Stümpel, J., Harlos, K., & Eibl, H. (1980) *Biochim. Biophys. Acta 599*, 464-472.
Träuble, H., Teubner, M., Woolley, P., & Eibl, H. (1976) *Biophys. Chem. 4*, 319-342.
van Deenen, L. L. M., & de Haas, G. H. (1963) *Biochim. Biophys. Acta 70*, 538-553.
Watts, A., Harlos, K., Maschke, W., & Marsh, D. (1978) *Biochim. Biophys. Acta 510*, 63-74.

Copper(II)·Bleomycin, Iron(III)·Bleomycin, and Copper(II)·Phleomycin: Comparative Study of Deoxyribonucleic Acid Binding†

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ABSTRACT: The kinetics and mechanism of binding of Cu(II)·bleomycin, Fe(III)·bleomycin, and Cu(II)·phleomycin to DNA were studied by using fluorometry, equilibrium dialysis, electric dichroism, and temperature-jump and stopped-flow spectrophotometry. The affinity of Cu(II)·bleomycin for DNA was greater than that of metal-free bleomycin but less than that of Fe(III)·bleomycin. Cu(II)·bleomycin exhibited a two-step binding process, with the slow step indicating a lifetime of 0.1 s for the Cu(II)·bleomycin·DNA complex. Fe(III)·bleomycin binding kinetics indicated the presence of complexes having lifetimes of up to 22 s. DNA was lengthened by 4.6 Å/molecule of bound Cu(II)·bleomycin and by 3.2 Å/bound Fe(III)·bleomycin but not at all by Cu(II)·phleomycin, suggesting that both bleomycin complexes intercalate while the phleomycin complex does not. However, phleomycin exhibited nearly the same specificity of DNA base release as bleomycin. These results suggest that the coordinated metal ion plays a major role in the binding of metal–bleomycin complexes to DNA but that intercalation is neither essential for DNA binding and degradation nor primarily responsible for the specificity of DNA base release by these drugs.

Degradation of DNA by bleomycin is thought to be caused by radicals resulting from oxidation of DNA-bound Fe(II)·bleomycin to Fe(III)·bleomycin (Sausville et al., 1978a,b). To understand better the details of this process, we have studied the interaction of DNA with Fe(III)·bleomycin, Cu(II)·bleomycin, and Cu(II)·phleomycin. Cu(II)·bleomycin was chosen as a model for Fe(II)·bleomycin, whose oxygen lability makes study extremely difficult. Phleomycin is identical with bleomycin, except that it lacks one double bond in the bithiazole moiety (Figure 1) (Takita et al., 1972), which in bleomycin intercalates between DNA base pairs (Povirk et al., 1979). Although both drugs have similar DNA strand-breaking activity (Stern et al., 1974; Suzuki et al., 1969), this structural difference makes phleomycin a less likely candidate for intercalation.

Materials and Methods

Drugs. Blenoxane, the clinical mixture containing primarily bleomycins A_2 and B_2, was used in most experiments. Fluorescence studies (not shown) indicated that purified bleomycins A_2 and B_2 had the same affinity for DNA in both high and low salt as blenoxane.

Metal–bleomycin complexes were formed by mixing equimolar quantities of bleomycin and either $CuCl_2$ or $Fe^{II}(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (all at concentrations of at least 2 mM) in distilled water, pH 5. Fe(II)·bleomycin oxidized rapidly to Fe(III)·bleomycin (Povirk, 1979). Fe(III)·bleomycin was used within 3 h of its preparation because its ultraviolet–visible spectrum, although stable for several hours, showed measurable changes after incubation for 1 day at 25 °C. The clinical mixture of phleomycins (95% copper free) was used in base-release experiments. Chromatographically purified Cu(II)·phleomycin A_2, which was used in all binding studies, was a gift of Dr. T. Takita of the Institute of Microbial Chemistry, Tokyo. All drugs and drug solutions were stored at −20 °C.

DNA. Either high molecular weight repurified calf thymus DNA or sonicated, fractionated, 150 base pair long calf thymus DNA was used in binding studies. Details of these preparations have been described (Hogan et al., 1978; Povirk et al., 1979). Specifically labeled *Escherichia coli* DNA was isolated from cultures grown in the presence of [^{14}C]thymidine, [^{3}H]adenosine, or [^{3}H]cytidine, as previously described (Povirk et al., 1978). All DNA concentrations are expressed in moles of base pairs.

Equilibrium Dialysis. Sonicated, fractionated 2 mM calf thymus DNA (2 mL) was dialyzed against a known concentration of Cu(II)·bleomycin (50–100 mL). After 2 days of dialysis at 25 °C, 0.1 volume of 25% sodium dodecyl sulfate was added to solutions from inside and outside the dialysis bag, and the Cu(II)·bleomycin concentrations were determined from the A_{310}–A_{340} of the solutions. This procedure minimized the effect of small but variable amounts of ultraviolet-absorbing material which accumulated in DNA samples during dialysis. Dialysis was usually begun with equal Cu(II)·bleomycin concentrations inside and outside the dialysis bag; however, the same results were obtained with samples having an initial excess on either side.

Fluorescence Studies. Binding of bleomycin and phleomycin and their metal complexes to DNA was measured by fluorometry (Chien et al., 1977); the fluorescence of the bithiazole rings is quenched upon binding to DNA. For bleomycin studies in low-salt buffer, high molecular weight calf thymus DNA was used satisfactorily. however, for studies in high-salt buffer, where higher DNA concentrations were required, and for studies with Cu(II)·phleomycin, whose fluorescence was only $\sim 1/30$ as strong as Cu(II)·bleomycin, light scattering by DNA became a significant problem. Therefore, sonicated DNA (Hogan et al., 1978) was used instead. Emission was measured at 353 nm, and exitation was at either 300 or 310

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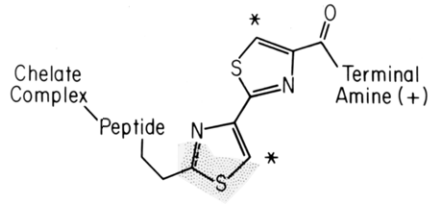

FIGURE 1: Bithiazole moiety of bleomycin, showing nonexchangeable hydrogens (*), double bond which is missing in phleomycin (dashed line), and region of aromatic overlap with DNA bases (shaded) as proposed by Murakami et al. (1973, 1976). Although the bithiazole is frequently drawn with the two nitrogens in a *cis* configuration with respect to the connecting bond, X-ray crystallography of a similar bithiazole derivative indicated a planar *trans* configuration, as shown above (Koyama et al., 1968).

nm. The temperature was 25 °C. The absorbance of all samples at the excitation wavelength was measured, and corrections for this absorbance were made by reference to calibration curves of fluorescence vs. absorbance of known bleomycin concentrations in the absence of DNA. This correction was never >15%. Cuvettes of 2-mm path length were used in most cases.

Stopped-Flow Studies. All measurements were taken on a Durrum stopped-flow spectrophotometer at 33 °C, unless otherwise stated. For association kinetics, DNA was rapidly mixed with a solution containing a metal–bleomycin complex. For dissociation kinetics, a solution containing DNA and a metal–bleomycin complex was mixed with one containing 2.5% sodium dodecyl sulfate. This detergent is thought to bind rapidly to any free positively charged ligand and prevent its reassociation with DNA, so that the relaxation signals observed spectrophotometrically upon mixing represent the intrinsic rates of dissociation of the ligand from DNA in the absence of detergent (Müller & Crothers, 1968).

Temperature-Jump Studies. The apparatus and procedure described by Li & Crothers (1969) were used. A 0.05 μF capacitor was discharged at 30 kV to give a temperature jump from 25 to 33 °C, and the absorbance changes at 290–340 nm were recorded. Sonicated, fractionated DNA was used in these studies.

DNA Length Measurements. Changes in DNA length were measured by determining the rotational diffusion coefficients of short rodlike DNA molecules (150 base pairs) in the presence and absence of bound metal–drug complexes. The DNA was oriented by rapid application of an electric field, and the orientation relaxation time was determined by recording the absorbance of light polarized parallel to the field, as described previously (Hogan et al., 1978).

DNA Base Release Assays. Reaction mixtures (0.12 mL) consisted of 0–44 μM phleomycin, 40 mM tris(hydroxymethyl)aminomethane (Tris) pH 8, 10 mM 2-mercaptoethanol, 50 μM ferrous ammonium sulfate, and 1.6–2.2 mM DNA, a mixture of [^{14}C]thymine–DNA (2000 or 4000 cpm) and either [^{3}H]cytosine–DNA (6000 cpm) or [^{3}H]adenine–guanine–DNA (34 000 cpm). After 3 h at 22 °C, release of various bases was measured by paper chromatography, as described previously (Povirk et al., 1978).

Results

Equilibrium Binding Measurements. Fluorescence quenching studies (Figure 2) indicated that in 20 mM Tris, complexation of bleomycin with either Cu(II) (Kasai et al., 1978) or Fe(III) enhanced its affinity for DNA. A Scatchard plot of the Cu(II)·bleomycin data was fitted to a neighbor exclusion binding isotherm (McGhee & Von Hippel, 1974; Jovin & Striker, 1977). The best fit was obtained by assuming K_{app} = 20 000 M^{-1} and n = 4, where K_{app} is the affinity for

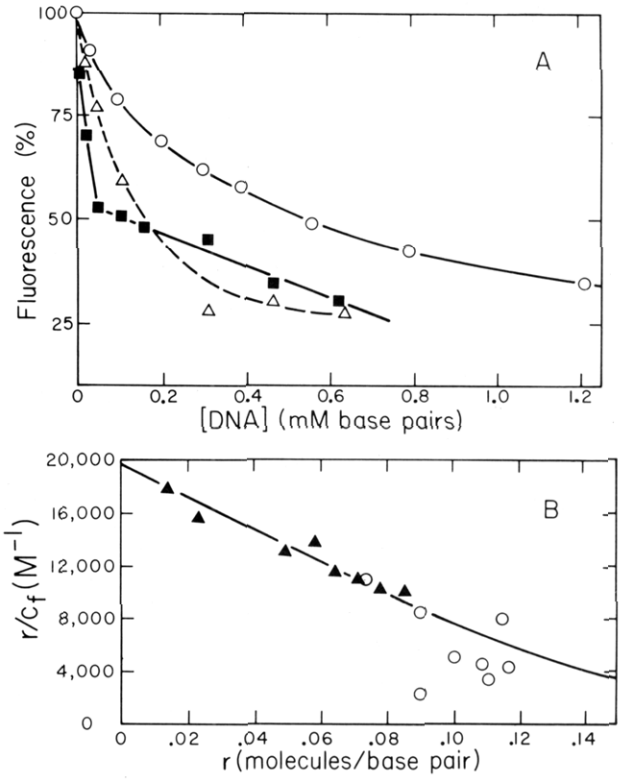

FIGURE 2: Binding of metal–bleomycin complexes to DNA in 20 mM Tris, pH 8. (A) Fluorescence of 17 μM bleomycin (○), Cu(II)·bleomycin (△), or Fe(III)·bleomycin (■) in the presence of various concentrations of DNA. (B) Scatchard plot of the binding of Cu(II)·bleomycin to DNA. Aliquots of bleomycin were added to 0.19 (▲) or 0.043 (○) mM DNA, and the fraction bound was calculated by assuming 80% quenching upon binding. r is the number of bound Cu(II)·bleomycin complexes per base pair, and c_f is the concentration of free Cu(II)·bleomycin. The data were fit to a neighbor exclusion isotherm, with K_{app} = 20 000 and n = 4. The dotted line in (A) indicates the curve expected from these binding parameters. The excitation wavelength was 300 nm.

the binding of Cu(II)·bleomycin to any given site on DNA in the limit of low r (i.e., the y intercept of the Scatchard plot) and n is the size of the binding site in base pairs (Jovin & Striker, 1977; Li & Crothers, 1969).

For Fe(III)·bleomycin, the fluorescence quenching curve had a steep negative slope, indicating very tight binding of this complex to DNA. A sharp knee occurred in the curve at a ratio of 2.5 base pairs/Fe(III)·bleomycin complex. The continued slow decrerase in fluorescence with increasing DNA concentration may be due to rearrangement of Fe(III)·bleomycin binding to sites of higher affinity and greater quenching.

In 50 mM NaCl–25 mM Tris, the affinity of Cu(II)·bleomycin for DNA was much lower (Figure 3A). A complete Scatchard plot could not be obtained, due to the high absorbance of bleomycin at 310 nm for large values of r. Since the fluorescence never reached a minimum value, but was still decreasing even at the highest DNA concentrations used, the degree of quenching upon binding of Cu(II)·bleomycin to DNA in this salt could not be independently determined. However, by fitting the fluorescence data to curves which assume various affinities, a reasonable estimate for K_{app} could be obtained. The value of n was assumed to be 4, as in the low-salt buffer, but the curves are relatively insensitive to the value of n chosen as long as the concentration of DNA is much greater than that of bleomycin.

These data indicated a K_{app} of ~1000 M^{-1} (Figure 3A), in good agreement with the value of 1200 M^{-1} (Figure 3B) obtained by equilibrium dialysis. Measurements at higher r values by dialysis were precluded by the large amounts of

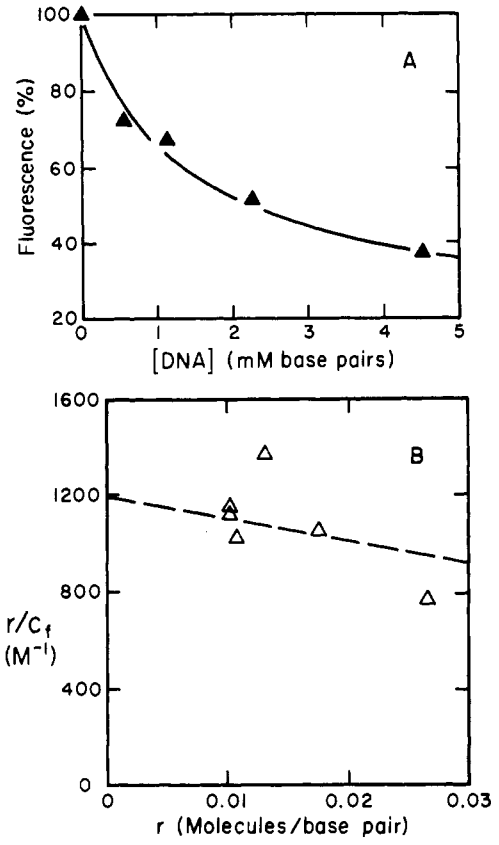

FIGURE 3: Binding of Cu(II)·bleomycin to DNA in 50 mM NaCl–25 mM Tris, pH 8. (A) Fluorescence of 52 μM Cu(II)·bleomycin (exitation at 310 nm) in the presence of various concentrations of DNA. With the assumption that $n = 4$, binding curves expected from various values of K_{app} were fit to the data. The best fit was obtained with a K_{app} of 1000 M^{-1} and an intrinsic quenching of 75% (solid line). (B) Binding of Cu(II)·bleomycin as measured by equilibrium dialysis. The dotted line indicates the best fit to the data under the constraint that $n = 4$.

bleomycin required. In a similar fluorescence titration (not shown), Cu(II)·phleomycin showed roughly the same degree of quenching by DNA as Cu(II)·bleomycin, but due to its weak fluorescence, an accurate estimate of K_{app} could not be obtained.

DNA Lengthening. Binding of both Cu(II)·bleomycin and Fe(III)·bleomycin increased the length of DNA (Figure 4) as measured by its orientation time upon application of an electric field (Hogan et al., 1978). Fluorescence studies (Figure 4B) with Cu(II)·bleomycin indicated the DNA concentration was sufficient so that very nearly all the added Cu(II)·bleomycin would be bound. It is likely that the observed lengthening was due to intercalation of the bithiazole rings, as we have shown for lengthening by metal-free bleomycin (Povirk et al., 1979). The length change per bound molecule was the same for Fe(III)·bleomycin as for metal-free bleomycin, ~3.2 Å. However, Cu(II)·bleomycin lengthened DNA by 4.6 Å, implying that it differs somewhat from the Fe(III) and metal-free complexes in the stereochemistry of its interaction with DNA.

Cu(II)·phleomycin did not lengthen DNA at all at either pH 5.5 or pH 8 (Figure 4A), even though its affinity for DNA was comparable to that of bleomycin (Figure 4B). This result suggests that phleomycin does not intercalate. Using phleomycin A_2 whose copper had been removed by dithizone extraction, we also failed to observe unwinding of closed circular DNA, another diagnostic for intercalation (Waring, 1970), under conditions where bleomycin did induce unwinding [see Povirk et al. (1979)]. Phleomycin increased the sedimentation

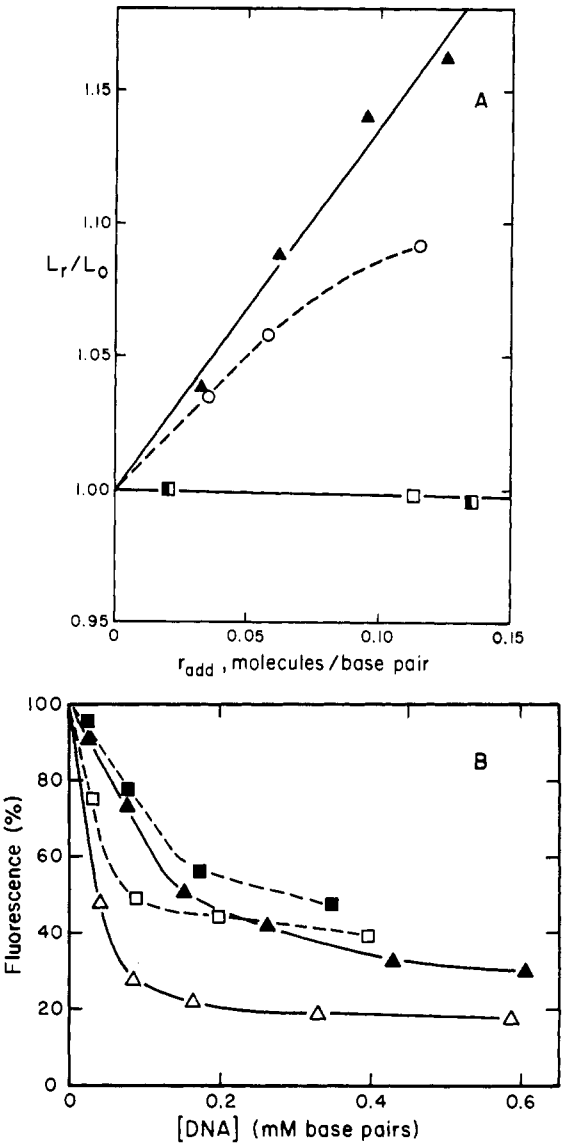

FIGURE 4: (A) Length changes induced in DNA by binding of Cu(II)·bleomycin (▲), Fe(III)·bleomycin (○), or Cu(II)·phleomycin (■, □). r_{add} is the number of moles of complex added to the solution per mole of base pairs. The buffer was either 2.5 mM sodium cacodylate, adjusted to pH 5.4 with HCl (▲, ○, ■), or 2 mM NaCl–2 mM Tris, pH 8 (□). The DNA concentrations were 1.0×10^{-4}–6×10^{-4} M base pairs. (B) Fluorescence quenching of 19 μM (△) or 84 μM (▲) Cu(II)·bleomycin or 84 μM Cu(II)·phleomycin (■, □) by DNA at pH 5.4 (△, ▲, ■) or pH 8 (□) in the same buffer solutions as in (A). Excitation was at 300 nm.

rate of relaxed DNA to about the same extent as bleomycin, so it was clearly binding to DNA, yet the relative sedimentation rates of supercoiled and relaxed DNA molecules remained unchanged (data not shown).

Binding Kinetics. When a temperature jump was produced by electrical discharge in a solution containing Cu(II)·bleomycin and DNA, three relaxation times in the absorbance at 318 nm were observed. No additional relaxation times were found at any wavelength from 300 to 360 nm. The fastest relaxation time was independent of both temperature and concentration and probably was not associated with binding. The other two relaxation times, opposite in sign and of comparable amplitudes, showed a concentration dependence characteristic of a two-step intercalative binding mechanism (Figure 5).

The expected relaxation kinetics of such a mechanism have been treated by Li & Crothers (1969) and Jovin & Striker

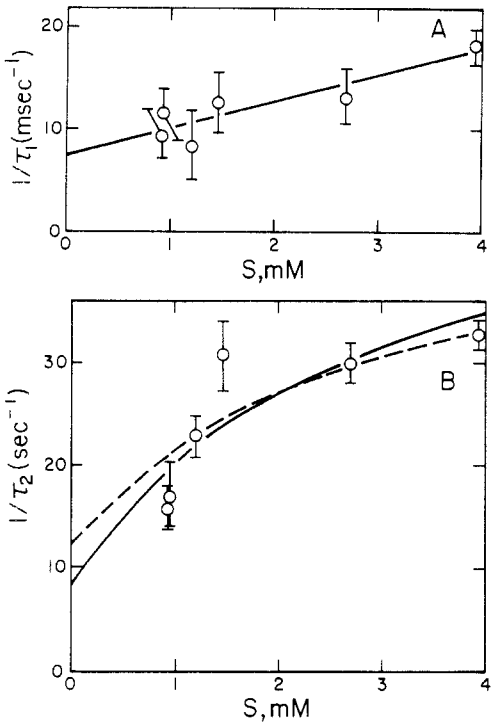

FIGURE 5: Concentration dependence of the fast (A) and slow (B) relaxation times for binding of Cu(II)·bleomycin to DNA, seen after a temperature jump of 8.45 °C. The ratio of Cu(II)·bleomycin to DNA (r_{add}) was held at 0.015, and the buffer was 50 mM NaCl–25 mM Tris, pH 8. Solid lines show least-squares fits of the data to equations for a two-step binding process, while dashed line shows the best fit under the constraint that $k_{32} = 12.5$ s^{-1} (see text). S is the concentration of free binding sites.

(1977). The binding equilibrium may be represented by

$$\text{DNA} + \text{Cu(II)·bleomycin} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{C}_{\text{out}} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \text{C}_{\text{in}}$$

where C_{out} and C_{in} are the outside-bound and intercalated forms of the Cu(II)·bleomycin·DNA complex. If the first step in binding is much faster than the second, a small instantaneous perturbation from equilibrium should result in two relaxation times ($\tau_2 \gg \tau_1$) given by

$$\frac{1}{\tau_1} = k_{12}[S - f'(r)L] + k_{21}$$

$$\frac{1}{\tau_2} = k_{32} + \frac{k_{23}[S - f'(r)L]}{K_{12} + [S - f'(r)L]}$$

$$S = S_0 f(r)$$

where S_0 is the total DNA concentration in base pairs, $f(r)$ is a statistical function [calculated from the neighbor exclusion binding model of Jovin & Striker (1977)] giving the concentration of remaining free binding sites S at a given value of r, $f'(r)$ is its first derivative, L is the concentration of free ligand, r is the number of ligands bound per base pair, and $K_{12} = k_{12}/k_{21}$. In our case, the second term $[f'(r)L]$ is small compared to S, and most of the Cu(II)·bleomycin is bound, so that $f(r)$ may be approximated by $f(r_{add}) = f(0.015) = 0.91$ for $n = 4$ where r_{add} is the total number of moles of ligand per mole of DNA base pairs (Jovin & Striker, 1977). Least-squares fits of the data to the above equations (Figure 4) gave $k_{12} = 2.6 \times 10^6$ M^{-1} s^{-1}, $k_{21} = 7.5 \times 10^3$ s^{-1}, $k_{23} = 45$ s^{-1}, and $k_{32} = 8.4$ s^{-1}. K_{app} should be predicted by $K_{app} = K_{12}(1 + K_{23})$ = 2200 M^{-1}, where $K_{23} = k_{23}/k_{32}$ (Li & Crothers, 1969).

This value is somewhat larger than those obtained from equilibrium measurements by dialysis (1200 M^{-1}) or fluorescence (1000 M^{-1}). However, the scatter in the kinetic

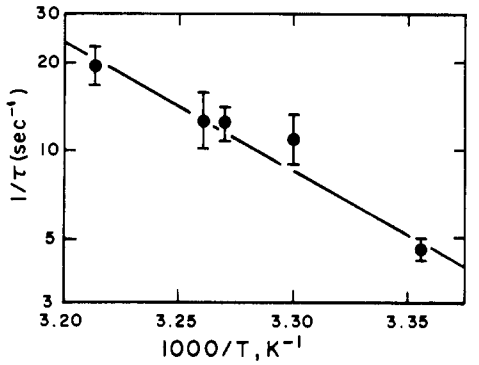

FIGURE 6: Arrhennius plot of the temperature dependence of the slow step in the dissociation of Cu(II)·bleomycin from DNA. A solution containing 2 mM DNA and 0.04 mM Cu(II)·bleomycin was rapidly mixed with one containing 2.5% sodium dodecyl sulfate, both in 50 mM NaCl–25 mM Tris, pH 8, and the A_{290} was recorded as a function of time. A signal of opposite sign was seen at 320 nm. The slope indicates an activation energy of 19 kcal/mol.

data is considerable. In particular, the estimate for K_{23} is very sensitive to the y intercept (k_{32}) of the τ_2 plot (Figure 5B) which is not very accurately determined. An independent measurement of k_{32} was therefore obtained by dissociating Cu(II)·bleomycin from DNA by mixing with sodium dodecyl sulfate in a stopped-flow spectrophotometer and recording the A_{290} or A_{320} (Müller & Crothers, 1968). At 33 °C, the data showed a single relaxation time, giving $k_{32} = 12.5$ s^{-1}. The temperature-jump data, when constrained to this estimate of k_{32}, predict $k_{23} = 35$ s^{-1}, $K_{23} = 2.8$, and $K_{app} = 1320$ M^{-1}, in good agreement with equilibrium measurements (Figure 5). An Arrhennius plot (Figure 6) of k_{32}, as estimated from stopped-flow data, gave an activation energy of 19 kcal/mol for the slow dissociation step, similar to that measured for proflavin (Li & Crothers, 1969).

Temperature-jump studies with DNA and Cu(II)·phleomycin yielded a single relaxation time $\tau \approx 100$–250 μs, but the data were too noisy to predict reasonably K_{app}. The lack of a slow relaxation time with Cu(II)·phleomycin is consistent with the proposition that the slow step in the Cu(II)·bleomycin kinetics is the intercalation step. However, considering the lack of quantitative data on binding of Cu(II)·phleomycin to DNA, the presence of a slow step with little absorbance change or whose equilibrium has little temperature dependence cannot be ruled out.

With DNA and Fe(III)·bleomycin, no temperature-jump signal was observed except for the very fast component (τ = 18 μs) probably not associated with any binding step. Stopped-flow measurements at 290 nm and at concentrations similar to those used for Cu(II)·bleomycin (2 mM DNA and 0.04 mM (Fe(III)·bleomycin) indicated a single association time of τ = 115 ± 29 ms and three dissociation times of approximately equal amplitude: 0.25 ± 0.06, 2.4 ± 0.9, and 22 ± 4 s. In the absence of DNA, no relaxation signal was observed. Although it is not known whether these three processes are independent or sequential, it is clear that there are steps in the dissociation of Fe(III)·bleomycin from DNA which are considerably slower than those of Cu(II)·bleomycin.

DNA Base Release by Phleomycin. Because of the apparent differences in the binding of bleomycin and phleomycin to DNA, we compared their specificities of base release and found them to be very similar (Table I). The only unequivocal difference was the smaller cytosine to thymine ratio seen with phleomycin. A redetermination of this ratio for bleomycin by using the same DNA preparation and exact experimental conditions as the phleomycin experiments confirmed our earlier

Table I: Release of DNA Bases and Corresponding Minor Products by Phleomycin[a]

product	relative yield	
	phleomycin	bleomycin[c]
thymine	(1.00)	(1.00)
thymine minor product	0.09 ± 0.03	0.10 ± 0.03
cytosine	0.41 ± 0.04	0.63 ± 0.07[e]
cytosine minor product	0.05 ± 0.01[b]	0.09 ± 0.03
adenine	0.12 ± 0.02	0.15 ± 0.02
guanine	0.05 ± 0.02	0.08 ± 0.01
purine minor product	0.02 ± 0.01[d]	0.04 ± 0.02[d]

[a] Yields were normalized to the release of [^{14}C]thymine (between 2 and 20%) from [^{14}C]thymine–DNA, which was included in each experiment as an internal control. All other bases bore a ^{3}H label. Each entry is the average of four to six determinations, except where noted otherwise. [b] Two determinations. [c] From Povirk et al. (1978). [d] As a fraction of total purine label. [e] A repetition of this experiment, using the same DNA preparations as were used in the phleomycin experiments, gave 0.62 ± 0.07 for three determinations.

results (Povirk, et al., 1978), suggesting that this difference, though small, is real.

Discussion

Of the three complexes, only for Cu(II)·bleomycin were we able to obtain a reasonably complete description of the kinetics of binding to DNA. Since the two binding steps observed spectrophotometrically (Figures 5 and 6) were sufficient to account for the measured equilibrium binding constant (Figure 3), it is unlikely that any major binding steps were overlooked. These data, combined with DNA-lengthening studies (Figure 4), suggest a classical two-step intercalation process (Li & Crothers, 1969) with the slow step representing transfer between the outside-bound and intercalated forms. The small value of $K_{23} = 3$ (the ratio of intercalated to outside-bound complexes) is much smaller than that seen with proflavin (K_{23} = 10–20; Li & Crothers, 1969) and implies that the contribution of intercalation to the total binding energy is small. This is not a surprising conclusion considering the small aromatic surface of the bithiazole and the possible steric hindrances of its side chains.

Even the slow step for binding of Cu(II)·bleomycin to DNA ($k_{23} = 35\ s^{-1}$) is much faster than the rate of oxidation of free Fe(II)·bleomycin ($\tau = 35$ s, $k_0 = 1/\tau = 0.03\ s^{-1}$) (Povirk, 1979). The fast association step is concentration dependent but will be faster than the oxidation reaction as long as the DNA concentration is $> k_0/k_{12} = 0.03\ s^{-1}/(2.6 \times 10^5\ M^{-1}\ s^{-1}) \cong 10^{-7}$ M. Thus, except at extremely low DNA concentrations, Fe(II)·bleomycin (with the assumption that its binding kinetics are similar to those of Cu(II)·bleomycin) will have ample time to equilibrate between free and DNA-bound forms before oxidizing. Although the equilibrium binding constant may be low ($K_{app} \simeq 10^3\ M^{-1}$, with the assumption that it is similar to that of Cu(II)·bleomycin), it is compensated by the fact that oxidation of DNA-bound Fe(II)·bleomycin is 60 times faster than oxidation of the free complex (Povirk, 1979). Thus, most of the oxidations will occur when the complex is bound to DNA as long as the DNA concentration is $> 1/(60K_{app}) \cong 17$ μM (in the high-salt buffer). Since in a eukaryotic nucleus the DNA concentration is 3 orders of magnitude higher than this figure (Novikoff, 1976), it would appear that any Fe(II)·bleomycin formed in the nucleus has a very high probability of attacking DNA. Fe(II)·bleomycin could be formed either by spontaneous combination of Fe(II) with metal-free bleomycin (Sausville et al., 1976) or by reduction of Fe(III)·bleomycin by sulfydryls (Povirk, 1979).

Although the assumption that Cu(II)·bleomycin and Fe(II)·bleomycin have similar binding kinetics is a tentative one, there is considerable evidence that the two complexes are similar. Nuclear magnetic resonance and polarographic studies (Dabrowiak et al., 1978, 1979) indicate that most of the same ligands are involved. The degree of proton release upon chelation is similar for both complexes, so they probably have the same final net charge (Oppenheimer, 1979). Iitaka et al. (1978) have determined the structure of the Cu(II) complex of a bleomycin fragment by X-ray crystallography, and Takita et al. (1978) have proposed a nearly identical structure for Fe(II)·bleomycin. However, more recent nuclear magnetic resonance studies of the tertiary CO·Fe(II)·bleomycin complex (Oppenheimer et al., 1979) suggest differences in the identity or arrangement of three of the chelating ligands, compared to the structure determined by Iitaka et al. (1978). It is difficult to predict what effect specific ligand changes might have on binding of metal–bleomycin complexes to DNA, but a clear demonstration of substantial differences between Fe(II)·bleomycin and Cu(II)·bleomycin would of course weaken arguments on an analogy between them.

Our description of the binding kinetics of Fe(III)·bleomycin is much less complete. This complex appears more tightly bound to DNA than Cu(II)·bleomycin (Figure 2A). The higher affinity may be a consequence of the additional positive charge on the chelated ion. The multiple dissociation times observed suggest that there are several forms of the Fe(III)·bleomycin·DNA complex, with lifetimes of up to 22 s. These long-lived complexes may play a role in sequestering radicals generated during Fe(II)·bleomycin oxidation, increasing the probability that these radicals will attack DNA.

The differences seen in the DNA binding properties of the two bleomycin complexes indicate significant interaction between DNA and the chelating groups of the metal–bleomycin complex. Such interactions are also suggested by the increase in the oxidation rate of Fe(II)·bleomycin upon binding to DNA (Povirk, 1979) and by the fact that damage to DNA by Fe(II) plus bleomycin is different from, and more specific than, damage by Fe(II) alone (Takeshita et al., 1978; D'Andrea & Haseltine, 1978). Furthermore, electron spin resonance studies by H. Shields and F. Hutchinson (unpublished experiments) have shown that the plane of symmetry of the Cu(II)·bleomycin coordination complex [see Iitaka et al. (1978)] is always aligned nearly perpendicular to the DNA helix axis, again suggesting stereospecific interaction between DNA and the Cu(II) chelating groups of bleomycin.

DNA lengthening studies (Figure 4) strongly suggest that the two bleomycin complexes intercalate, while Cu(II)·phleomycin does not. The length change seen with Cu(II)·bleomycin (4.6 Å) is larger than that of most intercalators but similar to that of actinomycin (Hogan et al., 1979). If only three-fourths of the bound Cu(II)·bleomycin is intercalated, as indicated by the kinetic studies, the actual length change per intercalated molecule would be 6.2 Å, much larger than any other intercalator and nearly double the van der Waals thickness of an aromatic ring. More likely, the much lower ionic strength used in DNA lengthening studies increases DNA phosphate repulsion such that a lengthening process such as intercalation is greatly favored, and nearly 100% of the added Cu(II)·bleomycin is intercalated. Thus, 4.6 Å is probably the actual length change.

Recently, Chen et al. (1980) reported nuclear magnetic resonance studies with metal-free bleomycin which they considered inconsistent with intercalation. Specifically, they found that the upfield shifts of the bithiazole hydrogens in the

poly(dA-dT)·bleomycin complex were much smaller than those of the proflavin ring hydrogens in the poly(dA-dT)·proflavin complex (Patel & Canuel, 1977), implying that at least the hydrogens of the bithiazole (Figure 1) are not in intimate contact with the aromatic surfaces of DNA bases. However, in the same study the chemical shift seen for the thymine H-6 proton in poly(dA-dT)·bleomycin suggests that interactions between this proton and ring currents of the adjacent 5′-adenine have been eliminated [see Patel & Tonelli (1975)], as would be expected from insertion of a small intercalator between base pairs at each $A^{(3'-5')}T$ site. If poly(dA-dT)·bleomycin is an intercalated complex, absence of any effects of DNA base ring currents on bithiazole proton resonances would imply a very restricted aromatic overlap but would not exclude intercalation of at least the N–C double bond nearest the chelating end of bleomycin (see Figure 1), since this bond is well removed from both hydrogens. The nonintercalation of phleomycin (Figures 1 and 4) and model building studies (Murakami et al., 1973, 1976) also suggest that bleomycin intercalation involves this double bond. It is possible that poly(dA-dT) due to its unique structural properties, binds bleomycin differently than natural DNA, but for calf thymus DNA, it is difficult to explain DNA lengthening and linear dichroism data (Figure 4, Povirk et al., 1979) without proposing at least partial intercalation of the bithiazole.

The finding that phleomycin has nearly the same specificity of base release as bleomycin (Table I) suggests that intercalation is not primarily responsible for this specificity. The detailed sequence preference of bleomycin (e.g., the prefernce for a guanine adjacent to the released base) (Takeshita et al., 1978; D'Andrea & Haseltine, 1978) is also closely mimicked by phleomycin (A. P. Grollman, personal communication). Considering the structural differences between drug–DNA complexes having intercalated and nonintercalated geometries, it is surprising that phleomycin shows almost exactly the same sequence preference and distribution of products as bleomycin. Changes in the terminal amine also do not appear to significantly alter the sequence preference of bleomycin (Takeshita et al., 1978). Thus, interactions between DNA and the chelating groups of iron–bleomycin complexes may be the more important determinants of bleomycin specificity. If the sole function of the bithiazole and terminal amine is to help bind the drug to DNA, it might be possible to substitute for them other DNA-binding moieties without destroying drug activity.

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References

Chen, D. M., Sakai, T. T., Glickson, J. D., & Patel, D. J. (1980) *Biochem. Biophys. Res. Commun. 92*, 197.

Chien, M., Grollman, A. P., & Horwitz, S. B. (1977) *Biochemistry 16*, 3641.

Dabrowiak, J. C., Greenaway, F. T., & Grulich, R. (1978) *Biochemistry 17*, 4090.

Dabrowiak, J. C., Greenaway, F. T., Santillo, F. S., & Crooke, S. T. (1979) *Biochem. Biophys. Res. Commun. 91*, 721.

D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608.

Hogan, M., Dattagupta, N., & Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 195.

Hogan, M., Dattagupta, N., & Crothers, D. M. (1979) *Biochemistry 18*, 280.

Iitaka, Y., Nakamura, H., Nakatani, T., Muraoka, Y., Fujii, A., Takita, T., & Umezawa, H. (1978) *J. Antibiot. 31*, 1070.

Jovin, T. M., & Striker, G. (1977) in *Chemical Relaxation in Molecular Biology* (Pecht, I., & Rigler, R., Eds.) pp 245–281, Springer-Verlag, Berlin.

Kasai, H., Naganawa, H., Takita, T., & Umezawa, H. (1978) *J. Antibiot. 31*, 1316.

Koyama, G., Nakamura, H., Muraoka, Y., Takita, T., Maeda, K., & Umezawa, H. (1968) *Tetrahedron Lett. 44*, 4635.

Li, J. L., & Crothers, D. M. (1969) *J. Mol. Biol. 39*, 461.

McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469.

Müller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251.

Murakami, H., Mori, H., & Taira, S. (1973) *J. Theor. Biol. 42*, 443.

Murakami, H., Mori, H., & Taira, S. (1976) *J. Theor. Biol. 59*, 1.

Novikoff, A. B. (1976) *Cells and Organelles*, 2nd ed., p 61.

Oppenheimer, N. J. (1979) in *Bleomycin: Chemical, Biochemical and Biological Aspects* (Hecht, S., Ed.) p 124, Springer-Verlag, New York.

Oppenheimer, N. J., Rodriguez, L. O., & Hecht, S. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5616.

Patel, D. J., & Tonelli, A. E. (1975) *Biochemistry 14*, 3990.

Patel, D. J., & Canuel, L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2624.

Povirk, L. F. (1979) *Biochemistry 18*, 3989.

Povirk, L. F., Köhnlein, W., & Hutchinson, F. (1978) *Biochim. Biophys. Acta 521*, 126.

Povirk, L. F., Hogan, M., & Dattagupta, N. (1979) *Biochemistry 18*, 96.

Sausville, E. A., Peisach, J., & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun. 73*, 814.

Sausville, E. A., Peisach, J., & Horwitz, S. B. (1978a) *Biochemistry 17*, 2740.

Sausville, E. A., Stein, R. W., Peisach, J., & Horwitz, S. B. (1978b) *Biochemistry 17*, 2747.

Stern, R., Rose, J. A., & Friedman, R. M. (1974) *Biochemistry 13*, 307.

Suzuki, H., Nagai, K., Yamaki, H., Tanaka, N., & Umezawa, H. (1969) *J. Antibiot. 22*, 446.

Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983.

Takita, T., Muraoka, Y., Yoshioka, T., Fujii, A., Maeda, K., & Umezawa, H. (1972) *J. Antibiot. 25*, 755.

Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Iitaka, Y., & Umezawa, H. (1978) *J. Antibiot. 31*, 1073

Waring, M. (1970) *J. Mol. Biol. 54*, 247.