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The mechanism of DNA breakage by phleomycin in vitro.

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ABSTRACT

Phleomycin induces DNA breakage *in vitro* in the presence of the sulphydryl compound dithiothreitol. The reaction appears to be free radical-mediated, and requires oxygen and metal ions. Reaction rate is limited by the concentration of oxygen, which is converted to hydrogen peroxide during DNA breakage. However there is no net change in the sulphydryl compound. The proposed reaction mechanism involves metal ion/oxygen-catalysed oxidation of dithiothreitol to its free radical form, which reacts with phleomycin, leading to formation of activated phleomycin and regeneration of free sulphydryl. Free phleomycin is converted to an inactive form, but activation of phleomycin bound to DNA leads to DNA breakage.

INTRODUCTION

Phleomycin, a mixture of copper-containing glycopeptides isolated from *Streptomyces verticillus*[1] binds to DNA[2] and stimulates DNA breakage *in vitro* by both enzymic and non-enzymic mechanisms[3,4]. The non-enzymic reaction is dependent on the presence of sulphydryl compounds such as dithiothreitol or 2-mercaptoethanol. The closely related antibiotic bleomycin can break DNA under similar conditions, and studies on the mechanism of breakage have been carried out mainly with this antibiotic[5-8]. A free radical reaction mechanism was proposed [7,8]. Muller *et al*[7] found that free radical scavengers failed to block DNA breakage, but the scavengers used were specific for only some of the possible species of radicals being formed, so that their results do not exclude a free radical mechanism for the breakage reaction.

We have studied DNA breakage in vitro by phleomycin. The reaction conditions in general appear to be similar to those for bleomycin, and the mechanism we propose is consistent with data for DNA breakage by both antibiotics.

MATERIALS AND METHODS

Phleomycin (batch A9331-648, Cu^{2+}-containing) was supplied by Dr. W. Bradner, Bristol Laboratories, Syracuse, N.Y, U.S.A, and bleomycin A2 (Cu-free) was obtained from Dr. H. Umezawa, Institute of Applied Microbiology, University of Tokyo, Japan. Beef liver catalase (E.C. 1.11.1.6) was obtained from Koch Light Laboratories, Bucks, U.K, and had an activity of 4000 units/mg protein. Superoxide dismutase, a gift from Dr. J.M. Gebicki, Macquarie University, N.S.W, was prepared from bovine blood by the method of McCord and Fridovich[9]. T2 bacteriophage DNA, labelled with [^{14}C] thymidine (60mCi/mmol) or [^{3}H] thymidine (1.0Ci/mmol) (both products of The Radiochemical Centre, Amersham, Bucks., U.K) was prepared as described previously[3]. All other reagents were obtained from commercial sources.

For measurement of phleomycin-induced DNA single-strand breakage in vitro, the standard reaction mixture contained ^{3}H-labelled native T2 phage DNA (5µl, 7µg), phleomycin, dithiothreitol (10mM) and 0.01M Tris-HCl/0.01M NaCl/1mM, pH7.6 in a total volume of 0.1ml. Incubation was at 37^{o} for 30 mins. after addition of dithiothreitol. DNA was then denatured by heating the sample in a boiling water bath, followed by rapid cooling in ice. ^{14}C-labelled T2 marker DNA (20µl, 6µg) was added 2 min. after heating began. The samples were diluted with 0.1ml 20% (w/v) sucrose in buffer containing 0.04M Tris/0.036 Na_2HPO_4/1mM EDTA, pH7.7, and applied to the top of a 1x25cm column of Biogel A50 (100-200 mesh, 2% agarose, operating range $1x10^5$ - $5x10^7$ daltons, from Bio-Rad. Laboratories, Richmond, California). Elution was with the same buffer, without the addition of sucrose. Fractions (0.6ml) were collected every 4.5 min. and radioactivity of the aqueous samples was measured in a Packard Tri-Carb Liquid Scintillation spectrometer, using 10ml PPO/POPOP/toluene counting mixture containing 34% (v/v) Triton X-100.

Sulphydryl groups were estimated by the method of Ellman[10]. Samples containing 0-1.0mM sulphydryl (0.1ml) were added to 2.5ml buffer containing 0.01M Tris-HCl, 0.01M NaCl, 0.5mg/ml 5,5'-dithiobis (2-nitrobenzoic acid) and 0.5mg/ml EDTA, pH7.6. After 2 min. at room temperature, absorbance at 412 nm was measured against a reagent blank.

RESULTS AND DISCUSSION

Phleomycin-induced DNA breakage *in vitro* depended on the presence of dithiothreitol, and this was included in all incubation mixtures. Breakage was accelerated by the addition of the free radical-generating system ammonium persulphate/ tetraethylene methylene diamine (Fig.1). This effect was

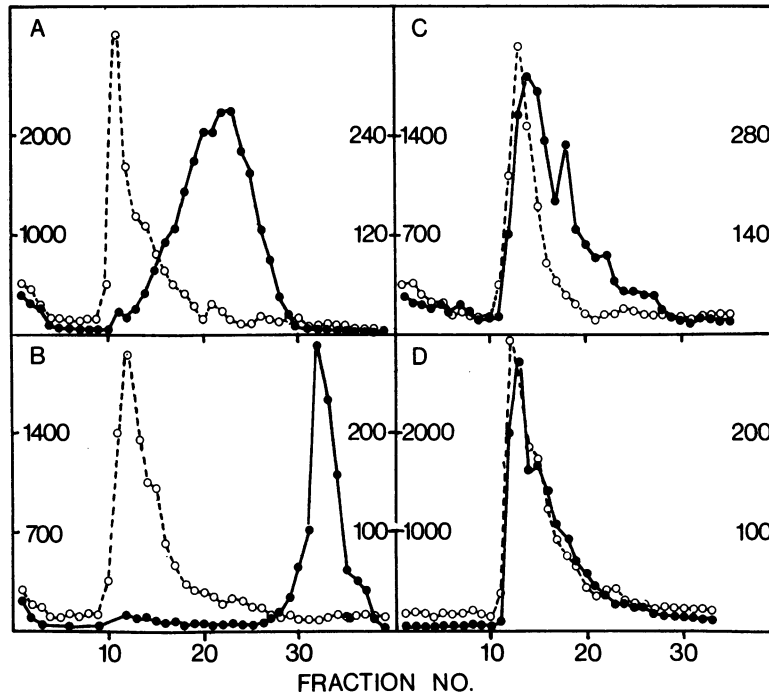

Figure 1: Enhancement of phleomycin-induced DNA breakage by free radical generation. The figure shows the elution pattern obtained from filtration of T2 DNA through a column of agarose beads. The left hand scale shows c.p.m. from ^{3}H-labelled, phleomycin-treated DNA (●——●) and the right hand scale shows c.p.m. from ^{14}C-labelled untreated DNA (0 -- 0). A - DNA breakage by 2µg/ml phleomycin; B - 2µg/ml phleomycin with the addition of 0.07% (w/v) ammonium persulfate and 0.1% (w/v) tetraethylene methylene diamine; C - same as B with phleomycin omitted; D - same as B with dithiothreitol omitted.

completely dependent on the presence of both phleomycin and dithiothreitol, suggesting acceleration of an existing free radical breakage reaction.

To test the involvement of hydroxyl radicals, $OH^{\cdot}$, in DNA breakage, the $OH^{\cdot}$ scavengers histidine and ethanol[11] were included in the incubation mixture. Neither histidine (0.2M) nor ethanol (0.5M) in any way affected phleomycin-induced DNA damage, demonstrating that hydroxyl radicals were not involved in the breakage mechanism.

DNA breakage by phleomycin was completely blocked by KI (0.1M) (Fig.2A). KI interferes with lipid autoxidation reactions which involve a free radical mechanism[12] suggesting that some similar oxidation reaction may be occurring to produce DNA breakage.

DNA damage was wholly dependent on the sulphydryl moiety of dithiothreitol, since breakage was blocked by titration of the sulphydryl groups with N-ethylmaleimide (Fig.2B). Sulphydryl groups are readily oxidised to a free radical form $RS^{\cdot}$ in the

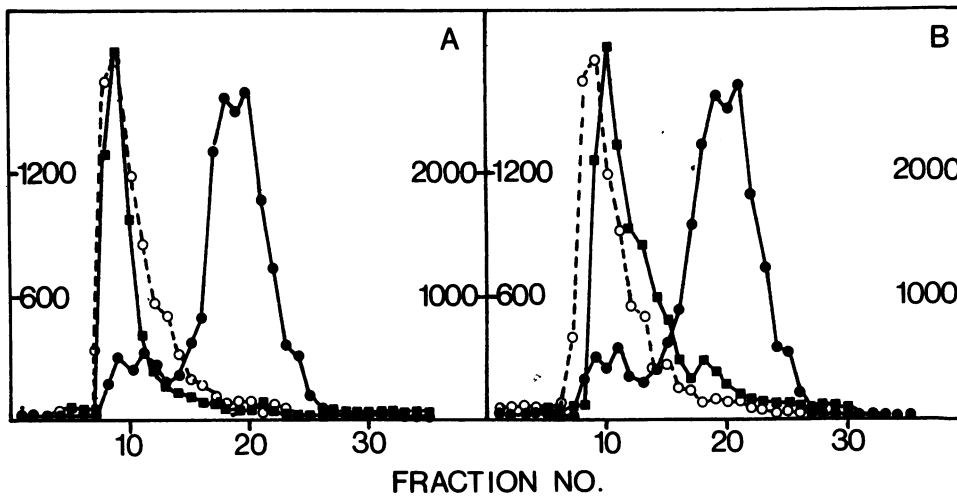

Figure 2: Sulphydryl involvement in phleomycin-induced DNA breakage. The left hand scale shows c.p.m. from ^{3}H-labelled, phleomycin-treated DNA (solid lines) and the right hand scale shows c.p.m. for ^{14}C-labelled untreated DNA (0 -- 0). A - DNA breakage by 1µg/ml phleomycin alone (●——●) and in the presence of 0.1M KI (■——■); B - 1µg/ml phleomycin alone (●——●) and with 20mM N-ethylmaleimide (■——■).

presence of a suitable catalyst e.g. Fe^{2+} or Cu^{2+}. This catalytic activity of metal ions can be inhibited by EDTA but not by 8-hydroxyquinoline[13]. EDTA inhibited DNA damage by both bleomycin[6] and phleomycin (Fig.3A), demonstrating that metal ions were required for DNA breakage to occur. Phleomycin itself contains Cu^{2+}, and this can be removed by 8-hydroxyquinoline but not by EDTA[14]. 8-hydroxyquinoline also inhibited phleomycin-induced DNA damage (Fig.3B), showing that metal ions

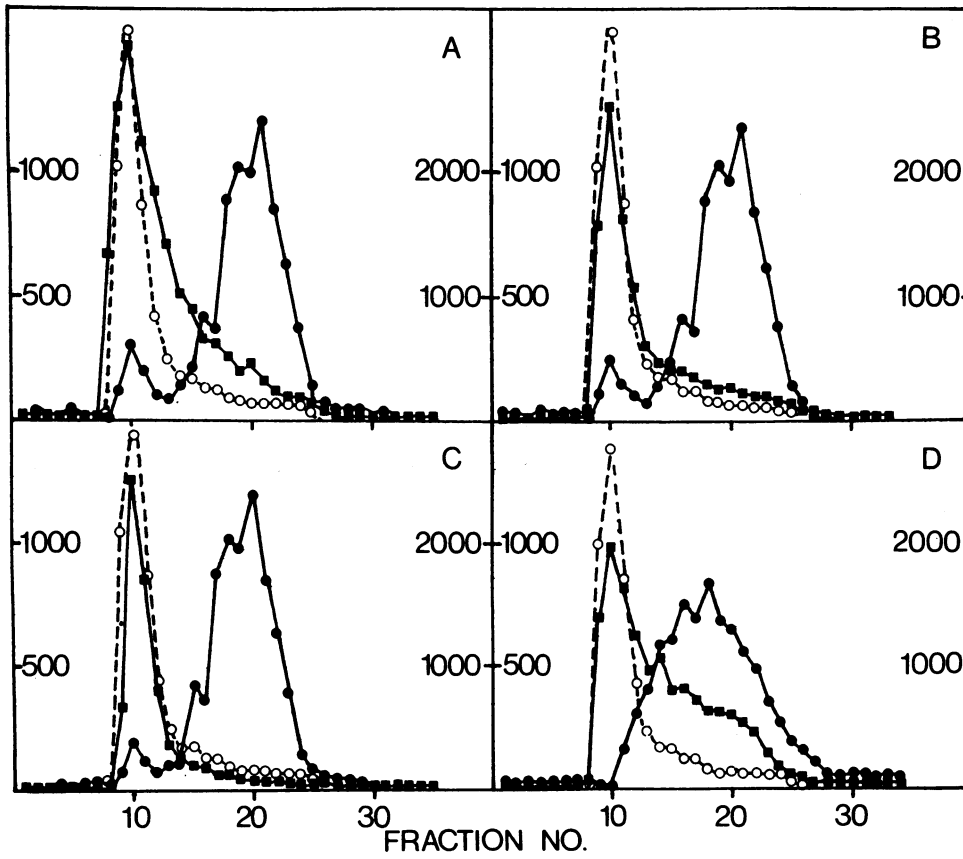

Figure 3: Effect of metal ions on phleomycin-induced DNA breakage. The left hand scale shows c.p.m. from ^{3}H-labelled phleomycin-treated DNA (solid lines) while the right hand scale shows ^{14}C c.p.m. from untreated DNA (0 -- 0). A - DNA breakage by 0.5µg/ml phleomycin alone (●——●) and with 10mM EDTA (■——■); B - 0.5µg/ml phleomycin alone (●——●) and with 10mM 8-hydroxyquinoline and 5.0mM $MgCl_2$ (■——■); D - 0.5µg/ml phleomycin in the presence of 1mM (●——●) and 2mM (■——■) $CuSO_4$.

must be attached to phleomycin, and so held close to DNA to bring about DNA breakage. Inhibition by 8-hydroxyquinoline was not reversed by addition of a slight excess of Mg^{2+} (Fig.3C).

The amounts of Cu-free bleomycin used to produce DNA breakage in previous studies[5-8] were much higher than the phleomycin concentrations used here. However this is due to the greater sensitivity of our method for detecting DNA breakage, rather than to the relative amounts of Cu^{2+} present, since bleomycin A2, reported to be Cu-free, was just as active as phleomycin in producing DNA damage in our system. Evidently sufficient metal ions are contained in bleomycin or other reagents to allow the breakage reaction to proceed, suggesting that only very small amounts of metal ions are required. High concentrations of Cu^{2+} actually inhibited DNA breakage by both phleomycin (Fig.3D) and bleomycin[6] apparently due to a reduction in antibiotic binding[6]. Bleomycin appeared to be affected by lower concentrations of Cu^{2+} (0.1mM)[6] than were required to block the phleomycin response (Fig.3D).

Thiol oxidation is dependent, not only on a metal ion catalyst, but also on the presence of O_2, which maintains the Cu^{2+} in its catalytically active state. Superoxide radical ions may be formed in the reaction, and these can take part in further thiol oxidations[13]. Superoxide radical ions, $O_2^{\cdot -}$, react rapidly in aqueous solution, producing a series of highly reactive species e.g. $HO_2^{\cdot}$, singlet O_2, H_2O_2, any of which may be involved in producing DNA damage[15].

That O_2 is required for DNA breakage by phleomycin as well as by bleomycin[8] is shown in Fig.4, since carrying out the incubation under N_2 blocked DNA damage (Fig.4A), while bubbling O_2 through the system increased breakage (Fig.4B). No DNA breakage was obtained with O_2 in the absence of phleomycin.

The enzyme catalase, which converts hydrogen peroxide to oxygen and water, increased the DNA breakage rate (Fig.5A). This increase was attributed to reconversion of H_2O_2 formed to O_2, effectively increasing the O_2 concentration, since the increase in breakage observed was much less than that obtained by adding extra O_2 to the system under similar conditions (Fig.5B).

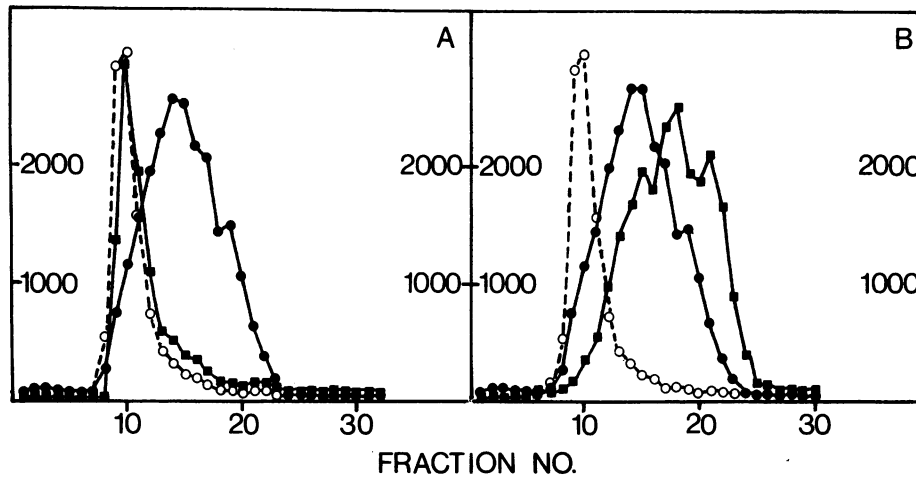

Figure 4: 0_2 requirement for phleomycin-induced DNA breakage. The left hand scale shows c.p.m. from ^3H-labelled phleomycin-treated DNA (solid lines) and the right hand scale shows ^{14}C c.p.m. from untreated DNA (0 -- 0). A - DNA breakage by 0.4µg/ml phleomycin in air (●——●) and under N_2 (■——■); B - 0.4µg/ml phleomycin in air (●——●) and under 0_2 (■——■).

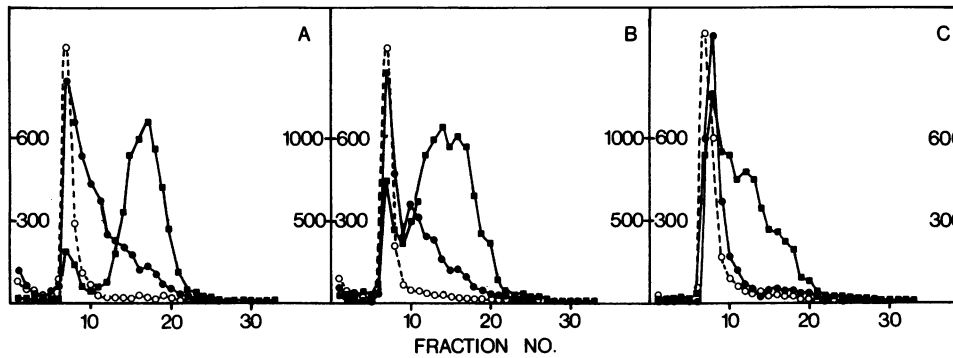

Figure 5: Effect of catalase and superoxide dismutase on phleomycin-induced DNA breakage. The left hand scale shows c.p.m. from ^3H-labelled phleomycin-treated DNA (solid lines) and the right hand scale shows ^{14}C c.p.m. from untreated DNA (0 -- 0). A - DNA breakage by 0.25µg/ml phleomycin alone (●——●) and in the presence of 50µg/ml catalase (■——■); B - 0.2µg/ml phleomycin in the presence of air and 50µg/ml catalase (●——●) or in the presence of O_2 (■——■); C - 0.2µg/ml phleomycin with 50µg/ml superoxide dismutase (●——●) or superoxide dismutase (50µg/ml) and catalase (50µg/ml) (■——■).

The involvement of other oxygen-containing reactive species in DNA breakage is not yet clear. Addition of the enzyme superoxide dismutase, which catalyses the conversion of superoxide radical ions to O_2, did not significantly increase DNA damage by a low concentration of phleomycin (Fig.5C), nor did it decrease breakage in the presence of a higher phleomycin concentration. Simultaneous addition of superoxide dismutase and catalase resulted in a slight increase in DNA over that observed with catalase alone, apparently due to complete reconversion of $O_2^{\cdot -}$ to O_2 by the two enzymes (Fig.5C). Since $O_2^{\cdot -}$ is normally a short-lived species in aqueous solution, the results with superoxide dismutase do not exclude the transient formation of greater amounts of the radical ion. While it is evident that $O_2^{\cdot -}$ itself is not involved in production of DNA damage by phleomycin, a dismutation product of the radical ion may cause DNA damage.

Free Cu^{2+}-catalayzed thiol oxidation leads to a net decrease in the amount of free sulphydryl present (Fig.6) due to disulphide formation[13]. However no decrease in free -SH was produced by Cu-phleomycin, either in the presence or absence of DNA (Fig.6). Evidently thiyl radicals were reconverted to thiol in the presence of phleomycin, explaining why bleomycin-induced DNA breakage was not limited by sulphydryl concentration[8].

Incubation of bleomycin with sulphydryl in the absence of DNA resulted in inactivation of bleomycin, an effect reported to be independent of O_2 and not blocked by the free radical scavenger S-(2-aminoethyl) isothiouronium bromide hydrobromide[8]. However, this scavenger is itself converted to a sulphydryl compound in solution, and is able to participate in thiol oxidation reactions[16]. Thus, while it is apparently unable to catalyse DNA breakage, it may be as effective in inactivating bleomycin as other sulphydryl compounds.

Phleomycin was similarly inactivated after incubation with dithiothreitol (Fig.7A), but little inactivation was observed when the amount of O_2 present was reduced by bubbling N_2 through the reaction mixture (Fig.7B). Oxygen was restored to the system after DNA was added to measure phleomycin activity.

My results are consistent, therefore, with a mechanism

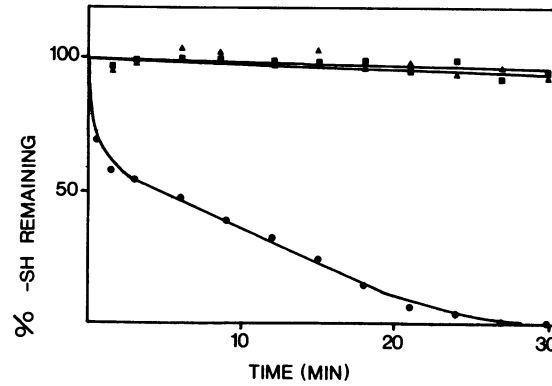

Figure 6: Effect of phleomycin and Cu^{2+} on free sulphydryl concentration. Reaction mixtures contained 70mM 2-mercaptoethanol, with 140mM $CuSO_4$, (●——●), 70µg/ml (approx. 70mM) phleomycin (■——■), or 70µg/ml phleomycin with 45µg/ml T2 DNA (▲——▲).

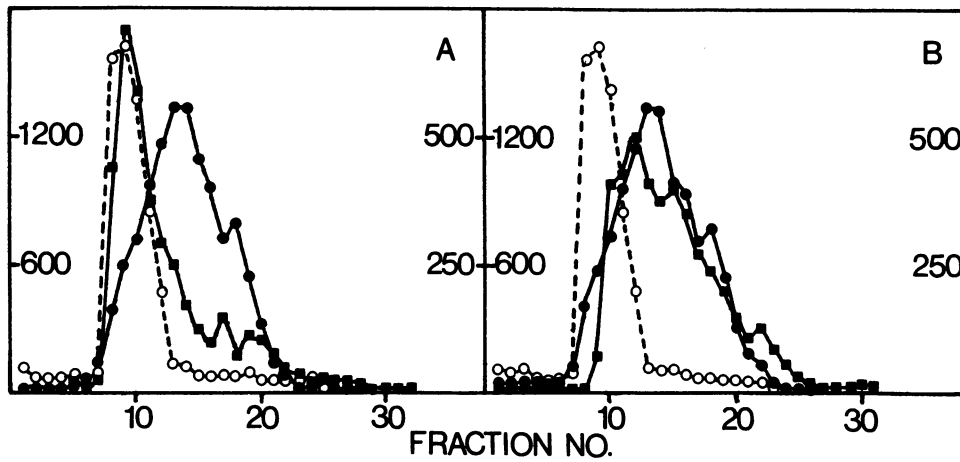

Figure 7: Inactivation of phleomycin by dithiothreitol. The left hand scale shows c.p.m. from ^3H-labelled phleomycin-^{14}C treated DNA (solid lines) and the right hand scale shows ^{14}C c.p.m. from untreated DNA (0 -- 0). A - DNA breakage by 0.5µg/ml phleomycin where dithiothreitol was added after (●——●) or 30 min. before addition of DNA to the reaction mixture (■——■); B - 0.5µg/ml phleomycin alone (●——●) or after 30 min. preincubation with dithiothreitol under N_2, followed by addition of DNA and O_2 (■——■).

for DNA breakage by phleomycin in which free thiol is oxidised to its radical form RS$^{\cdot}$. This reaction is catalysed by metal ions and O_2, and result in the formation of H_2O_2[13]. Free phleomycin is in activated by sulphydryl compounds, perhaps subsequent to or during this thiol oxidation, since inactivation also requires O_2. However, in the presence of DNA, phleomycin produces DNA breakage, with no net decrease in free thiol concentration.

The contribution of this direct DNA breakage to DNA damage induced *in vivo* by phleomycin and bleomycin is not yet clear. Addition of sulphydryl compounds to phleomycin-treated *E.coli* resulted in inhibition of DNA breakage[17]. This effect of sulphydryl compounds in whole cells may be due to reduction in cellular permeability to phleomycin, or inactivation of phleomycin before it can become attached to DNA, since phleomycin-treated spheroplasts of *E.coli* B showed *increased* DNA breakage on addition of 10mM dithiothreitol (Sleigh and Grigg, unpublished) demonstrating that, *in vivo*, *E.coli* DNA is susceptible to breakage by phleomycin via a thiol-mediated pathway.

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