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Bleomycin-Specific Fragmentation of Double-Stranded DNA†

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ABSTRACT: Brief exposure of covalently closed circular duplex PM2 DNA to low concentrations of the clinical bleomycin mixture (Blenoxane) resulted in specific fragmentation of the genome that does not depend on the presence of superhelical turns. The double-strand breaks are in fact produced at several discrete sites on the PM2 genome but frequently occurring near the *Hpa*II restriction endonuclease cleavage site. Initial rates of formation of nicked circular and linear duplex PM2 DNAs are reduced to different extents as the ionic strength of the reaction is increased. Increasing ionic strength is most effective in reducing the initial rate and overall yield of apparent double-strand scissions compared with single-strand scissions in the bleomycin-treated PM2 DNA.

The bleomycins are a group of glycopeptide antibiotics produced by *Streptomyces verticillus* (Umezawa et al., 1966) and have been used in clinical treatment of human malignancies (Ichikawa, 1970; Shastri et al., 1971; Blum et al., 1973). An extensive review of the action of bleomycin has been published recently (Müller & Zahn, 1977). Although the physiological basis of drug action has not yet been defined, the antibiotic mixture has been shown to cause extensive single-strand breakage of DNA, in vitro, when the reaction products are assayed by velocity sedimentation in alkaline sucrose gradients (Suzuki et al., 1969; Haidle, 1971). It has also been shown that DNA strand scissions are produced more efficiently with single-stranded compared with double-stranded DNAs (Suzuki et al., 1969; Haidle, 1971). Furthermore, shifts in the velocity sedimentation profiles at neutral pH suggested the possibility that bleomycins also produce double-strand breaks in duplex DNA (Haidle, 1971). This earlier study did not, however, determine if the putative double-strand breaks may not have arisen by an accumulation of single-strand scissions. This latter consideration is particularly significant since high concentrations of bleomycin (more than approximately 1.9 mol of bleomycin per mol of DNA phosphate) and extensive reaction conditions had been employed (Haidle, 1971). Double-strand breaks also occur in DNA extracted from bleomycin-treated mammalian cells, but again an accumulation of single-strand scissions could have accounted for these observations (Saito & Andoh, 1973; Byfield et al., 1976).

Recently, a study of bleomycin treated T2 and ColEl DNAs has demonstrated that double-strand breaks are produced in proportion to the number of single-strand breaks accumulated, but are much more frequent than the number expected simply from random coincidence of single-strand breaks (Povirk et al., 1977). Regardless of the mechanism of production of the double-strand breaks, it was deemed important to determine whether there were specific sites on the DNA molecule at which these breaks occurred and to determine some of the properties of the chemical reaction. Possible approaches in defining the modes of action for different component bleomycins which are applied in mixture for clinical chemotherapy may be indicated by the findings of these studies.

Materials and Methods

Preparation of PM2 DNAs. Covalently closed circular PM2 form I DNA (Espejo & Canelo, 1968) was isolated by extraction of purified bacteriophage according to a modification (Strong & Hewitt, 1975) of the procedure described by Salditt et al. (1972). The closed circular DNA was isolated from the lower band in buoyant CsCl[1] gradients containing saturating levels of ethidium bromide (Radloff et al., 1967). After removal of ethidium bromide (EthBr) by extraction with NaCl-saturated isopropyl alcohol, the DNA samples were dialyzed against several changes of 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) and stored at 4 °C. Covalent closed circular PM2 form I° DNA (relaxed form I DNA) was prepared by treatment of purified form 1 DNA with the calf thymus nicking-closing enzyme (Pulleyblank & Morgan, 1974; Hancock, 1974; Germond et al., 1975) using the reaction conditions described by Champoux & McConaughy (1976). The form I° DNA was isolated from the lower band in EthBr–CsCl gradients as described above. The EthBr was

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[1] Abbreviations used: CsCl, cesium chloride; EthBr, ethidium bromide; EDTA, ethylenedinitrilotetraacetic acid; SSC, standard saline citrate.

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removed as before and the samples were dialyzed against several changes of 0.1 × SSC and stored at 4°C. The form I° DNA was shown to migrate with form II DNA by agarose gel electrophoresis. If 0.5 μg/mL EthBr were added to the gel and electrophoresis buffer, form I° DNA completely separated from the form II DNA. The form I° DNA also was shown to be resistant to denaturation at pH 12.1 which distinguishes it from form II DNA which completely denatures at this pH. Preparations of both forms I and I° DNAs were generously provided by Dr. R. R. Hewitt, Dr. M. L. Dodson, and J. Harless.

Electrophoretic Analysis of Bleomycin Treated PM2 DNAs. Solutions containing 1.25 μg of PM2 DNA in a volume of 2.5 μL of 0.1 × SSC were brought to a final volume of 27 μL by the addition of Tris buffer, pH 8.0. The final concentrations were as follows: Tris buffer, 20 mM; $CaCl_2$, 0.27 mM; 2-mercaptoethanol, 25 mM; and bleomycin, from 0.2 to 5.0 μg/mL. Since EDTA is known to inhibit the fragmentation reaction, $CaCl_2$ was included to saturate the binding capacity of any traces of EDTA remaining in the solution. Bleomycin (Blenoxane), provided as a lyophilized powder, was dissolved in water approximately 2 weeks before use and stored at 4 °C. Blenoxane was the generous gift of Bristol Laboratories (Syracuse, N.Y.). Solutions were incubated at 37 °C for selected times and the reaction stopped by addition of 30 μL of 20 mM Na_2EDTA adjusted to pH 7.6 by the addition of NaOH. When electrophoresis was to be done, the EDTA solution also contained 10% (w/v) sucrose and 0.025% (w/v) bromophenol blue. The samples were layered onto 1.4% or 0.9% cylindrical agarose gels, 10 cm in length prepared as described by Sharp et al. (1973). Electrophoresis was conducted at 2 mA per gel for 7.5 h (0.9% gels) or at 100 V for 6.0 h (1.4% gels). Experiments involving form I° PM2 DNA were conducted with 0.5 μg/mL EthBr in the gel and the electrophoresis buffer, in order to separate form I° DNA from form II DNA. In 0.9% gels, form I DNA (covalent closed circular native PM2 DNA) migrated faster than form III DNA (full-length linear duplex PM2 DNA) which in turn migrated faster than form II DNA (nicked circular PM2 DNA). In 1.4% gels, form III DNA migrated faster than form I DNA which in turn migrated faster than form II DNA. Also in 1.4% gels, form I° DNA complexed with EthBr migrated faster than form II DNA, but slower than form III DNA. After electrophoresis, the gels were stained for at least 4 h in the dark by immersion in a solution of EthBr (0.5 μg/mL) which had been prepared in electrophoresis buffer (40 mM Tris, 5 mM sodium acetate, 1 mM disodium EDTA, pH 7.8).

The mass of PM2 DNA at each position of the gel was determined from spectrofluorometric scans using an Aminco-Bowman spectrofluorometer (American Instruments Co., Silver Spring, Md.). The gel was placed in a glass cuvette and mechanically driven at a constant rate past an incident beam of 510-nm wavelength light in the apparatus described previously by Grdina et al. (1973). Fluorescence was monitored at a wavelength of 590 nm. Fluorescence intensity was recorded as a function of distance along the gel using a Gilford strip chart recorder and the areas under the DNA bands (Figure 1, at left) were measured with a Dietzgen compensating polar planimeter. Under these conditions, the integral fluorescence intensity of form I DNA is a linear function of DNA mass in the range of 0.25–1.50 μg per band (determined spectrophotometrically with concentrated solutions prior to dilution for electrophoresis) (Figure 1, at right).

Enhanced fluorescence intensity is expected to be less for the form I DNA in comparison to an equal mass of forms II or III DNA, neither of which are topologically restricted in

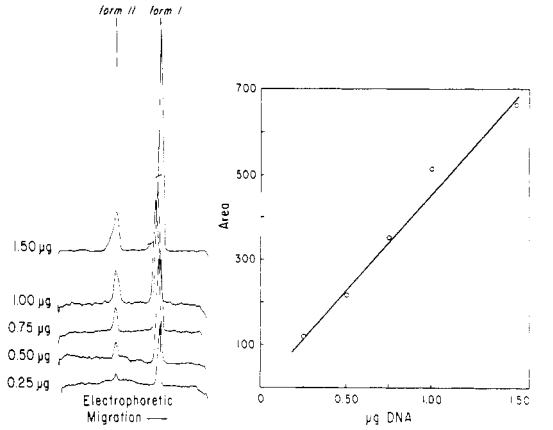

FIGURE 1: Linear dependence of EthBr enhanced fluorescence intensity on mass of PM2 DNA forms. Spectrofluorometric scans of agarose gels containing form I and form II PM2 DNAs (at positions indicated by arrows) after electrophoresis and staining with EthBr are shown at the left. A plot of the total area under the peaks in these scans as a function of total mass of DNA (form I plus form II) is shown in the panel at the right. The area under form I DNA fluorescence peaks in each case has been multiplied by the factor 1.42 to correct for reduced binding of EthBr. The area was measured in arbitrary units where 1 cm^2 equaled 23 units.

binding of EthBr. In order to estimate the true mass of form I DNA in a gel band, it was necessary to correct the integral fluorescence intensity by a multiplicative factor which we have determined in the following way. First, nine identical samples of PM2 DNA containing a high percentage of form I DNA molecules were subjected to agarose gel electrophoresis, EthBr staining, and spectrofluorometric scanning as described above. Next, nine identical samples containing the same mass of DNA converted to forms II and III DNA by DNase I treatment as described by Sierakowska & Shugar (1977) were also subjected to electrophoresis, staining, and scanning as previously described. The requirement for the conservation of DNA mass after DNase I conversion of form I to forms II and III DNA permitted calculation of the multiplicative factor of 1.41 ± 0.14 by which the integral fluorescence intensity of form I PM2 DNA is reduced by topologically restricted uptake of EthBr. This correction factor compared favorably with the increased integral fluorescence intensity measured after irradiation of the gel with short wavelength (254 nm) ultraviolet light to nick the DNA in the form I DNA band, followed by restaining and rescanning (correction factor of 1.42 ± 0.005). These factors are also in good agreement with the mass of form I PM2 DNA determined by scintillation spectrometry of forms I and II DNA (correction factor of 1.42 ± 0.09) which had been uniformly labeled by incorporation of [^{32}P]phosphate prepared under conditions described previously by Strong & Hewitt (1975). Analysis was performed by taking 2-mm gel slices, treating with 2 mL of Nuclear Chicago tissue solubilizer for 3 h at 60 °C followed by the addition of 10 mL of toluene-based scintillation fluid. A similar analysis of form I° DNA using irradiation with short wavelength ultraviolet light (254 nm) to nick the DNA followed by restaining and rescanning gave a multiplicative correction factor of 2.04 ± 0.07.

Isolation of Form III PM2 DNA Produced by Bleomycin Treatment. A total of 50 μg of PM2 form I DNA in a total reaction volume of 1 mL was incubated for 30 min at 37 °C with bleomycin as described above. The DNA was then subjected to electrophoresis in 1.4% agarose gels as described. After staining with EthBr, the form III DNA bands were visualized by fluorescence stimulated by long wavelength (356 nm) ultraviolet light and the band excised by slicing the gel.

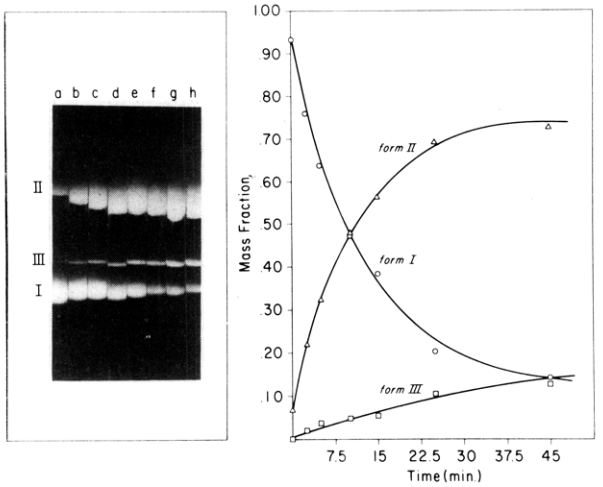

FIGURE 2: Time course of bleomycin reaction with PM2 form I DNA. PM2 form I DNA was treated with bleomycin for increasing times of incubation after which the reaction products, form II and form III DNA, were resolved from form I DNA by agarose gel electrophoresis. The 0.9% gels were stained with EthBr to reveal the positions of each topological form, as indicated by Roman numerals. Incubation times of the bleomycin reaction increase from left to right (a–h corresponding to 0, 1, 2.5, 5, 10, 15, 25, and 45 min, respectively). In the panel at right, the mass fraction of each topological form of PM2 DNA is plotted as a function of reaction time with bleomycin. Mass fractions of DNA were determined from spectrofluorometric scans of individual cylindrical gels and corrected for reduced binding of EthBr by form I DNA.

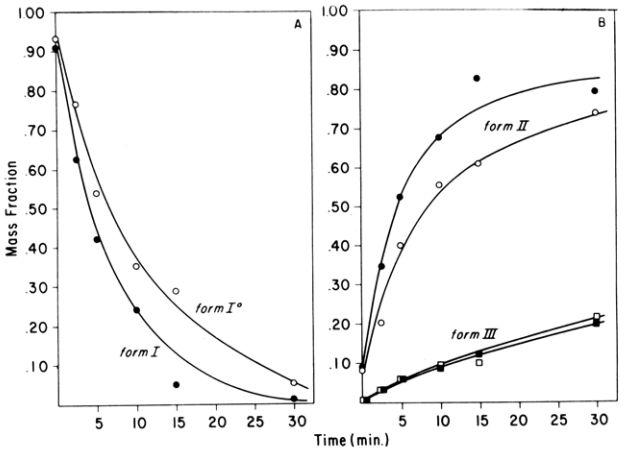

FIGURE 3: Dependence of superhelical turns in PM2 DNA on reactivity with bleomycin. Reduction in the mass fraction of total DNA as either form I (solid circles) or form I° (open circles) as a function of reaction time with bleomycin is shown at the left. At the right, the increases in mass fractions of total DNA are plotted as a function of reaction time with bleomycin: form II (solid circles, for bleomycin reaction with form I DNA and open circles for bleomycin reaction with form I° DNA) or form III (solid squares, for bleomycin reaction with form I DNA and open squares, for bleomycin reaction with form I° DNA).

The slices were combined in a dialysis tube together with 2.5 mL of electrophoresis buffer. Electrophoretic elution (McDonell et al., 1977) of the DNA from the gel slices was then conducted for 3 h at 100 V. After removal of the slices, the sample was placed in a second dialysis tubing and concentrated to a final volume of 100 μL by dialysis against dry Sephadex G-100.

Restriction Endonuclease Cleavage of PM2 DNA. Samples of PM2 form I DNA or form III DNA produced by bleomycin treatment were digested with *Hpa*II (Sharp et al., 1973) or *Hin*dIII (Smith & Wilcox, 1970) restriction endonucleases, respectively, essentially as described by Brack et al. (1976a). In other experiments, the DNA was reacted with the restriction endonuclease prior to bleomycin treatment. The restriction enzymes were obtained commercially from New England Biolabs (Beverly, Mass.).

Electron Microscopy of Bleomycin-Treated PM2 DNA. Samples of bleomycin-treated PM2 DNA were prepared for electron microscopy by the Kleinschmidt basic protein technique as described by Davis et al. (1971). Grids were rotary-shadowed with Pt–Pd (80:20) and examined in a Philips 300 electron microscope.

Results

Bleomycin-Specific Fragmentation of Covalently Closed Circular PM2 DNA. Treatment of covalently closed circular PM2 DNA with bleomycins at an approximate molar ratio of 2.6×10^{-3} bleomycins per DNA phosphate resulted in the progressive conversion of form I DNA to forms II (nicked circular) and III (linear duplex) DNAs (Figure 2). The three topological forms of DNA are resolved by agarose gel electrophoresis and visualized by fluorescence after staining with EthBr (Figure 2, at left). Spectrofluorometric scans of the individual cylindrical gels permitted a quantitative estimate of the fraction of form I DNA mass that was progressively reduced as a result of bleomycin treatment with concomitant accumulation of equivalent mass fractions as form II plus form III DNAs (Figure 2, at right). The relative order of migration rates in agarose gel electrophoresis was established in independent experiments in which PM2 form I DNA was progressively converted to form II and then form III DNA by DNase I digestion (Materials and Methods).

Examination of DNA samples treated with bleomycin for equivalent reaction times by the basic protein Kleinschmidt technique for electron microscopy (Davis et al., 1971) revealed the presence of three topological forms of DNA identified as closed circular, nicked circular, and linear duplex forms, respectively. The time-dependent accumulation of form II and form III DNAs determined by electron microscopy was in general in good agreement with the mass fractions of each form of DNA determined by scanning spectrofluorometry. Length measurements of randomly selected examples of 30 molecules each of form III and form II PM2 DNA observed at 5 and 15 min of bleomycin treatment, respectively, gave ratios of average length of form III to form II DNA of 0.98 ± 0.04 and 0.97 ± 0.03 ($\pm$ standard deviations of ratios). Thus, the linear duplex DNAs produced at these reaction times were near full length, but may be slightly shortened. At the longest reaction times used (30 min), we noted that some linear duplexes less than approximately two-thirds of full length were formed. Linear duplex DNAs less than approximately 90% of the full-length PM2 genome have not been monitored in either gel electrophoretic analyses (Figure 2) or electron microscopic scorings.

Bleomycin-Specific Double-Strand Scissions Do Not Require Superhelical Turns. Either single-strand or double-strand scissions in form I PM2 DNA could occur as a result of breakage of single-stranded or weakly hydrogen bonded regions of the superhelical DNA molecule. This type of DNA substrate preference for cleavage is exhibited by several well-characterized single-strand specific deoxyriboendonucleases (Sierakowska & Shugar, 1977). In order to test for this possible requirement in the bleomycin reaction, the superhelical turns of form I PM2 DNA were relaxed by treatment with calf thymus nicking–closing enzyme (Pulleyblank & Morgan, 1974; Hancock, 1974; Germond et al., 1975) as described by Champoux & McConaughy (1976). Bleomycin treatment of the covalently closed circular form I° PM2 DNA prepared in

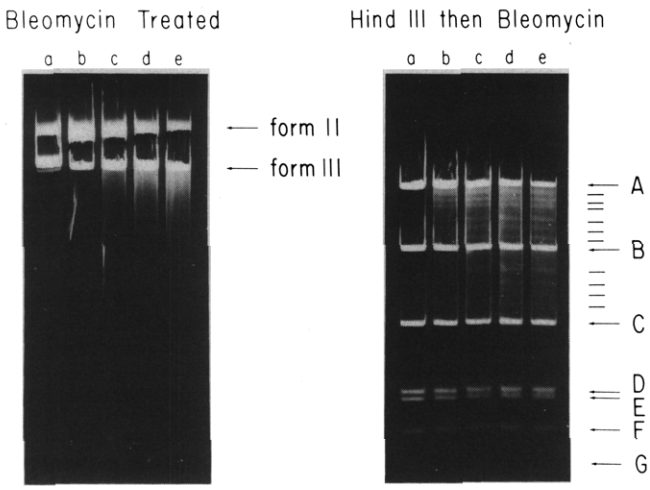

FIGURE 4: Bleomycin fragmentation at discrete sites on PM2 DNA. PM2 form I DNA was treated with bleomycin for increasing reaction times (a–e corresponding to 0, 15, 30, 60, and 180 min) that would produce mixtures of forms II and III DNAs resolved by agarose gel electrophoresis (at left). PM2 DNA was digested with the *Hin*dIII restriction endonuclease followed by bleomycin treatment for increasing reaction times to give fragments of discrete sizes which were resolved by agarose gel electrophoresis (at right). Tracks a–e (corresponding to 0, 15, 30, 60, and 180 min) in the slab gel shown at the right contain the DNA mixtures corresponding to gel tracks a–e for increasing time of bleomycin treatment shown at left. The *Hin*dIII fragments of PM2 DNA are indicated in order of decreasing sizes, A–G, by arrows at the right. Predominant intermediate size fragments produced by *Hin*dIII digestion followed by bleomycin treatment are indicated by lines at the right.

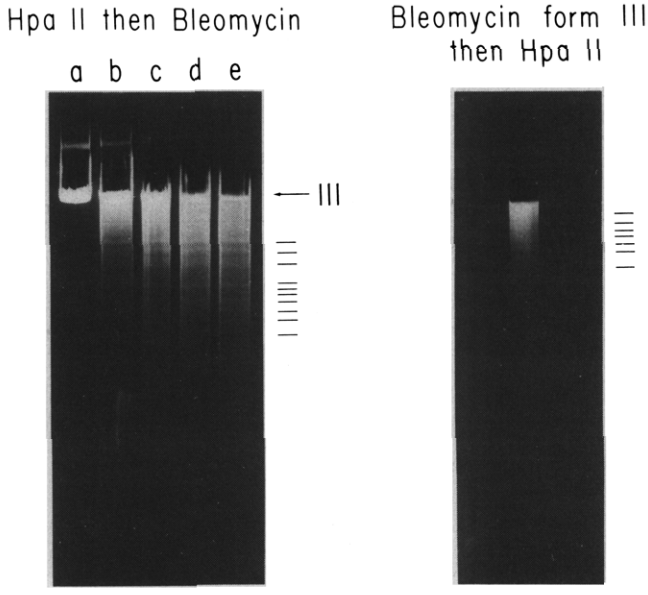

FIGURE 5: Bleomycin fragmentation of PM2 DNA at discrete sites near the *Hpa*II restriction enzyme cleavage site. PM2 form I DNA was exhaustively digested with *Hpa*II restriction endonuclease followed by bleomycin treatment for increasing times of incubation and the reaction products resolved by agarose slab gel electrophoresis (at left, tracks a–e corresponding to 0, 15, 30, 60, and 180 min). The position of form III PM2 DNA is indicated by an arrow. Form III PM2 DNA derived from bleomycin treatment of form I DNA was purified and then exhaustively digested (12 h) with *Hpa*II restriction endonuclease to give discrete fragments resolved by agarose slab gel electrophoresis (at right). The positions of predominant fragment sizes in both agarose slab gels are indicated by lines to the right of each photograph.

this manner resulted in a time-dependent nicking of the substrate DNA which was nearly as efficient as when superhelical form I DNA was used as a reactant (Figure 3A). The difference in nicking rate was found to result essentially from a reduced rate of formation of form II DNA (Figure 3B). The rates of formation of form III DNA remained the same when either form I or form I° DNAs were used as reactants (Figure 3B). Thus, double-strand scissions produced by bleomycin treatment are not dependent on the presence of negative superhelical turns in the reactant DNA.

The rate of bleomycin-promoted single-strand scissions was, however, slightly reduced when the reactant DNA contained superhelical turns. These differences in rates of formation of form II DNA were relatively small and correspond to a large difference in the number of superhelical turns from zero (form I° DNA) to −113 [form I native PM2 DNA with a superhelix density of −0.11 (Upholt, 1977) and length of 10 250 base pairs (D. L. Robberson, unpublished results)].

Bleomycin-Promoted Double-Strand Scissions Occur at Many Discrete Sites in PM2 DNA. Bleomycin-promoted double-strand scissions could occur at specific sites on the genome. At the other extreme, the double-strand scissions could be introduced at sites selected at random throughout the genome. In order to examine the spectrum of possible site-specific double-strand scissions in bleomycin-treated PM2 DNA, the pattern of fragmentation produced by restriction endonuclease digestions in combination with bleomycin fragmentation was determined. When PM2 DNA was digested with *Hin*dIII restriction endonuclease as described by Brack et al. (1976a,b), the limit products were seven linear duplex fragments which were resolved by agarose gel electrophoresis (Figure 4, at right, A–G). These fragments range in molecular weight from 3.4×10^6 (fragment A) to 4.8×10^4 (fragment G) (Figure 4, at right, track a). When the *Hin*dIII PM2 DNA fragments were subsequently treated with 2.0 μg/mL bleomycin for reaction times which, under equivalent conditions, would progressively convert a population of form I and II DNA molecules (at left, track a) to mixtures of forms II and III DNA (Figure 4, at left, tracks b–e), a series of discrete DNA fragments with intermediate sizes became apparent (Figure 4, at right). The intermediate size fragments migrated to positions in the agarose gel primarily between *Hin*dIII fragments A and B and between fragments B and C (Figure 4, at right). Those fragments which migrate between *Hin*dIII fragments A and B must have been derived from bleomycin promoted fragmentation within *Hin*dIII fragment A, since these fragments are smaller than fragment A but larger than fragments B–G. Ten discrete molecular species are indicated in Figure 4 at the right and a total of 15 species have been detected by visual inspection of the agarose gels after staining with EthBr and illumination with short wavelength ultraviolet light. This observation requires that bleomycin-promoted double-strand scissions occur at specific sites on the PM2 genome and that many such sites are available for reaction.

The restriction endonuclease, *Hpa*II, is known to introduce one double-strand cleavage at a specific site in PM2 DNA (Brack et al., 1976a). When PM2 DNA was digested with *Hpa*II and subsequently treated with bleomycin as described above, we again observed a spectrum of molecular species with discrete fragment sizes apparent in agarose gel electrophoresis (Figure 5A); however, many of these fragments migrated to positions in the gel corresponding to near full-length linear duplexes of PM2 DNA (Figure 5, position marked by arrow). When purified form III PM2 DNA, derived from bleomycin treatment, was subsequently digested with *Hpa*II restriction endonuclease, discrete fragment sizes were again detected (Figure 5B). As before, many of these fragments remain near the position of full-length PM2 linear duplex DNA. These combined observations suggest that, although bleomycin-promoted double-strand scissions occur at many discrete sites

TABLE I: Molecular Weights of PM2 DNA Fragments Produced by *Hin*dIII Digestion followed by Bleomycin Treatment.

Fragments with sizes between	Mol wt of intermediate size fragment[b]	Fragment pair	Mol wt of fragment pair
*Hin*dIII fragments A & B[a]			
1	3.13×10^6	1 + 15	3.53×10^6
2	2.87×10^6	2 + 13	3.37×10^6
3	2.60×10^6	3 + 8	3.44×10^6
4	2.12×10^6		
5	1.86×10^6	5 + 6	3.45×10^6
6	1.59×10^6	6 + 5	3.45×10^6
*Hin*dIII fragments B & C[a]			
7	1.03×10^6		
8	0.84×10^6		
9	0.74×10^6		
10	0.67×10^6		
11	0.65×10^6		
*Hin*dIII fragments C & D[a]			
12	0.55×10^6		
13	0.50×10^6		
14	0.45×10^6		
15	0.40×10^6		

[a] Fragments of intermediate sizes were identified as bands in agarose gels stained with EthBr and examined under 254-nm wavelength ultraviolet light. [b] Molecular weights of fragments were determined from distance migrated in agarose gel electrophoresis relative to the positions of *Hin*dIII fragments of PM2 DNA of known molecular weight (Brack et al., 1976a) as in Figure 4.

on the PM2 genome, a significant number of these breaks occur near a site corresponding to the *Hpa*II restriction endonuclease cleavage site. From spectrofluorometric scans of the gel depicted in Figure 5B, we estimate that approximately 50% of the bleomycin-promoted double-strand scissions occur within a distance of 30% of the PM2 genome length from the *Hpa*II restriction endonuclease cleavage site.

On a physical map of the restriction enzyme cleavage sites of PM2 DNA (Brack et al., 1976a,b), the *Hpa*II cleavage site lies within the *Hin*dIII fragment A at a distance of 33.4% of the genome length from the fragment A–fragment B junction and a distance of 23.6% of the genome length from the fragment A–fragment D junction. We therefore predict that *Hin*dIII digestion of PM2 DNA followed by bleomycin-promoted double-strand scission will result in pairs of fragments whose sizes lie between *Hin*dIII fragments A and B and between B and C, respectively, and whose summed molecular weights for each pair will be equal to the PM2 DNA molecular weight. The molecular weights of the intermediate size fragments apparent in Figure 4 calculated using the known molecular weights and gel positions of the *Hin*dIII fragments are presented in Table I. These fragment sizes are summarized in Table I for DNA fragments at gel positions between either *Hin*dIII fragments A and B or between fragments B and C. In each case, the fragment sizes are presented in order of decreasing molecular weight. We observe that pairwise combinations of either two fragments between the *Hin*dIII A and B fragments or one fragment between *Hin*dIII A and B fragments with one fragment between the *Hin*dIII B and C fragments, sum to the molecular weight expected for the intact *Hin*dIII A fragment (Table I). In particular, the molecular

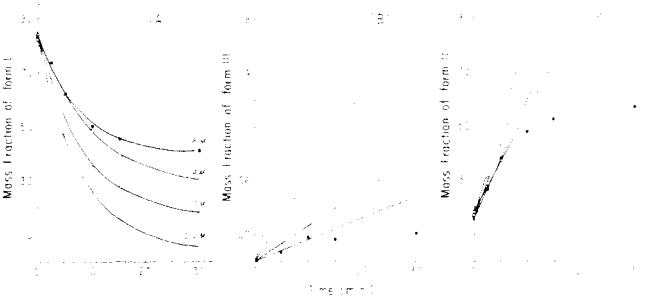

FIGURE 6: Salt concentration dependence of bleomycin reactivity. (A) The time-dependent loss of form I PM2 DNA, expressed as fraction of total mass by reaction with bleomycin in solutions containing 0.017 (open circles), 0.2 (open triangles), 0.4 (open squares), and 0.8 M (solid circles) NaCl, respectively. B and C show corresponding increases in mass fraction of total DNA as forms II and III PM2 DNAs, respectively, after reaction with bleomycin in solutions containing NaCl concentrations as indicated in A. The lines in B and C show initial rates of formation.

weights of these pairs of fragments also permit an estimate of the position of bleomycin-promoted double-strand scission. If bleomycin-promoted double-strand scission occurred predominately at a site identified with the *Hpa*II cleavage site, then one would predict the appearance of only one pair of intermediate size fragments after *Hin*dIII digestion followed by bleomycin treatment. These fragments would have molecular weights of 1.97×10^6 and 1.39×10^6, respectively. In fact, we observe a series of discrete fragment sizes which can be paired to give the molecular weight of *Hin*dIII fragment A. This result requires that at least five bleomycin-promoted double-strand scissions occur at different sites which are within approximately 20% (1.2×10^6) of the PM2 genome length on either side of the *Hpa*II restriction enzyme cleavage site. Thus, both *Hin*dIII and *Hpa*II cleavages in conjunction with bleomycin-promoted double-strand scissions give fragmentation patterns that are mutually compatible. This indicates that several sites of the breakage of duplex PM2 DNA are localized near the *Hpa*II region but that other specific cleavage sites do exist.

Ionic Strength Dependence of Bleomycin Promoted Fragmentation. As noted earlier, the bleomycin used in clinical chemotherapeutic protocols is a mixture of bleomycin components which differ in primary chemical structure through different terminal amine groups. Differences in the rates of formation of form II and form III PM2 DNAs, respectively, when either form I or form I° DNA is used as reactant (Figure 3), suggest that perhaps different components of the bleomycin mixture used here may be separately promoting either single-strand or double-strand scissions. If different components predominate in the formation of single-strand and double-strand scissions, respectively, one might expect to find an experimental condition which differentially alters these two activities. We have found that increasing the NaCl concentration in the reaction reduces the rate of nicking of form I PM2 DNA (Figure 6A). The initial rate of formation of form III DNA was markedly reduced as the NaCl concentration was increased from 0.017 M to 0.8 M (Figure 6B). By way of contrast, the initial rate of formation of form II DNA was much less affected by increasing salt concentration (Figure 6C). The ratios of initial rates of formation of form III DNA to form II DNA were 0.19, 0.115, 0.06, and 0.055 in solutions of ionic strengths of 0.019, 0.2, 0.4, and 0.8, respectively. These ratios of initial rates of formation indicate a strong preferential inhibition of the bleomycin reaction to produce form III DNA compared with form II DNA as the ionic strength of the reaction is increased. The results are consistent with the existence

of two bleomycin components, or at least one of two activities in each of two components of bleomycin, which are acting individually in the production of single-strand and double-strand scissions, respectively. In the case of double-strand scissions or closely spaced single-strand scissions that produce form III DNA, we also observe that after 30 min of reaction the mass fraction of total DNA as form III DNA was dramatically reduced from 18%, when the reaction was performed in 0.017 M NaCl to 2% when the reaction was performed in 0.8 M NaCl solutions.

Discussion

Bleomycin-promoted damage to DNA structure appears to comprise a spectrum of chemical alterations. Modifications that result in depurination or depyrimidation, as well as single-strand scissions of the phosphodiester backbone, are expected to be repaired and would not necessarily constitute lethal cellular damage. By way of contrast, bleomycin-promoted double-strand scissions are expected to be largely irreparable alterations. In fact, cytological studies have demonstrated that fragmentation of metaphase chromosomes occurs when cultured mouse fibroblasts are exposed to high concentrations of bleomycin (Paika & Krishan, 1973). Thus, bleomycin-specific double-strand or closely spaced single-strand breaks in DNA may represent an important physiological aspect for effective clinical application of the drug to control cellular growth in certain human malignancies. Alternatively, it is possible that double-strand scissions in cellular DNA may cause the pulmonary toxicity that frequently accompanies administration of bleomycin and that other types of cellular DNA damage, as yet unidentified, are primarily responsible for arrest of cell proliferation.

In the present study, it is demonstrated that bleomycin reaction with PM2 DNA results in double-strand scissions which cannot be accounted for by simple accumulation of single-strand scissions at random sites on the genome. Although bleomycin-promoted double-strand scissions do not require superhelical turns (Figure 3), it is clear that the breaks are introduced in PM2 DNA at several specific sites which are most frequently located near the *Hpa*II cleavage site (Figures 4 and 5). These regions of the PM2 genome are not, however, particularly distinguished in base composition (Brack et al., 1976b). Specific fragmentation by bleomycin in this region of the genome may, nevertheless, reflect the presence of particular deoxyribonucleotide sequences that permit binding of the drug in a configuration that results in scission of complementary strands at closely spaced sites. A minimum of at least five and possibly six different sites for bleomycin-specific double-strand scissions occur within the *Hin*dIII fragment A. For a random sequence of deoxyribonucleotides with the length of PM2 DNA (taken as 10 250 deoxyribonucleotide pairs), the probability of finding a specific sequence n nucleotides in length is $(\frac{1}{4})^n$. For a sixfold repetition of a particular unique sequence in the PM2 genome, we would therefore expect the sequence to contain 5–6 nucleotide pairs. It is also possible that six separate unique sequences each comprising 7 nucleotide pairs could account for the observed frequency of double-strand scissions. In either case, the lengths of PM2 DNA sequences that are calculated to account for the observed frequency of specific fragmentation produced by bleomycin are approximately one-half the number of nucleotides that are thought to be involved in, or at least blocked by, the binding of bleomycin (Kuo et al., 1973). Chien et al. (1977) has also postulated that one bleomycin molecule could bind to every 4–5 nucleotides. Thus, it is possible to account for bleomycin-promoted fragmentation of PM2 DNA on the basis of sequence specific-strand breakage even for a random arrangement of deoxyribonucleotides in the genome.

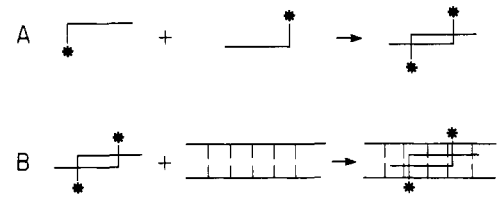

FIGURE 7: Model of bleomycin binding to double-stranded DNA that leads to double-strand scissions. In A, assymmetric monomer units of bleomycin associate to form a dimer species. In B, the dimer species binds to double-stranded DNA to provide a complex in which chemically reactive groups (indicated by asterisks) are juxtaposed along complementary strands. The separation of reactive groups illustrated here is 2–3 base pairs but could be greater.

It is particularly relevant that our unpublished observations with bleomycin A_2 purified to homogeneity indicate that both single-strand and double-strand scissions also occur with approximately the same yield and kinetics as was found here for the bleomycin mixture. The bleomycin mixture (Blenoxane) contains two major components, bleomycins A_2 and B_2 (Crooke & Bradner, 1977) and at least nine minor components. We therefore must resolve the paradox that a purified component of bleomycin reacts with PM2 DNA in a manner that is essentially indistinguishable from the bleomycin mixture. One explanation to resolve this paradox would be to postulate the existence of a monomer molecular species of bleomycin A_2 which reacts with DNA to produce a single-strand scission, whereas a dimer species of bleomycin A_2 reacts with DNA to produce double-strand scissions (Figure 7). Such a dimer species necessarily requires an asymmetric arrangement of the two bleomycin molecules that would interact with double-stranded DNA as illustrated diagrammatically in Figure 7. In the double-strand breakage reaction, two bleomycin A_2 molecules would dimerize and bind to double-stranded DNA such that the reactive groups which lead to phosphodiester bond scissions are juxtaposed on the complementary strands. If the region of juxtaposition encompasses less than approximately four nucleotide pairs, then strand scissions would result in a double-strand break upon dissociation of the intervening base pairs under the reaction conditions used. We consider this scheme to be the simplest in explaining the spectrum of properties for bleomycin-specific fragmentation of double-stranded DNA, although other, more complicated schemes may be found to apply to these results. Experiments are now in progress in search of such a dimer species in solutions of bleomycin A_2.

The possibility of different chemical reactions with DNA mediated by dimer or even higher oligomeric species of bleomycin components is an intriguing though speculative concept and emphasizes the need to experimentally define the spectrum of chemical damage to DNA promoted by a mixture of pharmacologically active agents that are routinely applied in chemotherapy. The methods of analyses applied in this study provide a first step in defining the different modes of action expressed by one such mixture of bleomycins and can provide insights into the possible basis for cytotoxicity of drug action.

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Immunological Detection of O^6-Methylguanine in Alkylated DNA†

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ABSTRACT: Antibodies to O^6-methyldeoxyguanosine were produced in rabbits and utilized in a radioimmunoassay to detect this nucleoside at picomole levels. The specificity of the antibodies was demonstrated by the use of nucleoside analogues as inhibitors in the radioimmunoassay. The antibodies cross-reacted with O^6-methylguanosine, O^6-methylguanine, and O^6-ethylguanosine. There was 10^4 to 10^6 times less sensitivity to inhibition by deoxyadenosine, deoxyguanosine, and guanosine than by O^6-methyldeoxyguanosine. The radioimmunoassay also detected O^6-methylguanine in DNA alkylated by agents known to produce O^6-methylguanine, such as N'-methyl-N-nitrosourea. DNA alkylated with dimethyl sulfate, which does not produce O^6-methylguanine in DNA, cross-reacted with the antibodies to a very limited extent. Such an assay system for modified nucleic acid components would be very useful in following the production, persistence, and repair of these lesions in a variety of cells and tissues treated with a broad spectrum of carcinogens and suspected carcinogens.

In the investigation of the mode of action of carcinogens, much work has centered around the effects of alkylating agents on DNA (Singer, 1975). Recently, a specific alkylation product, O^6-methylguanine, has been found in DNA treated with N'-methyl-N-nitrosourea (MNU)[1] (Loveless, 1969; Kirtikar & Goldthwait, 1974). O^6-Methylguanine accumulated preferentially in rat brain DNA upon in vivo administration of this potent neurocarcinogen (Goth & Rajewsky, 1974; Kleihues & Margison, 1974; Margison & Kleihues, 1975). This modified base also appears to be mutagenic at the level of transcription (Gerchman & Ludlum, 1973). However, O^6-methylguanine is relatively unstable under conventional DNA hydrolysis procedures (Singer, 1976) and is difficult to

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[1] Abbreviations used: MNU, N'-methyl-N-nitrosourea; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; TS buffer, 0.14 M NaCl, 0.01 M Tris, pH 7.5.

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