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# DNA double-strand breaks and alkali-labile bonds produced by bleomycin

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## ABSTRACT

Both in linear T2 DNA, analyzed by velocity sedimentation, and in supercoiled Col E1 DNA, analyzed by gel electrophoresis, the number of double-strand breaks produced by bleomycin was directly propotional to the number of single-strand breaks and was far greater than the number expected from random coincidence of single-strand breaks, suggesting that the bleomycin-induced double-strand breaks occur as an independent event. In Col E1 DNA, at least twice as many single-strand breaks were found under alkaline assay conditions as were found under neutral conditions, showing the production of alkaline-labile bonds by bleomycin.

## INTRODUCTION

The antitumor drug bleomycin has been shown to produce both single-strand breaks[1,2] and double-strand breaks[3-5] in DNA. However, it has not been determined whether the double-strand breaks result from random coincidence of single-strand breaks or whether they occur as an independent event. One approach is to measure double-strand break production under conditions where the expected production from coincidence of single-strand breaks is negligible. This requires quantitative measurement of a small number of breaks per unit length of DNA. We present here results in two systems, linear T2 DNA and supercoiled Col E1 DNA, which permit such an analysis.

## MATERIALS AND METHODS

Preparation of T2 DNA and treatment with bleomycin: T2 phage were grown on *E. coli* C 321 *thy*$^-$ in M 9 medium[6] with 1 x $10^{-6}$ M $FeCl_3$, 2.5 μg per ml thymine, 1.5 μCi/ml (methyl-$^3$H)-thymine (Amersham). The phage were purified by differential centrifugation including CsCl-step gradients and kept at 5 x $10^{11}$/ml in 0.15 M NaCl, 0.015 M sodium citrate (SSC). Phage DNA was prepared by adding 1% Sarcosyl and extracting several times with

water-saturated phenol freshly distilled under vacuum, then dialyzing extensively against SSC. The DNA was handled with care to avoid shearing; it contained no measurable double-strand breaks and less than 0.2 single-strand breaks per strand. The DNA preparations were stored at 40 μg/ml in SSC.

T2 DNA at 20 μg/ml was treated with 250 μg/ml bleomycin in 0.5 SSC, pH 7.3 at 21°C. At various times the action of bleomycin was stopped by changing the pH to 5.6 or 12 by adding appropriate amounts of HCl or NaOH.

Preparation of Col E1 DNA and treatment with bleomycin: E. coli JC411 thy⁻ cells harboring the Col E1 plasmid were grown in M9 medium[6] containing 1% Casamino acids, 1% glucose, 180 μg/ml chloramphenicol and 10 μg/ml thymine or 2 μg/ml ($^{14}$C)-thymine (New England Nuclear). Supercoiled Col E1 DNA was isolated from the high-speed supernatant of the lysate by banding in equilibrium CsCl-ethidium bromide gradients as described by Blair, et al.[7]

To study the reaction of bleomycin with DNA, a solution of supercoiled Col E1, usually 100 μg/ml in 0.01 M Tris, pH 8, was added to an equal volume of a solution containing various concentrations of bleomycin in 0.01 M Tris, or 0.08 M Tris, 0.05 M 2-mercaptoethanol. The mixtures were incubated at 37°C for 30 min in glass shell vials. To stop the reaction, 0.2 volume of 0.05 M EDTA in either 25% sucrose or 50% glycerol was added. Samples were then frozen for later analysis or immediately subjected to gel electrophoresis and/or neutral and alkaline velocity sedimentation.

In a similar experiment, DNA samples (50 μg/ml) in 0.1 M Tris, pH 8, were irradiated with $^{60}$Co γ-rays at 22°C and 450 rads/min. Samples were then incubated for 2 hr at 37°C and subjected to gel electrophoresis.

Neutral and alkaline gradient centrifugation: About 50 μl samples containing 0.8 μg T2 DNA or less were placed on top of 5 ml 5-20% sucrose gradients, pH 12 or 5.6, for single- and double-strand break analysis respectively. Alkaline gradient samples were first denatured by adding 0.2 volume 2 M NaOH and holding 10 min at room temperature. The alkaline gradients contained 0.5 M NaCl, 0.05 M NaOH, and 0.01 M EDTA. The pH 5.6 gradients contained 0.5 M NaCl, 2.6 mM NaOH, 7.5 mM disodium citrate, and both pH 12 and pH 5.6 gradients were centrifuged 150 min at 35,000 rpm, 18°C, in an SW50.1 rotor.

Col E1 DNA containing 5% sucrose was centrifuged on 10-20% sucrose gradients. The pH 12 gradients were centrifuged at 45,000 rpm for 60 min, the pH 5.6 gradients at 45,000 rpm for 180 min, but otherwise the conditions were identical to those used for the T2 DNA.

Gradients were fractionated from the bottom into 33 equal fractions, either on Whatman No. 17 paper strips for T2 DNA, or directly into scintillation vials for Col E1 DNA, and radioactivity of each fraction determined in a liquid scintillation counter.

Agarose gel electrophoresis: 0.9% agarose slab gels, 14 cm in length were formed with a buffer containing 0.04 M Tris, 0.001 M EDTA and 0.005 M sodium acetate, pH 7.8, and were run on an apparatus similar to that described by Studier[8]. Samples were electrophoresed for 4 - 4.5 hr at 7 volts/cm at 22°C. The gels were then stained in the same buffer containing 0.25 μg/ml ethidium bromide, a concentration at which supercoiled and relaxed molecules exhibited the same amount of fluorescence (within ± 10%). Gels were exposed to 254 nm light, and the amount of DNA in each band was determined by quantitative fluorescence photography[9,10] or by dissolving gel slices in 8 M $NaClO_4$ and counting the radioactivity.

## RESULTS

1. Strand breaks in T2 DNA: Samples of T2 DNA were treated with 250 μg/ml bleomycin for various times without sulfhydryl reagents, and sedimented on neutral and alkaline sucrose gradients. The number of strand breaks was determined by fitting calculated curves to the sedimentation profiles[11]. The number of double-strand breaks per T2 genome is plotted against the number of single-strand breaks per single strand in Fig. 1. The linear relation shows that double-strand breaks are not the result of coincidences between random breaks in the complementary strands, which would give a quadratic. The dashed line shows the number of double-strand breaks expected from the equation of Freifelder and Trumbo[12], assuming that breaks in each of the complementary single strands and within 15 base pairs of each other give a double-strand break.

2. Strand breaks in Col E1 DNA: A single-strand break converts a supercoiled ( form I ) plasmid to a relaxed circle ( form II ), while a double-

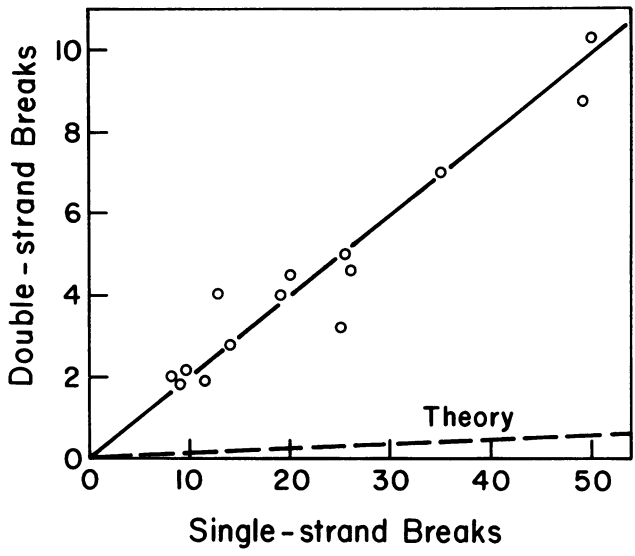


Figure 1: Number of double-strand breaks per T2 genome plotted versus the number of single-strand breaks per single strand for DNA treated with bleomycin. The data can be fitted best by a linear relation with a slope of 5 ± 1 single-strand breaks per double-strand break (solid line). The dashed line is the Freifelder-Trumbo relation[12].

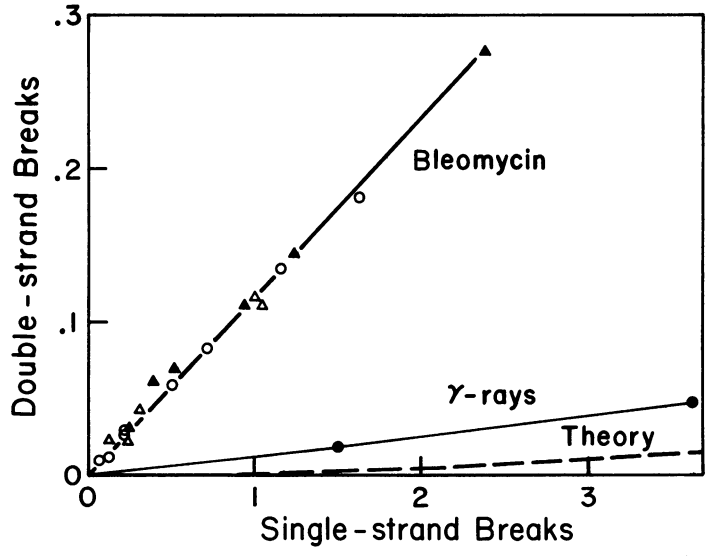


Figure 2: Production of single- and double-strand breaks in Col E1 DNA, as determined from conversion of supercoils into linear and relaxed molecules. Bleomycin-treated and γ-irradiated DNA were analyzed on agarose gels using fluorescence photography. Data from three experiments with bleomycin (different symbols) gave a ratio of 9±.4 single-strand breaks per double-strand break. Dashed line is the Freifelder-Trumbo relation[12].

strand break converts either of these forms to a linear molecule ( form III). Under our conditions , 0.9% neutral agarose gels resolve the three different molecular forms into distinct and well separated bands. Assuming that breaks are randomly distributed, the number $n_2$ of double-strand breaks per molecule can be determined from the fraction $f_{III}$ of full length linear molecules appearing after treatment. These molecules have exactly one break and are given by the first term of a Poisson distribution.

$$f_{III} = n_2 \exp(-n_2) \quad (1)$$

The sum of single- plus double-strand breaks per molecule, $n_1 + n_2$, can be determined from the fraction $f_I$ of supercoils remaining after treatment.

$$f_I = \exp -(n_1 + n_2) \quad (2)$$

When Col E1 supercoils were exposed to various concentrations of bleomycin in the presence of mercaptoethanol for a fixed time and analyzed on agarose gels, the number of double-strand breaks was roughly proportional to the bleomycin concentration, but the data showed considerable scatter. However, if $n_2$ was plotted against $n_1$, the relation was linear ( Fig. 2 ). The number of double-strand breaks expected from coincidences of random single-strand breaks is less than 0.01 per molecule, as shown by the dashed line in the figure. Gamma rays produced many fewer double-strand breaks per single-strand scisson than did bleomycin, as shown in Fig. 2.

3. Alkali-labile bonds: Because single-strand breaks in T2 linear DNA were assayed under alkaline conditions while those in Col E1 supercoils were assayed at neutral pH, we determined whether exposure to alkali after bleomycin treatment produced additional breaks. Several samples of Col E1 supercoils were treated with bleomycin, and portions of each sample were analyzed on neutral and alkaline sucrose gradients and on neutral gels. For alkaline gradients, samples were first treated 10 min with 0.4 M NaOH at 22°. As shown in Table 1, the number of strand breaks caused by bleomycin increased two- to four-fold after treatment with alkali. Therefore, bleomycin induces alkali-labile bonds in addition to true single-strand breaks.

Treatment of Col E1 DNA with the 0.4 M NaOH by itself typically introduced 0.2 - 0.3 single-strand breaks in the ten-minute period, by hydrolysis of the ribose linkages known to be present in these supercoils when

Table 1: Sum of single- plus double-strand breaks in Col E1 DNA treated with bleomycin and assayed under neutral and alkaline conditions, using equation ( 2 )

| DNA µg / ml | Bleomycin µg/ml | 2-mercapto-ethanol, mM | Breaks per Molecule: alkaline gradients | neutral gradients | neutral gels |
|---|---|---|---|---|---|
| 5 | 0.015 | 25 | 0.53 | 0.14 | - |
| 50 | 0.015 | 25 | 0.26 | 0.12 | 0.13 |
| 50 | 0.08 | 25 | 0.20 | 0.12 | 0.14 |
| 50 | 0.16 | 25 | 1.10 | 0.50 | 0.57 |
| 50 | 6.0 | 0 | 0.92 | 0.21 | - |

prepared in the presence of chloramphenicol[7]. Such breaks were subtracted, assuming that the same number of such hydrolyzed ribose linkages occurred in the samples treated with bleomycin and in controls; this is equivalent to measuring bleomycin-induced breakage of only those molecules with no hydrolyzed ribose linkages.

## DISCUSSION

For both linear T2 DNA treated with bleomycin without added sulfhydryl reagent, and Col E1 supercoiled DNA treated with bleomycin in the presence of sulfhydryl reagents, the number of double-strand breaks was linearly related to the number of single-strand breaks ( Figs. 1 and 2 ). Thus, only a small fraction of the measured double-strand breaks are the result of coincidences between randomly produced single-strand breaks in the complementary strands. This is true even if the specificity of bleomycin should confine its action to relatively short stretches of the DNA.

There are data in the literature which support the selective formation of double-strand breaks by bleomycin. Onishi, et al[13] give median molecular weights for E. coli DNA treated with bleomycin in the presence of various reducing agents and sedimented in neutral and alkaline sucrose gradients. Their data are consistent with the linear production of about 1/6 double-strand break per $10^6$ daltons for each single-strand break per $10^6$ daltons. Stern, Rose and Friedman[14] show neutral and alkaline sucrose gradients for λ and adenovirus type 2 DNA treated with phleomycin, a drug closely related to bleo-

mycin. Their Figs 1 and 2 show 0.4 - 0.6 double-strand breaks for 1.5 single-strand breaks per single strand. In both studies, the number of double-strand breaks is much larger than could be expected from coincidences between randomly produced single-strand breaks.

Table 1 demonstrates that bleomycin produces alkali-labile bonds in DNA. Since bleomycin releases free bases (rather than nucleosides or nucleotides) from DNA[15, 16], these alkali-labile bonds may be backbone sugars from which bases have been released. Such sites are well known to open in alkali [17]. Müller, et al[18] have shown that for poly (dA-dT) treated with bleomycin and heat-denatured, the size of the single strands is much greater than the average distance between released thymines.

Fig. 1 shows one double-strand break per five single-strand breaks in the T2 single-strand, or ten single-strand breaks in both strands. This is very similar to the ratio for Col E1 DNA ( Fig. 2), but the agreement is only apparent. The single-strand breaks for the T2 DNA are measured in alkali, and if Col E1 DNA were measured in the same way, Table 1 shows that there would be about one double-strand break per 18-40 single-strand breaks (including both alkali-labile and 'true' breaks). The reasons for the differences in this ratio are not known.

It has not escaped our notice that the formation of double-strand breaks immediately suggests a possible explanation for the effect of bleomycin on the copying mechanism of the genetic material.

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