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Phleomycin-Induced Cleavage of Deoxyribonucleic Acid†

Robert Stern,* James A. Rose, and Robert M. Friedman

ABSTRACT: Phleomycin, a polypeptide with antibiotic and antitumor activity, produces single-strand breaks in purified adenovirus and phage λ deoxyribonucleic acid. A reducing agent was required and both native and single-stranded deoxyribonucleic acids were substrates for this activity. The limit reaction products were acid insoluble and sedimented at 5 S or less in sucrose density gradients. The deoxyribonucleic acid cleaving activity of phleomycin could be measured by a nitrocellulose filter binding assay and characteristics of this assay are described. Ribonucleic acid did not appear to be cleaved by phleomycin.

Phleomycin is a water-soluble, copper-containing protein, obtained from the culture medium of *Streptomyces verticillus* (Maeda *et al.*, 1956). It is a specific inhibitor of DNA synthesis in bacteria (Falaschi and Kornberg, 1964) and it has been found to act as an antitumor agent (Bradner and Pindell, 1962). These activities are thought to occur through a direct effect on DNA though a precise mechanism has not been established. It is known that phleomycin binds to DNA and causes *in vitro* inhibition of DNA polymerase I of *Escherichia coli* (Falaschi and Kornberg, 1964). In a survey of reported inhibitors of DNA synthesis, instead of inhibition, we observed a marked stimulation of [^{3}H]TTP incorporation into the DNA of isolated HeLa cell nuclei when incubated in the presence of phleomycin (R. M. Friedman, R. Stern, and J. A. Rose, manuscript in preparation). Furthermore, an associated marked decrease in size of the extracted DNA suggested that increased [^{3}H]TTP incorporation might have resulted from repair synthesis secondary to phleomycin-induced DNA breakage.

Degradation of DNA in the presence of phleomycin has also been observed previously in bacteria (Grigg, 1969) and in viruses (Iwata and Consigli, 1971). In addition, chromosome breaks have been detected in human lymphocytes cultured in the presence of the drug (Jacobs *et al.*, 1969). Phleomycin may act either directly in breaking DNA or, as suggested previously (Grigg, 1969), indirectly by activating an endogenous DNA endonuclease. In the present communication, the effect of phleomycin on isolated DNA was examined to distinguish between these two possibilities. We present data which indicate that pheleomycin is a low molecular weight polypeptide which is able to cleave purified viral DNA directly. Some other physical properties of phleomycin are also described.

Materials and Methods

Viral DNA. Adenovirus type 2 (Ad2)[1] DNA, ^{14}C, ^{3}H, or ^{32}P labeled, and ^{32}P-labeled phage λ p gal DNA were prepared as described previously (Kelly and Rose, 1971; Nissley *et al.*, 1972).

Phleomycin. Phleomycin (lot no. A9 331-909 and 64L-1238) was the kind gift of Dr. W. T. Bradner of Bristol Laboratories. Phleomycin was stored as a frozen solution at 0.1 mg/ml in 50 mM Tris-Cl (pH 7.5) and was stable for several months at −20° with repeated freezing and thawing.

DNA Incubation Mixtures for Sucrose Density Gradient Analysis. DNA preparations (1–3 μg, 3000–5000 cpm of ^{32}P or ^{14}C/μg) were incubated with phleomycin at 37° in the presence of 50 mM Tris-Cl (pH 8.0)–30 mM NaCl–20 mM dithiothreitol. Incubation volumes were 0.1 ml and phleomycin concentrations were 0.125 or 1.25 μg/ml, as indicated.

Sucrose Density Gradient Analysis. The 0.1-ml incubation mixture of DNA was laid over 5–20% neutral or alkaline sucrose gradients in a 5-ml cellulose nitrate tube. The gradients were sedimented for 2.75 hr at 20° at 42,000 rpm in an SW 50 rotor. Neutral gradients contained 1.0 M NaCl, 10 mM Tris-Cl (pH 7.2), 0.15% sarkosyl, and 1 mM EDTA. Alkaline gradients contained 0.7 M NaCl, 0.3 M NaOH, and 0.15% sarkosyl. Addition of sarkosyl to these gradients enhanced recovery of the single-stranded DNA from the cellulose nitrate tubes.

The radioactivity in gradient fractions was assayed in a Triton X-100 emulsion in a scintillation spectrometer.

Assay for DNase Activity. The assay was a modification of

† From the Laboratory of Biochemistry, National Institute of Dental Research (R. S.), the Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases (J. A. R.), and the Laboratory of Pathology, National Cancer Institute (R. M. F.), National Institutes of Health, Bethesda, Maryland 20014. *Received June 27, 1973.*

[1] Abbreviations used are: Ad2, adenovirus type 2; DNase, deoxyribonuclease; RNase, ribonuclease.

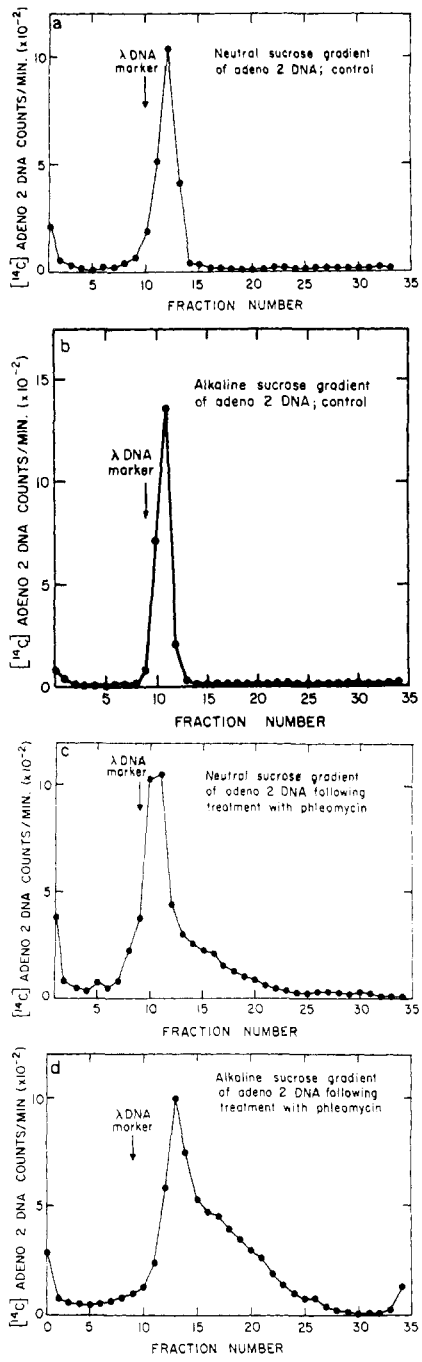

FIGURE 1: Phleomycin action on Ad2 DNA. Radioactive ^{14}C-labeled Ad2 DNA (3000 cpm) was incubated in a 0.1-ml incubation mixture for 1 hr at 37° in the presence or absence of 1.25 μg/ml of phleomycin. Samples were layered over a neutral or an alkaline sucrose gradient (5–20%) and sedimented for 2.7 hr at 42,000 rpm in an SW 50 rotor together with ^{32}P-labeled λ DNA as marker: (a) untreated Ad2 DNA (31 S) sedimented in a neutral sucrose density gradient; (b) neutral sucrose sedimentation of Ad2 DNA following phleomycin treatment; (c) untreated Ad2 DNA sedimented in an alkaline sucrose density gradient; and (d) phleomycin-treated Ad2 DNA in an alkaline sucrose density gradient.

that described by Geiduschek and Daniels (1965) based on the ability of large, denatured DNA molecules to bind to nitrocellulose filters (Nygaard and Hall, 1963). ^{32}P-labeled Ad2 DNA was used with a specific activity of 300–1000 cpm/μg. Stock preparations of bovine pancreatic DNase (Worthington Biochemical Corp.) containing 1 mg/ml in 50 mM Tris-Cl (pH 7.5) were frozen and stored in small aliquots. Once thawed, aliquots were not reused. Schleicher and Schuell nitrocellulose membrane filters (Grade B6) were presoaked in 0.5 M KCl–10 mM Tris-Cl (pH 7.5) and then washed with 10 ml of the same buffer. The radioactively labeled DNA substrate was diluted in 10 mM Tris-Cl (pH 8.0)–1 mM EDTA. For alkaline denaturation of the DNA substrate, NaOH was added to a final concentration of 0.2 M and the sample incubated at 23° for 10 min. Tris-Cl (pH 8.0) (0.2 M) and sufficient 1 N HCl were then added to adjust the pH to 8.0. The assay reaction mixture contained 1.5 mM $MgCl_2$, 5 mM Tris-Cl (pH 7.5), 1–5 μg of native or denatured Ad2 DNA, and the indicated amounts of pancreatic DNase in a final volume of 0.5 ml. After incubation at 37° for 15 min, samples were diluted with 10 ml of 10 mM Tris-Cl (pH 7.5), 0.5 M KCl, and 10 mM EDTA at 0° and immediately filtered through the presoaked, prewashed membrane filters. Filters were then rinsed with an additional 50 ml of dilution buffer, dried, and counted in Liquifluor–toluene in a liquid scintillation counter. The assay for the endonuclease activity of phleomycin was the same as that described for bovine pancreatic DNase except that reaction mixtures contained 20 mM dithiothreitol.

RNase Activity. To determine whether phleomycin could cleave RNA, 5-[^3H]uridine-labeled polysomal RNA from KB cells was incubated with and without phleomycin (10 μg) in 0.1-ml reaction mixtures containing 50 mM Tris-Cl (pH 8.0), 30 mM NaCl, and 20 mM dithiothreitol. Following incubation at 37° for 20 min, the mixtures were layered onto 10–30% linear sucrose gradients containing 0.1 M NaCl, 10 mM Tris-Cl (pH 7.5), 10 mM EDTA, and 0.5% sodium dodecyl sulfate, and centrifuged at 65,000 rpm for 90 min at 20° in an SW 65 Ti rotor. The radioactivity in gradient fractions was determined as above.

Amino Acid Analysis. Phleomycin was dialyzed against H_2O and hydrolyzed under a nitrogen atmosphere for 12 or 24 hr in 6 N NCl at 106°. Hydrolysates were analyzed on an automatic instrument (Piez and Morris, 1960) modified for high-speed analysis (Miller and Piez, 1966). In each case 1 mg of phleomycin was used for analysis.

Results

Cleavage of Purified Ad2 and Phage λ DNA by Phleomycin. The effect of phleomycin was examined on Ad2 and λ phage DNAs. When purified native Ad2 DNA was incubated with phleomycin, a slower sedimenting shoulder was observed in both neutral (Figure 1c) and alkaline (Figure 1d) sucrose gradient profiles. This material was not seen in samples of untreated DNAs (Figures 1a and 1b), suggesting that cleavage of DNA was occurring in the presence of phleomycin. No material appeared at the top of the gradients, consistent with little, if any, exonucleolytic activity in the phleomycin preparation. The amount of trailing material was clearly greater in the alkaline sucrose gradient, suggesting that molecular breakage resulted from single-strand scissions which occurred in both DNA strands.

It is known that double-stranded DNA endonuclease is associated with the adenovirus virion (Burlingham *et al.*, 1971). Therefore, it was necessary to exclude the possibility that the effect of phleomycin was in some way related to this enzyme. We therefore examined the effect of phleomycin on purified phage λ DNA. The neutral and alkaline sucrose gradients in Figure 2 demonstrated the increasing endonucleolytic-like effect of phleomycin at two levels of the drug. Again, DNA cleavage appeared to result from single-strand breaks. The finding of a similar activity with phage λ DNA makes it unlikely that the phleomycin effect on Ad2 DNA was due to

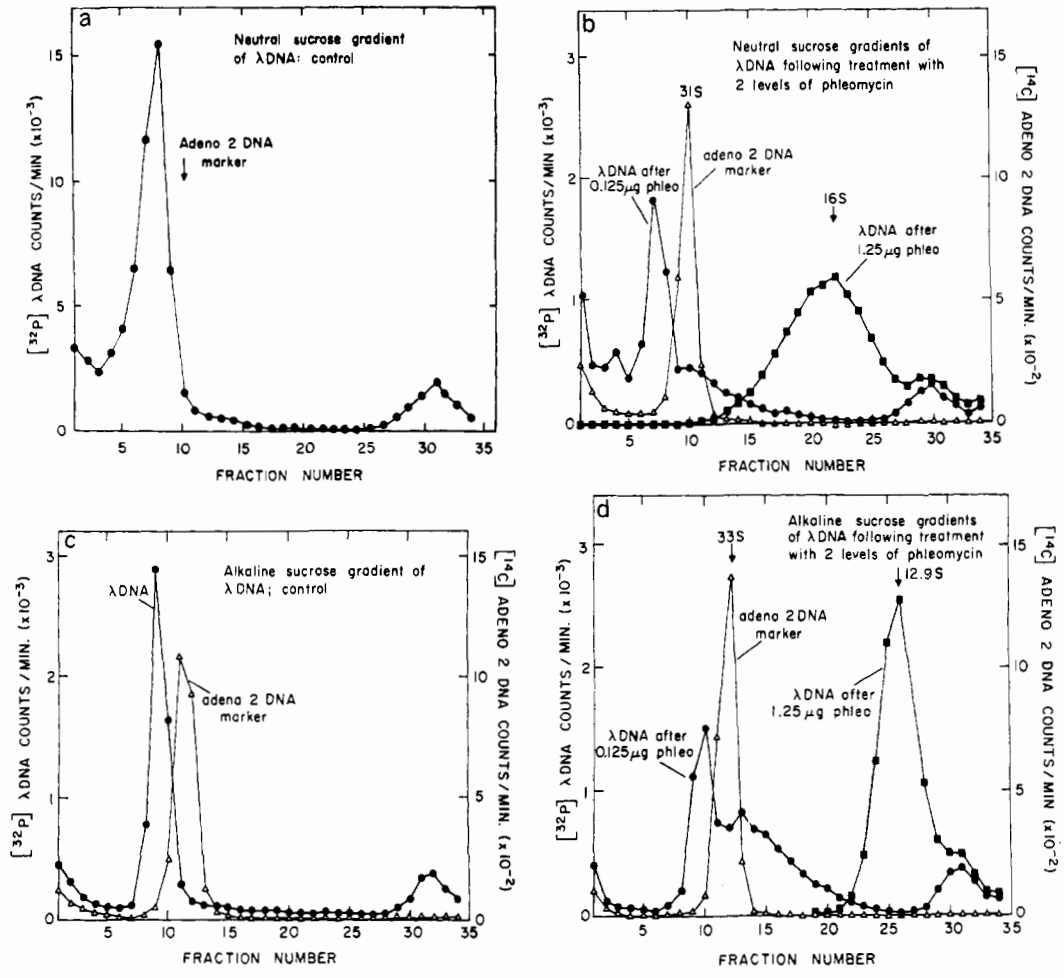

FIGURE 2: Phleomycin action on λ DNA. Radioactive [^{32}P]DNA (6000 cpm) was incubated 1 hr at 37° in a 100-μl reaction mixture without phleomycin or containing 0.125 or 1.25 μg/ml of phleomycin. Samples were then layered directly over a neutral or an alkaline sucrose gradient and sedimented together with ^{14}C-labeled Ad2 DNA as marker: (a) profile of untreated DNA in a neutral sucrose gradient; (b) phleomycin-treated λ DNA, a composite of profiles from two neutral sucrose density gradient centrifugations; (c) untreated DNA in an alkaline sucrose gradient; and (d) phleomycin-treated λ DNA, a composite of profiles from two alkaline sucrose density centrifugations. A slow sedimenting ^{32}P-labeled component was present in the λ DNA preparation (a). The nature of this material was not clear. However, its sedimentation characteristics were unaffected by alkali (c) or phleomycin treatment (b, d).

the virion endonuclease. This experiment also demonstrated that both bacteriophage and animal virus DNA were substrates for phleomycin cleavage. Some of the properties of this reaction were readily demonstrable. The phleomycin-associated decrease in Ad2 DNA size was unaffected by heating the drug preparation to 60° for 1 hr and only partially inhibited by 1 hr at 90°. This correlated well with the drug's heat-stable ability to stimulate [^{3}H]TTP incorporation into isolated nuclei and, coupled with the observed DNA cleaving activity, suggested that this phenomenon is probably secondary to drug-induced cleavage of DNA (R. M. Friedman, R. Stern, and J. A. Rose, manuscript in preparation). Incubating λ DNA with dithiothreitol alone or phleomycin alone did not decrease the size of λ DNA. However, incubation in the presence of both dithiothreitol and phleomycin resulted in a marked decrease in size (data not shown). This indicated that dithiothreitol alone was not activating a DNA-bound endonuclease.

In other studies it was found that the phleomycin-associated decrease in DNA size was enhanced by increasing phleomycin concentration or the length of incubation. In Figure 3a, a neutral sucrose density gradient profile of untreated λ DNA (expt 1) was compared with that of λ DNA incubated for 1 or 2 hr at 37° with 1.25 μg/ml of phleomycin (expt 2 and 3). In addition, λ DNA incubated for 1 hr with 1.25 μg/ml of phleomycin was divided into equal aliquots, phleomycin re-added, and incubation continued for 30 and 60 min (expt 4 and 5). However, if λ DNA was incubated for 1 hr with 1.25 μg/ml of phleomycin, and then additional λ DNA added and the incubation continued for 30 or 60 min (Figure 3b, expt 6 and 7), degradation of the added DNA was not observed. Since phleomycin was not completely inactivated under the incubation conditions, this finding would indicate that the drug binds firmly to DNA, *i.e.*, all the phleomycin was bound to the initial DNA and once bound did not detach and bind to the subsequently added DNA. No degradation was observed when λ DNA was incubated for 2 hr at 37° in the absence of phleomycin.

The above experiments suggested that the observed breakage of DNA was due to a direct action of phleomycin. This cleavage activity was quantitated in the following experiments.

Assay for DNA Cleavage Reactions. Nygaard and Hall (1963) devised a method for detecting nucleic acid hybrids on nitrocellulose filters based on the ability of the membranes to retain denatured DNA. Geiduschek and Daniels (1965), observing that only large single-stranded DNA chains are retained by the filters, adapted the procedure as a simple assay for DNA endonucleases. Using denatured Ad2 DNA, we confirmed that retention of DNA was proportional to the in-

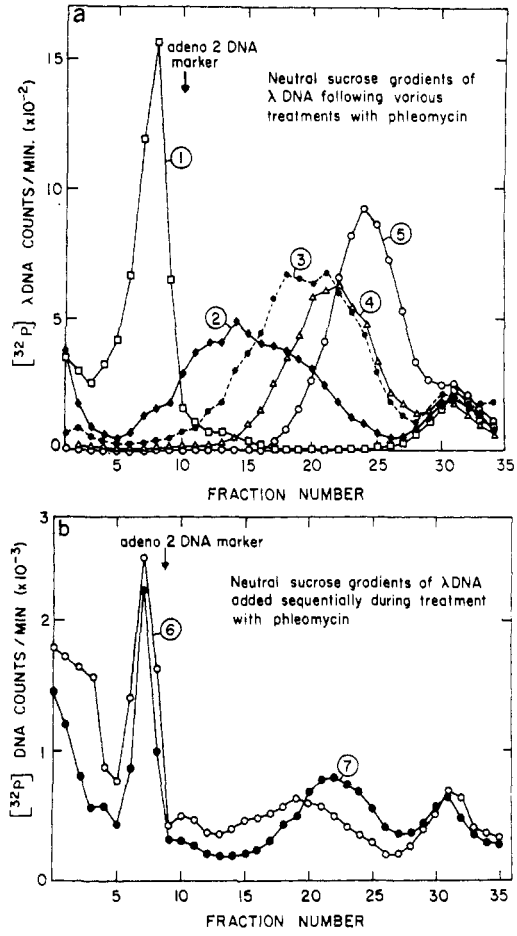

FIGURE 3: Effect of increasing length of incubation, increasing levels of phleomycin, and sequential addition of substrate on λ DNA cleavage. Radioactive, [^{32}P-labeled λ DNA was divided into eight aliquots of 6000 cpm and each was placed in a 100-μl reaction mixture (a) containing no addition (expt 1) or 1.25 μg/ml of phleomycin (expt 2–7). Aliquots were incubated at 37° for 1 (expt 2) and 2 hr (expt 3). Additional phleomycin was added, 2.5 μg final concentration, and the incubation continued for 30 (expt 4) or 60 min (expt 5). (b) An additional equivalent of ^{32}P-labeled λ DNA was added after 1 hr, and the incubation continued for 30 (expt 6) or 60 min (expt 7). Samples were then layered onto neutral sucrose gradients together with ^{14}C-labeled Ad2 DNA as marker and sedimented as described in Figure 1.

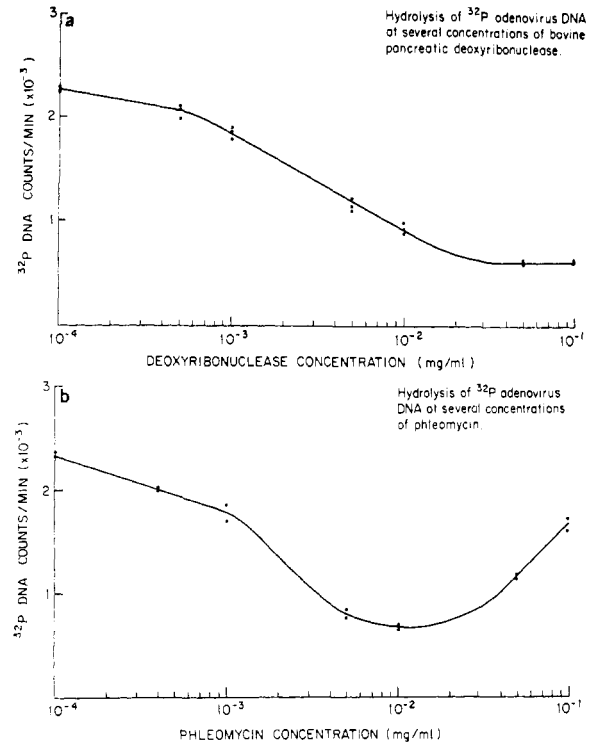

FIGURE 4: Assays for DNA cleavage activities with bovine pancreatic DNase and phleomycin preparations. (a) Bovine pancreatic DNase, 1 mg/ml, was diluted in 10 mM Tris (pH 7.5) containing 3 mM $MgCl_2$ and 200 μg/ml of bovine serum albumin. Samples (0.1 ml) containing levels of DNase indicated on the abscissa were added to the standard reaction mixture containing 2 × 10^3 cpm of denatured ^{32}P-labeled Ad2 DNA in a final volume of 0.5 ml and incubated for 15 min at 37°. The reaction mixture was then diluted and filtered and the filters were washed, dried, and counted as described under Materials and Methods. (b) Phleomycin was diluted and DNA retention assayed as in a.

put DNA over a wide range of DNA concentrations, from 0.01 to more than 10 μg.

The action of bovine pancreatic DNase on alkali-denatured Ad2 DNA was determined. The retention of DNA on the filters was proportional to the concentration of pancreatic DNase in the standard reaction mixture (Figure 4a). A concentration of 3 μg/ml of DNase in the standard reaction mixture was sufficient to decrease the retention of adenovirus DNA by 50% in 15 min at 37°. The DNA retention assay was proportional to enzyme concentration between 3 × 10^{-2} and 6 × 10^{-4} mg/ml. The discontinuity in the curve at low enzyme concentration may be due to the instability of the enzyme at high dilutions.

The effect of phleomycin on the retention by filters of denatured adenovirus DNA is shown in Figure 4b. This experiment demonstrates that phleomycin preparations contained activity comparable on a weight basis to that of bovine pancreatic DNase. Using 2 μg/ml of phleomycin in the standard reaction mixture 50% of the DNA was retained on the filter after 15 min at 37°. The reason for increased DNA retention at phleomycin concentrations above 10 μg/ml is not clear. It may be possible that aggregation and DNA fragment–phleomycin binding to the filter may be occurring at high concentrations of the drug, or that some inhibitory substance is present in the preparation which prevents cleavage of DNA.

Requirements of DNA Cleavage by Phleomycin. Some of the properties of the phleomycin cleavage reaction were examined in the *in vitro* assay (Table I). The reaction had an absolute requirement for a reducing agent, and either β-mercaptoethanol or dithiothreitol was effective. Magnesium was not required for the reaction. Omission of bovine serum albumin also had no effect on cleavage activity.

Alkaline and neutral sucrose density gradient analyses of phleomycin-treated DNA (Figure 1) suggested that the drug was causing single-strand breaks in native DNA. The ability of phleomycin to cleave denatured DNA was examined using the nitrocellulose filter binding assay. ^{3}H-Labeled Ad2 DNA was treated with phleomycin with and without preliminary denaturation of the DNA. Figure 5 demonstrates that native double-stranded and denatured single-stranded viral DNA were equally good as substrates for phleomycin action over a wide range of concentrations of the drug (Figure 5a). In addition, no difference was observed in the kinetics of the reaction over a 30-min incubation period between the two preparations of DNA (Figure 5b). These experiments demonstrated that it was not necessary for DNA to be in a double-stranded configuration for phleomycin cleavage to take place.

Activity of Phleomycin against RNA. The ability of phleo-

TABLE I: Requirements of the Phleomycin-Induced DNA Cleavage Reaction.

Reaction Mixture[a]	Act.[b]
Complete	1450
Without dithiothreitol	72
Without dithiothreitol plus 20 mM mercaptoethanol	1308
Without $MgCl_2$	1855
Without $MgCl_2$ plus 10 mM EDTA	1427
Without bovine serum albumin	1380

[a] The complete reaction mixture is described under Materials and Methods and contained 6500 cpm of ^{3}H-labeled Ad2 DNA and 20 μg/ml of phleomycin. [b] Activity is expressed as cpm not retained by the filter, in 15 min.

mycin to cause cleavage of RNA was also examined. Profiles of KB cell ^{3}H-labeled RNA were compared from sucrose density gradient centrifugations in the presence and absence of preliminary treatment with phleomycin. No RNA cleavage activity could be detected under these conditions.

Column Chromatography of Phleomycin. Some physical properties of the phleomycin preparation were also examined. Phleomycin (5 mg) was dissolved in 5 ml of 10 mM Tris-Cl (pH 7.5) and placed on a column (75 cm × 1.8 cm i.d.) of Sephadex G-100 at 23°. The elution rate with the same buffer was 0.25 ml/min. Fractions of 5.0 ml were collected with continuous recording at 254 mμ. One peak of optically absorbing material was obtained. One milligram of phleomycin contained approximately 14 A_{254} units. Eighty per cent of the material was recovered in this peak and no contaminating peaks were observed (data not shown). The absorption maximum of phleomycin is 254 nm (Ikekawa *et al.* 1964). Some separation of 280-nm from 254-nm absorbing material was achieved by Sephadex G-100 column chromatography.

The elution position of phleomycin on Sephadex G-100 suggested a low molecular weight for the drug. Phleomycin was then chromatographed by gel filtration on a column of agarose Bio-Rad P-2 and compared with the elution position of molecular weight markers including cytochrome *c*, Bacitracin, and dithiothreitol. Phleomycin had an apparent mol wt of 815 by this criterion. Again a single symmetric peak of optically absorbing material was eluted from the column. An assumption implicit in this estimate was that phleomycin did not interact with the column resin and was not thereby retarded from the column in its elution position.

Phleomycin was also chromatographed on a column of CM-Sephadex as described previously (Ikekawa *et al.*, 1964) except that a linear rather than a discontinuous gradient was applied. CM-Sephadex (C-25) was treated with 5% ammonium formate and then suspended in 0.5% ammonium formate. Phleomycin (5 mg) was dissolved in 3.0 ml of 9.5% ammonium formate, placed on the column (25 × 2.5 cm i.d.), and eluted with a 500-ml linear gradient of from 0.5 to 5.0% ammonium formate at 23°. Fractions (5 ml) were collected and absorbancy at A_{254} recorded continuously. Eleven peaks of A_{254} absorbing material were partially resolved compared to eight components observed with a discontinuous gradient elution reported by Ikekawa *et al.* (1964). The peak fractions were pooled, lyophilized, and dissolved in small volumes of 10 mM Tris-Cl (pH 7.5). The specific cleaving activities of peaks were comparable to each other and to the original material.

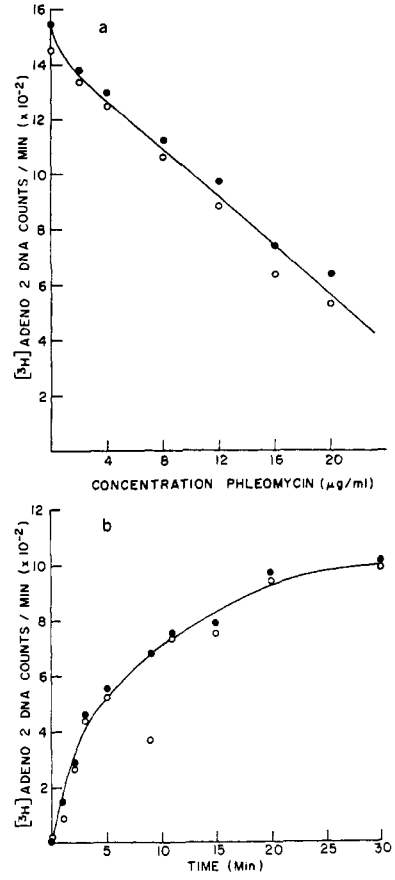

FIGURE 5: Comparison of native and denatured DNA as substrates in the phleomycin-induced cleavage reaction. A radioactive ^{3}H-labeled Ad2 DNA preparation was divided into two aliquots. For native DNA the preparation was made 0.5 M in KCl, 0.4 M in NaCl and 50 mM in Tris-Cl (pH 8.0). Aliquots (0.1 ml) of the preparation were used in the standard reaction mixture. At the termination of the reaction, the DNA was denatured as described under Materials and Methods. Preparation of denatured DNA was also prepared as described under Materials and Methods for the reaction substrate. Reactions were carried out as described in Figure 4 using (a) varying concentrations of phleomycin, as indicated, utilizing a 15-min reaction time, and (b) using increasing lengths of reaction time in the presence of 10 μg/ml of phleomycin: (○) native DNA; (●) denatured DNA. In a the data are presented as the amount of [^{3}H]-DNA which binds to the filter and in b as the amount of [^{3}H]DNA which is hydrolyzed and no longer binds to the filter.

The heterogeneity of phleomycin by CM-Sephadex criterion compared to molecular sieve column profiles is unexplained. It may be due to different sugar moieties associated with the molecule, or single amino acid substitutions.

Amino Acid Analysis. Amino acid analysis of dialyzed acid-hydrolyzed phleomycin contained seven well-defined peaks plus a peak of NH_3. No difference was observed between 12 and 24 hr of hydrolysis. Two of these peaks corresponded to the elution positions of L-phenylalanine and L-threonine. The remaining peaks were not identified. It is apparent that the phleomycin was a far more homogeneous preparation than has been assumed previously (Falaschi and Kornberg, 1964).

Discussion

These experiments indicate that phleomycin is a low molecular weight polypeptide with the apparent ability to act directly upon DNA, producing single-strand breaks. If double-strand breaks had occurred, the quantity of DNA sedimenting as smaller fragments in alkaline sucrose gradients would not

have differed substantially from the quantity seen in neutral sucrose gradients (Burlingham *et al.*, 1971). Both single- and double-stranded DNA chains were substrates for this reaction (Figures 1d and 2d). The activity of the drug appeared to be stoichiometric with DNA and was not a catalytic enzyme-like reaction (Figure 4, expt 6 and 7). The absence of acid-soluble products in limit digests of the phleomycin reaction indicated that some specificity may be involved in the cleavage. The reaction of phleomycin with several synthetic deoxyribonucleotide oligomers is being examined currently to investigate the possible specificity of the cleavage reaction. It is possible that phleomycin and other agents such as bleomycin, which also causes single-strand breaks in DNA (Nagai *et al.*, 1969; Shirakawa *et al.*, 1971; Müller *et al.*, 1972), may have some use in sequence determination of DNA.

In addition to the effects of phleomycin on DNA, there are reports of inhibition of bacterial RNA synthesis and inhibition of the replication of RNA phage (Watanabe and August, 1968). We failed to observe a direct effect of phleomycin *in vitro* on purified animal cell RNA. Thus, the reported inhibitory action of phleomycin on RNA synthesis is unlikely to be related to RNA cleavage *per se*.

Polypeptide antibiotics are produced only by spore-forming microorganisms. Mutational loss of antibiotic production is associated with a loss of the ability to form spores, suggesting that polypeptide antibiotics have an essential role in sporulation. They may function in the regulation of gene transcription, during the transition from vegetative growth to sporulation. Such a mechanism has been demonstrated for the antibiotic tyrothricin produced by *Bacillus brevis* (Sarkar and Paulus, 1972). Regulation of transcription, by creating specific nicks in DNA, could be invoked as the role for phleomycin in the life cycle of *Streptomyces verticillus*.

Acknowledgment

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