INDUCTION OF LOCAL DENATURATION IN DNA IN VITRO BY PHLEOMYCIN AND CAFFEINE

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1. Introduction

Phleomycin, a copper-containing antibiotic isolated from *Streptomyces verticillus* [1] has antibacterial [2–4] antitumour [5,6] and antiviral [7–9] activity DNA strand breakage following phleomycin treatment has been reported in *Escherichia coli* [2,10,11] and *Bacillus subtilis* [3] as well as in viruses [9] and human cells [12,13]. To explain this effect in *E. coli*, we have proposed [10,11] that phleomycin, which binds to thymine residues of DNA both in vitro [14] and in vivo [15], distorts the secondary structure of DNA in the bacterial cell. A number of such complexes within a short region of the DNA would cause local denaturation, leading to sensitisation of the DNA to cellular endonucleases.

This suggestion is supported to some extent by the fact that phleomycin appears to bind to DNA at discrete regions, rather than being distributed with all A–T base pairs [14] and also by the observations of Falaschi and Kornberg [16] on the effect of phleomycin on DNA melting profiles. An increase in the range of temperatures over which melting occurred (σ_T) suggested that in the presence of phleomycin, some regions of the DNA were more readily denatured than others.

Caffeine and other ligands binding preferentially to single stranded DNA [11], do not themselves cause DNA degradation, but enhance the extent of cell death and breakdown of DNA following treatment of *E. coli* B cells with phleomycin [10,11]. This effect could be explained by an ability to increase denaturation around single phleomycin–thymine complexes in DNA, again leading to sensitisation of the DNA to breakage by single strand-specific endonucleases and subsequent degradation to small fragments [11].

In this paper we report a specific test of our propositions. We have examined the sensitivity of native DNA, treated in vitro with phleomycin and/or caffeine, to the action of a *Neurospora crassa* endonuclease which is highly specific for single stranded DNA [17]. Both phleomycin and caffeine have been shown to produce in native DNA sites sensitive to this endonuclease.

2. Materials and methods

The phleomycin used was Batch A9331-616 kindly supplied by Dr. W. Bradner of Bristol Laboratories, Syracuse, N.Y. DNA labelled with [^{3}H]- or [^{14}C]thymidine was prepared from T2 phage grown in *E. coli* B thy$^-$ as described for λ and T5 phages by Hayward and Smith [18]. Single strand specific endonuclease was purified from frozen *Neurospora crassa* conidia (Miles Laboratories) by the method of Rabin and Fraser [19], with the omission of the hydroxyapatite chromatography step. At 37°C, the preparation degraded 1.3 mg of calf thymus DNA per mg of protein to acid soluble form in 20 min when single stranded DNA was the substrate, but had only 1/330 of this activity when the substrate was double stranded DNA.

[^{3}H]DNA samples (10 μl, 8 μg) were incubated at 37°C with various concentrations of phleomycin and/or caffeine, in 0.01 M Tris–HCl buffer, pH 7.5, containing 0.01 M NaCl and 1 mM $MgCl_2$. Total volume of the incubation mixture was 0.08 ml. After 30 min, single strand specific endonuclease (20 μl, 26 μg protein) was added. After incubation for a further 30 min, the reaction was stopped and the DNA denatured by heating the sample in a boiling water bath for 15 min, followed by rapid cooling in ice. ^{14}C-labelled T2 marker

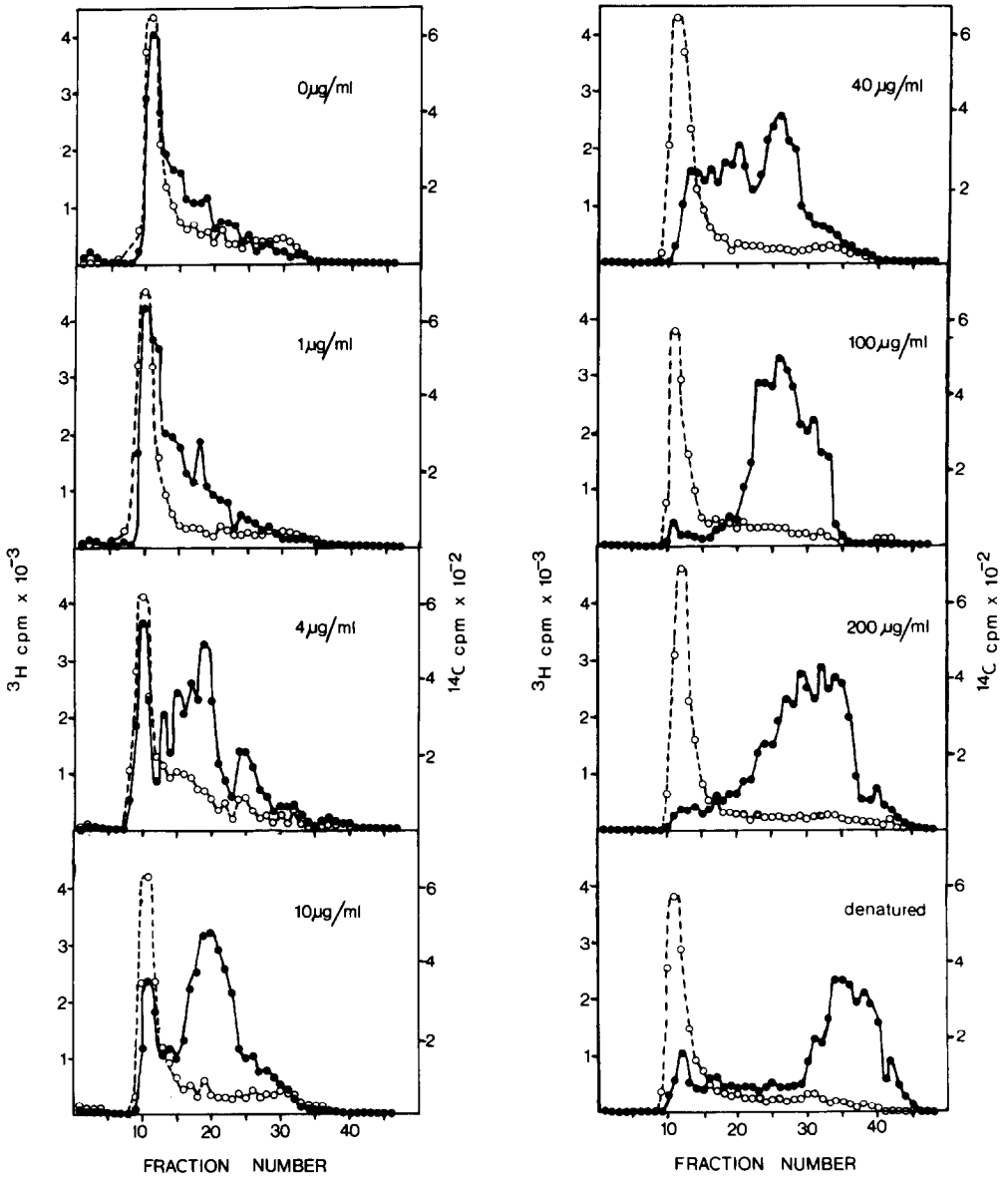

Fig. 1. Strand breakage by single strand-specific endonuclease in phleomycin-treated DNA. Samples of ^{3}H-labelled native T2 DNA were incubated with phleomycin. After digestion with single strand-specific endonuclease (see text for details), samples were filtered through an agarose column with operating range $1 \times 10^5 - 5 \times 10^7$ mol. wt., together with samples of untreated, denatured, ^{14}C-labelled T2 DNA. Elution patterns are shown above, with the concentration of phleomycin shown for each case, and native and denatured DNA samples treated with enzyme included for comparison. Reference DNA (mol. wt. 1.2×10^8 in the native state) began to elute with the void volume. (○—○—○) ^{14}C cpm = reference DNA: (●—●—●) ^{3}H cpm = treated DNA.

DNA (20 μl, 6 μg) was added 5 min after heating was begun. The samples were diluted with 0.1 ml 20% (w/v) sucrose in buffer containing 0.04 Tris, 0.036 M NaH_2PO_4, 1 mM EDTA, pH 7.7, and applied to the top of a 1 × 25 cm column of Biogel A50 (100–200 mesh, 2% agarose, operating range $1 \times 10^5 - 5 \times 10^7$ mol. wt.). Elution was with the same buffer. Fractions of 0.6 ml were collected every 4.5 min, and radioactivi-

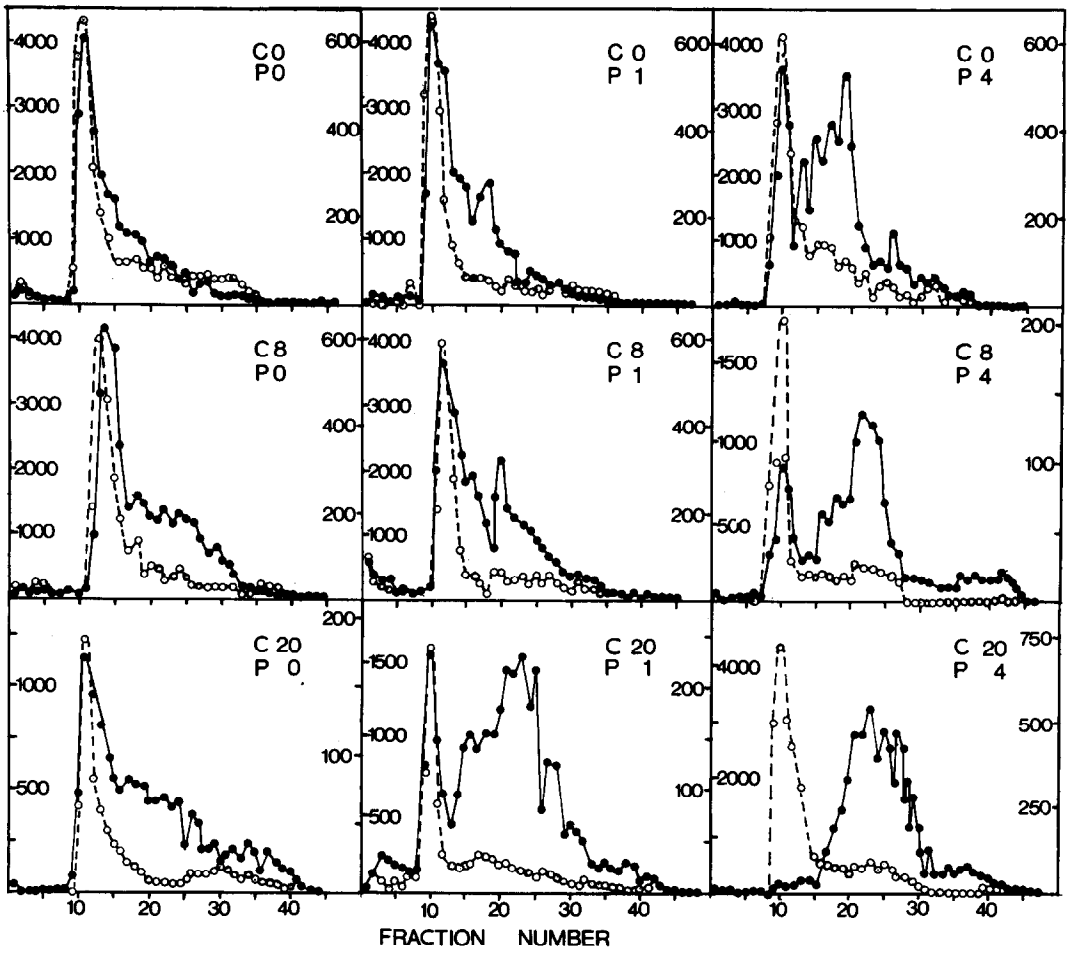

Fig. 2. Strand breakage by single strand-specific endonuclease in DNA treated with phleomycin and caffeine. Samples of ^{3}H- labelled native T2 DNA were incubated with phleomycin and caffeine together, and treated as described for the samples in fig. 1. The concentrations of caffeine (C) as mM and phleomycin (P) as μg/ml are shown for each case. The scale for the left hand ordinate shows ^{3}H cpm (●—●—●) and for the right hand ordinate ^{14}C cpm (○—○—○).

ty of the aqueous samples was measured in a Packard Tri-Carb Liquid Scintillation spectrometer using 10 ml PPO/POPOP/toluene counting mixture containing 34% (v/v) Triton X-100.

3. Results and discussion

3.1. *Effects of phleomycin*

Native T2 DNA incubated in the presence or absence of single strand-specific endonuclease, or with phleomycin but no enzyme, showed no change in size from the control DNA, as seen in the agarose gel elution patterns. However, when native DNA samples treated with increasing concentrations of phleomycin were incubated with the endonuclease, progressive degradation of the DNA was observed (fig. 1).

Kato and Fraser [20] have recently used this single strand-specific enzyme to examine nuclease sensitivity of ultraviolet-irradiated DNA. They concluded that the enzyme recognised specific regions of the DNA as single stranded where clusters of pyrimidine adducts occurred. Our results confirm the suggestion [10,11] that phleomycin–thymine complexes are similar in

their effects of the secondary structure of DNA. In situations where phleomycin can enter the cell and bind to DNA, strand breakage produced in this manner would be the first step in the DNA degradation which occurs in vivo [10].

3.2. *Effects of caffeine and its interactions with phleomycin*

When native T2 DNA was treated with caffeine in vitro, some breakage by single strand-specific endonuclease occurred (fig. 2). No change in elution pattern from control was observed in DNA samples incubated with caffeine but no endonuclease, or with endonuclease but no caffeine. These results were in contrast with those obtained in vivo, where *E. coli* B cells treated with caffeine showed no breakdown of their DNA to small fragments [10].

Cells treated with a combination of caffeine and phleomycin however, showed considerably greater breakdown than cells treated with phleomycin alone [10]. Likewise, when native T2 DNA was treated in vitro with phleomycin and caffeine, the amount of DNA degradation produced by *Neurospora* endonuclease was much greater than in DNA treated with phleomycin alone, particularly at high caffeine concentrations (fig. 2). Our suggestion that caffeine, which binds preferentially to single stranded DNA, increases local denaturation by binding to DNA at sites provided by phleomycin–thymine complexes [11], could explain this interaction.

The frequency of DNA strand breaks detected in vivo is the resultant of breakage and repair events. In addition, DNA degradation following such breaks is dependent on further variables, such as the sensitivity of the break points to exonuclease attack, and the availability of degradative exonucleases. Although caffeine inhibits *E. coli* exonucleases in vitro [22], it does not reduce DNA degradation following UV- and X-irradiation in *E. coli* B [21] at the concentrations used here. The absence of DNA degradation following caffeine treatment of *E. coli* B in vivo, despite the appearance of endonuclease sensitive sites in vitro, could be explained by the existence of a mechanism to repair caffeine-induced DNA strand breaks. This repair system would presumably be absent in strains such as Bs-11, which degrades its DNA in response to caffeine treatment, and also has reduced ability to repair DNA damage resulting from UV- and X-irradiation [21].

Simple nicks in DNA should differ in ease of repair from nicks in the region of large phleomycin–thymine complexes. Thus caffeine-induced DNA strand breaks would be less likely to be repaired, and more likely to act as sites for exonuclease action in phleomycin-treated, than in untreated DNA. Breakage of DNA induced by a combination of caffeine and phleomycin would then appear additive in vitro, but synergistic in vivo, where the normal repair of caffeine-induced breaks was influenced by the presence of phleomycin.

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