Binding of Bleomycin to DNA: Intercalation of the Bithiazole Rings†

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ABSTRACT: At pH 5.5, binding of bleomycin relaxed supercoiled ColE1 DNA without breaking it. Binding of tripeptide S, a fragment of the drug containing the bithiazole rings, also relaxed and then recoiled supercoiled DNA, at pH 5.5 and at pH 8.0, where bleomycin is normally active. The unwinding angle was 12°. Both compounds lengthened linear DNA by 3.1 Å per molecule bound, and linear dichroism (303–315 nm) of bleomycin bound to linear DNA oriented in an electric field indicated the presence of a chromophore making an angle of 59–61° with the helix axis. These results strongly suggest that bleomycin binding to DNA involves intercalation of the bithiazole rings. In 0.1 M NaCl at pH 8, supercoiled ColE1 DNA was broken at a rate 50% greater than relaxed closed circular ColE1 DNA. Since supercoiling increases the affinity of DNA for intercalators, this result suggests that intercalative binding is involved in bleomycin-induced breakage of DNA.

Bleomycin is an antitumor drug which produces single- and double-strand breaks in DNA (Suzuki et al., 1969; Haidle, 1971; Povirk et al., 1977) and releases free bases (Haidle et al., 1972; Müller et al., 1972). A free radical mechanism for these reactions (Sausville et al., 1976; Lown & Sim, 1977) and detailed enzyme-like mechanisms (Müller & Zahn, 1977; Murakami et al., 1973) have been proposed, but none have been either proven or ruled out. Bleomycin binds to both DNA and to divalent metal ions (Chien et al., 1977; Nunn, 1976; Sausville et al., 1976), and knowledge of the stereochemistry of these interactions could aid greatly in elucidating the reaction mechanism. The bithiazole rings of bleomycin are coplanar (Koyama et al., 1968), and Murakami et al. (1976) proposed, on the basis of model studies, that they intercalate. Chien et al. (1977) showed that these rings interact strongly with DNA, but considered intercalation unlikely. However, we find that the rings exhibit properties expected of an intercalator.

Materials and Methods

Buffers. Sodium citrate (0.8 mM and 8 mM) was adjusted to pH 5.5 with HCl. NaCl (1 M)–10 mM Tris base–1 mM Na_2EDTA was adjusted to pH 7.1 with HCl. Tris base (2 mM)–1 mM free EDTA was adjusted to pH 8.0 with NaOH. All pHs are those measured at 22 °C with a glass–calomel electrode. The pH of sucrose solutions was adjusted after addition of sucrose.

DNA. Supercoiled ColE1 was purified from *E. coli* JC411 using CsCl–ethidium bromide gradients (Blair et al., 1972) and stored frozen in 10 mM Tris, pH 8.0. Relaxed closed circular ColE1 was prepared by treating supercoils (50 μg/mL) with *Agrobacterium tumefaciens* DNA relaxing enzyme (Bethesda Research) at a concentration of 1 unit of enzyme/μg of DNA, for 1 h at 37 °C in 20 mM Tris–2 mM $MgCl_2$, pH 7.9. For partially relaxed DNA, various amounts of ethidium bromide were added to the reaction mixture. At this DNA concentration, nearly quantitative binding of ethidium is expected (LePecq & Paoletti, 1967). Ethidium was removed by phenol extraction, phenol removed by diethyl ether extraction, and ether removed by heating to 60 °C for 5 min. Samples to be used in strand-breakage experiments were dialyzed overnight to remove $MgCl_2$.

Calf thymus DNA (Sigma) was dissolved in 0.05 M sodium phosphate, pH 8.0, extracted four times with phenol and dialyzed against the appropriate buffer. Preparation of 150-base-pair-long calf thymus DNA for use in dichroism experiments has been described (Hogan et al., 1978). DNA concentrations are expressed in moles of nucleotides per liter.

Drugs. Bleomycin was clinical Blenoxane (Bristol lot A6X00 or BL19125). Tripeptide S was prepared from purified bleomycin A_2 sulfate (Bristol lot 531) by acid hydrolysis and Sephadex CM-25 chromatography as described by Chien et al. (1977). Concentrations were determined from the A_{290} (ϵ_{290} = 14 100 for bleomycin (Dabriowak et al., 1978); ϵ_{290} = 12 850 for tripeptide S (Muraoka et al., 1972)).

Binding Studies. Bleomycin binding to DNA was determined using two procedures. Bleomycin and 0.5–1.0 mM DNA, both in 0.8 mM citrate buffer, were placed in opposite 2-mL chambers of a Plexiglas dialysis cell, separated by a membrane cut from dialysis tubing. After 2 days of constant agitation at 11 °C, bleomycin concentrations in both chambers were determined from the A_{290}, after addition of 0.1 volume of 25% sodium dodecyl sulfate to dissociate bleomycin from DNA. This method was accurate only if the free bleomycin concentration was greater than 10 μM. For lower concentrations, 2 mL of 0.2–0.5 mM DNA was dialyzed against a large reservoir of bleomycin at a known concentration. After 2 days, DNA samples were removed and the bleomycin was dissociated by addition of 0.1 volume of 5 M NaCl–0.5 M Tris base. The fluorescence intensities (Turner 430 spectrofluorometer; excitation at 300 nm; emission at 353 nm (Chien et al., 1977)) from solutions both inside and outside the dialysis bag were measured and subtracted to give the bound bleomycin concentration. Since the free bleomycin concentration was too low to measure accurately, it was calculated by subtracting the amount of bound bleomycin from the amount initially added to the reservoir. Dialysis tubing was prepared by successive boiling in 1% Na_2EDTA, in water, in 5% $NaHCO_3$, and then in three more changes of distilled water (Huang & Ts'o, 1966). In samples where the free bleomycin concentration was measurable, the total A_{290} was conserved, indicating that there was no tendency for bleomycin to stick to the dialysis tubing.

The difficulty of purifying large quantities of tripeptide S precluded the use of equilibrium dialysis. Binding was instead measured at 22 °C in 10% sucrose and either 0.8 mM citrate or 2 mM Tris–1 mM EDTA by fluorescence quenching as

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described by Chien et al. (1977), using the same wavelengths as for bleomycin.

Superhelix Titrations. Sucrose gradients (5.3 mL of 5–20%) containing various drug concentrations were preformed in the appropriate buffer. Twenty-five microliters of a mixture (1.5 μg or less) of ^{14}C-labeled ColE1 DNA containing 70% supercoils and ^{3}H-labeled ColE1 DNA containing 70% nicked circles was layered atop the gradients and centrifuged for 190–270 min at 45 000 rpm in an SW50.1 rotor. Thirty-four equal volume fractions were collected and their radioactivity was counted in a liquid scintillation spectrometer. Since the ^{3}H and ^{14}C profiles should each be a linear combination of the supercoiled and nicked circular DNA profiles, one can use them to calculate the profile expected for a pure population of either supercoils or nicked circles. Profiles derived in this manner were used to calculate median sedimentation rates for these two topological forms. Each titration was done in a single centrifuge run.

Dichroism Studies. Details of electric linear dichroism measurements have been described (Hogan et al., 1978). Briefly, mixtures of bleomycin or tripeptide S and 150-base-pair-long molecules of DNA (10^{-5} to 10^{-4} M) in a modified temperature-jump apparatus were exposed to an electric field of 12–32 kV/cm, which oriented the DNA parallel to the field. The absorbance parallel to the field was measured before (A_0) and after ($A_{\parallel}$) orientation to give the reduced dichroism $\rho = {}^{3}/_{2}(A_{\parallel} - A_0)/A_0$. The angle α between the transition moment of the chromophore and the DNA helix axis is then given by $\rho = {}^{3}/_{2}(3\cos^2\alpha - 1)\Phi$, where Φ is a factor reflecting the degree of orientation of the DNA. ρ was determined at several field strengths, E, and extrapolated to $1/E = 0$ (i.e., to perfect DNA orientation). In these experiments, at least 97% of the bleomycin was bound.

Changes in DNA length upon bleomycin binding were measured by recording the kinetics of rise of dichroism after application of the field. The characteristic rotational correlation time taken for the DNA to orient in the field is $\tau_C = {}^{1}/_{6}\Theta$, where Θ is the rotational diffusion coefficient. For rod-like DNA, Θ can be used to calculate the length by use of the Broersma relation (Broersma, 1960)

$$\Theta = \frac{3kT}{8\pi\eta_0 L^3}\left\{\ln\left(\frac{L}{b}\right) - 1.57 + 7\left(\frac{1}{\ln(L/b)} - 0.28\right)^2\right\}$$

where L is the length, b is the rod radius of the macromolecule, and η_0 is the solvent viscosity. The accuracy of this relation for determining DNA length in the range of 130–230 base pairs has been verified using DNAs whose lengths were standardized against sequenced restriction fragments (Hogan et al., 1978). Thus, our measurements do not depend on any assumptions about the mechanism of orientation. All dichroism measurements were performed at 11 °C.

DNA Strand Break Measurements. Reaction mixtures contained, in 40 μL, 50 μM DNA (a mixture of supercoiled ^{14}C-labeled ColE1 and relaxed closed circular ^{3}H-labeled ColE1), 0–0.12 μM bleomycin, 0.1 M NaCl, 25 mM 2-mercaptoethanol, and 20 mM Tris, pH 8.0. After incubation for 30 min at 37 °C, the reaction was stopped by addition of 0.25 volume of 0.25 M EDTA, pH 8.0. The samples were layered atop 5–20% sucrose gradients containing 12 μM ethidium bromide and 8 mM citrate buffer, pH 5.5, and centrifuged at 45 000 rpm for 255 min at 11 °C as described above. Presence of ethidium induced strong positive superhelicity in both native supercoiled and "relaxed" closed circular forms, causing them to cosediment 40% faster than the nicked circular form, allowing accurate determination of strand-breakage rates in the two closed circular forms.

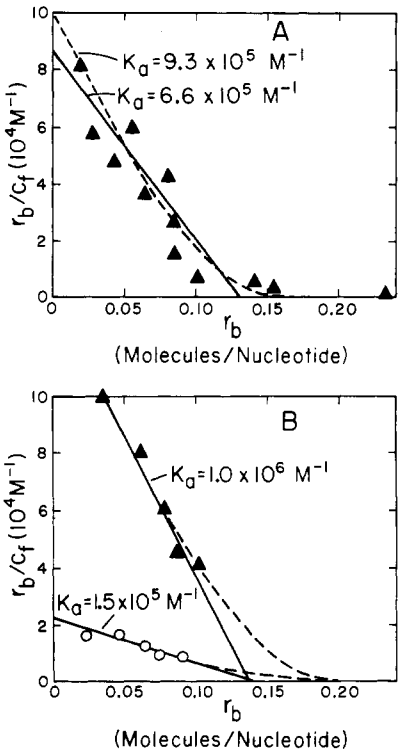

FIGURE 1: Scatchard plots of binding data obtained for (A) bleomycin by equilibrium dialysis at 11 °C or for (B) tripeptide S by fluorescence quenching at 22 °C. Buffers were 0.8 mM citrate, pH 5.5 (▲), and 2 mM Tris–1 mM EDTA, pH 8.0 (○). For tripeptide S binding, 10% sucrose was also included in the buffers. Data were fit to both linear (—) and neighbor exclusion (---) isotherms (Crothers, 1974). In the neighbor exclusion fits, the number of base pairs between potential intercalation sites was 3 for bleomycin and 2.5 for tripeptide S.

Results

Binding. At pH 5.5, affinities of bleomycin and tripeptide S for DNA were comparable, although they were measured by different methods under slightly different conditions (Figure 1). Increasing the pH to 8.0 decreased the affinity of tripeptide S by a factor of 6. The sizes of the binding sites obtained by linear fits to the data were 3.6 base pairs for tripeptide S and 3.9 pairs for bleomycin, in reasonable agreement with previous studies (Chien et al., 1977). The binding constant for the pH 8 buffer is also similar to that obtained by Chien et al. (1977) for 2.5 mM Tris, pH 8.4. The neighbor exclusion model (Crothers, 1974) appeared to give a slightly better fit to the bleomycin binding data.

Relaxation of Supercoiled DNA. One test for an intercalator is its ability to relax supercoiled DNA without nicking the DNA strand (Waring, 1970). At low pH, bleomycin binds to DNA, but its nicking activity is greatly inhibited (Umezawa et al., 1973; Chien et al., 1977). When ColE1 supercoils were sedimented in gradients at pH 5.5, presence of bleomycin caused a movement of the supercoil peak to coincidence with that of nicked circles (Figures 2 and 3A). It is unlikely that this effect was due to nicking of the supercoils. In that case, one would expect, with increasing bleomycin concentration, a gradual loss of material from a stationary supercoil peak, rather than the observed rather abrupt movement of the entire peak, without broadening or loss of material. At higher bleomycin concentrations (not shown), we did not observe the recoiling of the supercoils and resulting increase in their sedimentation coefficients normally expected with an intercalator. Since we are working near saturation binding, high

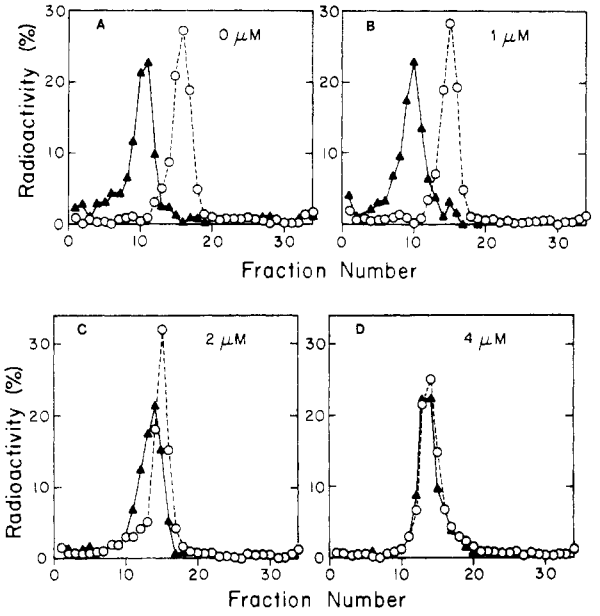

FIGURE 2: Sedimentation profiles of supercoiled (▲) and nicked (○) ColE1 DNA in the presence of the indicated concentrations of bleomycin in 0.8 mM citrate, pH 5.5. Centrifugation was for 255 min at 45 000 rpm at 11 °C. Sedimentation is to the left.

concentrations (10–20 μM) of bleomycin were required to raise the binding ratio (r_b) above 0.09 molecule per nucleotide, and these high concentrations may have caused enough nicking, even at low pH, to obscure the recoiling effect.

To circumvent this problem, and to implicate the bithiazole rings as the intercalating moiety, similar experiments were performed with tripeptide S, an inactive fragment of bleomycin containing the bithiazole rings as its sole aromatic component (Umezawa, 1974). At pH 5.5 and at pH 8.0, where bleomycin is highly active, tripeptide S binding first relaxed and then recoiled supercoiled DNA (Figures 3B and 3C), eliminating any possibility that relaxation was due to nicking. Presence of a chelator (EDTA or citrate) in the gradients was found to be necessary for DNA relaxation, possibly due to inhibition of tripeptide S binding by divalent cations (Chien et al., 1977).

Like other small intercalators, bound tripeptide S decreased the sedimention coefficient of relaxed circles slightly (Figures 3B and 3C). The slight increase in this coefficient seen with bleomycin (Figure 3A) is similar to that seen for other intercalators having large sugar or peptide adducts (Waring, 1970).

The angle ϕ of DNA unwinding caused by each bound tripeptide S molecule can be calculated from the superhelix titration data, if the superhelicity σ of the DNA is known (Waring, 1970). To determine σ, a set of partially relaxed closed circular DNAs was prepared using DNA relaxing enzyme. Fully relaxed DNA prepared in the absence of ethidium cosedimented with nicked circular DNA in 0.8 mM citrate gradients, while a series of samples prepared in the presence of 0.002–0.008 ethidium molecule per nucleotide sedimented progressively faster, indicating that the superhelicity was the same in the sedimentation buffer as in the relaxation buffer. Both native supercoiled and fully relaxed closed circular DNAs were then titrated with ethidium (data not shown) in gradients containing 1 M NaCl–10 mM Tris–1 mM Na_2EDTA, pH 7.1. Using the binding data of Hinton & Bode (1975a,b), it was determined that native supercoiled DNA was relaxed at r_b = 0.075 ethidium molecule per nucleotide while "fully relaxed" DNA was relaxed in this buffer at r_b = 0.025. Assuming an unwinding angle of 26° (Wang,

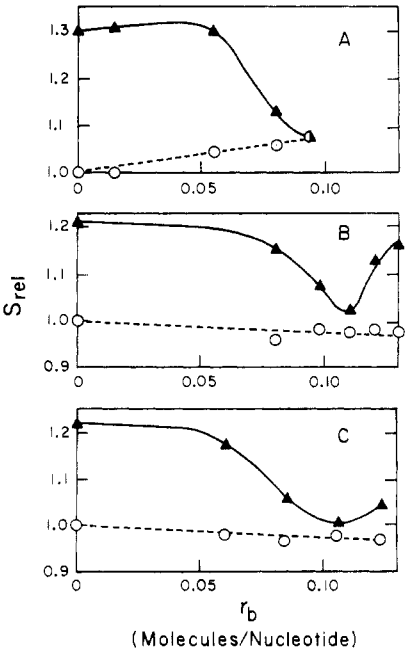

FIGURE 3: Unwinding of supercoiled ColE1 DNA as measured by changes in its sedimentation rate. (A) Bleomycin in 0.8 mM citrate, pH 5.5, 11 °C; (B) tripeptide S in 0.8 mM citrate, pH 5.5, 22 °C; (C) tripeptide S in 2 mM Tris, 1 mM EDTA, pH 8.0, 22 °C; (▲) native supercoiled DNA; (○) nicked DNA. All sedimentation coefficients are normalized to that of nicked DNA at zero r_b. r_b was calculated from the known free concentration in the gradient using linear fits to the binding data (Figure 1).

1974) for ethidium, the difference in superhelicity (Pulleyblank et al., 1975) of these two DNAs was

$$\Delta\sigma = \frac{\phi \Delta r_b^\circ}{18} = \frac{26(0.050)}{18} = 0.072$$

where r_b° is the binding ratio at full relaxation, and σ is expressed in superhelical turns per ten base pairs. This value is, as expected, slightly lower than that measured by DeLeys & Jackson (1976) at somewhat higher ionic strength. Since native DNA was relaxed by tripeptide S in 0.8 mM citrate at r_b = 0.108, the unwinding angle of tripeptide S was

$$\phi = \frac{18\sigma}{r_b^\circ} = \frac{18(0.072)}{0.108} = 12°$$

All three titrations (Figure 3) achieved full relaxation at roughly the same r_b value, indicating that the unwinding angles of tripeptide S at pH 8.0 and of bleomycin at pH 5.5 were also about 12°. This value is smaller than that of most intercalators but is similar to that of daunomycin (Waring, 1970).

Dichroism of DNA and Bleomycin. Chromophores which bind to DNA in an ordered manner exhibit linear dichroism when bound to rod-like DNA molecules oriented in an electric field. The aromatic rings of bound intercalators are roughly parallel to the DNA bases. Therefore, for optical transition moments in the plane of the ring, the absorbance $A_\parallel$ parallel to the helix axis is smaller than the absorbance A_0 at random orientation, giving a strong negative dichroism. The dichroism of bound bleomycin at 303–320 nm, presumably due to the in-plane π–π^* transition (Dabrowiak et al., 1978), indicated an angle of 59–61° between the bithiazole rings and the helix axis (Table I). This value is within the range measured for other intercalators and is the same as that of actinomycin (Hogan, et al., 1979).

The angle measured was the same at r_b = 0.018 as at r_b = 0.05, suggesting that a single mode of binding strongly predominates in this range of r_b.

Table I: Electric Dichroism of Bleomycin-DNA Complexes

chromophore	r_b[a]	λ (nm)	ρ[b]	α[c] (deg)
bithiazole	0.018	303	−0.25	58.2
		310	−0.32	59.2
		315	−0.47	61.4
		320	−0.46	61.3
	0.050	306	−0.36	59.8
		315	−0.40	60.4
DNA bases	0	265	−0.87	68.0
	0.050	265	−0.88	68.2

[a] Bleomycin molecules per nucleotide. [b] Reduced linear dichroism $\frac{3}{2}(A_{\parallel} - A_0)/A_0$ extrapolated to 100% orientation. [c] Angle between the transition moment and the DNA helix axis.

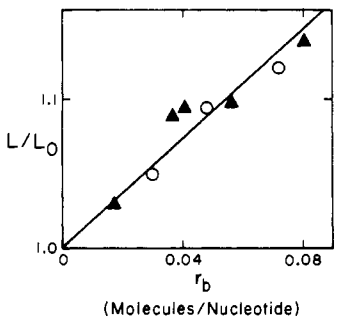

FIGURE 4: Changes in DNA length induced by binding of bleomycin (▲) and tripeptide S (○). The buffer was 0.8 mM citrate, pH 5.5, at 11 °C. Lengths were determined from the rotational correlation times of 150-base-pair-long DNA molecules oriented in an electric field as monitored by their linear dichroism. r_b was calculated from the known total concentrations of drug and DNA, using linear fits to the binding data (Figure 1). At least 70% of the drug was bound in all cases.

Bleomycin binding did not change the dichroism of the DNA bases, indicating that the DNA retained a rod-like configuration. In an analogous experiment, irehdiamine A, which is thought to kink DNA by partial intercalation (Sobell et al., 1977), induces a monotonic change toward zero dichroism with increasing r_b (Dattagupta et al., 1978). Thus, bleomycin binding is unlike that of irehdiamine and more similar to that of other classical intercalators.

DNA Lengthening. The characteristic time (typically 3–5 μs) needed for rod-like DNA to orient in an electric field, as monitored by the linear dichroism, can be used to determine the length of the DNA (Hogan et al., 1978). Both bleomycin and tripeptide S induced a linear increase in DNA length (Figure 4). The slope indicated a fractional length change of 1.84 nucleotides per bound molecule. Assuming a DNA length of 3.4 Å per base pair, each bleomycin or tripeptide S bound lengthened DNA by (1.84 × 3.4 Å)/2 = 3.1 Å. This lengthening is most likely due to insertion between base pairs of the bithiazole rings, which are the only aromatic moiety in tripeptide S. Similar changes are seen for other intercalators (Hogan et al., 1979; Lerman, 1961; Müller & Crothers, 1968).

Enhanced Strand Breakage in Supercoiled DNA. Because binding of an intercalator reduces the torsional stress in a supercoiled DNA molecule, supercoiled DNA has a higher affinity for intercalators at low r_b values than does relaxed DNA (Bauer & Vinograd, 1968). Thus, if intercalative binding were involved in the breakage of DNA by bleomycin, one would expect supercoils to be more susceptible to bleomycin-induced breakage than relaxed DNA molecules. This was in fact found to be the case for mixtures of supercoiled

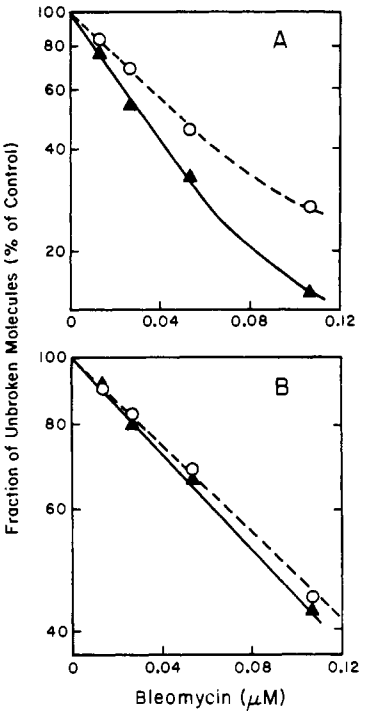

FIGURE 5: Breakage of supercoiled (▲) and relaxed (○) closed circular ColE1 DNA by bleomycin. (A) Incubation in 0.1 M NaCl–20 mM Tris; (B) incubation in 20 mM Tris. Analysis of breakage was by neutral sucrose gradient sedimentation.

^{14}C-labeled ColE1 DNA and relaxed closed circular ^{3}H-labeled ColE1 DNA. In 0.1 M NaCl, 50% more breaks occurred in supercoiled than in relaxed molecules (Figure 5). Although "relaxed" molecules will be slightly supercoiled in this salt, stronger binding to the native highly supercoiled form is still expected, since affinity is a continuously increasing function of superhelicity (Hinton & Bode, 1975a). In 20 mM Tris, a smaller but measurable 10% enhancement was observed, which is consistent with the smaller enhancement of binding observed by Hinton & Bode (1975a) at lower salt concentration. Recently, Lloyd et al. (1978) have also reported a slightly greater rate of strand breakage for supercoils than for relaxed circles in 20 mM Tris.

Discussion

The major problem in studying the binding of a drug like bleomycin is that, to obtain meaningful results, the integrity of the DNA must be preserved. We have found it very difficult to stop completely the DNA degrading activity of bleomycin, but lowering the pH appears to reduce it considerably (Umezawa et al., 1973). From the gradients (Figure 2B), it appeared that there may have been a small amount of nicking at C_f = 1 μM (r_b = 0.055), but clearly much less than one nick per ColE1 molecule. Since the DNA used in dichroism experiments was only $^1/_{40}$th the size of ColE1, and since the overall bleomycin to DNA ratio was much smaller, it is unlikely that DNA damage could have influenced the dichroism results. The nicking does complicate interpretation of the supercoil relaxation experiments, but the similar results seen with tripeptide S, which apparently has no nicking activity, strongly suggest that unwinding, not nicking, was responsible for DNA relaxation. Thus, at least under conditions of low pH and low ionic strength, bleomycin and tripeptide S exhibited properties expected of an intercalator: unwinding of supercoiled DNA, DNA lengthening, and strong negative electric dichroism. Our results do not indicate whether the detailed stereochemistry of intercalation suggested by Mu-

rakami et al. (1973, 1976) is correct. Further, we cannot completely eliminate the unlikely possibility that bleomycin binds in some unusual way which produces these effects without insertion of an aromatic ring.

In measurement of the DNA unwinding, calf thymus and ColE1 DNAs were assumed to be similar in their bleomycin-binding properties. Also, the r_b values calculated for partially relaxed and recoiled ColE1 (Figure 3) are slightly in error, due to supercoil-dependent changes in affinity. However, the r_b value at full relaxation should be correct (Waring, 1970), and the symmetry of the descending and ascending portions of the S_{rel} curve suggests that good approximations of r_b were obtained. Sucrose (10%) was included in solutions used in measuring tripeptide S binding, in order to approximate conditions in a 5–20% sucrose gradient; the sharpness of the relaxation curve (Figure 3B) suggests that r_b did not change as the DNA sedimented through the gradient. For bleomycin (Figure 3C) there is some doubt about the exact value of r_b at full relaxation, since the DNA did not recoil and since sucrose was not included during the binding experiments. However, this quantity for bleomycin appeared to be approximately the same as for tripeptide S.

Similarly, the DNA lengthening experiments for tripeptide S were done under slightly different conditions (11 °C, no sucrose) than the binding experiments (22 °C, 10% sucrose). However, the linearity of the lengthening curve, its similarity to the bleomycin-induced lengthening, and the fact that most of the tripeptide S (70–90%) is expected to be bound suggest that good approximations for r_b were obtained.

For simplicity, linear fits to the binding data have been used to calculate r_b. Use of neighbor exclusion fits would not significantly affect the results since the two fits are nearly coincident for most r_b values (Figure 1). The largest effect would be to reduce the estimated unwinding angle by half a degree.

Only one previous study has dealt experimentally with the question of bleomycin intercalation. Chien et al. (1977) measured DNA length changes upon bleomycin binding using viscometry. They found a maximum fractional length increase of 5% and concluded that intercalation was unlikely. However, since they did not measure binding in the same buffer as viscosity, the amount of bound bleomycin in their viscosity experiment is uncertain. In fact, their binding data show that an increase in the ionic strength can lead to a large decrease in r_b at saturation, perhaps large enough to explain the smallness of the length change they observed.

An important question is whether bleomycin intercalates under conditions where it degrades DNA. Our results and those of Chien et al. (1977) suggest that tripeptide S is the component of bleomycin primarily responsible for DNA binding. They also showed that the bithiazole fluorescence quenching upon binding is similar for bleomycin and tripeptide S at pH 4.4 and at pH 8.0, in 2.5 mM Tris and in 25 mM Tris. This result suggests that the mode of binding of the bithiazole is similar under all conditions and agrees with our findings that tripeptide S unwound DNA at pH 8 (Figure 2C), as did bleomycin at pH 5.5 in the presence of 24 mM Na^+ (not shown). While these results suggest that intercalation occurs under a variety of conditions, they do not deal with the possibility that binding of bleomycin·Fe(II), thought to be the active form, may be quite different from that of the metal-free compound. However, Dabrowiak et al. (1978) have shown that there is no strong interaction between the bithiazole rings and metal ions, at least for the bleomycin·Cu(II) and bleomycin·Zn(II) complexes.

A more direct approach to the question of involvement of intercalation in bleomycin-induced DNA breakage is comparison of breakage rates in supercoiled and relaxed DNA. The observed 50% enhancement in breakage of DNA in 0.01 M NaCl (Figure 5) is about what one would expect from increased intercalative binding to supercoils. Assuming that increased binding is due to reduction of torsional stress in DNA upon intercalation (Bauer & Vinograd, 1968), the ratio r_b'/r_b of binding to supercoiled and relaxed forms at a given C_f should be given by a partition function

$$r_b'/r_b = e^{-A\phi/kT}$$

where ϕ is the unwinding angle and A is the torsional free energy change per degree of unwinding. For ethidium and λ DNA in 0.1 M NaCl, $r_b'/r_b \sim 2.5$ at low r_b (Hinton & Bode, 1975a), and $\phi = 26°$ (Wang, 1974). For bleomycin $\phi = 12°$, therefore r_b'/r_b should be about 1.4–2.1, which is similar to the observed ratio of 1.5 for DNA breakage. At low salt (10 mM Tris), the data of Hinton & Bode (1975a) are less accurate, but do indicate a smaller r_b'/r_b in qualitative agreement with our observation of a smaller enhancement of supercoil breakage (~10%) at low salt (20 mM Tris). Thus, intercalative binding appears to be involved in DNA breakage.

Our results also suggest that DNA breakage occurs primarily in the part of the molecule to which the bleomycin is bound, an important consideration for models involving free radical intermediates. Unfortunately, we cannot completely eliminate the possibility that increased sensitivity of supercoils is due to presence of denatured regions of DNA, although this would require that denatured DNA be much more sensitive than native DNA. Despite reports that denatured DNA is more sensitive (Haidle, 1971) or less sensitive (Onishi et al., 1975), no quantitative data on this subject are available.

The exact role of the bithiazole in the reaction is unknown. It may simply help bind bleomycin to DNA, it may interact chemically with DNA, or it may make certain DNA moieties more accessible to radicals or to other parts of the bleomycin molecule. Since intercalators interact strongly with DNA bases, its seems likely that intercalation at specific base sequences may be involved in determining the sequence specificity of DNA breakage (Lloyd et al., 1978). Bleomycin, like other intercalators (Krugh & Reinhardt, 1975; Krugh, 1972), shows a strong preference for binding to alternating purine-pyrimidine sequences (Asakura et al., 1978). The sequence GpC, which is the preferred sequence for antinomycin D intercalation (Krugh, 1972), is also one of the two most frequent sites of bleomycin-induced DNA breakage, the other being GpT (Takeshita et al., 1979; D'Andrea & Haseltine, 1978).

Acknowledgments

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