Properties of the Binding of Copper by Bleomycin

Daniel Solaiman, Eswara A. Rao, William Antholine, and David H. Petering

University of Wisconsin–Milwaukee

ABSTRACT

The glycopeptide, bleomycin, binds metal ions including Cu^{2+}. It is the copper complex of this material that is isolated from *Streptomyces verticillus*. Both free ligand and copper complex are excellent antitumor agents in animals. The biochemical and pharmacological relationship between these compounds has not been established. The present study begins an analysis of the chemistry and biochemistry of copper-bleomycin with structural and equilibrium properties of the complex. Potentiometric and fluorometric titrations of bleomycin confirm three acidic groups with pK_a values of 7.50, 4.93, and 2.72. The conjugate nitrogen bases of these groups, comprise three of the binding sites for Cu^{2+} according to similar titrations of copper–bleomycin. The fourth is a conjugate base of an acid with a very large pK_a that cannot be measured by these techniques. The participation of a fourth such group is inferred from both proton release studies of the binding of metal and ligand above pH 8 and from several studies of the thermodynamic stability of copper bleomycin. At low pH binding of copper to bleomycin occurs in two steps, as observed by several independent techniques which monitor either the metal or the ligand. Log stability constants for the reactions $Cu^{2+} + H_kBlm \rightleftharpoons CuH_{k-n}Blm + nH^+$ and $CuH_{k-n}Blm \rightleftharpoons CuH_{k-n-r}Blm + rH^+$ are 1.32 and −4.31, respectively, with n of 2.21 in the first equation and r of 2.07 in the second equation. The derived logarithm of the pH independent stability constant for copper bleomycin multiplied by the protonation constant for the unknown fourth ligand in the binding site is 12.16. This agrees closely with values obtained from measurements of conditional formation constants. One of the groups which binds in the second reaction is the substituted pyrimidine.

INTRODUCTION

Bleomycin constitutes a family of glycopeptides existing in *Streptomyces verticillus* as copper complexes, which are distinguished by a variable, positively charged R group (Figure 1) [1]. They have the unusual feature of containing one of the few naturally occurring copper complexes to have been isolated from microorganisms. In addition, the mixture of copper bleomycins as well as isolated components have strong anti-

Address reprint requests to: David H. Petering, Department of Chemistry and the Laboratory for Molecular Biomedical Research (Contribution 110), University of Wisconsin-Milwaukee, Milwaukee, WI 53201.

Journal of Inorganic Biochemistry 12, 201–220 (1980)

52 Vanderbilt Ave., New York, New York 10017

0162-0134/80/030201-20$02.25

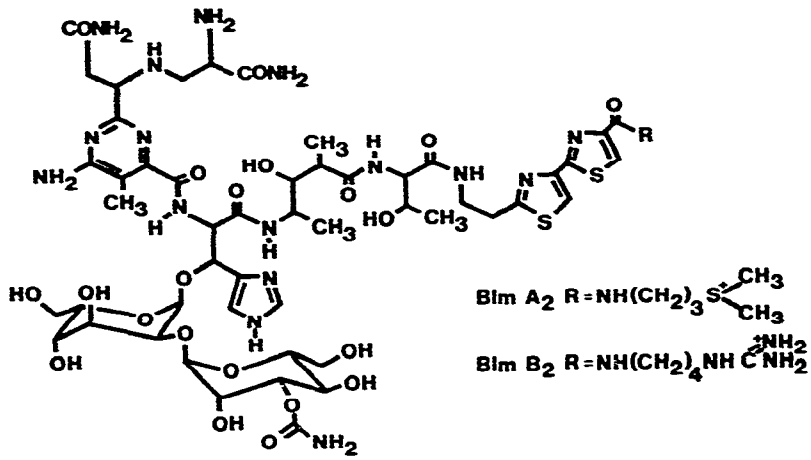


FIGURE 1. Structure of bleomycin with variable R group. The *N*-Bromosuccinimide cleaves the molecule at the dashed line.

tumor properties [2]. Metal-free bleomycin is now exclusively used in clinical cancer chemotherapy and little attention has been given to the relationship of copper bleomycin and the free ligand. Nevertheless, from studies of mono- and bis (thiosemicarbazones), there is precedent to hypothesize that bleomycin is activated in vivo through its binding to available copper or perhaps another metal such as iron [3-7]. Recently, Umezawa et al. have suggested that the copper complex does form as part of the reaction of the ligand with organisms [8]. Complicating this picture is the fact that copper ion inhibits the bleomycin-dependent scission of double stranded DNA [9]. This reaction may be involved in the mechanism of action of the uncomplexed drug. Radiolabeled metal complexes of bleomycin have been used as diagnostic scanning agents with little information available about the properties of the metal binding site [10]. The x-ray structure determination of a biosynthetic intermediate of bleomycin shows Cu^{2+} bound to five nitrogen atoms, which complete a square pyramidal coordination [11]. The in-plane nitrogens are: a secondary amine, a ring nitrogen from 4-amino pyrimidine, a peptide amide nitrogen, and an imidazole nitrogen (Figure 2). To provide a foundation for analyzing and understanding the results in these areas a study of the properties of the metal center in bleomycin has been initiated [12]. In this paper, binding properties of copper bleomycin are examined. Dabrowiak and coworkers have also begun such a study of metal bleomycins using complementary methods [13, 14].

EXPERIMENTAL

Materials

Blenoxane, the clinically used mixture of bleomycins, was supplied by Bristol Laboratories of Syracuse, New York. It is comprised principally of bleomycin-A_2, with bleomycin-B_2 as a second component, and appears to be the material described as bleomycin-A_2 in other investigations that depended on this source for the antibiotic (Figure 1). This material was further purified according to the procedure of Umezawa and coworkers [15]. The ligand, 3-ethoxy-2-oxobutyraldehyde bis (thiosemicarbazone) was a gift from Dr. Harold Petering, Department of Environmental Health, University

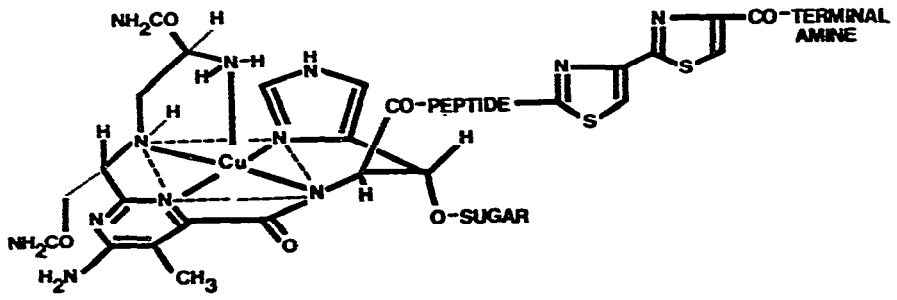


FIGURE 2. Proposed structure of Cu Bleomycin (Ref. 11).

of Cincinnati. Gold label EDTA was obtained from Aldrich Chemical Co. All other reagents used were reagent grade or highest purity available.

Definitions and Abbreviations. Generally, in the text bleomycin is used as a generic term for either blenoxane or bleomycin-A_2, both of which were used in this study. Tables and legends distinguish these as blenoxane (Blx) and bleomycin A_2. 3-Ethoxy-2-oxobutyraldealdehyde bis (thiosemicarbazone) is designated H_2KTS. Blm is the deprotonated form of the ligand which binds to Cu^{2+} above pH 3. H_kBlm is a protonated form of the ligand.

Methods

Electron Paramagnetic Resonance Spectroscopy. epr spectra were obtained on a Varian E-9 spectrometer at the NIH national Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, Wisconsin. Samples were frozen in liquid nitrogen in quartz tubes and inserted into a standard finger Dewar. The variation in the spectrum of CuBlm as a function of pH was done by titrating the complex at room temperature, removing samples, and freezing them at 77 K for spectral analysis at points along the titration. Peak heights of hyperfine lines in the $g_{\parallel}$ region were then taken to be proportional to the concentration of the species of copper which generated the particular spectrum.

Potentiometric Titrations and pH Measurements. A Radiometer TTT60 autotitrator equipped with a PHM 64 pH meter and ABU 12 autoburette was used to record continuous titrations of bleomycin and Cu-bleomycin. The titration vessel was jacketed so that constant temperature could be maintained. Some measurements were made manually with a Radiometer PHM-26 pH meter.

In the proton release experiments the titrator was used to set the initial pH of Cu^{2+} and bleomycin solutions and then to back titrate the final solution of Cu^{2+} plus bleomycin to the same pH. Fresh sodium hydroxide standardized with potassium hydrogen phthalate served as the titrant. The amount of base required in the back titration is then equivalent to the hydrogen ion released upon complexation. The volume of NaOH used was small compared with the volume of complex. For points above pH 4 where copper solutions would precipitate as $Cu(OH)_2$ only the ligand was initially adjusted to the starting pH. In the final calculation the equivalents of H^+ released equal the equivalents of OH^- added in the back titration minus the equiva-

lents of OH^- which would be necessary theoretically to adjust the pH of the initial Cu^{2+} solution to that of the initial bleomycin solution, in the absence of any reaction of base with the metal ion. These experiments were conducted in 0.1 M NaCl at 25°. At large hydrogen ion concentration, the readings of the pH meter were checked with standardized solutions of acid and found to agree within error.

To determine the proton dissociation constants of the imidazole and one of the amine groups of bleomycin, first derivative plots of potentiometric titration curves were made. The inflection point at which $\Delta pH/\Delta$ equivalents OH^- is 0 is taken to be the pK_a value of the group undergoing dissociation. The pK related to the substituted pyrimidine was measured after observing either the dependence of bleomycin fluorescence or uv absorbance upon pH. The data were fit to the equation

$$pK_a = pH + \log \frac{F - F_i}{F_f - F} \quad \text{or} \quad pK_a = pH + \log \frac{\Delta - \Delta_i}{\Delta_f - \Delta}, \tag{1}$$

where F_i and Δ_i, F_f and Δ_f, and F and Δ are initial, final, and intermediate relative fluorescence intensity, and difference absorbances, respectively.

There appear to be five constants of interest related to the five nitrogen atoms that can interact with the copper center according to the x-ray structure [11]. These are designated $K_{a1}, K_{a2}, K_{a3}, K_{a4}$, and K_{a5} for the five equilibria beginning with

$$H_5Blm^{5+} \overset{K_{a1}}{\rightleftharpoons} H_4Blm^{4+} + H^+. \tag{2}$$

Hence in the conditional formation constants for CuBlm considered below, all forms of bleomycin include H_5Blm, H_4Blm, H_3Blm, H_2Blm, HBlm, and Blm.

Instrumentation. Fluorescence excitation and emission spectra were recorded on an Amino Bowman spectrofluorimeter equipped for photon counting. Electronic spectra were taken on a Beckman Acta V or Cary 17 spectrophotometer having constant temperature cell holders.

N-*bromosuccinimide Cleavage of Bleomycin*. Cleavage of the peptide linkage adjacent to the imidazole shown in figure 1, followed a published method [16]. Chromatography of the products on Cm-Sephadex (C-25-120) separated the components as expected. One peptide contained only the pyrimidine chromaphore absorbing at 282 and 235 nm and fluorescing at 400 nm and another only the chromaphore corresponding to the dithiazole group, having a uv spectral maximum at 290 nm and a fluorescence emission band maximum at 355 nm.

Treatment of Data for Binding Constants. Figure 6 describes the pH dependence of the *d-d* band absorbance as bleomycin becomes associated with the metal. The process occurs in two steps in sufficiently different pH ranges so that they can be separately treated. The equilibria involved are analyzed as follows:

$$Cu^{2+} + H_kBlm \rightleftharpoons CuH_{k-n}Blm + nH^+, \tag{3}$$

$$CuH_{k-n}Blm \rightleftharpoons CuH_{k-n-r}Blm + rH^+. \tag{4}$$

To examine the reaction represented by equation 3, absorbance changes are recorded as a function of pH at a given wavelength. Thus, if A_i and A_f are initial and final absorbance at 650 nm in the titration and A is the intermediate absorbance at a given pH, then for equation 3

$$K_{CuH_{k-n}}{}^{Cu}Blm = \frac{[CuH_{k-n}Blm]\,[H^+]^n}{[Cu^{2+}]\,[H_kBlm]} = \frac{(A-A_i)[H^+]^n}{(A_f-A)^2[Cu_T{}^{2+}]}\,(A_f-A_i), \quad (5)$$

in which

$$[CuH_{k-n}Blm] = [(A-A_i)/(A_f-A_i)][Cu_T] \quad (6)$$

$$[Cu^{2+}] = [H_kBlm] = [(A_f-A)/(A_f-A_i)]\,[Cu_T]. \quad (7)$$

A plot of $\log (A-A_i)/(A_f-A)^2$ vs pH should yield a straight line for which the slope is n and the intercept is $\log K_{CuH_{k-n}Blm}{}^{Cu}[Cu_T{}^{2+}]/(A_f-A_i)$. This treatment implies that over the pH range examined, 0.2–1.2, other states of protonation of complex and ligand affecting the binding process do not exist.

As discussed below it is thought that the second binding step involves a ligand with a pK_a of 2.72. Thus, in the pH range in which binding occurs, pH 1.5–3.0, significant amounts of both acid and conjugate base may exist as indicated in the denominator of equation 8. It is assumed that this pK_a is not perturbed measurably in $CuH_{k-n}Blm$, for as described in the Results, the dipositive copper ion has displaced two protons in the first binding step. Hence the net charge on the ligand has not changed. The equilibrium constant for equation 4, $K_{CuH_{k-n-r}Blm}{}^{CuH_{k-n}Blm}$, can be calculated from absorbance vs pH data as follows: a conditional formation constant, K_1, can be defined at any pH

$$K_1 = \frac{[CuH_{k-n-r}Blm]}{[CuH_{k-n}Blm] + [CuH_{k-n-1}Blm]} = \frac{A-A_i}{A_f-A}. \quad (8)$$

The ratio of concentrations of all protonated and unprotonated forms of product and reactants is equal to the ratio of absorbance terms, where A is the absorbance at a given pH and A_i and A_f are initial and final absorbances at the beginning and endpoint of the titration, then

$$\log\left(\frac{A-A_i}{A_f-A}\right)(1+10^{-2.72}/[H^+]) = r\,pH + \log K^{CuH_{k-n}Blm}_{CuH_{k-n-r}Blm}. \quad (9)$$

A plot of $\log (A-A_i/A_f-A)(1+10^{-2.72}/[H^+])$ vs pH will yield values of r and $\log K_{CuH_{k-n-r}Blm}{}^{CuH_{k-n}Blm}$. A completely analogous treatment of fluorescence data has been used to calculate $\log K_{CuH_{k-n-r}Blm}{}^{CuH_{k-n}Blm}$. Fluorescence intensities of copper bleomycin, gathered over the same pH range, directly substitute for absorbances in equation 9.

The conditional stability constant for copper bleomycin in the pH range where $CuH_{k-n}Blm$ is formed was measured by the direct spectrophotometric titration of bleomycin with Cu^{2+} at pH 1.23. In this pH dependent equilibrium

$$Cu^{2+} + \text{bleomycin}_{\text{all forms}} \overset{K_2}{\rightleftharpoons} \text{Cu bleomycin}_{\text{all forms}}. \quad (10)$$

all forms of each molecule, protonated and unprotonated, are summed together to give an effective total concentration. Figure 10 is plotted according to the equation,

$$1/Y = 1/K_2 \cdot 1/[Cu^{2+}] + 1, \tag{11}$$

in which

$$Y = [\text{Cu bleomycin}_{\text{all forms}}]/\{[\text{bleomycin}_{\text{all forms}}] + [\text{Cu bleomycin}]\}_{\text{all forms}}. \tag{12}$$

To obtain Y and the free copper concentration, $[Cu^{2+}]$,

$$Y = (A - A_i)/(A_f - A_i), \tag{13}$$

$$[Cu^{2+}] = C_{T,Cu} - YC_{T,\text{Bleomycin}}, \tag{14}$$

in which the absorbances are defined as in equation 5, $C_{T,\text{Bleomycin}}$ is the total concentration of bleomycin in solution, and $C_{T,Cu}$ is the total concentration of copper added to the system at any point in the titration.

To measure K_3, conditional stability constant for copper bleomycin at hydrogen ion concentrations at which proton does not compete well with Cu^{2+} for the ligand, equilibria were established between the complex and the competing ligands for copper, H_2KTS, or EDTA. These ligands were chosen because of their high affinity for Cu^{2+} which can be modulated by pH and because their conditional stability constants are known. Further, CuKTS has a charge transfer band at 469 nm that can be used for quantitation of this complex [17]. Similarly, CuEDTA does not fluoresce, so that the increase in bleomycin fluorescence upon liberation from Cu^{2+} can be used to follow the equilibration process. Considering the general competitive reaction, in

$$\text{CuBlm} + \text{L} \rightleftharpoons^{K_4} \text{CuL} + \text{Bleomycin}_{\text{all forms}}, \tag{15}$$

which L represents all forms of the competing ligand, it follows that

$$K_4 = K_5/K_3, \tag{16}$$

where K_5 is the conditional stability constant for CuL. For the determination of K_4 and calculation of K_3 using H_2KTS in the pH range indicated in Table 1, its copper complex exists as the sum of two forms $\{[CuKTS] + [CuKTSH^+]\}$. The sum can be spectrophotometrically determined. Furthermore, $[H_2KTS] = C_{T,H\,2KTS} - \{[CuKTS] + [CuKTSH^+]\}$; $[CuBlm] = C_{T,CuBlm} - \{[CuKTS] + [CuKTSH^+]\}$ and [Bleomycin all forms] = $[CuKTS] + [CuKTSH^+]\}$. As above, C_T denotes the total concentration of a given compound placed in solution at the beginning of the reaction. According to previous work, $K_5 = (2.1\ \text{pH} + 2.85)(1 + [H^+]/K_a)$, in which K_a is the acid dissociation constant of $CuKTSH^+$ and is equal to $10^{-2.75}$ [17, 18].

When EDTA was used as the competing ligand, K_4 for equation 15 was determined after monitoring fluorescent changes of the CuBlm solution upon reaction with EDTA. If F_i, F_f, and F represent the fluorescent intensities of free bleomycin, of the same concentration of copper complex, and of the equilibrium state of equation 15, respec-

tively, then the following expressions hold:

$$[\text{Bleomycin}_{\text{all forms}}] = \frac{F - F_f}{F_i - F_f} C_{T,\text{Bleomycin}} = YC_{T,\text{Bleomycin}}, \quad (17)$$

in which Y is defined as in equation 12, except the numerator becomes [bleomycin all forms].

$$[\text{Cu EDTA}] = YC_{T,\text{Bleomycin}} \quad (18)$$

$$[\text{CuBlm}] = (1 - Y)C_{T,\text{Blm}} \quad (19)$$

$$[\text{EDTA}_{\text{all forms}}] = C_{T,\text{EDTA}} - [\text{Cu EDTA}]. \quad (20)$$

Since C_T for each compound and Y are known, K_4 can be determined. The conditional stability constant for Cu EDTA is obtained from the following known constants for EDTA: $\log_{\text{CuEDTA}}{}^{\text{Cu}}$ 18.80 and the pK_a values 10.26, 6.16, 2.77, 2.21 [19]. Over the pH range utilized in Table 1 the complex exists as Cu EDTA^{2-}. Finally, equation 17 is solved for K_3.

The pH independent formation constant

$$K^{\text{Cu}}_{\text{CuBlm}} = [\text{CuBlm}]/[\text{Cu}^{2+}][\text{Blm}] \quad (21)$$

is related to the conditional constants, K_3, determined above pH 3 through a set of equations.

$$K_3 = [\text{CuBlm}]/[\text{Cu}^{2+}]\{[\text{Blm}] + [\text{HBlm}] + [\text{H}_2\text{Blm}] + \cdots + [\text{H}_k\text{Blm}]\} \quad (22)$$

$$= K^{\text{Cu}}_{\text{CuBlm}}\{(1 + [\text{H}^+]/K_{a,k} + [\text{H}^+]^2/K_{a,k}K_{a,k-1} + \cdots + [\text{H}^+]^k/K_{a,k}K_{a,k-1}\cdots K_{a1})\}^{-1}, \quad (23)$$

in which k represents the number of binding sites with protons dissociating upon reaction with Cu^{2+}. All experiments were carried out in 0.1 M NaCl at 25°. Care was taken to ensure that equilibrium had been attained in all reactions studied.

RESULTS

Acid-Base Chemistry of Bleomycin

Potentiometric titrations of bleomycin were run at 25.0° and 0.10 M NaCl. Two pK_a values obtained from the calculated first derivatives of three such titrations are 4.93 ± 0.08 and 7.50 ± 0.05. These have been assigned by others to the imidazole and one of the amine groups of bleomycin, respectively [20, 21]. Titration of CuBlm reveals no titratable groups over the pH range 3–11. Assuming that complex formation has not shifted these pK_a values outside of this range, these data are consistent with the strong binding of amine and imidazole to the copper.

An examination of the pH dependence of the fluorescence emission of bleomycin is consistent with pK_a of 2.72 [Figure 3(a)]. The emission intensities in Figure 3(a) were generated with excitation at 250 nm. This band, not previously described, principally excites the pyrimidine group with only a small contribution to dithiazole

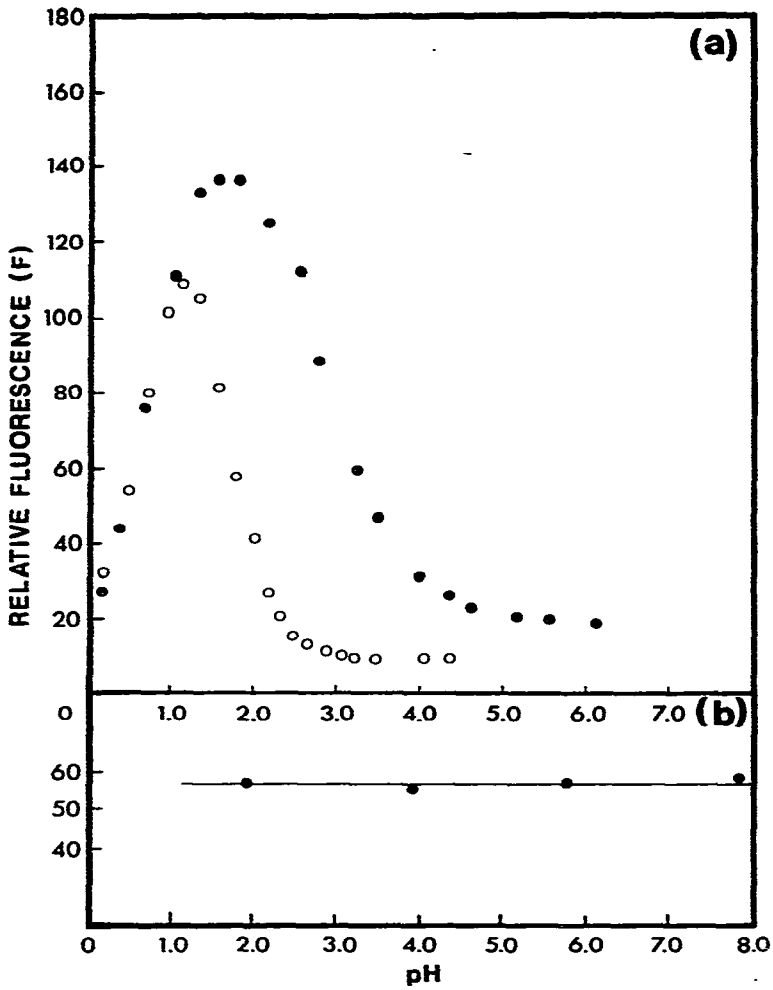


FIGURE 3. Relative fluorescent intensities of $H_kBlm\text{-}A_2$, $CuBlm\text{-}A_2$, and the dithiazole fragment vs pH. Conditions: 0.10 M NaCl and (25 ± 1)°. (a) (•) $Blm\text{-}A_2$ and (○) $CuBlm\text{-}A_2$ are 3.50×10^{-5} M. (b) The dithiazole fragment is 6.5×10^{-5} M. Different machine settings used in (a)—upper panel and (b)—lower panel. Using equation 1, the least linear regression of the bleomycin data for pH 4.36-2.78 is $2.73 = 0.93 + \log F - F_i/F_f - F$ (cc. = 0.99).

fluorescence. This result was established by examining the *N*-bromosuccinimide cleavage products of bleomycin, which can be separated into fragments containing only the dithiazole or pyrimidine fluorophares. As seen in figure 3(b), the fluorescence of the dithiazole fragment has no pH dependence. Similarly, excitation of bleomycin at 320 nm, where both groups contribute to the intensity of fluorescence, gives the same behavior as seen in Figure 3(a). Thus changes observed in the whole peptide at low pH are attributable to the acid base chemistry related to the pyrimidine group. There is also a small enhancement of fluorescence in the region of pH 7.5, near an amine pK_a. However, no changes are observed as the imidazole is protonated. No interpretation is given for this effect.

That the pK_a at 2.72 is due to pyrimidine related acid-base chemistry is further supported by the changes in uv absorbance at 237 and 252 nm of the ligand as a

function of pH shown in Figure 4. Ultraviolet spectral changes associated with two processes are observed as the pH is reduced below three. The first centered at pH 272, is accompanied by isosbestic points at 252 and 280 nm. The second, which commences below pH 1.2, is also characterized by an isosbestic point at 237 nm. These changes are also observed in the fluorescence emission spectrum of the substituted pyrimidine group (Figure 3).

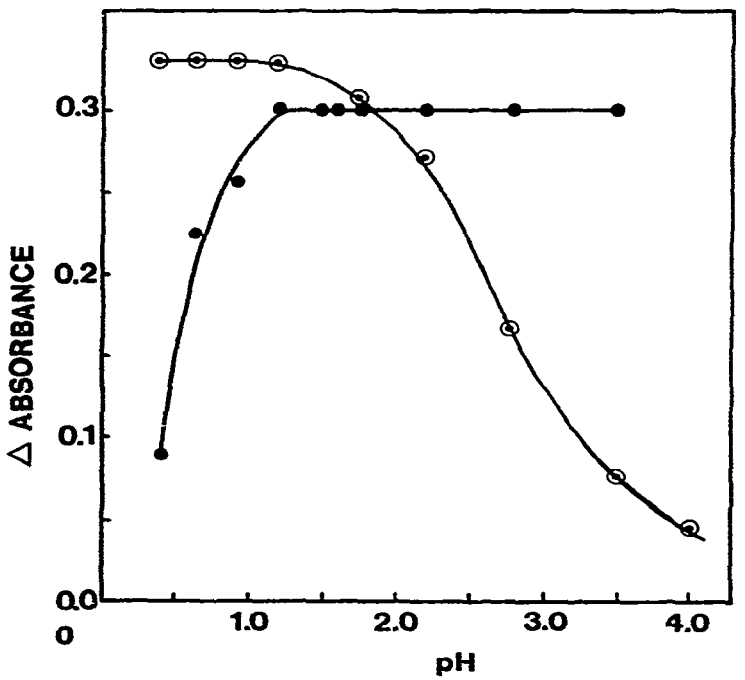
Mode of Ligation of Copper by Bleomycin

Figure 2 shows a proposed structure of the metal center of copper bleomycin [11]. Solution chemistry supporting aspects of this structure is described here. As stated above, the potentiometric titration of CuBlm provides strong evidence that an amine and imidazole are part of the copper binding site.

These assignments are extended by a study of the protons released upon complex formation as a function of pH (Figure 5). It is noted that approximately one proton is released upon complexation at pH values well above the amine pK_a. Along with the experimental data is the calculated line assuming the presence of a nitrogen with a very large pK_a, such as an amide at the binding site as well as an amine and imidazole.

Several properties of the copper complex vary with pH and thereby provide insight into the binding process. Figure 6 shows the pH dependence of the *d-d* band of copper as the complex is progressively dissociated. Three forms of copper can be detected here: fully ligated CuBlm, an intermediate form, and aquated Cu^{2+}. The first is charac-

FIGURE 4. pH Dependence of uv spectra for blenoxane. Conditions: 0.1 mM Blx, 0.1 M NaCl at 25°C. Absorbance changes at 252 nm (•); at 237 nm (⊙). Using equation 1, the linear regression data at 237 nm is $2.72 = 0.97\ pH + \log(\Delta - \Delta_i/\Delta_f - \Delta)$, in which $\Delta_i = 0.029$ and $\Delta_f = 0.329$ (cc. = 0.99).



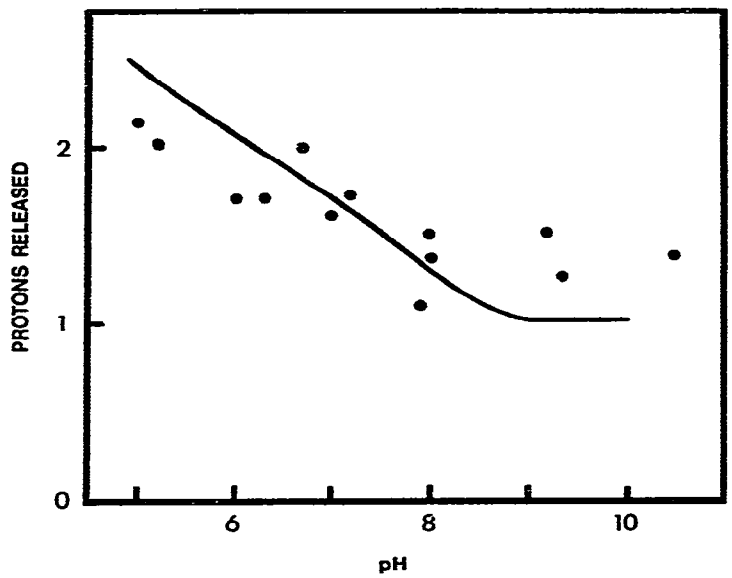


FIGURE 5. Protons released upon complexation of blenoxane with Cu^{2+} as a function of pH. Conditions: Equal concentrations of metal and ligand, 1 mM metal and ligand, 0.10 M NaCl, and 25.0°. The solid line is calculated using assumptions listed in the text.

terized by a *d-d* band maximum at 605 nm with an extinction coefficient of 130 M^{-1} cm^{-1}. Accompanied by an isosbestic point at 655 nm, the spectrum of this species is converted to a second form over the pH range 3-1.6. The absorbance maximum of this intermediate occurs at lower energy, 658 nm, with an extinction coefficient of 100 M^{-1} cm^{-1}. The *d-d* band shift indicates a weakening of the ligand field, presumably as the chelate becomes partially dissociated. In a second step between pH 1.6 and 0.3, this form undergoes complete dissociation to metal and ligand. Between pH 3 and 6 there are small changes in *d-d* band width, and band maximum, the nature of which have not been defined. Above pH 6, progressive changes in band position and intensity occur which would seem consistent with the formation of CuBlm-OH or possible axial ligation.

The electronic state of copper in copper bleomycin has also been examined by epr spectroscopy. The X-band spectrum of CuBlm has been published and its $g_\parallel$ and $A_\parallel$ values identified with those of copper complexes containing 3-4 in plane nitrogen atoms [13]. When the $g_\perp$ and $g_\parallel$ were separated with Q band, it was evident that the copper ion exists in an axial magnetic environment in which $g_x \cong g_y \cong 2.07$ and $g_z = 2.21 \pm 0.02$, with $A_z = 180$ *G*. No superhyperfine structure was observed in the $g_\parallel$ hyperfine lines when isotopically pure 63 Cu (I = 3/2) was used. However, second derivative scans in the $g_\perp$ region of the X-band spectrum were made, and evidence of splittings seen, also indicating that nitrogen ligands (I = 1) are bound to the copper.

The pH-dependent stepwise dissociation of CuBlm is observed in the epr spectrum of the complex (Figure 7). As the pH is decreased 3 different sets of hyperfine splittings in the $g_\parallel$ region of the spectrum can be detected: the first for native complex, an intermediate one having $g_\parallel = 2.25$ and $A_\parallel = 170$ G, consistent with copper bound to 2 nitrogens and two oxygens such as water, and the third for Cu^{2+} itself, with $g_\parallel = 2.33$ and $A_\parallel = 140$ G. Since these spectra are taken at 77 K and there may be

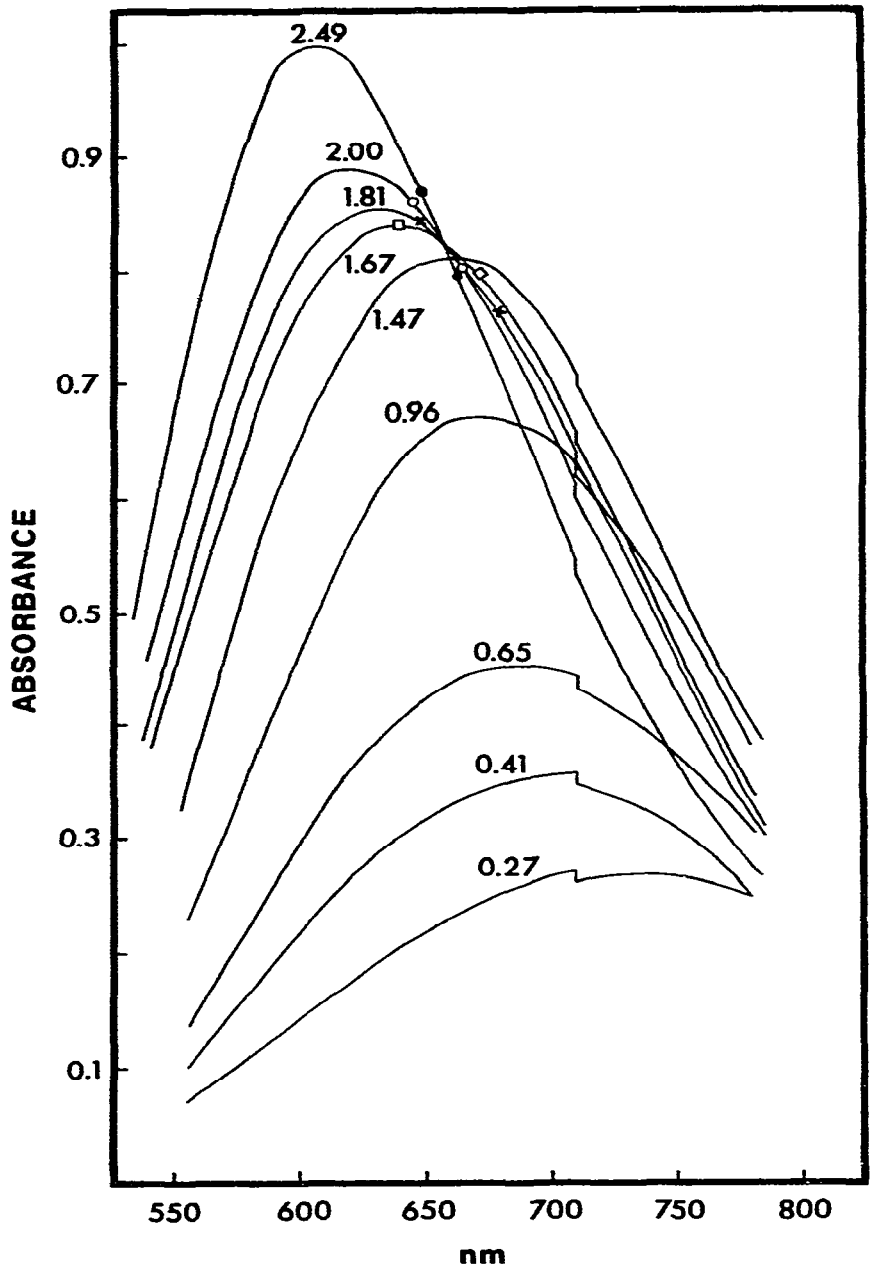


FIGURE 6. *d-d* band spectra of Cu^{2+} plus blenoxane as a function of pH. Spectra labeled with pH values. Initial CuBlm concentration is 5.7 mM in 0.10 M NaCl at 25.0°. Symbols are a guide in the region of the isosbestic point.

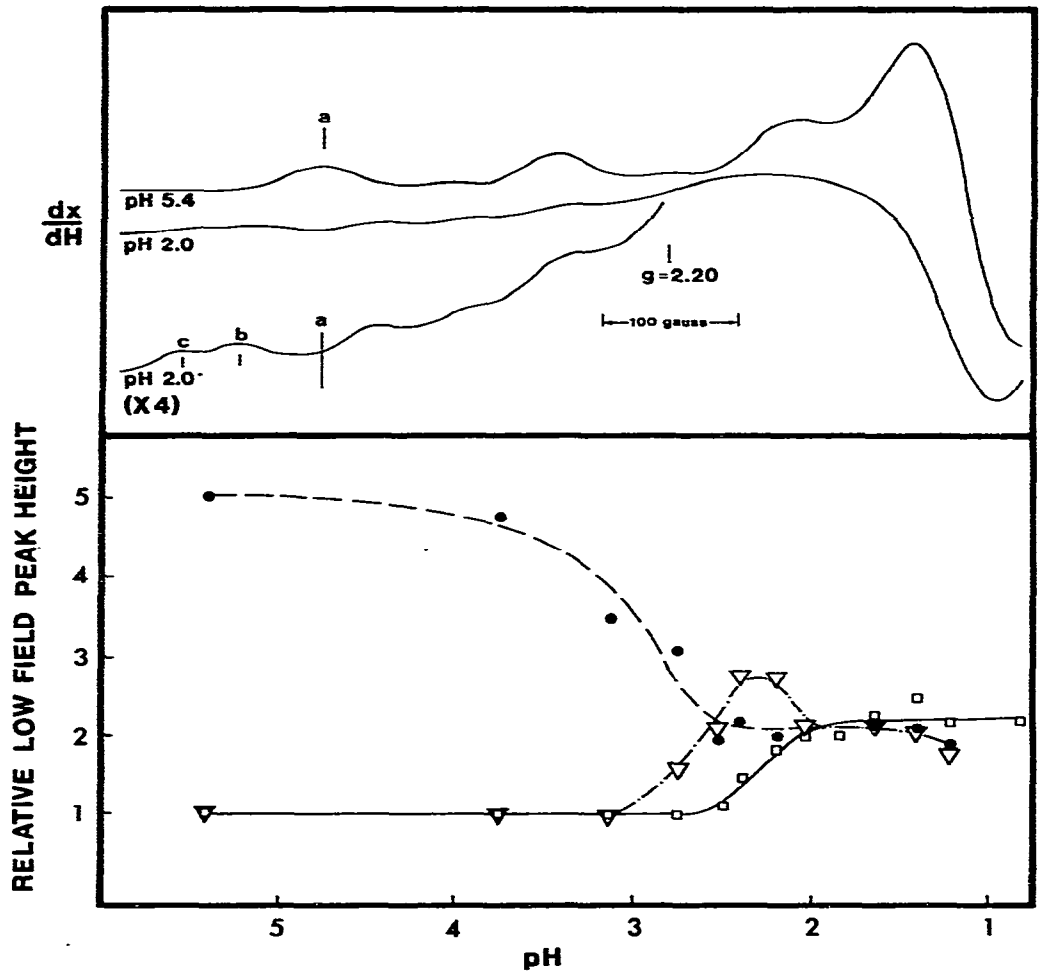


FIGURE 7. epr spectra of Cu blenoxane as a function of pH. Upper panel: Sample spectra of 0.8 mM CuBlm taken at −196°C. Spectrometer settings: incident power 5 mW, mod 5 G, scan 8 min, time const 1 sec, gain 5 X 10^2. Lower panel: Peak height changes in $g_{\parallel}$ vs pH. (a) from upper panel is (●), (b) is (▽), and (c) is (□).

shifts in pH dependent equilibria in the transition from 25° to these low-temperature frozen solutions, it is the presence of these forms, not the details of the conditions of their appearance, that is significant.

Another technique which monitors changes in the bleomycin ligand also reveals this stepwise dissociation of the complex. As changes in the ultraviolet region of the absorbance spectrum, due mainly to the pyrimidine and dithiazole groups, are monitored at low pH, two isosbestic points are seen at 240 and 250 nm. As seen in figure 8, the absorbance change at 240 nm corresponds to the first binding reaction and the change at 250 nm to the second. Other changes in absorbance at 290 and 325 nm also correlate with the second binding step of equation 4.

Finally, the relative intensity of fluorescent emission due to excitation of the pyrimdine group at 245 nm has been plotted as a function of pH in figure 3(a). It can be

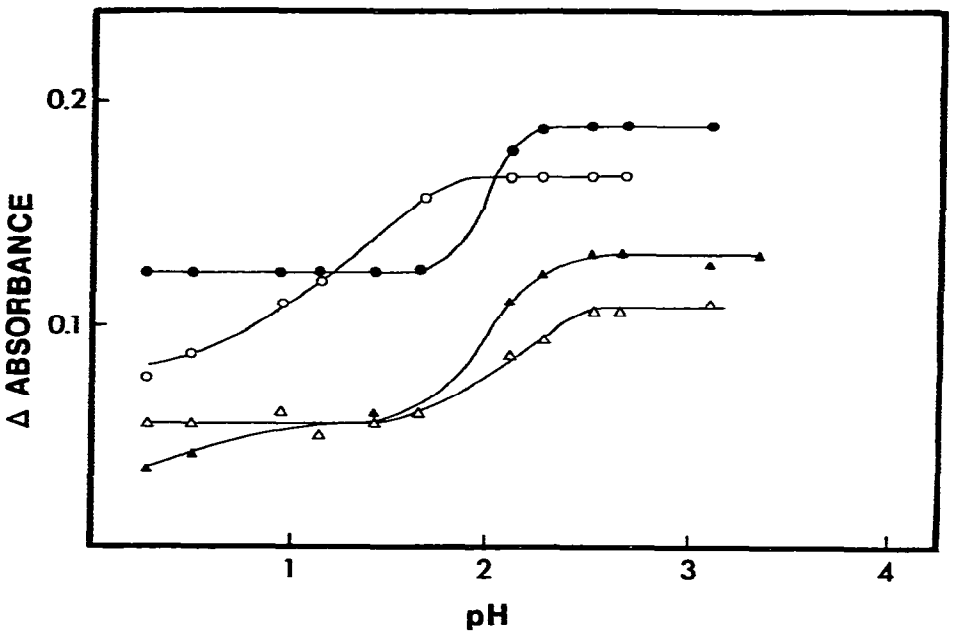


FIGURE 8. pH dependence of uv absorbance spectrum of Cu blenoxane. Conditions 25.0 and 0.10 M NaCl. (•) Absorbance changes at 250 nm; (○) 240 nm; (▲) 290 nm; (△) 325 nm. Isosbestic points occur between pH 0.28 and 1.62 at 250 nm and between pH 2.05 and 2.64 at 240 nm. 0.03 mM CuBlx was titrated in 0.10 M NaCl at 25.0°.

seen that formation of the complex quenches pyrimidine fluorescence. The enhancement of pyrimidine fluorescence begins about pH 3. This corresponds to the shift from fully to partially ligated CuBlm. The intensity profile then approaches that for free bleomycin at even lower pH values were complete dissociation is occurring. These data suggest that a pyrimidine nitrogen is initially present in the binding site and is released in the first pH dependent dissociation step. It will be noted that the maximum fluorescence intensity at about pH 1.2 is less than that for the free ligand, so that some quenching of pyrimidine by copper occurs even when this ligand is not directly bound to the metal.

Stability Constants for Copper-Bleomycin

Spectral data such as shown in figure 6 have been analyzed in terms of two equilibria (equations 3 and 4). Using equations 5 and 9 developed in the Experimental Section, the data are plotted in figures 9 and 10. The linear regression lines describing these data are

$$\log (A - A_i)/(A_f - A)^2 = 2.21\ \text{pH} - 0.659$$

[Correlation coefficient (cc) 0.99] for equation 5, in which $\log K_{\text{CuH}_k - n\text{Blm}}{}^{\text{Cu}} = 1.32$, and

$$\log (A - A_i)/(A_f - A)(1 + 10^{-2.72}[H^+]) = 2.07\ \text{pH} - 4.31$$

(correlation coefficient 0.99) for equation 9 with $\log K_{\text{CuH}_{k-n-r}\text{Blm}}{}^{\text{CuH}_k - n\text{Blm}} = -4.31$. In each reaction, 2 protons are released in the binding step. Both blenoxane and bleomysin-A_2 are used to generate the data for figure 9.

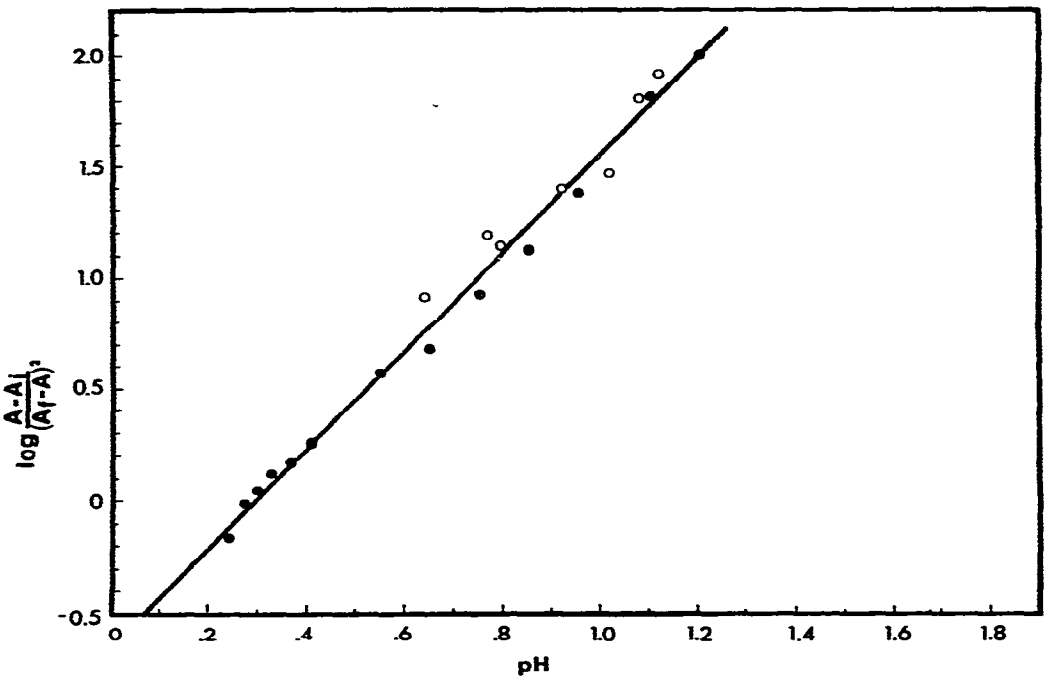


FIGURE 9. Spectrophotometric pH titration of copper bleomycin: the determination of $K_{CuH_k-_nBlm}^{Cu}$. (•) Cu blenoxane, (○) Cu bleomycin A_2. Titrations were carried out with 5.7 mM CuBlx and CuBlm-A_2 in 0.10 M NaCl at 25.0°. Solid line is linear regression cited in text.

The validity of these results is supported by two other sets of data. The conditional stability constant, K_2, describing the equilibium state of reaction 10 was measured using equation 11 to treat the data from the spectrophotometric titration of bleomycin with Cu^{2+} at pH 1.23. The linear regression (cc = 0.99) fitting the experimental points shown in figure 11 is $1/Y = 8.06 \times 10^{-5}/[Cu^{2+}] + 0.999$. Therefore, K_2 is 1.24×10^4 M^{-1}. This agrees closely with the value for K_2 of 1.1×10^4 calculated by inserting the hydrogen ion concentration corresponding to pH 1.23 into the equation $K_2 = K_{CuH_k-_nBlm}^{Cu}/[H^+]^{2.21}$ based on the reaction shown in equation 3 and calculated according to equation 5.

Secondly, the fluorescence changes of copper-bleomycin-A_2 between 3 and 1.2 shown in figure 3 have been used to calculate $K_{CuH_k-_n-_rBlm}^{CuH_k-_nBlm}$. Figure 10 contains a plot of the data. The linear regression describing the results is

$$\log \frac{F-F_i}{F_f-F}(1 + 10^{-2.72}/[H^+]) = 2.28\ \text{pH} - 4.18\ (\text{cc} = 0.99),$$

in which $\log K_{CuH_k-_n-_r}^{CuH_k-_nBlm}$ is −4.18. Thus, two methods utilizing blenoxane and bleomycin-A_2, one monitoring the copper center and the other the pyrimidine residue, give similar results.

Of interest are the conditional stability constants K_3, as a function of pH at hydrogen ion concentrations at which copper is completely ligated by bleomycin. To obtain these constants, CuBlm was reacted with competing ligands, 3-ethoxy-2-oxobutyraldehyde bis (thiosemicarbazone) or EDTA, which have large affinities for copper. Table 1

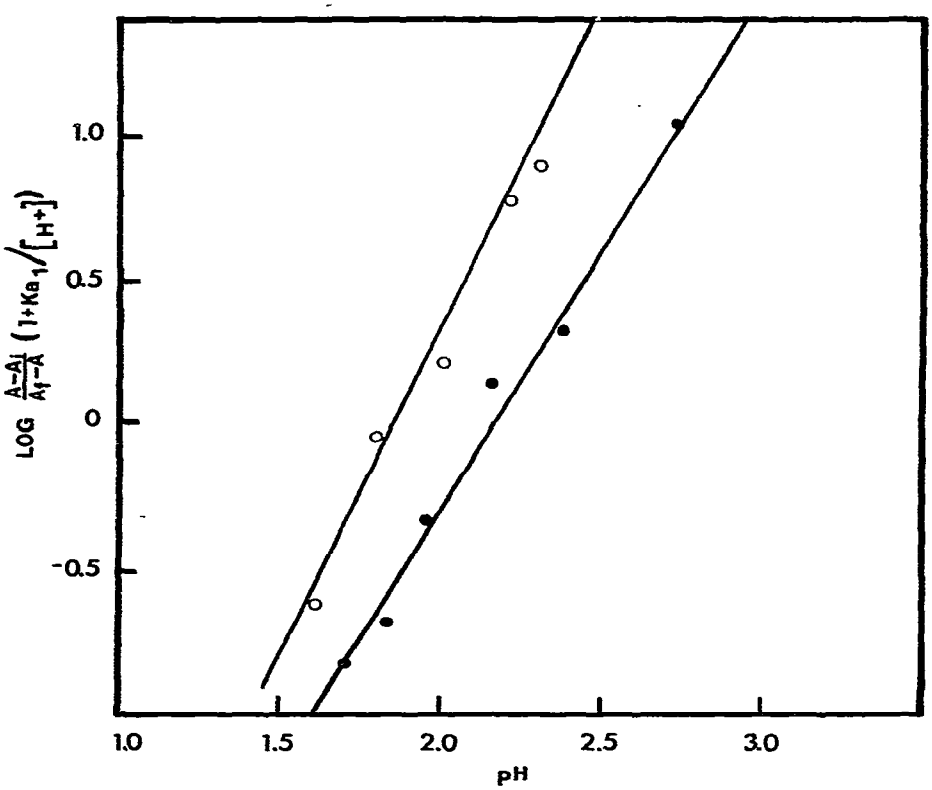


FIGURE 10. Spectrophotometric pH titration of copper bleomycin: the determination of $K_{CuH_{k-n-r}Blm}{}^{CuH_{k-n}Blm}$. (•) Cu blenoxane, conditions: 11.5 mM CuBlm in 0.10 M NaCl at 25.0°. (○) Fluorimetric titration of Cu bleomycin A_2 in which $\log(F - F_i)/(F_f - F)(1 + 10^{-2.72}/[H])$ replaces the ordinate in the figure. Data taken from figure 3. Solid lines are linear regressions cited in text.

summarizes the data for the corresponding equilibrium constants that can be derived from equations 16 and 17. All of the data points give rise to closely similar values for $\log K_{a,k}K_{CuBlm}{}^{Cu}$ obtained from equation 23.

This calculation assumes that the fully protonated binding site contains four or five protons, including one with a very large pK_a. A similar calculation to determine $\log K_{CuBlm}{}^{Cu}$ in which only 3 or 4 protonated ligands are included, all with pK_as less than or equal to 7.50, leads to widely divergent values for the stability constant and thus cannot be correct. Hence, these findings corroborate the data of Figure 5 that a group such as an amide nitrogen with a very weakly dissociable hydrogen ion is involved in the binding site.

DISCUSSION

Although the history of investigation of the biological and biochemical effects of bleomycin contains a number of references to the metal binding capacity of the molecule, systematic studies of this aspect of bleomycin have only recently begun. The publication of the conclusions of an x-ray crystallographic study of the complex of a biosynthetic intermediate of bleomycin shown in figure 2 shows the binding site to be square

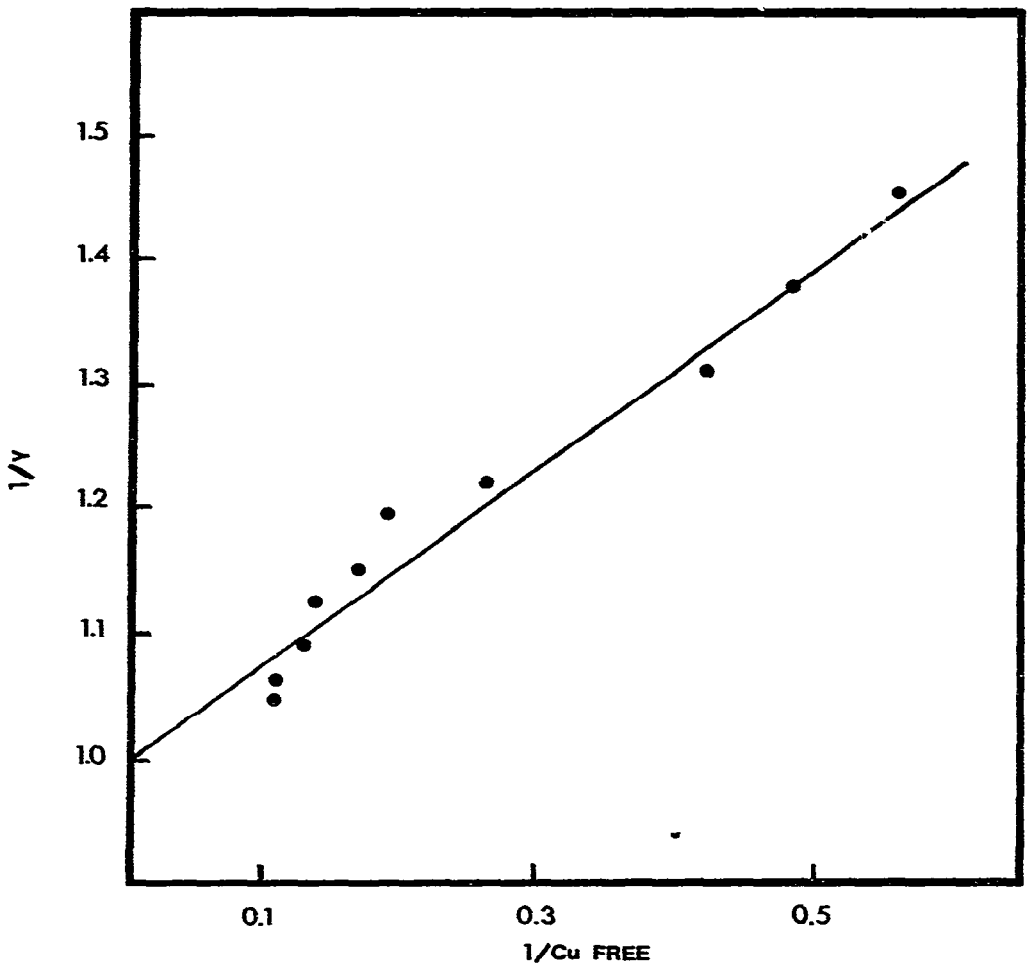


FIGURE 11. Titration of blenoxane with Cu^{2+} at pH 1.23. Conditions: 5.7 mM bleomycin in 0.10 M NaCl at 25.0°. Solid line is linear regression cited in text.

pyramidal and makes it clear that the in-plane coordination sites are nitrogen atoms from the secondary amine, pyrimidine, amide linkage, and imidazole groups of bleomycin [11]. Also, axially bound to copper is the primary amine of the structure.

Is this structure consistent with the solution data for copper bleomycin? In a preliminary report Solaiman et al. indicated on the basis of electron paramagnetic resonance studies that CuBlm appeared to contain a typical axial binding site for copper [12]. Independently, based on similar epr results, Dabrowiak and coworkers concluded that CuBlm has four nitrogen atoms as ligands for the metal [13]. In this identification they make use of the model studies of Peisach and Blumberg [22, 23]. Unfortunately, this work, in which g and A values of epr spectra of various copper complexes were correlated with the nature of the ligating atoms, did not examine 5-coordinate complexes, as exists with the Cu bleomycin derivative shown in figure 2. Dabrowiak et al. also concluded that the pyrimidine was part of the coordination sphere after observing changes in the ultraviolet absorbance spectrum of the ligand upon complexa-

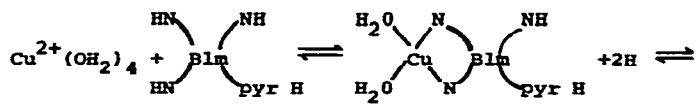
TABLE 1. Conditional Stability Constant for Cu-Blm[a]

pH	K_4	log K_3	log $K_{ak}K_{CuBlm}{}^{Cu}$[b]	log $K_{CuBlm}{}^{Cu}$[c]
2.94[d]	20.5	7.93	11.75	14.13
3.87[d]	0.159	10.81	11.63	15.75
4.48[d]	0.495	12.57	11.56	16.17
5.93[d]	0.396	15.71	11.35	17.32
2.94[e]	1.09	8.00	11.82	14.20
3.71[e]	0.60	10.02	11.34	15.09
3.93[e]	0.15	11.06	11.72	15.69
5.02[e]	0.0295	13.95	11.67	16.67

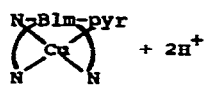
[a] Conditions: 0.10 M NaCl and 25.0°.
[b] $k = 4$ with pK_a values 2.72, 4.93, 7.50, and pK_{a4} or $k = 5$ with pK_a values <1, 2.72, 4.93, 7.50, and pK_{a5} give identical results.
[c] Calculation as in *b* with $k = 3$ or 4 and no indeterminately large pK_a ≥ 7.50.
[d] Range of initial concentrations: Cu bleomycin-A_2, 0.15 mM; H_2KTS, 0.28–0.74 mM.
[e] Initial conditions: Cu blenoxane, 0.064 mM; EDTA, 0.57 mM.

tion with copper [13]. Further, these researchers showed that pyrimidine and imidazole protons underwent marked chemical shifts upon interaction of zinc and bleomycin, whereas the dithiazole moiety was relatively unaffected. With the assumption that the zinc and copper binding sites are identical, pyrimidine and imidazole groups appear to be involved in copper complexation.

Although the results of this work provide information about the mode of ligation of copper to bleomycin, the focus of this paper is on the equilibrium properties of copper bleomycin. The pH-dependent formation of the complex serves as a useful point of organization for this discussion. As presently envisioned, two equilibria occur:



I II



III

Results summarized in figures 9 and 10 indicate that four protons are released during this process. These originate from four groups shown in figure 2. Two of these have pK_a values of 7.50 and 4.93 according to potentiometric studies and can reasonably be identified as amine and imidazole groups. A third has a pK_a of 2.72 and is defined as the pyrimidine moiety as discussed below. A fourth group with an indeterminantly large pK_a is also bound according to proton-release data. This may be the in-plane amide nitrogen shown in figure 2.

In this analysis it is striking that the complex forms in two discrete steps instead of by a concerted chelation process. Thus, a bidentate chelate complex has significant stability over the pH range 0.3–1.6. For example, at pH 1.23 the conditional formation constant is 1.2×10^4 M^{-1}. If the amine and imidazole ligands are chosen as the

pair binding in this pH region and the conditional formation constant for a model complex containing the same ligands, CuH histidine, is calculated, one obtains a number 2 orders of magnitude smaller (24). Hence, even though the histidine ligands are ideally suited to form a five-membered chelate ring, whereas the same groups in bleomycin have more degrees of rotational freedom, the CuBlm structure is more stable. Further studies on the effect of the rest of the structure upon chelation will be necessary to understand this finding.

Of the four groups binding in these two steps, only the binding of pyrimidine in the conversion of II to III can be specified. This is shown in the quenching of pyrimidine fluorescence as Cu bleomycin is titrated in the second step between pH 1.2 and 3. This conclusion is also consistent with changes in uv absorbance versus pH. The spectrum of CuBlm has a shoulder at about 325 nm attributed to a $d\pi \rightarrow \pi^*$ interaction of the metal ion with pyrimidine [13]. It is seen in figure 8 that this feature of the spectrum only appears in the pH region in which species II is converted to species III and the pyrimidine group binds to the copper center according to figure 3. It should be noted that the use of changes in the bleomycin absorption spectrum in the 230–250 nm range upon metal complexation to argue that pyrimidine is bound to copper or zinc, as has been done previously, must be done carefully [13]. Substantial changes occur in the low pH range, 0.3–1.6, as species II forms and before pyrimidine binds to copper.

The calculations of $K_{\mathrm{CuBlm}}{}^{\mathrm{Cu}}K_{a,k}$ in Table 1 from conditional formation constant data gathered between pH 3 and 6 all lead to closely similar numbers when a minimal value of $k = 4$ dissociable protons is used in equation 23. The results do not change if $k = 5$, in which axial ligation by the primary amine is also included and a fourth pK_a of less than 1 as defined by Takita et al. is entered into equation 23 [25]. This is because in the pH range considered the acid with this pK_a must be deprotonated and therefore does not affect the functional dependence of K_3 upon hydrogen ion concentration.

The average value of $\log K_{\mathrm{CuBlm}}{}^{\mathrm{Cu}}K_{a,k}$ of 11.60 obtained from conditional formation constant data can be compared with the same constant calculated as $\log K_{\mathrm{CuH}_k-n\mathrm{Blm}}{}^{\mathrm{Cu}}K_{\mathrm{CuH}_k-n-r\mathrm{Blm}}{}^{\mathrm{CuH}_k-n\mathrm{Blm}}K_{a1}K_{a2}K_{a3}K_{a4}$ the value derived is 12.16. It is seen that there is satisfactory agreement, particularly considering the diverse measurements which go into the determination of each number.

In the measurement of these equilibria constants, blenoxane and bleomycin-A_2 were used, yielding comparable results. This is expected since the principal components of bleomycin from Bristol Co., the A_2 and B_2 derivatives, are quite similar in structure (Figure 1). They differ only in the R group which, however, in each case contributes one positive charge to the molecule over the pH range investigated. There is an electrostatic effect on the metal binding process from the R group, but it is similar for the mixture and bleomycin-A. Since virtually all of the clinical work on bleomycin has used the mixture, the studies on the A_2 component apply to the mixture as well.

Some comment must now be directed to unresolved questions about the bleomycin and copper-bleomycin structures. Is there axial ligation in solution? This study provides no positive evidence for this and is entirely consistent with a basically square planar chelation of copper by bleomycin. Thus, in the various copper-binding studies only a four proton-donating ligands can be observed. It may be, as Umezawa and coworkers suggest in a recent report, that one of the ligands has such a small pK_a that it

is deprotonated in the pH range of present studies [25]. Hence, a square pyramidal structure could be consistent with the results. However, the data summarized in their report are not confirmed in the present investigation, as is discussed below.

Of equal importance is the question of the identification of the groups in bleomycin associated with each pK_a value. Having revised the structure of bleomycin, Takita et al. also changed these assignments [25]. The new secondary amine was given the pK_a of 2.72 based on potentiometric titration. The pyrimidine constant was lowered to less than 1, presumably on the basis of such titrations as shown in figure 4. However, these authors show no data and fail to point out that the pH dependence of uv absorbance has a pK_a value at 2.7 which nicely fits equation 9, while the further changes in absorbance below pH 1 may represent a second acid-base process involving the pyrimidine. A consistent plot to obtain the stability constant $K_{H_k-_nBlm}^{Cu}$ can be made without reference to such a small pK_a value for the ligand. Thus, if it exists, it probably has a value of less than 0.5. Both absorbance and fluorimetric examination of this group indicates two pH dependent changes in its electronic structure.

It is also difficult to rationalize the anomalously low pK_a values for the primary and secondary amine groups. Inspection of the bleomycin structure suggests that these should behave as strong bases such as one encounters in polyamines, certainly stronger than the imidazole group. In particular the assignment of the pK_a 2.72 to the secondary amine involves the following equilibrium according to equation 2

$$H_4Blm^{4+} \rightleftharpoons H_3Blm^{3+} + H^+,$$

in which the charges include the fixed positive charge on the R group of bleomycin A_2 and B_2. The corresponding pK_a in tetraethylene pentamine, involving the same charges on acid and conjugate base forms is 4.72 [26]. Thus, an unfavorable electrostatic environment would not explain this assignment. Furthermore, hydrogen bonding between the secondary amine and an adjacent pyrimidine ring nitrogen would not be expected to greatly perturb the amine pK_a. The suggestion of Takita et al. that the secondary amine lone pair can H-bond instead to the terminal carboxamide group would uncouple the secondary amine from the pyrimidine and thus make it difficult to see how the titration of the amine could systematically perturb the electronic structure of the pyrimidine group [25]. In the absence of more supporting data, it appears that one of the amine pK_a values remains to be determined. Finally, at present there is no compelling evidence to assign the pK_a = 7.50 to the primary amine instead of the secondary amine, which in many cases behaves as a stronger base.

The authors gratefully acknowledge the support of the National Cancer Institute through Grant CA 22184, the Milwaukee section of the American Cancer Society, and RR 01008 for the EPR facilities. Bleomycin was generously supplied by Bristol Laboratories, Syracuse, New York, through the courtesy of Dr. William Bradner.

REFERENCES

1. H. Umezawa, *Fed. Proc.* 33, 2296 (1974).
2. M. Ishizuka, H. Takayama, T. Takeuchi, and H. Umezawa, *J. Antibiotics Ser. A* 20, 15 (1967).
3. E. A. Sausville, J. Peisach, and J. B. Horwitz, *Biochemistry* 17, 2740 (1978).
4. T. Takita, Y. Muraoka, T. Nakatani, A. Fujii, Y. Iitaka, and H. Umezawa, *J. Antibiotics* 31, 1073 (1978).

5. W. E. Antholine, J. M. Knight, and D. H. Petering, *J. Med. Chem.* 19, 339 (1976).
6. H. G. Petering, H. H. Buskirk, and J. A. Crim, *Cancer Res.* 27, 1115 (1967).
7. G. J. Van Giessen, J. A. Crim, D. H. Petering, and H. G. Petering, *J. Nat. Cancer Inst.* 51, 139 (1973).
8. K. Takahashi, O. Yoshioka, A. Matsuda, and H. Umezawa, *J. Antibiotics* 30, 861 (1977).
9. H. Suzuki, K. Nagai, E. Akutsu, H. Yamaki, N. Tanaka, and H. Umezawa, *J. Antibiotics* 23, 473 (1970).
10. U. Y. Ryo, R. D. Ice, J. D. Jones, and W. H. Beierwaltes, *J. Nucl. Med.* 16, 127 (1975).
11. Y. Iitaka, H. Nakamura, T. Nakatani, Y. Muraoka, A. Fujii, T. Takita, H. Umezawa, *J. Antibiotics* 31(10), 1070 (1978).
12. D. Solaiman, W. Antholine, L. A. Saryan, and D. H. Petering, *Lloydia* 39, 370 (1976).
13. J. C. Dabrowiak, F. T. Greenway, W. E. Longo, M. Van Hussen, and S. T. Crooke, *Biochim. Biophys. Acta* 517, 517 (1978).
14. J. D. Dabrowiak, F. T. Greenway, and R. Grulich, *Biochemistry* 17, 4090 (1978).
15. H. Umezawa, Y. Suhara, T. Takita, and K. Maeda, *J. Antibiotics Ser. A.* 19, 210 (1966).
16. Y. Murioka, T. Takita, K. Maeda, and H. Umezawa, *J. Antibiotics* 22, 185 (1972).
17. D. H. Petering, *Bioinorg. Chem.* 1, 255 (1972).
18. D. H. Petering, *Biochem. Pharmacol.* 23, 567 (1972).
19. L. G. Sillen, and A. E. Martell, in *Stability Constants of Metal-Ion Complexes.* The Chemical Society, Burlington House, London, p. 634 (1964).
20. H. Umezawa, *Biomedicine* 18, 459 (1973).
21. D. M. Chen, B. I. Hawkins, and J. D. Glickson, *Biochemistry* 16, 2731 (1976).
22. J. Peisach, and W. E. Blumberg, *Mol. Pharmacol.* 5, 200 (1969).
23. J. Peisach, and W. E. Blumberg, *Arch. Biochem. Biophys.* 165, 691 (1974).
24. H. C. Freeman, and R. P. Martin, *J. Biol. Chem.* 244, 4825 (1969).
25. J. Takita, Y. Muraoka, T. Natani, A. Fujii, Y. Umezawa, H. Naganawa, and H. Umezawa, *J. Antibiotics* 31, 1023 (1978).
26. R. Paoletti, A. Vacca, and I. Giusti, *Ricera Sci.* 33(II-A), 523 (1963).

Received April 9, 1979; revised August 27, 1979