Bleomycin–DNA Interactions: Fluorescence and Proton Magnetic Resonance Studies†

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ABSTRACT: The interaction of bleomycin A_2 with DNA has been examined by fluorescence spectroscopy and proton magnetic resonance techniques. Fluorescence bands observed at 353 and 405 nm in the spectrum of bleomycin were assigned to the bithiazole and 4-aminopyrimidine rings, respectively. Quenching of bithiazole fluorescence by DNA was used to determine apparent equilibrium constants for the complex which, in 2.5 mM tris(hydroxymethyl)aminomethane buffer, pH 8.4, are 1.2×10^5 M^{-1} for bleomycin and 1.4×10^5 M^{-1} for tripeptide S, a partial acid hydrolysis product of the antibiotic. Under these conditions, one molecule of bleomycin binds for every five to six base pairs in DNA. In the proton magnetic resonance spectrum of bleomycin, resonances emanating from the bithiazole rings and dimethylsulfonium groups are preferentially broadened and reduced in intensity in the presence of DNA, suggesting that these moieties bind most tightly to the polymer.

The bleomycins, extracted from cultures of *Streptomyces verticullus* by Umezawa and his collaborators, were isolated as their copper chelates. This family of glycopeptide antibiotics exhibits a wide spectrum of antimicrobial activities and is currently used in the therapy of certain neoplasms in man (Umezawa, 1975). The structure of bleomycin A_2 is shown in Figure 1.

Bleomycin decreases the melting temperature of isolated DNA and causes single strand scissions in the polymer (Suzuki et al., 1968, 1969; Nagai et al., 1969a,b; Shirakawa et al., 1971). Fragmentation of DNA is enhanced in the presence of sulfhydryl compounds or hydrogen peroxide (Nagai et al., 1969c); during this process, free bases are released (Haidle, 1971; Müller et al., 1972; Haidle et al., 1972; Kuo and Haidle, 1974).

Bleomycin inhibits replication of vaccinia virus in HeLa cells, protects mice against the effects of vaccinia infection, and induces fragmentation of vaccinia DNA (Takeshita et al., 1974, 1976). The extent of bleomycin-induced degradation of DNA is determined by the concentrations of bleomycin, ATP, metal ions, and sulfhydryl agents present; however, the precise mechanism by which this effect occurs is unclear (Sausville et al., 1976; Takeshita et al., 1977).

The fluorescent properties of bleomycin have not previously been reported. In the present report, we use fluorescence spectroscopy and proton magnetic resonance techniques to examine the binding of bleomycin and its hydrolysis products to DNA. These experiments allow us to propose certain features of the bleomycin-DNA complex.

Materials and Methods

A sample of copper-free bleomycin A_2 (lot No. 71L489) was kindly provided by Bristol Co. and stored at −20 °C. This material contained several components (instability of bleomycin has been reported (Umezawa, 1971)) and was purified by column chromatography at 4 °C on a CM[1]-Sephadex C-25 column. The column was eluted using a pH gradient with 25 mM ammonium formate, pH 6.4, and 25 mM ammonium bicarbonate, pH 9.5, as initial and final buffer solutions, respectively. The major peak eluting at pH 8.4–8.6 was lyophilized and used for all experiments. Calf thymus DNA, purchased from Worthington Biochemical Corp., was dissolved in 0.1 M NaCl and dialyzed exhaustively, first against 0.1 M EDTA and then against 0.1 M NaCl at 4 °C. The dialyzed solution of DNA (approximately 2 mg/mL) was stored at 4 °C and the final concentration determined by phosphate analysis (Ames, 1966).

Hydrolysis of Bleomycin. Partial acid hydrolysis of bleomycin was conducted according to the procedure of Umezawa (1973). Ten milligrams of bleomycin was dissolved in 25 mL of 6 N HCl, incubated with mild stirring at 37 °C for 4 days, and then evaporated to dryness at 25 °C. The hydrolysis products were adsorbed on a column of CM-Sephadex C-25 and eluted at 4 °C with a linear gradient (0.025 to 0.5 M) of ammonium formate, pH 6.4.

A major component was eluted at 0.26 M ammonium formate. The properties of this compound, as tested by paper chromatography (1-butanol-acetic acid-water, 4:1:2) and paper electrophoresis (formic acid-acetic acid-water, pH 1.7 (3:1:6), and 0.065 M potassium phosphate buffer, pH 8.4), compared closely with an authentic sample of tripeptide S (Figure 1) provided by Professor Umezawa. Hydrolysis of this compound in 12 N HCl for 24 h at 100 °C yielded three spots with R_f values identical with the hydrolysis products of tripeptide S.

Material excluded by the CM-Sephadex C-25 column was purified on a column of DEAE-Sephadex A-25. The compound eluting at 0.1 M ammonium formate showed an ultraviolet absorption spectra similar to that of 4-aminopyrimidine. A pK_a of 5.3 was demonstrated by plotting intensities of fluorescence emission at 405 nm against pH in the presence of DNA. Thus, this compound, referred to in the Results section as compound

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[1] Abbreviations used: CM, carboxymethyl; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane.

I, most likely includes the part of bleomycin that contains the 4-aminopyrimidine and imidazole moieties.

Sonication of DNA. A solution of 4.5 mM calf thymus DNA was prepared in a buffer composed of 2.5 mM Tris-HCl–30 mM NaCl, pH 8.4. Nitrogen was bubbled through the solution for 5 min which was then sonicated for 5 min at 4 °C with a Branson W 185 sonifier set at full power (Richards and Boyer, 1965). The sonication procedure was repeated once.

Spectroscopic Measurements. Proton NMR spectra were recorded at 25 °C on a JEOLCO 100-MHz pulsed Fourier transform spectrometer equipped with a Nicolet 1080 digital computer for signal accumulation and Fourier transformation. Eight-millimeter NMR tubes with coaxial inserts containing hexamethyldisiloxane as an external reference were used for spectral studies. All samples were prepared in 1 mL of D_2O and the pD was adjusted by adding small amounts of DCl or NaOD.

Uncorrected fluorescence spectra were recorded at 25 °C on an Aminco-Bowman spectrophotofluorometer equipped with an X–Y recorder; corrected spectra were taken on a Perkin-Elmer fluorescence spectrophotometer, Model MPF-3. All measurements were made in a cuvette with a 1-cm light path. When the samples contained DNA, absorbance of the added DNA at 300 nm was less than 0.05. Quantum yields were calculated by comparing the absorbance at the excitation wavelength, 300 nm, and the emission spectra of the unknown sample with a standard of known quantum yield. Quinine bisulfate, dissolved in 1 N H_2SO_4 (quantum yield, 0.55), was used as a primary standard (Melhuish, 1961).

Calculation of Binding Constants. Quantitative measurements of the binding of bleomycin and tripeptide S to DNA were based on fluorescence studies. As discussed in the Results section of this paper, the fluorescence of bleomycin and tripeptide S is partially quenched by DNA, thereby providing a sensitive method to determine apparent equilibrium constants and stoichiometry of binding.

Assuming that all binding sites on DNA are equivalent and noninteracting, i.e., binding of one molecule of bleomycin to DNA does not affect binding of a second molecule, an apparent equilibrium constant for the reaction can be expressed by eq 1

$$\frac{1}{(C)} = \frac{1}{nK(D_0)(B)} + \frac{1}{n(D_0)} \tag{1}$$

which was derived from a more general expression of equilibrium

$$K = \frac{(C)}{n(D)(B)} \tag{2a}$$

and the relation

$$(C) = n[(D_0) - (D)] \tag{2b}$$

In these equations, (B), (C), and (D) represent the concentration of free bleomycin, bound bleomycin, and free DNA, respectively. (D_0) represents the total concentration of DNA, n is the number of bound drug molecules per DNA nucleotide, and K is the apparent equilibrium constant.

(B) and (C) are obtained from fluorescent measurements as follows: two solutions were prepared, one containing DNA, NaCl, and Tris-HCl buffer, pH 8.4, and the other containing only the buffer. Small amounts of bleomycin, covering a wide range of concentrations, were added to both solutions and the fluorescent intensities were measured at 355 nm following excitation at 300 nm. The solution lacking DNA gives the fluorescent intensity of free bleomycin (F_b). The fluorescent

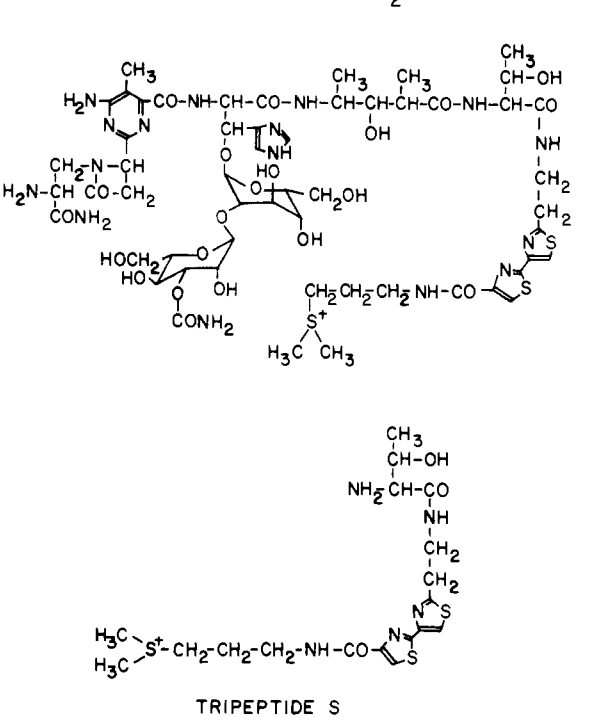

FIGURE 1: Structure of bleomycin A_2 and tripeptide S.

intensity of bound bleomycin (F_c) is assumed to be $0.5F_b$ since the fluorescence intensity of bleomycin was quenched by 50% in the presence of excess DNA. The solution containing DNA and bleomycin gives fluorescent intensity F; that is, F represents an average of the free and bound bleomycin in the cuvette. (B) and (C) were calculated from eq 3 and 4 as follows:

$$(B) = \frac{F - F_c}{F_b - F_c}(B_0) \tag{3}$$

$$(C) = \frac{F_b - F}{F_b - F_c}(B_0) \tag{4}$$

where (B_0) represents the total concentration of bleomycin.

The apparent equilibrium constant (K) and number of ligand sites per DNA base (n) were determined by plotting 1/(C) against 1/(B). Principles and assumptions involved in using these types of measurements are discussed by Goldstein et al. (1974).

Viscosity Studies. Viscosity measurements were conducted with a Cannon-Ubbelohde four-bulb shear solution viscometer, size 50. The viscosity of sonicated DNA in 2.5 mM Tris-HCl, pH 8.4, and 5 mM NaCl was measured over a range of 0.31 to 1.56 mM. The relation of viscosity to DNA concentration (D_0) is given by eq 5 (Zimm, 1971; Cohen and Eisenberg, 1969)

$$\frac{\eta_{sp}}{(D_0)} = [\eta] + k(D_0) \tag{5}$$

where η_{sp} is the specific viscosity and k is Huggins' coefficient; the latter is generally a function of salt concentration. Using eq 5, $[\eta]$, the intrinsic viscosity, and k for sonicated DNA were determined to be 0.147 mM^{-1} and 0.0028 mM^{-2}, respectively. The molecular weight of the sonicated DNA, estimated from the intrinsic viscosity by the experimental equation of Doty et al. (1958), was 6.2×10^5. Changes in viscosity are related to changes in the length of the DNA as given in eq 6

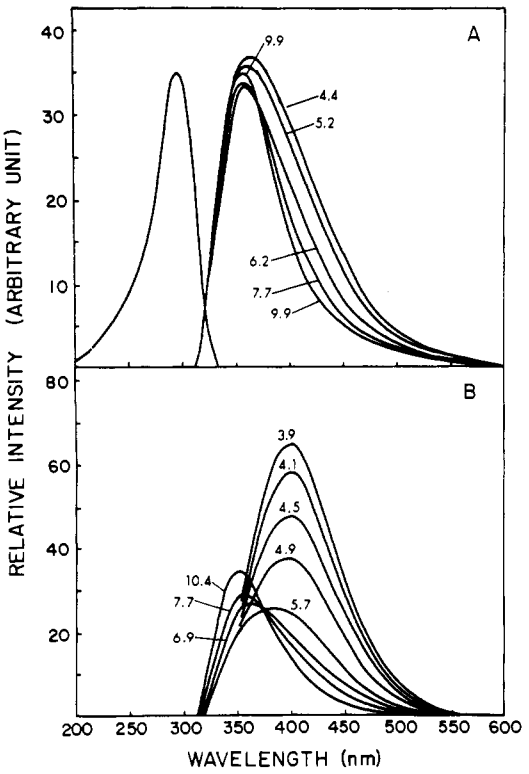

FIGURE 2: Effect of varying pH on the fluorescence spectra of bleomycin in the presence and absence of DNA. Excitation wavelength is 300 nm. Emission spectra are shown at different pH values: (A) 0.02 mM bleomycin (OD_{300} = 0.21); (B) 0.02 mM bleomycin and 0.18 mM calf thymus DNA dissolved in 3 mM NaCl.

$$\frac{L}{L_0} = \left(\frac{[\eta]}{[\eta_0]}\right)^{1/3} = 1 + \gamma r' \qquad (6)$$

where L and L_0 are the length of the DNA rod in the presence and absence of drug, respectively, $[\eta]$ and $[\eta_0]$ are the corresponding intrinsic viscosities, and r' represents the number of bleomycin molecules bound per DNA base. The parameter, γ, reflects alterations in the length of DNA in the presence of the drug and varies with the experimental conditions.

Viscosity of solutions of DNA were measured as a function of the concentration of bleomycin and ethidium bromide. Ethidium bromide and bleomycin were used over a range of concentrations up to 200 μM and 250 μM, respectively. Values for r' ($r' = (C)/(D_0)$ were calculated from r, ($r = (B_0)/(D_0)$), n, and the apparent equilibrium constants: 1.2×10^5 M^{-1} for bleomycin and 2.5×10^6 M^{-1} for ethidium bromide (LePecq and Paoletti, 1967). All measurements were performed at 21 °C in 2.5 mM Tris-HCl, pH 8.4.

Results

Fluorescence Spectra of Bleomycin. The corrected emission maximum for bleomycin (3 μM) in 0.01 M Tris buffer, pH 7.5, is at 350 nm; the excitation maximum is at 292 nm. All spectra described below are uncorrected. Uncorrected fluorescence spectra of bleomycin at different pH values are shown in Figure 2A. At pH 9.9, excitation and emission maxima are 297 and 353 nm, respectively. Quantum yield is approximately 0.01. At pH 6.2, the excitation peak remains unchanged while the emission peak shows decreasing intensity and a small red shift; an isoemissive point was obtained at 257 nm. At pH 5.2 and 4.4, the excitation spectrum remains unchanged but the emission peak is shifted by 17 to 370 nm with a small increase in intensity.

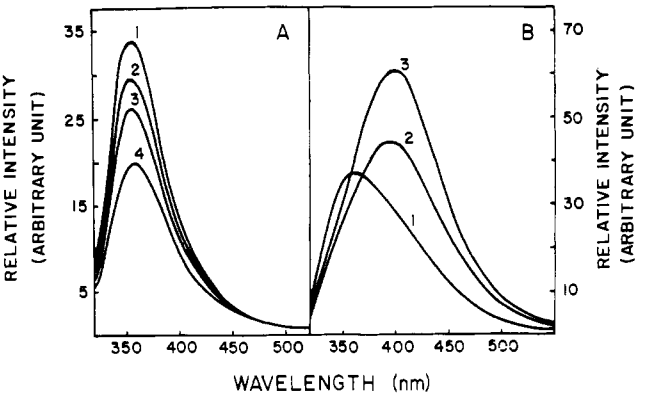

FIGURE 3: Effect of varying concentrations of DNA on the fluorescence emission spectra of bleomycin. Excitation wavelength is 300 nm. (A) With 0.02 mM bleomycin (OD_{300} = 0.31) in 2.5 mM Tris-HCl, pH 8.4. Concentration of DNA is: (curve 1) 0; (2) 0.062 mM; (3) 0.16 mM; (4) 0.62 mM. (B) With 0.02 mM bleomycin: aqueous solution adjusted to pH 4.4 with HCl. Concentration of DNA is: (curve 1) 0; (2) 0.019 mM; (3) 0.30 mM.

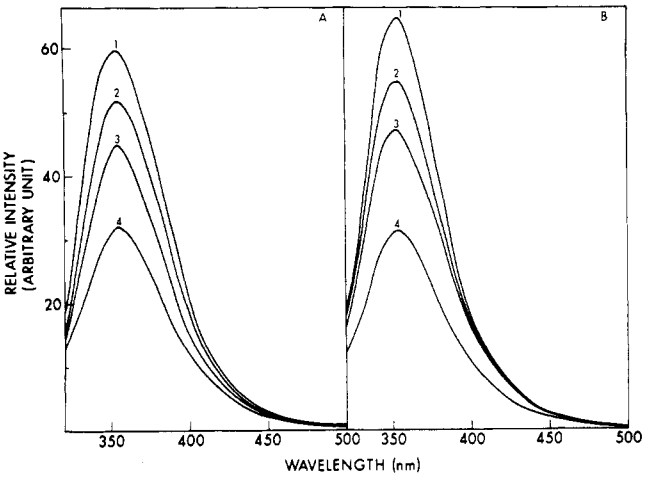

FIGURE 4: Effect of varying concentrations of DNA on the fluorescence emission spectra of tripeptide S. Excitation wavelength is 300 nm. (A) With 0.025 mM tripeptide S (OD_{300} = 0.31) in 2.5 mM Tris-HCl, pH 8.4. Concentration of DNA is: (curve 1) 0; (2) 0.062 mM; (3) 0.16 mM; (4) 0.62 mM. (B) With 0.025 mM tripeptide S; aqueous solution adjusted to pH 4.4. Concentration of A is: (curve 1) 0; (2) 0.062 mM; (3) 0.16 mM; (4) 0.62 mM.

The fluorescence spectrum of bleomycin was determined as a function of pH in the presence of DNA (Figure 2B). The isoemissive point is at 375 nm. At pH 10.4, the emission peak and spectrum is similar to that of bleomycin. At pH 5.7, a red shift accompanied by decreasing peak intensity is observed; this shift is somewhat greater than observed in the absence of DNA. Below pH 5, a new emission peak, which dominates the spectrum, appears at 405 nm. No emission at 405 nm is observed in the absence of DNA.

The effect of varying concentrations of DNA on the fluorescence of bleomycin was measured (Figure 3). At pH 8.4, addition of DNA to bleomycin quenches fluorescence at 353 nm; quenching did not exceed 50% even when the molar ratio of DNA to bleomycin was increased to 175 (Figure 3A). There was no shift in emission wavelength. At pH 4.4, emission was enhanced following addition of DNA and the emission peak shifted to 405 nm (Figure 3B).

Fluorescence Spectra of Tripeptide S and Other Hydrolysis Products of Bleomycin. The fluorescence spectra of tripeptide S at pH 8.4 is shown in Figure 4A; excitation (not shown) and

TABLE I: Binding of Bleomycin and Tripeptide S to DNA.

Buffer concn[a] (mM)	Ligand	Magnesium concn (mM)	App equilibrium constant ($K \times 10^5$) (M^{-1}) I[b]	II[c]	Nucleotides per ligand I[b]	II[c]
2.5	Bleomycin	0	1.2	1.20 ± 0.20	11	11 ± 1
	Tripeptide S	0	1.4	1.95 ± 0.04	8	8 ± 0
25	Bleomycin	0	0.42	0.92 ± 0.06	45	52 ± 4
	Tripeptide S	0	0.70	0.59 ± 0.06	29	35 ± 4
2.5	Bleomycin	0.05	0.27	0.33 ± 0.01	11	9.5 ± 0.2
	Tripeptide S	4.0	0.17	0.15 ± 0.01	11	6.5 ± 0.13

[a] Tris-HCl, pH 8.4. [b] Data calculated from double-reciprocal plots. [c] Data calculated from Scatchard plots.

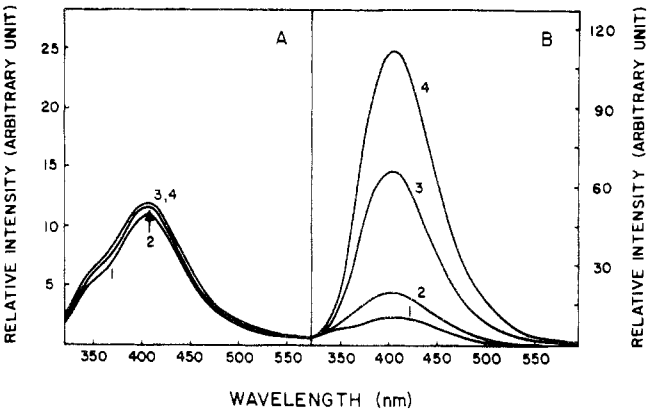

FIGURE 5: Effect of DNA on fluorescence emission spectra of compound I. Excitation wavelength is 300 nm. (A) Compound I (OD_{300} = 0.42) in water, pH adjusted to 7.7. Concentration of DNA is: (curve 1) 0; (2) 0.045 mM; (3) 0.12 mM; (4) 0.23 mM. (B) Compound I (OD_{300} = 0.43) in water, pH adjusted to 4.4. Concentration of DNA is: (curve 1) 0; (2) 0.045 mM; (3) 0.12 mM; (4) 0.23 mM.

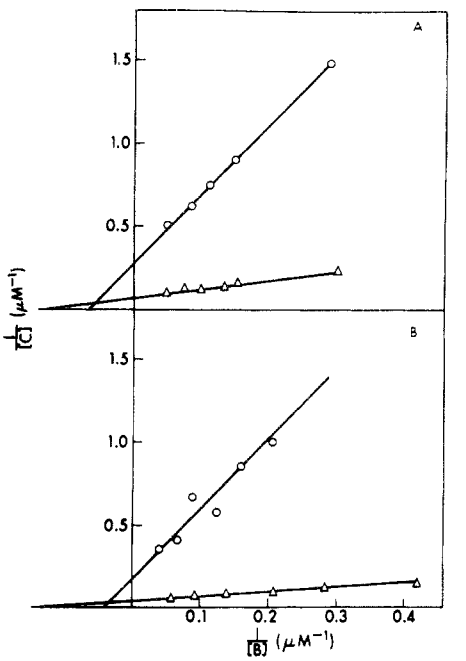

FIGURE 6: Binding of bleomycin and tripeptide S to DNA. Reaction mixture contained 0.165 mM calf thymus DNA, 1.2 mM NaCl and Tris-HCl, pH 8.4. Bleomycin (panel A) or tripeptide S (panel B) was added to the reaction mixture and the fluorescence emission measured at 355 nm after excitation at 300 nm. (Δ) With 2.5 mM Tris-HCl; (O) 25 mM Tris-HCl.

emission maxima are at 302 and 353 nm, respectively. The quantum yield for this compound is approximately 0.02. When DNA was added, the fluorescence of tripeptide S at pH 8.4 was quenched in a manner similar to that observed with bleomycin; maximum quenching was approximately 50%. Unlike bleomycin, there is little difference in the emission spectra of tripeptide S at pH 8.4 and 4.4 and no new emission at 405 nm was observed in the presence of DNA (Figure 4B).

Compound I emits a different type of fluorescence (Figure 5) in which the excitation (not shown) and emission maxima are at 306 and 405 nm, respectively. The quantum yield for compound I is 0.002. At pH 7.7, addition of DNA has little effect on the fluorescence spectra of I (Figure 5A); at pH 4.4, the emission was enhanced but the emission maxima remained at 405 nm (Figure 5B). This enhanced emission is similar to that observed when bleomycin was added to a solution of DNA at this pH. Titration curves were obtained (data not shown) by plotting the intensities of emission at 405 nm against pH; the apparent pK_a values for bleomycin and compound I were 4.7 and 5.3, respectively.

Quantitative Fluorescence Studies on the Binding of Bleomycin and Tripeptide S to DNA. Measurements of the fluorescence quenching of bleomycin and tripeptide S by DNA at 353 nm allowed the concentration of free and bound bleomycin and free and bound tripeptide S to be determined. Double-reciprocal and Scatchard plots of the concentrations of free and bound bleomycin were utilized to determine apparent equilibrium constants and the stoichiometry of binding of bleomycin and tripeptide S to DNA (Figure 6). Fluorescence intensity increased linearly over the range of ligand concentrations used in the binding experiment. The values obtained are summarized in Table I. When the ionic strength is increased, binding of both compounds is reduced. Magnesium ion competitively inhibits binding of bleomycin to DNA (data not shown).

NMR Studies of the Binding of Bleomycin to DNA. The high-resolution ^1H NMR spectrum of a solution of bleomycin dissolved in D_2O is shown in Figure 7. The low-field portion of the spectrum, composed of four single proton peaks, has been analyzed as follows (Takita et al., 1972a). Resonances at 8.53 and 8.34 ppm are assigned to the two protons on the bithiazole rings and those at 8.13 and 7.58 ppm to the C-2 and C-4 protons of imidazole. The peak at 3.22 ppm, composed of six protons, is assigned to the dimethylsulfonium moiety since this group contains the only six equivalent protons in bleomycin; a chemical shift of 3.22 ppm is typical for this kind of proton (Ramirez et al., 1973) and the narrowness of this peak compared with other peaks suggests that these protons are located

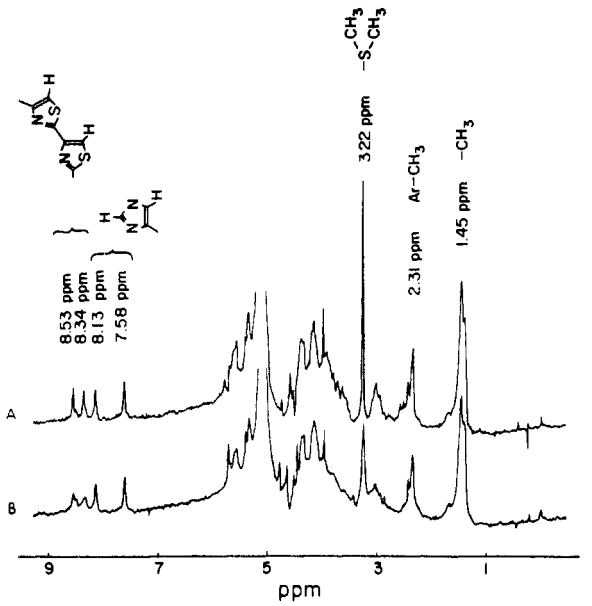

FIGURE 7: ^{1}H NMR spectra of bleomycin at 100-MHz resolution. Each spectrum is an average of 512 scans. (A) With 6 mM bleomycin in D_2O at pD 8.4; (B) 6 mM bleomycin and 3.5 mM calf thymus DNA in D_2O, pD 8.4.

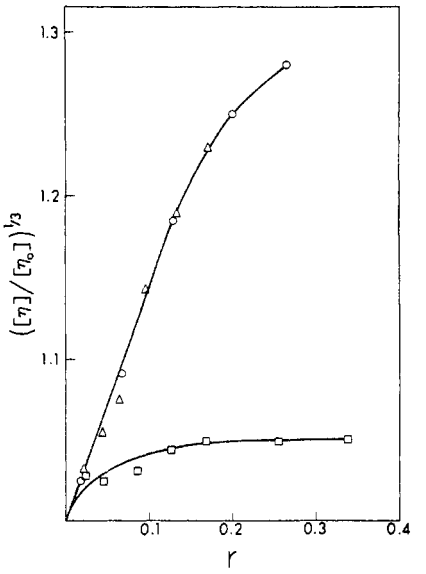

FIGURE 8: Effect of bleomycin on viscosity of sonicated DNA. The viscosity of DNA in the presence of bleomycin or ethidium bromide was measured in 5 mM NaCl as described in Materials and Methods. $r = B_0/D_0$. (□) With 0.738 mM DNA and bleomycin; (○) 0.755 mM DNA and ethidium bromide; (△) 0.805 mM DNA and ethidium bromide.

at the end of the side chain. The partially resolved resonances at 1.45 ppm are assigned to the methyl and methylene protons.

Resonances ascribed to the two bithiazole protons and six dimethylsulfonium protons of bleomycin are greatly reduced when the antibiotic is added to a solution of DNA. Other resonances identified, including the imidazole protons and methyl protons, were affected to a much lesser extent. No changes in chemical shift were observed on addition of DNA at pD 8.4 or 7.0; at lower pD values, DNA precipitated from the solution.

Effect of Bleomycin on the Viscosity of DNA. The effect of bleomycin and ethidium bromide on the viscosity of sonicated DNA is shown in Figure 8. In this figure $([\eta]/[\eta_0])^{1/3}$ is plotted against r, the ratio of total bleomycin molecules per DNA base in solution. The calculations and equations are described in Materials and Methods. Under our experimental conditions, almost all ethidium bromide molecules are bound to DNA. This dye is known to intercalate with DNA (Gale et al., 1972) and induces a linear increase in the viscosity of DNA (Zimm, 1971). Our results with ethidium bromide are in good agreement with previous reports (Festy and Daune, 1973); in contrast, the interaction of bleomycin with DNA results in only a very small change in the viscosity of the solution.

Discussion

Assignment of Bands in the Fluorescence Spectrum of Bleomycin. The fluorescence spectrum of bleomycin has not previously been reported or analyzed. Of the three chromophores present in this antibiotic, the bithiazole rings would be expected to fluoresce strongly, the 4-aminopyrimidine group is known to fluoresce weakly after η-π^* excitation (Cohen et al., 1965). Bithiazole rings are present in tripeptide S, the main product of acid hydrolysis; thus, an emission at 353 nm, which dominates the fluorescence spectra of this fragment, can clearly be assigned to this chromophore.

When DNA is added to a solution of bleomycin at pH 4.0 or less, a fluorescent emission appears at 405 nm. Compound I also fluoresces at 405 nm; enhanced fluorescence at this wavelength is observed at acid pH in the presence of DNA. The ultraviolet absorption spectra of compound I display peaks corresponding to π-π^* and η-π^* transitions at 245 and 285 nm; similar results were reported for derivatives of 4-aminopyrimidine (Muraoka et al., 1970; Toth and Billes, 1968). The fluorescent excitation spectrum of compound I overlaps the η-π^* transition bands in the ultraviolet absorption spectra; thus, the emission at 405 nm observed at acid pH in bleomycin apparently originates from the 4-aminopyrimidine group of the molecule.

Bleomycin-DNA Interaction. (A) Evidence from Fluorescence Studies. At physiological pH, fluorescence spectra of the bithiazole rings in bleomycin and tripeptide S have similar excitation and emission maxima and line shapes. Binding parameters for their respective complexes with DNA, obtained by measuring the quenching of bithiazole fluorescence, are of the same order of magnitude. The apparent equilibrium constants for bleomycin and tripeptide S in 2.5 mM Tris buffer, pH 8.4, are 1.2×10^5 and 1.4×10^5 M^{-1}, respectively. Approximately 1 molecule of bleomycin or tripeptide S binds to every 4 to 5 base pairs in DNA.

At pH 8.4, bleomycin exists as a cation by virtue of the positively charged sulfonium group on the side chain. Increasing ionic strength reduces the apparent equilibrium constant and the number of bleomycin molecules bound to DNA. Magnesium ions interact with DNA (Eichorn et al., 1974) and competitively inhibit binding of bleomycin, also suggesting that charged interactions contribute to the binding forces. One such ionic bond could involve the sulfonium group of bleomycin and a phosphate residue in DNA.

Fluorescence of bleomycin may be affected by its state of ionization. The apparent pK_a of the imidazole group in bleomycin, obtained from ^{1}H NMR titration curves, is 4.7 (Takita et al., 1972b). We confirmed this observation by fluorescence quenching measurements and detected a titratible group in compound I (probably imidazole) with a pK_a of 5.3. At physiological pH, fluorescence of the 4-aminopyrimidine group is not altered by the addition of DNA; however, at pH values below 5, changes in its fluorescence are observed.

(B) Evidence from ^{1}H NMR Studies. Line widths of proton magnetic resonances are related to the spin–spin relaxation

time, which reflects the rotational motion of the molecules, and to the chemical exchange rate, which reflects changes in their environment. Measurements of line broadening and reduction of intensity were used to indicate those protons of bleomycin that are involved in binding to DNA. Among the resonances identified, those originating from the bithiazole and dimethylsulfonium protons are selectively broadened in the presence of DNA. This suggests that the bithiazole and dimethylsulfonium groups are bound more tightly to DNA than other parts of the molecule. Proton resonances originating from the imidazole moiety, the aromatic methyl group of 4-aminopyrimidine, and the three methyl groups of bleomycin are affected to a lesser extent by the addition of DNA, suggesting that rotational motion of these parts of the molecule remains rapid when the antibiotic is bound. These data argue against the participation of the 4-aminopyrimidine and imidazole residues in the binding reaction.

The possibility that the observed broadening of resonances results from the presence of contaminating ions seems unlikely. To induce the observed preferential broadening, paramagnetic metals would have to bind to the cationic disulfonium group and to the bithiazole rings. Studies reviewed by Umezawa (cf., 1976) demonstrate that cupric ion binds to the β-aminoalaninamide and carbamoyl moieties in bleomycin. Thus, metal ions would account for the observed effects only if such ions were introduced adventitiously in the NaCl or DNA and a new high affinity metal binding site on bleomycin was created after the antibiotic itself binds to DNA.

Conclusions

The experiments reported in this paper provide evidence for the involvement of the bithiazole rings and dimethylsulfonium ions in bleomycin A_2 in the binding of the antibiotic to DNA. Binding of bleomycin was not dependent on the presence of cofactors or reducing agent. Although tripeptide S has no biological activity (Asakura et al., 1975; Takeshita et al., 1977) and represents only a fragment of bleomycin A_2, this compound also binds to DNA.

Studies of structure-activity relationships using SV40 DNA in vitro (Asakura et al., 1975) indicate the importance of certain parts of the bleomycin molecule; namely, the integrity of the 2,3-diaminopropionic acid moiety (enzymatic hydrolysis of the amide bond abolishes the ability to cleave circular DNA), the correct position of the 2-*O*-carbamoyl-D-mannose group (an isomer with the 3-*O*-carbamoylmannose linkage is inactive), and the requirement for a neutral or positively charged side chain linked to the bithiazole moiety (bleomycinic acid is inactive but derivatives containing a variety of side chains are active). Hydrogenation of the bithiazole rings (as in phleomycin) does not affect biological activity. From this type of information, one cannot determine if the groups implicated bind directly to DNA or if conformational or structural changes associated with inactive derivatives interfere with the binding of other bleomycin groups that are essential for activity. Parts of the bleomycin molecule that contribute to the biological activity may be involved in stabilizing the bleomycin–DNA complex or in reactions that break covalent bonds in DNA. Further information is needed to fully elucidate the nature of the bleomycin–DNA interaction.

Finally, crystallographic analysis has shown that the portion of bleomycin containing the bithiazole rings are planar (Koyama et al., 1968). It is possible that the bithiazole group intercalates between base pairs of DNA. To test this hypothesis, the effect of bleomycin on the viscosity of low-molecular-weight calf thymus DNA (6.2×10^5) was examined. No significant increase in the viscosity was observed. Although additional data would be required to rigorously exclude intercalation, the data suggest that the bithiazole rings in bleomycin interact with DNA in a manner other than intercalation.

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Mechanism of Ethidium Bromide Fluorescence Enhancement on Binding to Nucleic Acids†

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ABSTRACT: The mechanism of the enhancement of the fluorescence of ethidium bromide on binding to double helical RNA and DNA has been investigated. From an examination of the effect of different solvents on the fluorescence lifetime, quenching of fluorescence by proton acceptors, and the substantial lengthening of lifetime observed upon deuteration of the amino protons, regardless of the medium, we conclude that proton transfer from the excited singlet state is the process primarily responsible for the low fluorescence yield in most polar solvents. Enhancement of fluorescence upon intercalation is attributed to a reduction in the rate of excited state proton transfer to solvent molecules. The proposed mechanism accounts for the ~3.5-fold increase in the lifetime of free ethidium bromide in going from H_2O to D_2O; the fact that addition of small amounts of water to nonaqueous solvents decreases the fluorescence whereas addition of small amounts of D_2O enhances the fluorescence; and the enhancement of the ethidium bromide triplet state yield on binding to DNA. Other proposed mechanisms are shown to be inconsistent with our findings.

Ethidium bromide, a cationic dye (structure I) which interacts strongly and specifically with double helical RNAs and DNAs, is widely used in spectrofluorimetric studies because of the striking fluorescence enhancement it displays upon binding (Waring, 1965; LePecq and Paoletti, 1967; Bittman, 1969; Burns, 1969, 1971; Tao et al., 1970; LePecq, 1971; Angerer et al., 1974; Bontemps and Fredericq, 1974; Genest et al., 1974; Gatti et al., 1975). It is generally agreed that strong fluorescence enhancement accompanies intercalation of the dye into the double helix conformation of the nucleic acid but there is also evidence for additional nonintercalative, less fluorescence-enhanced sites which are presumed to involve electrostatic binding (Waring, 1965; LePecq and Paoletti, 1967; Bittman, 1969). Recently, ethidium bromide has been used to probe tRNA structure (Bittman, 1969; Urbanke et al., 1973), 5S RNA (Gray and Saunders, 1971; Feunteun et al., 1975), circular DNA (Hudson et al., 1969), chromatin structure (Ide and Baserga, 1976), ribosomal RNA (Lawrence and Daune, 1976), synthetic DNA (Aktipis and Martz, 1974), tRNA protein interactions (Rigler et al., 1971), and to determine the molecular weight of DNA (Weissman et al., 1976). Ethidium bromide also elicits a wide range of biochemical effects (Lurquin and Buchet-Mahieu, 1971; Avadhani et al., 1973; Kramer et al., 1974; Criddle et al., 1976).

Despite extensive studies of ethidium bromide complexes with polynucleotides, a satisfactory mechanism explaining the high degree of fluorescence enhancement upon binding has not yet emerged. When ethidium bromide intercalates into a double helix, both its solvent environment and equilibrium conformation are modified, and either one or both of these changes might be involved in the enhancement mechanism. LePecq and Paoletti (1967) suggested the enhancement resulted from immersion of ethidium bromide in a hydrophobic region where it would no longer be quenched by aqueous solvent but made no proposal with respect to the quenching mechanism. Burns (1969) attributed the enhancement to a change in the conformation of ethidium bromide which renders a previously forbidden transition allowed. Recently, Hudson and co-workers (Hudson and Jacobs, 1975; Waleh et al., 1976) presented evidence for a triplet state nearly degenerate with the lowest excited singlet in ethidium bromide and postulated that the fluorescence enhancement is due to environment-induced shifts in the relative singlet–triplet energy separation leading to a reduction in the singlet-to-triplet intersystem crossing rate.

The experimental evidence presented to date is insufficient to decide unequivocally among these or other possible enhancement mechanisms. Without such knowledge, the usefulness of ethidium bromide fluorescence assaying of the extent of double helical structure (LePecq, 1971) remains somewhat open to question, especially in view of reports that the fluorescence parameters of ethidium bromide vary with extent of binding (Bontemps and Fredericq, 1974). A proper interpretation of ethidium bromide fluorescence studies of the interaction of nucleic acid with proteins such as the synthetases (Rigler et al., 1971), ribosomal proteins, and histones (Lawrence and Daune, 1976) also requires an understanding of the enhancement mechanism and how it may be affected by such interactions. Once the enhancement mechanism is known, it

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