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Nonspecific Cleavage of ϕX174 RFI Deoxyribonucleic Acid by Bleomycin†

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ABSTRACT: The covalently closed circular duplex deoxyribonucleic acid (DNA) of ϕX174 underwent progressive conversion to nicked and linear DNA with increasing bleomycin/ϕX174 RFI DNA molecule ratios. The formation of linear DNA (a double-strand break) occurred under limited reaction conditions as low as an average of 0.2 single-strand break/ϕX174 RFI DNA molecule. As bleomycin-produced linear DNA was further fragmented by bleomycin, a broad distribution of DNA fragments without notable concentrations of unique size was formed. Restriction enzymes *Pst*I and *Sst*II did not generate discrete fragments from bleomycin-produced full-length linear ϕX174 DNA, nor did bleomycin cleavage generate discrete fragments from *Hpa*II or *Pst*I digests of ϕX174 RFI. These findings suggest that bleomycin does not act at a few specific sites on ϕX174 RFI DNA. The single-strand nick appeared to be the preferred site for bleomycin action for a second cleavage in a ϕX174 molecule.

Bleomycin is a complex, low molecular weight, glycopeptide antibiotic used clinically as an antitumor agent, primarily against a variety of solid tumors (Umezawa, 1975). The precise mechanism by which bleomycin causes cell death is unknown but is thought to be mediated by its interaction with DNA. Bleomycin is known to produce a variety of different effects on DNA, including loss of free base (Haidle et al., 1972; Muller & Zahn, 1976), production of alkali-sensitive sites (Ross & Moses, 1978), and single- and double-strand breaks (Suzuki et al., 1969; Haidle, 1971; Umezawa et al., 1973). At low bleomycin concentrations, thymine is selectively released (Muller & Zahn, 1976), and thymine 3′ to guanine is preferred [Maxam & Gilbert (1977), reported in Poon et al. (1977)]. At high concentrations, all four bases are released (Haidle et al., 1972). Under the appropriate reaction conditions, both single- and double-strand breaks in DNA are produced by bleomycin action. The breaks formed by bleomycin action are not simple phosphodiester bond nicks, because they appear to bear either 3′- or 5′-phosphoryls (Kuo & Haidle, 1973; Shapiro & Chargaff, 1964) and contain gaps where thymine and other bases have been removed (Poon et al., 1977; Ishida & Takahashi, 1976). At high bleomycin/nucleotide ratios, breaks occur preferentially 3′ to GT and GC sequences and to a lesser extent at other sequences containing thymine (D'Andrea & Haseltine, 1978; Takeshita et al., 1978).

It has been suggested that at low bleomycin concentrations double-strand breaks occur at specific sites in the genome of the closed circular bacteriophage PM2 (Lloyd et al., 1978a). Thus far, 11 apparent regions of BLM preference have been identified and mapped on the PM2 genome (Lloyd et al., 1978a,b). In view of the site preferences of bleomycin-produced double-strand breaks, we thought it of interest to analyze the preferred sites for common nucleotide sequences. We have examined bleomycin action on ϕX174 RFI DNA, a closed circular duplex for which the entire base sequence has been published (Sanger et al., 1977). To facilitate sequence analysis, we decided to investigate bleomycin action leading to the first DNA double-strand break. In this report, we demonstrate the lack of site-specific double-strand break formation on the ϕX174 RFI genome under limited reaction conditions. In accord with previously published results, we find that double-strand breaks occur more rapidly than would be predicted by random accumulation of single-strand breaks (Lloyd et al., 1978a; Povrik et al., 1977).

Experimental Procedures

Materials. Blenoxane, clinical bleomycin sulfate, was obtained from Bristol Laboratories. Agarose, acrylamide (recrystallized), *N,N′*-methylenebis(acrylamide), *N,N,N,N′*-tetramethylethylenediamine, and ammonium persulfate were products of Bio-Rad Laboratories. Nitrocellulose filters (BA 85) were obtained from Schleicher & Schüll. All restriction enzymes were obtained from Bethesda Research Labs. [γ-^{32}P]Adenosine triphosphate was obtained from Amersham/Searle.

Preparation of ϕX174 RFI DNA and [^{3}H]ϕX174 RFI DNA. The procedure employed in isolation of the covalently closed circular form of ϕX174 was that of Schekman et al. (1971), with the modifications of Ross & Moses (1978). Following the final precipitation of the supercoiled fraction from cesium chloride–ethidium bromide gradients, the DNA

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was redissolved in 50 mM Tris-HCl,[1] pH 8.1, and 1 mM EDTA and stored at 4 °C.

Filter Retention Assay. The assay for DNA nicking activity based on separation of broken DNA from covalently closed circular DNA following alkali denaturation and renaturation is based on the binding of nonreannealing broken [^{3}H]DNA to nitrocellulose filters under high salt conditions (Kuhnlein et al., 1976). A Poisson distribution correction factor was applied to calculate nicking. The modifications for examining bleomycin-treated DNA were previously described (Ross & Moses, 1978).

Bleomycin Reactions. The standard reaction conditions for bleomycin action used in these studies were 20 mM Tris-HCl, pH 8.1, 20 mM $MgCl_2$, 25 mM dithiothreitol, and 1–10 μg of φX174 RFI in a total reaction volume of 30 μL. For preparing large quantities of bleomycin-treated DNA, 100 μg of φX174 RFI was used in a total reaction volume of 100 μL. Reactions were at 4 °C for 10 min unless otherwise indicated. Reactions were stopped by the addition of EDTA (25 mM final) for gel electrophoresis and by the addition of 0.5 mL of 2.5 mM EDTA and 0.01% $NaDodSO_4$ for filter retention.

Agarose Gel Electrophoresis. Gel and electrophoresis buffer for agarose gels (0.9% agarose, 40 cm × 7.5 cm × 0.2 cm) was 40 mM Tris, 50 mM sodium acetate, and 10 mM EDTA, pH 7.8. Samples were layered on the gels in 10% sucrose, 0.02% xylene cyanol FF, and 0.02% bromophenol blue. Electrophoresis was for 16 h at 150 V (approximately 16 mA), unless otherwise indicated. Following electrophoresis, gels were stained with 1 μg/mL ethidium bromide, 50 mM Tris–borate, pH 8.3, and 1 mM EDTA for 1 h.

Polyacrylamide Gel Electrophoresis. The gel electrophoresis buffer for polyacrylamide gels (4% acrylamide, 40 × 7.5 × 0.2 cm) was 50 mM Tris–borate and 10 mM Na_2EDTA, pH 8.3. Denaturing gels contained 7 M urea in the gel buffer only. After polymerization, gels were preelectrophoresed at 300 (nondenaturing) or 1700 V (denaturing). All samples were layered in 10% sucrose, 0.02% xylene cyanol FF, and 0.02% bromophenol blue. Sample electrophoresis was at 300-V constant voltage for nondenaturing gels and 1700-V constant current for denaturing gels. Polyacrylamide gels were stained and photographed as were agarose gels, or autoradiography was on standard X-ray film (Kodak, X-omat XS) at 4 °C for the times indicated in the figure legends.

Stained agarose and polyacrylamide gels were photographed by using Polaroid Type 55 Positive/Negative film. Exposure time was 3 min. Gels were illuminated from below with 254-nm ultraviolet light, and the fluorescence of the ethidium bromide–DNA complex was photographed from above through a yellow gelatin filter. This system was capable of detecting a band of nicked, linear, or closed DNA containing as little as 30 ng of DNA. When desired, the Polaroid negative was scanned in a Quick Scan Flur/Vis densitometer (Helena Laboratories) to determine the relative peak intensities. The area under each peak was determined by photocopying the densitometric scans, cutting out the peaks, and weighing them on an analytical balance. We determined that the fluorescent intensity of DNA bands on Type 55 film under our exposure conditions was linearly proportional to the amount of DNA added over the range of DNA concentrations used in these studies. It has been previously demonstrated that the fluorescence intensity of DNAs containing a terminus to allow free rotation of one strand about the other (i.e., nicked and linear) is greater than that of an equimolar quantity of covalently closed circular DNA (Paoletti et al., 1971). We did not correct the data for relative fluorescence of supercoiled DNA and freely rotating DNA. In the scanning system we used, supercoiled DNA showed 90% of the fluorescence of linear or relaxed DNA, tested over the range 0.5–5 μg (S. H. Robison and R.E. Moses, unpublished).

[1] Abbreviations used: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

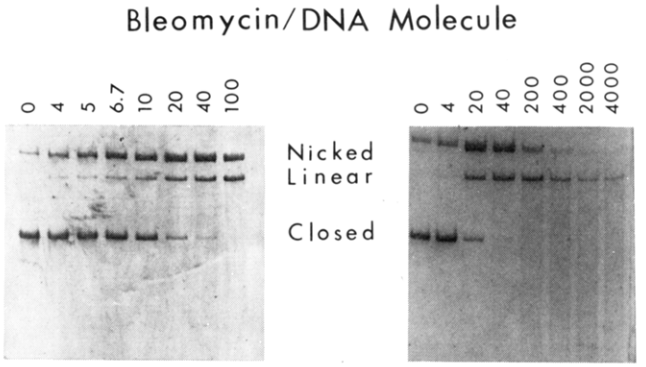


FIGURE 1: Cleavage of φX174 RFI DNA at varying bleomycin/DNA molecule ratios. DNA was treated for 10 min at 5 °C at the bleomycin/DNA molecule ratio indicated in a total reaction volume of 30 μL containing 5 μg of φX174 DNA. The reaction was stopped by addition of EDTA (25 mM final) plus the addition of sucrose layering solution. Electrophoresis was at 150 V for 16 h on a 0.9% agarose gel. DNA was visualized by staining with ethidium bromide.

End Labeling. Termini produced by the action of bleomycin on φX174 RFI DNA were labeled by the phosphate exchange reaction catalyzed by T4 polynucleotide kinase (Berkner & Folk, 1977). This procedure transfers the γ-phosphate from ATP to the 5′ terminus of DNA without prior treatment by dephosphorylating enzyme.

Restriction Endonuclease Cleavage of DNA. Reaction conditions for restriction of various DNAs are described in the figure legends.

Results

Cleavage of φX174 RFI DNA at Varying Bleomycin/DNA Molecular Ratios. Cleavage of covalently closed circular φX174 RFI DNA was by bleomycin action in the presence of dithiothreitol at ice–water bath temperature. Kinetic studies of RFI DNA breakage by bleomycin using the filter retention assay indicated that the reaction was nearly complete within the first 1–2 min and did not show additional breakage after 30 min at low doses. In these studies, all reactions were for 10 min unless otherwise indicated. We were able to detect the conversion of RFI DNA to nicked and full-length linear forms at various bleomycin to RFI DNA ratios (Figure 1). Full-length linear DNA, the result of a double-strand break per φX174 RFI DNA molecule, first appeared after treatment with as little as four bleomycin molecules per DNA circle. These conditions generated an average of only 0.2 nick/DNA molecule as measured by the filter retention assay. Thus, double-strand breaks rapidly formed after limited bleomycin cleavage, a finding similar to those of Lloyd et al. (1978a,b) and Povrik et al. (1977). Full-length linear DNA was further degraded to smaller fragments of no distinct size (i.e., a smear) at higher drug to RFI DNA ratios, and at very high ratios (greater than 20000), no DNA fluorescence was observed in gels.

We were unable to detect discrete bands of DNA at any of the bleomycin/RFI DNA ratios used. These results are in contrast to previously published results on PM2 DNA (Lloyd et al., 1978b). Reactions contained 5 μg of φX174 DNA, and we could have detected a band containing as little as 0.6% of the DNA on the gel (30 ng). If bleomycin promotes

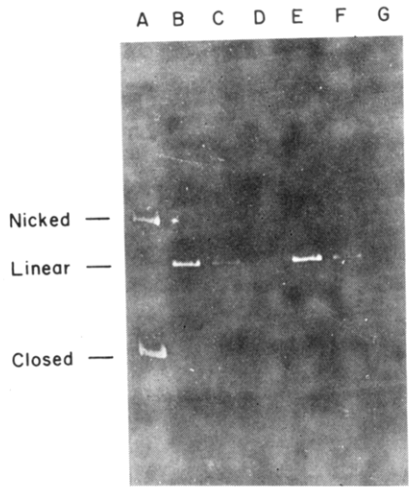


FIGURE 2: Single-site cleavage of bleomycin-produced full-length linear ϕX174 DNA. Treatment with bleomycin in a total reaction volume of 0.1 mL containing 100 μg of DNA was at 5 °C for 10 min at a ratio of 20 bleomycin/DNA molecule. One-half of the sample (E–G) was subsequently treated twice with an equal volume of isoamyl alcohol–chloroform at 5 °C for 30 min with continuous shaking. The DNA in the aqueous layer was then brought to 0.3 M sodium acetate and precipitated with ethanol. Precipitated DNA was resuspended in electrophoresis buffer and electrophoresed in 4% polyacrylamide at 300 V for 16 h. A small portion in a separate channel was stained with ethidium bromide to localize the DNA bands to be extracted. After extraction of the linear DNA from the gel, the DNA was ethanol precipitated and resuspended in appropriate buffer for enzyme reaction: 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 6 mM 2-mercaptoethanol for *Pst*I and 14 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 90 mM NaCl, and 6 mM 2-mercaptoethanol for *Sst*II. Enzymes were added (10 units), and incubation was for 16 h at 37 °C. Electrophoresis was at 150 V for 16 h on 0.9% agarose. DNA was visualized by staining with ethidium bromide. Each track contained approximately 2 μg of DNA. Track A contains control ϕX174 DNA as a marker. Tracks B–D were not extracted with chloroform–isoamyl alcohol; tracks E and G were extracted prior to electrophoresis. Tracks B and E contain bleomycin-produced full-length linear DNA isolated from 4% polyacrylamide gels. Tracks C and F contain *Sst*II-digested linear DNA, and tracks D and G contain *Pst*I-digested linear DNA.

double-strand break formation at a limited number of specific sites in the ϕX174 genome, we should have observed discrete bands by this technique. Therefore, it appeared that bleomycin did not cause double-strand breaks at a limited number of specific sites on ϕX174 RFI DNA.

Restriction Enzyme Digestion of Bleomycin-Linearized DNA. To further test for site specificity of bleomycin-produced double-strand breaks, we digested full-length bleomycin-linearized DNA with restriction enzymes. If bleomycin causes double-strand breaks at a few specific sites, then cleavage of bleomycin-produced linear DNA by restriction enzymes which break ϕX174 DNA at a single site should result in two discrete fragments per bleomycin site, permitting unambiguous determination of the existence and number of unique termini. For example, 20 discrete bands after complete restriction would represent 10 bleomycin sites. On the other hand, if the bleomycin sites are not specific or if there are large numbers of specific sites, then restriction analysis should generate so many fragments that no specific band should be observed on the gel. This kind of analysis should fail only if the bleomycin site and the restriction site were very close together, in which case the bleomycin-produced linear DNA might appear resistant to enzyme digestion. To guard against this possibility, we have performed the analysis with two different restriction enzymes, *Pst*I and *Sst*II, each of which cleave ϕX174 DNA only once and at widely separated sites on the genome (Smith et al., 1976; Goff & Rambach, 1978).

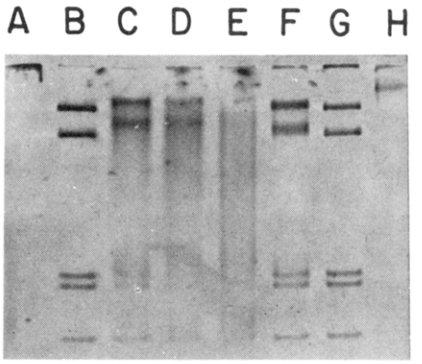


FIGURE 3: Time course of bleomycin action on *Hpa*II digest of ϕX174 RFI DNA. DNA (5 μg) was treated with *Hpa*II (5 units) for 16 h at 37 °C in 20 mM Tris-HCl, pH 7.4, 7 mM $MgCl_2$, and 1 mM dithiothreitol. DNA was then ethanol precipitated, resuspended in bleomycin buffer, and treated with bleomycin at 5 °C. Electrophoresis was on 4% polyacrylamide gels at 300 V for 8 h without removal of bleomycin. Track A contains ϕX174 RFI DNA, and track B contains *Hpa*II-digested ϕX174 DNA. Track H contains ϕX174 DNA treated only with bleomycin (5 μg/mL) for 30 min. Tracks C and E show *Hpa*II fragments treated with bleomycin (20 μg/mL) for 30, 60, and 90 min, respectively. Tracks F and G show *Hpa*II fragments treated for 30 min with 5 and 1 μg/mL bleomycin, respectively.

RFI DNA was treated with bleomycin under conditions which converted 20% of the total DNA to full-length linear DNA, and after the reaction, the DNA was extracted with chloroform–isoamyl alcohol (24:1 v/v) to prevent bleomycin interference with subsequent analysis. This DNA was then run on a 4% polyacrylamide gel. The full-length linear DNA was localized by comigration with *Pst*I-digested RFI DNA, extracted from the gel, digested with the appropriate restriction enzyme, and reelectrophoresed on a 0.9% agarose gel (Figure 2). We did not detect any discrete subspecies after either *Pst*I or *Sst*II treatment, again indicating a lack of site specificity in bleomycin-produced double-strand breaks in ϕX174 RFI DNA. We could have detected a band containing 0.5% of the applied DNA. A faint band was still apparent in the *Sst*II-treated DNA (Figure 2, tracks C and F). Therefore, this reaction did not go to completion as did the *Pst*I reaction.

Bleomycin Degradation of Restriction Fragments. We also examined the effect of bleomycin on fragments of the ϕX174 genome produced by the restriction enzymes *Pst*I and *Hpa*II. The rationale was similar to previous experiments. Specific bleomycin sites would be indicated by discrete bands of intermediate size as demonstrated previously by Lloyd et al. (1978a,b) on PM2 DNA. In addition, it was possible that one or more of the five *Hpa*II fragments might not contain a bleomycin site and, therefore, would be resistant to bleomycin degradation. This might aid in localization of bleomycin cuts.

We first produced 100% linear DNA from ϕX174 RFI by the action of *Pst*I followed by bleomycin treatment under conditions that convert 20% of RFI DNA to linear (i.e., limited incisions). We detected only a broad and continuous distribution of fragments under these conditions of limited double-strand cleavage. No discrete bands were observed that were smaller in size than the full-length linear phage DNA. Figure 3 shows the results of exposure of *Hpa*II fragments to bleomycin. No single fragment was more resistant to treatment than any of the others, and no discrete intermediate size fragments were detected. At higher bleomycin concentrations, we observed a slight decrease in the mobility of the larger *Hpa*II fragments, which could be due to bleomycin binding to the fragments. This appears to be the case for electrophoresis in polyacrylamide but not in agarose (compare to Figure 2). Thus, bleomycin treatment resulted in a smear of

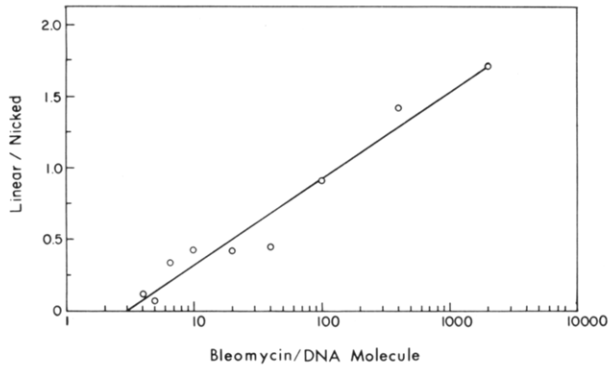


FIGURE 4: Ratio of linear DNA to nicked DNA as a function of increasing bleomycin/DNA molecule. Amounts of different DNA forms were determined from densitometry scans of agarose gels as described under Experimental Procedures. The straight line was determined by least-squares fit and had a correlation coefficient of 0.98.

DNA fragments whether treatment preceded or followed restriction enzyme digestion. These results are in contrast to those of Lloyd et al. (1978a,b) with PM2 DNA.

Formation of Single- and Double-Strand Breaks by Bleomycin. The relative rates of formation and degradation of nicked circular and linear DNAs may be related to the mechanism of bleomycin degradation of DNA. The quantity of nicked circular DNA at any time is a function of competing rates of (1) formation from closed circular DNA and (2) subsequent conversion to the linear form. The quantity of nicked circular DNA reached a maximum at a 10-fold lower bleomycin/DNA molecule ratio than linear DNA (see Figure 1). However, the ratio of full-length linear DNA to nicked circular DNA continued to increase in proportion to the bleomycin concentration until no nicked circles remained (Figure 4). The intercept on the abscissa suggests that no double-strand breaks should occur at less than three bleomycin/RFI DNA molecule. We were not able to investigate this by the gel assay due to the background level of nicked circles present in our preparations of ϕX174 DNA. However, under these conditions (i.e., three bleomycin/DNA molecule), approximately 10% of the RFI DNA was nicked over background according to the filter retention assay (not shown). Thus, single-strand breaks may occur prior to the initiation of double-strand breaks, but clearly double-strand breaks were formed at limited extents of reaction. To verify this conclusion, we looked for the presence of additional single-strand breaks in bleomycin-linearized ϕX174 DNA. Following limited treatment of RFI DNA with bleomycin to convert about 20% of the total DNA to linear chains, the termini were labeld with ^{32}P by the phosphate exchange reaction utilizing T4 polynucleotide kinase (Berkner & Folk, 1977). Labeled DNA was electrophoresed on a 4% polyacrylamide gel under either denaturing or nondenaturing conditions (Figure 5). We observed two main regions of dense label on nondenaturing gels (track A), and these regions correspond to areas containing nicked and linear ϕX174. The band containing linearized DNA was cut out, and the DNA was extracted, denatured in 80% formamide, and electrophoresed in a denaturing gel containing 7 M urea (track D).

Primarily, only large DNA equivalent in size to full-length single-strand ϕX174 (track E) was observed. This result indicates that bleomycin-linearized ϕX174 DNA, under these limiting reaction conditions, contained few additional single-strand breaks. This supports the idea that subsequent bleomycin cleavage occurs preferentially in the region of a single-strand cleavage.

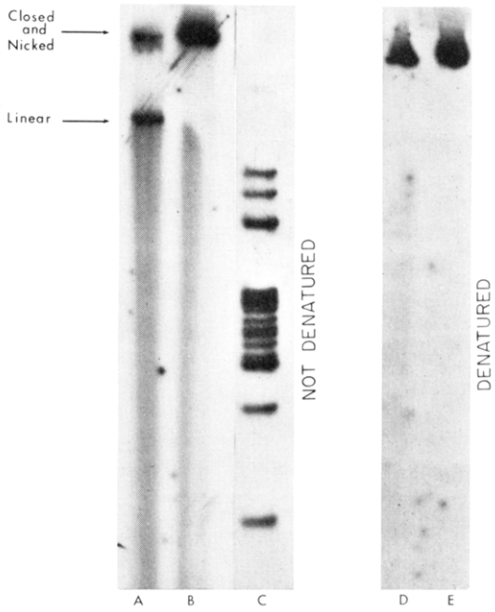


FIGURE 5: Polyacrylamide gel analysis of bleomycin-treated ϕX174 DNA. DNA (30 μg) was treated at a bleomycin/DNA molecule ratio of 100 for 10 min at 4 °C. The DNA was then precipitated with ethanol. The precipitate was rinsed once with ice-cold 70% ethanol, resuspended in a total reaction volume of 45 mM KCl, 18 mM $MgCl_2$, 5 mM dithiothreitol, 25 mM imidazole, pH 6.6, 0.3 mM adenosine diphosphate, 10-fold diluted T4 polynucleotide kinase (Boehringer Mannheim), and 0.3 mCi of [γ-^{32}P]adenosine triphosphate (3000 Ci/mmol), and incubated 15 min at 37 °C. Samples were electrophoresed on 4% polyacrylamide gels as described under Experimental Procedures. Gels were autoradiographed for 18 h at 4 °C (nondenaturing) and 7 days at −20 °C (denaturing). Track A contains bleomycin-treated ϕX174 DNA, and track B contains untreated ϕX174 DNA. Track C contains molecular weight markers (*Hin*fI fragments of plasmid pOV230) varying in size from 3000 to 50 base pairs. Track D is bleomycin-produced full-length linear DNA that was prepared as in Figure 2, heat denatured in a boiling H_2O bath for 1 min in 80% formamide, and quick cooled in an ice bath immediately prior to electrophoresis. Track E is *Pst*I-digested ϕX174 RFI DNA (i.e., full-length linear DNA) that was heat denatured in the same manner.

We can predict the number of molecules having 0, 1, 2, ..., *n* cleavages at any given level of bleomycin activity from the Poisson distribution. Table I shows the predicted percentage of molecules receiving 1, 2, or more single-strand breaks on the basis of the measured percentage of closed circles at different bleomycin/DNA molecule ratios. The measured quantity of nicked circles (i.e., a single hit) approximately equalled the number of molecules calculated by the Poisson distribution to have one hit[2] up to a bleomycin/DNA molecule ratio of 10. There was also a correlation between the quantity of linear DNA measured on gels (i.e., double-strand breaks) and the percentage of DNA predicted to receive two or more hits (Table I). At low ratios (10 or less), these measured and predicted values agreed quite well. Our observation is comparable with previous studies (Lloyd et al., 1978a,b) but suggests to us that there is no preference for bleomycin attack on DNA molecules containing a nick; this is supported by the progressive appearance of nicked DNA molecules in agreement with the dose relationships shown here (Figure 1). However, when a molecule containing a nick receives a second nick, there is a marked preference for bleomycin to act near the nick in the opposite strand, producing a linear molecule. An alter-

[2] We are using the Poisson distribution to evaluate the probability of events occurring in a ϕX174 DNA molecule. The events are theoretically both single- and double-strand breaks, and there is no difference in the way these breaks are scored (i.e., the Poisson distribution does not distinguish various types of events).

Table I: Bleomycin-Produced DNA Forms and Poisson Distribution of Cleavages[a]

bleomycin/ ϕX174 molecule	closed (%)	nicked (%)	linear (%)	single-strand breaks/ϕX174 molecule	closed (P_0) (%)	P_1 (%)	P_2 (%)	$>P_2$ (%)
4	73.2	23.7	3.1	0.31	73.2	22.7	3.5	0.6
5	70.0	27.8	2.3	0.36	70.0	25.2	4.5	0.6
6.7	62.9	27.8	9.4	0.46	62.9	28.9	6.7	1.5
10	52.0	33.9	14.2	0.65	52.0	33.9	11.0	3.1
20	18.5	57.9	23.5	1.69	18.5	31.2	26.4	23.9
40	6.8	64.4	28.9	2.69	6.8	18.3	24.6	50.3

[a] Percentages of closed, nicked, and linear DNAs were determined from agarose gels by densitometry. The percentage of molecules expected to have 0, 1, 2, ... n single-strand breaks were determined by the Poisson distribution. No correction was made for relative fluorescence of DNA forms, as described under Experimental Procedures. $P_{n+1} = P_n[x/(n+1)]$, where x is the average number of single strand breaks per molecule. P is the individual probability of single-strand breaks. When $n = 0$, the probability of not receiving an event (P_0) is equal to the percent of surviving closed circular DNA.

native explanation, which cannot be ruled out, is that bleomycin occasionally directly causes double-strand breaks, possibly by acting in the form of a dimer as suggested by Lloyd et al. (1978a). However, the latter choice does not explain the good agreement with predictions for two or more hits. That is, there would be a number of "hidden" second and third hits in the linear molecule. The data in Figure 5 do not support this argument. Resolution of these two possibilities requires demonstration of whether linear molecules are formed from nicked circular molecules.

At higher ratios, the total DNA content detected on the gels was not constant, due to loss of material in the linear band by further degradation, as discussed (Figure 1). This would lead to an overestimate of closed circles and nicked circles relative to linear forms. For some circles to remain intact or just receive one nick while linear forms were further degraded to less than unit length (and lost from view on the gel) raises the question of whether linear DNA may be attacked more readily by bleomycin.

Discussion

Results presented here indicate that bleomycin apparently does not interact with ϕX174 RFI DNA in the manner reported for PM2 DNA. Double-strand breaks were formed rapidly, in accordance with previously published observations on PM2, but these breaks were not associated with a limited number of specific sites on the ϕX174 genome. Our results argue that at limited extents of reaction bleomycin shows no site preference in ϕX174 DNA. These results are compatible with the analyses of bleomycin breakage following extensive DNA degradation in which moderate preference of bleomycin for the pyrimidines T and C over A and G has been demonstrated (D'Andrea & Haseltine, 1978; Takeshita et al., 1978). We cannot explain the differences between bleomycin action on ϕX174 DNA and on PM2 DNA. One possible basis for such a difference in observations could lie in the treatment of the DNA during preparation. While we are not aware of significant variances, it should be noted that PM2 supercoiled DNA represents a mature product while ϕX174 RFI represents a replicative intermediate.

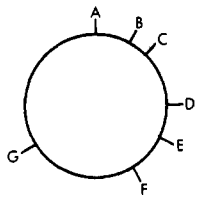
One can predict the number of discrete bands that would be formed if bleomycin acted on specific sites in a closed circular genome. This prediction allows one to interpret the banding pattern on gels with regard to a number of associated specific sites. The following relation can be demonstrated empirically:

$$F = C^2 - C + 1$$

where F is the number of possible fragments, but includes one full-length linear fragment, and C is the number of double-strand cuts. Thus, for example, if there were 11 specific sites

Table II: Fragments Formed from Limited Cleavage at Seven Discrete Sites

	A	B	C	D	E	F	G
A	■	A-B	A-C	A-D	A-E	A-F	A-G
B	B-A	■	B-C	B-D	B-E	B-F	B-G
C	C-A	C-B	■	C-D	C-E	C-F	C-G
D	D-A	D-B	D-C	■	D-E	D-F	D-G
E	E-A	E-B	E-C	E-D	■	E-F	E-G
F	F-A	F-B	F-C	F-D	F-E	■	F-G
G	G-A	B-B	G-C	G-D	G-E	G-F	■



42 Fragments
■1 Full-Length Linear Arising From Each Site

and all were broken by bleomycin reactions, then one should observe 111 bands on the gel or 110 if no full-length molecules remain. This expression is true only for reactions that do not go to completion. It accounts for the overlap of random fragments prior to cleavage of an intervening site when more than two sites per circular genome are present. As an example, Table II shows all of the possible fragments which would be produced if seven cleavage sites were present on the genome. The sites are labeled with letters and the squares on the diagonal represent full-length linear molecules. Thus, banding patterns for site-specific DNA degradation can be predicted for a reaction, such as bleomycin degradation, which is examined under limited conditions. These interactions differ from those of restriction enzymes because there is no obvious completion of the reaction which yields a single set of fragments with combined length the size of the original phage DNA.

We have examined here the bleomycin reaction leading to the formation of the first few double-strand breaks in the ϕX174 RFI DNA molecule whereas the 11 apparent sites reported on PM2 were demonstrated under more extensive reaction conditions. The time course of bleomycin reaction used for studies on PM2 DNA (Lloyd et al., 1978a,b) should have revealed the multiple banding pattern just discussed. In this manner, the 11 discrete bands observed by bleomycin treatment of PM2 could be the result of only a few (e.g., four) specific sites. In the studies reported here, we were assured of only one double-strand break per ϕX174 RFI genome because we identified full-length linear DNA that was formed by bleomycin action, and the DNA could not have been full length if more than one double-strand break had occurred. This linear DNA could have contained additional single-strand nicks, but data in Figure 5 indicate these were minimal.

The relative rates of appearance and disappearance of nicked and linear DNAs suggest that bleomycin may prefer a nicked site on a substrate, producing a double-strand break, in distinction to observations of other authors (Lloyd et al., 1978a). Since double-strand breaks did not result in unique termini, single-strand ("first hit") nicks were distributed nonuniquely,

as demonstrated in Figure 5. The rapid degradation of linear DNA to smaller fragment suggests that other double-strand breaks might also be formed in a similar manner, with random cleavage of one strand of duplex DNA followed by a separate cleavage event in close proximity on the opposite strand to yield fragments. It is not necessary to invoke a model using molecular dimers of bleomycin to account for rapid accumulation of double-strand breaks; a preference for nicked sites would be sufficient.

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Biosynthesis of *o*-Succinylbenzoic Acid in a *men*⁻ *Escherichia coli* Mutant Requires Decarboxylation of L-Glutamate at the C-1 Position†

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ABSTRACT: A *men*⁻ mutant of *Escherichia coli*, AN 209, which accumulates *o*-succinylbenzoic acid, has been used for a direct study of the biosynthesis of this benzenoid compound. Samples of labeled glutamic acids were added to growth media, and the *o*-succinylbenzoic acid was isolated and converted to a dimethyl derivative. This dimethyl derivative was purified on thin-layer chromatograms and by gas chromatography. When the glutamic acid used as precursor contained ^{14}C at position 5, or was uniformly labeled, the dimethyl *o*-succinylbenzoate contained radioactivity (as shown by radiogas chromatography). However, from [1-^{14}C]glutamate, the dimethyl *o*-succinylbenzoate was without radioactivity. Hence, in the biosynthesis of *o*-succinylbenzoate, carbon atom 1 of glutamate is lost, and carbon atoms 2–5 are retained. It was also shown that this mutant lacked the enzyme dihydroxynaphthoic acid synthase. It should, therefore, continue to be classified as a *menB* mutant, rather than as a member of the newly created *menE* group (lacking *o*-succinylbenzoate-CoA synthetase).

That menaquinones are shikimate-derived materials has been known since the early experiments of Cox & Gibson (1964, 1966). Seven of the ten carbon atoms of the naphthalenoid nucleus are derived from the intact shikimate skeleton (Campbell et al., 1967, 1971; Guêrin et al., 1970); the remaining three atoms originate in either α-ketoglutarate or glutamate with both of the carboxyl carbon atoms of these precursors being lost (Robins et al, 1970; Campbell et al., 1971; Robins & Bentley, 1972). To account for the observed role of the C_5 compounds, Campbell (1969) proposed that a thiamin pyrophosphate complex of succinic semialdehyde (formed from α-ketoglutarate) condensed with shikimate in a reaction analogous to the Michael addition. Subsequent evidence implicated chorismate rather than shikimate as the material reacting with the C_5 precursor; it was also suggested that *o*-succinylbenzoic acid (OSB)[1] was a product of the conden-

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