The Mechanism of Sensitivity to Phleomycin in Growing *Escherichia coli* Cells

By MERILYN J. SLEIGH and GEOFFREY W. GRIGG

Division of Animal Genetics, C.S.I.R.O., P.O. Box 90, Epping, N.S.W. 2121, Australia

(Received 8 September 1975)

Stationary-phase *Escherichia coli* B cells transferred to new growth medium are initially resistant to net DNA breakage by low concentrations of phleomycin, and become sensitive as DNA replication commences. From studies with inhibitors of various stages of the DNA replication cycle it is evident that it is not DNA synthesis itself that is required for induction of DNA breakage by phleomycin, but events associated with the initiation of DNA replication. Termination of replication in the absence of further initiation results in resistance to phleomycin. The cellular change responsible for changes in sensitivity to phleomycin could be the attachment of the bacterial chromosome to the cell membrane at initiation and detachment on termination of replication, suggesting an alteration in the balance between cellular DNA breakage and repair processes for membrane-associated compared with non-membrane-associated DNA.

Phleomycin is made up of a group of closely related compounds isolated from *Streptomyces verticillus* (Maeda *et al.*, 1956) with anti-bacterial (Grigg, 1969; Pietsch & Clapper, 1969; Reiter *et al.*, 1972), anti-tumour (Bradner & Pindell, 1962; Umezawa *et al.*, 1962) and anti-viral (Pitts & Sinsheimer, 1966; Tevethia & Rapp, 1969; Iwata & Consigli, 1971) activity. DNA strand breakage after treatment with phleomycin has been described in *Escherichia coli* (Grigg, 1969, 1970; Grigg *et al.*, 1971), *Bacillus subtilis* (Reiter *et al.*, 1972) as well as in viruses (Iwata & Consigli, 1971) and human cells (Jacobs *et al.*, 1969; Kleijer *et al.*, 1973).

It has been reported (Grigg, 1969) that exponentially growing *E. coli* B *thy* cells degrade their DNA much more rapidly in response to low concentrations of phleomycin than do cells just commencing growth. We have examined the transition of *E. coli* cells from the resting to the growing state, in an attempt to relate the change in sensitivity to phleomycin to a specific event in this transition.

Experimental

Phleomycin

Phleomycin (batch A9331-648) was kindly supplied by Dr. W. T. Bradner, of Bristol Laboratories, Syracuse, NY, U.S.A. A fresh solution was made up for each experiment in a medium of pH 7.4, the composition of which is described below. (It is our experience that phleomycin loses activity below pH 7.0.) All other materials used were obtained from commercial sources.

Bacterial strains

Bacterial strains, obtained from the late Dr. Ruth Hill, were *E. coli* B and the Trp strain WP-2. *E. coli* B

is *lon*, *sul* and is filament forming, whereas WP-2, derived from *E. coli* B, is *lon*, *sul*⁺, *trp*. Thymine-requiring mutants were prepared from the parent strains by the method of Okada *et al.* (1960).

Growth of cells

The growth medium used was a modified Gray and Tatum (GT) glucose-salts medium containing 5.0g of NH₄Cl, 1.0g of NH₄NO₃, 3.0g of K₂HPO₄, 1.0g of KH₂PO₄, 2.0g of Na₂SO₄, 0.1g of MgSO₄, 1.0mg of CaCl₂, 1.0g of glucose, 0.1mg of Na₂B₄O₇, 0.4mg of CuSO₄, 1.0mg of FeCl₃, 0.07mg of MnCl₂, 0.05mg of Na₂MoO₄ and 0.01mg of ZnSO₄ per litre. Hydrated salts were used in all cases, and pH was adjusted to 7.4 with 0.5M-NaOH. Cultures were grown to stationary phase (16h at 37°C), with addition of thymidine (20 µg/ml) and tryptophan (20 µg/ml) as required.

DNA was labelled in thymine-requiring strains by the addition of either [¹⁴C]thymidine (20 µg/ml, 60mCi/mmol) or [³H]thymidine (1.0mCi/mmol, diluted 100 times with unlabelled thymidine). Both were from The Radiochemical Centre, Amersham, Bucks., U.K. Labelled thymidine was incorporated by Thy⁺ cells grown in the presence of deoxyadenosine (250 µg/ml) (Boyce & Setlow, 1962).

Stationary-phase cells were harvested by filtration on prewashed Millipore membrane filters (pore size, 0.45 µm), washed with GT medium containing unlabelled thymidine and resuspended in the same medium. Exponentially growing cells were obtained by incubating the cells for 2h at 37°C after resuspension.

Measurement of DNA damage

Two different measures of DNA damage were used. (1) DNA breakdown was assessed by the appearance

of acid-soluble DNA fragments. This method has been outlined previously (Grigg, 1970). (2) The appearance of single-strand breaks and/or alkali-labile sites in cellular [^{14}C]thymidine-labelled DNA was measured by alkaline sucrose-density-gradient centrifugation (McGrath & Williams, 1966). *E. coli* samples containing approx. 10^8 cells in a volume of 0.1 ml were incubated at room temperature with 10 μl of lysozyme (2 mg/ml) (Calbiochem, Spring Valley, NY, U.S.A.) and 10 μl of 0.01 M-Tris/0.01 M-EDTA, pH 8.3. After 1 min, 10 μl (10^8 cells) of ^3H -labelled control cells was added. After a further 10 min, the spheroplasts were transferred to a layer (0.4 ml) of 0.5 M-NaOH and 0.5% sodium lauryl sarcosinate on 5–20% alkaline sucrose gradients (in 0.1 M-NaOH/0.9 M-NaCl/1 mM-EDTA). After 20 min at room temperature, gradients were centrifuged in a Beckman SW41 rotor at 111 700g for 90 min at 21°C. Fractions (0.22 ml) were pumped from the bottom of the tube on to glass-fibre paper (Whatman GF187). The papers were dried, transferred to vials containing 10 ml of scintillator (5.5 g of Packard Permablend I/litre of toluene) and counted for radioactivity in a Packard liquid-scintillation spectrometer.

Labelling of the replication origin

The method used was similar to that described by Lark (1972) and Beppu & Arima (1971). Stationary-phase cells of *E. coli* WP-2, grown for 16 h in GT tryptophan medium supplemented with [^{14}C]thymidine and 250 μg of deoxyadenosine/ml, were transferred to fresh medium containing thymidine (20 $\mu\text{g}/\text{ml}$), but lacking the required amino acid tryptophan. After 80 min at 37°C, to allow termination of DNA replication, tryptophan (20 $\mu\text{g}/\text{ml}$) was restored, and [^3H]thymidine (50 μCi , 22 Ci/mmol) was added. After 25 min [^3H]thymidine was removed by membrane filtration (pore size, 0.45 μm) and the cells were resuspended in medium containing unlabelled thymidine (20 $\mu\text{g}/\text{ml}$), but lacking tryptophan, so that the replication cycles initiated from the origin during [^3H]thymidine treatment would be completed. After a further 80 min, tryptophan was restored, phleomycin added, and the appearance of ^3H - and ^{14}C -labelled acid-soluble DNA was followed.

For control cells, the same series of incubations was carried out, except that tryptophan was present at all stages, so that no particular point on the chromosome was being replicated either when [^3H]thymidine was added or when phleomycin treatment was begun.

Preparation of a synchronous cell culture by amino acid starvation

E. coli WP-2 cells were grown for 16 h in GT tryptophan medium supplemented with [^{14}C]thymidine and 250 μg of deoxyadenosine/ml and then transferred to fresh medium containing un-

labelled thymidine and tryptophan. After 90 min at 37°C, cells were transferred to medium containing no tryptophan. Tryptophan was added after 90 min, when cell cycles in progress should have been completed. Incorporation of added [^3H]thymidine into DNA was measured by pipetting 0.1 ml on to a washed glass-fibre paper disc and immediately washing with ice cold 5% (w/v) trichloroacetic acid/2.5% (v/v) acetone to remove acid-soluble material. The discs were then washed with ethanol, dried, and the radioactivity was measured.

Results

DNA breakage in stationary-phase and exponentially growing cells

Exposure to low concentrations of phleomycin (2 $\mu\text{g}/\text{ml}$) for 30 min induced a greater number of DNA single-strand breaks (or alkali-labile sites; the two are not differentiated by this method) in exponen-

Fig. 1. Phleomycin-induced DNA single-strand breakage in stationary-phase and exponentially growing *E. coli* B thy

At 30 min after addition of phleomycin (2 $\mu\text{g}/\text{ml}$) to exponentially growing cells (b), or to stationary-phase cells resuspended in growth medium (a), cells were filtered through a Millipore filter to remove phleomycin, and after cell lysis the [^{14}C]thymidine-labelled DNA was separated by alkaline sucrose-density-gradient centrifugation. Sedimentation is from right to left with results plotted as percentage of total c.p.m. for ^3H - and ^{14}C -labelled DNA. $\square$ — $\square$, ^{14}C -labelled phleomycin-treated DNA; $\blacksquare$ — $\blacksquare$, ^3H -labelled marker DNA.

tially growing *E. coli* B *thy* cells than in stationary-phase cells transferred to new growth medium. Moreover, a significant proportion of the DNA in stationary-phase cells appeared to be unbroken (Fig. 1).

Phleomycin-induced DNA breakage in cells emerging from stationary phase

A transition from low to high sensitivity to phleomycin was observed when stationary-phase *E. coli* B *thy* cells were transferred to new growth medium. Fig. 2 illustrates the extent of DNA single-strand breakage in samples taken sequentially from the germinating culture and treated with phleomycin (6 µg/ml, 10 min). For the first 30 min after resuspension, the cells showed little DNA damage in response to phleomycin, but after this DNA strand breakage increased until 100 min after resuspension, when a constant response was obtained. The cells began to synthesize DNA after 50 min, and the first replication cycle was completed at the end of 110 min (Fig. 2). Thus the DNA of the cells became sensitive to phleomycin-induced damage just before the start of DNA replication. We therefore examined the role of DNA replication in induction of DNA damage by phleomycin.

DNA degradation associated with replication

Phleomycin-induced DNA degradation preferentially associated with the replication point was examined by labelling the replication origin of one DNA strand of *E. coli* WP-2 with [³H]thymidine, and then restarting replication at the origin as phleomycin was added (Fig. 3). The rate of appearance of ³H-labelled acid-soluble DNA fragments (expressed as a proportion of total [³H]thymidine incorporated) was the same as that for fragments labelled with [¹⁴C]-thymidine, which was incorporated throughout the chromosome. Therefore it appeared that production of small DNA fragments was not limited to the region of the replication point as it moved along the chromosome from the origin.

The possible association of DNA replication with phleomycin-induced DNA damage was further tested

Fig. 2. Phleomycin-induced DNA single-strand breakage in *E. coli* B *thy* cells growing from stationary phase

Stationary-phase cells were transferred to new growth medium and at various times samples were removed and incubated with phleomycin (6 µg/ml); (b) 20 min; (c) 40 min; (d) 60 min; (e) 100 min. After the 10 min treatment, cells were filtered to remove phleomycin, and the DNA was separated by alkaline sucrose-density-gradient centrifugation. Results are plotted as for Fig. 1. ▽ ---- ▽, ¹⁴C-labelled DNA from phleomycin-treated cells; ▾ — ▾, ³H-labelled marker DNA. Incorporation of [³H]thymidine into ¹⁴C-labelled acid-insoluble DNA is shown in (a) by the ratio of ³H:¹⁴C c.p.m. incorporated at various times.

Fig. 3. Phleomycin-induced DNA degradation associated with the replication point in *E. coli* WP-2

Appearance of acid-soluble fragments from ^{14}C -labelled DNA of cells starting replication from the origin, which is labelled with $[^3\text{H}]$ thymidine (a), is compared with DNA degradation in cells with $[^3\text{H}]$ thymidine incorporated randomly in the chromosome (b). Cells were treated with phleomycin: $\circ, \bullet, 0 \mu\text{g/ml}$; $\nabla, \blacktriangledown, 2 \mu\text{g/ml}$; $\square, \blacksquare, 20 \mu\text{g/ml}$. —, ^3H ; ----, ^{14}C .

by observing the effects of inhibiting DNA replication. Four different methods were used.

(1) *Addition of 1- β -D-arabinofuranosyl-cytosine.* Incubation of *E. coli* B *thy* cells with this inhibitor (1 mg/ml) for 60 min before phleomycin (2 $\mu\text{g/ml}$) was added altered neither the extent nor the time-course of phleomycin-induced DNA degradation. This concentration of 1- β -D-arabinofuranosyl-cytosine decreased the incorporation of $[^3\text{H}]$ thymidine into acid-insoluble DNA below detectable values within 20 min.

(2) *Thymine starvation.* Inhibition of DNA synthesis by starving *E. coli* B *thy* cells of thymine for 60 min was ineffective in preventing DNA breakdown when phleomycin (2 $\mu\text{g/ml}$) was added to the cell suspension.

(3) *Addition of hydroxyurea.* Hydroxyurea (0.025 M) failed to affect the appearance of DNA single-strand breaks in *E. coli* B *thy* cells treated with phleomycin (2 $\mu\text{g/ml}$). Cells were pretreated with hydroxyurea for 60 min, during which time the rate of incorporation of $[^3\text{H}]$ thymidine into acid-insoluble DNA decreased to less than 5% of the previous value.

(4) *Addition of phenethyl alcohol.* Phenethyl alcohol (2-phenylethanol, 0.25%) decreased incorporation of $[^3\text{H}]$ thymidine into acid-insoluble DNA below detectable values within 10 min, and its addition immediately inhibited phleomycin-induced DNA breakage in *E. coli* B *thy* cells. A lower concentration (0.22%) of phenethyl alcohol considerably decreased the rate of DNA synthesis and also slowly induced resistance to DNA damage by phleomycin (Fig. 4).

These results show that DNA replication itself is not required for the production of DNA damage by

Fig. 4. Inhibition by phenethyl alcohol of phleomycin-induced DNA single-strand breakage in *E. coli* B *thy* cells

Growing cells were treated with phenethyl alcohol (0.22% or 0.25%) and samples were removed at various times to measure DNA single-strand breakage during 10 min treatment with phleomycin (6 $\mu\text{g/ml}$). (b), Exponentially growing cells; (c), time 0 after addition of 0.25% phenethyl alcohol; (d), time 0 after addition of 0.22% phenethyl alcohol; (e), 30 min after addition of 0.22% phenethyl alcohol; (f), 60 min after addition of 0.22% phenethyl alcohol. Results are plotted as described for Fig. 1. Δ ---- Δ , ^{14}C -labelled treated DNA; $\blacktriangle$ — $\blacktriangle$, ^3H -labelled marker DNA. Phenethyl alcohol alone produced no DNA strand breakage in the absence of phleomycin. (a) shows the inhibition of DNA synthesis after addition of phenethyl alcohol (marked by the arrow). Results are plotted as the ratio of $^3\text{H}:^{14}\text{C}$ incorporated into ^{14}C -labelled acid-insoluble DNA after addition of $[^3\text{H}]$ thymidine. $\circ$, Phenethyl alcohol (0.25%); $\bullet$, phenethyl alcohol (0.22%).

Fig. 5. Inhibition by rifampicin of induction of DNA damage by phleomycin in *E. coli* B *thy*

The response to treatment with phleomycin (6 $\mu\text{g/ml}$, 10 min) was measured in stationary-phase cells 60 min after resuspension in new growth medium with (b) or without (a) rifampicin (100 $\mu\text{g/ml}$). Growing cells were treated with rifampicin (100 $\mu\text{g/ml}$) and the response to phleomycin (6 $\mu\text{g/ml}$, 10 min) 10 min (d) and 30 min (e) after addition of rifampicin was compared with the response in cells not treated with rifampicin (c). Results are plotted as described for Fig. 1. $\square$ ---- $\square$, ^{14}C -labelled DNA from phleomycin-treated cells; $\blacksquare$ — $\blacksquare$, ^3H -labelled marker DNA.

Fig. 4

Fig. 5

Fig. 6. Inhibition by amino acid starvation of phleomycin-induced DNA breakage in *E. coli* WP-2

Stationary-phase cells were resuspended in growth medium lacking the required amino acid tryptophan, and after 30 min (b), and 60 min (c) DNA breakage induced by phleomycin (6 $\mu\text{g}/\text{ml}$, 10 min) was compared with breakage in a sample of stationary-phase cells resuspended and incubated for 60 min in growth medium containing tryptophan (a). Growing cells were deprived of tryptophan. The response to phleomycin (6 $\mu\text{g}/\text{ml}$, 10 min) after 10 min (e) and 30 min (f) of amino acid starvation is compared with DNA breakage in cells incubated with tryptophan (d). Results are plotted as for Fig. 1. $\square$ — $\square$, ^{14}C -labelled phleomycin-treated DNA; $\blacksquare$ — $\blacksquare$, ^3H -labelled marker DNA.

Fig. 7. Effect of chloramphenicol treatment on induction of DNA single-strand breakage in *E. coli* B thy by phleomycin. Stationary-phase cells were transferred to new growth medium and were tested for induction of DNA breakage by phleomycin (2 μ g/ml, 30 min) before (a), and at 0, 10, 30 and 60 min after addition of 25 μ g of chloramphenicol/ml (b-e). Exponentially growing cells were treated similarly (f-j). Results are plotted as for Fig. 1. $\circ$ --- $\circ$, 14 C-labelled phleomycin-treated DNA; $\bullet$ — $\bullet$, 3 H-labelled marker DNA.

phleomycin. However, phenethylalcohol, an inhibitor of the initiation of DNA replication (Lark & Lark, 1966; Silver & Wendt, 1967), inhibited phleomycin-induced DNA damage as DNA replication ceased, so that an event or events associated with the initiation of DNA replication may be important for phleomycin-induced DNA damage. Initiation of DNA replication involves a number of discrete processes including a RNA-synthesis step occurring at the time of initiation, a protein-synthesis step sensitive to low concentrations of chloramphenicol occurring 20 min before initiation and a second protein-synthesis step, sensitive to high concentrations of chloramphenicol, occurring 5–10 min before initiation (Lark & Lark, 1966; Lark, 1972; Messer, 1972). We therefore examined the role of RNA and protein synthesis in sensitivity of *E. coli* cells to phleomycin.

Inhibition of RNA synthesis

Addition of rifampicin (100 µg/ml) to stationary-phase *E. coli* B *thy* cells suspended in growth medium completely prevented the development of sensitivity to phleomycin (Fig. 5). However, in growing cells, a period of preincubation with rifampicin was required before cells became completely resistant to the induction of DNA damage by phleomycin (Fig. 5).

Inhibition of protein synthesis

Amino acid starvation. Starvation of *E. coli* WP-2 cells of the required amino acid tryptophan decreased the appearance of phleomycin-induced DNA damage in growing cells, with complete resistance to phleomycin coinciding with cessation of DNA synthesis (Fig. 6). Suspension of stationary-phase *E. coli* WP-2 in growth medium lacking tryptophan greatly decreased the appearance of sensitivity to DNA damage by phleomycin, and after 60 min, cells were again completely resistant to phleomycin.

Chloramphenicol treatment. Addition of chloramphenicol (25 µg/ml) to stationary-phase *E. coli* B *thy* cells at the same time as phleomycin (2 µg/ml) considerably decreased the amount of DNA-strand breakage obtained during the 30 min treatment time, whereas in growing cells the same concentration of chloramphenicol had little effect on the response to phleomycin (Fig. 7). As the time of treatment with chloramphenicol before phleomycin addition was increased sensitivity to phleomycin disappeared in both stationary-phase and exponentially growing cells (Fig. 7). Comparison of amino acid starvation and chloramphenicol as inhibitors of phleomycin-induced DNA degradation in *E. coli* WP-2 confirmed that amino acid starvation was more effective than chloramphenicol treatment in decreasing phleomycin-induced DNA degradation in growing cells (Fig. 8).

DNA breakage in cells recovering from amino acid starvation. When tryptophan was restored to *E. coli* WP-2 cells starved of the required amino acid for

120 min there was a lag of 10–20 min before full sensitivity to phleomycin was attained, and before DNA replication commenced (Fig. 9). Subsequently, sensitivity to phleomycin remained approximately constant in the synchronously growing culture, up to the 50–60 min sample, which marked the end of a round of DNA replication.

In these experiments, samples of ³H-labelled T2 bacteriophage DNA were included in the sucrose gradients as molecular-weight markers. Methods of calculating the number of single-strand breaks in DNA assume that breakage occurs randomly, but in these experiments the sedimentation patterns obtained for phleomycin-treated DNA suggested that breakage was not random, with some DNA relatively resistant to breakage. For that reason we have not attempted to estimate the frequency of DNA single-strand breaks in this or other experiments reported here.

If the resistance to phleomycin, induced by inhibiting RNA or protein synthesis, is due specifically to failure of initiation of chromosome replication (Lark & Lark, 1966; Lark, 1972; Messer, 1972), then interruption of replication before termination should prevent any subsequent effect of inhibition of protein synthesis on induction of DNA damage by phleomycin.

Inhibition of protein synthesis in cells whose DNA replication cycle is interrupted before termination. Growing *E. coli* WP-2 cells were prevented from terminating the replication cycle in progress by

Fig. 8. Comparison of chloramphenicol and tryptophan starvation as inhibitors of phleomycin-induced DNA degradation in *E. coli* WP-2

Growing cells were treated with chloramphenicol (■), or deprived of tryptophan (▼) at the same time as phleomycin (2 µg/ml) was added. Results show the appearance of acid-soluble DNA fragments. Control results are shown for phleomycin-treated cells (●) and corresponding cells not treated with phleomycin (○).

Fig. 9. Phleomycin-induced DNA breakage in cells recovering from amino acid starvation

Cells of *E. coli* WP-2 were synchronized by tryptophan starvation. Tryptophan was restored at zero time and at various times (0, 5, 10, 20, 30 and 50 min) samples were removed and DNA single-strand breakage by phleomycin ($6\text{ }\mu\text{g/ml}$, 10 min) was tested (b–g). Results show alkaline density-gradient-sedimentation profiles, plotted as described for Fig. 1. ●—●, ^3H -labelled marker DNA; ○—○, ^{14}C -labelled phleomycin-treated DNA. (a) shows the ratio of $^3\text{H}:^{14}\text{C}$ in acid-insoluble DNA of an identical sample of ^{14}C -labelled cells incubated with [^3H]thymidine after restoration of tryptophan.

treatment with hydroxyurea and deprivation of thymine in the presence of deoxyadenosine. Subsequent inhibition of protein synthesis by amino acid starvation failed to induce resistance to phleomycin (Fig. 10).

During thymine starvation cells accumulate the ability to initiate new rounds of DNA replication for

several generations in the absence of protein synthesis (Kogoma & Lark, 1970; Masker & Eberle, 1971). When DNA synthesis in growing *E. coli* WP-2 cells was first inhibited for a period, as described above, and then DNA-synthesizing ability was restored at the same time as protein synthesis was suppressed by amino acid starvation, the cells retained their sensi-

Fig. 10. Inhibition of amino acid starvation-induced resistance to phleomycin by inhibition of DNA synthesis

Growing *E. coli* WP-2 cells were deprived of thymidine in the presence of deoxyadenosine and hydroxyurea (0.025 M) for 75 min before the required amino acid tryptophan was removed. Induction of DNA single-strand breakage by phleomycin (6 µg/ml, 10 min) was measured in these cells 0 min (c) and 30 min (e) after removal of tryptophan, and compared with breakage in growing cells before (a), at the time of (b), and 30 min after (d) removal of tryptophan. Results are plotted as in Fig. 1. ▽----▽, ¹⁴C-labelled treated DNA; ▼——▼, ³H-labelled marker DNA.

tivity to phleomycin-induced DNA damage, despite the immediate resumption of DNA synthesis (Fig. 11).

Discussion

E. coli B cells, starting to grow from stationary phase, or after amino acid starvation, acquired increasing sensitivity to DNA breakage by phleomycin as DNA replication commenced. However, DNA damage was not decreased by inhibiting the progress of the replication fork, and seemed to be unrelated to the position of the replication fork on the chromosome.

Factors that inhibit the initiation of DNA replication (phenethyl alcohol, protein-synthesis inhibition, RNA-synthesis inhibition) inhibited phleomycin-induced DNA damage. Three distinct steps in the chronology of initiation of DNA replication have been identified in *E. coli* (Lark & Lark, 1966; Lark, 1972; Messer, 1972). One protein, whose synthesis is sensitive to 2 µg of chloramphenicol per ml, is made 20 min before initiation, and another, whose synthesis is sensitive to 100 µg of chloramphenicol per ml, is made 5–10 min before initiation. Finally, RNA synthesis is required at the time of initiation. Treatment of cells with rifampicin, or inhibition of protein synthesis by amino acid starvation, were more effective in preventing initiation of new DNA-replication cycles than treatment with low concentrations of chloramphenicol (Messer, 1972), and their relative effectiveness in inhibiting induction of DNA damage by phleomycin in cells growing from stationary phase was of the same order. Similarly, in exponentially growing cells, resistance to DNA damage by phleomycin was achieved sooner on inhibition of steps occurring late in the initiation process (i.e. those sensitive to inhibition of RNA synthesis and amino acid starvation) than when an early event in initiation was inhibited by treatment with 25 µg of chloramphenicol/ml. The time at which complete resistance to phleomycin was achieved corresponded to the time required for completion of DNA replication cycles in progress, in the absence of further initiation (Messer, 1972). Therefore it seems that sensitivity to phleomycin develops at the same time as DNA replication is initiated, and disappears as the round of replication terminates in the absence of further initiation.

Where termination of DNA replication was prevented by inhibiting DNA synthesis part of the way around the cycle, cells failed to become resistant to phleomycin-induced DNA damage, even though their ability to initiate new rounds of DNA replication was inhibited (by amino acid starvation). Moreover, when the ability to synthesize DNA was restored at the same time as protein synthesis was inhibited, cells remained sensitive to phleomycin. Thus the cells seemed able to accumulate 'phleomycin-sensitivity

Fig. 11. Accumulation of phleomycin sensitivity during inhibition of DNA synthesis in *E. coli* WP-2

DNA synthesis of growing cells was inhibited as described for Fig. 10. After 75 min hydroxyurea was removed and thymidine was restored, and simultaneously cells were deprived of the required amino acid tryptophan. (d) shows incorporation of [^3H]thymidine into acid-insoluble DNA in these cells, the first arrow marking inhibition of DNA synthesis, and the second, restoration of DNA synthesis in the absence of tryptophan. Induction of DNA single-strand by phleomycin (6 $\mu\text{g}/\text{ml}$, 10 min) in these cells 0 min (e) and 30 min (f) after removal of tryptophan is compared with breakage in growing cells before (a), 0 min (b) and 30 min (c) after removal of tryptophan, in the absence of the DNA-synthesis-inhibition treatment. Results are plotted as for Fig. 1. $\circ$ --- $\circ$, ^{14}C -labelled phleomycin-treated DNA; $\bullet$ — $\bullet$, ^3H -labelled marker DNA.

capacity' just as they are known to accumulate the ability to initiate subsequent rounds of DNA replication, when DNA synthesis is inhibited (Kogoma & Lark, 1970; Masker & Eberle, 1971).

The mechanism of inhibition of replication initiation by phenethyl alcohol has not been clarified, but it may act at the time of the second protein-synthesis step (Lark & Lark, 1966). In *E. coli* 15 *thy* DNA synthesis continues for 30–40 min in the presence of 0.25% phenethyl alcohol (Masker & Eberle, 1972), but we found that in *E. coli* B *thy* all DNA synthesis had ceased 10 min after addition of this concentration of phenethyl alcohol, and resistance to DNA damage by phleomycin was achieved immediately. This may be associated with the ability of high concentrations of phenethyl alcohol to release DNA from its association with cell membrane (Masker & Eberle, 1972). The results with both high and low concentrations of phenethyl alcohol are consistent with the suggestion that appearance of resistance to phleomycin-induced DNA damage is associated with the termination of DNA replication.

What particular cellular change, occurring in conjunction with initiation and termination of DNA replication, is responsible for the appearance and disappearance of phleomycin sensitivity? Alteration in cellular nuclease activity could be responsible, since some nucleases, particularly endonucleases, have higher activity in growing than in stationary-phase cells (Shortman & Lehman, 1964).

Changes in membrane structure may also affect the response to phleomycin. Maintenance of membrane protein and lipid is essential for the initiation of new DNA replication cycles (Fielding & Fox, 1970; Fralick & Lark, 1973) and under conditions where initiation is inhibited large changes in cell permeability and membrane structure are observed (Lark & Lark, 1966; Knox *et al.*, 1967; Silver & Wendt, 1967; Shapiro *et al.*, 1970; Normark & Westling, 1971; Crowfoot *et al.*, 1972; Wada & Yura, 1974).

If a change in membrane structure is responsible for altered sensitivity to phleomycin, then it must be a highly specific change associated with replication initiation or cell division, rather than a non-specific change produced, for example, by amino acid starvation, since amino acid starvation did not result in phleomycin resistance under all conditions, but only where DNA replication was not impeded. Cell permeability is greatest under conditions where phleomycin-induced DNA damage is least, so that a general change in permeability is unlikely to explain the altered response to phleomycin, although phleomycin may be transported into the cell by some specific mechanism, whose operation is dependent on the maintenance of membrane structure.

It is possible that phleomycin induces DNA damage from outside the cell, in a manner analogous to the proposed mechanism for the induction of DNA

damage by colicin E2 (Ringrose, 1970), so that disruption of cell membrane structure may interfere with the mechanism for transmission of the phleomycin message. The evidence, however, points to an intracellular interaction between phleomycin and DNA (Falaschi & Kornberg, 1963; Pietsch & Garrett, 1968; Pietsch & Corbett, 1968; Pietsch & Garrett, 1969; Stern *et al.*, 1974).

Increased sensitivity to DNA degradation in growing cells occurs, not only in response to phleomycin, but also after ionizing radiation (Town *et al.*, 1970; Yatvin *et al.*, 1972), heat treatment (Woodcock & Grigg, 1972) and treatment with the alkylating agent ethyl methanesulphonate (Howell-Saxton *et al.*, 1974). After u.v.-irradiation, amino acid starvation protects the cells from DNA degradation, a phenomenon which appears to require termination of DNA-replication cycles (Sedliakova *et al.*, 1971). This suggests that the difference in response between stationary-phase and growing cells may be dependent on the operation of DNA breakage and repair mechanisms in general, rather than on the activity of, or permeability to, a particular DNA-damaging agent.

During initiation of DNA replication in *E. coli* the chromosome becomes attached to the cell membrane (Deliuss & Worcel, 1974; Worcel & Burgi, 1974), probably by means of the initiator proteins, and is released only on termination of the replication cycle. The times at which the chromosome is released from the cell membrane during amino acid starvation, and re-attached on restoration of amino acids, closely parallel the disappearance and re-appearance of sensitivity to phleomycin under similar conditions. Masker & Eberle (1972) reported that a concentration of phenethyl alcohol sufficient to arrest DNA replication immediately causes the release of DNA from the cell membrane. The immediate appearance of resistance to phleomycin in cells treated with a high concentration of phenethyl alcohol may be explained if only DNA attached to cell membrane is sensitive to damage by phleomycin.

Membrane-associated synthesis of bacteriophage ϕ X174 replicative-form DNA was inhibited by phleomycin, but synthesis of DNA for progeny phage, independent of the membrane, was unaffected (Pitts & Sinsheimer, 1966). During thymine starvation, despite disruption of cell membrane structure (Silver & Wendt, 1967) there was no alteration in DNA-cell membrane association (Dankberg & Cummings, 1973), and the response to phleomycin was unaffected. In general it seems that membrane-bound DNA is more sensitive to DNA-breakage mechanisms (Shuster, 1973).

We suggest that the cellular change possibly responsible for the appearance and disappearance of sensitivity to phleomycin is the attachment of the chromosome to the cell membrane at initiation, and

detachment from the membrane on termination of DNA replication (Delius & Worcel, 1974; Worcel & Burgi, 1974). Either the membrane attachment itself, or the accompanying change in chromosome structure (Pettijohn & Hecht, 1973; Ryder & Smith, 1974; Worcel & Burgi, 1974) may increase phleomycin binding to DNA, reveal more phleomycin-bound sites for breakage, or decrease the efficiency of repair enzymes. Membrane-bound DNA may be accessible to nucleases or other breakage mechanisms located in the membrane region (Heppel, 1967; Costerton *et al.*, 1974) leading to a rate of DNA breakage in excess of the capacity of DNA-repair mechanisms.

We thank Dr. B. McAuslan for his critical review of the manuscript. M. J. S. is a candidate for the degree of Doctor of Philosophy at Macquarie University and is grateful to Dr. J. Gebicki for helpful discussions.

References

- Beppu, T. & Arima, K. (1971) *J. Biochem. (Tokyo)* **70**, 263–271
- Boyce, R. P. & Setlow, R. B. (1962) *Biochim. Biophys. Acta* **61**, 618–620
- Bradner, W. T. & Pindell, M. H. (1962) *Nature (London)* **196**, 622–623
- Costerton, J. W., Ingram, J. M. & Cheng, K. J. (1974) *Bacteriol. Rev.* **38**, 87–110
- Crowfoot, P. D., Esfahani, M. & Wakil, S. J. (1972) *J. Bacteriol.* **112**, 1408–1415
- Dankberg, F. & Cummings, D. J. (1973) *J. Bacteriol.* **113**, 711–717
- Delius, H. & Worcel, A. (1974) *J. Mol. Biol.* **82**, 107–109
- Falascchi, A. & Kornberg, A. (1964) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **23**, 940–945
- Fielding, P. & Fox, C. F. (1970) *Biochem. Biophys. Res. Commun.* **41**, 157–162
- Fralick, J. A. & Lark, K. G. (1973) *J. Mol. Biol.* **80**, 459–475
- Grigg, G. W. (1969) *Mol. Gen. Genet.* **104**, 1–11
- Grigg, G. W. (1970) *Mol. Gen. Genet.* **107**, 162–172
- Grigg, G. W., Edwards, M. J. & Brown, D. J. (1971) *J. Bacteriol.* **107**, 593–598
- Heppel, L. A. (1967) *Science* **156**, 1451–1455
- Howell-Saxton, E., Smith, D. C., Zamenhof, R. J. & Zamenhof, S. (1974) *Mutat. Res.* **24**, 227–237
- Iwata, A. & Consigli, R. A. (1971) *J. Virol.* **7**, 29–40
- Jacobs, N. F., Neu, R. L. & Gardner, L. I. (1969) *Mutat. Res.* **7**, 251–253
- Kleijer, W. J., Hoeksma, J. L., Sluyter, M. L. & Bootsma, I. D. (1973) *Mutat. Res.* **17**, 385–394
- Knox, K. W., Culleen, J. & Work, E. (1967) *Biochem. J.* **103**, 192–201
- Kogoma, T. & Lark, K. G. (1970) *J. Mol. Biol.* **52**, 143–164
- Lark, K. G. (1972) *J. Mol. Biol.* **64**, 47–60
- Lark, K. G. & Lark, C. (1966) *J. Mol. Biol.* **20**, 9–19
- Maeda, K., Kosaka, H., Yagishita, K. & Umezawa, H. (1956) *J. Antibiot. (Ser. A)* **9**, 82
- Masker, W. E. & Eberle, H. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2549–2553
- Masker, W. E. & Eberle, H. (1972) *J. Bacteriol.* **109**, 1170–1174
- McGrath, R. A. & Williams, R. W. (1966) *Nature (London)* **212**, 534–535
- Messer, W. (1972) *J. Bacteriol.* **112**, 7–12
- Normark, S. & Westling, B. (1971) *J. Bacteriol.* **108**, 45–50
- Okada, F. J., Yanagisawa, K. & Ryan, F. J. (1960) *Nature (London)* **188**, 340–341
- Pettijohn, D. E. & Hecht, R. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 31–41
- Pietsch, P. & Clapper, G. (1969) *Cytobios* **2**, 145–152
- Pietsch, P. & Corbett, C. (1968) *Nature (London)* **219**, 933–934
- Pietsch, P. & Garrett, H. (1968) *Nature (London)* **219**, 488–489
- Pietsch, P. & Garrett, H. (1969) *Cytobios* **1A**, 7–15
- Pitts, J. D. & Sinsheimer, R. L. (1966) *J. Mol. Biol.* **15**, 676–680
- Reiter, H., Milewski, M. & Kelley, P. (1972) *J. Bacteriol.* **111**, 586–592
- Ringrose, P. (1970) *Biochim. Biophys. Acta* **213**, 320–334
- Ryder, O. A. & Smith, D. W. (1974) *J. Bacteriol.* **120**, 1356–1365
- Sedliakova, M., Stefunkova, E. & Bernatova, L. (1971) *J. Bacteriol.* **107**, 16–20
- Shapiro, B. M., Siccardi, A. G., Hirota, Y. & Jacob, F. (1970) *J. Mol. Biol.* **52**, 75–89
- Shortman, K. & Lehman, I. R. (1964) *J. Biol. Chem.* **239**, 2964–2974
- Shuster, R. C. (1973) *J. Bacteriol.* **116**, 1067–1070
- Silver, S. & Wendt, L. (1967) *J. Bacteriol.* **93**, 560–566
- Stern, R., Rose, J. A. & Friedman, R. M. (1974) *Biochemistry* **13**, 307–312
- Tevethia, S. S. & Rapp, F. (1969) *Cancer Res.* **29**, 912–917
- Town, C. D., Smith, K. C. & Haplan, H. S. (1970) *J. Bacteriol.* **105**, 127–135
- Umezawa, H., Mori, M., Ishizuka, H. & Takeuchi, T. (1962) *J. Antibiot. (Tokyo)* **15A**, 274–275
- Wada, C. & Yura, T. (1974) *Genetics* **77**, 199–220
- Woodcock, E. & Grigg, G. W. (1972) *Nature (London)* **237**, 76–79
- Worcel, A. & Burgi, E. (1974) *J. Mol. Biol.* **82**, 91–105
- Yatvin, M. B., Wood, P. G. & Brown, S. M. (1972) *Biochim. Biophys. Acta* **287**, 390–403