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# Fragmentation of Deoxyribonucleic Acid by Bleomycin

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SUMMARY

The ability of the antibiotic bleomycin to cause single- as well as double-strand breaks in deoxyribonucleic acid *in vitro* has been confirmed. Heat-denatured, single-stranded DNA is considerably more sensitive to the action of bleomycin than is native DNA. The rate of the reaction of DNA breakage by bleomycin is enhanced in the presence of 2-mercaptoethanol; however, 2-mercaptoethanol also inactivates bleomycin. This inactivation reaction is especially rapid at 80°, although bleomycin itself is stable to heating at 100° for 10 min. Bleomycin alone causes the fragmentation of DNA upon prolonged incubation, and therefore the presence of the reducing agent is not obligatory. In the presence of high concentrations of bleomycin, the DNA is degraded to the level of free nucleotides, nucleosides, or bases.

## INTRODUCTION

The antibiotic bleomycin was first described by Umezawa *et al.* (1). The bleomycins were subsequently shown to be a group of related glycopeptides separable by carboxymethyl-Sephadex chromatography (2). The peptide portion of bleomycin was found to contain six amino acids and an amine (3), and the sugar portion contained L-gulose and 3-*O*-carbamoyl-D-mannose (4).

The antibiotic has antineoplastic activity toward the ascitic form of Ehrlich carcinoma (5) and several strains of transplantable mouse tumors (6). Bleomycin has also been found effective against squamous cell carcinoma in man (7, 8).

Bleomycin inhibits DNA and protein synthesis in *Escherichia coli*, Ehrlich carcinoma, and HeLa cells (9). In addition, the drug causes a decrease in the melting temperature of DNA and also produces scission of DNA strands both *in vitro* and *in vivo*, the former reaction requiring 2-mercaptoethanol or hydrogen peroxide (10–12).

The present study was initiated to learn more details of the reaction of fragmentation of DNA *in vitro* in order to gain insight into the mechanism of strand breakage of DNA by bleomycin.

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## MATERIALS AND METHODS

*Chemicals.* Bleomycin was a gift from Bristol Laboratories, Syracuse, N. Y. Thymidine-2-$^{14}$C (48.4 mCi/mmole) and thymidine-*methyl*-$^{3}$H (11.9 Ci/mmole) were purchased from Schwarz BioResearch, Orangeburg, N. Y. All other chemicals were purchased from commercial sources.

*Labeled DNA. Bacillus subtilis* 168 (*thy trp* C2), which requires thymine and tryptophan for growth, was used as the source of labeled DNA. The cells were grown overnight in a medium containing 2.0 g of

$(NH_4)_2SO_4$, 14.0 g of $K_2HPO_4$, 6.0 g of $KH_2PO_4$, 1.0 g of trisodium citrate, 0.2 g of $MgSO_4$, 5.0 g of glucose, 0.05 g of glutamic acid, 0.2 g of casein hydrolysate, 0.04 g of DL-tryptophan, and 0.004 g of thymine or thymidine in 1 liter of distilled water. The overnight culture was transferred to fresh medium containing 0.5 μCi/ml of $^3$H-thymidine or 0.2 μCi/ml of $^{14}$C-thymine. The culture was allowed to grow to the late logarithmic phase and was harvested by centrifugation. The cells were lysed by incubation at 37° for 30 min with 100 μg/ml of egg white lysozyme. DNA was isolated by a modification of the method of Marmur (13). The labeled DNA, in a final volume of 2–5 ml, was dialyzed overnight in the cold against 2 liters of 0.15 M NaCl and 0.015 M trisodium citrate. DNA concentration was assayed by the method of Burton (14).

*Gradient centrifugation.* Linear 4.5-ml, 5–20% (w/v) sucrose gradients containing 0.3 N NaOH and 0.7 N NaCl (alkaline) or 1.0 N NaCl (neutral) were prepared using a Beckman density gradient former. For alkaline gradients, 0.4 ml of the reaction mixture was mixed with 0.1 ml of a solution containing 3.5 N NaCl and 1.5 N NaOH, and the entire 0.5 ml was layered on top of the gradient; for neutral gradients, 0.4 ml of sample was mixed with 0.1 ml of 5.0 N NaCl. Gradients were centrifuged in the SW 50.1 rotor of a Beckman L265B ultracentrifuge at 40,000 rpm (149,000 × *g*) for 4 hr at 5°. After centrifugation, 0.15-ml fractions were collected onto 2.3-cm Whatman No. 3MM filter paper discs, using a Buchler Auto Densi-Flow gradient collector. The discs were dried, treated with cold 5% (w/v) trichloroacetic acid for 20 min (10 ml/disc), and washed with cold 5% trichloracetic acid (10 ml/disc) followed by 95% ethanol (10 ml/disc). The discs were dried and placed in scintillation vials with 10 ml of toluene containing 5.0 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxzolyl)]benzene per liter. Radioactivity was measured using a Beckman liquid scintillation spectrometer.

*Molecular weight determination.* Molecular weights of DNA were determined by sedimentation velocity in a Beckman model E analytical ultracentrifuge. Molecular weights were calculated using the equation of Studier (15).

*Paper chromatography.* Descending paper chromatography was performed on Whatman No. 3MM chromatography paper. Radioactive samples (0.02 ml) were spotted, and the chromatograph was developed for 16 hr with methanol–ethanol–concentrated HCl–water (50:25:6:19). After development and drying, 2-inch strips for each spot were cut into 0.5-inch fractions, placed in scintillation vials with 10 ml of the toluene mixture described above and counted.

*Bleomycin reaction mixtures.* The standard reaction mixture contained 10–40 μg/ml of DNA, 1–25 mM 2-mercaptoethanol, and bleomycin in a total volume of 0.4 ml of 0.05 M Tris, pH 8.0. Variations in this reaction mixture are given in the legends to

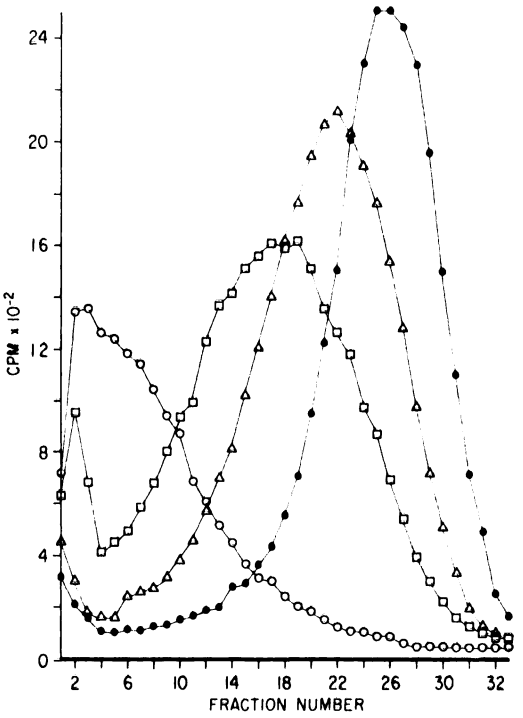


FIG. 1. *Effect of increasing bleomycin concentration on fragmentation of DNA*

Alkaline sucrose gradients; sedimentation was to the left. The reaction mixtures contained 25 mM 2-mercaptoethanol, 15 μg/ml of $^3$H-thymidine-labeled DNA, and bleomycin as follows: ○, control (no bleomycin); □, 10 μg/ml; △, 40 μg/ml; ●, 60 μg/ml. The reaction mixtures were incubated for 1 hr at 37°.

the figures. All reaction mixtures were incubated in tightly stoppered tubes.

## RESULTS

The fragmentation of DNA in the presence of increasing concentrations of bleomycin is shown in Fig. 1. The control DNA (bottom of gradient, untreated with bleomycin) has a molecular weight of $1.7 \times 10^6$ daltons; at the top of the gradient, the DNA has a molecular weight of approximately $3.1 \times 10^4$ daltons, both being determined as single-stranded DNA. In the density gradient experiments, we usually obtained approximately 90% recovery of radioactivity when using bleomycin concentrations up to 100 μg/ml. In some experiments, however, the control DNA recovery was less than expected. This occurred when the control DNA had a high molecular weight and sedimented to form a pellet, which was not recovered in these experiments. If the bleomycin concentration was held constant and the concentration of 2-mercaptoethanol was increased (Fig. 2), a dose-response effect similar to that found with increasing bleomycin concentrations was observed. The control, untreated DNA used for Fig. 2 was of smaller molecular size than in Fig. 1. This was caused by differences in the amount of shear during the isolation process and did not reflect any fragmentation caused by bleomycin alone during the 1-hr incubation of the reaction mixtures. In an attempt to determine the temperature optimum for the reaction, the experiment represented by Fig. 3 was performed. The greatest amount of fragmentation was seen in the reaction mixture incubated at 5°, and little or no fragmentation was observed at 80°; i.e., less breakage of DNA occurred with increasing temperature. That bleomycin alone did not lose its ability to fragment DNA when heated is shown in Fig. 4. In this experiment, bleomycin was boiled for 10 min and allowed to react with DNA and 2-mercapto-

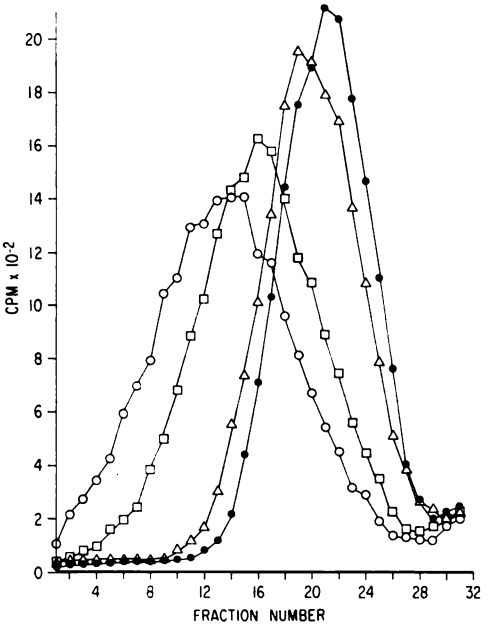


FIG. 2. *Effect of increasing 2-mercaptoethanol concentration on fragmentation of DNA*

Alkaline sucrose gradients; sedimentation was to the left. The reaction mixtures contained 40 μg/ml of bleomycin, 15 μg/ml of $^3$H-thymidine-labeled DNA, and 2-mercaptoethanol as follows: ○, control (no 2-mercaptoethanol); □, 0.01 mM; △, 1.0 mM; ●, 20.0 mM. DNA alone, without bleomycin or 2-mercaptoethanol, sedimented together with the control. The reaction mixtures were incubated for 1 hr at 37°.

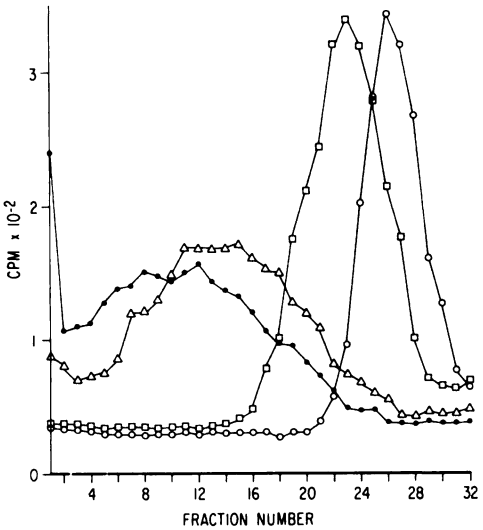


FIG. 3. *Effect of temperature on fragmentation of DNA by bleomycin*

Alkaline sucrose gradients; sedimentation was to the left. The reaction mixtures contained 40 μg/ml of bleomycin, 1.0 mM 2-mercaptoethanol, and 10 μg/ml of $^3$H-thymidine-labeled DNA, and were incubated for 1 hr at the following temperatures: ○, 5°; □, 20°; △, 40°; ●, 80°.

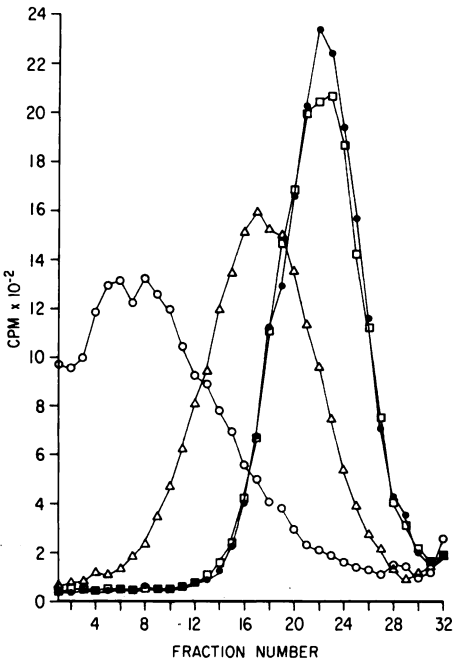


FIG. 4. *Effect of heat and Pronase on bleomycin activity*

Alkaline sucrose gradients; sedimentation was to the left. The reaction mixtures contained 40 μg/ml of bleomycin (where indicated), 1.0 mM 2-mercaptoethanol, and 15 μg/ml of $^3$H-thymidine-labeled DNA. ○, control (no bleomycin); □, control (untreated bleomycin); △, bleomycin (1.0 mg/ml) after reaction with Pronase (0.5 mg/ml) for 1 hr at 37° before incubation of reaction mixture; ●, bleomycin (1.0 mg/ml) heated at 100° for 10 min before incubation of reaction mixture. The Pronase was self-digested for 1 hr at 37° prior to use. In addition, the DNAs used were treated with self-digested Pronase (100 μg/ml) during isolation. The reaction mixtures were incubated for 1 hr at 37°.

ethanol. The heat-treated bleomycin maintained exactly the same ability to fragment DNA as the unheated control. When bleomycin was heat-treated for 60 min at 80°, the conditions of least fragmentation from Fig. 3, it maintained complete ability to fragment DNA. Figure 4 also shows that bleomycin lost some of its ability to fragment DNA after treatment with the proteolytic enzyme Pronase.

When bleomycin was mixed with 2-mercaptoethanol and allowed to stand for several hours before reaction with DNA, very little fragmentation of DNA occurred. It was thought that 2-mercaptoethanol had inactivated the bleomycin. This is clearly illustrated in Fig. 5. After reaction with 2-mercaptoethanol for as little as 5 min at 80°, bleomycin dramatically lost its ability to cause breakage of DNA. After 15 min at 80°, the fragmentation reaction was completely abolished. A comparison of the degree of fragmentation of DNA by bleomycin alone with that produced by bleomycin plus 2-mercaptoethanol when incubated for 16 hr (Fig. 6) reveals that bleomycin alone was able to fragment DNA. The degree of fragmentation was not as great as in the presence of 2-mercaptoethanol, but this experiment shows that 2-mercaptoethanol is not obligatory for the reaction. The effect of temperature on the breakage of DNA by bleomycin alone (Fig. 7) was

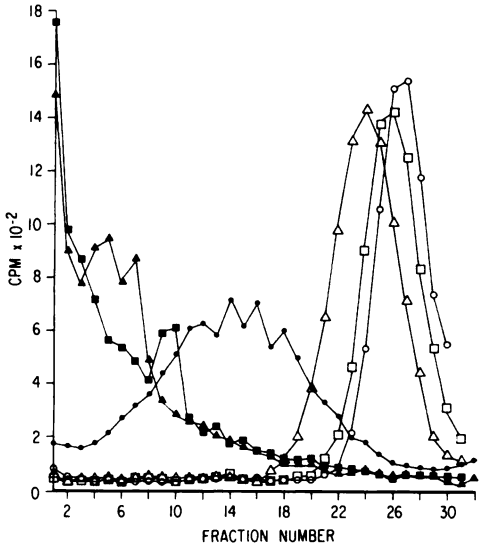


FIG. 5. *Time course of inactivation of bleomycin by 2-mercaptoethanol*

Alkaline sucrose gradients; sedimentation was to the left. Bleomycin (80 μg/ml) was mixed with 20 mM 2-mercaptoethanol and incubated at 80° for the following times: ○, 0 min (unincubated control); □, 1 min; △, 3 min; ●, 5 min; ■, 10 min; ▲, 15 min. The bleomycin–2-mercaptoethanol mixtures were added to reaction mixtures at the following final concentrations; 40 μg/ml of bleomycin, 10 mM 2-mercaptoethanol, and 15 μg/ml of $^3$H-thymidine-labeled DNA. Incubation was carried out for 1 hr at 5°.

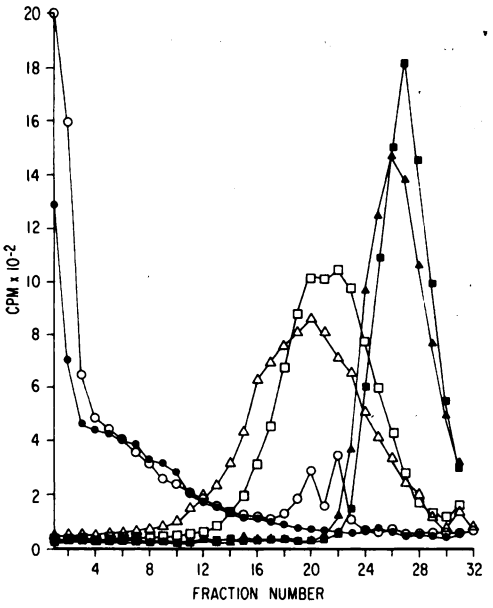


FIG. 6. *Effect of long incubation of bleomycin minus 2-mercaptoethanol on fragmentation of DNA*

Alkaline sucrose gradients; sedimentation was to the left. All reaction mixtures contained 15 μg/ml of $^3$H-thymidine- labeled DNA in addition to the following: ○, control (no additions); □, 80 μg/ml of bleomycin; △, 40 μg/ml of bleomycin; ●, control, 2 mM 2-mercaptoethanol; ■, 80 μg/ml of bleomycin plus 2 mM 2-mercaptoethanol; ▲, 40 μg/ml of bleomycin plus 2 mM 2-mercaptoethanol. All reaction mixtures were incubated at 5° for 16 hr.

the reverse of that seen in the presence of 2-mercaptoethanol (Fig. 3), the greatest amount of fragmentation being observed at 80°.

That bleomycin caused double- as well as single-strand breaks in DNA is shown by the experiment represented by Fig. 8. When this same experiment was performed on an alkaline sucrose gradient, the profiles were shifted further to the right. Treatment of heat-denatured DNA with increasing concentrations of bleomycin (Fig. 9) resulted in considerable fragmentation, even at the lowest concentration (10 μg/ml) of the antibiotic.

When DNA was treated, in the presence of 2-mercaptoethanol, with bleomycin at 5–20 mg/ml, there was a marked loss of trichloroacetic acid-precipitable counts from the gradient profiles. The corresponding percentage increase of acid-soluble counts from such a gradient is shown in Fig. 10. Finally, when DNA treated with 5 mg/ml of bleomycin was subjected to paper chromatography (Fig. 11), free nucleosides, nucleotides, or bases could be detected.

## DISCUSSION

The results of the dose-response experiment with bleomycin and DNA (Fig. 1) suggest that the points of breakage probably are randomly distributed along the DNA molecule. If these sites were located nonrandomly along the molecule, one would expect the appearance of more than one molecular size after incubation with the drug. Although homogeneous preparations of DNA, such as purified phage DNA, have not been tested, there seems no reason to

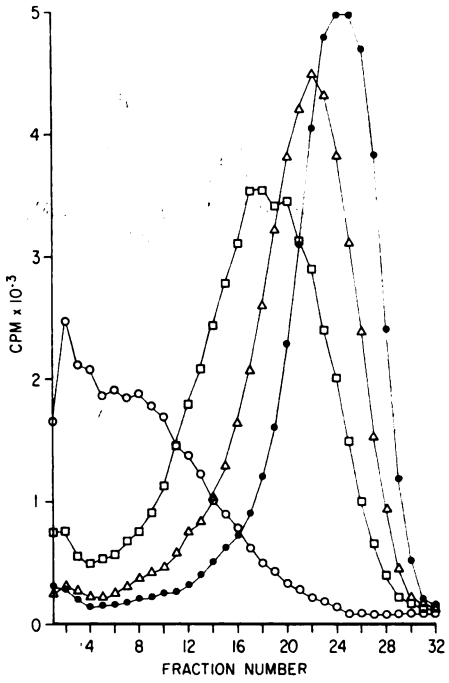


FIG. 7. *Effect of temperature on fragmentation of DNA by bleomycin minus 2-mercaptoethanol*

Alkaline sucrose gradients; sedimentation was to the left. The reaction mixtures contained 40 μg/ml of bleomycin and 10 μg/ml of $^3$H-thymidine-labeled DNA, and were incubated for 16 hr at the following temperatures; ○, 5° (unincubated control); □, 20°; △, 40°; ●, 80°.

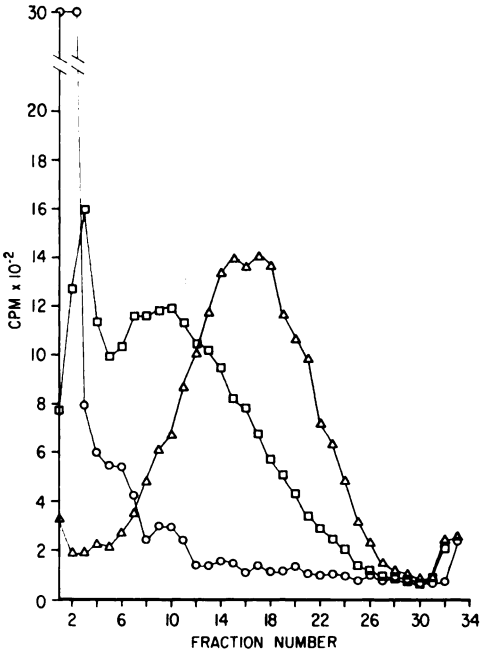


FIG. 8. *Effect of bleomycin on production of double-strand breaks in DNA*

Neutral sucrose gradients; sedimentation was to the left. The reaction mixtures contained 2 mM 2-mercaptoethanol, 10 μg/ml of $^3$H-thymidine-labeled DNA, and bleomycin as follows; ○, control (no bleomycin); □, 40 μg/ml; △, 80 μg/ml. The reaction mixtures were incubated for 1 hr at 37°.

expect results different from those observed using *B. subtilis* DNA. Since approximately 90% of the labeled DNA was rendered soluble in trichloracetic acid by treatment with high concentrations of bleomycin (Fig. 10), the bleomycin-sensitive sites must be situated rather close together, of the order of 10 or fewer nucleotides distant. The results presented in Fig. 11 suggest that many sites are adjacent, since approximately 30% of the label was released as free nucleotide, nucleoside, or base. This same result was obtained when the DNA was labeled with $^{14}$C-guanine.[1] This indicates that the sensitive site might be either the phosphodiester bond or deoxyribose. Experiments in progress indicate that only free base is liberated after treatment of DNA with high concentrations of the drug.

[1] Unpublished observations.

The bleomycin-induced fragmentation of DNA resembles the action of an endonuclease. Since there is little information available concerning the commercial production of this antibiotic, it is impossible to rule out the presence of contaminating nuclease from this source. It is known, however, that the activity of the bleomycin used in the present study is dialyzable and also heat-stable. In addition, experiments using $^3$H-thymidine-labeled cell lysates as the DNA source show essentially the same degree of fragmentation by bleomycin as obtained with highly purified, Pronase-treated DNA. Thus it seems unlikely that bleomycin contains nuclease or activates any endogenous nuclease present in the DNA preparations.

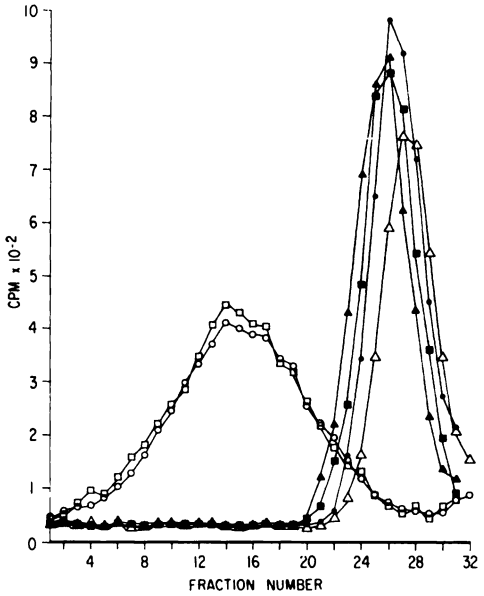


FIG. 9. *Effect of bleomycin on fragmentation of heat-denatured DNA*

Alkaline sucrose gradients; sedimentation was to the left. The DNA was heated at 100° for 10 min and quickly cooled in an ice bath. The reaction mixtures contained 10 μg/ml of $^3$H-thymidine-labeled DNA plus the following: ○, control (no additions); □, control, 1 mM 2-mercaptoethanol; △, 80 μg/ml of bleomycin plus 1 mM 2-mercaptoethanol; ●, 40 μg/ml of bleomycin plus 1 mM 2-mercaptoethanol; ■, 20 μg/ml of bleomycin plus 1 mM 2-mercaptoethanol; ▲, 10 μg/ml of bleomycin plus 1 mM 2-mercaptoethanol. The reaction mixtures were incubated for 1 hr at 5°.

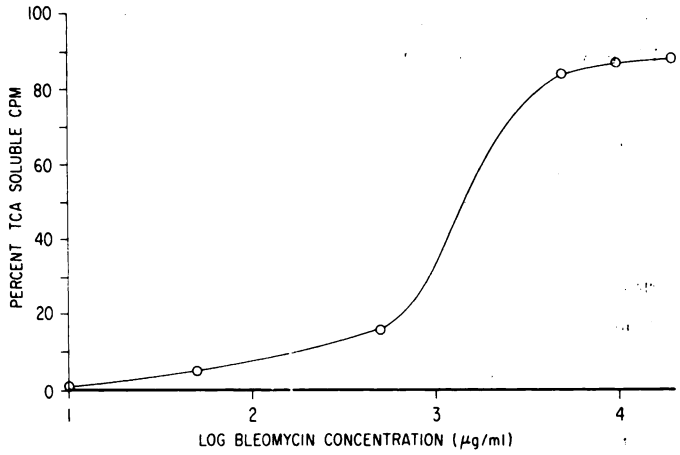


FIG. 10. *Effect of high concentrations of bleomycin on release of trichloracetic acid (TCA)-soluble material from DNA*

The acid-soluble radioactivity released upon treatment of $^{3}$H-thymidine-labeled DNA with bleomycin was determined by totaling the acid-precipitable counts from an alkaline sucrose gradient. The reaction mixtures contained 37 μg/ml of $^{3}$H-thymidine-labeled DNA, 25 mM 2-mercaptoethanol, and bleomycin at the indicated concentrations.

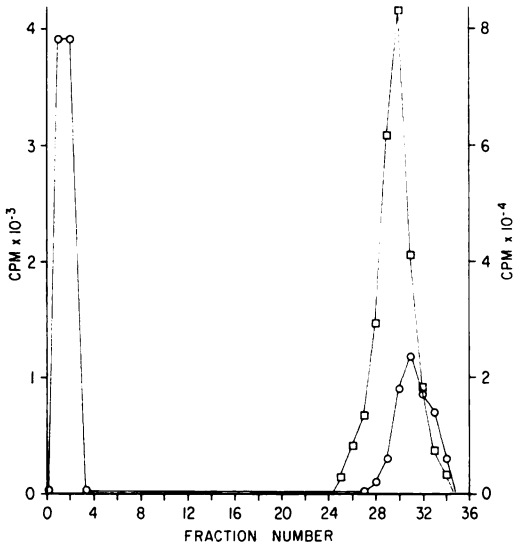


FIG. 11. *Paper chromatograph of $^{14}$C-thymine-labeled DNA treated with bleomycin*

○, 0.02 ml of $^{14}$C-labeled DNA (42 μg/ml) treated with 5 mg/ml of bleomycin plus 1 mM 2-mercaptoethanol for 3 hr at 22° (left ordinate); □, 0.005 ml of $^{3}$H-thymidine spotted as standard (right ordinate).

The paradoxical behavior of 2-mercaptoethanol in the reaction is not understood. That this compound rapidly inactivates bleomycin is quite clear (Fig. 5); it is equally clear that it greatly accelerates the reaction. Bleomycin, after treatment with 2-mercaptoethanol, is also inactive biologically; i.e., it does not inhibit growth of *B. subtilis*.[1] Experiments are planned to determine the structural integrity of bleomycin after reaction with 2-mercaptoethanol. Presently it is impossible to determine whether 2-mercaptoethanol reacts transiently with bleomycin to produce a more reactive species, with the subsequent production of an unreactive species, or whether 2-mercaptoethanol reacts in some way with DNA to render it more susceptible to the action of bleomycin. It is known that DNA incubated with 2-mercaptoethanol, followed by dialysis, is readily degraded by the subsequent addition of bleomycin alone (16). However, this preliminary treatment of DNA with 2-mercaptoethanol, followed by rather extensive dialysis, still yields the same temperature profile shown in Fig. 3;[1] i.e., the bleomycin is inactivated at the higher temperatures. From this it would appear either that the 2-mercaptoethanol is not easily dialyzed away from the DNA or that the reaction of 2-mercaptoethanol with DNA renders the DNA capable of inactivating bleomycin.

This point is under investigation. In any case it is apparent that 2-mercaptoethanol is not required for bleomycin-induced DNA fragmentation.

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