which small molecules recognize and interact with given binding sites be understood. The difficulty of rationalizing why drugs favor one site over another has stimulated interest in ligands which can interact with DNA in different ways depending on conditions. The complex nature of DNA–ligand interactions has been demonstrated in recent studies of "classical" minor-groove binders such as DAPI and Hoechst with GC sequences where non-minor-groove binding modes have been observed.[1,2] Such studies demonstrated the heterogeneity of native DNA as a target for a drug, with 10 different base-pair steps, composing AT- and GC-rich tracts in addition to regions of random sequence. Crystallographic and NMR data for drug/oligonucleotide complexes provide structural information about binding sites, but for solution structures with longer DNA, spectroscopic data can be much more revealing despite representing an average. By examining spectroscopically the interactions of a drug with polynucleotides of defined sequence under varying conditions, a model may be constructed of how the drug may interact in different ways with heterogeneous DNA.

It is a common conception that the phenothiazinium dye methylene blue (1) has only one binding mode, which is intercalation. The dye has been used extensively as an optical



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probe of biophysical systems including nucleic acids, chromatin, and nucleosomes,[3-6] since its high extinction coefficient allows it to be used in low concentrations, thus ensuring minimial perturbations of the host structure. It also photosensitizes damage (base modifications and strand breaks) in DNA,[3,7-9] which has resulted in extensive scrutiny of its binding, since the binding mode of a photosensitizer is expected to influence the photoreaction

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mechanism.[10] Although methylene blue appears to photoinduce damage principally via a singlet oxygen-mediated mechanism, it has also been shown that its singlet state can undergo direct reactions with the nucleobases when bound to mononucleotides or polynucleotides.[11-15] The deactivation mechanism is a matter of current debate, and suggestions have included electron-, successive electron/proton-, H atom-, or charge-transfer and the actual mechanism may differ with sequence depending on the binding mode.

It is clear that for the photophysical behavior of methylene blue with nucleic acids to be rationalized, the binding must be well characterized. However, despite the abundance of information accumulated about methylene blue/DNA binding to date,[9,16-20] questions still remain regarding the possibility of external binding at high P/D ratios.[40] External binding of methylene blue with double- and single-stranded nucleic acids at low P/D ratios and low ionic strength has been previously reported.[19,20] However, such studies have always relied on the observation of dye–dye interactions which occur under such conditions: the characterization of any isolated externally bound dye is hampered if strong intercalative binding also occurs extensively and dominates the optical and physical properties of the system.

Negative LD, and LD^r values of the same order as for the DNA bases have been reported under various conditions for methylene blue with different polynucleotides and DNA, which has been interpreted in terms of intercalation with all nucleic acids.[6c,17,18,21,22] However, Lyng *et al.* reported that the CD of methylene blue/$[poly(dA\text{-}dT)]_2$ increased with increasing ionic strength while that of methylene blue/$[poly(dG\text{-}dC)]_2$ remained unaffected, suggesting that the dye might be displaced to an external site on $[poly(dA\text{-}dT)]_2$ as salt was added.[21b] This supported earlier conclusions by Kelly and co-workers from studies on the ionic strength dependence of the triplet-state properties of bound methylene blue[15] and of its unwinding of DNA.[9] In our complete study here, the absorption, CD, LD, and LD^r characteristics of methylene blue in complexes with several representative polynucleotides at low binding ratios are presented under conditions of extremely low and high ionic strengths. It will become clear from the results presented that, even for a simple model intercalator such as methylene blue, there are many complexities in the interpretation of the binding data and that the mode of binding strongly depends on both the nucleic acid substrate and the experimental conditions.

Materials and Methods

Materials. Methylene blue (Fluka puriss grade, 98%) was purified by gravity column chromatography on Sephadex LH-20 with methanol as eluent.[15] Multiple separations were performed, and purity was confirmed by TLC on silica plates with 9:1 methanol/acetic acid as solvent[22] and by absorption, emission, and excitation spectroscopy. Dye concentration was determined using the extinction coefficient in phosphate buffer ϵ_{665} = 81 600 M^{-1} cm^{-1}.[16] Highly polymerized calf thymus DNA (Type I, sodium salt, Sigma) was dialysed against 5 mM phosphate buffer (pH 6.9) prior to use. The synthetic polynucleotides $[poly(dG\text{-}dC)]_2$ (750 bp), $[poly(dA\text{-}dT)]_2$ (2658 bp), poly(dG)·poly(dC) (8560 bp), poly(dA)·poly(dT) (349 bp), and poly(dA) (349 bases) were purchased from Pharmacia and used as received. The concentrations of all nucleic acids are presented in terms of phosphate concentration. $[Poly(dG\text{-}dC)]_2$, $[poly(dA\text{-}dT)]_2$, and poly(dA) samples were dissolved in 5 mM phosphate (pH 6.9) while poly(dG)·poly(dC) and poly(dA)·poly(dT) were reconstituted in 100 mM NaCl, 5 mM phosphate (pH 6.9), according to manufacturers instructions to prevent denaturation; some samples were also prepared in 1 mM phosphate (pH 7). Polynucleotide concentrations were determined using the following molar absorptivities: ϵ_{260} = 6600 M^{-1} cm^{-1} for CT-DNA,[23] ϵ_{254} = 8400 M^{-1} cm^{-1} for $[poly(dG\text{-}dC)]_2$,[24] ϵ_{262} = 6600 M^{-1} cm^{-1} for $[poly(dA\text{-}dT)]_2$,[24] ϵ_{253} = 7400 M^{-1} cm^{-1} for poly(dG)·poly(dC),[24] ϵ_{260} = 6000 M^{-1} cm^{-1} for poly(dA)·poly(dT),[24] and ϵ_{257} = 8600 M^{-1} cm^{-1} for poly(dA).[24] Measurements were conducted at room temperature on solutions buffered with 5 mM phosphate (2.5 mM Na_2HPO_4 and 2.5 mM NaH_2PO_4, pH 6.9), 200 mM phosphate (100 mM of each salt, pH 6.9), or 5 mM phosphate containing 0.1 or 0.2 M NaCl (pH 6.9). Buffers were prepared using chemical grade salts and water purified in a Millipore Milli-Q purification system. Experimental samples were prepared using calibrated micropipets.

Absorption. Isotropic UV/vis absorption spectra were recorded on a Varian Cary 2300 spectrophotometer. All data were normalized to a 1-cm path length.

Circular Dichroism (CD). CD spectra were measured on a Jasco J-720 instrument, and data were normalized to a 1-cm path length. The data are presented as collected in mdeg—these data can be converted to absorbance units through division by the factor 32 980 mdeg. Although methylene blue is achiral, it exhibits an induced CD signal when bound to nucleic acids as a result of coupling of its electric-dipole-allowed transition with the transitions of the chirally organized nucleobases.[17,21]

Linear Dichroism (LD). LD spectra were measured on a Jasco J-500A spectropolarimeter, adapted as described previously with an Oxley prism to convert the incident circularly polarized light to linear.[25] Orientation of the dye/nucleic acid samples was achieved in a flow Couette cell with an outer rotating cylinder. The experimental path length was 1 mm, and data were normalized to a 1-cm path length. LD is the differential absorptoin of light plane polarized parallel and perpendicular to the flow direction in the Couette cell. The magnitude of the LD signal depends on the degree of orientation of the sample as well as the molar absorptivity and concentration of the sample. Since the different polyelectrolytes used had different flexibilities and chain lengths, it was necessary to use quite different shear gradients for the different systems which are stated in each case in the text.

Reduced Linear Dichroism (LD^r). The reduced dichroism (LD^r) is defined as

$$LD^r(\lambda) = LD(\lambda)/A_{iso}(\lambda) \quad (1)$$

where A_{iso} is the absorption of the sample without orientation. It is related to the orientation of the chromophore as

$$LD^r(\lambda) = \frac{3}{2}S\,(3\langle\cos^2\alpha\rangle - 1) \quad (2)$$

where α represents the angle between the absorbing transition moment

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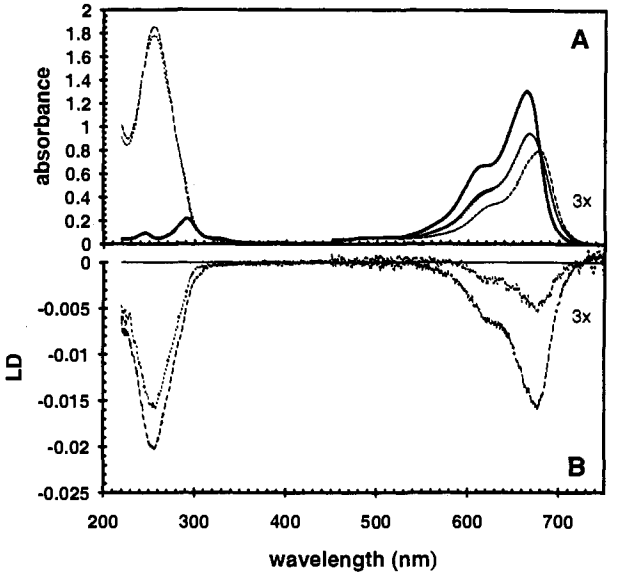

Figure 1. (A) Absorption and (B) LD spectra for methylene blue with $[poly(dG\text{-}dC)]_2$ at low binding ratios. [MB] = 4 μM, $[[poly(dG\text{-}dC)]_2]$ = 199 μM, P/D = 50, shear gradient 3140 s^{-1}, low and high salt correspond to 5 and 200 mM phosphate (pH 6.9), respectively. The spectra shown are (a) free dye in solution (▬); (b) MB/$[poly(dG\text{-}dC)]_2$, low salt and (c) MB/$[poly(dG\text{-}dC)]_2$, high salt (···). In A, the generated spectrum (—) shows an attempted fitting of c with a sum of 60% (b) and 40% (a). The region between 450 and 750 nm is expanded by a factor of three for comparative purposes.

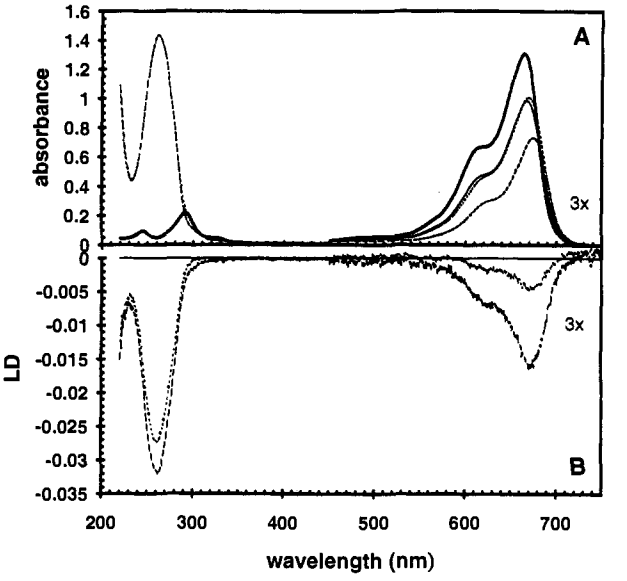

Figure 2. (A) Absorption and (B) LD spectra for methylene blue with $[poly(dA\text{-}dT)]_2$ at low binding ratios. [MB] = 4 μM, $[[poly(dA\text{-}dT)]_2]$ = 199 μM, P/D = 50, shear gradient 3140 s^{-1}, low and high salt correspond to 5 and 200 mM phosphate (pH 6.9), respectively. The spectra shown are (a) free dye in solution (▬); (b) MB/$[poly(dA\text{-}dT)]_2$, low salt (- - -); and (c) MB/$[poly(dA\text{-}dT)]_2$, high salt (···). In A, the generated spectrum (—) shows an attempted fitting of c with a sum of 50% (b) and 50% (a). The region between 450 and 750 nm is expanded by a factor of 3 for comparative purposes.

and the DNA helix axis. S is an orientation function describing the degree of orientation of the DNA helix such that $S = 1$ is equivalent to perfect orientation and $S = 0$ to random orientation. $\langle \cos^2 \alpha \rangle$ represents an average over the angular distribution. S depends on the DNA stiffness and length, the flow rate, and the viscosity of the medium. S can be determiend from the dichroism of DNA at 260 nm, where the π–π^* transitions are polarized in the plane of the bases; previous studies have indicated an effective value of $\alpha = 86°$ for B-DNA.[26]

Results

Alternating Nucleic Acid Polymers at Low Mixing Ratios. (a) Absorption. Figures 1A–3A show the absorption spectra for methylene blue (4 μM) with each of the polynucleotides $[poly(dG\text{-}dC)]_2$, $[poly(dA\text{-}dT)]_2$, and CT-DNA under conditions of low (5 mM phosphate) and high (200 mM phosphate) ionic strength at P/D ratios of 50. Although at this concentration there is a small amount of methylene blue dimer present in free solution,[28] this is eliminated when the dye binds to the polynucleotides.[15] In the presence of the polymers, red shifts and hypochromicities were observed in the methylene blue visible absorption band. At low ionic strength it is estimated that most of the dye is bound (95–97%). Increasing the ionic strength reduces the intercalative binding constant: consequently, less dye is bound at high ionic strength.[11] With $[poly(dG\text{-}dC)]_2$, an isosbestic point is observed in the visible band (Figure 1A) while with CT-DNA and $[poly(dA\text{-}dT)]_2$ there are none. It appears that with $[poly(dG\text{-}dC)]_2$ only one binding mode exists at both ionic strengths while with $[poly(dA\text{-}dT)]_2$ and CT-DNA the binding modes are different at low and high ionic strengths. Thus, with $[poly(dG\text{-}dC)]_2$ the measured absorption spectrum at high ionic strength could be fitted reasonably well assuming contributions of 60% from the experimental dye spectrum at low ionic strength, where it is expected to be almost completely bound, and 40% from the free dye spectrum (Abs_{est}; as shown in Figure 1A).

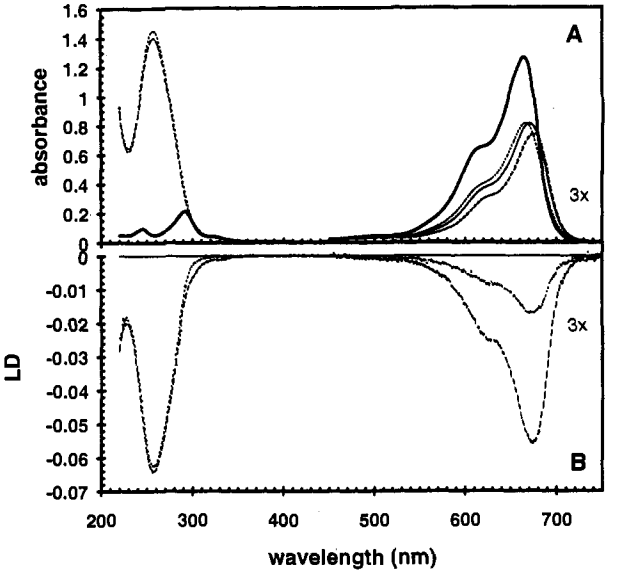

Figure 3. (A) Absorption and (B) LD spectra for methylene blue with CT-DNA at low binding ratios. [MB] = 4 μM, [CT-DNA] = 200 μM, P/D = 50, shear gradient 314 s^{-1}, low and high salt correspond to 5 and 200 mM phosphate (pH 6.9), respectively. The spectra shown are (a) free dye in solution (▬); (b) MB/CT-DNA, low salt (- - -); and (c) MB/CT-DNA, high salt (···). In A, the generated spectrum (—) shows an attempted fitting of c with a sum of 80% (b) and 20% (a). The region between 450 and 750 nm is expanded by a factor of 3 for comparative purposes.

This fit gave the correct wavelength shift as well as amplitude for the fitted spectrum. In contrast, such manipulations could not be performed on the spectra with $[poly(dA\text{-}dT)]_2$ or CT-DNA: combinations of free and bound dye spectra adjusted to the correct intensities for the high ionic strength spectra did not give the correct spectral shifts, suggesting that with both $[poly(dA\text{-}dT)]_2$ and CT-DNA more than one binding mode exists at high ionic strength and that the different modes have different effects on the optical absorption spectra. At a higher dye

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(28) $K_D = 6 \times 10^3$ M^{-1} (ref 27); therefore, *ca.* 4–5% of the methylene blue exists as dimer in solution.

Table 1. Effect of Dye Binding on the LD in the DNA Absorption Band (260 nm)

	low salt (5 mM phosphate)			high salt (0.2 M phosphate[d] or 0.1 M NaCl[e])		
polynucleotide	10^3LD_{DNA}[a]	$10^3LD_{MB^+}$[b]	% change[c]	10^3LD_{DNA}[a]	$10^3LD_{MB^+}$[b]	% change[c]
$[poly(dG\text{-}dC)]_2$	−8.7	−10.0	+15% ↑	−6.6	−6.5	−2% ↓
$[poly(dA\text{-}dT)]_2$	−14.3	−16.8	+17% ↑	−11.4	−13.7	+20% ↑
CT-DNA	−56.7	−63.4	+12% ↑	−59.6	−62.0	+4% ↑
poly(dG)·poly(dC)				−21.3	−20.7	−3% ↓
poly(dA)·poly(dT)	−63.3	−53.5	−15% ↓	−113.3	−102.3	−10 ↓

[a] Magnitude of LD signal at 260 nm for the free polynucleotide; for shear gradients and concentrations see captions for relevant figures. [b] Magnitude of LD signal at 260 nm with dye bound to the polynucleotide; see figure captions for details. [c] Percentage change of the LD signal at 260 nm when the dye binds. ↑ indicates an increase in the magnitude of the LD signal when the dye binds, and ↓, a decrease. [d] High salt is 200 mM phosphate for the alternating polynucleotides and DNA. [e] High salt is 5 mM phosphate/100 mM NaCl for the nonalternating polynucleotides.

Table 2. LD^r Data for Methylene Blue Bound to Alternating Polynucleotides

	5 mM phosphate (pH 6.9)				200 mM phosphate (pH 6.9)			
polynucleotide	LD^r_{DNA}	$LD^r_{MB^+}$	DR[a]	α_{MB^+}[b]	LD^r_{DNA}	$LD^r_{MB^+}$	DR[a]	α_{MB^+}[b]
$[poly(dG\text{-}dC)]_2$	−0.011	−0.020	1.82	90	−0.009	−0.0045	0.5	
						−0.010[c]	1.11	90
$[poly(dA\text{-}dT)]_2$	−0.0225	−0.0225	1.00	≤86	−0.019	−0.004	0.21	
						−0.013[d]	0.68	≤71
CT-DNA	−0.046	−0.077	1.67	90	−0.043	−0.018	0.42	
						−0.031[e]	0.72	≤72

[a] DR (dichroism ratio) = $LD^r_{DNA}(260\text{ nm})/LD^r_{MB^+}(660\text{ nm})$. [b] α_{MB^+} (orientation angle) is the *average* angle in deg between the absorbing methylene blue transition moment and the DNA helix axis calculated from eq 2 assuming $\alpha = 86°$ for the DNA bases and the orientation factor S is not affected by the binding of the dye. [c] Calculated assuming 60% of the dye is bound. [d] Calculated assuming 50% of the dye is bound. [e] Calculated assuming 80% of the dye is bound.

concentration (13 μM), but comparable P/D of 46, similar effects were observed with these alternating polynucleotides and CT-DNA: under such conditions a greater amount of dye is bound at both low and high ionic strength.

(b) Linear Dichroism and LD^r. The LD spectra measured for the dye/polynucleotide samples are shown in Figures 1B–3B. In each case, the LD in the visible band due to the absorption of bound dye is clearly negative at both ionic strengths examined. LD is observed *only* for dye which is bound and is thus of a lower magnitude under conditions of high ionic strength. However, it is not possible to estimate the amount of dye bound at high ionic strength from the magnitude of the LD signal, since the possibility that some of the dye may be displaced to an alternative binding mode with different LD characteristics cannot be excluded; *e.g.*, a fraction of the bound dye could give rise to positive LD if it were minor-groove bound.

The LD spectra at high and low ionic strength had the same maximum wavelengths within error in all cases in spite of the fact that the absorption spectra with $[poly(dA\text{-}dT)]_2$ and CT-DNA indicated more than one binding mode with different spectral shifts. This suggests that the new binding modes observed at high ionic strength make insignificant contributions to the visible LD spectra.

Binding of the dye to the polymers caused small increases in the LD of the nucleic acids (Table 1) which can be due to stiffening or lengthening of the DNA, consistent with intercalation. However, methylene blue has a small but significant absorption in the DNA band which could make either a positive or negative contribution to the LD at 260 nm, depending on the orientation of the dye in a given binding site.

The reduced linear dichroism (LD^r) spectra were calculated from the LD and absorption spectra (LD/A_{iso}, supplementary material), and the LD^r data in the visible and UV bands are presented in Table 2 along with the calculated orientation angles. Methylene blue has a single long-axis polarized transition in its visible absorption band. There has been speculation that there may also exist a weak short-axis or out-of-plane polarized transition at the red edge of this band,[6d,29] but recent magnetic circular dichroism experiments in solution and LD experiments on methylene blue oriented in stretched PVA films have failed to confirm this.[30]

At low ionic strength with each polymer, the LD^r in the dye visible band (680 nm) was more negative than that observed in the polynucleotide band (260 nm). This was most pronounced with $[poly(dG\text{-}dC)]_2$ and CT-DNA while with $[poly(dA\text{-}dT)]_2$ the LD^r of the dye was only slightly more negative than that of the nucleobases. A more negative LD^r than that of the DNA bases has been observed for several intercalators including ethidium and acridines,[2a] ruthenium polypyridyl complexes,[31] and the cyanine dye oxazole yellow (YO)[32] and implies that on average the bound dye molecules are oriented more perpendicular to the helix axis than the DNA bases. This suggests that there may be either (i) significant inclination of the bases from perpendicular orientation and that ligands are intercalated with their transition moments parallel to the tilt axis or (ii) local stiffening of the helix at the intercalation sites. That the LD^r for the dye is more negative than that of the bases with $[poly(dA\text{-}dT)]_2$, although it is with $[poly(dG\text{-}dC)]_2$ and DNA, has several possible explanations: firstly, even at low ionic strength there may be some amount of the dye bound in a mode which gives rise to a more positive LD than intercalation does; secondly, the dye may be intercalated with its long axis oriented more parallel to the base-pair long axis in $[poly(dA\text{-}dT)]_2$ than in the other polymers; and, finally, the base inclinations in $[poly(dA\text{-}dT)]_2$ may not be as large as those in the GC polymer and CT-DNA, although this would be inconsistent with published data on DNA base twist and tilt angles.[33]

At high ionic strength, determination of the correct values for LD^r is not trivial, since the LD must be divided by the absorption of the *bound* dye, which under the conditions used can only be estimated. However, it can be assessed for the $[poly(dG\text{-}dC)]_2$ polymer as *ca.* 60% that of the low-salt spectrum (Figure 1A), and the LD/Abs_{est} in this case gives an LD^r which is indeed more negative than that of the DNA bases. LD^r data can also be

(29) (a) Nordén, B. *Linear Dichroism Spectroscopy: Proceedings of a Nobel Workshop in Lund*; Lund University Press: Sweden, 1977; pp 22–24. (b) Kubista, M. Ph.D. Thesis, Chalmers University of Technology, Gothenburg, 1988.

(30) Tuite, E. Unpublished results.

(31) Hiort, C.; Lincoln, P.; Nordén, B. *J. Am. Chem. Soc.* **1993**, *115*, 3448–3454.

(32) Larsson, A.; Carlsson, C.; Jonsson, M.; Albinsson, B. *J. Am. Chem. Soc.*, in press.

(33) Chou, P.-J.; Johnson, W. C., Jr. *J. Am. Chem. Soc.* **1993**, *115*, 1205–1214.

estimated for [poly(dA-dT)]$_2$ and CT-DNA by dividing the measured LD at high ionic strength by a percentage of the measured absorption spectrum at low ionic strength. However, in order for the dye LD^r in the presence of [poly(dA-dT)]$_2$ to be even slightly more negative than that of the DNA bases, it would be required that only 35% of the dye be bound: the best estimate from fitting the experimental absorption spectrum used 50% bound dye, and previous binding data[11] suggests that even more may be bound.[34] Thus, it appears probable that under these conditions a fraction of the dye is bound to [poly(dA-dT)]$_2$ in a mode which gives an LD signal which is less negative than that for intercalation. Certainly, there could also be a small amount of dye bound in such a mode at low ionic strength, giving an LD^r for the dye which is not more negative than that of the DNA bases. In CT-DNA, there may be complex equilibria between possible binding sites, and while the best fit to the absorption spectrum at high ionic strength occurs using about 80% bound dye, no more than 50% of the dye should be bound to make the LD^r in the dye band more negative than that in the DNA band.

At high ionic strength with 13 μM methylene blue, where more of the dye is bound, the LD^r for MB is more negative than that of the DNA bases with [poly(dG-dC)]$_2$, and it is about the same as that of the DNA bases with CT-DNA, while with [poly(dA-dT)]$_2$ it is still less negative than that of the bases, supporting the model of a second binding mode with [poly(dA-dT)]$_2$ and CT-DNA under high-salt conditions.

(c) Circular Dichroism. The CD spectra in the visible region for MB (13 μM) with the three polymers are presented in Figure 4 and were found to be very similar to those reported previously.[17,21] The induced CD signals were generally very small and could not be clearly distinguished from noise if a lower dye concentration was used. As with LD, only bound dye gives rise to a signal; the spectra are thus presented in millidegrees (mdeg), as they were measured, due to the difficulties in determining the exact amount of bound dye in all cases, which prevented conversion to standard molar units ($\Delta\epsilon$).

The CD spectra of methylene blue with [poly(dG-dC)]$_2$ are biphasic at both low and high ionic strength and have practically the same magnitude in the two samples although less of the dye is expected to be bound in 200 mM phosphate. At these higher concentrations of dye and polymer it was estimated that about 85–90% of the dye should be bound: indeed, the spectrum at high ionic strength has a magnitude about 85% that of the spectrum at low ionic strength. Possible explanations for the biphasic behavior invoke exciton interactions or two different binding sites which give different CD signals. The bisignate shape of the CD (positive maximum at 705 nm, negative maximum at 670 nm, and change of sign at 695 nm) has the form of an exciton CD but is different from that of the true exciton spectrum observed with this polynucleotide at high binding ratios (supplementary material). It is possible that interactions between dye molecules intercalated in next nearest neighbor sites or between an intercalated and an externally bound dye could give rise to a weak exciton-type CD spectrum, but such a proposal is discounted here for several reasons. The CD changes sign at a higher wavelength than the maximum absorption of the bound dye, and the CD spectrum is nonconservative. Also, there is no evidence of any changes in the absorption, LD, or fluorescence[11b] characteristics of this system that indicate any dye–dye interactions under these high P/D conditions, and Scatchard plots[11b] for methylene blue with [poly(dG-dC)]$_2$ do not show evidence of cooperative binding at high P/D. Finally, the shape of the spectrum is independent of ionic strength and of the P/D ratio, both of which would be expected to influence the extent of dye–dye interactions.

(34) Using the values for the binding constant and binding number of $K_b \sim 10^5$ M^{-1} and $n \sim 0.125$, it is calculated that about 65% of the dye should be bound.

(35) Lerner, D. B.; Kearns, D. R. *Biopolymers* **1981**, *20*, 803–816.

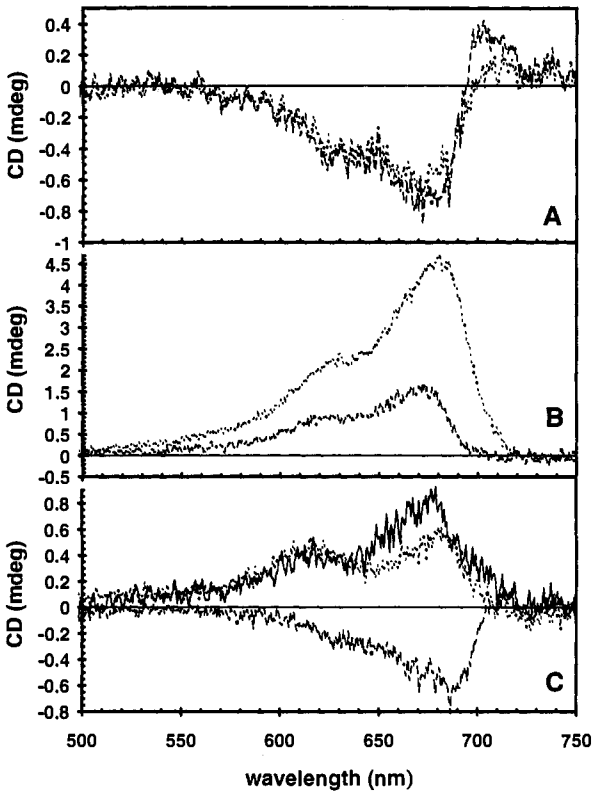

Figure 4. CD spectra for methylene blue with (A) [poly(dG-dC)]$_2$, (B) [poly(dA-dT)]$_2$, and (C) CT-DNA at low binding ratios. [MB] = 13 μM; (A) [[poly(dG-dC)]$_2$] = 509 μM, P/D = 39; (B) [[poly(dA-dT)]$_2$] = 503 μM, P/D = 39; (C) [CT-DNA] = 600 μM, P/D = 46. Low and high salt correspond to 5 and 200 mM phosphate (pH 6.9), respectively. The spectra shown are as follows. (A): (a) MB/[poly(dG-dC)]$_2$, low salt (- - -); (b) MB/[poly(dG-dC)]$_2$, high salt (···). (B): (c) MB/[poly(dA-dT)]$_2$, low salt (- - -); (d) MB/[poly(dA-dT)]$_2$, high salt (···). (C): (e) MB/CT-DNA, low salt (- - -); (f) MB/CT-DNA, high salt (···). In C, the generated spectrum (—) represents a crude attempt to fit f with contributions from b, c, and d, *i.e.*, 0.5[0.33(d) + (c)] + (b).

Thus, it appears that the spectrum is due to two different sites with CD signals of opposite sign and shift. These two sites could be intercalation and groove binding, two different intercalation orientations, or intercalation at the two different base-pair steps 5′ G-C 3′ and 5′ C-G 3′: these possibilities are considered in greater detail in the Discussion.

The behavior with [poly(dA-dT)]$_2$ is quite different, as previously observed with the absorption and LD spectra. The CD signal is positive over the entire spectrum under all ionic strength conditions, and the magnitude of the CD signal increases significantly with increasing ionic strength. There is a slight redshift of the maximum CD wavelength at the higher ionic strength, however, which suggests that the two spectra do not arise from the same species. Indeed, it is expected that less rather than more dye be bound at the higher ionic strength, and the larger CD signal in 200 mM phosphate strongly suggests a shift of the bound dye from an intercalative to an external, possibly groove-bound, site.[21]

The CD signals for methylene blue with CT-DNA are extremely small, and the analysis of the spectra is far from straightforward. It had been reported previously from this lab that the induced CD for methylene blue bound to CT-DNA changes sign on going from low to high ionic strength.[17] This is confirmed by the spectra shown in Figure 4C, which have been recorded with more precision than allowed previously. However, it is clear that in neither case has the CD signal exactly the shape of the dye visible absorption band. The CD spectrum observed at low ionic strength is quite similar to that for the dye bound to [poly(dG-dC)]$_2$ while that at high ionic strength appears to represent a combination of the

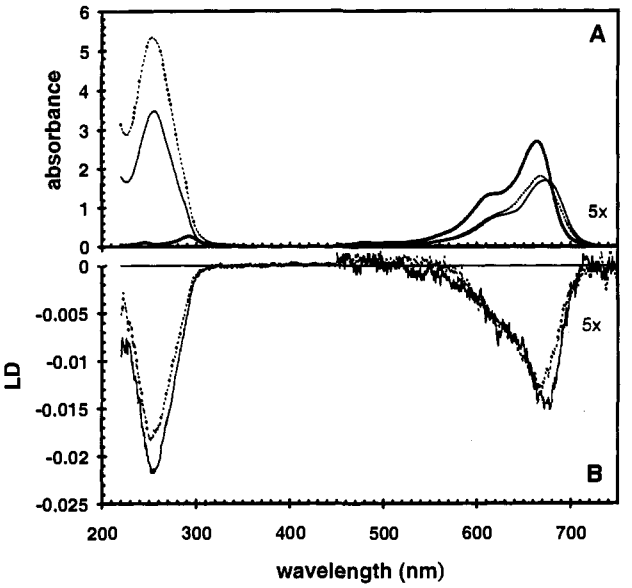

Figure 5. (A) Absorption and (B) LD spectra for methylene blue with GC polynucleotides at low binding ratios. [MB] = 6.6 μM, [GC] = 393 μM, P/D = 60, shear gradients [poly(dG-dC)]$_2$ 3140 s^{-1} and poly(dG)·poly(dC) 785 s^{-1}, buffer was 5 mM phosphate/100 mM NaCl (pH 6.9). The spectra shown are (a) free dye in solution (▬); (b) MB/[poly(dG-dC)]$_2$ (—); and (c) MB/poly(dG)·poly(dC) (···). The region between 450 and 750 nm is expanded by a factor of 5 for comparative purposes.

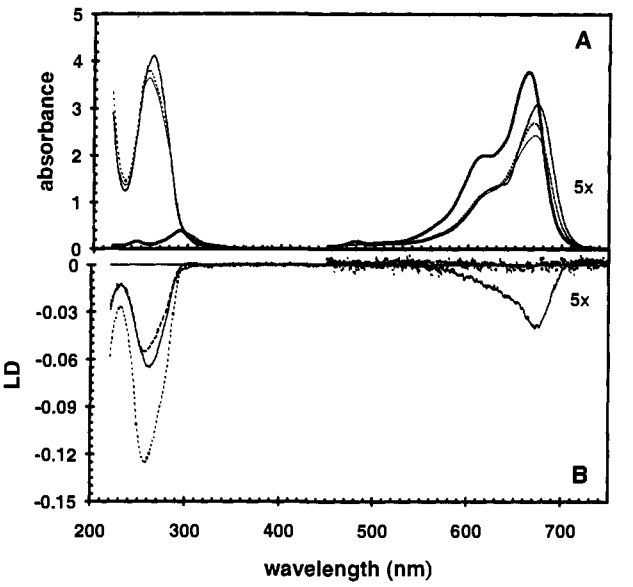

Figure 6. (A) Absorption and (B) LD spectra for methylene blue with AT polynucleotides at low binding ratios. [MB] = 10 μM, [AT] = 600 μM, P/D = 60, shear gradients [poly(dA-dT)]$_2$ 3140 s^{-1} and poly(dA)·poly(dT) 314 s^{-1}, low and high salt correspond to 5 mM phosphate and 5 mM phosphate/100 mM NaCl (pH 6.9), respectively. The spectra shown are (a) free dye in solution (▬); (b) MB/[poly(dA-dT)]$_2$, high salt (—); (c) MB/poly(dA)·poly(dT), low salt (- - -); and (d) MB/poly(dA)·poly(dT), high salt (···). The region between 450 and 750 nm is expanded by a factor of 5 for comparative purposes.

CD spectra of the dye when bound to [poly(dG-dC)]$_2$ and to [poly(dA-dT)]$_2$: in fact, with a crude combination of polynucleotide spectra, it is possible to approximately fit the DNA spectrum (Figure 4C). Hence, in view of the results obtained with the synthetic polynucleotides, the data shown here suggest a redistribution of the dye between GC- and AT-like sites as the ionic strength is raised rather than a rotation in the intercalation pocket, which was the model proposed initially before CD data with the polynucleotides were available.[17]

Nonalternating Nucleic Acid Polymers at Low Mixing Ratios. (a) Absorption. The absorption spectra for methylene blue with the nonalternating homopolymers poly(dG)·poly(dC) in 100 mM NaCl and poly(dA)·poly(dT) in both 5 mM phosphate and 100 mM NaCl are shown in Figures 5A and 6A, where they are compared with the spectra of the dye with the analogous alternating polymers. For both GC and AT polymers, the band shapes, hypochromicities, and spectral shifts in the visible absorption band are somewhat different for the alternating and nonalternating polynucleotides.

(b) Linear Dichroism and LD^r. Figures 5B and 6B show the comparative LD spectra of the dye with the alternating and nonalternating copolymers. With each of the GC polymers, negative LD in the dye band is observed: both signals are of approximately the same magnitude although the maximum wavelengths of the bound dye spectra are clearly different. With the AT polymers, on the other hand, negative LD in the visible region is observed only with the alternating polymer: no significant LD signal was obtained in the methylene blue visible absorption region with poly(dA)·poly(dT) for any ionic strengths greater than 1 mM phosphate (up to 200 mM NaCl). In 1 mM phosphate a very small negative LD was observed which was calculated to give an angle of 56–58° between the helix axis and the dye long axis. This absence of LD may result from any one of four phenomena: (1) the dye not binding to the polymer, (2) its long axis being oriented at the magic angle (54.7°), (3) the presence of two or more binding modes so that the average LD signal is close to zero, or (4) a nearly random orientation of the bound dye. The first of these proposals is eliminated considering the shifts in the absorption spectra and the observation of induced CD (below). It is more difficult to distinguish between the other three possible explanations, although binding with a magic angle orientation is less probable and the LD independence from ionic strength suggests that two binding modes are unlikely unless they have identical ionic strength responses.

Although the polymer was reconstituted at quite high salt to ensure it remained double-stranded, it was still possible that the absence of LD for the dye was due to unspecific binding to locally denatured poly(dA) and poly(dT) strands. Hence, the binding of methylene blue to the single-stranded polynucleotide poly(dA) was checked.[36] At low ionic strength (5 mM phosphate), negative LD was observed in both the dye and the DNA absorption bands, but when the ionic strength was raised to 100 mM NaCl, there was no LD signal for either the dye or the DNA bases. Since poly(dA)·poly(dT) had strong LD, even though there was none for the dye, and since the CD spectrum of methylene blue bound to poly(dA) at low ionic strength (supplementary material) was completely different from the CD when it was bound to poly(dA)·poly(dT), it is clear that the absence of LD in the dye band is not due to binding to denatured polynucleotide. These results contrast wtih two previous reports on the binding of methylene blue to poly(dA)·poly(dT), both using electric dichroism,[2a,6c] which found significant negative LD in the dye absorption band. However, electric dichroism measurements demand that the experiments be carried out at low ionic strength, *e.g.*, 10 mM Tris/10 mM EDTA[6c] or 1 mM sodium cacodylate,[22] under which conditions poly(dA)·poly(dT) could conceivably be denatured.

The LD^r data for the dye/polynucleotide systems were calculated and are presented in Table 3. As previously mentioned, it is difficult to estimate exactly how much dye is bound in each system although, in 100 mM phosphate and at the higher dye concentrations used in these experiments, it is estimated that at least 90% of the dye should be bound in each case.

With both the GC polynucleotides, the LD^r in the dye visible band is more negative than that at 260 nm. The LD$^r_{DNA}$/LD$^r_{DYE}$

(36) The homopolynucleotide poly(dA) is single-stranded under the conditions used here (pH 6.9) although it does self-associate when protonated (ref 35).

Table 3. LD^r Data for Methylene Blue Bound to Nonalternating Polynucleotides

	5 mM phosphate				100 mM NaCl			
polynucleotide	LD^r_{DNA}	$LD^r_{MB^+}$	DR[a]	α_{MB^+}[b]	LD^r_{DNA}	$LD^r_{MB^+}$	DR[a]	α_{MB^+}[b]
poly(dG)·poly(dC)					–0.0035	–0.007	2.00	90
poly(dA)·poly(dT)	–0.033	–0.006	0.18	58	–0.027	0		54.7 or none
poly(dA)	–0.004	–0.0058	1.45	90	0	0		

[a] DR (dichroism ratio) = $LD^r_{DNA}(260\text{ nm})/LD^r_{MB^+}(660\text{ nm})$. [b] α_{MB^+} (orientation angle) is the *average* angle in deg between the absorbing methylene blue transition moment and the DNA helix axis calculated from eq 2 assuming $\alpha = 86°$ for the DNA bases and the orientation factor S is not affected by the binding of the dye.

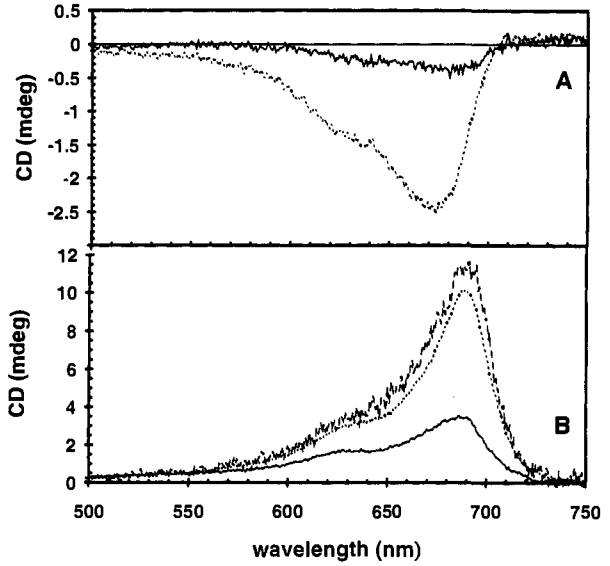

Figure 7. CD spectrum for methylene blue with (A) GC polynucleotides and (B) AT polynucleotides: (A) [MB] = 6.6 μM, [GC] = 393 μM; (B) [MB] = 10 μM, [AT] = 600 μM. Low and high salt correspond to 5 mM phosphate and 5 mM phosphate/100 mM NaCl, respectively. The spectrum shown are as follows. (A): (a) MB/[poly(dG-dC)]$_2$, high salt (– –); (b) MB/poly(dG)·poly(dC), high salt (···). (B): (c) MB/[poly-(dA-dT)]$_2$, high salt (– –); (d) MB/poly(dA)·poly(dT), low salt (- - -); and (e) MB/poly(dA)·poly(dT), high salt (···).

is greater for poly(dG)·poly(dC) than for [poly(dG-dC)]$_2$: this may be a result of more dye being bound to poly(dG)·poly(dC), of different angular orientations of intercalated dye in the two polymers, of the bases being more inclined on average in the nonalternating polymer, or of a greater local stiffening of the poly(dG)·poly(dC) helix compared with [poly(dG-dC)]$_2$ when the dye binds. These possibilities cannot be easily distinguished, but what can be concluded is that the dye is intercalated in each of these polynucleotides.

With [poly(dA-dT)]$_2$, the LD^r of the dye is somewhat less negative than that of the bases while with poly(dA)·poly(dT) there is no LD^r for the dye although the LD^r for the bases is very strongly negative. This reflects the better orientation of the nonalternating polynucleotide, probably due to it being stiffer than the nonalternating polymer, which is longer and experiences a higher shear moment. The LD^r of the poly(dA)·poly(dT) bases is reduced on binding of the dye, which could be caused by increased flexibility of the polynucleotide when the dye binds and is consistent with nonintercalative binding.

(c) Circular Dichroism. In Figure 7A and B the CD spectra for methylene blue with the GC and the AT polynucleotides are shown. In both cases the magnitudes of the CD signals are much greater with the nonalternating polymers. While the CD signal with [poly(dG-dC)]$_2$ is bisignate, that with poly(dG)·poly(dC) is negative over the whole absorption band. By contrast, the spectra with both the AT polymers are entirely positive. The CD spectra have their maxima at more red-shifted wavelengths with the AT polymers compared to the GC polymers. The large positive CD signals for methylene blue with poly(dA)·poly(dT) indicate nonintercalative binding while the smaller signals with poly-(dG)·poly(dC) are consistent with intercalation as discussed below.

Discussion

Binding to (GC) Polynucleotides at Low Binding Ratios. The absorption and LD evidence presented here clearly indicate that methylene blue binds to [poly(dG-dC)]$_2$ in only one mode, which is independent of the binding ratio and the ionic strength conditions: the isosbestic point in absorption, LD spectra of identical shape, and the LD^r of the bound dye being more negative than that of the nucleobases both at low and (after correction for free dye) at high ionic strength all support this proposal. These observations are consistent with intercalation of the dye, which is the expected binding mode for a planar heteroaromatic tricycle such as methylene blue.

However, the CD spectra are not immediately indicative of such a simple picture. An intercalative binding mode should give an induced CD for the dye which has the same shape as the absorption spectrum. The magnitude of the signal is expected to be quite small for intercalation ($\Delta\epsilon/\epsilon < ca.\ 1 \times 10^{-5}$) and may be positive or negative, depending on the orientation of the dye with respect to the surrounding base pairs. By contrast, groove binding should induce a positive CD of the same shape as the absorption band but generally larger in magnitude, although there are also certain groove sites where the induced CD could be as low as that for intercalation.[21,37] In detailed computational studies of the induced CD expected for intercalators,[21] Lyng has shown how the induced CD of a dye intercalated into an alternating polynucleotide changes as a function of the angular orientation of the dye relative to the surrounding base pairs, of the lateral displacement of the dye relative to the helix axis, and of whether a 5′ pur-pyr 3′ or a 5′ pyr-pur 3′ step is considered. It is calculated that a methylene blue molecule centered on the helix axis with its long axis parallel to the surrounding base pairs should have positive induced CD in both the 5′ G-C 3′ step and the 5′ C-G 3′ step. If its long axis is perpendicular to the surrounding base pairs, then the induced CD should be positive in the 5′ G-C 3′ step and negative in the 5′ C-G 3′ step. However, small lateral displacements from the center are calculated to cause sign changes too; hence, it is impossible to determine from the sign of the induced CD exactly the orientation of the dye in the intercalation pocket.

The bisignate CD observed with [poly(dG-dC)]$_2$ was suggested to arise from concurrent occupancy of two different sites with different signs and shifts. These could be an intercalation site and a groove-bound site, which would give rise to CD of different signs;[21] however, this is discounted because of the ionic strength independence of the spectrum, since it is expected that the salt effects on the two sites would be different. Instead, we suggest that there exist two types of intercalation site which give CD signals of different signs and strengths and slightly different red shifts. These could correspond to two different angular orientations of the dye although it is difficult to imagine that only two of all possible angular orientations would be preferred and that the relative proportions of dye bound in each of these orientations would be unaffected by the ionic strength and the overall amount

(37) Kubista, M.; Åkerman, B.; Nordén, B. *J. Phys. Chem.* **1988**, *92*, 2352–2356.

of dye bound. However, if there is a dynamic equilibrium between the two orientations and the dye spends more time in these orientations compared to other angular orientations, then this could give rise to the observed CD spectrum. It must be reiterated that although we can say from these data that the dye is intercalated, its absolute orientation in the intercalation pocket cannot be determined: we cannot say whether the dye long axis is oriented parallel or perpendicular to the base-pair long axis or whether the dimethylamino groups reside in the major or minor groove of the helix. An alternative proposal is that the two sites correspond to the 5′ G-C 3′ and the 5′ C-G 3′ intercalation pockets. Calculations indicate that methylene blue in these two sites could have induced CD signals differing in both sign and magnitude even for the same orientation in each of the pockets.[21] It is also reasonable that different electronic interactions with the surrounding bases in each pocket could give rise to different spectral shifts. If the binding constants for the two sites are approximately equal, then at high P/D a single methylene blue molecule will not distinguish between the two sites which are present in equal amounts and thus would be equally distributed between them. Hence, the CD spectrum, as well as the absorption and LD spectra, would be independent of the amount of dye bound when the polymer is in excess. At lower P/D ratios where nearest neighbor exclusion becomes more important and it might be possible to observe the filling of one site in preference to the other, interpretation of the CD is complicated by additional external binding of the dye and thus dye–dye interactions. It is almost impossible to distinguish between these alternatives, but in an attempt to clarify the situation, binding of methylene blue to the nonalternating polynucleotide poly(dG)·poly(dC), which has only one type of intercalation pocket, a 5′ G-G 3′ one, was examined.

The LD and absorption data indicate that methylene blue binds strongly to poly(dG)·poly(dC) in an intercalative manner just as it does with [poly(dG-dC)]$_2$. One other piece of evidence in favor of intercalation in poly(dG)·poly(dC) is that fluorescence quenching observed in this system[6c,38] is as efficient as that observed with [poly(dG-dC)]$_2$, where the dye is intercalated. Since the fluorescence quenching is assigned to electron (or charge) transfer to the methylene blue singlet state from the guanine base, if the dye were externally bound, the fluorescence quenching would be less efficient. The CD spectrum of methylene blue with poly(dG)·poly(dC) is completely negative and of the same shape as the absorption band. The signal is stronger than with [poly(dG-dC)]$_2$, but the molar ellipticity remains relatively low ($\Delta\epsilon/\epsilon$ = *ca.* 1×10^{-4}), consistent with intercalation.[21] The presence of a strong, negative signal with the nonalternating polynucleotide with only one base-pair step supports a model in which the low magnitude and biphasic nature of the spectrum with [poly(dG-dC)]$_2$ were assigned to the presence of two possible intercalation sites, possibly the 5′ G-C 3′ and 5′ C-G 3′ steps of the polynucleotide.

Binding to (AT) Polynucleotides at Low Binding Ratios. Characterization of the binding of methylene blue to [poly(dA-dT)]$_2$ is less straightforward. In this case, the absorption and LD data suggest that the binding mode varies with ionic strength: with increasing ionic strength there is no isosbestic point in absorption and the LD^r for the bound dye appears to become less negative compared to that for the bases although the LD spectrum has the same shape. This suggests that at higher salt concentrations some of the dye is moved from intercalation sites to external sites which have a different absorption spectral shift but make a negligible contribution to the LD.

This proposal is supported by the induced CD of the MB/[poly(dA-dT)]$_2$ system under conditions of changing ionic strength. At low ionic strength the CD is small in magnitude ($\Delta\epsilon/\epsilon = 3 \times 10^{-5}$), positive in sign, and of the same shape as the absorption spectrum of the bound dye. At high ionic strength the CD is still positive but much larger in magnitude ($\Delta\epsilon/\epsilon \geq 3 \times 10^{-4}$), consistent with nonintercalative binding, and more red-shifted. This is similar to the behavior of DAPI, which is minor-groove-bound in AT sequences and shows strong positive induced CD but is intercalated in the methylated polynucleotide [poly(dG-dm^{5}C)]$_2$, where its CD is only weakly positive.[1] Hence, the spectroscopic data all point to the presence of both intercalated and groove-bound methylene blue at high ionic strength while at low ionic strength most of the dye appears to be intercalated; however, the fact that the LD^r for the dye is not significantly more negative than that of the DNA bases suggests that even in 5 mM phosphate a fraction of the dye may be externally bound. It cannot be concluded from these data where the dye may be bound—in a groove or at the phosphates—or what its orientation in this nonintercalative mode could be although the apparent lack of an LD signal from the second mode suggests either binding at the magic angle or in a random orientation.

With poly(dA)·poly(dT), the binding is clearly quite different than with [poly(dA-dT)]$_2$. There is no LD in the dye band although the absorption and CD spectra clearly indicate that under these conditions the dye does interact with the polynucleotide. Binding purely by intercalation or in the minor groove is excluded, since these would give strong negative and positive LD signals, respectively. It is possible that binding in both these modes could occur and that the dye is distributed between sites in such a manner as to result in zero LD; however, under such circumstances changing the ionic strength would be expeced to shift the equilibrium between the binding sites to result in either a positive or negative LD signal, contrary to experimental observations. In principle, it is possible that the dye could be bound in a single binding mode so that its long axis is oriented at the magic angle: in practice, such binding has not previously been reported but it cannot be excluded. If this were the binding mode, it could occur only in the major groove. The final alternative is that the dye is bound rather loosely in a large site which allows it to adopt a range of orientations relative to the helix axis such that the average LD becomes zero—such a site could exist in the major groove of the polynucleotide or at the sugar–phosphate backbone. Although backbone binding is possible, since methylene blue does interact with polyphosphate, it is discounted here because binding to polyphosphate leads to strong stacking even at P/D = 50 and at yet higher P/D values there is no shift of the absorption band.[11b] Thus, either interpretation of the spectroscopic data places methylene blue in the major groove of poly(dA)·poly(dT), although we cannot distinguish between the possibility of the dye being bound in a specific site with approximately magic angle orientation or in a nonspecific mode with a broad distribution of orientations. It remains unclear why the dye should favor this groove site over intercalation and why it occurs only in AT sequences. Certainly, poly(dA)·poly(dT) does not possess a normal B-type DNA structure, and intercalation of methylene blue is possibly inhibited by the bifurcated hydrogen bonds between consecutive base-pairs:[39] however, other "classical" intercalators appeared to intercalate with poly(dA)·poly(dT) under the same conditions (data not shown). Methylene blue has no hydrogens available for hydrogen-bond formation in the AT minor groove, but the ring nitrogen could potentially be involved in H-bonding to the free adenine amino hydrogen in the

(38) In ref 6c experiments were carried out in 10 mM Tris/0.01 mM EDTA and I/I_0 was reported to be 0.025 at high P/D; in our laboratory this efficient fluorescence quenching was confirmed to occur also in 100 mM NaCl, as used for the spectroscopic experiments reported herein.

(39) Nelson, H. C. M.; Finch, J. T.; Luisi, B. F.; Klug, A. *Nature* **1987**, *330*, 221–226.

(40) Abbreviations: MB, methylene blue; CT-DNA, calf thymus-deoxyribonucleic acid; [poly(dA-dT)]$_2$, polydeoxy(adenylic-thymidylic) acid; [poly(dG-dC)]$_2$, polydeoxy(guanylic-cytidylic) acid; poly(dA)·poly(dT), polydeoxyadenylic acid–polydeoxythymidylic acid; poly(dG)·poly(dC), polydeoxyguanylic acid–polydeoxycytidylic acid; poly(dA), polydeoxyadenylic acid (single stranded); dGMP, deoxyguanosine monophosphate; dAMP, deoxyadenosine monophosphate; bp, base pair; P/D, ratio of concentration of polynucleotide phosphate groups to dye concentration.

major groove: this could stabilize binding of the dye in this groove compared to the minor groove. It is also suggested that similar groove binding is the second binding mode observed with [poly(dA-dT)]$_2$ at high ionic strength, although we cannot say why an intercalation to groove-binding shift should occur with increasing ionic strength. It may be noted that binding in the major groove with a H-bond between the methylene blue ring nitrogen and the adenine amino hydrogen could protect the triplet-state dye from solvent protonation, consistent with previously reported observations.[15]

Binding to CT-DNA at Low Binding Ratios. Native DNA possesses 10 possible base-pair steps: four of these are represented in [poly(dG-dC)]$_2$ and [poly(dA-dT)]$_2$, and two more in the nonalternating polynucleotides. Together with potential binding sites in the grooves, this makes DNA an extremely heterogeneous target for binding ligands. CT-DNA contains 42% GC base pairs, and it has been reported previously that methylene blue shows a preference for binding to DNAs with high GC contents;[16] hence, it probably prefers to bind beside GC base pairs, where it is likely to be intercalated. At one methylene blue molecule for 25 base pairs, as used in the studies reported here, and under low ionic strength conditions, it is likely that the dye will intercalate beside a GC base pair. As the ionic strength is raised, the situation changes: the spectroscopic evidence suggests that, as with [poly(dA-dT)]$_2$, the dye changes binding mode possibly to external sites, since the LD^r of the dye relative to that of the bases is reduced and the maximum LD wavelength is not changed despite the lack of an isosbestic point in absorption. The CD spectra are more difficult to interpret but essentially support this proposal. The CD at low ionic strength is bisignate and very similar to that observed with [poly(dG-dC)]$_2$, suggesting that 5′ G-C 3′ and 5′ C-G 3′ sites may be filled preferentially under these conditions. At high ionic strength the CD spectrum is completely positive but markedly different in shape from the absorption spectrum. This spectrum resembles the spectrum which would result if the CD spectra for methylene blue with [poly(dG-dC)]$_2$ and [poly(dA-dT)]$_2$ were combined and thus appears to suggest a shift of the dye from GC- to AT-like sites as the ionic strength is raised. This analysis is of course a simplification, since the binding to mixed sequence sites has not been characterized, but it serves to illustrate how the binding of even a simple dye like methylene blue to DNA is complex and depends very much on the conditions used.

Intercalation versus Groove Binding. It is not clear why methylene blue should not intercalate into AT polymers to the same extent as it does with GC polymers, although it has often been found that intercalators exhibit some GC specificity. As discussed above, the external binding site with AT sequences appears to be in the major rather than the minor groove, perhaps because methylene blue lacks the H-bonding ability to facilitate minor groove binding while it has the potential to form one H-bone in the major groove of AT polymers. However, the dye also has opportunities to form H-bonds in both the major and the minor groove of GC polymers, but in these cases intercalation appears to be far more favored. One possible explanation for groove binding is that the dye intercalates from the major groove but is somehow blocked by steric hindrance between the thymine methyl group and the dimethylamino substituents. In poly(dA)·poly(dT), intercalation may also be strongly disfavored by the unusual polymer structure with bifurcated hydrogen bonds between adjacent base pairs. Studies on the related dye thionine, which has exocyclic amino hydrogens available for H-bonding, and experiments with modified polynucleotides are currently in progress in an attempt to understand what causes methylene blue to chose this unusual binding mode.

Conclusions

The present spectroscopic studies have allowed a number of important conclusions to be drawn regarding the modes of interaction of methylene blue with nucleic acids.

(1) Binding of methylene blue to both the alternating [poly(dG-dC)]$_2$ and the nonalternating poly(dG)·poly(dC) is purely intercalative, and the mode of binding is insensitive to the ionic strength. The bisignate CD spectra observed with [poly(dG-dC)]$_2$ are suggested to arise from intercalation in the two different base-pair steps, since the CD with poly(dG)·poly(dC) is entirely negative.

(2) Binding to the alternating [poly(dA-dT)]$_2$ is mainly intercalative at low ionic strength, but with increasing ionic strength the dye moves to external sites which appear to make no contribution to the LD. These sites are suggested to lie in the major groove with either random or magic angle orientation of the dye.

(3) This major-groove binding appears to be the only mode of interaction for methylene blue with the nonalternating polynucleotide poly(dA)·poly(dT), suggested by the absence of LD but rather strong induced CD in the dye absorption band.

(4) CT-DNA with 42% GC base pairs presents a very heterogeneous target for methylene blue. At low ionic strength, the binding appears to be intercalative and to occur principally at GC-like sites, judging from the CD spectrum. With increasing ionic strength, however, the dye appears to change preference and move to AT-like sites, where it may be both intercalated and externally bound.

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Supplementary Material Available: Complete reduced linear dichroism spectra for each sample and circular dichroism spectra with poly(dA) and [poly(dG-dC)]$_2$ (P/D = 3) (8 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.