and orbital symmetry rules are weakened. Thus, symmetry-forbidden complexes (e.g., triplet $[{}_{\pi}4_s + {}_{\pi}2_s]$) may become as stable as symmetry-allowed ones (e.g., triplet $[{}_{\pi}2_s + {}_{\pi}2_s]$) and spin inversion may dictate the [4 + 2]/[2 + 2] competition (in polar cases). For a discussion on orbital-symmetry effects vs. polarity effects see ref 4a, Chapter 22.
(26) In a polar case the T_1 wave function for the 2s + 2s complex is mainly $^3D^+A^-$; namely, the individual reactants are not in their locally excited states but are in their ionic states (D^+ and A^-, respectively). Thus, there will be a barrier for disrotation of a reactant in the 2 + 2ID (90°) mechanism. This barrier will increase as the reactant pair is made more polar and may eventually suppress 2s + 2a product formation. The same conclusions apply to $4D_y$ (90°) + $2D_z$ (90°), which require disrotation of both reactants.
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Interactions of Gallium(III) with Bleomycin Antibiotics[1a]

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Abstract: The addition of Ga(III) to an aqueous solution of bleomycin results in the observation of several new peaks in the ^1H NMR spectrum, concomitant with the reduction in intensity of several of the peaks of the antibiotic. By measuring the relative intensity of the resonance of the C_4 proton of the imidazole at various Ga(III) concentrations, we have found that the Ga(III)-bleomycin complex has a 1:1 stoichoimetry. The lifetime of the Ga(III)-bleomycin complex was found to be ca. 15 s at 343 K (70 °C) from NMR transfer of magnetization and spin-lattice relaxation time measurements. On the basis of a combination of fluorescence binding studies and proton-displacement experiments, we conclude that the binding of Ga(III) to the bleomycins (A_2 and B_2) displaces a single proton from the α-amino group of the diaminopropionamide moiety of the drugs. This evidence implicates this portion of the molecule directly in metal binding.

Introduction

The clinical uses of the radioisotope gallium-67 have developed from the observation that this isotope selectively localizes in a broad range of tumors and lymphomas.[2,3] Current applications are directed at the detection and staging of solid tumors and lymphomas[4] as well as the use of gallium nitrate as a therapeutic agent[5] in the treatment of human neoplasias. In our laboratories, investigations into the mechanism of gallium-67 localization have proceeded along two lines: in vitro studies of the uptake of gallium by normal and malignant cells[6-10] as well as NMR investigations of the aqueous chemistry of gallium.[11-14] The present study is directed at the characterization of complexes of gallium with the bleomycin antibiotics.

The bleomycins are a family of glycopeptide antibiotics isolated from *Streptomyces verticillus* by Umezawa and coworkers.[15] The revised primary structures[16] of these molecules are shown in Figure 1. The various congeners of the bleomycins differ from each other in their terminal amine moiety (R group in Figure 1). Blenoxane, the commercial form of these drugs, marketed by Bristol Laboratories (Syracuse, N.Y.), contains ca. 70% bleomycin A_2 and 25% bleomycin B_2, with trace amounts of other congeners. These drugs have been employed clinically in the treatment of a wide variety of human carcinomas and lymphomas.[17] In addition, these molecules have been used in tumor scanning as carriers for various radionucleotides including gallium-67.[4] The impetus for the present study arises from the following considerations: (1) the observation that the bleomycins inhibit the uptake of gallium-67 in L1210 leukemic cells;[18] (2) gallium-67 complexes of the bleomycins have been employed as radiopharmaceuticals;[4] (3) metal ions (especially Fe(II))[19] have been proposed to play an important role in the mechanism of action of these drugs.[20]

Previous spectroscopic investigations into the interactions of the bleomycins with polyvalent metal ions have focused on the Zn(II),[21-23] Cu(II),[24] and Fe(II)[19] complexes. Umezawa and co-workers have proposed a square pyrimidal coordination geometry for the Cu(II) complex on the basis of UV evidence[24] and X-ray crystallographic data[25] on a metabolite of these antibiotics. In contrast, Dabrowiak et al. have inferred a square-planar geometry for the Zn(II) and Cu(II) complexes from the results of ^{13}C NMR studies,[23] ^1H NMR investigations, and UV difference spectra.[21] On the basis of our previous studies on the kinetics of dissociation of the Zn(II) complex of bleomycin, we have suggested that the coordination geometry is tetrahedral.[26] Thus, there is still some controversy surrounding the coordination geometry around various metal ions in their complexes with bleomycin and, in fact, there probably are different geometries for different metal ions.

In the present study we have monitored the interactions of gallium(III) with the bleomycins using ^1H NMR, fluorescence spectroscopy, and potentiometric titrations. We have also determined the kinetics of dissociation of the Ga(III)-bleomycin complex from NMR transfer of magnetization and spin-lattice relaxation time (T_1) measurements. These results are compared with the results of similar experiments conducted on the Zn(II)-bleomycin complex.[26]

Experimental Section

Blenoxane, a generous gift from Drs. W. T. Bradner and S. T. Crooke of Bristol Laboratories, was separated into its component

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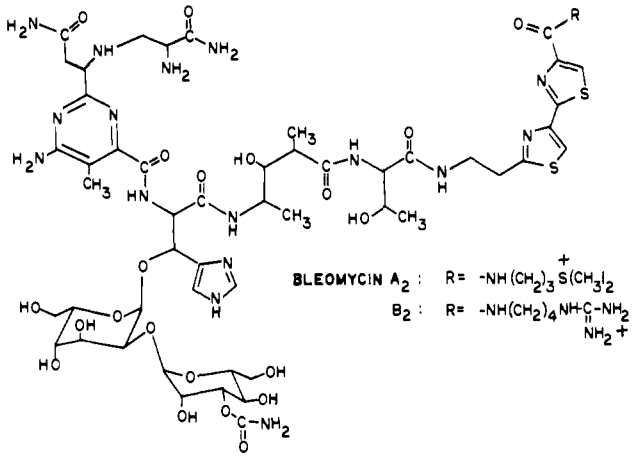

Figure 1. The primary structure of the bleomycin antibiotics.

congeners by chromatography on carboxymethyl-Sephadex CM-25 using a linear gradient of 0.0–0.5 M NaCl. The fractions were dissolved in methanol to remove bulk salt and passed over an R-10 Amberlite ion exchange column using neutral H_2O as an eluent. The concentration of each congener and blenoxane was determined spectrophotometrically using a molar absorptivity of $1.3 \pm 0.2 \times 10^4$ M^{-1} cm^{-1} at 290 nm. The concentration of Ga(III) in solution was determined spectrophotometrically at 600 nm using the arsenazo complex.[27] In practice, a concentrated stock solution of $Ga(NO_3)_3$ was prepared and appropriate dilutions were made as needed. In D_2O, we report as the pD the pH meter reading which has not been corrected for the deuterium isotope effect.

NMR experiments were conducted at 90 MHz on a Bruker HX-90 spectrometer operating in the Fourier transform (FT) mode. The transfer of magnetization experiments were performed on a modified Bruker HX-360 (Stanford University) operating at 360 MHz in the FT mode. Proton T_1s at 360 MHz were determined by the inversion-recovery method using a Nicolet NTC-360 spectrometer (Purdue University). For the latter two sets of experiments, the temperature was determined to ±1 °C from the chemical shifts of a standard sample of ethylene glycol.

Fluorescence experiments (uncorrected) were performed at ambient temperature on a Perkin-Elmer MPF-3 fluorimeter. For the proton-release experiments, a solution of NaOH of the appropriate concentration was prepared from concentrated standard NaOH solutions (Anachemia Chemicals Ltd., Montreal, Quebec, Canada). All computations were performed on an IBM-370 computer.

Results and Discussion

NMR Experiments at 90 MHz. The 90-MHz ^1H NMR spectrum of 10 mM Blenoxane at pD 6.8 is shown in Figure 2. Addition of Ga(III) results in the appearance of several new peaks in the spectrum concomitant with the reduction in intensity of several peaks in the Blenoxane spectrum. From this observation, we infer that for some of the protons in Blenoxane the rate of dissociation of the Ga(III)–bleomycin complex is much slower than their chemical-shift differences in the free and complexed states. Similar observations have been reported by Dabrowiak et al.[21,23] for both the ^{13}C and ^1H NMR spectra of the Zn(II)–bleomycin A_2 complex and by Cass et al.[22] for the ^1H NMR spectrum of the Zn(II) complex of Blenoxane.

In order to establish the stoichiometry of the complex, we determined the relative intensities of the imidazole C_4 proton resonances of Blenoxane at various Ga(III) concentrations (Figure 3). The intercept on the abscissa of ca. 1.0 clearly demonstrates the formation of a 1:1 complex. In addition, we wish to point out that the nearly linear variation in the intensities indicates that the dissociation constant for the Ga(III)–bleomycin complex is several orders of magnitude smaller than the concentration of this antibiotic used in this experiment, i.e., binding is quantitative.

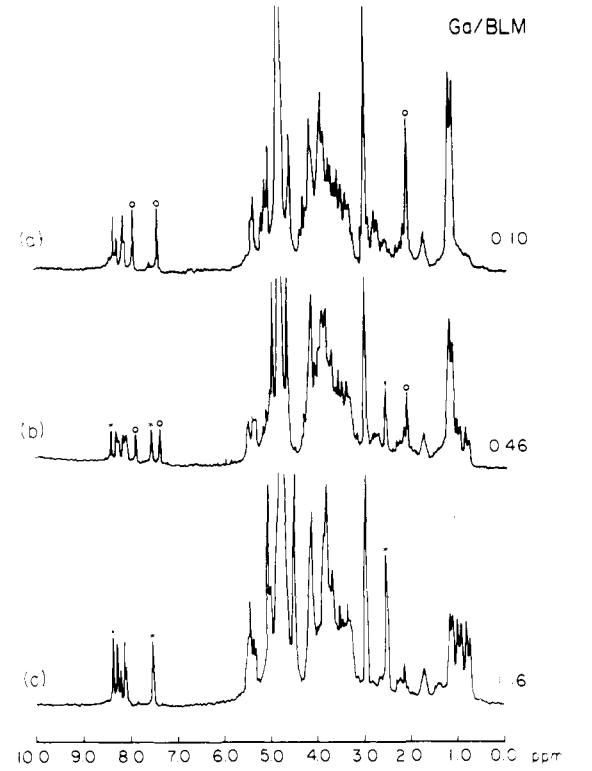

Figure 2. The 90-MHz ^1H spectrum of 10 mM Blenoxane at a pD of 6.8 in the presence of (a) 0, (b) 4.6, and (c) 11.6 mM Ga(III) present. Circles denote resonances of the free antibiotics; asterisks denote the resonances of the bound antibiotics.

In the spectra shown in Figure 2, the largest perturbations induced by Ga(III) are downfield shifts of the resonances of the imidazole C_2 (0.2 ppm) and the C_4 (0.09 ppm) protons and the pyrimidine methyl group (0.4 ppm). These perturbations are almost identical with the ones induced by Zn(II).[21,22,26] As we have pointed out in our study of the Zn(II)–bleomycin complex,[26] these perturbations do not necessarily implicate either the nitrogens of the pyrimidine ring or the imidazole group as the sites of metal chelation.

360-MHz Transfer of Magnetization Experiments. Transfer of saturation is governed by the equation

$$(M_0^\alpha - M_z^\alpha)/M_0^\alpha = [T_{1_\alpha}/(T_{1_\alpha} + \tau_\alpha)][M_0^\beta - M_z^\beta)/M_0^\beta \quad (1)$$

which was derived from modified Bloch equations using a procedure analogous to that used by Gupta and Redfield.[28] The α and β states refer to two nuclei which are chemically exchanging. T_{1_α}, τ_α, M_z^α, and M_0^α are the spin–lattice relaxation time, lifetime, observed magnetization, and equilibrium magnetization of the α nucleus, respectively. Analogous notation is used for the β nucleus. $(M_0^\alpha - M_z^\alpha)/M_0^\alpha$ is the fractional decrease in resonance intensity of the α resonance resulting from the double irradiation of the β resonance, whose intensity is diminished by a factor of $(M_0^\beta - M_z^\beta)/M_0^\beta$. Complete saturation of the β state causes a fractional decrease of the α resonance equal to $T_{1\alpha}/(T_{1\alpha} + \tau_\alpha)$, which is significant only if the pseudo-first-order rate constant for exchange of the α nucleus, $1/\tau_\alpha$, is comparable to or greater than its relaxation rate, $1/T_{1\alpha}$.

The aromatic region of the 360-MHz ^1H NMR spectrum of Blenoxane at 343 K in the presence of Ga(III) is shown in Figure 4a. The spectrum obtained on complete saturation of the imidazole C_2 proton of the Ga(III)–bleomycin complex (at 8.25 ppm) is shown in Figure 4b. There is a slight decrease in intensity in the C_2 resonance of the free bleomycin. When

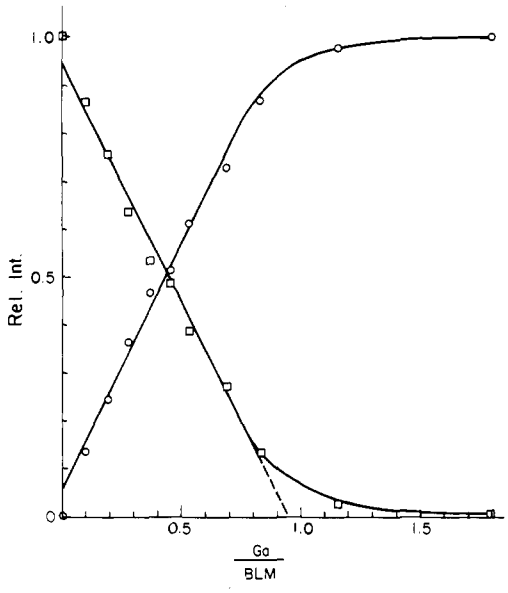

Figure 3. The relative intensities of the C_4 imidazole resonances of Blenoxane in the free (○) and bound (□) states as a function of the relative concentration of Ga(III) present. The Blenoxane was 10 mM at a pD of 6.8. Spectra were recorded at 28 °C.

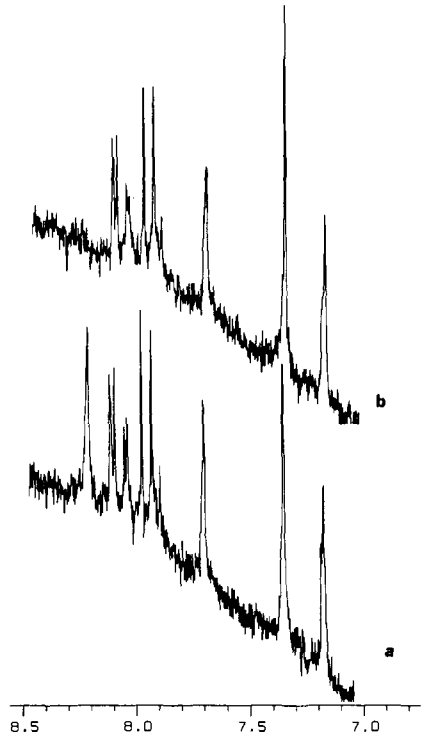

Figure 4. The 360-MHz ^1H spectrum of 10 mM Blenoxane in the presence of 5 mM Ga(III) at a pD of 6.8: (a) with no decoupling radiation; (b) with the decoupler set at 8.25 ppm.

the frequency of the decoupler was shifted to 6.8 ppm, the spectrum obtained was identical with that shown in Figure 4a. From these spectra we calculate that there is a ca. 20% transfer of magnetization observed at 343 K. Similar spectra obtained at 303 K indicate no significant perturbations in the intensity of the C_2 resonance of the free bleomycin on saturation of the bound peak. This observation demonstrates the temperature dependence of the extent of saturation transfer.

We have previously reported the variation in T_1 of the C_2 proton resonance of bleomycin as a function of temperature.[26] Using the appropriate value of T_1 for 343 K (2.86 s) in eq 1, we calculate a value for τ of ca. 15 s. At this same temperature, the value of τ for the Zn(II)-bleomycin complex is 1.61 s. Thus the Ga(III)-bleomycin complex is approximately an order of magnitude more stable kinetically than the Zn(II)-bleomycin complex. We can obtain a rough estimate of the lifetime of the Ga(III)-bleomycin complex at 310 K (37 °C) by assuming that the energy of activation for dissociation is approximately the same as for the Zn(II) complex. Under this assumption we calculate the lifetime of the complex to be ca. 3 min at 37 °C. We have previously reported that the rates of dissociation of gallium-citrate complexes are also slow on the NMR time scale.[11,12] Investigations of the kinetics of dissociation of these latter complexes are currently in progress in our laboratory.

Proton-Release Experiments. The addition of Ga(III) to an unbuffered solution of bleomycin at pH 7.0 results in a decrease in the pH of the solution. This decrease in pH results from competition between Ga(III) and hydrogen ions for an ionizable group or groups in the drug. The concentration of hydrogen ions released per equivalent of Ga(III) added can be determined by titrating the solution to pH 7.0 after each addition of metal ion. The results of these experiments for bleomycin A_2 are shown in Figure 5. These data clearly demonstrate that the binding of Ga(III) to this congener displaces a single proton from an ionizable group in the antibiotic. We obtained similar results for the same experiments conducted with bleomycin B_2.

Since a single proton is displaced from the bleomycins on metal binding, we can describe the competition between metal ions and protons by the following equilibria:

$$M + B \rightleftarrows MB \qquad (2)$$

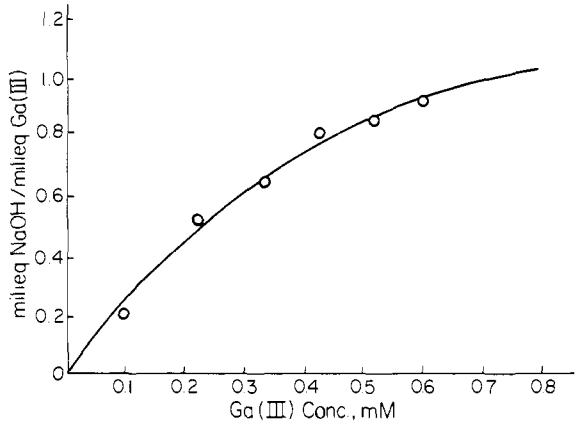

Figure 5. The number of milliequivalents of sodium hydroxide per milliequivalent of Ga(III) added (initial pH 7.0) for a 1×10^{-4} M solution of bleomycin A_2 in the presence of increasing concentrations of Ga(III).

with

$$K_M = [M][B]/[MB] \qquad (3)$$

and

$$H^+ + B \rightleftarrows BH^+ \qquad (4)$$

with

$$K_H = [B][H^+]/[BH^+] \qquad (5)$$

where M, B, and MB refer to the metal ion, bleomycin, and the complex, respectively, K_M and K_H are the dissociation constants for the complex and protonated form of bleomycin, respectively, BH^+ and H^+ refer to the protonated form of bleomycin and protons, respectively, and brackets denotes equilibrium concentration. Note that K_H equals the K_a of the ionizable group of bleomycin which dissociates on binding of the metal.

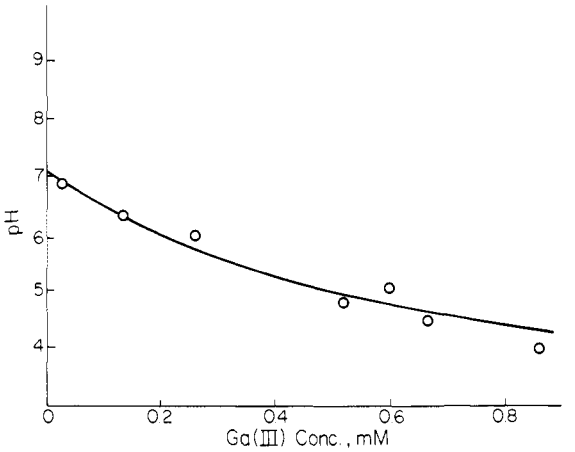

Figure 6. The variation in pH of a solution of bleomycin A_2 (1×10^{-4} M) at an initial pH of 7.1 in the presence of increasing concentrations of Ga(III).

Combining these equations with the mass law expressions for all of the species present and solving for [B] gives the cubic equation

$$[B]^3 + (K_M + K_H + H_T^+ + M_T - B_T)[B]^2 + [K_M K_H + M_T K_H + H_T^+ K_M - B_T(K_M + K_H)][B] - B_T K_M K_H = 0 \quad (6)$$

where M_T, B_T, and H_T^+ refer to the total concentrations of metal ions, antibiotic, and protons present, respectively. The value of H_T^+ can be calculated from

$$pH = pK_a + \log([B]/[BH^+]) \quad (7)$$

$$H_T^+ = [H^+] + [BH^+] \quad (8)$$

$$B_T = [B] + [BH^+] \quad (9)$$

Since the values of B_T and M_T are known experimentally, the solution of eq 6 and 7-9 requires the specification of two parameters, K_M and K_H. The fitting of experimental data to these equations is simplified by the availability of the pK_as of the three ionizable groups of bleomycin B_2, which Takita et al.[29] have reported to be 2.9, 4.7, and 7.3. Our approach to the data-fitting procedure was to begin our nonlinear least-squares algorithm[30] with a value of K_H corresponding to each of the three pK_a values. The value of H_T^+ was calculated from eq 7-9 and then eq 6 was solved. The values of $[H^+]$ can be calculated from

$$[H^+] = H_T^+/[1 + ([B]/K_H)] \quad (10)$$

The variation in pH of a solution containing bleomycin A_2 in the presence of various concentrations of Ga(III) is shown in Figure 6. Nonlinear least-squares minimization[30] of the data in Figure 6 gave values of K_M of $1.5 \pm 0.3 \times 10^{-5}$ M and K_H of $6.5 \pm 0.5 \times 10^{-8}$ M. Similar experiments performed with bleomycin B_2 yielded K_M and K_H values of $1.8 \pm 0.4 \times 10^{-5}$ and $6.3 \pm 0.5 \times 10^{-8}$ M, respectively. The rather good agreement between the values of K_M obtained for the Ga(III) complexes of bleomycin A_2 and bleomycin B_2 indicates that the terminal amine moiety (R group in Figure 1) is not involved in metal binding. The values of K_H obtained for both of the congeners correspond to a pK_a of 7.2 ± 0.1, implicating the α-amino group of the diaminopropionamide moiety in metal binding. Umezawa and co-workers have found that in the presence of Cu(II) titration of the α-amino group is suppressed.[31] More recently, these investigators have reported an X-ray crystallographic study of a Cu(II) complex of a metabolite of bleomycin whose α-amino group is coordinated to this transition metal.[25]

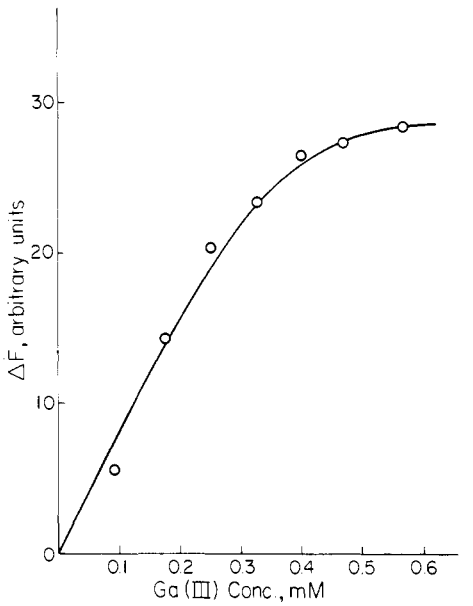

Figure 7. The variations in the fluorescence intensity of bleomycin A_2 (2×10^{-4} M, pH 7.0) in the presence of increasing concentrations of Ga(III).

Fluorescence Experiments. The fluorescence properties of bleomycin A_2 have been described by Chien et al.[32] This congener has maxima at 352 and 302 nm in its emission and excitation spectra, respectively. We have estimated the quantum yields of blenoxane (0.080 ± 0.005), bleomycin A_2 (0.083 ± 0.005), and bleomycin B_2 (0.070 ± 0.005) at pH 7.0 relative to tryptophan,[32] which has similar emission and absorption properties.[33,34]

The addition of Ga(III) to bleomycin A_2 brings about an increase in the intensity of the fluorescence of the antibiotic concomitant with a shift in the position of the emission maximum from 352 to 358 nm. This bathochromic shift reflects the more polar environment of the fluorophore (bithiazole) in the metal complex than in its free or uncomplexed state.[35] The variation in intensity of the fluorescence of this congener with the concentration of Ga(III) is shown in Figure 7. These data were analyzed in terms of the equilibria described by eq 2-5. However, this particular application does not involve making the substitution for $[H^+]$ described in eq 10. Combining eq 3 and 5 with the mass law expressions for M_T and B_T and solving for [B] gives the quadratic equation

$$[B]^2(K_H + [H^+]) + [B][(K_H + [H^+])K_M + K_H(M_T - B_T)] - K_H K_M B_T = 0 \quad (11)$$

Note that the value of K_H is known from the results of proton-displacement experiments. Also, if the experiment is performed in a buffered system, the value of $[H^+]$ is kept constant. Thus, the computation of the values of [B] requires the specification of one parameter, K_M. Once the values of [B] are known, the concentrations of MB, M, and BH^+ can be calculated. The change in fluorescence intensity of the bleomycin, ΔF, obeys the relationship

$$\Delta F = \Delta F_{max}[MB]/B_T \quad (12)$$

where ΔF_{max} is the maximum increase in fluorescence intensity and all other symbols have been defined previously.

The data in Figure 7 were fit to eq 11 and 12 using the Marquardt algorithm.[30] The best fit value of K_M obtained in this manner was $2.0 \pm 0.3 \times 10^{-5}$ M. The experiments and analysis were repeated for the B_2 congener. A value for K_M of $2.2 \pm 0.3 \times 10^{-5}$ M was obtained from the fluorescence data. Note the good agreement between these values for K_M and

those obtained from an analysis of proton-displacement experiments. This agreement serves as a check of the two methods confirming the consistency of the two approaches.

Conclusions

1. The binding of Ga(III) to the bleomycins (A_2 and B_2) displaces a single proton from the α-amino group of the diaminopropionamide moiety in the antibiotics. This evidence strongly implicates this portion of the molecule directly in metal binding.

2. The Ga(III) complex of bleomycin is fairly long lived. We have previously reported that the Zn(II) complex of bleomycin is also relatively long lived. In fact the kinetic parameters for the dissociation of the Zn(II) ion from the bleomycin complex are more similar to those parameters for Zn(II) containing metalloenzymes than to parameters obtained for Zn(II) complexes of low molecular weight ligands.[26] These observations together with NMR data for various lanthanide complexes of the bleomycins[36] suggest that these antibiotics form anomalously long-lived complexes with polyvalent metal ions.

Acknowledgments. We acknowledge the use of the Stanford Magnetic Resonance Laboratory located at Stanford University which is operated under the sponsorship of the National Science Foundation (Grant 23633) and Division of Research Resources, National Institutes of Health (Grant RR00711) and the use of the Purdue Biochemical Magnetic Resonance Laboratory located at Purdue University, West Lafayette, Ind., which is operated under the sponsorship of the National Institutes of Health (Grant RR01077). We are grateful to Dr. T. T. Sakai for his advice regarding the purification of the bleomycin congeners. We also thank Drs. N. R. Krishna and D. M. Chen for helpful advice and preliminary work on this project.

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(1) (a) This investigation was supported by Public Health Service Grants CA-13148 (J. R. Durant) and CA-24411 (J. D. Glickson) from the National Cancer Institute and Grant PDT-71B (R. E. Lenkinski) and Faculty Research Award FRA-162 (J. D. Glickson) from the American Cancer Society. (b) Department of Chemistry, Purdue University, West Lafayette, Ind. (c) Department of Biochemistry. (d) Department of Chemistry.
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