Two Actions of Bleomycin on Superhelical DNA†

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ABSTRACT: Bleomycin is an antitumor and antimicrobial drug which appears to exert a direct effect on DNA in vivo. A shift to lower molecular weight as defined by alkaline isokinetic analysis after exposure to bleomycin has been interpreted as indicating that the drug causes single-strand nicks in the DNA. We have used φX174 RFI DNA as a substrate for bleomycin since a single nick in the supercoil produces a marked change in sedimentation properties. We find that bleomycin-treated φX174 DNA sediments through alkaline sucrose in a manner indicating strand breakage. However, we do not observe any change between bleomycin-treated and untreated DNA when sedimentation is done on neutral gradients. This result indicates that bleomycin induces alkaline-sensitive sites in the DNA but does not cause a single-strand break. The alkaline sensitivity of bleomycin-treated DNA is not increased by heating, a different result than with alkylation of DNA. Phosphodiester bond cleavage does occur in neutral conditions if dithiothreitol is added to the incubation mixture. Our results are compatible with removal of a base moiety by bleomycin and with strand scission when dithiothreitol is present.

Bleomycin (BLM)[1] is a mixture of glycopeptide antibiotics discovered by Umezawa (Umezawa et al., 1966). Initially, it was investigated as both an antimicrobial and antitumor agent. It reduces cell proliferation in cultures of the mouse lymphoma L51784 and is an effective agent in the treatment of Hodgkin's disease (Terasima et al., 1970; Yagada et al., 1972).

Since BLM-treated DNA from a variety of cells sediments more slowly through an alkaline sucrose gradient than untreated DNA, it has been suggested that the mechanism of action of BLM is to induce single- or double-strand breaks in the DNA, lowering its molecular weight (Suzuki et al., 1969; Umezawa et al., 1973; Haidle, 1971; Fujiwara & Kondo, 1973). However, these studies have not distinguished between a site which is sensitive to the high pH of the gradient or an actual break in the DNA. Loss of a nitrogenous base from a polydeoxyribonucleotide creates an alkaline-sensitive site at the deoxyribose which remains (Tamm et al., 1953). Several laboratories have suggested that DNA exposed to BLM does suffer loss of a base (Haidle, 1971; Muller et al., 1972; Haidle et al., 1972). Therefore, we felt that the observed reduction in molecular weight of BLM-treated DNA could be due to the introduction of alkali-sensitive sites into the DNA.

Our interest in the mechanism of action of BLM was stimulated by our observation of the effect of the drug on DNA synthesis in toluene-treated *E. coli* cells. We found that BLM induces extensive repair activity but appears to have no effect on replication (Ross & Moses, 1976). Broken strands ought to result in repair synthesis, which we observed when toluene-treated cell reaction mixtures included BLM. However, replication appeared to continue unhindered as judged by density analysis. Since strand breakage inhibits replication in this system, this raised the possibility of a repair mechanism preventing the accumulation of breaks.

In this paper we present the results obtained when supercoiled DNA was treated with BLM and analyzed by isokinetic gradients under both alkaline and neutral conditions. We chose a supercoiled DNA (φX174 RFI) because a single cleavage produces a readily measurable change in sedimentation properties (Vinograd, 1965). We find no change in the sedimentation of BLM-treated DNA analyzed under neutral conditions. In contrast the sedimentation of DNA through an alkaline gradient indicates cleavage after exposure to BLM. The presence of a sulfhydryl reagent (dithiothreitol) in the reaction mixture greatly increases the fragmentation of the DNA under neutral and alkaline conditions.

Materials and Methods

Chemicals. BLM mixture (lot no. C4175) was from Bristol Laboratories, Syracuse, N.Y. The antibiotic was dissolved in deionized water at concentrations of 15 mg/mL or less and stored in the refrigerator at 4 °C.

Bacteria and Bacteriophage. Bacteriophage φX174 (Eam 3, lysis-deficient) and *E. coli* C (SuI^-) and Cr (SuI^+) were from Dr. David Denhardt, McGill University.

Preparation of φX174 RFI [^{3}H]DNA. The procedure used was modified from the preparation of labeled φX174 RFI DNA (Schekman et al., 1971). *E. coli* C was grown to 4×10^8 cells/mL in 50 mL of mT 3XD medium and infected with φX174 (Eam 3) at a multiplicity of 6 at zero time ($t = 0$). After 5 min ($t = 5$), 0.5 mCi of [^{3}H]thymidine (specific activity, 66 Ci/mmol) was added. After 5 additional min ($t = 10$) chloramphenicol was added to a final concentration of 20 μg/mL. After 5 min ($t = 15$) a second 0.5-mCi aliquot of [^{3}H]thymidine was added. The culture was aerated for 3 h at 37 °C and harvested by centrifugation at 10 000 rpm for 10 min. The cells were resuspended in 2 mL of 50 mM Tris-HCl (pH 8.1) and lysed by incubation in EDTA-lysozyme for 15 min at 37 °C. The suspension was diluted fivefold with 50 mM Tris-HCl (pH 8.1) and the sodium chloride concentration brought to 0.5 M. After lysis a poly(ethylene glycol) phase extraction was done prior to phenol extraction. After precipitation by isopropyl alcohol-sodium acetate, the RFI [^{3}H]DNA was isolated by

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[1] Abbreviations used are: BLM, bleomycin; RFI DNA, covalently closed circular φX174 RFI DNA supercoils; MMS, methylmethanesulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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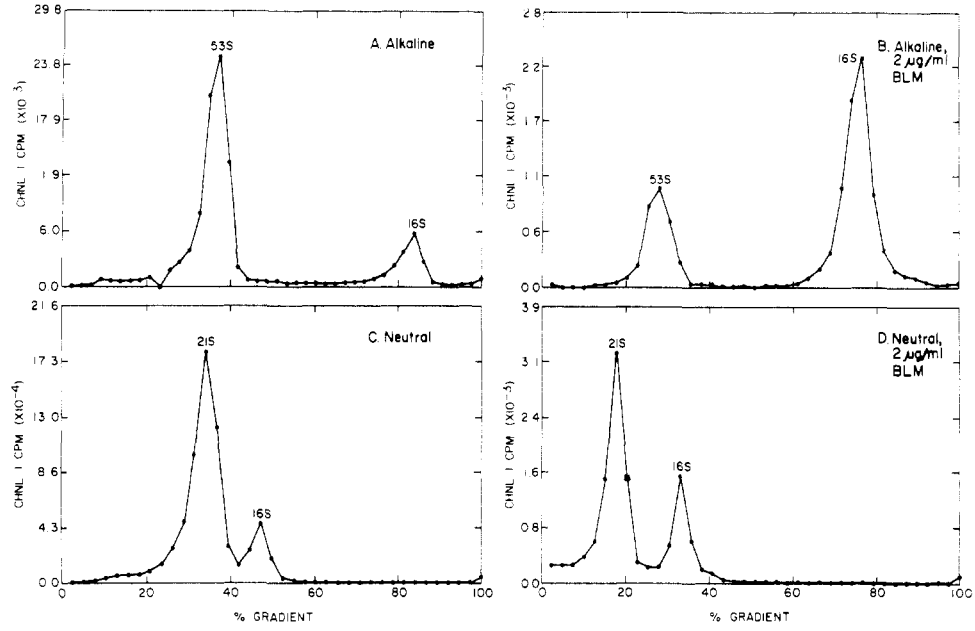

FIGURE 1: Isokinetic sedimentation of [^{3}H]-ϕX174 DNA supercoils. Panels A and B: sedimentation under alkaline conditions. Panels C and D: sedimentation under neutral conditions. Results shown in panels B and D are from DNA treated with BLM. Sedimentation was as in Materials and Methods. Reaction temperature was 0 °C. The direction of sedimentation is from right to left.

ethidium bromide-cesium chloride centrifugation and reprecipitated and the precipitate was dissolved in 1.0 mL of 50 mM Tris-HCl (pH 8.1)-20 mM EDTA, stored at 0-4 °C, and used without further purification. The specific activity of the final product was about 2×10^4 [^{3}H cpm/μg] and its concentration was about 120 μg/mL.

BLM Treatment of DNA. Reaction mixtures (100 μL) contained: 0.6 μg (5 μL) RFI [^{3}H]DNA, BLM, 10 mM Tris-HCl (pH 8.1), 100 mM sodium chloride and, when present, 2 mM dithiothreitol. The reaction was initiated by addition of BLM at 0 °C, the temperature of incubation.

MMS Treatment of DNA. Reaction mixtures (80 μL) contained in water: 0.6 μg (5 μL) RFI [^{3}H]DNA and were 6.25 mM in MMS. Incubations were at 37 °C for 15 min or 54 °C for 30 min. After reaction the DNA was used immediately, without removal of MMS.

Isokinetic Gradient Analysis. A 40-μL aliquot of each sample was layered on a 5-mL, 5-20% sucrose gradient containing either 0.3 N NaOH or 0.3 N NaCl, 100 mM EDTA, 50 mM Tris-HCl (pH 8.1), and 0.15% Sarkosyl. All centrifugation experiments were done in a SW50.1 rotor at 20 °C and conditions were either 40 000 rpm for 120 min (alkaline) or 48 000 rpm for 160 min (neutral). The gradients were collected and assayed for acid-precipitable DNA by the paper strip method (Carrier & Setlow, 1971).

Acid Depurination of DNA. RFI DNA was depurinated as outlined (Kirtikar et al., 1976). One volume of DNA was mixed with 3 volumes of 0.05 M sodium citrate (pH 3.5) and incubated at 50 °C for 30 min. This treatment produced 1.7 breaks per molecule after alkaline hydrolysis, as calculated by the method of Kuhnlein et al. (1976).

Filter Retention Assay. The procedure used has been published (Kuhnlein et al., 1976). Reaction mixtures (100 μL) contained 100 mM sodium chloride, 10 mM Tris-HCl (pH 8.0), 0.6 μg/mL of RFI DNA, 2 mM dithiothreitol when present, and various concentrations of BLM. After incubation at 0 °C for 15 min, 200 μL of 0.3 M K_2HPO_4 (pH 12.3) was added. After 6 min at room temperature, hydrolysis of the depurinated DNA was not detectable and this length of exposure was used for the portions of the experiment corresponding to neutral conditions. Alkaline hydrolysis of the DNA appeared to be complete after 60 min of exposure at pH 12.3 and room temperature. After the appropriate incubation period, 100 μL of 1.0 M KH_2PO_4 (pH 4.0), 200 μL of 5.0 M NaCl, and 5 mL of 1.0 M sodium chloride-50 mM Tris-HCl (pH 8.0) were added. The solution was passed through a Schleicher and Schull BA85 filter (pore size, 04.5 μm) at a flow rate of 10 mL/min. The filters were washed with 2-3-mL aliquots of 0.3 M sodium chloride-0.03 M sodium citrate, dried, and added to 10 mL of scintillant. DNA which is superhelical passes through the filter.

Results

ϕX174 RFI DNA (supercoiled form) can be separated from ϕX174 RFII DNA (relaxed circle form) on neutral or alkaline isokinetic gradients (Figure 1A and 1C). Analysis under both conditions allows differentiation between the breaking of DNA strands and the generation of alkali-sensitive sites. Alkali-sensitive sites are scored as breaks if alkaline conditions are used. When ϕX174 supercoils were exposed to BLM (2 μg/mL) and analyzed by neutral isokinetic gradients, we found no change in sedimentation compared to untreated controls (Figure 1C and 1D). In both cases greater than 70% of the DNA sedimented as the supercoiled form. However, when BLM-treated ϕX174 supercoils were sedimented under alkaline conditions, we found 27% of the DNA migrating as the supercoil (Figure 1B). Before treatment, greater than 70% of the DNA was in the supercoiled form (Figure 1A). These results indicate that BLM induces alkali-sensitive sites in the DNA which are subsequently hydrolyzed when the DNA is exposed to alkaline conditions and that BLM does not cause strand scissions directly. The lack of fragments smaller than unit length (16S) suggests that most supercoils have few breaks per duplex under these conditions (Figure 1B).

Since we had shown that BLM-treated supercoils do not break under neutral conditions, we measured the amount of supercoils lost as a function of BLM concentration on alkaline isokinetic gradients. From 0.2 μg/mL BLM to 2.0 μg/mL BLM, the number of alkali-sensitive sites induced per molecule of BLM was constant. This is in agreement with our previous

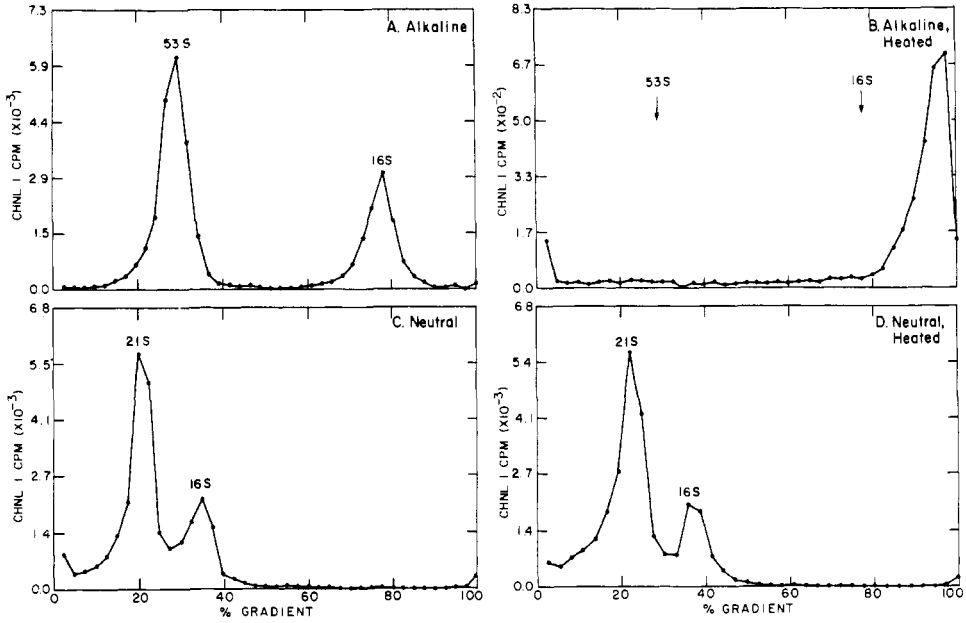

FIGURE 2: Isokinetic sedimentation of alkylated [^{3}H]-ϕX174 DNA supercoils. Panels A and B: sedimentation under alkaline conditions. Panels C and D: sedimentation under neutral conditions. Results shown in panels A and C are with alkylated RFI DNA prepared by incubation in MMS as in Materials and Methods. Results shown in panels B and D are from depurinated RFI DNA prepared by heating alkylated RFI DNA at 54 °C for 30 min. Sedimentation was as in Materials and Methods.

experiments with BLM in toluene-treated cells (Ross & Moses, 1976). When we increased the BLM concentration to 10 μg/mL, the number of alkali-sensitive sites induced per molecule BLM decreased (Table I).

Umezawa reported an inverse temperature effect for BLM activity showing greater BLM activity at lower temperature (Umezawa et al., 1973). We tested this effect and found that at 37 °C BLM had less than 5% the activity measured at 0 °C (data not shown). Therefore, we always exposed the DNA to BLM at ice bath temperature.

Haidle et al. have shown that one effect of BLM on DNA is to cause the loss of the nitrogenous base portion of a nucleotide within a duplex (Haidle et al., 1972). Depurinated DNA resulting from alkylation is alkali-sensitive (Tamm et al., 1953), so we compared BLM-treated DNA with alkylated DNA. We treated RFI DNA with 6.25 mM MMS and then heated part of the sample at 54 °C for 30 min to remove alkylated purines (Ljungquist et al., 1974). RFI DNA depurinated in this manner and sedimented under both alkaline and neutral conditions gave results similar to those obtained for BLM-treated DNA (Figure 2). We did not find any change in the sedimentation of alkylated RFI DNA on either the neutral or the alkaline gradient (Figures 2A and 2C); alkylating the DNA does not appear to create measurable alkali-sensitive sites. After the alkylated RFI DNA was heated and sedimented under alkaline conditions, more than 75% of the DNA layered on the gradient had a value of less than 9 S. Sedimenting depurinated ϕXRFI DNA under neutral conditions did not produce any change in the percentage of DNA migrating as the 21S species (Figures 2C and 2D). Thus heating alkylated DNA does not introduce a high level of breaks. The results of this experiment are consistent with our conclusion that exposure to BLM does not break DNA strands but does induce the loss of nitrogenous bases from a duplex.

If BLM interacts with bases in a manner identical with an alkylating agent (*N*-glycosidic bond destabilization), then heating BLM-treated DNA should create more alkali-sensitive sites than just exposing DNA to the drug without heating. When BLM-treated RFI DNA was heated and analyzed on an alkaline, isokinetic gradient, we obtained the result shown in Figure 3. Under alkaline conditions 70% of the BLM-treated DNA sedimented as the 16S species. After heating the BLM-treated DNA as described (54 °C for 30 min), we found no increase in the amount sedimenting as the 16S species under alkaline conditions; nor was there any change in the relative amount of 53S species before or after heating. Similar results were obtained at lower BLM concentrations (data not shown). We conclude that few alkali-sensitive sites were introduced into the BLM-treated DNA as a result of the heat treatment. This suggests that BLM does not function in a manner identical with an alkylating agent, but that those bases BLM interacts with are destabilized sufficiently to be lost completely at reaction temperatures. Since we found no new sites appeared during the 30-min incubation at 54 °C, it appears that the reaction is terminated at this temperature (BLM was still present).

Sulfhydryls have been reported to potentiate the action of BLM on DNA (Suzuki et al., 1969; Umezawa et al., 1973; Haidle, 1971). When we exposed RFI DNA to BLM in the presence of 2 mM dithiothreitol and analyzed the product by alkaline isokinetic gradients, we found that the DNA was

TABLE I: Conversion of [^{3}H]-ϕX174 DNA Supercoils to Relaxed Circles after Exposure to BLM.[a]

BLM (μg/mL)	% loss supercoil	Nicks per supercoil
0.0	0	0.0
0.2	9	0.1
1.0	39	0.5
2.0	75	1.4
10.0	97	3.5

[a] Percent loss of supercoil was measured as the loss of radioactivity from peak I of an alkaline isokinetic gradient compared with an unexposed control. Reaction conditions were as described in Materials and Methods. The reaction temperature was 0 °C. Nicks/supercoil were calculated from the Poisson distribution as in Kuhnlein et al. (1976).

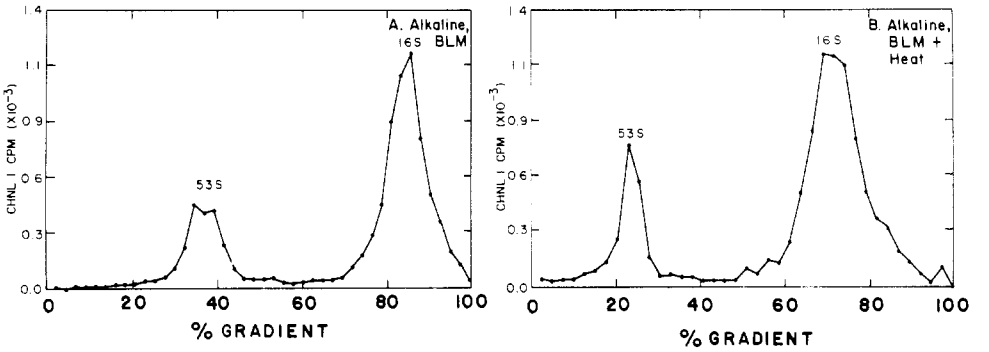

FIGURE 3: Isokinetic sedimentation of BLM-treated [^{3}H]-ϕX174 RFI DNA. The DNA was treated with BLM (2 μg/mL) at 0 °C for 15 min. Panel A: no heat treatment. Panel B: heated according to protocol used for heat depurination of alkylated DNA at 54 °C for 30 min. Sedimentation was as in Materials and Methods.

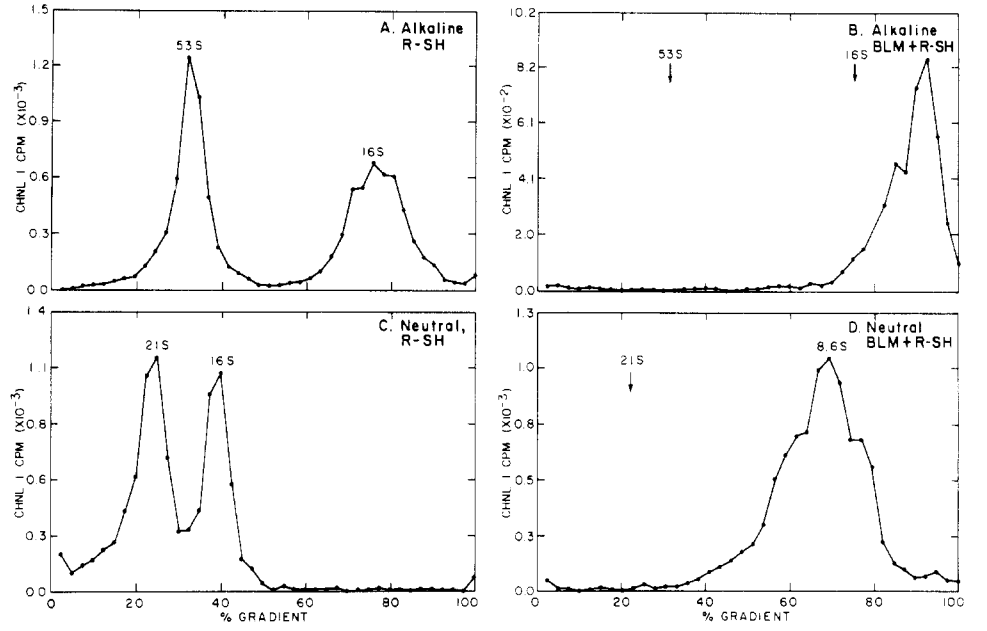

FIGURE 4: Isokinetic sedimentation of BLM and dithiothreitol (DDT)-treated [^{3}H]-ϕX174 DNA supercoils. Panels A and B: sedimentation under alkaline conditions. Panels C and D: sedimentation under neutral conditions. Results in panels B and D are from DNA treated with BLM (2 μg/mL) in the presence of dithiothreitol at 0 °C for 15 min. Sedimentation was as in Materials and Methods.

fragmented to less than unit length and that the recovery of acid precipitable radioactivity from the gradient was reduced to 50% (Figure 4B). Therefore, including dithiothreitol in the reaction mixture appeared to cause at least a 25-fold increase in the number of alkali-labile sites induced by BLM. This agrees with the 20-fold increase reported by Umezawa (1974).

When a portion of the same sample was sedimented under neutral conditions, we found that the DNA was broken (Figure 4D). This result is in contrast to the result without dithiothreitol (Figure 1). We felt that there were three possible interpretations of this result: (i) dithiothreitol increased the activity of BLM, producing long apurinic and apyrimidinic regions in the DNA sensitive to shear during sedimentation, (ii) dithiothreitol catalyzed a strand scission at apurinic and apyrimidinic sites, or (iii) in the presence of dithiothreitol, the mechanism of action of BLM is altered.

We tested ii by sedimenting acid-depurinated RFI DNA after exposure to dithiothreitol (Figure 5). The upper panels (alkaline) demonstrate that the DNA was depurinated. Exposure of the same DNA to dithiothreitol did not appear to cause any loss of the RFI DNA under neutral conditions (Figure 5E). Thus, dithiothreitol-catalyzed strand scission at apurinic and apyrimidinic sites was probably not a contributing factor to cleavage after BLM exposure in the experiment described in Figure 4.

To distinguish between the remaining possibilities, we repeated the dithiothreitol experiment at lower BLM concentrations so that the degradation of DNA was reduced. For convenience with numerous concentrations of BLM, we used the nitrocellulose filter retention assay to measure scissions. This assay measures single-stranded DNA after superhelical DNA has been treated, denatured, and renatured. Any DNA that does not renature is bound to the filter as an indication of strand scission (Kuhnlein et al., 1976).

Figure 6 shows the result of treating RFI DNA with BLM in the presence or absence of 2 mM dithiothreitol followed by exposure to pH 12.3 for 60 min at 22 °C (complete alkaline hydrolysis, Figure 6A) or 6 min at 22 °C (minimal alkaline hydrolysis, Figure 6B). The DNA was converted to a form that bound to the filter with increasing BLM but to a much lesser extent when dithiothreitol was omitted. When higher BLM concentrations were used, the amount of DNA binding to the filter decreased due to extensive degradation and generation of oligonucleotides in alkali (Figure 6A). In the presence of 2 mM dithiothreitol, 0.05 μg/mL BLM induces 0.5 alkali-labile

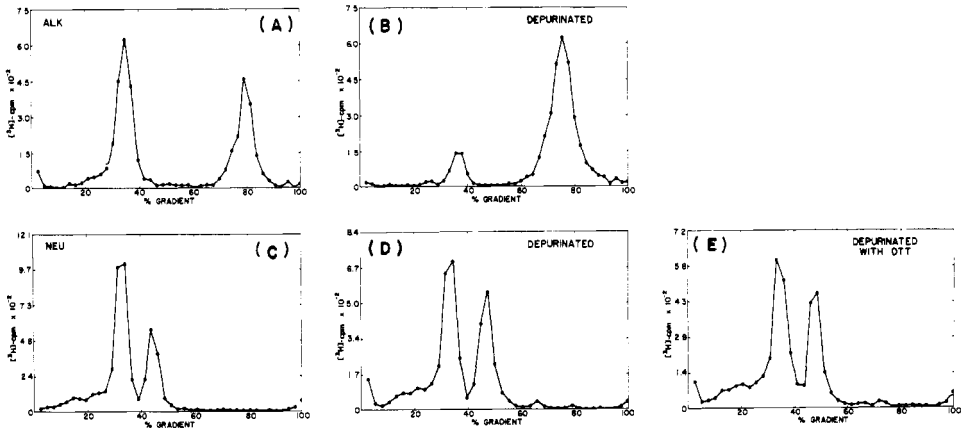

FIGURE 5: Isokinetic sedimentation of depurinated [^{3}H]-ϕX174 RFI DNA supercoils after exposure to dithiothreitol (DDT). Top panels: alkaline sedimentation before (A) and after (B) acid depurination. Bottom panels: neutral sedimentation before (C) and after (D) acid depurination and after acid depurination followed by exposure to dithiothreitol (2 mM) for 15 min at 0 °C (E). Acid depurination was for 30 min at 50 °C and pH 3.5. Sedimentation was as in Materials and Methods.

sites per supercoil while in the absence of the sulfhydryl 2 μg/mL BLM causes 1.4 alkali-labile sites per supercoil. Therefore, in the presence of dithiothreitol, the activity of the BLM is increased about 20-fold.

When the experiment was repeated under conditions which did not permit hydrolysis of alkaline-sensitive sites, we obtained the result shown in Figure 6B. In the presence of dithiothreitol, the low concentrations of BLM used caused the conversion of the DNA to a form which bound to the filter. Dithiothreitol by itself does not cause strand scissions at apurinic sites (Figure 5). Thus, the combination of BLM and dithiothreitol causes strand cleavage in the DNA whereas BLM by itself does not. The extent of strand scission appears to be the same in the presence of BLM and dithiothreitol, whether measured under alkaline or neutral conditions. We conclude that the mechanism of action of BLM is different in the presence of sulfhydryl reagent, resulting in complete strand scission at sites of BLM action. Similar results were obtained by isokinetic gradient analysis (data not shown).

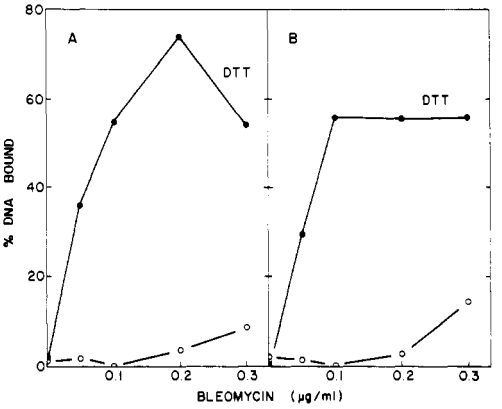

FIGURE 6: Retention of DNA on nitrocellulose after reaction of [^{3}H]RFI DNA with BLM and dithiothreitol (DTT). Panel A: retention of BLM- and BLM plus dithiothreitol-treated RFI DNA after incubation under conditions for complete hydrolysis of alkali-sensitive sites. Panel B: retention of BLM- and BLM plus dithiothreitol-treated RFI DNA after incubation under conditions at which no hydrolysis of alkali-sensitive sites was detectable. See Materials and Methods. Symbols are: (○) BLM and (●) BLM plus 2 mM dithiothreitol. Reaction with BLM (2 μg/mL) was at 0 °C for 15 min.

Discussion

Treating DNA with BLM introduces two new properties to the DNA: (i) the DNA sediments more slowly in an alkaline gradient (Suzuki et al., 1969; Umezawa et al., 1973; Haidle, 1971; Fujiwara & Kondo, 1973) and (ii) aldehyde groups can be detected in the DNA (Muller et al., 1972). The reduction in sedimentation velocity indicates a reduction in the single-strand molecular weight and on this evidence it has been concluded that breaks are introduced into the duplex when it is exposed to the drug. However, the introduction of aldehyde groups into the DNA by BLM suggests that this initial interpretation should be modified. The appearance of the aldehyde group in the DNA indicates that the nitrogenous base portion of a deoxyribonucleotide is lost after exposure to the drug. Haidle et al. have quantitated this loss by paper chromatography and reported that only the nitrogenous base is lost and that no detectable amounts of nucleosides, nucleotides, deoxyribose, deoxyribose phosphate or inorganic phosphate were found after BLM treatment (Haidle et al., 1972). These results argue against a strand-breaking activity for BLM but do suggest it has a base-removing activity.

If exposing DNA to BLM causes the loss of a base, then the duplex ought to contain sites which have the properties of apurinic acid. When we analyzed BLM-treated ϕX174 RFI DNA on both alkaline and neutral isokinetic gradients, we found a decrease in sedimentation on the alkaline gradient but not on the neutral gradient (Figure 1). We interpret this result as indicating that BLM modifies DNA to contain alkali-sensitive sites but does not cause a strand break in the duplex. Our results indicate that even though a base is lost from the DNA under neutral conditions the phosphodiester backbone remains intact (Figure 1D). Our assay should detect one event per approximately 11 000 bases. Thus we were observing "first-hit" processes. Recently other investigators (Muller & Zahn, 1977) have drawn a similar conslusion from studies with *E. coli* DNA by a filter-retention assay, noting strand cleavage of the linear duplex DNA only following alkaline treatment. In alkaline solution chain cleavage due to β elimination occurs at apurinic sites in DNA (Bayley et al., 1961). We believe that strand breaks in BLM-treated DNA occur as a result of exposure to the high pH used to denature the DNA, not as a result of exposure to the drug. In experiments with SV40 supercoils which indicated strand scission on neutral gradients, the reactions were stopped by the addition of 5 N sodium hydroxide (Umezawa et al., 1973).

We do not believe that BLM acts on DNA in a manner identical with an alkylating agent. When alkylated DNA was

heated at 54 °C for 30 min, we found that alkali-sensitive sites were introduced into the duplex (Figure 2). When BLM-treated DNA was heated as above, no increase in alkali-sensitive sites occurred (Figure 3). Instead, we found the same percentage of DNA migrating as the supercoil both before and after the heat treatment. Our results suggest that BLM induces a specific type of base removal but does not produce modified bases with increased lability remaining in the DNA.

We observed an increase in activity when DNA was treated with BLM in the presence of a sulfhydryl agent, in agreement with the results of other laboratories (Suzuki et al., 1969; Umezawa et al., 1973; Haidle, 1971). However, our results suggest that the increase is not merely a potentiation of the effect of BLM on DNA. Instead, a different reaction mechanism appears to be involved. From consideration of space-filling models, the primary interaction of BLM with DNA may be an insertion of the bithiazole between the stacked bases of the duplex. The subsequent increase in the strain and polarization of the *N*-glycosidic bond probably results in an increase in the rate of spontaneous loss of bases from the DNA. This intercalation has been suggested previously (Murakami et al., 1973). The mechanism in the presence of sulfhydryl might involve a different portion of the BLM molecule (Murakami et al., 1973). The β-hydroxyhistidine group is directed toward the ionized oxygen of the phosphate by an electrophilic attraction. However, the distance separating the two groups is too great to allow an interaction. When a proton donor, such as a sulfhydryl compound, is present, the proton can be transferred to the 3′ oxygen resulting in hydrolysis of the ester linkage.

Because a different portion of the BLM is involved in each of these suggested mechanisms, both reactions may occur in the presence of a sulfhydryl. Hydrolysis under neutral conditons allows the measurement of phosphodiester breaks resulting from treatment with BLM and dithiothreitol. Alkaline hydrolysis should allow the measurement of both alkali-sensitive sites and phosphodiester breaks.

Acknowledgments

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