JOURNAL OF VIROLOGY, Feb. 1975, p. 363-371

Vol. 15, No. 2
Printed in U.S.A.

Inhibition of Bacteriophage PBS2 Replication in *Bacillus subtilis* by Phleomycin

LEONARD POST AND ALAN R. PRICE*
Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48104

Received for publication 11 October 1974

Phleomycin is an effective inhibitor of the replication of *Bacillus subtilis* bacteriophage PBS2, whose DNA contains uracil instead of thymine. Phleomycin does not affect the induction of the known phage enzymes involved in deoxyribonucleotide metabolism. But phage DNA synthesis is severely inhibited by phleomycin, and late virion protein synthesis is eliminated. These effects appear to result from a phleomycin-induced degradation of the parental phage DNA. Similar inhibitory and degradative effects on DNA are seen in phleomycin-treated, uninfected cells. This system is unaffected by the related antibiotic, bleomycin.

In our study of the synthesis of uracil-containing DNA by phage PBS2 in its thymine-DNA host, *Bacillus subtilis*, we searched for a potent and specific inhibitor of PBS2 DNA synthesis. Nalidixic acid, which inhibits *B. subtilis* DNA synthesis by an unknown mechanism (2), was relatively ineffective against PBS2 (23). Hydroxyphenylazouracil (HPUra), which specifically blocks *B. subtilis* DNA polymerase III (6), did not alter the course of PBS2 infection (24). Hydroxyurea, an inhibitor of *B. subtilis* ribonucleotide reductase (3), blocked PBS2 DNA synthesis only at high concentrations which had nonspecific effects (23). Therefore, we examined a different class of inhibitor, a DNA-binding antibiotic.

Commercially available phleomycin is a mixture of at least 10 different antibiotics excreted by *Streptomyces verticullus* (11). Its components share a complex multiring structure (31), reversibly bind copper ions (30), and have different potencies in inhibiting the growth of various bacterial and animal cells (12). Phleomycin has been shown to bind to several DNA species in studies with thermal denaturation profiles (4), infrared spectra (18), electron microscopy (19), gel filtration (17), and ultraviolet and circular dichroism difference spectra (14). Interaction of one lot of phleomycin with certain RNAs was indicated in studies on the infectivity of single-stranded polio RNA (13) and on the melting of double-stranded reovirus RNA (34). An elaborate model of phleomycin binding to the 2-oxygen of thymine rings in poly (dT) stretches of DNA helixes has been devised (7).

Phleomycin has been reported to be an inhibitor of growth in prokaryotic cells (30), specifically reducing the rate of DNA synthesis in *B. subtilis* (27) and in *Escherichia coli*, either uninfected (5, 32) or early in ϕX174 phage infection (20). In these studies, little effect of phleomycin addition was observed on RNA and protein synthesis (27, 32). Degradation of DNA after phleomycin treatment of uninfected cells was found to be slight (32) or nearly complete (5, 8, 27). DNA as the target of phleomycin action was also supported by the partial resistance to phleomycin of *E. coli* DNA repair (host cell reactivation-negative) mutants (8); mutants selected for phleomycin resistance were often also very sensitive to ultraviolet light (8) or subject to high spontaneous mutation rates (10). Furthermore, drugs like caffeine which bind single-stranded DNA were found to enhance phleomycin-induced DNA degradation (9).

In contrast to the above reports, others (34) have found essentially identical degrees of inhibition of *E. coli* DNA, RNA, protein, and specific enzyme synthesis by phleomycin. These authors (34) also observed inhibition of the in vitro activity of *E. coli* DNA polymerase (see also ref. 4), *E. coli* RNA polymerase, and phage Qβ RNA polymerase by high concentrations of phleomycin. Differences in results among the above authors may well reflect differences in the bacterial strains or the phleomycin lots employed, as Pietsch (16) has demonstrated. To confirm the specificity of phleomycin and study its effects in our system, we have added phleomycin during PBS2 infection of *B. subtilis*. Our results suggest that phleomycin acts specifically to prevent PBS2 DNA replica-

tion by stimulating the degradation of the DNA template.

MATERIALS AND METHODS

Cells and phage. Methods for growing *B. subtilis* SB19 and PBS2 phage in Difco Penassay broth and procedures for making dialyzed infected cell extracts have been described (22, 25).

Enzyme assays. Assays of PBS2 phage-induced DNA polymerase (22), dTMP phosphohydrolase (25), dCTP deaminase (21), and dUTP pyrophosphatase inhibitor (A. R. Price, manuscript in preparation) were performed as before.

DNA analyses. The indole colorimetric assay for DNA in washed, trichloroacetic acid culture precipitates was used (24, 33). In other experiments, [2-^{3}H]adenine (Schwarz/Mann) was added, and samples (50 μliters) were removed at intervals and spotted on Whatman 3 MM paper disks for alkaline hydrolysis and/or washing (35) for scintillation counting in 10 ml of toluene-based fluid (22) to determine incorporation into DNA and/or RNA. Alternatively, the 50-μliter samples were precipitated in tubes with carrier salmon sperm DNA (100 μg) in trichloroacetic acid, processed in alkali, and centrifuged as before (33); after resuspension in 1 ml of 0.1 N NaOH, the radioactive DNA was counted in 5 ml of Triton-toluene scintillation fluid (25).

Antibiotics. Phleomycin (Bristol, lot A9331-909) was stored at −20 C in water at 1 mg/ml. Solutions of rifampin (Schwarz/Mann; 50 mg/ml) and HPUra (Imperial Chemical Industries; 9.3 mg/ml) were freshly prepared in dimethyl sulfoxide.

PBS2 phage with [^{3}H]DNA. To a 2-ml culture of cells infected for 5 min with PBS2 phage at a multiplicity of 5, [^{3}H]adenine was added as above. Two hours after infection, the lysate was clarified by centrifugation for 15 min at 10,000 × *g*; the labeled phage were pelleted at 25,000 × *g* for 1 h and resuspended in 1 ml of adsorption medium (22). This stock contained 4.8×10^9 phage and 7×10^5 counts/min.

Sucrose gradients. Infected cells were lysed and centrifuged in sucrose gradients as described by Reiter et al. (27). Standards included 23*S* Col E$_1$ ^{14}C-labeled DNA, 63*S* T4 phage ^{32}P-labeled DNA, and 86*S* *Salmonella* cryptic plasmid ^{14}C-labeled DNA.

SDS-polyacrylamide gel electrophoresis. Slab gels (100 mm long) were prepared as described by Ames (1) with the following percentages of acrylamide: 5% stacking gel, 7% separating gel, and 15% front-retaining gel. *N,N'*-methylene-bis-acrylamide was used as cross-linker at a constant percentage (2.7%, wt/wt) of the acrylamide. All buffers contained 0.1% sodium dodecyl sulfate (SDS).

PBS2-infected cells (1 ml) were labeled for 10 min at 37 C with ^{14}C-labeled amino acids (New England Nuclear, ^{14}C-labeled algal hydrolysate) at 10 μCi/ml. Cells were diluted with 2 ml of 2% Difco Casamino Acids at 0 C, centrifuged at 7,500 × *g* for 10 min, and suspended for 20 min at 37 C in 24 μliters of Tris-hydrochloride buffer (pH 6.8, 70 mM) containing lysozyme at 1 mg/ml. Lysis and protein dissociation were completed by the addition of 6 μliters of 5% SDS solution containing 50% glycerol and 0.7 M 2-mercaptoethanol, followed by heating for 2.5 min in a boiling water bath. Aliquots (10 μliters) were layered under the SDS buffer on the slab gels for electrophoresis. Other aliquots (5 μliter) were precipitated at 0 C with 1 ml of 10% trichloroacetic acid containing 100 μg of albumin carrier, filtered on Gelman GA-6 membranes, dried, and counted in toluene-based scintillation fluid.

Marker proteins for electrophoresis included bovine serum albumin (mol wt = 68,000), ovalbumin (45,000), chymotrypsinogen (23,000), myoglobin (17,000), and lysozyme (14,300). Bromophenol blue was used to follow the front boundary during electrophoresis (2.5 h at 15 mA at 22 C). Gels were stained with Coomassie blue in 10% acetic acid to detect the standard proteins or were immediately dried unfixed on filter paper and subjected to autoradiography with Kodak NoScreen film for 4 days.

^{14}C-labeled PBS2 virion proteins. To a 4-ml culture of cells infected for 20 min by PBS2 phage, ^{14}C-labeled amino acids were added as above. Two hours after infection, the lysate (6×10^9 phage/ml) was clarified by centrifugation at 0 C for 10 min at 10,000 × *g*. The supernatant fluid was centrifuged at 25.000 × *g* for 1 h to pellet the phage, which were allowed to resuspend overnight in adsorption medium (22). The phage (density = 1.43 g/cm^3) were purified in a CsCl density gradient in adsorption medium; centrifugation was for 40 h at 15 C in a 50 Ti rotor at 40,000 rpm. After dilution with citrate buffer (22), the labeled phage were pelleted at 25,000 × *g* for 2 h at 0 C, resuspended in 50 μliters of the above SDS buffer, and boiled for 2 min. To reduce viscosity, 5 μliters of 10 mM $MgCl_2$ containing pancreatic DNase I (Worthington code D, 10 mg/ml) was incubated with the dissociated phage for 25 min at 37 C; a 10-μliter sample was subjected to SDS-polyacrylamide gel electrophoresis as above.

RESULTS

Phleomycin inhibition of PBS2 phage production. Figure 1 indicates the inhibitory effect on intracellular phage production of increasing concentrations of phleomycin added at the time of infection. Phleomycin at as little as 1 μg/ml detectably reduced PBS2 reproduction, and at 25 μg/ml inhibited it over 99%. This effect was dependent upon both the phleomycin concentration and the time of its addition (Table 1). Inhibition was greatest when the antibiotic was added before or within 5 min after infection. Adding phleomycin at times later than 20 min after infection, when intracellular phage are accumulating (26), had a progressively smaller inhibitory effect. Phage did continue to be assembled in the presence of phleomycin at 10 μg/ml; its addition at 20 min after infection still allowed a fivefold increase in the level of infec-

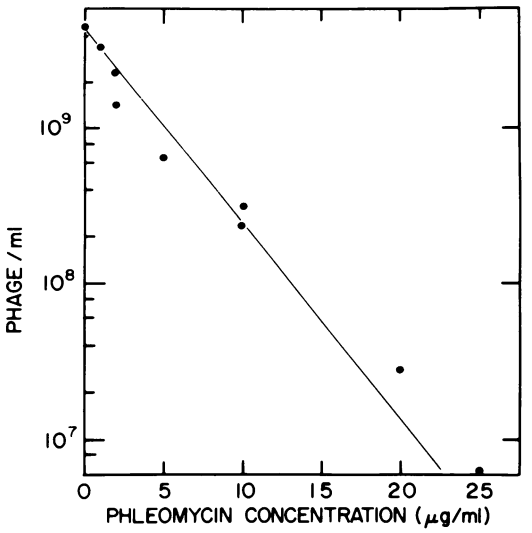

FIG. 1. *Inhibition of PBS2 phage production by phleomycin. B. subtilis cultures grown to A_{660} = 1.2 were infected with a multiplicity of 7.5 PBS2 phage per cell. Phleomycin at the indicated concentration was added at the time of infection. After 150 min, lysis was completed by addition of lysozyme and chloramphenicol (24), and the phage were titered. All cultures lysed prematurely at 15 min by this treatment showed an eclipse of infecting phage to less than 10^7/ml.*

tive particles during the following 40 min (data not shown).

Control experiments demonstrated that phleomycin did not prevent the normal adsorption of PBS2 phage to the cell (see legend to Fig. 1). Furthermore, the titer of PBS2 phage stocks was not reduced by incubation with even 500 μg of phleomycin per ml.

The sensitivity of phage production to phleomycin was relatively independent of the multiplicity of infection. Addition of 2 μg of phleomycin per ml at the time of infection at multiplicities of 2, 4, 6, and 8 PFU per colony-forming unit resulted in 30 to 35% of normal burst size at 2 h. Infection at a multiplicity of 2, 6, or 10 followed at 25 min by phleomycin treatment at 25 μg/ml gave 7, 12, and 16% of normal burst sizes, respectively.

Phleomycin effect on cell lysis. The absorbance of uninfected cells treated with phleomycin at 10 μg/ml (absorbance at 660 nm [A_{660}] was 1.0) continued to increase to A_{660} = 1.7 in 30 min, and then it remained constant for 2 h. Infected cultures normally continue to increase in turbidity until lysis begins after 45 min and is almost complete by 120 min (see Fig. 2). Phleomycin reduced the rate of lysis of infected cells by only 5% at 2 μg/ml and 20% at 10 μg/ml; however, 25 μg of phleomycin per ml added at the time of infection essentially prevented lysis (Fig. 2). Addition of 25 μg of phleomycin per ml at 25 min after infection only slightly reduced the extent of lysis.

Phleomycin and phage enzyme synthesis. To determine whether phage genes were being expressed in the presence of phleomycin, we assayed extracts of infected cells for all the known PBS2-induced proteins involved in deoxyribonucleotide metabolism (Fig. 2). Phleomycin at 25 μg/ml, sufficient to prevent any detectable phage production (less than 0.1%), had no appreciable effect on the time course of induction of the PBS2 DNA polymerase (22), the PBS2 dTMP phosphohydrolase which produces thymidine and inorganic orthophosphate (25), and the PBS2 protein which inhibits the host's dUTP pyrophosphatase from producing dUMP (A. R. Price, manuscript in preparation). These extracts could not be assayed for the PBS2 dCTP deaminase which produces dUTP (21), since that enzyme was found to be inactivated by the axide in the dialysis buffer (see legend to Fig. 2). However, an independent experiment with azide-free extracts prepared 45 min after infection gave dCTP deaminase specific activities of 200 and 220 nmol/h per mg of protein for control and phleomycin-treated (25 μg/ml), added with phage) cultures, respectively.

Even prior addition of phleomycin had little

TABLE 1. *Effect of concentration and time of addition on phleomycin inhibition of PBS2 phage production*[a]

Time of phleomycin addition (min after infection)	Phage yield in the presence of phleomycin at:	
	2 μg/ml	10 μg/ml
No phleomycin	(100%)[b]	(100%)[c]
−20	7	1
−5	21	
0	25	1
5	33	
20	72	11
25		17
30		37
35		54
40	90	71
60	106	105

[a] Cells were grown to A_{660} = 1.0 and infected with PBS2 phage at a multiplicity of 7.5. The burst of progeny phage was determined as in Fig. 1.

[b] Phleomycin was added at 2 μg/ml at the indicated times, and all cells were lysed for titering at 120 min after infection (100% = 7.5×10^9 phage/ml).

[c] Phleomycin was added at 10 μg/ml at the indicated times, and all cells were lysed for titering at 60 min after infection (100% = 1.1×10^{10} phage/ml).

effect on PBS2 enzyme synthesis. Cultures treated with 25 μg of phleomycin per ml at 5 min before infection gave extracts at 20 and 35 min after infection with 90 to 120% of normal PBS2 DNA polymerase and dTMP phosphohydrolase specific activities. Cultures treated at 20 min before infection still gave 60 to 75% of control levels of these enzymes. Thus, phleomycin did not appear to interfere appreciably with the synthesis of PBS2 mRNA and the known proteins believed to be necessary for uracil-DNA synthesis.

Measurement of PBS2 DNA synthesis. Initial experiments with colorimetric DNA analysis suggested that the addition of 25 μg of phleomycin per ml with PBS2 phage prevented the normal rise (23, 24, 26) in DNA level from 13 μg/ml at 20 min to 30 μg/ml by 90 min; instead, a decline in DNA to 8 μg/ml of culture was observed. However, this assay is tedious and insensitive to small increases in DNA synthesis.

Therefore, we tested other methods for detecting DNA synthesis, utilizing the incorporation of [^{3}H]adenine into acid-precipitable material. Figure 3 shows the amount of incorporation in aliquots from uninfected and infected cells as measured on paper disks by the method of Watson and Yamazaki (35). Uninfected cells incorporated [^{3}H]adenine immediately into DNA (4%) and RNA (96%) and continued until

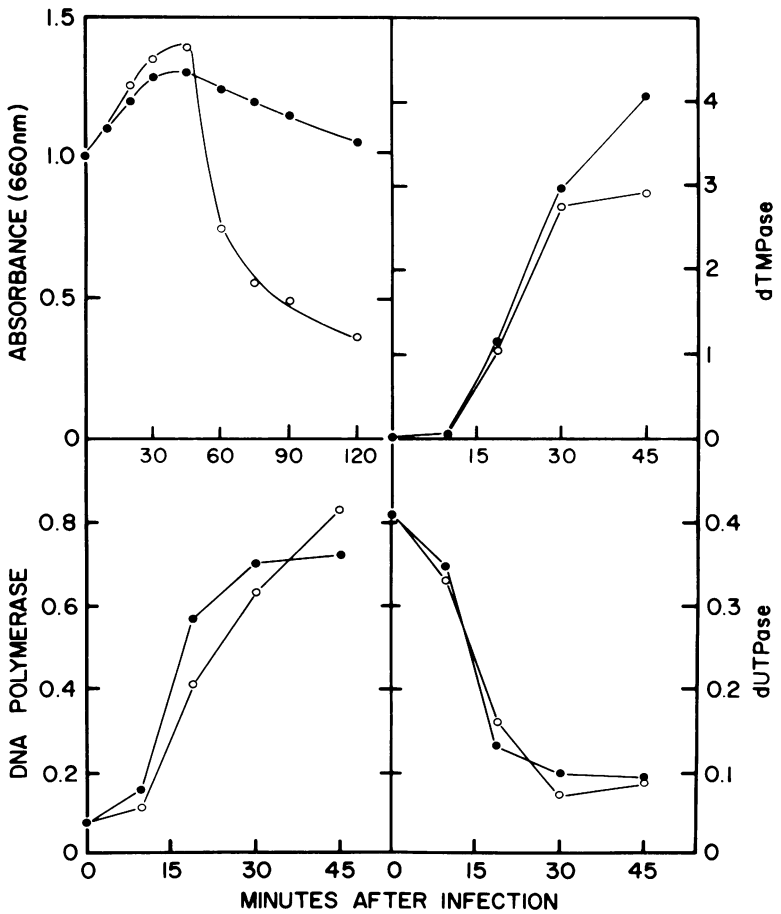

FIG. 2. *Induction of PBS2 enzymes in the presence of phleomycin. Cells were infected at 37 C in the absence* (○) *or presence* (●) *of 25 μg of phleomycin per ml. Culture absorbance readings (1-cm path) and 35-ml aliquots for extract preparation were taken at the indicated times. Extracts were dialyzed in buffer G (25) containing 0.02% sodium azide as preservative; they had protein concentrations of 1.7 to 2.1 mg/ml. They were assayed at 37 C to determine the specific activities of DNA polymerase (nanomoles of [^{3}H]dATP incorporated per minute per milligram of protein), dTMP phosphohydrolase (micromoles of inorganic orthophosphate produced in 15 min per milligram of protein), and dUTP pyrophosphatase (micromoles of [^{3}H]dUMP plus deoxyuridine produced per hour per milligram of protein).*

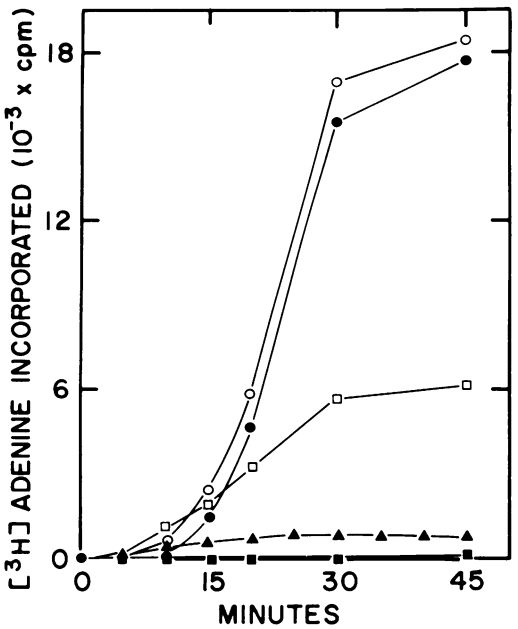

FIG. 3. *Measurement of DNA synthesis by [^{3}H]adenine incorporation in uninfected and PBS2-infected cells. A culture of B. subtilis was grown to A_{660} = 1.0 and divided for infection of one half by PBS2 phage at a multiplicity of 10. Five minutes later, aliquots (1 ml) were added to tubes containing [^{3}H]adenine (50 nmol, 12.5 μCi) in the presence of the following: untreated (□, uninfected; ○, infected); HPUra, 19 μg/ml (■, uninfected; ●; infected); or phleomycin, 10 μg/ml (▲, uninfected). Samples (50-μliter) were taken at the indicated times after ^{3}H addition onto paper disks for processing to determine radioactivity incorporated into DNA. Complete incorporation of the [^{3}H]adenine added gave 150,000 counts/min per 50-μliter aliquot into acid-insoluble DNA plus RNA.*

the [^{3}H]adenine supply was exhausted at 45 min. Incorporation into host DNA (but not into RNA) was completely blocked by the addition of HPUra (Fig. 3), which inhibits the host's DNA polymerase III (6). Phleomycin at 10 μg/ml inhibited the rate of host DNA synthesis by 75% and stopped net incorporation into DNA by 20 min.

In contrast, there is a 10-min delay in the onset of DNA synthesis in PBS2-infected cells, since host DNA synthesis is shut off (see ref. 26 and 28). [^{3}H]adenine was incorporated into DNA (25%) and RNA (75%). Labeling of DNA (and RNA) was unaffected by HPUra (Fig. 3), confirming the resistance of PBS2 infection to HPUra (24). The apparent rate of DNA synthesis in infected cells is about threefold higher than in uninfected cells (Fig. 3), confirmed by previous colorimetric DNA results (23, 24). The apparent rate of RNA synthesis (data not shown) after infection is two- to fourfold lower than before infection (see also ref. 26). But the results show that the incorporayion of [^{3}H]adenine after PBS2 infection is an accurate measure of phage DNA synthesis and could be used to determine the effects of phleomycin.

Phleomycin inhibition of phage DNA synthesis. Phleomycin at 25 μg/ml added at the time of infection reduced the rate of phage DNA synthesis by 90% (Fig. 4). Net DNA synthesis began at the normal time, 15 min after infection, but stopped 20 to 30 min later. These experiments were performed by using acid precipitation of DNA in tubes (Fig. 4; see text). Similar results were obtained by using precipitation on paper disks as in Fig. 3, but the radioactivity precipitable on disks was found to decrease late in infection, due to a failure of DNA packaged into phage to stick to the disks. Addition of phleomycin 10 min after phage DNA synthesis had begun (Fig. 4) allowed continued DNA synthesis and phage assembly (see above) for another 25 min. This is consistent with in vitro assays of the PBS2 DNA polymerase activity in DNA-free extracts of *pol*$^-$ cells (22); a 45-min preincubation and 15-min assay with salmon sperm DNA or PBS2 DNA as template was inhibited less than 10% by the presence of 250 μg of phleomycin per ml.

DNA synthesis in uninfected cells displayed a

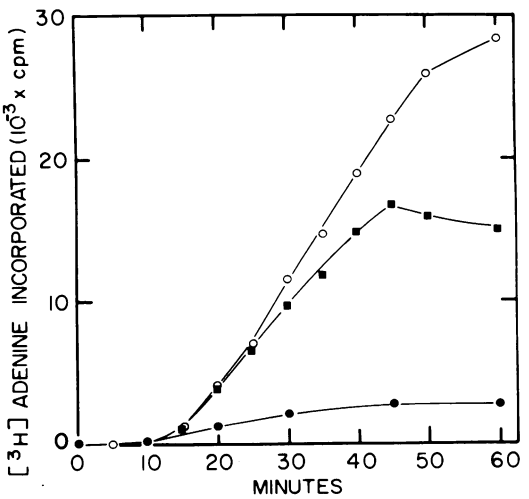

FIG. 4. *Inhibition by phleomycin of PBS2 phage DNA synthesis. Cells were grown, infected, and incubated with [^{3}H]adenine at 5 min after infection as in Fig. 3. Cultures were untreated (○) or treated with phleomycin at a final concentration of 25 μg/ml, added at the time of infection (●) or at 25 min after infection (■). Samples (50 μliter) were taken into tubes at the indicated times after ^{3}H addition and processed.*

sensitivity and pattern of inhibition similar to infected cells, when phleomycin was added with [^{3}H]adenine (Fig. 3) or 20 min after [^{3}H]adenine (data not shown).

PBS2 DNA degradation induced by phleomycin. The effect of phleomycin on the stability of parental phage DNA during infection is shown in Fig. 5. While untreated infected cells maintained all of the prelabeled ^{3}H-labeled phage DNA in an acid-precipitable form, addition of phleomycin at the time of infection resulted in degradation of the DNA. At 25 μg of phleomycin per ml, 20% of the parental DNA became acid-soluble within 80 min; at 250 μg/ml, 40% was solubilized. As a control, DNA extracted from prelabeled phage and incubated with phleomycin (250 μg/ml for 1 h) was shown to remain completely acid insoluble.

Using uninfected cells whose DNA had been prelabeled with [^{3}H]thymidine, we observed a progressive 35% and 70% loss in acid-precipitable radioactivity during a 2-h incubation with 25 and 250 μg of phleomycin per ml, respectively (data not shown). This is confirmed by the 40% decline in colorimetrically assayable DNA (see above) after PBS2 infection in the presence of phleomycin. This level of *B. subtilis* DNA degradation is much more than the slight loss observed by Tanaka et al. (32), but it is less than the almost total degradation seen by Reiter et al. (27). In all cases observed here, DNA degradation appeared to cease within 1 to 2 h after phleomycin treatment, even though the cultures do not lyse (see above).

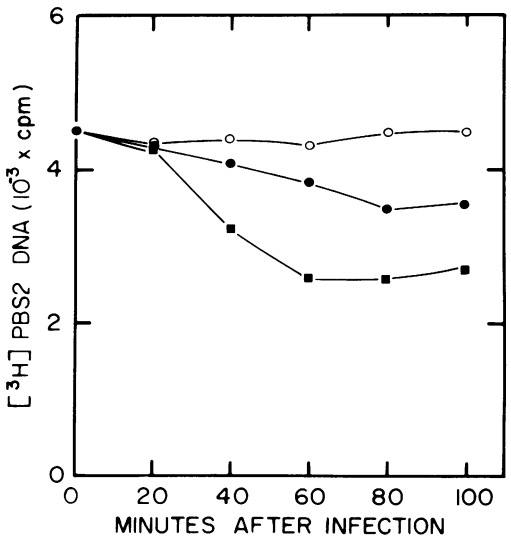

FIG. 5. *Degradation of parental PBS2 DNA during infection in the presence of phleomycin. Cells were grown and infected at a multiplicity of 2.5 with a PBS2 stock (100 μliter) containing phage whose DNA had been prelabeled with [^{3}H]adenine (see text). Cultures were untreated (○) or treated with phleomycin at 25 μg/ml (●) or 250 μg/ml (■) added at the time of infection. Samples (100 μliters) were taken into tubes at the indicated times and processed to determine the radioactive DNA remaining acid insoluble.*

Analysis by sucrose gradient sedimentation of infecting ^{3}H-labeled parental PBS2 DNA in the presence of 25 μg of phleomycin per ml also showed this degradation. The 70*S* DNA present in virions was converted to acid-insoluble fragments in the range of 10 to 30*S* during the first 15 min after infection in phleomycin. Reiter et al. (5, 27) observed a similar endonucleolytic cleavage of uninfected cellular DNA.

Phleomycin inhibition of PBS2 virion protein synthesis. Since phleomycin degraded parental PBS2 DNA during infection (Fig. 5) without affecting the production of the known PBS2 enzymes (Fig. 2), it was of interest to determine whether virion protein synthesis was occurring. Therefore, cells were incubated with ^{14}C-labeled amino acids to synthesize proteins (Table 2) which could be separated by SDS-slab gel electrophoresis and visualized by autoradiography (Fig. 6). These experiments were performed in the presence of rifampin, an antibiotic which specifically blocks the *B. subtilis* RNA polymerase but has no effect on PBS2 replication (26). The extensive labeling of uninfected cell proteins (slot B) was completely eliminated by prior treatment of the cells with rifampin (slot A) (Table 2 and Fig. 6).

However, PBS2-infected cells did incorporate ^{14}C-labeled amino acids in the presence of rifampin (Table 2), and this incorporation was inhibited by phleomycin added at the time of infection. Incorporation at 5 to 15, 15 to 25, and 25 to 35 min after infection was reduced by 50%, 70%, and 87%, respectively, by 25 μg of phleomycin per ml.

^{14}C-labeled amino acids added late during infection in the presence of rifampin do label certain regions of the SDS-slab gel more intensely (slot C; Fig. 6). Several of these bands correspond in size to proteins found in dissociated purified PBS2 phage particles (slot D). Synthesis of these proteins was essentially eliminated during infection in the presence of phleomycin (slot E). Earlier labeling of infected cells gave similar but less intense patterns; no distinct and different bands were visible early (5 to 15 min) in infection in the presence or absence of phleomycin. Therefore, we are unable to distinguish true "early" and "late" classes of PBS2 proteins (see also Fig. 2), as

TABLE 2. *Levels of cellular protein synthesis in the presence of rifampin and phleomycin*

Cells labeled[a]	Rifampin (μg/ml)	Phleomycin (μg/ml)	^{14}C-labeled amino acid incorporation (counts/min)
Uninfected			
Control	0	0	61,600
Pre-treated	100	0	2,300
PBS2-infected			
5 to 15 min	100	0	13,300
5 to 15 min	100	25	10,800
15 to 25 min	100	0	13,400
15 to 25 min	100	25	6,900
25 to 35 min	100	0	23,700
25 to 35 min	100	25	3,000

[a] Uninfected cells were grown to A_{660} = 1.0, and one aliquot was pretreated for 30 min with rifampin before labeling for 10 min with ^{14}C-labeled amino acids; acid-precipitable radioactivity was determined. Another aliquot was treated with rifampin for 5 min before infection with PBS2 phage at a multiplicity of 10 in the absence or presence of phleomycin. Infected cells were labeled for 10 min at the indicated times after infection and processed as above. The radioactivity measured in these 5-μliter aliquots represents a small fraction of the maximum possible incorporation of 2.5×10^6 counts/min of ^{14}C added per aliquot.

found in other viruses like *E. coli* phage T4 and *B. subtilis* phage SPO1 (15).

Although we have not used protein markers over 70,000 mol wt in these SDS gels, we can estimate the sizes of labeled proteins found in PBS2 phage by extrapolation of a plot of log M versus migration distance for our standards. Thus, we estimate the six most intensely labeled bands in dissociated PBS2 phage (slot D) to correspond to peptide mol wt of 290,000, 260,000, 85,000, 68,000, 32,000, and 20,000. The first two are surprisingly large relative to other phages (15), but large proteins have been independently observed in SDS-dissociated PBS2 phage by S. Clark and R. Losick (personal communication). Further work is needed to accurately determine the size, amount, and particle location of these virion proteins.

DISCUSSION

In contrast to other inhibitors tested (23, 24), phleomycin (Bristol lot #909) was both a potent and specific inhibitor of phage PBS2 replication in *B. subtilis*. Phleomycin appears to inhibit PBS2 DNA synthesis (Fig. 4) by inducing the degradation of the DNA template (Fig. 5), allowing normal PBS2 enzyme synthesis (Fig. 2) but preventing virion protein synthesis late in infection (Fig. 6). This specificity for DNA and proposed mechanism of action are consistent with previous results by: (i) Reiter et al. by using the same phleomycin lot on uninfected *B. subtilis* (27) or *E. coli* (5); (ii) Grigg et al. by using a different lot (#648) on *E. coli* (8, 9); and (iii) Tanaka et al. by using phleomycin (source not stated) on *E. coli* (32). However, these authors had not tested the specificity of phleomycin by the sensitive measurement of induction of enzymes (see Fig. 2).

Our results and others (5, 8, 9, 27, 32) are inconsistent with the nonspecific inhibition of all types of macromolecular synthesis in *E. coli* by phleomycin lot #616 (34). The different effects on *E. coli* growth and RNA phage replication between lots #616 and #909 (16, 34) have been attributed to the presence of a nitrile contaminant in lot #616 (16). However, it must be recalled that commercial phleomycin is a mixture of many related antibiotics (11, 12, 31), and critical comparisons should be performed with the same phleomycin lot or, better, with individually purified components when they become available.

In a related matter, phleomycin lots #909 (16; L. Post, unpublished data) and #648 (14) have ultraviolet absorption spectra similar to the reported major phleomycin components D_1, E, G, H, and I (11) and unlike the "bleomycin-type" spectra of minor components C, D_2, and F (11). Since the bleomycin series of antibiotics had been shown to differ from the basic phleomycin ring structure only by the presence of an additional double bond (31), and since bleomycin had been shown to inhibit growth and degrade DNA in *B. subtilis* (29), we tested bleomycin complex and bleomycin A_2 in our system. Although we could confirm the sensitivity of *B. subtilis* 168 to 25 μg of bleomycin per ml (29), we found that DNA synthesis by strain SB19, our host for phage PBS2, was unaffected by the presence of 100 μg of bleomycin per ml. Thus, we were not surprised to find that PBS2 phage production in strain SB19 was insensitive to bleomycin.

The endonuclease(s) presumed to be responsible for the phleomycin-induced degradation of intracellular DNA has not been identified. It is likely that PBS2 DNA is being degraded by the same nuclease system present in uninfected phleomycin-treated *B. subtilis*; Reiter et al. (27) suggest that this system is energy dependent, inactivated on protoplast preparation, and insensitive to simultaneous addition of chloram-

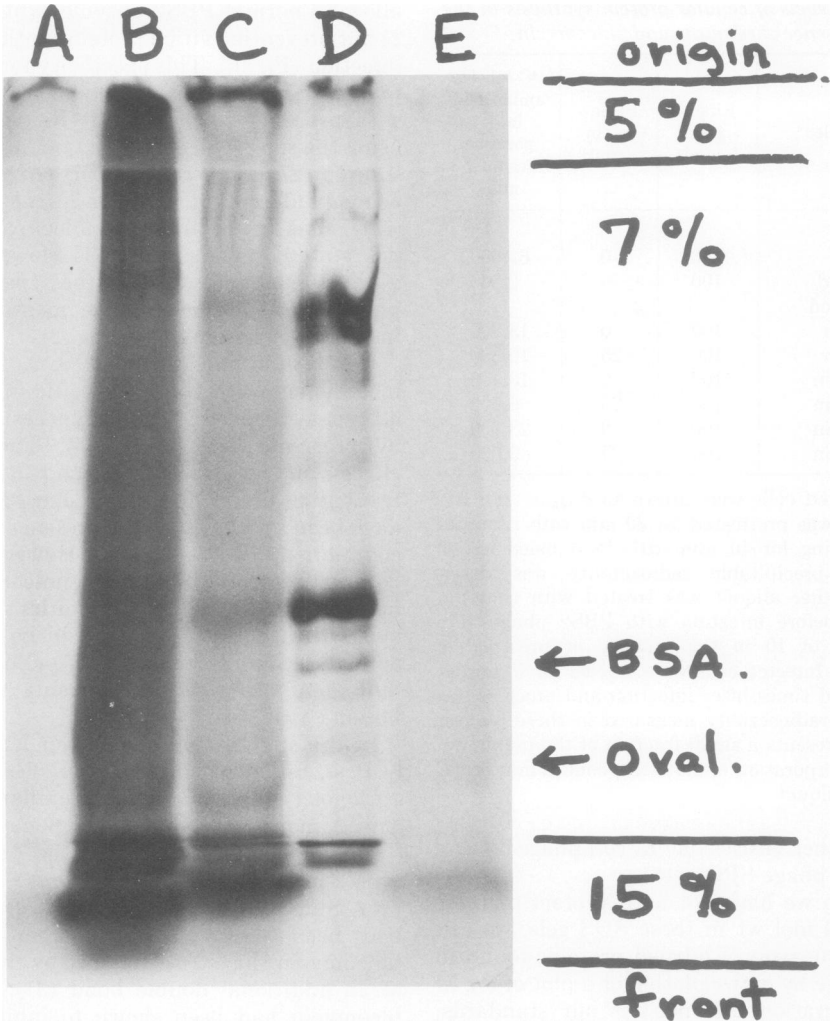

FIG. 6. *SDS-polyacrylamide slab gel electrophoresis of proteins synthesized in uninfected and PBS2-infected cells. Uninfected cells were grown to* A_{660} *= 1.0 and incubated with* ^{14}C*-labeled amino acids for 10 min, either immediately (slot B) or 30 min after treatment with 100 μg of rifampin per ml (slot A). Other cells were treated with the same level of rifampin for 5 min and then infected with PBS2 phage at a multiplicity of 10, either alone (slot C) or in the presence of 25 μg of phleomycin per ml (slot E); infected cells were incubated for 25 min, followed by the addition of* ^{14}C*-labeled amino acids for another 10 min. For comparison,* ^{14}C*-labeled proteins found in CsCl gradient-purified PBS2 phage are shown (slot D). See text for a description of sample, gel, and autoradiogram preparation. The origin of the stacking gel, the percentage of acrylamide in each gel layer, the position of the bovine serum albumin (BSA) and ovalbumin (Oval) standards, and the bromophenol blue dye front are indicated.*

phenicol and rifampin. In *E. coli*, this system appears to be the same one involved in Col E_2-induced DNA degradation, and it is somehow turned off after T4 phage infection (5).

Although the syntheses of PBS2 enzymes, DNA, and progeny phage all begin at approximately the same time (10 to 15 min after infection) and continue until cell lysis (21, 22, 23, 24, 25, 26, 28; Fig. 2, 3, and 4), we find that phleomycin seems to differentiate between certain PBS2-induced proteins. Phleomycin had no effect throughout infection on the level of PBS2 enzymes of deoxyribonucleotide metabolism (Fig. 2). However, during the same period, total protein synthesis is being progressively reduced (Table 2) and virion protein synthesis late in infection is essentially eliminated (Fig. 6). One possible explanation is that mRNAs for PBS2 enzymes are actually transcribed very early in infection but not translated (or the enzymes somehow not activated) until late in infection, when mRNA and protein synthesis

for virions is occurring. Another possibility is that the mRNAs for enzymes are shorter, or that they are transcribed off of smaller operons or phleomycin-insensitive regions of the DNA (Fig. 5), different from the genetic regions coding for virion proteins. Alternatively, mRNA for enzyme synthesis may occur off of a limited number of templates, so destruction of a major fraction of the input DNA may not affect the number of available templates. It will be of interest, by using other drugs and mutants, to determine whether PBS2 phage DNA synthesis is a necessary prerequisite for late virion protein synthesis as in *E. coli* phage T4 (15).

ACKNOWLEDGMENTS

We thank W. F. Minor and W. T. Bradner of Bristol Laboratories for supplying phleomycin and bleomycin. Marker DNAs for sucrose gradients were the gifts of D. Clewell, M. Wovcha, and H. Whitfield. M. Wovcha and R. L. Armstrong gave us assistance with slab gels and sucrose gradients, and P. P. Saunders sent us the bleomycin-sensitive strain. This study was supported by the U.S. Atomic Energy Commission (Report COO-2101-23).

LITERATURE CITED

1. Ames, G. F. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249**:634-644.
2. Bauernfeind, A. 1971. Mode of action of nalidixic acid. Antibiot. Chemother. (Basel) **17**:122-136.
3. Bazill, G. W., and D. Karamata. 1972. Temperature-sensitive mutants of *B. subtilis* defective in deoxyribonucleotide synthesis. Mol. Gen. Genet. **117**:19-29.
4. Falaschi, A., and A. Kornberg. 1964. Phleomycin, an inhibitor of DNA polymerase. Fed. Proc. **23**:940-945.
5. Farrell, L., and H. Reiter. 1973. Phleomycin-stimulated degradation of deoxyribonucleic acid in *Escherichia coli*. Antimicrob. Agents Chemother. **4**:320-326.
6. Gass, K. B., R. L. Low, and N. R. Cozzarelli. 1973. Inhibition of a DNA polymerase from *Bacillus subtilis* by hydroxyphenylazopyrimidines. Proc. Nat. Acad. Sci. U.S.A. **70**:103-107.
7. Gorman, T., and P. Pietsch. 1969. A strategy for crystallographic analysis of phleomycin-DNA complexes: fourier transformations. Physiol. Chem. Phys. **1**:312-316.
8. Grigg, G. W. 1969. Induction of DNA breakdown and death in *Escherichia coli* by phleomycin. Mol. Gen. Genet. **104**:1-11.
9. Grigg, G. W., M. J. Edwards, and D. J. Brown. 1971. Effects of courmarin, thiopurines, and pyronin Y on amplification of phleomycin-induced death and deoxyribonucleic acid breakdown in *Escherichia coli*. J. Bacteriol. **107**:599-609.
10. Iijima, T., and Y. Ikeda. 1970. Mutability of the phleomycin-resistant mutants of *Bacillus subtilis*. I. Isolation of genetically unstable mutants. J. Gen. Appl. Microbiol. **16**:419-427.
11. Ikekawa, T., F. Iwami, H. Hiranaka, and H. Umezawa. 1964. Separation of phleomycin components and their properties. J. Antibiot. **17**:194-199.
12. Ishizaka, M., H. Tokayama, T. Takeuchi, and H. Umezawa. 1966. Studies on antitumor activity, antimicrobial activity, and toxicity of phleomycin. J. Antibiot. **19**:260-271.
13. Koch, G. 1971. Differential effect of phleomycin on the infectivity of poliovirus and poliovirus-induced ribonucleic acids. J. Virol. **8**:28-34.
14. Krueger, W. C., L. M. Pschigoda, and F. Reusser. 1973. Interaction of DNA with zorbamycin, phleomycin, and bleomycin. J. Antibiotics **26**:424-428.
15. Levinthal, C., J. Hosoda, and D. Shub. 1967. The control of protein synthesis after phage infection, p. 71-78. *In* J. S. Coulter and W. Paranchych (ed.), The molecular basis of virology. Academic Press Inc., New York.
16. Pietsch, P. 1969. Phleomycin: biological and chemical variations in different batches. Microbios **4**:387-392.
17. Pietsch, P. 1973. Biopolymers in drug technology: the use of DNA in purifying phleomycin. Biotechnol. Bioeng. **15**:1039-1044.
18. Pietsch, P., and H. Garrett. 1968. Primary site of reaction in the in vitro complex of phleomycin in DNA. Nature (London) **219**:488-489.
19. Pietsch, P., and H. Garrett. 1969. Phleomycin: evidence of in vivo binding to DNA. Cytobios **1A**:7-15.
20. Pitts, J. D., and R. L. Sinsheimer. 1965. Effect of phleomycin upon replication of bacteriophage ϕX174. J. Mol. Biol. **15**:676-680.
21. Price, A. R. 1974. Bacteriophage PBS2-induced deoxycytidine triphosphate deaminase in *Bacillus subtilis*. J. Virol. **14**:1314-1317.
22. Price, A. R., and S. J. Cook. 1972. New deoxyribonucleic acid polymerase induced by *Bacillus subtilis* bacteriophage PBS2. J. Virol. **9**:602-610.
23. Price, A. R., and S. M. Fogt. 1973. Effect of nalidixic acid on PBS2 bacteriophage infection of *Bacillus subtilis*. J. Virol. **12**:405-407.
24. Price, A. R., and S. M. Fogt. 1973. Resistance of bacteriophage PBS2 infection to 6-(*p*-hydroxyphenylazo)-uracil, an inhibitor of *Bacillus subtilis* deoxyribonucleic acid synthesis. J. Virol. **11**:338-340.
25. Price, A. R., and S. M. Fogt. 1973. Deoxythymidylate phosphohydrolase induced by bacteriophage PBS2 during infection of *Bacillus subtilis*. J. Biol. Chem. **248**:1372-1380.
26. Price, A. R., and M. Frabotta. 1972. Resistance of bacteriophage PBS2 infection to rifampicin, an inhibitor of *Bacillus subtilis* RNA synthesis. Biochem. Biophys. Res. Commun. **48**:1578-1585.
27. Reiter, H., M. Milewskiy, and P. Kelley. 1972. Mode of action of phleomycin on *Bacillus subtilis*. J. Bacteriol. **111**:586-592.
28. Rima, B. K., and I. Takahashi. 1973. The synthesis of nucleic acids in *Bacillus subtilis* infected with phage PBS1. Can. J. Biochem. **51**:1219-1224.
29. Saunders, P. P., and G. A. Schultz. 1972. Mechanism of action of bleomycin. I. Growth studies. Biochem. Pharmacol. **21**:1657-1666.
30. Takita, T. 1959. Studies on purification and properties of phleomycin. J. Antiobiot. **12**:285-289.
31. Takita, T., Y. Muraoka, T. Yoshioka, A. Fuji, K. Maeda, and H. Umezawa. 1972. The chemistry of bleomycin. IX. The structures of bleomycin and phleomycin. J. Antibiot. **25**:755-758.
32. Tanaka, N., H. Yamaguchi, and H. Umezawa. 1963. Mechanism of action of phleomycin. I. Selective inhibition of the DNA synthesis in *E. coli* and HeLa cells. J. Antibiot. **16**:86-91.
33. Warner, H. R., and J. E. Barnes. 1966. Deoxyribonucleic acid synthesis in *Escherichia coli* infected with some deoxyribonucleic acid polymeraseless mutants of bacteriophage T4. Virology **28**:100-107.
34. Watanabe, M., and J. T. August. 1968. Replication of RNA bacteriophage R23. II. Inhibition of phage-specific RNA synthesis by phleomycin. J. Mol. Biol. **33**:21-33.
35. Watson, R., and H. Yamazaki. 1973. Alkali hydrolysis of RNA of *Escherichia coli* deposited on filter paper discs. Anal. Biochem. **51**:312-314.