

## Ethidium bromide·(dC-dG-dC-dG)<sub>2</sub> complex in solution: Intercalation and sequence specificity of drug binding at the tetranucleotide duplex level

(drug-tetranucleotide duplex/helix-coil transition/intercalation at dC-dG sites/drug-base pair overlap geometries)

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**ABSTRACT** The binding of ethidium bromide (EtdBr) to the dC-dG-dC-dG self-complementary duplex has been monitored at the resolvable drug and nucleic acid protons and backbone phosphates at high nucleotide/drug (N/D) ratios by nuclear magnetic resonance (NMR) spectroscopy in aqueous solution. We observe averaged resonances (25°-95°) for the nucleic acid and drug nonexchangeable protons in the presence of excess tetranucleotide (N/D = 24), indicative of rapid exchange relative to the chemical shifts in the free and complexed states. Complex formation results in upfield shifts for the base protons at the terminal and internal base pairs and an increase in the transition midpoint for the duplex-to-strand conversion. We observe upfield chemical shift changes of 1.2 ppm at the Watson-Crick guanine N-1 proton(s) on complex formation (N/D = 24), with slow exchange between (dC-dG-dC-dG)<sub>2</sub> and EtdBr·(dC-dG-dC-dG)<sub>2</sub> relative to this chemical shift difference at -5°. The EtdBr phenanthridine ring protons shift upfield by about 0.9 ppm (H-2, H-4, H-7, H-9) and >0.5 ppm (H-1, H-10) on complex formation, with the chemical shifts versus temperature plots (25°-95°) monitoring the dissociation of the EtdBr·(dC-dG-dC-dG)<sub>2</sub> structure. These upfield shifts at the exchangeable and nonexchangeable base protons and phenanthridine ring (but not side chain) protons demonstrate intercalation of the phenanthridine ring of EtdBr into the dC-dG-dC-dG duplex in solution. The intercalation model may be supported by the observation of downfield shifts (up to 1 ppm) at the internucleotide phosphate(s) of the tetranucleotide duplex on addition of EtdBr at low temperatures. We observe stronger binding of EtdBr to the self-complementary dC-dG-dC-dG (2 dC-dG intercalation sites) and dC-dC-dG-dG (1 dC-dG site) duplexes compared to the dG-dG-dC-dC (no dC-dG sites) as monitored by UV absorbance changes at 480 nm. These studies extend to the tetranucleotide duplex level earlier observations that EtdBr exhibits a selectivity for formation of complexes to dinucleoside monophosphates with a pyrimidine (3'-5') purine sequence in the crystal and in solution. The experimental proton NMR upfield shifts at the phenanthridine protons on formation of the EtdBr·(dC-dG-dC-dG)<sub>2</sub> complex compare favorably with calculated values (atomic diamagnetic anisotropy and ring current contributions) based on the overlap geometry for EtdBr intercalated into the pyrimidine (3'-5') purine dinucleoside monophosphate duplex in the crystal.

Ethidium bromide (EtdBr) is a trypanocidal drug (Fig. 1) which interferes with nucleic acid synthesis (1). Spectroscopic, hydrodynamic, and sedimentation studies have demonstrated strong binding by intercalation of the EtdBr into double-stranded nucleic acids (2-6).

Direct visualization of the intercalation model has been reported for crystals of the 2:2 complex of EtdBr and self-complementary dinucleoside monophosphates with a pyrimidine (3'-5') purine sequence (7). X-ray fiber diffraction studies have also demonstrated intercalation of a Pt-substituted derivative

of EtdBr into calf-thymus DNA according to the neighbor exclusion model (8).

Nuclear magnetic resonance (NMR) studies have investigated structural aspects of complex formation between EtdBr and dinucleoside monophosphates (9, 10), transfer RNA (11), and poly(dA-dT) (12) while absorption (13) and fluorescence (14) temperature-jump studies have investigated kinetic aspects of the EtdBr-DNA complex.

We report below on NMR studies of drug-nucleic acid interactions at the dC-dG-dC-dG tetramer duplex level (15) which enable one to monitor the proton resonances of the nucleic acid in the absence and presence of bound drug. Since the drug is gradually added to the tetranucleotide duplex, spectra can be recorded at high nucleotide/drug (N/D) ratios to yield the NMR parameters of the drug in the bound state. The well-resolved drug resonances can be utilized to monitor the dissociation of the EtdBr·(dC-dG-dC-dG)<sub>2</sub> complex as a function of temperature.

### EXPERIMENTAL

**Materials.** The dC-dG-dC-dG tetranucleotide was purchased from Collaborative Research, Waltham, Mass. Details on its synthesis and purity have been summarized earlier (15). Ethidium bromide was purchased from Sigma. The reported pH values of the drug-nucleic acid solutions in H<sub>2</sub>O and D<sub>2</sub>O are direct pH meter readings.

**Spectra.** High resolution NMR spectra were recorded on a Bruker HX-360 spectrometer interfaced with a BNC-12 computer system. 360 MHz NMR spectra of the exchangeable protons (H<sub>2</sub>O, continuous wave mode) and the nonexchangeable protons (D<sub>2</sub>O, Fourier transform mode) were recorded in 0.1 M phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA) solution (referenced relative to internal standard 2,2-dimethyl-2-silapentane-5-sulfonate, DSS). 145.7 MHz phosphorus NMR spectra (H<sub>2</sub>O, Fourier transform mode) were recorded in 0.1 M sodium cacodylate, 0.05 M EDTA solution [referenced relative to internal trimethylphosphate, (CH<sub>3</sub>)<sub>3</sub>PO].

Optical spectra were recorded in 5 mm cells on a Cary 118 C UV-visible spectrophotometer. The tetranucleotides (mM solutions) were added in 5 μl aliquots to 52.5 μM ethidium bromide (based on ε<sub>480</sub> = 5600 M<sup>-1</sup> cm<sup>-1</sup>) solution in 0.02 M sodium phosphate, H<sub>2</sub>O, pH 6.5, and the absorbance changes (corrected for dilution effects) were monitored at 480 nm.

### RESULTS AND DISCUSSION

It has been recently demonstrated that self-complementary tetranucleotides containing G-C base pairs form stable duplexes at room temperature (15, 16). The dC-dG-dC-dG duplex ex-

Abbreviations: NMR, nuclear magnetic resonance; EtdBr, ethidium bromide; N/D, nucleotide-to-drug molar ratio.

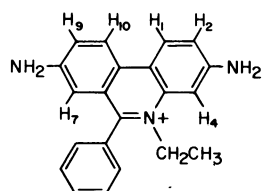
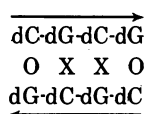


FIG. 1. Structure and numbering system for ethidium bromide.

hibits 2-fold symmetry and progress has been made towards differentiation between resonances located at the terminal and internal base pairs (15).



Since strong binding of EtdBr is observed predominantly at high N/D ratios (1), we have restricted our studies to conditions where the duplex is in excess to insure intercalation of the bound drug.

**Nonexchangeable Nucleic Acid Protons.** The 360 MHz proton NMR spectrum (5.5–8.0 ppm) of the EtdBr + dC-dG-dC-dG (N/D = 24) complex in 0.1 M phosphate, D<sub>2</sub>O, pH 6.74, 49.5°, is presented in Fig. 2. The major resonances correspond to the nucleic acid (5 mM duplex concentration) and the minor resonances correspond to bound EtdBr (1.67 mM).

The two guanine H-8 singlets resonate at 7.90 and 7.92 ppm, the four sugar H-1' triplets resonate at 5.90, 5.91, 6.01, and 6.10 ppm, the terminal cytosine doublets resonate at 7.59 ppm (H-6) and 5.86 ppm (H-5), and the internal cytosine doublets resonate at 7.47 ppm (H-6) and 5.64 ppm (H-5) (Fig. 2).

The temperature dependence of the nucleic acid base and sugar protons in the complex (N/D = 24) are plotted between 25° and 95° in Fig. 3. Since the nucleic acid is in excess (1 drug per 3 duplexes), the tetranucleotide resonances are in fast exchange (25°–95°) between free and complexed states relative to their respective chemical shift separation. Line width contributions due to uncertainty broadening are observable at room temperature, with intermediate exchange between states at lower temperatures. The (terminal and internal) guanine H-8 and terminal cytosine H-6 protons exhibit small temperature-dependent chemical shifts (Fig. 3). The terminal cytosine H-5 resonance and one sugar H-1' resonance shift upfield by about 0.1 ppm while the internal cytosine H-5, internal cytosine H-6, and three sugar H-1' resonances shift upfield by 0.3–0.4 ppm

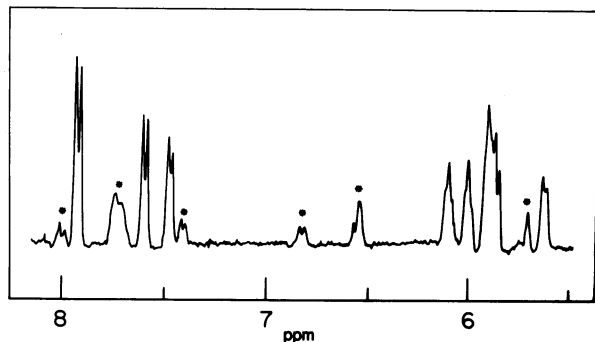


FIG. 2. The 360 MHz proton NMR spectrum of the EtdBr + dC-dG-dC-dG (N/D = 24; 5 mM duplex concentration) complex in 0.1 M phosphate, 1 mM EDTA, D<sub>2</sub>O, pH 6.74, 49.5°. The spectral resolution was improved by convolution difference techniques. The EtdBr resonances are denoted by \*.

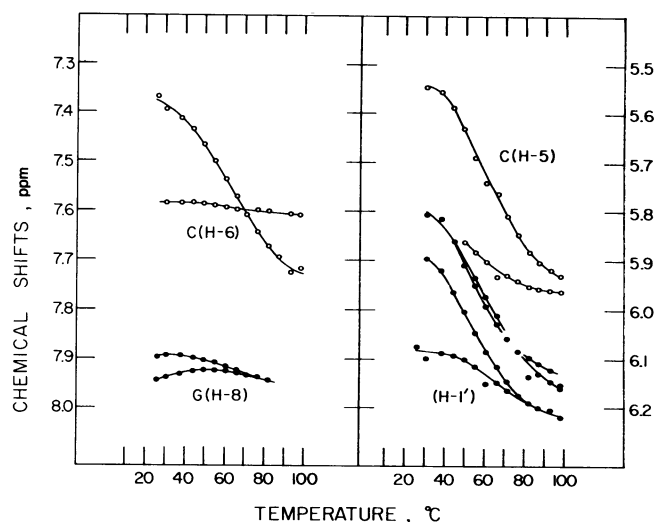


FIG. 3. The temperature dependence of the base and sugar nucleic acid protons in the EtdBr + dC-dG-dC-dG (N/D = 24; 5 mM duplex concentration) complex in 0.1 M phosphate, 1 mM EDTA, D<sub>2</sub>O, pH 6.74.

(95°–25°) (Fig. 3). The midpoints of the transition,  $t_{1/2}$ , (derived from the chemical shift versus temperature curves in differentiated form) increase from their values in the dC-dG-dC-dG duplex [ $C(H-5)_{int} = 50 \pm 2^\circ$ ;  $C(H-6)_{int} = 55 \pm 1^\circ$ ], (15), to their values in the EtdBr + dC-dG-dC-dG (N/D = 24) complex [ $C(H-5)_{int} = 59^\circ$ ;  $C(H-6)_{int} = 63.5^\circ$ ], (Fig. 3), consistent with stabilization of the tetramer duplex in the presence of bound drug in 0.1 M salt solution.

**Nonexchangeable EtdBr Protons.** We can monitor all the protons on the phenanthridine ring (Fig. 1) and the CH<sub>3</sub> and phenyl protons of the side chain of EtdBr (denoted by \* in Fig. 2). In the complex (N/D = 24) presented in Fig. 2 the H-7 singlet resonates at 5.72 ppm while the H-4 singlet is superimposed on a doublet at 6.55 ppm. The H-2/H-9 pair of protons is observed as doublets at 6.55 and 6.84 ppm while the H-1/H-10 pair of protons is observed as doublets at 7.99 ppm (60°) and 8.00 ppm (Fig. 2). The CH<sub>3</sub> protons of the ethyl side chain resonate at 1.40 ppm while the phenyl protons are superimposed at 7.72 ppm and at 7.42 ppm (Fig. 2).

Identical chemical shifts have been observed for the phenanthridine H-2/H-9 (6.75 ± 0.02 ppm), H-4 (6.47 ± 0.02 ppm) and H-7 (5.65 ± 0.02 ppm) protons in the EtdBr + dC-dG-dC-dG complex (5 mM duplex concentration), 26°, at N/D ratios of 91, 59, 34, and 24, even though the EtdBr concentration varies from 0.44 mM to 1.67 mM. Thus, in the presence of excess nucleic acid, all the EtdBr is in the bound form and the observed chemical shifts of the drug reflect values for a strong complex of one drug bound per duplex [EtdBr·(dC-dG-dC-dG)<sub>2</sub>]. Dimer formation is negligible at 0.44 mM EtdBr concentration (9, 10), so that the large shifts of phenanthridine protons on formation of complexes are not a consequence of monomer-dimer equilibrium of the drug. Weak surface binding of EtdBr to nucleic acids is negligible for N/D ratios of >15 (1) so that the large shifts of phenanthridine on complex formation which persist to an N/D ratio of 91 cannot arise from surface binding of EtdBr to the dC-dG-dC-dG duplex.

The temperature dependence (25°–95°) of the EtdBr protons in the complex (N/D = 24) is plotted in Fig. 4. We observe large upfield shifts at the H-7, H-4, H-2, and H-9 (about 0.9 ppm,  $t_{1/2} = 73 \pm 1^\circ$ ) and the H-1 and H-10 (>0.5 ppm,  $t_{1/2} = 76^\circ$ ) phenanthridine protons between 95° (free EtdBr) and 25° [EtdBr·(dC-dG-dC-dG)<sub>2</sub>] complex, with rapid exchange be-

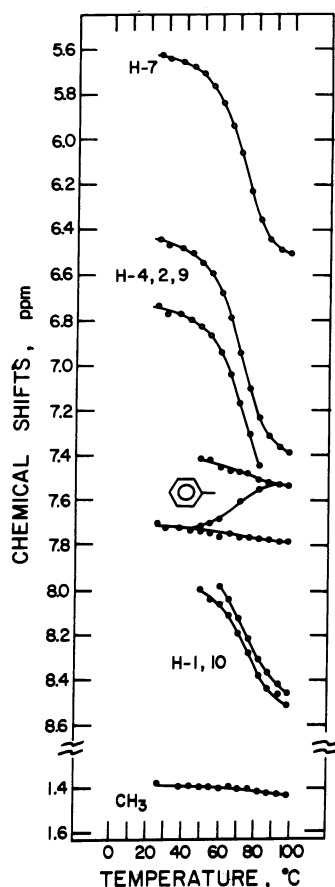


FIG. 4. The temperature dependence of the drug protons in the EtdBr + dC-dG-dC-dG (N/D = 24; 5 mM duplex concentration) complex in 0.1 M phosphate, 1 mM EDTA, D<sub>2</sub>O, pH 6.74.

tween free and complexed states between 25° and 95° (Fig. 4).

The EtdBr proton assignments discussed above are based on the multiplicity of the resonances and correlation of the chemical shifts in the EtdBr + dC-dG-dC-dG (N/D = 24) complex at 95° with those reported for the EtdBr monomer in dilute solution (9, 10).

**Intercalation.** We assign the upfield shifts of the phenanthridine ring protons in the EtdBr + dC-dG-dC-dG complex relative to their values in the free state to ring current contributions (17) arising from overlap between the phenanthridine ring and nucleic acid base pair(s). The large magnitude of these shifts (up to 0.9 ppm) and their distribution throughout the phenanthridine ring require overlap with two nucleic acid base pairs, as would result from intercalation of the EtdBr into the tetramer duplex. By contrast, the absence of upfield shifts at the EtdBr methyl and phenyl side chain protons on complex formation (Fig. 4) suggests that these groups are not intercalated between base pairs.

**Exchangeable Protons.** The 360 MHz continuous wave proton NMR spectra of the dC-dG-dC-dG duplex and the EtdBr + dC-dG-dC-dG (N/D = 24) complex in 0.1 M phosphate, 1 mM EDTA, H<sub>2</sub>O, pH 6.65, -5°, are presented in Fig. 5. These downfield-shifted resonances are characteristic of the duplex state and originate in the guanine N-1 H imino Watson-Crick hydrogen-bonded protons (18). The resonances at 13.245 ppm (width, 22 Hz) and 13.335 ppm (50 Hz) in the absence of drug are assigned to the internal and terminal dC-dG-dC-dG base pairs, respectively (Fig. 5). New resonance(s)

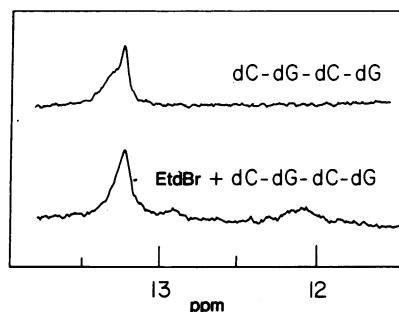


FIG. 5. The 360 MHz proton NMR spectrum of dC-dG-dC-dG (5 mM duplex concentration) and EtdBr + dC-dG-dC-dG (N/D = 24; 5 mM duplex concentration) complex in 0.1 M phosphate, 1 mM EDTA, H<sub>2</sub>O, pH 6.65, -5°.

are observed at 12.1 ppm on gradual addition of EtdBr to the dC-dG-dC-dG duplex at -5° (slow exchange relative to the 1.2 ppm chemical shift separation), with the spectrum in Fig. 5 characteristic of 1 drug per 3 duplexes. The resonance(s) at 12.1 ppm are assigned to guanine N-1 protons in G-C base pairs directly adjacent to the intercalated drug in the EtdBr-(dC-dG-dC-dG)<sub>2</sub> complex, with the large upfield shift primarily due to nearest-neighbor ring currents from the intercalated phenanthridine ring (11, 19). The resonance(s) at 12.92 ppm (Fig. 5) probably originate in guanine N-1 protons in G-C base pairs distant from the intercalation site in the EtdBr-(dC-dG-dC-dG)<sub>2</sub> complex.

No additional exchangeable resonances were observable between 8.5 and 12 ppm that could be attributed to the phenanthridine NH<sub>2</sub> protons in the EtdBr + dC-dG-dC-dG complex (N/D = 24) at low temperatures.

**Backbone Phosphates.** The 145.7 MHz phosphorus NMR spectra of the dC-dG-dC-dG duplex (pH 7.35, 7°) and the EtdBr + dC-dG-dC-dG (N/D = 24) complex (pH 6.95, -3°) in 0.1 M cacodylate, 0.05 M EDTA, H<sub>2</sub>O, are presented in Fig. 6. The three internucleotide phosphates on one strand (4.01 ppm and 4.19 ppm) are related by symmetry to those on the partner strand in the dC-dG-dC-dG tetranucleotide duplex. Additional resonances are observed between 3 and 4 ppm on complex formation (1 EtdBr per 3 duplexes) resulting in downfield shifts of up to 1 ppm in the presence of bound drug

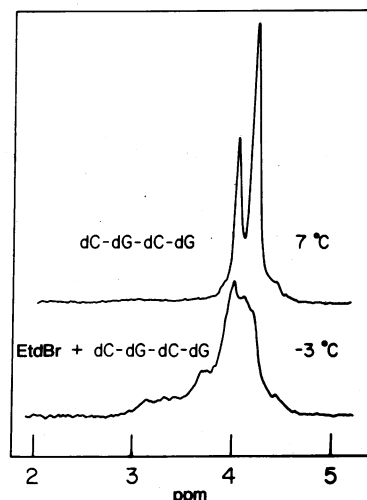


FIG. 6. The 145.7 MHz phosphorus NMR spectrum [2-5 ppm upfield from standard (CH<sub>3</sub>O)<sub>3</sub>PO] of dC-dG-dC-dG (3.3 mM duplex concentration), pH 7.35, 7°, and EtdBr + dC-dG-dC-dG (N/D = 24; 6 mM duplex concentration) complex, pH 6.9, -3°, in 0.1 M cacodylate, 5 mM EDTA, H<sub>2</sub>O.

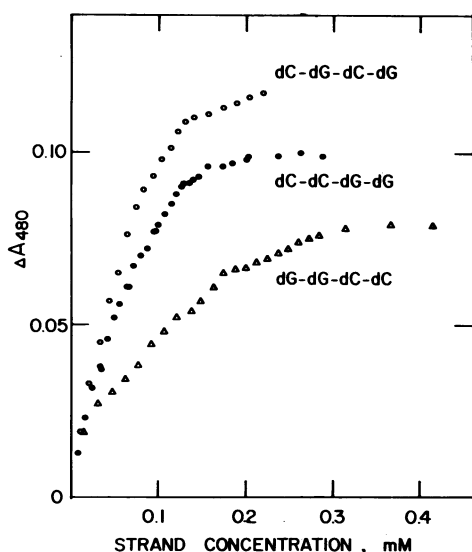


FIG. 7. Changes in the 480 nm absorption of EtdBr (52.5  $\mu$ M) on gradual addition of the self-complementary tetranucleotide duplexes dC-dG-dC-dG, dC-dC-dG-dG, and dG-dG-dC-dC in 0.02 M phosphate, H<sub>2</sub>O, pH 6.5.

at  $-3^\circ$  (Fig. 6). We have previously demonstrated that intercalation of actinomycin D into deoxy tetra- and hexanucleotide duplexes results in downfield shifts of 1.5 and 2.5 ppm for the phosphates at the intercalation site (15, 19). The resonances between 3 and 4 ppm in the EtdBr + dC-dG-dC-dG complex ( $N/D = 24$ ) are tentatively assigned to the phosphates at the intercalation site. They broaden when the temperature is raised and coalesce with the 4 ppm resonance(s) at room temperature, suggestive of the onset of slow exchange between free and bound states at  $-3^\circ$ .

**Sequence Specificity.** We have monitored the chemical shift of the nonexchangeable nucleic acid resonances on gradual addition of EtdBr to the dC-dG-dC-dG duplex in 0.1 M phosphate, at  $26^\circ$ . The base pair under observation at the intercalation site would experience the difference in upfield ring current contributions due to the intercalating group at a distance of 3.4 Å and an adjacent base pair which shifts from 3.4 Å to 6.8 Å as a result of the intercalation. Experimentally, the upfield base proton shifts on EtdBr + dC-dG-dC-dG ( $N/D = 24$ ) complex formation are 0.07 and 0.08 ppm for the guanine H-8 resonances, 0.07 and 0.065 ppm for the terminal and internal cytosine H-6 resonances, respectively, and 0.07 ppm for the internal cytosine H-5 resonance. These values would be three times as large on extrapolation to 1 EtdBr bound per dC-dG-dC-dG duplex ( $N/D = 8$ ).

EtdBr can intercalate at the dC-dG and/or dG-dC site(s) in the dC-dG-dC-dG tetranucleotide duplex. Intercalation at the dG-dC site should primarily affect the base resonances at the internal base pairs, in contrast to experimental shifts at both terminal and internal base pairs on complex formation. Intercalation at the dC-dG site should affect the base resonances at the terminal and internal base pairs.

We have compared the absorbance changes at the 480 nm EtdBr transition with increasing tetranucleotide concentration for the self-complementary duplex sequence dC-dG-dC-dG (two potential dC-dG intercalation sites), dC-dC-dG-dG (one dC-dG site) and dG-dG-dC-dC (no dC-dG site) (Fig. 7). The strongest binding is observed for the dC-dG-dC-dG and dC-dC-dG-dG sequences (Fig. 7), which contain dC-dG intercalation sites (Fig. 8).

These results extend to the stable tetranucleotide duplex level

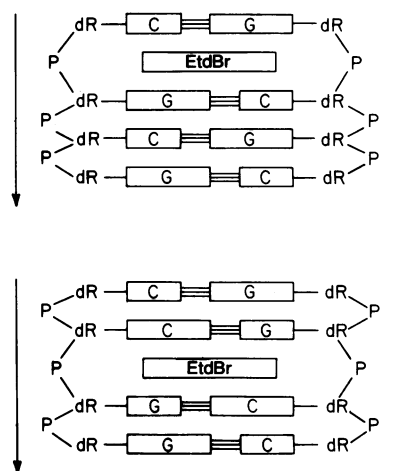


FIG. 8. Intercalation of EtdBr At the dC-dG site in the dC-dG-dC-dG duplex (top) and the dC-dC-dG-dG duplex (bottom).

the recent observation that EtdBr exhibits a specificity for pyrimidine (3'-5') purine sequences at the dinucleoside monophosphate level in the crystal (7) and in solution (10).

**Overlap Geometry.** Giessner-Pretre and Pullman have reported on the upfield chemical shift contours at a distance of 3.4 Å from nucleic acid bases due to atomic diamagnetic anisotropy and ring current contributions (20). We apply these contours to the published overlap geometry perpendicular to the base pair planes for EtdBr intercalated into the self-complementary pyrimidine (3'-5') purine dinucleoside monophosphate duplex in the crystal (7). The calculated upfield contributions at the phenanthridine protons are H-1 (0.65 ppm), H-10 (0.55 ppm), H-2 (0.75 ppm), H-4 (0.8 ppm), H-7 (0.75 ppm), and H-9 (0.75), and may be compared with the experimental values of >0.5 ppm at H-1 and H-10 and about 0.9 ppm at H-2, H-4, H-7, and H-9 for the EtdBr-(dC-dG-dC-dG)<sub>2</sub> complex. There are additional calculated contributions of a smaller magnitude at the phenanthridine protons due to next-nearest-neighbor interactions (21) at the tetranucleotide duplex level, so that the experimental and calculated values are in good agreement.

These results suggest that the EtdBr intercalated at the dC-dG site in the dC-dG-dC-dG duplex in solution exhibits the same overlap geometry as observed for the complex at the dinucleoside monophosphate level in the crystal.

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