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Mode of Action of Phleomycin on *Bacillus subtilis*

HARVARD REITER, MARIANNE MILEWSKIY, AND PHILIP KELLEY

Department of Microbiology, University of Illinois at the Medical Center, Chicago, Illinois 60680

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Phleomycin appears to act on the cell wall and membrane to induce the release of membrane-associated deoxyribonucleic acid (DNA) and the degradation of the DNA. Degradation occurs in a series of energy-requiring endonuclease and exonuclease reactions. These produce, first, single-strand breaks, then double-strand breaks, and finally almost complete solubilization of the cellular DNA. The in vivo inhibition of DNA synthesis by phleomycin is probably a secondary effect caused by the destruction of template DNA.

Phleomycin is a copper-containing antibiotic that is lethal to a wide variety of gram-negative and gram-positive bacteria as well as to a variety of mammalian viruses and cells (6, 7, 12-14). It inhibits bacterial deoxyribonucleic acid (DNA) synthesis in vivo (7) and it induces the solubilization of the DNA in treated cells (3). It also blocks the activity of DNA polymerase in vitro, apparently by binding to the DNA primer (2).

If phleomycin binds to DNA in the cells as it does to DNA in vitro, its effects can be understood as being similar to those of ultraviolet (UV) irradiation which induces pyrimidine dimers in DNA, thereby arresting DNA synthesis and causing some DNA solubilization. However, the different sensitivities of some DNA syntheses to phleomycin are an indication that DNA synthesis in vivo is not inhibited in this way. At concentrations of phleomycin that totally block the synthesis of *Escherichia coli* DNA, the synthesis of T4 phage DNA (Farrell and Reiter, *manuscript in preparation*) and of the late DNA of ϕX174 in *E. coli* cells is not affected (9).

In this paper we describe some of the effects of phleomycin on the DNA of *Bacillus subtilis*. We present evidence showing that it causes the destruction of cellular DNA by inducing a series of energy-dependent nuclease reactions in Uvr^+ and Uvr^- (excisionless) strains and, that during this destruction, the cells retain the ability to synthesize DNA. The effects of phleomycin on *B. subtilis* are similar to its effects on *E. coli* (Farrell and Reiter, *manuscript in preparation*). The data indicate that the mode of action of phleomycin in both *B. subtilis* and in *E. coli* is similar to the mode of action of the colicin E2 in *E. coli*.

MATERIALS AND METHODS

Strains and media. *B. subtilis* 168*thyA*, *thyB* and 168*uvr*$^-$ were described previously (10). The cells were grown at 37 C with aeration, in Spizizen's minimal medium (1) supplemented with 0.5% glucose, 0.04% acid-hydrolyzed casein, and 4 μg of thymidine per ml ($CHthy_4$). Phleomycin, lot no. A9331-909, was supplied by Bristol Laboratories, Syracuse, N.Y., and *m*-chlorophenyl carbonyl cyanide hydrazone (CCCP) was purchased from Calbiochem. Chloramphenicol was purchased from Parke, Davis & Co., and rifampin from Mann Research Laboratories.

DNA labeling. Cell DNA was labeled by growing the cells for the indicated times in $CHthy_4$ plus 4 μCi of ^{3}H-thymidine per ml (Schwarz/Mann, 6.0 Ci/mmole). All experiments were done with exponentially growing cells at concentrations less than 10^8 cells/ml. Labeling was terminated by washing the cells on membrane filters and resuspending them in $CHthy_4$. Phleomycin (20 μg/ml) was added to the cells at the indicated times after resuspension. DNA, ribonucleic acid (RNA), and protein syntheses were measured by determining the amounts of ^{3}H-thymidine, ^{3}H-uracil, or ^{3}H-leucine incorporated into material insoluble in cold, 5% trichloroacetic acid. Samples (0.1 ml) of culture were precipitated, collected and washed with water on fiberglass filters, and then dried and counted in 2 ml of scintillation fluid (5 g Packard premix "P" per liter of toluene).

DNA solubilization. Acid-soluble radioactivity was determined by treating cells plus medium for 30 min with ice-cold 5% trichloroacetic acid, centrifuging at 10,000 $\times$ *g* for 10 min, extracting the trichloroacetic acid from the supernatant fluid with ether, and then counting 0.1 ml of the supernatant fluid in 5 ml of scintillation fluid (5 g of 2,5-diphenyloxazole, 80 g of naphthalene, 360 ml of toluene,

360 ml of dioxane, and 216 ml of absolute ethyl alcohol). The total radioactivity in a culture was measured by solubilizing the DNA with 10% trichloroacetic acid at 90 C for 20 min, ether-extracting the trichloroacetic acid, and counting the radioactivity in 0.1 ml as above.

Cell lysates. Volumes (5 ml) of cells to be lysed were chilled, centrifuged, suspended in 1 ml of cold 0.01 M ethylenediaminetetraacetate (EDTA)-0.01 M tris(hydroxymethyl)aminomethane (Tris) (*p*H 8.3), and then treated at 37 C with 200 μg of lysozyme per ml for 2 min, 500 μg of Pronase per ml for 1 min, and then 0.05% sodium lauryl sulfate for 5 to 10 min. Cells to be lysed for DNA-membrane experiments were centrifuged and suspended in 1 ml of 20% sucrose in 0.05 M Tris, *p*H 8.1. They were then treated at 37 C for 1 min with 500 μg of lysozyme per ml, chilled, and centrifuged at 2,000 × *g* for 5 min, resuspended in 0.1 ml of 20% sucrose in 0.05 M Tris. A 0.4-ml amount of 0.1 M EDTA-0.05 M Tris, *p*H 8.1, was added to the cells followed by 0.02 ml of $MgSO_4$ at a concentration of 2 mg/ml. The cells were warmed to 37 C and treated with 0.15 ml of 20% Brij-58 for 5 min. DNA in 0.15 ml of cell lysate was denatured by adding 0.15 ml of 0.8 M KH_2PO_4 in 1/10 NET buffer (4) and then 0.03 ml of 1 N NaOH for 1 min at room temperature.

Sucrose gradients. A 0.1- or 0.2-ml amount of cell lysate was layered on 5 to 20, or 10 to 30% sucrose gradients in 1/10 NET buffer and then centrifuged in a Spinco SW50.1 rotor as indicated for each experiment. After centrifugation, the distribution of radioactivity in the gradients was determined by collecting drops from the bottoms of the tubes, precipitating the DNA with 5% trichloroacetic acid, washing, and counting as above. Alkaline sucrose gradients were 10 to 30% gradients of sucrose in 0.4 M NaCl, 0.01 M EDTA, and 0.1 M NaOH.

DNA synthesis in toluenized cells. Cells were grown in $CHthy_4$ to a concentration of 5 × 10^7 to 8 × 10^7 cells/ml, washed on a membrane filter, and resuspended in 1 ml of cold 0.05 M KH_2PO_4, *p*H 7.4. The cells were agitated with 2% toluene for 10 min at room temperature and then chilled. A 0.1-ml amount of ^{3}H-deoxythymidine triphosphate (^{3}H-dTTP), 0.1 ml of triphosphate stock (330 mM deoxyadenosine triphosphate [dATP], 330 mM guanosine triphosphate, 330 mM deoxycytidine triphosphate, 20 mM TTP), and 0.5 ml of basic buffer (330 mM; 0.07 M KH_2PO_4, 0.013 M $MgSO_4$, 0.002 M dithiothreitol), *p*H 7.4, and 0.1 ml of either water or 13 mM ATP were added to 0.2 ml of washed cells at 4 C. DNA synthesis was initiated and measured by warming the culture to 37 C and adding 0.05-ml samples to 0.6 ml of 5% trichloroacetic acid. The precipitated material was caught on a fiberglass filter, washed, and counted.

RESULTS

Inhibition of DNA and RNA synthesis in phleomycin-treated cells. Because there was some contradictory evidence in the literature about the specificity of phleomycin inhibition of DNA synthesis (15), our first experiment was to determine whether our lot of phleomycin did indeed inhibit DNA synthesis specifically. Figure 1 shows that cells treated with 20 μg of phleomycin per ml continued to incorporate ^{3}H-thymidine into acid-insoluble material for approximately 4 min. There was then an abrupt end to further incorporation. Incorporation of ^{3}H-uracil was slowed after about 4 min, but it was not completely inhibited until 20 min after treatment. ^{3}H-leucine incorporation into protein is apparently unaffected by phleomycin. Phleomycin appears to inhibit both DNA and RNA synthesis, but its primary or most rapid effect is on DNA synthesis. The secondary inhibition of RNA synthesis may be due to the destruction of DNA (*see below*) which serves as a template for RNA synthesis. We cannot explain the long continued synthesis of proteins in the absence of RNA synthesis unless it is due to the continued activity of stable messenger RNA in the cells.

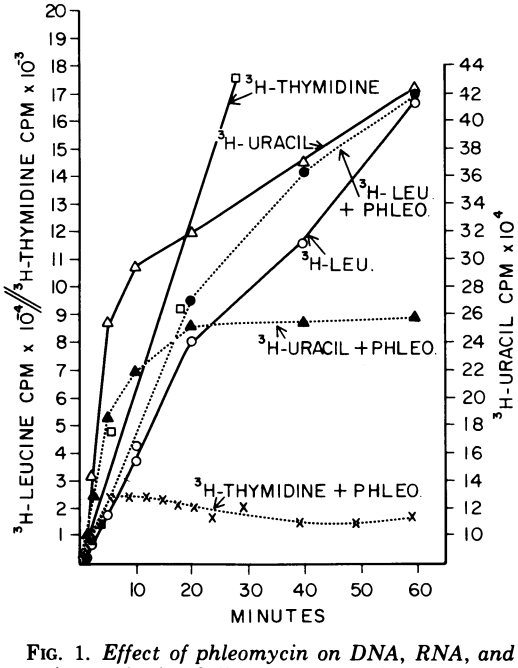

FIG. 1. *Effect of phleomycin on DNA, RNA, and protein synthesis. Cells were grown in $CHthy_4$ to concentrations of 5 × 10^7 to 8 × 10^7 cells/ml. At zero time, 4 μCi of ^{3}H-thymidine, 2 μCi of ^{3}H-uracil, or 2 μCi of ^{3}H-leucine per ml, with and without 20 μg of phleomycin per ml, was added to the logarithmically growing cells. At the times indicated, 0.1-ml samples of the cultures were withdrawn, treated with cold 5% trichloroacetic acid, and assayed for precipitated ^{3}H-labeled material.*

Our second experiment (Fig. 2) showed that, in 60 min, phleomycin caused the degradation of 80 to 90% of the DNA in cells that had been uniformly labeled with ^{3}H-thymidine. Figure 2 also shows that degradation occurred in Uvr$^-$ cells that were unable to excise pyrimidine dimers as well as in Uvr$^+$ cells. Degradation appeared to require energy metabolism in the cells, since it was arrested by CCCP added to the culture at any time during the experiment. Degradation did not appear to require the synthesis of new enzymes, since neither chloramphenicol at 50 μg/ml nor rifampin at 10 μg/ml added to the cells along with phleomycin significantly altered the ensuing degradation.

Production of double-strand scissions in the DNA prior to solubilization. The rapid rate of solubilization of the DNA made it appear likely that degradation was due to exonucleases acting on a number of new sites made throughout the chromosome by prior endonuclease activity. To determine whether this was the case, cells were uniformly labeled with ^{3}H-thymidine and then treated with phleomycin. At intervals after the onset of phleomycin treatment, samples of the cells were withdrawn from the incubating culture and lysed with lysozyme, Pronase, and Dupanol as described in Materials and Methods. Samples of each lysate were layered and centrifuged in neutral 5 to 20% sucrose gradients. The distribution of labeled DNA in these gradients is shown in Fig. 3. There was a clear shift in the band position of the DNA within 5 min of treatment, and by 10 min the shift in band position was largely completed. The loss of acid-insoluble radioactivity in each of the lysates was also determined, and the calculated solubilization of DNA was plotted along with a

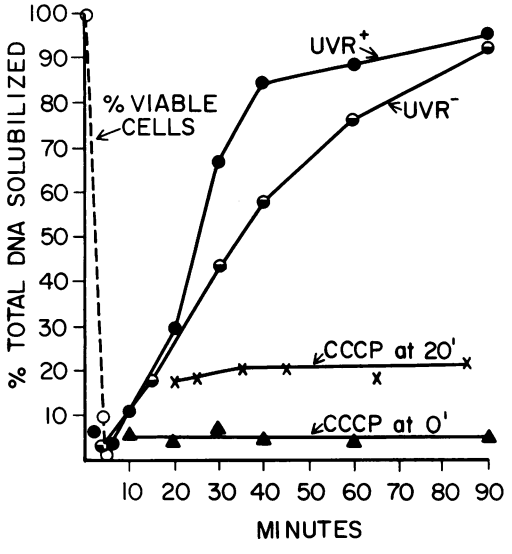

FIG. 2. *Solubilization of DNA in phleomycin-treated cells. Both Uvr$^+$, and Uvr$^-$ cells were grown for more than four generations in CHthy$_4$ plus 4 μCi of ^{3}H-thymidine per ml, washed, and resuspended in ice-cold CHthy$_4$. Phleomycin (20 μCi/ml) was added to both cultures, which were held at 4 C for 30 min. Uvr$^+$, Uvr$^-$: A sample of each culture was incubated with aeration at 37 C and sampled for cold acid-soluble ^{3}H radioactivity at the indicated times. CCCP at 0': 5 × 10^{-4} M CCCP was added to a sample of the chilled Uvr$^+$ cells. The cells were then incubated with aeration at 37 C and assayed for cold, acid-soluble radioactivity. CCCP at 20': 5 × 10^{-4} M CCCP was added to a sample of the Uvr$^+$ cells that had been incubated at 37 C for 20 min.*

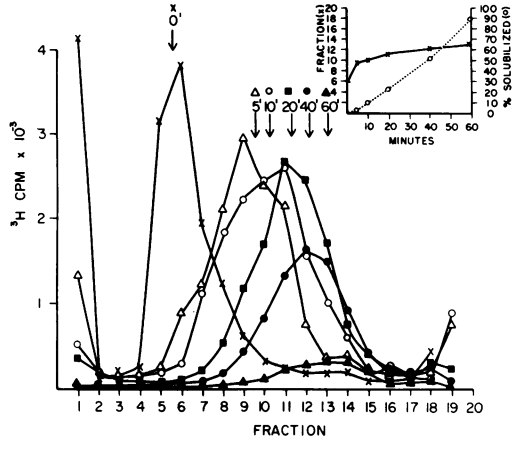

FIG. 3. *Neutral sucrose gradients of DNA from cells treated with phleomycin for increasing lengths of time. Cells were labeled and washed and resuspended as described in Fig. 2. A sample of the untreated cells (0-min curve) was lysed directly as described in Materials and Methods. Phleomycin (20 μg/ml) was added to the remaining cells which were then incubated with aeration at 37 C. At 5, 10, 20, 40, and 60 min after warming, cells in a sample of the culture were lysed. Samples (0.2 ml) of the lysates were layered on 5 to 20% gradients of sucrose and centrifuged at 35,000 rev/min at 18 C, for 2 hr. Sixteen-drop fractions were collected, and the distribution of acid-precipitable radioactivity in the gradients was determined. Inset: The amount of acid-precipitated radioactivity collected from the gradient of the untreated lysate was set at 100%. The differences between this amount and the amounts collected in the gradients of the other lysates were calculated and plotted as percent (of total DNA) solubilized during the indicated period of phleomycin treatment. The positions of the DNA bands in the sucrose gradients of each lysate were also calculated, and the band positions were plotted as a function of time of phleomycin treatment.*

graph showing the positions of the band peaks in the sucrose gradients. Clearly, extensive double-strand breaks were made in the DNA before the occurrence of most of the DNA solubilization.

Presence of single-strand breaks in DNA prior to double-strand scissions. We found that numerous single-strand breaks were present in the DNA of phleomycin-treated cells after only 1 min of treatment, a time when double-strand scissions were not very evident in the neutral sucrose gradients (Fig. 4). The single-strand breaks were detected by denaturing the DNA in lysates of cells treated for 0, 1, and 10 min and then centrifuging the denatured samples through 5 to 20% alkaline sucrose gradients.

Release of membrane-bound DNA. Sucrose gradients such as those shown in Fig. 3 had a rapidly sedimenting band of DNA in the lysates of control cells, whereas there was no such band in the lysates of phleomycin-treated cells. The possibility that this rapidly sedimenting DNA was membrane-bound DNA and that phleomycin treatment caused the dissociation of this complex was investigated by lysing the cells with lysozyme and Brij-58 and then centrifuging the lysate through a 5 to 20% neutral sucrose gradient, over a shelf of 62% sucrose. DNA that is generally considered to be membrane-bound banded on the shelf, ahead of free DNA. Figure 5 shows that 56% of the total DNA in control cells may band on the shelf. Only 3.6% of the DNA banded on the shelf as membrane-associated material after the cells were treated with phleomycin for 10 min. Figure 5 also shows that when CCCP was added to cells 5 min before the addition of phleomycin 25% of the DNA banded on the shelf. That is, the release of DNA from the membrane was partially inhibited. Although the degree of inhibition varied somewhat from experiment to experiment, there appeared to be an energy requirement for the release of DNA from the membrane complex. It is noteworthy that CCCP not only inhibited the phleomycin-triggered release of DNA but also increased to 64% the amount of DNA retained in the membrane fraction in untreated cells. CCCP may inhibit a normally occurring, low-level, spontaneous release of DNA.

Phleomycin-induced nuclease activity in disrupted cells. A number of attempts were made to disrupt cells and to recover a nuclease that acted on phleomycin-treated DNA. We found, however, that solubilization was immediately and completely arrested in cells treated with lysozyme, even when the cells were osmotically protected in 20% sucrose. The simple disruption of the cell wall, therefore, seems sufficient to arrest the effect of phleomycin.

When cells were treated with toluene so that the cell walls remained intact but the cell

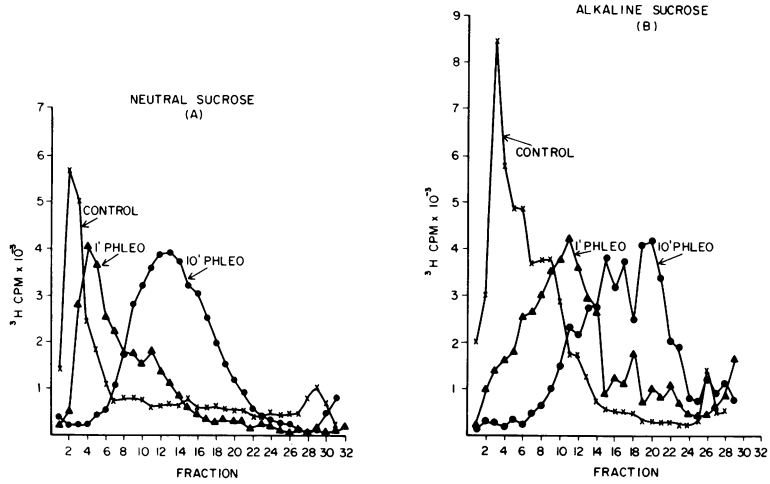

FIG. 4. *Centrifugation of DNA from phleomycin-treated cells, in neutral and in alkaline-sucrose gradients. A, Labeled cells were prepared as described in Fig. 2. Untreated cells and cells treated with phleomycin for 1 min and for 10 min were lysed. A 0.1-ml amount of each lysate was centrifuged through a 5 to 20% gradient of neutral sucrose as before, except that the time of centrifugation was extended to 3 hr to increase the resolution of the different band positions. B, Samples of each lysate were denatured, layered on 10 to 30% gradients of alkaline sucrose, and centrifuged at 35,000 rev/min for 3 hr. Ten-drop fractions were collected, and the distribution of acid-precipitable radioactivity in the gradients was determined.*

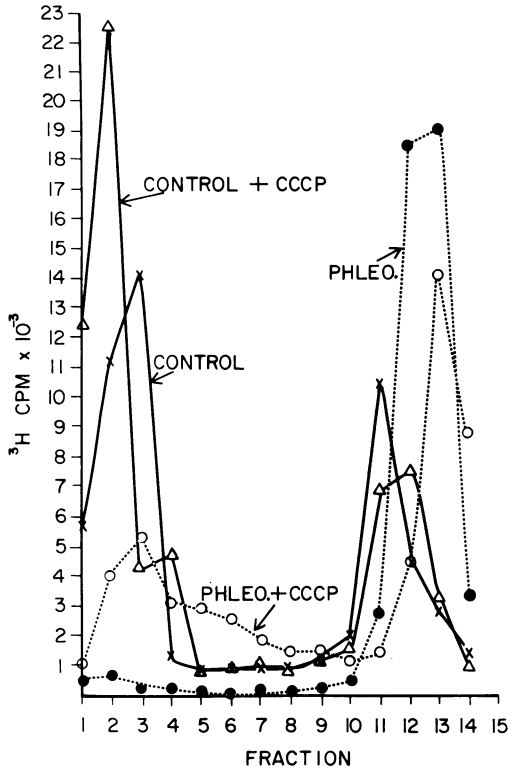

FIG. 5. *Membrane-associated DNA in normal and in phleomycin-treated cells. Labeled cells, prepared as described in Fig. 2, were divided into four tubes and held at 4 C. CCCP at 5 × 10^{-4} M was added to one tube, 20 μg of phleomycin per ml to another, and 5 × 10^{-4} M CCCP plus 20 μg of phleomycin per ml to a third. All four tubes were then incubated with aeration at 37 C for 10 min. The cells were then chilled, washed, and lysed with Brij-58 as described in Materials and Methods. A 0.2-ml amount of each lysate was layered on 4 ml of a 5 to 20% gradient of neutral sucrose over a 1-ml shelf of 62% sucrose. The gradients were centrifuged at 18 C, 25,000 rev/min, for 40 min, and the distribution of acid-precipitable radioactivity in the gradients was determined.*

membrane was disrupted, we found that solubilization continued at only a slightly reduced rate (Fig. 6). The addition of ATP to these toluenized cells had the effect of reducing the amount of measurable solubilization rather than increasing it, as would be expected if the nucleases involved in degradation were ATP-dependent.

Increased DNA synthesis in toluenized, phleomycin-treated cells. The apparent reduction in solubilization caused by ATP in toluenized, phleomycin-treated cells could be explained if ATP increased the reutilization of solubilization products in the synthesis of new DNA. To test this hypothesis, we first established that toluenized cells could carry on DNA synthesis in both the presence and absence of added ATP (Fig. 7). We then toluenized cells that had been uniformly labeled with ^{14}C-thymidine. We added ATP, ^{3}H-TTP, and the other requirements for DNA synthesis, and then incubated the cells with and without phleomycin. Samples of each culture were taken at intervals to determine the amounts of ^{14}C solubilized and of ^{3}H incorporated into acid-insoluble material. The results of such an experiment are shown in Fig. 7. There was a measurable phleomycin-induced solubilization of ^{14}C-labeled DNA at the same time that ^{3}H was being incorporated into newly synthesized DNA. Not only did DNA synthesis occur in

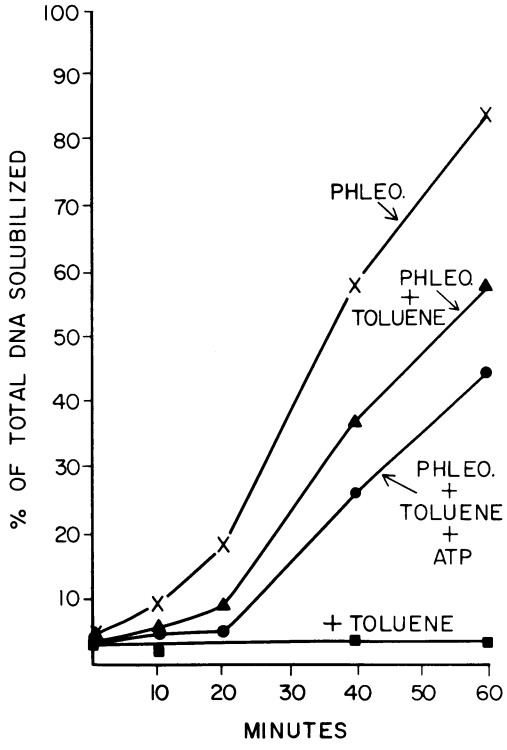

FIG. 6. *Solubilization of DNA in toluenized, phleomycin-treated cells. Labeled cells, prepared as described in Fig. 2, were divided into four tubes and held at 4 C. Phleomycin at 20 μg/ml was added to one tube, 2% toluene to a second tube, 20 μg of phleomycin per ml plus 2% toluene to a third tube, and 20 μg of phleomycin per ml plus 2% toluene plus 1.0 mM ATP to the fourth tube. All tubes were then incubated with aeration at 37 C, and, at the times indicated, samples of each culture were taken and assayed for cold, acid-soluble radioactivity.*

the presence of phleomycin, but the rate of synthesis in the phleomycin-treated cells was noticeably increased over the rate in the control cells.

DISCUSSION

The data show clearly that phleomycin initiates a sequence of events leading to the ultimate solubilization of 80 to 90% of the DNA in the cells. Single-strand breaks, nicks, appear in the DNA duplex almost immediately after exposure to phleomycin. The production of nicks is accompanied by the release of the DNA that, in control cells, is attached to the membrane. It is not clear whether dissociation of the DNA-membrane complex precedes or follows nicking, or whether nicking and the release are the same event. In any case, both production of nicks and the release of membrane-bound DNA are at least partially inhibited by CCCP, indicating that both reactions are energy-dependent.

Some double-strand breaks in the DNA are also detected almost immediately after the start of phleomycin treatment. Nicks and double-strand scissions may be made independently and simultaneously in the DNA. However, after 1 min of phleomycin treatment, when double-strand breaks are just detectable, the amount of nicking is extensive so that the primary event caused by phleomycin could be the nicking of the DNA. Double-strand breaks could occur when two nicks are made close together, on opposite strands of the DNA molecule. In this case the production of double-strand breaks should be, as indeed they are (data not shown), more sensitive to CCCP inhibition than the production of nicks. Double-strand breaks are made until the DNA segments approach a lower size limit of about 1×10^7 molecular weight. Degradation beyond this point seems to be caused by an energy-requiring (that is, CCCP-inhibited) nuclease. Our reason for believing that endonuclease activity stops after the DNA has reached a molecular weight of about 1×10^7 is the absence of smaller DNA segments in cells that were treated with phleomycin for longer than 40 min.

The nature of the nuclease or nucleases responsible for the two or three different degradation steps is not clear. They require cellular energy metabolism to function, but this does not necessarily mean that they are ATP-dependent. They appear to be activated rather than induced by phleomycin since they function in chloramphenicol-inhibited, phleomycin-treated cells. They are not, however, recoverable from cells by routine methods used to isolate DNA nucleases. They are not responsible for the excision of pyrimidine dimers, since excisionless cells degrade their DNA normally upon phleomycin treatment. They may be analogous to the nucleases that act in colicin E2-treated *E. coli* cells, since phleomycin has the same effects on *E. coli* cells as it does on *B. subtilis* cells (Farrell and Reiter, *manuscript in preparation*), and these effects are almost identical to those described for colicin E2 on *E. coli* cells (11).

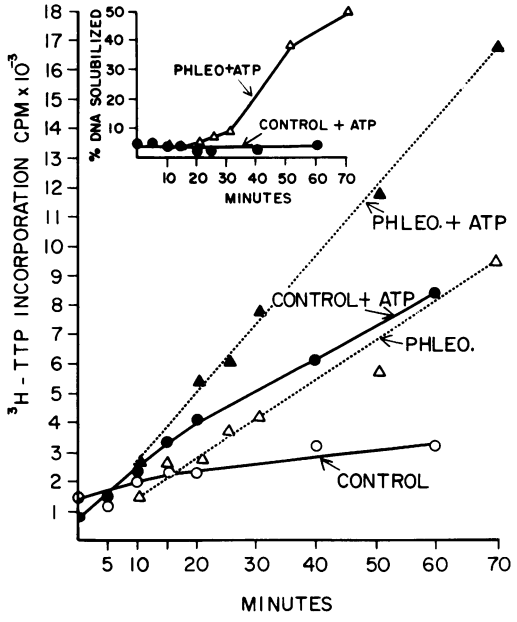

FIG. 7. *DNA synthesis in toluenized, phleomycin-treated cells. Two 0.2-ml samples of toluenized cells were incubated at 37 C with triphosphates plus ^{3}H-TTP but no ATP. After 10 min of incubation, 40 μg of phleomycin per ml was added to one of the cultures. Two 0.2-ml samples of cells were incubated with triphosphates plus ^{3}H-TTP and 1.3 mM ATP. After 10 min of incubation, 40 μg of phleomycin per ml was added to one of these cultures. Each culture was sampled at the indicated times to determine the amounts of ^{3}H-TTP incorporated into cold, acid-insoluble material. Inset: Cells were grown in CHthy$_4$ plus 4 μCi of ^{3}H-thymidine per ml, washed, treated with toluene, and divided into two. One culture of labeled, toluenized cells was incubated with triphosphates plus ATP and unlabeled TTP; the other with the triphosphates, ATP, unlabeled TTP, and 40 μg of phleomycin per ml. The amounts of labeled DNA solubilized during the course of incubation were determined as described previously.*

The arrest of DNA solubilization when lysozyme was added to the cells after the DNA

had separated from the membrane, and after extensive single- and double-strand breaks had been made in the DNA, indicates that both the early endonuclease and the late exonuclease activities are activated by phleomycin. It also indicates that there is a continuous activation of late exonuclease activity. There is a similar effect with colicin E2-induced solubilization of DNA (5). When E2 is removed from the cells by trypsin, the E2-induced solubilization of DNA decreases. E2 has no effect on spheroplasts, presumably because the cell wall provides necessary receptor sites for the colicin (8). It is possible that phleomycin is retained on the cell wall and that it acts continuously through the cell wall on the membrane in much the same way as colicin E2.

This cell membrane target theory is consistent with the observation that synthesis of the replicative form of ϕX174 DNA which occurs on the membrane is inhibited by phleomycin, but that synthesis of the mature, nonmembrane-associated ϕX174 DNA is not sensitive to phleomycin (9). Our experiments with toluenized cells show that they can be stimulated by phleomycin to degrade their DNA. These cells, while degrading their DNA, can, at the same time, synthesize new DNA. Most of the new synthesis is probably repair synthesis occurring at the sites of phleomycin-induced nicks. Since our experiment does not differentiate between repair and replication synthesis, it is possible that increased repair synthesis masks the arrest of replication synthesis. However, other experiments (*manuscript in preparation*) show that replication synthesis on the membranes is altered but not inhibited by phleomycin.

DNA synthesis in nontoluenized cells is inhibited after 4 to 6 min of phleomycin treatment. This is after single-strand breaks and some double-strand breaks have been made in the DNA. This, plus the fact that DNA polymerase activity continues in toluenized cells, leads to the conclusion that the arrest of DNA synthesis in vivo is due to the destruction of template DNA rather than to the stearic interference of template-adsorbed phleomycin with polymerase.

ACKNOWLEDGMENTS

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