Interactions of a Fragment of Bleomycin with Deoxyribodinucleotides: Nuclear Magnetic Resonance Studies†

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ABSTRACT: Proton NMR spectroscopy was used to establish certain geometrical parameters of the complexes formed between *N*-(3-aminopropyl)-2′-(2-acetamidoethyl)-2,4′-bithiazole-4-carboxamide hydrochloride (BLMF), a fragment of bleomycin, and various deoxyribodinucleotides. All proton resonances in these compounds have been assigned; chemical shifts were recorded as functions of their concentration. In the complex formed between BLMF and pdG-dC, chemical shifts of the bithiazole protons (measured with respect to values extrapolated to infinite dilution) were displaced upfield by 0.4 ppm. Other proton resonances of BLMF were shifted upfield but to a lesser extent. After corrections are made for self-stacking, maximum values for induced chemical shifts of the bithiazole protons are reached at a dinucleotide/BLMF ratio of 2. Coupling sums for dinucleotides (12.5–13.7 Hz) were unchanged following complexation, suggesting that there is no marked change in sugar conformation when BLMF is bound. On the basis of these results and of molecular model building studies, we propose a three-dimensional structure for a BLMF:pdG-dC complex in which the thiazole rings are intercalated in the duplex and stack preferentially on the purines. Projected on the same plane, the horizontal axis connecting the center of both bithiazole rings in this configuration superimposes on the axis connecting the centers of the purine bases. In this complex, both thiazole protons extend into the minor groove and the positively charged terminal amine binds to the negatively charged phosphate group of DNA.

The bleomycins, a family of structurally related glycopeptide antibiotics, possess antitumor, antimicrobial, and antiviral properties (cf. Haidle & Lloyd, 1979). Bleomycin binds to DNA (Nagai et al., 1969) and, in the presence of Fe(II) and molecular oxygen (Onishi et al., 1973; Sausville et al., 1978a,b), causes preferential strand scission at guanosine–pyrimidine (5′ → 3′) sequences (Takeshita et al., 1978; D'Andrea & Haseltine, 1978). The latter effect presumably relates to the chemotherapeutic and toxic properties of the bleomycin antibiotics.

In a previous study, Chien et al. (1977) utilized fluorescence quenching techniques to study binding of bleomycin and related compounds to DNA. One molecule of antibiotic was shown to bind for each 5–6 base pairs. Tripeptide S, a partial hydrolysis product of bleomycin which contains the bithiazole and terminal amine moieties of the parent compound but lacks the metal-binding ligands (Asakura et al., 1975), also forms a complex with DNA (Chien et al., 1977). This observation suggested that the primary groups involved in binding bleomycin to DNA are represented in the tripeptide S moiety.

Povirk et al. (1979) reported that tripeptide S and bleomycin lengthen linear DNA and relax supercoiled DNA, suggesting that part of the drug intercalates between base pairs. The thiazole rings, which can assume a coplanar conformation, are logical candidates for such a process.

Chien et al. (1977) reported that proton resonances of the bithiazole and dimethylsulfonium moieties of bleomycin are selectively broadened in the presence of DNA, indicating that these functional groups participate in forming the drug–DNA complex. These preliminary observations suggested that useful information might be obtained by analyzing interactions of a model complex in which all proton resonances could be resolved. NMR studies of this type have been useful in defining other drug–nucleic acid complexes (Kearns, 1977; Krugh & Chen, 1975; Patel & Shen, 1978; Krugh & Neely, 1973; Patel, 1974; Krugh & Reinhardt, 1975; Kreishman et al., 1971). Information so obtained correlates with results of X-ray diffraction studies.

The experiments described in the present paper define geometrical parameters of complexes formed between BLMF[1] (a fragment of bleomycin that resembles tripeptide S) and various deoxyribodinucleotide phosphates. These complexes appear to be stabilized by intercalation of the bithiazole moiety with the thiazole rings stacked primarily on purines. Results are interpreted in light of recent biochemical data describing sequence-specific strand scission of DNA by bleomycin (Takeshita et al., 1978; D'Andrea & Haseltine, 1978; Giloni et al., 1981).

Materials and Methods

Materials. Crystalline BLMF, generously provided by Professor Sidney Hecht, was stored at −20 °C. Deoxyribodinucleotides, purchased from Collaborative Research, were treated with Chelex-100 to remove contaminating metal ions and then lyophilized twice from 100% D_2O before use.

Preparation of Samples. Compounds were prepared for spectral studies by dissolving them in a 10 mM solution of potassium phosphate prepared in 100% D_2O containing 0.1 M NaCl. The pD was adjusted to 7.0 with NaOD or DCl. Concentrations of nucleotides were determined spectrophotometrically. After obtaining initial spectra at low nucleotide/drug ratios, we added lyophilized deoxyribonucleotides to produce concentrated solutions for subsequent measurements.

Spectral Measurements. Proton magnetic resonance spectra were measured on a Bruker WH 360 spectrometer operating at 360 MHz in the Fourier transform mode with a probe temperature of 278 ± 1 K. The spectrophotometer was locked on the deuterium resonances of solvent D_2O. DSS was used as internal reference standard. Spectra were accumulated with

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[1] Abbreviations used: BLMF, *N*-(3-aminopropyl)-2′-(2-acetamidoethyl)-2,4′-bithiazole-4-carboxamide hydrochloride; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

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Table I: Chemical Shifts of BLMF Proton Resonances after Complexation with Deoxyribonucleotides

nucleotide added[b]	concn (mM)	chemical shift (ppm)[a] u	v	b	d	c	f	e	a
none		8.234[c]	8.124[c]	3.600[c]	3.555[c]	3.270[c]	3.090[c]	2.024[c]	1.940[c]
		8.134	8.009	3.570	3.529	3.213	3.102	2.030	1.960
pdG-dC	10	8.051	7.922	3.531	3.479	3.149	3.092	2.007	1.950
	100	7.829	7.711	3.435	3.353	2.994	3.084	1.984	1.940
pdC-dG	10	8.071	7.937	3.541	3.499	3.171	3.120	2.021	1.956
	80	7.917	7.781	3.466	3.416	3.054	3.082	1.990	1.940
pdG-dA + pdT-dC	10[f]	8.074	7.925	3.547	3.503	3.177	3.105	2.028	1.963
	50[f]	7.960	7.800	3.486	3.440	3.090	3.108	2.000	1.972
pdA-dC	10	8.107	7.963	3.562	3.525	3.205	3.105	2.033	1.964
	100	8.032	7.851	3.562	3.521	3.194	3.132	2.055	1.991
pdC-dA[d]	10	8.120	7.974	3.570	3.527	3.213	3.102	2.029	1.957
	100	7.981	7.811	3.548	3.512	3.174	3.132	2.056	1.990
pdG-dT	10	8.108	7.966	3.569	3.521	3.206	3.111	2.036	1.969
	100	8.058	7.880	3.571	3.513	3.196	3.151	2.060	2.007
pdT-dG[e]	10	8.123	7.972	3.573	3.527	3.215	3.106	2.029	1.964
	100	8.062	7.867	3.571	3.511	3.195	3.140	2.053	2.000
dGMP	10	8.119	7.983	3.571	3.522	3.212	3.106	2.033	1.964
	100	8.019	7.855	3.547	3.464	3.168	3.106	2.019	1.976

[a] Values are reported in parts per million relative to DSS and are accurate to 0.002 ppm. Lettering of protons is given in Figure 1. [b] BLMF (20 mM) was present in all experiments. [c] Value extrapolated to infinite dilution (see Figure 2). [d] This experiment was carried out at 281 K. Reference values not shown. [e] This experiment was carried out at 280 K. Reference values not shown. [f] Concentration for each dinucleotide.

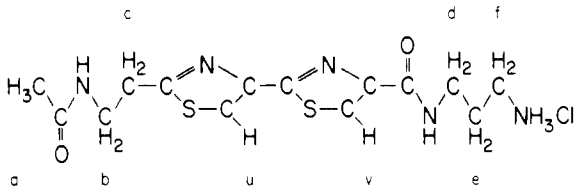

FIGURE 1: Molecular structure of BLMF. Proton resonances observed in the NMR spectrum are identified by lower case letters.

2-s intervals between pulses. Line position determinations were accurate to ±0.1 Hz. H′–H′ spin decoupling was performed in the Fourier transform mode utilizing a Bruker frequency synthesizer.

Results

Assignment of Proton Resonances. The structure of BLMF is shown in Figure 1. In the 360-MHz spectrum of this compound (not shown), the low-field resonances are assigned to the two bithiazole protons, and the methyl protons are identified by the high-field resonance at 1.94 ppm (Chien et al., 1977; Chen et al., 1977). Proton resonances of the various methylene groups were assigned by homonuclear decoupling techniques. The f and d protons were distinguished by the relatively greater chemical shift of the former observed when the pH is decreased. Table I summarizes chemical shifts for all nonexchangeable protons in BLMF. The contribution of intermolecular stacking forces to the chemical shifts observed at various concentrations of BLMF is shown by the titration data in Figure 2.

Chemical shifts of the proton resonance of dGMP, pdG-dC, and other deoxyribodinucleotides[2] studied are listed in Table II. Assignments for most of the base protons have been reported (Cheng & Sarma, 1977). Criteria for distinguishing between the H1′ protons of the deoxyribodinucleotides are described under Discussion.

In dilute solution, dinucleotides exist predominantly as monomers. Dimers form at higher concentrations and chemical shifts for the base protons generally move upfield (Young & Krugh, 1975). For example, when the concentration of pdG-dC is increased from 5 to 100 mM (Figure 3), chemical shifts for the C-H5, C-H6, and G-H8 protons (calculated from values extrapolated to infinite dilution) decrease by 0.3, 0.15, and 0.13 ppm, respectively. The chemical shift of one of the H1′ protons does not change with increasing concentrations of dinucleotide while the other shifts upfield by 0.15 ppm. The H1′ protons found in dinucleotides appear as pseudotriplets; the sum of $J_{1'2'}$ and $J_{1'2''}$ ranges from 12.0 to 14.7 Hz for complementary dinucleotides and from 9.0 to 14.0 Hz for noncomplementary dinucleotides (Table II).

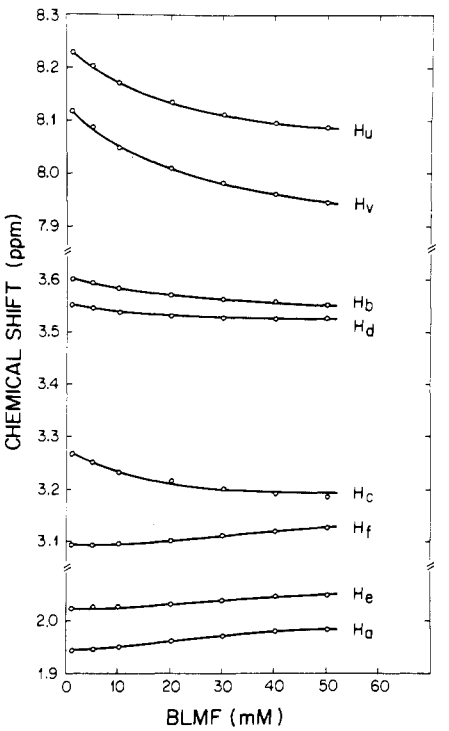

FIGURE 2: Chemical shifts of BLMF proton resonances measured at various concentrations of BLMF.

Complexes of BLMF with Complementary Dinucleotides. Chemical shifts of the BLMF protons were measured as a

[2] Dinucleotides were used in these experiments rather than dinucleosides because of the limited solubility of the latter at the concentrations required for these experiments.

Table II: Chemical Shifts of Deoxyribonucleotide Proton Resonances after Complexation with BLMF

nucleotide added	concn (mM)	chemical shift (ppm)[a]								
					pyrimidine		purine			
		H8	H2	H6	H1′	(J_{sum}[b])	H1′	(J_{sum}[b])	H5	CH3
pdG-dC	10	8.131		7.745	6.284	(12.2)	6.189	(12.8)	5.751	
pdG-dC[c]	10	8.067		7.628	6.254	(13.2)	6.022	(13.2)	5.530	
pdG-dC[c]	100	8.023		7.594	6.216	(12.7)	6.029	(12.7)	5.457	
pdC-dG	10	8.039		7.746	6.140	(13.7)	6.211	(13.7)	6.013	
pdC-dG[c]	10	7.982		7.703	5.929		6.087	(12.0)	5.9	
pdC-dG[c]	80	7.974		7.677	5.877	(13.9)	6.066	(13.9)	5.862	
pdG-dA[j] +	10	8.002[f]		7.899[h]	6.30[h]	(12.0)	6.30[f]	(12.0)	6.09[i]	1.90[h]
pdT-dC	10	8.369[g]	7.780	7.873[i]	5.840[i]	(14.7)	6.361[g]	(14.7)		
pdG-dA[c,j] +	10	7.898[f]		7.876[h]	6.24[h]	(13.0)	6.24[f]	(13.0)	6.059[i]	1.875[h]
pdT-dC	10	8.314[g]	7.739	7.855[i]	5.79[i]	(12.6)	6.28[g]	(12.8)		
pdG-dA[c] +	50	7.883[f]		7.865[h]	6.24[h]		6.24[f]		6.06[i]	1.87[h]
pdT-dC	50	8.293[g]	7.736	7.865[i]	5.824[i]		6.27[g]	(13.0)		
pdA-dC	10	8.523	8.119	7.677	6.151	(12.8)	6.400	(12.8)	5.732	
pdA-dC[c]	10	8.430	7.996	7.676	6.132	(12.6)	6.266	(12.2)	5.735	
pdA-dC[c]	100	8.441	8.008	7.699	6.150	(11.5)	6.317	(14.4)	5.760	
pd-dA	10	8.457	8.083	7.697	6.064	(14.2)	6.415	(13.7)	6.011	
pdC-dA[d]	10	8.385	8.063	7.697	6.054	(14.0)	6.333	(12.7)	5.991	
pdC-dA[c]	100	8.372	8.006	7.681	6.038	(13.0)	6.326	(13.7)	5.979	
pdG-dT	10	8.084		7.547	6.265	(13.2)	6.231	(12.6)		1.687
pdG-dT[c]	10	8.002		7.532	6.234	(13.4)	6.109	(13.2)		1.660
pdG-dT[c]	100	8.042		7.571	6.255	(13.1)	6.169	(13.2)		1.689
pdT-dG[e]	10	8.044		7.548	6.190	(13.6)	6.117	(9.3)		1.880
pdT-dG[c,e]	10	7.991		7.524	6.123	(13.2)	6.082	(9.3)		1.846
pdT-dG[c]	100	8.017		7.526	6.123	(14.0)	6.101	(9.0)		1.857
dGMP	10	8.157					6.307	(13.7)		
dGMP[c]	10	8.081					6.171	(13.2)		
dGMP[c]	100	8.088					6.177	(13.1)		

[a] Values are reported in parts per million relative to DSS. [b] J_{sum} (Hz) = $J(H_{1'}H_{2'}) + J(H_{1'}{}_{,2''})$. Values for broad resonances are not recorded. [c] BLMF (20 mM) added. [d] This experiment was carried out at 281 K. [e] This experiment was carried out at 280 K. [f] Value for guanine. [g] Value for adenine. [h] Value for thymine. [i] Value for cytidine. [j] Assignments of $H_{1'}$ proton resonances in this experiment are tentative.

Table III: Change in Chemical Shifts of BLMF Proton Resonances after Complexation with Deoxyribonucleotides

nucleotide added[b]	concn (mM)	change in chemical shift (ppm)							
		u	v	b	d	c	f	e	a
pdG-dC	10	0.083	0.087	0.039	0.050	0.064	0.010	0.023	0.010
	100	0.305	0.298	0.135	0.176	0.219	0.018	0.046	0.020
	100	0.405[a]	0.413[a]	0.165[a]	0.202[a]	0.276[a]	0.006[a]	0.041[a]	0.000[a]
pdC-dG	10	0.063	0.072	0.020	0.030	0.042	−0.002	0.009	0.004
	80	0.217	0.228	0.104	0.113	0.159	0.020	0.040	0.020
pdG-dA + pdT-dC	10[c]	0.089	0.111	0.048	0.051	0.061	0.019	0.023	0.019
	50[c]	0.174	0.209	0.084	0.089	0.123	−0.006	0.020	−0.012
pdA-dC	10	0.056	0.073	0.033	0.026	0.033	0.019	0.018	0.018
	100	0.102	0.158	0.008	0.008	0.019	−0.030	−0.025	−0.031
pdC-dA	10	0.041	0.061	0.003	0.007	0.014	0.005	0.004	0.004
	100	0.180	0.224	0.019	0.021	0.053	0.035	0.031	0.037
pdG-dT	10	0.055	0.070	0.026	0.033	0.032	0.013	0.015	0.013
	100	0.076	0.129	0.001	0.016	0.017	−0.049	−0.030	−0.047
pdT-dG	10	0.027	0.052	0.002	0.005	0.006	0.007	0.001	0.008
	100	0.088	0.156	0.004	0.021	0.026	0.040	0.025	0.040
dGMP	10	0.015	0.026	−0.001	0.007	0.001	−0.004	−0.003	−0.004
	100	0.115	0.154	0.023	0.065	0.045	−0.004	0.011	−0.016

[a] Values calculated by using chemical shifts extrapolated to infinite dilution (Table I). [b] BLMF (20 mM) added to all experiments. [c] Concentration for each dinucleotide.

function of dinucleotide concentration (Figure 4). In these experiments, the concentration of BLMF was maintained at 20 mM while concentrations of pdG-dA + pdT-dC and the self-complementary dinucleotides, pdG-dC and pdC-dG, were varied between 5 and 100 mM.

Chemical shifts for the various complexes were recorded at different ratios of dinucleotide/BLMF (Table I). The bithiazole protons (Figure 1, u and v) in the pdG-dC:BLMF complex, measured with respect to their chemical shifts at infinite dilution, were displaced upfield by 0.4 ppm. Protons c, b, and d in this complex are likewise shifted upfield by 0.28, 0.17, and 0.12 ppm, respectively, while chemical shifts of protons a, e, and f do not change significantly.

Figure 5 portrays the induced changes in chemical shifts ($\Delta\delta$) for BLMF protons in dinucleotide complexes. The titration curves do not reach an asymptote at the highest dinucleotide/BLMF ratios studied; therefore, precise stoichiometry of complex formation could not be directly determined. During titrations with pdG-dC or pdC-dG, the two bithiazole protons display similar $\Delta\delta$; however, they differ from one another when BLMF is titrated with pdG-dA + pdT-dC (Table III and Figure 5).

Chemical shifts for the pdG-dC protons were measured during complexation with the analogue at varying ratios of

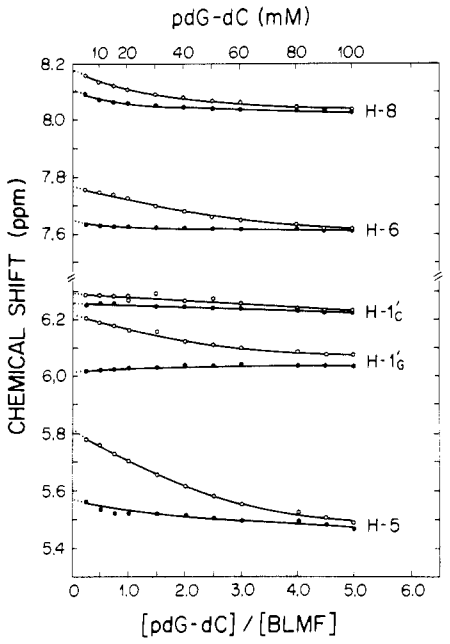

FIGURE 3: Chemical shifts of base and H1′ proton resonances in pdG-dC. The open circles represent values in absence of drug (top scale). Solid circles represent values after complexation with 20 mM BLMF.

dinucleotide/BLMF (Figure 3). Induced chemical shifts for the C-H5, C-H6, and G-H8 protons reach maximal values at a pdG-dC/BLMF ratio of 0.5. Under these conditions (Table IV), C-H5 shows the largest $\Delta\delta$ (0.221 ppm) among the base protons, followed by C-H6 (0.117 ppm) and G-H8 (0.064). The $\Delta\delta$ for one of the two H1′ protons is 0.167 ppm; the other is relatively unchanged. Formation of the complex does not affect the J_{sum} of the pseudotriplet representing the H1′ protons. As the ratio of dinucleotide/BLMF increases, chemical shifts of the pdG-dC protons in the complex move upfield, eventually becoming indistinguishable from chemical shifts represented by the free (predominantly dimerized) dinucleotides (Figure 3).

High ratios of dinucleotide/BLMF were achieved by titrating a 100 mM solution of pdG-dC with increasing concentrations of BLMF (Figure 6). Similar results were obtained in this experiment at dinucleotide/BLMF ratios which overlap the titration of the experiment shown in Figure 4. By combining results of these two experiments, the $\Delta\delta$ of protons u and v, induced by complex formation with pdG-dC, can be calculated (Figure 7). The lower curves in Figure 7 represent induced chemical shifts calculated at each concentration of BLMF; the upper curves are based on reference values for the u and v protons extrapolated to infinite dilution. The upper curves show an apparent stoichiometry of less than 2; the lower curves do not reach an asymptote at the maximum dinucleotide/BLMF ratio tested.

Complexes with Non-Self-Complementary Nucleotides. dGMP and the non-self-complementary dinucleotides, pdG-dT, pdT-dG, pdA-dC, and pdC-dA, do not form dimers. These nucleotides were used to assess the effect of stacking between the bithiazole rings and the nucleotide bases. Figure 8 portrays titration of BLMF in the presence of pdG-dT, pdT-dG, pdA-dC, pdC-dA, and dGMP; chemical shifts for proton resonances in these complexes are listed in Tables I and II. When BLMF forms a complex with non-self-complementary dinucleotides, values for $\Delta\delta$ are less than half of those observed in complexes with self-complementary dinucleotides. Only the bithiazole protons showed significant changes in chemical shifts. The $\Delta\delta$ for the bithiazole protons in the stacked complex with dGMP is comparable to those observed with pdG-dT, pdT-dG, pdA-dC, and pdC-dA. An apparent asymptote is reached for the purine–pyrimidine sequences, pdG-dT and pdA-dC, at lower concentrations of dinucleotide than for the pyrimidine–purine sequences, pdT-dG and pdC-dA.

At a nucleotide/BLMF ratio of 0.5, chemical shifts for G-H8 in guanosine move upfield by 0.082 ppm in the BLMF complex with pdG-dT and by 0.053 in the complex with pdT-dG while A-H8 in adenosine is shifted by 0.093 ppm in the complex with pdA-dC and by 0.072 ppm in the complex with pdC-dA (Table IV). The $\Delta\delta$ for the H2 proton of adenosine in pdA-dC (0.123 ppm) differs considerably from the $\Delta\delta$ of the same proton in pdC-dA (0.018 ppm). Chemical shifts of the pyrimidine protons (C-H5, C-H6, and methyl) show little or no change (<0.03 ppm). Of the two H1′ protons, one shifts upfield by 0.13 ppm.

Discussion

Assignment of Resonances. All proton resonances critical to the present analysis were resolved at 360 MHz. Most of

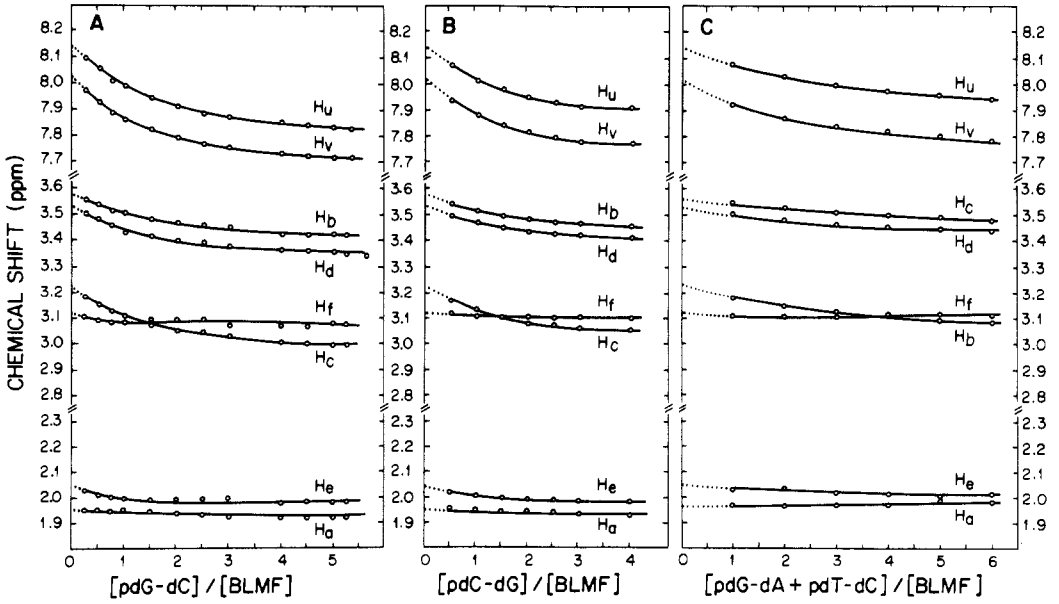

FIGURE 4: Chemical shifts of bithiazole proton resonances during titrations with pdG-dC, pdC-dG, and pdG-dA + pdT-dC.

Table IV: Change in Chemical Shifts of Deoxyribonucleotide Proton Resonances after Complexation with BLMF

		change in chemical shift (ppm)								
					pyrimidine		purine			
nucleotide added[b]	concn (mM)	H8	H2	H6	H1′	(ΔJ^a)	H1′	(ΔJ^a)	H5	CH3
pdG-dC	10	0.064		0.117	0.030	(1.0)	0.167	(0.55)	0.221	
	100	0.108		0.114	0.068	(0.5)	0.160	(0.05)	0.214	
pdC-dG	10	0.057		0.043	0.211		0.124	(1.7)	0.113	
	180	0.065		0.069	0.263		0.145	(0.2)	0.151	
pdG-dA + pdT-dC	10	0.104		0.023	0.060	(1.0)	0.060	(1.0)		
	10	0.055	0.041	0.018	0.050	(2.0)	0.081	(1.9)	0.031	0.025
	50	0.119		0.034	0.060		0.060			
	50	0.076	0.043	0.008	0.016		0.091	(1.6)	0.030	0.030
pdA-dC	10	0.093	0.123	0.001	0.019	(0.2)	0.134	(0.6)	0.003	
	100	0.082	0.135	0.022	0.001	(1.3)	0.083	(1.6)	0.028	
pdC-dA	10	0.072	0.020	0.000	0.017	(0.2)	0.082	1.0	0.020	
	100	0.085	0.077	0.0016	0.026	(1.2)	0.109	2.0	0.032	
pdG-dT	10	0.082		0.015	0.031	(0.2)	0.122	(0.6)		0.027
	100	0.042		0.024	0.010	(0.1)	0.062	(0.6)		0.002
pdT-dG	10	0.053		0.024	0.067	(0.4)	0.035	(0.0)		0.034
	100	0.027		0.022	0.067	(0.4)	0.016	(0.3)		0.023
dGMP	10	0.076					0.136	(0.5)		
	100	0.069					0.130	(0.6)		

[a] ΔJ in hertz is the difference of J_{sum} in Table III. [b] BLMF (20 mM) added to all experiments.

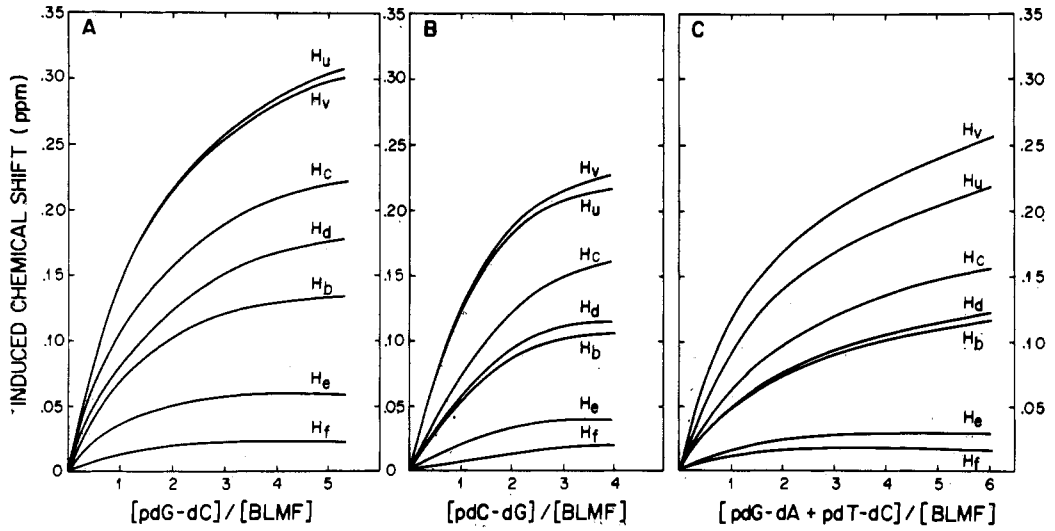

FIGURE 5: Induced chemical shifts of BLMF protons in titrations with pdG-dC, pdC-dG, and pdG-dA + pdT-dC. Calculations are made from data shown in Figure 4.

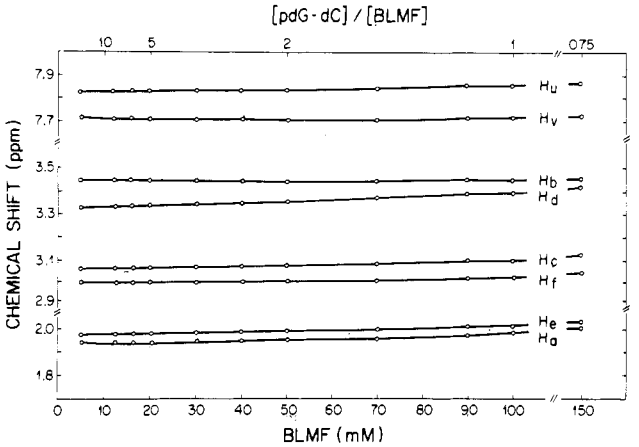

FIGURE 6: Chemical shifts of BLMF protons in the titration of pdG-dC (100 mM) with BLMF.

these could be assigned unequivocally on the basis of their chemical shifts and comparison to NMR spectra reported for bleomycin (Chien et al., 1977; Chen et al., 1977), for bleomycin analogues (J. M. Riordan and T. T. Sakai, unpublished data) and for dinucleotides (Cheng & Sarma, 1977). Additional experimental observations were required to distinguish between resonances emanating from the two bithiazole protons of BLMF and to assign H1′ resonances for the various dinucleotides studied.

Proton resonances of the bithiazole moiety were assigned as follows: The primary amino group of BLMF, positively charged under conditions of these experiments, is analogous to the sulfonium group of bleomycin A_2 and was assumed to bind, electrostatically, to one of the dinucleotide phosphate groups (Chien et al., 1977; Chen et al., 1980). As a result of such an interaction, the thiazole ring located closer to the amino group will stack on a base linked to this phosphate. The proton (v) on the stacked thiazole ring is subjected to greater ring-current effects than the analogous proton (u) on the more distal ring. Therefore, we have assigned the resonance which shifts further upfield on complexation to the H_v proton. The carbonyl group adjacent to H_v produces a small upfield shift in this proton with respect to H_u, further substantiating this assignment.

We base the relative assignments of the two H1′ protons in pdG-dC on the upfield change in chemical shift manifested by one of these protons when the concentration of dinucleotide is increased. This change, presumably due to duplex forma-

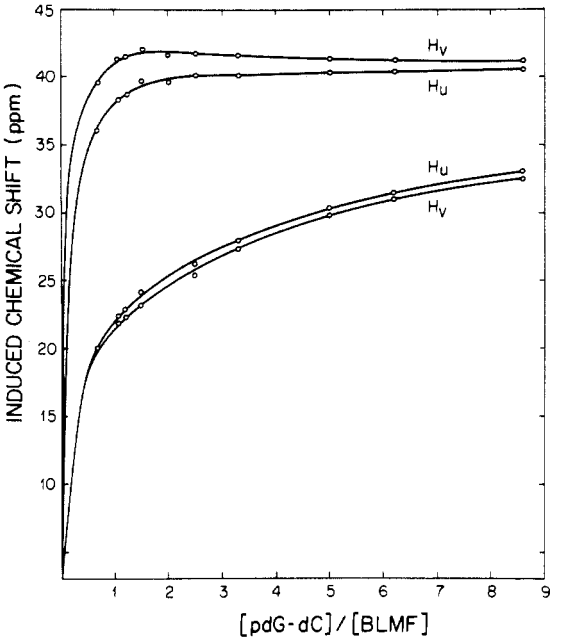

FIGURE 7: Changes in chemical shifts of the bithiazole protons observed when BLMF is titrated with pdG-dC. Data shown in the two upper curves were calculated relative to values of H_u and H_v measured at infinite dilution. Data shown in the two lower curves reflect data at each concentration. Values extrapolated from data shown in Figure 6 are used for concentrations higher than 50 mM.

tion, is also observed in the C-H5, C-H6, and G-H8 protons. The double-helical twist in B DNA is reflected by the magnitude of the chemical shift changes of C-H5, C-H6, and G-H8. Models of the pdG-dC dimer show that the ring-current effects of dC would have a greater effect on the H1′ proton of dG than those of dG on the H1′ proton of dC, assuming a twist angle of 36°. This assignment is consistent with an earlier study by Cheng & Sarma (1977).

Complex Formation. Stacking of BLMF on itself or on the nucleotide bases tends to delocalize the π electrons of the aromatic system, producing a secondary magnetic field which affects the chemical shifts of neighboring protons. The extent to which individual protons are affected by ring currents is determined by their position relative to the ring. Pullman and his colleagues have used molecular orbital calculations to predict chemical shifts for various nucleotide bases in a stacked configuration (Giessner-Prettre et al., 1976; Giessner-Prettre & Pullman, 1970). The upfield changes in chemical shift observed when BLMF complexes with various dinucleotides reveals the geometry of stacking between the bithiazole group and the nucleotide bases. Stacking may be facilitated by the electrostatic interaction between the positively charged amino group in BLMF and a negatively charged phosphate group of the dinucleotide. Complementary dinucleotides exist as monomers as well as dimerized Watson–Crick miniduplexes under the conditions of our experiments (Young & Krugh, 1975; Krugh & Young, 1975). The latter form intercalation complexes through stepwise association (Krugh & Reinhardt, 1975); hence, even at low concentrations of dinucleotides, considerable intercalative complexation may occur.

The most appropriate reference value for calculating induced changes in chemical shifts for intercalated or externally stacked complexes is the chemical shift extrapolated to infinite dilution.

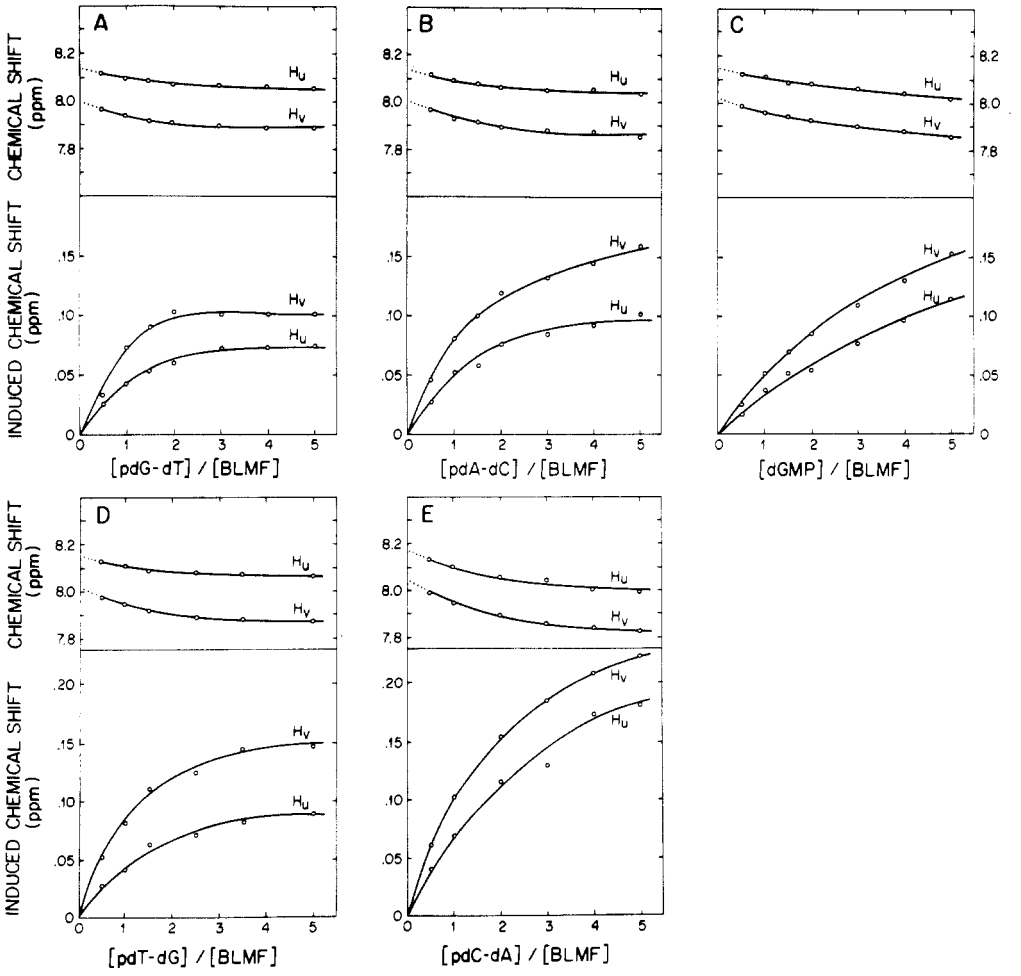

FIGURE 8: Chemical shifts (ppm) of BLMF protons in titrations with pdG-dT, pdT-dG, pdA-dC, pdC-dA, and dGMP.

Association constants for all possible complexes are not known with certainty; thus, the contribution of intermolecular stacking of BLMF molecules to the upfield chemical shift cannot be accurately calculated for complexes at low ratios (<2) of dinucleotide/BLMF. When dinucleotides are present in excess, all BLMF is presumably bound; therefore, values for $\Delta\delta$ extrapolated to infinite dilution were used as reference values to construct the upper curves shown in Figure 7. These curves reach an apparent asymptote at a dinucleotide/BLMF ratio of approximately 1.5. The lower curves, based on reference values for BLMF protons measured at a drug concentration of 20 mM, do not reach an asymptote even at dinucleotide/BLMF ratios of 9.

Because BLMF stacks on itself, the value of 1.5 is not considered to represent the precise stoichiometry of complex formation. When corrections are made for self-stacking, $\Delta\delta$ approaches an asymptote at a dinucleotide/BLMF ratio of 2, a value which might reasonably be expected to represent the stoichiometry of the complex formed between pdG-dC and BLMF.

Two preliminary reports have appeared in which NMR was used to study the interaction of bleomycin A_2 with polynucleotides. Chien et al. (1977) reported selective broadening of resonances associated with the bithiazole and terminal sulfonium groups of the antibiotic when complexed to DNA. Chen et al. (1980) confirmed these results in a bleomycin A_2–poly(dA-dT) complex and described additional spectral pertubations which led to the conclusion that two types of complexes were formed, one at room temperature, in which only small upfield shifts of the bithiazole protons were observed, and the second, observed at 50°, in which a 0.27-ppm upfield chemical shift was recorded. The upfield shift compares favorably with the result reported in this study. Similar results were obtained when the terminal fragment of bleomycin A_2 was used as a model for the intact antibiotic (Sakai et al., 1981).

It seems likely that electrostatic bonds form rapidly between bleomycin and a polynucleotide or between BLMF and dinucleotides. Similar initial reactions have been reported for ethidium bromide (Krugh & Reinhardt, 1975). Subsequent intercalation proceeds by way of the major or minor groove or its equivalent, in the case of a dinucleotide duplex. Molecular model building studies and the preferred geometry of stacking (see previous discussion) suggest that the bithiazole rings enter most easily through the major groove. The apparent failure of the bithiazole rings to intercalate with poly(dA-dT) at room temperature, as evidenced by lack of upfield chemical shifts (Chen et al., 1980), may reflect the presence of the methyl groups of thymine in the major groove of this polymer. At higher temperatures, unwinding of the poly(dA-dT) helix and thermally induced disorder in the polynucleotide may allow the ring to be intercalatively inserted.

Selectivity. Selective binding of BLMF is most clearly observed in the experiments using non-self-complementary dinucleotides. At a dinucleotide/drug ratio of 0.5, the significant difference in $\Delta\delta$ values between the base protons of the purines (0.053–0.123 ppm) and those of the pyrimidine protons (<0.02 ppm) suggests that the bithiazole ring stacks preferentially on purine bases at a dinucleotide/drug ratio of 0.5. At higher dinucleotide concentrations, base stacking contributes to the observed chemical shifts. One general exception was noted, A-H2 in pdC-dA. Possible reasons for this apparent discrepancy are discussed below.

Preferential stacking of BLMF on purines is not unexpected, considering the matching size of the two aromatic systems. Furthermore, bleomycin binds preferentially to guanine in DNA as revealed by studies which show sequence-specific strand-scission reactions (Takeshita et al., 1978; D'Andrea & Haseltine, 1978). Values for $\Delta\delta$ of the purine base protons in pyrimidine–purine (5′ → 3′) sequences are smaller than those in purine–pyrimidine (5′ → 3′) sequences. This difference in $\Delta\delta$, which depends on binding constants and geometrical considerations (Miller et al., 1980), may reflect the importance of an electrostatic interaction between a phosphate group of the dinucleotide and the positively charged amino group of BLMF. Under the assumption that the bithiazole binds preferentially to purines, there are two modes by which these rings can stack on purine in the purine–pyrimidine sequence and only one in the pyrimidine–purine sequence.

The difference in $\Delta\delta$ for A-H2 in complexes between BLMF and pdC-dA (<0.02) and BLMF and pdA-dC (0.123) at dinucleotide/drug ratios of 0.5 may reflect the shorter distance between the internucleotide phosphate and the purine base in the former. This arrangement prevents the thiazole rings from stacking fully on the purine. Weaker binding (compare pyrimidine–purine with purine–pyrimidine sequences) results in both thiazole protons being located more closely to the center of the purine base. In this conformation, A-H2 is located further from the thiazole rings. Again, the general preference for 5′-phosphoryl purines and for purine–pyrimidine sequences in noncomplementary dinucleotides is consistent with sequence-specific cleavage of DNA by bleomycin (Takeshita et al., 1978; D'Andrea & Haseltine, 1978; Takeshita et al., 1981).

The fact that both thiazole protons show significant $\Delta\delta$ in all complexes with noncomplementary dinucleotides supports the proposal that thiazole rings stack on purines rather than pyrimidines. Model building studies reveal that stacking of a bithiazole on a pyrimidine would either lead to equal but lower values for H_u and H_v or result in a marked inequality between chemical shifts of protons.

Conformational Considerations. Sugar puckering of deoxyribose in complementary dinucleotide duplexes can be represented as C2′ endo or C3′ endo, giving rise to several possible conformations. Some of these conformations have been recognized in dinucleotide/drug complexes (Patel & Canuel, 1976; Patel & Shen, 1978). The predominant form reported in crystalline complexes of drugs with ribodinucleotides is the mixed sugar puckering pattern: C3′ endo (3′ → 5′)–C2′ endo (Miller et al., 1980; Patel & Shen, 1978; Tsai et al., 1977; Sakore et al., 1979; Jain et al., 1979; Reddy et al., 1979).

In theory, coupling constants for H1′ and H2′ provide a method for determining the sugar conformation. Altona & Sundaralingam (1973) demonstrated that the coupling constant $J_{1',2'}$ is ~0 Hz for C2′-exo–C3′-endo sugar pucker geometry (designated as N type) and ~10 Hz for C2′-endo–C3′-exo sugar pucker geometry (designated as S type). The coupling sum of $J_{1',2'} + J_{1'2''}$ is ~7 Hz for N type geometry and ~16 Hz for the S type.

Coupling sums for dinucleotides reported in this paper varied from 12.5 to 13.7 Hz before complexation, suggesting the existence of a mixture of S and N type sugar puckering. After complexation, the coupling sum was minimally changed (<1.5 Hz), indicating that no marked change in puckering occurs when drugs are bound.

The BLMF molecule has several degrees of conformational freedom. For example, when aligned in a general plane, the two thiazole protons can be positioned on the same or on opposite sides of the bithiazole moiety. The presence of three methylene groups provides flexibility in the molecule, facili-

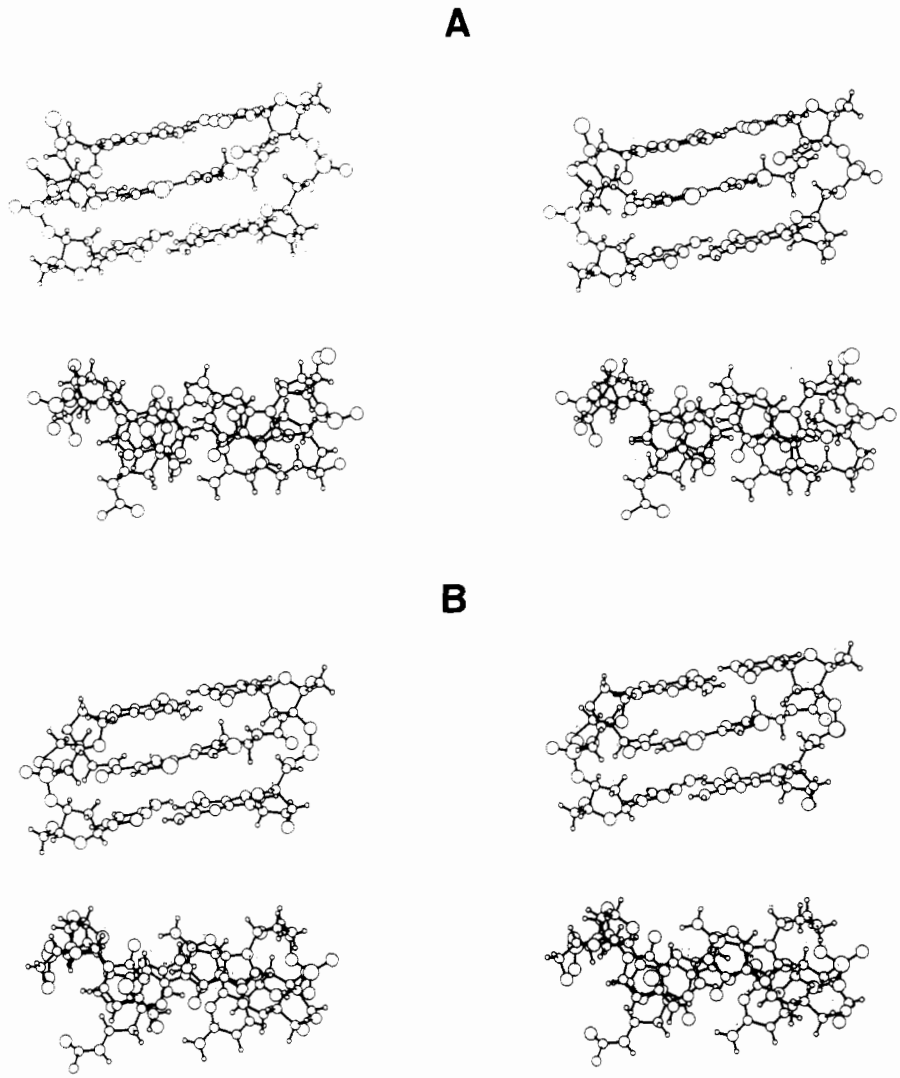

FIGURE 9: Computer-drawn molecular models of the proposed pdG-dC–BLMF complex. (A) Coordinates for the duplex provided by Sobell (C3′-endo–C2′-endo mixed sugar puckering). (B) Coordinates provided by Berman (C3′-endo–C3′-endo).

tating approach of the positively charged terminal amine to the phosphate groups of the dinucleotide while effectively retaining stacking forces between the thiazole rings and the base.

The preference of bithiazole rings for stacking on purines raises the possibility of an intercalative complex. Overlapping purines in dimers of complementary dinucleotides (viewed from the top of the miniature duplex) offers the most stable stacking complex. Retention of the sugar pucker geometry does not invalidate the arguments for intercalation. Berman & Neidle (1979) point out that intercalation geometry can be obtained while varying torsion angles in the DNA even if the sugar pucker geometry is fixed. The propeller-like base conformation in the DNA minature duplex proposed by Levitt (1978) and by Crothers et al. (1979) would fit well with this perception because of the conformational freedom of the two thiazole rings. Preference for purine bases allows both aromatic ring systems to be matched while retaining the propeller-like twist of the helix.

Geometry of the Proposed Complex. On the basis of the studies presented in this paper, we propose the following model for the BLMF-pdG-dC complex: the thiazole rings are positioned in a general plane equidistant (~3.4 Å) to the planes of the base pairs located immediately above and below. Both thiazole protons extend into the minor groove. The thiazoles stack approximately equally on the two purines. When projected on the same plane, the horizontal axis connecting the center of both thiazole rings in this configuration is almost superimposed on the axis that connects the centers of the purine bases. The positively charged terminal amine binds to the negatively charged phosphate group of DNA and the neutral acetyl terminal is arranged in a manner that provides a minimum of steric hindrance.

Two dinucleotide structures, the C2′-endo–C3′-endo mixed sugar pucker CpG duplex, which has an unwinding angle of 26° (Tsai et al., 1977; Sakore et al., 1979; Jain et al., 1979; Reddy et al., 1979), and the C3′-endo–C3′-endo CpG duplex, with no unwinding of the DNA backbone (Berman et al., 1977), have been fitted with BLMF by using a computer graphics display.[3] When the constraints described above are used, the BLMF molecule fits into both of these duplexes. Computer-drawn stereo pictures are shown in Figure 9. The acetyl terminal is positioned so as to reflect the $\Delta\delta$ of the methylene protons, H_b and H_c. H_d is the only methylene proton at the amine terminal subjected to ring-current effects from the base. With Berman's stretching duplex (Figure 9B), which has no unwinding angle, the thiazole protons should experience less ring-current effect than with Sobell's structure (Figure 9A) since the contribution of ring currents from py-

[3] Computer modeling was done at the Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD.

rimidine will be much higher in the latter case.

In proposing a three-dimensional structure to account for our experimental findings, we recognize that high-resolution studies of this type do not provide geometric information with the same degree of certainty as X-ray diffraction. Nevertheless, such analyses are useful in guiding experimental work and in defining the boundaries of NMR techniques. The configurations shown in Figure 9 are consistent with a recently proposed model based on the biochemical effects of bleomycin on DNA (Grollman & Takeshita, 1979; Giloni et al., 1981).

Evidence for Intercalation. The magnitude of the chemical shifts experienced by the bithiazole protons during complex formation and the fact that these shifts are equivalent represent important experimental data used to formulate the model shown in Figure 9. The largest chemical shift observed during complex formation (0.4 ppm) involves the bithiazole protons u and v (Figure 1). This value may be compared with results obtained with actinomycin D (Krugh & Chen, 1975; Krugh & Neely, 1973; Patel, 1974), proflavin, and ethidium bromide (Krugh & Reinhardt, 1975; Kreishman et al., 1971; Patel & Canuel, 1976), drugs shown by crystallographic techniques to form intercalation complexes. While a variety of factors, including aggregation, solvent exclusion, and charge effects, may induce changes in chemical shifts during complex formation, their contribution should be relatively small in the system under study.

Equivalent chemical shifts were induced in the u and v protons of BLMF when complexes were formed with pdG-dC and pdC-dG. Because of the 2-fold symmetry of such complexes, the bithiazole ring in our model is positioned so that the combined ring-current effects of the base pairs on the bithiazole protons is approximately equal. We have considered two arrangements, one in which the bithiazole rings are intercalated and the other in which they stack on the minihelix. The intercalation mode provides a more stable configuration while the stacking mode would show a large difference for $\Delta\delta$ between H_u and H_v.

A third arrangement, in which the bithiazole binds between two miniduplexes, cannot be rigorously excluded. However, energetic considerations and the observation that substantial aggregation of the miniduplex occurs only at high concentrations (Young & Krugh, 1975) suggest that the intercalation complex would be the preferred form over the range of experimental concentrations tested.

Evidence of backbone unwinding would provide strong supporting evidence for intercalation. However, ^{31}P NMR experiments, such as those described for ethidium bromide with pdC-dG (Reinhardt & Krugh, 1977), showed only small ($\sim$ 0.1 ppm) shifts in the ^{31}P resonances.

Our results indicate that the bithiazole rings stack preferentially on the purine bases. This conclusion is supported by data for complexes formed with BLMF and the noncomplementary dinucleotides pdG-dT and pdA-dC, in which the cytidine C-H5 and C-H6 protons do not display significant chemical shifts. The G-H8 proton shows an induced shift of about 0.1 ppm, comparable to that of dGMP.

The induced chemical shift for C-H5 of cytidine is twice as large as that for the C-H6 protons. This observation argues against full stacking of cytidine with the thiazole intercalated or stacked outside of the duplex. The acetyl group of BLMF effectively prevents the thiazole ring from stacking fully on cytidine in the intercalation mode while steric hindrance would not prevent stacking outside of the duplex.

The conformation of the deoxyribose ring in drug–nucleic acid complexes has been the subject of several investigations (Miller et al., 1980; Patel & Shen, 1978; Ezra et al., 1977; Rich et al., 1979). All complexes thus far reported have right-handed helices, although certain oligonucleotides crystallize in the left-handed configuration. In the crystal structure of dC-dG, sugar puckering assumes the C2′-endo (3′ → 5′)–C3′-endo configuration. This structure is consistent with the coupling constants observed in our experiments. Although coupling constants represent a weighted average between two forms, it appears that significant changes in sugar puckering do not occur during complex formation.

Other structural features of the complex involve the position of the bithiazole rings relative to one another. A relatively planar structure is required in any of the complexes considered. If ring-current effects are to be equivalently maintained, protons u and v must be on the same side of the bithiazole ring. This configuration also conforms better with the magnitude of chemical shifts induced in the other BLMF protons. Any reasonable model of an intercalation complex in which the u and v protons are on opposite sides would result necessarily in a significantly different $\Delta\delta$ for the two protons.

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