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Effect of Bleomycin on Deoxyribonucleic Acid Synthesis in Toluene-Treated *Escherichia coli* Cells

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The antibiotic bleomycin stimulates deoxyribonucleic acid (DNA) synthesis in toluene-treated *Escherichia coli* cells. The increase in synthesis is linear with bleomycin concentration. Bleomycin-stimulated DNA synthesis is independent of replication and dependent on DNA polymerase I. Replication is spared as the DNA polymerase I-dependent DNA synthesis increases. Bleomycin does not appear to have any effect on purified *E. coli* DNA polymerases I or II. Our results suggest that bleomycin causes nicking of the bacterial chromosome with subsequent DNA synthesis catalyzed by DNA polymerase I.

Bleomycin was first isolated and purified by Umezawa and co-workers in 1966 (30, 31, 33) and identified as a glycopeptide with a molecular weight of ~1,600. Initially, it was investigated as both an antimicrobial and antitumor agent (22, 25), and it has proved to be effective in the control of Hodgkin disease (34). Furthermore, the drug inhibits the growth and reduces the size of rat mammary carcinomas induced by 7,12-dimethylbenz-(α)-anthracene (29). Bleomycin inhibits deoxyribonucleic acid (DNA) synthesis in *Escherichia coli*, Ehrlich ascites cells, and HeLa cells (25). Fujimura et al. (6) and Haidle et al. (10) have shown that bleomycin can induce a prophage from a stable lysogen growing in liquid culture. Subsequent work by others indicates that bleomycin apparently causes single-stranded breaks in DNA, as determined by the sedimentation of bleomycin-treated DNA in neutral or alkaline sucrose density gradients (7, 9, 21, 26). Müller et al. (22) showed that bleomycin inhibits DNA-dependent DNA polymerase from Rauscher murine leukemia virus. Two different laboratories (11, 21) have reported that bleomycin is able to cause the loss of a base, leaving an aldehyde. Thus it is possible that bleomycin exerts its antibiotic effect by alterations in DNA metabolism.

Since the evidence indicated that bleomycin causes DNA strand scissions, we decided to test whether the drug would have any direct effect on DNA synthesis. For these experiments we chose the toluene-treated cell system. This in vitro DNA-synthesizing system exhibits replicative and enzymatic repair synthesis and allows use of cells that are defective in one or more of the enzymes of DNA synthesis (18).

We have examined the effect of bleomycin on DNA synthesis in toluene-treated *E. coli* and find that it stimulates DNA synthesis of a nonreplicative type. Our results indicate that an active DNA polymerase I must be present for this stimulation of synthesis to occur in the toluene-treated cell. In the presence of adenosine 5'-triphosphate (ATP) replication persists. Since bleomycin induces a polymerase I-mediated, nonreplicative type of synthesis, the drug should be a useful probe of DNA repair mechanisms in toluene-treated cells.

MATERIALS AND METHODS

Materials. Bleomycin (lot no. 72L570) was kindly provided by Alexander Lane of Bristol Laboratories. ^{3}H-labeled deoxythymidine 5'-triphosphate (dTTP) was obtained from New England Nuclear Corp. and α-^{32}P-labeled deoxycytidine 5'-triphosphate was purchased from Schwarz/Mann. The nonradioactive deoxyribonucleoside 5'-triphosphates and ATP were obtained from P-L Biochemicals. Bacterial culture media were purchased from Difco. Glass-fiber filters (Whatman GF/C) were obtained from Reeve-Angel Corp.

Media and growth conditions. *E. coli* strains (Table 1) were routinely grown in L broth (composition per liter: 10 g of tryptone [Difco], 5 g of yeast extract, and 5 g of NaCl) supplemented with thymine (10 mg/liter) and vitamin B_1 (0.2 mg/liter). Growth was measured by the increase in turbidity at 600 nm. Cells were collected by centrifugation in midlog phase (1.0 to 1.2 optical density units at 600 nm, corresponding to 6.0 to 7.5 $\times$ 10^8 cells/ml) and suspended in sufficient 0.05 M potassium phosphate buffer (pH 7.4) at 4 C to bring the cell concentration to 2.5 $\times$ 10^{10} cells/ml before toluene treatment.

Toluene treatment of cells. Toluene-treated cells were prepared as previously described (18). The cell suspension was made 1% in toluene and shaken at room temperature for time intervals that produced optimal activity, usually about 10 min. The cells

TABLE 1. *Strains examined for response to bleomycin*

Strain	Characteristics
ER22	*end I*$^-$ (18)[a]
W3110	Wild type (18)
P3478	*polA1* (5)
D110	*polA1, endo*$^-$ (18)
HMS85	*polB100* (4)
HMS83	*polA1, polB100* (4)
CRT 266	$dnaB_{ts}$ (12)
RS5064	$polA_{ex1}$ (15)

[a] Numbers in parentheses are reference numbers for each strain.

were immediately harvested by centrifugation at 10,000 × *g* for 15 min at 4 C, and the pellet surface was washed with 5 ml of buffer at 4 C. The pellet was resuspended at 2.5×10^{10} cells/ml in buffer and stored at −80 C.

Assay of DNA synthesis. The assay method has been previously described (18). The standard reaction mixture (300 μl) contained 67 mM potassium phosphate (pH 7.4), 13 mM $MgCl_2$, and 33 μM deoxyadenosine 5′-triphosphate, deoxycytidine 5′-triphosphate, deoxyguanosine 5′-triphosphate, and ^{3}H-labeled deoxythymidine 5′-triphosphate (specific activity, 10^4 counts/min per nmol). Reactions were begun by the addition of 10^9 toluene-treated cells and incubated at 37 C for time intervals given in the figure legends. The reaction was stopped by the addition of 3 ml of 10% trichloroacetic acid-0.1 M sodium pyrophosphate at 4 C. After standing 10 min in an ice-water bath, the precipitate was collected on a 2.4-cm glass-fiber filter (Whatman GF/C) and washed five times with 3 ml of 10% trichloroacetic acid-0.1 M sodium pyrophosphate followed by 10 ml of 0.01 M hydrochloric acid. The filter was dried, and radioactivity was determined in a scintillation counter with a scintillation fluid that contained Liquifluor (New England Nuclear Corp.) and toluene. Synthesis of DNA was measured as the trichloroacetic acid-precipitable counts per minute incorporated.

ATP-stimulated DNA synthesis in toluene-treated cells is linear for at least 30 min (18). Each preparation of toluene-treated cells was tested for linearity of bleomycin-stimulated DNA synthesis, and incubation times were kept within the linear range. In every case, the extent of the linear range of the bleomycin-stimulated synthesis was less than that of ATP-stimulated synthesis.

Purified enzyme assays. Reaction conditions for DNA polymerase I and DNA polymerase II have been described (19). The reaction mixture (300 μl) contained 67 mM tris(hydroxymethyl)aminomethane (Tris)hydrochloride buffer (pH 8.0), 6.6 mM $MgCl_2$, 1.6 mM dithiothreitol, 33 μM deoxyadenosine 5′-triphosphate, deoxycytidine 5′-triphosphate, deoxyguanosine 5′-triphosphate, and ^{3}H-labeled deoxythymidine 5′-triphosphate (specific activity, 10^4 counts/min per nmol), and 250 μM activated salmon sperm DNA (1). Reactions were begun by addition of enzyme and incubated at 37 C for 30 min. The reaction was stopped, and the extent of DNA synthesis was determined as in the previous section.

Isopycnic centrifugation. Cells were prepared for determination of DNA buoyant density by growth in L broth containing [^{3}H]thymidine (10 μCi/ml). After toluene treatment, newly synthesized DNA was measured by incorporation from α-^{32}P-labeled deoxycytidine 5′-triphosphate (140 counts/min per pmol). The DNA was density-labeled by substitution of bromodeoxyuridine 5′-triphosphate for deoxythymidine 5′-triphosphate. One set of samples was treated as above, and DNA synthesis was determined as the α-^{32}P counts per minute incorporated into acid-precipitable material after 30 min of incubation. Twenty microliters of 0.1 M *N*-ethylmaleimide and 2.0 ml of buffer containing 0.1 M sodium chloride, 10 mM ethylenediaminetetraacetate, and 10 mM Tris-chloride (pH 8) (NET) were added to a duplicate set of samples to stop the reaction. The cells were collected by centrifugation and suspended in 0.2 ml of 10% NET buffer. Twenty microliters of 10% Sarkosyl and 20 μl of Pronase (1 mg/μl, previously incubated at 37 C for 30 min) were then added, and the reaction mixture was incubated at 60 C for 2 h. The samples were mixed vigorously on a Vortex mixer to shear the DNA and were added to 6.5 ml of 65% (wt/wt) cesium chloride in 10% NET buffer. Ten percent NET buffer was added to a final density of 1.75 g/cm^3. Centrifugation was at 37,000 rpm for 48 h at 25 C in a Beckman 50 Ti rotor. Samples were collected on paper strips and sequentially washed with (i) 10% trichloroacetic acid-0.1 M sodium pyrophosphate, (ii) 5% trichloroacetic acid, and (iii) 0.01 M hydrochloric acid. Radioactivity was determined as above.

RESULTS

In vivo effect of bleomycin. We examined the effect of bleomycin on the growth of *E. coli* ER22 in a complex medium, L broth (Fig. 1). Both low (5 μg/ml) and high (100 μg/ml) concentrations of the drug appear to inhibit growth since the absorbance of a liquid culture stops increasing shortly after addition of bleomycin. Logarithmically growing liquid cultures of *E. coli* strains P3478 *(polA1)*, HMS83 *(polA1, polB100)*, HMS85 *(polB100)*, W3110 (wild type), and D110 *(polA1)* were tested with bleomycin, and similar results were obtained. The concentration of the drug required for an effect on growth varied with the strain, but no correlation could be made between the bleomycin sensitivity of a given strain and the known polymerase deficiency. In general, the arrest of growth occurred in progessively shorter times as the concentration of the antibiotic increased. Arrest of cell growth by bleomycin usually began in less than one generation and was complete in less than two generations.

Effect of bleomycin on DNA synthesis. Both replicative and repair DNA synthesis oc-

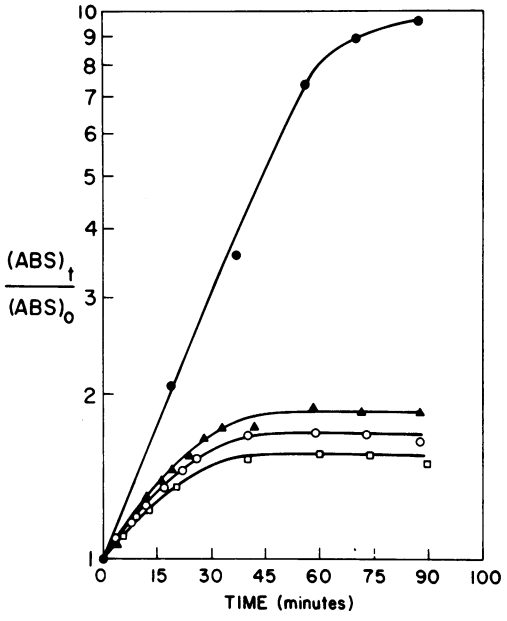

FIG. 1. *Antibiotic effect of bleomycin. Strain ER22 was grown in L broth as described in the text in the presence of bleomycin. Symbols: (●) 0 μg/ml, (▲) 5 μg/ml, (○) 25 μg/ml, and (□) 100 μg/ml of bleomycin. The ordinate is the ratio of the turbidity at 600 nm at time t divided by the turbidity at zero time.*

cur in toluene-treated cells (18). The availability of mutants defective in DNA polymerases as well as mutants conditionally defective in the replication pathway has allowed us to examine the effect of bleomycin on DNA synthesis in the absence of specific enzymatic activities. The toluene-treated cell system requires only Mg^{2+}, K^+, and the four deoxyribonucleoside-5'-triphosphates to exhibit repair synthesis. Replicative synthesis in toluene-treated cells requires ATP as well (18).

When bleomycin was added to a reaction mixture containing wild-type toluene-treated cells, we found a stimulation of DNA synthesis (Fig. 2). Drug concentrations of 2 μg/ml or less usually do not stimulate significant synthesis. Above this concentration, there is a dose-response relationship to a plateau level of synthesis. Bleomycin stimulation of DNA synthesis does not require exogenous ATP. Occurrence of a plateau of synthesis varies from strain to strain, but for a particular strain the concentration at which a plateau first occurs appears to be relatively constant.

Addition of ATP to a reaction mixture containing toluene-treated cells stimulates semiconservative DNA synthesis (18). As the bleomycin concentration is increased, the synthesis stimulated by ATP remains constant. This uniform difference suggested to us that replicative synthesis stimulated by ATP is not inhibited in the presence of the drug. To examine this possibility, we measured the buoyant density of DNA synthesized in the presence of a density label, bromodeoxyuridine 5'-triphosphate. In this experiment parental DNA has the normal density found in *E. coli* DNA (1.710 g/cm^3), and progeny DNA synthesized by a semiconservative mechanism has a higher density. Figure 3A shows the change in density to a hybrid position of the DNA synthesized when ATP is included in the reaction mixture. When the buoyant density of DNA synthesized in the presence of bleomycin, but not ATP, was measured, no difference in density between the parental and progeny DNA was observed (Fig. 3B). The conditions used in this reaction mixture correspond to the lower line in Fig. 2. The absence of a density shift in DNA synthesized under stimulation by bleomycin indicates a process different from replication, enzymatic repair. The DNA synthesized in the presence of both ATP and bleomycin, conditions that correspond to the upper line in Fig. 2, shows two

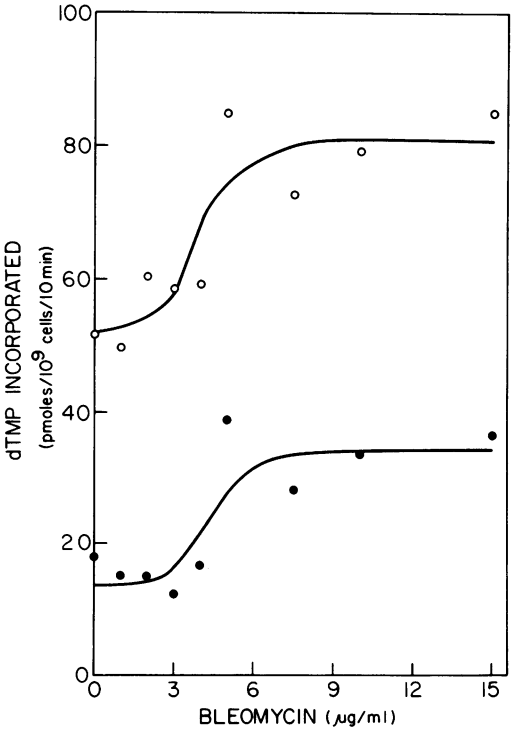

FIG. 2. *Effect of bleomycin on DNA synthesis in wild-type toluene-treated cells. Toluene-treated cells (ER22) were prepared and assayed as described in the text. Symbols: (●) 0 mM ATP and (○) 1.3 mM ATP. Incubation time was 10 min.*

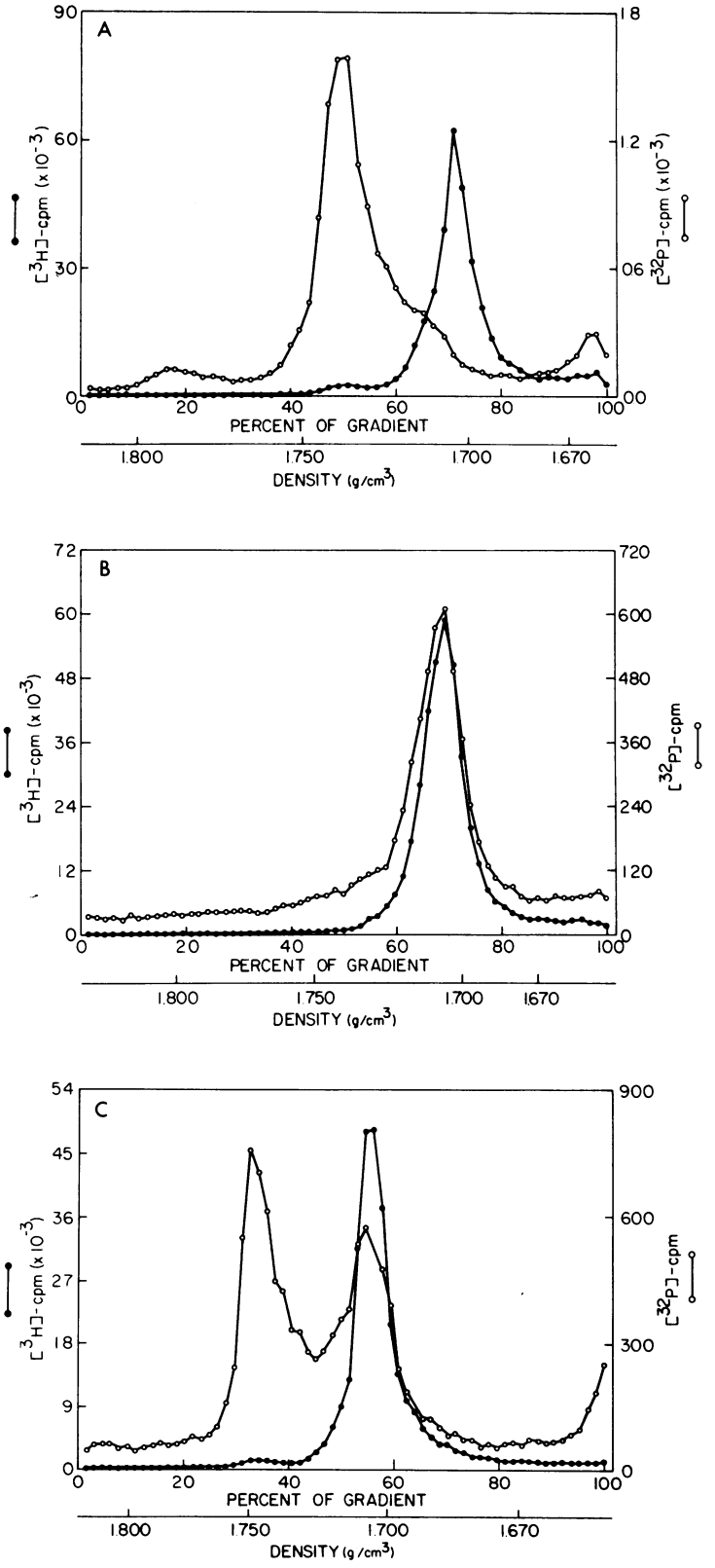

FIG. 3. *Isopycnic analysis of DNA synthesis in toluene-treated E. coli HMS85 (polB100). Incubation and analysis were performed as described in the text. (A) DNA synthesis in ATP, (B) DNA synthesis in bleomycin (30 μg/ml), and (C) DNA synthesis in both ATP and bleomycin. Symbols: (●) ^{3}H and (○) ^{32}P.*

distinct densities, one indicating enzymatic repair synthesis and one indicating replication (Fig. 3C). Therefore, addition of bleomycin to a reaction mixture containing ATP does not inhibit replication, as would be expected from the ability of the drug to introduce scissions into the phosphodiester backbone. Note that the concentration of bleomycin used in the gradient samples was twice the highest used in the samples in Fig. 2.

Effect with dnaB$_{ts}$ cells. Conditional lethal mutants defective in replicative DNA synthesis have been isolated. Strain CRT266 (*dnaB$_{ts}$*) is temperature sensitive for replicative DNA synthesis; at the restrictive temperature, elongation of the nascent polynucleotide chain is inhibited (12). The shutoff of synthesis occurs rapidly after a shift-up in temperature in vivo or in toluene-treated cells (20). Use of this class of mutants allowed us to test whether bleomycin stimulates DNA synthesis in the absence of residual levels of replication. At 32 C the response to bleomycin is qualitatively similar to that found in wild-type cells (cf. Fig. 2): bleomycin stimulates DNA synthesis in a linear dose-response relationship in the presence or absence of ATP (data not shown).

Figure 4 shows the result of adding bleomycin to a reaction mixture containing toluene-treated *dnaB$_{ts}$* cells at 42 C, the restrictive temperature. Bleomycin is able to stimulate DNA synthesis at the restrictive temperature in a dose-dependent manner. Addition of ATP to the reaction mixture at 42 C does not stimulate any additional synthesis (Fig. 4). Since bleomycin is able to stimulate DNA synthesis in the toluene-treated cell system, both in the absence of ATP and in the presence of a conditional block in replication, it appears that bleomycin-stimulated DNA synthesis is not the activation of residual levels of replication.

Effect of bleomycin in strains with deficiencies in DNA polymerases. When bleomycin is added to a reaction mixture containing toluene-treated cells prepared from a DNA polymerase I-deficient strain (Fig. 5), no stimulation of DNA synthesis occurs, in contrast to the experiments with wild-type cells. Thus, polymerase I is required for the bleomycin stimulation of DNA synthesis in the absence of ATP. When bleomycin is added to a reaction mixture containing ATP and toluene-treated cells prepared from a polymerase I-deficient strain, a minimal level of DNA synthesis is established and maintained as the drug concentration is increased.

polA$_{ex1}$ mutants (15) contain DNA polymerase I activity in nearly normal amounts but are conditionally defective in the 5′ → 3′ exonucle-

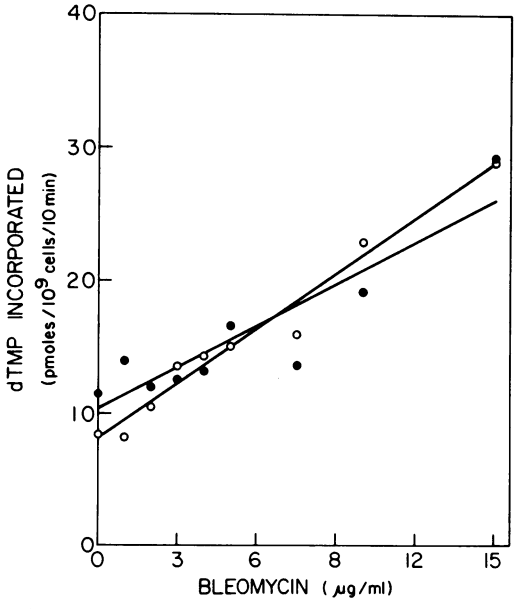

FIG. 4. *Effect of bleomycin on DNA synthesis in dnaB toluene-treated cells at 42 C. Toluene-treated cells of strain CRT 266 were prepared and assayed as described in the text, except that incubations were done at 42 C. Symbols: (●) 0 mM ATP and (○) 1.3 mM ATP. Incubation time was 10 min.*

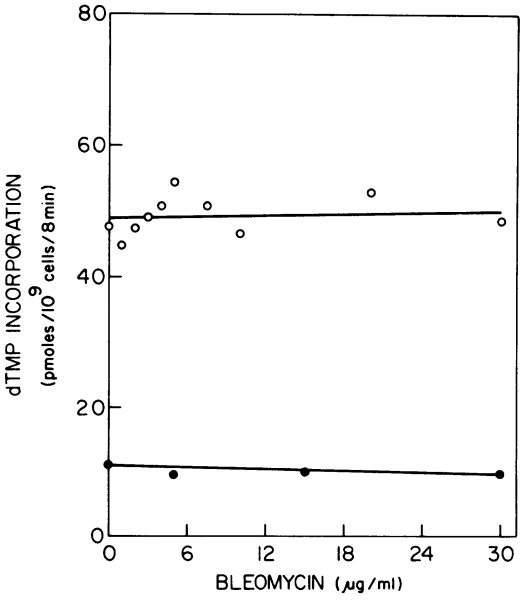

FIG. 5. *Effect of bleomycin on DNA synthesis in polA1 toluene-treated cells. Toluene-treated cells of strain P3478 were prepared and assayed as described in the text. Symbols: (●) 0 mM ATP and (○) 1.3 mM ATP. Incubation time was 8 min.*

ase thought to be involved in nick translation and repair synthesis (14). When toluene-treated cells of this strain are incubated at the permissive temperature (32 C) in a reaction mixture containing bleomycin, there is a normal stimulation of DNA synthesis (Fig. 6). At the restrictive temperature (42 C), qualitatively similar results are obtained (Fig. 6), indicating that the exonuclease is probably not involved in bleomycin stimulation of DNA synthesis. Thus, only the polymerizing function of polymerase I appears to be required for bleomycin to stimulate DNA synthesis.

When cells deficient in DNA polymerase II are toluene-treated and tested for a response to bleomycin in the absence of ATP, DNA synthesis is stimulated in a manner similar to wild-type cells (Fig. 7). Thus DNA polymerase II is not required for ATP-independent bleomycin stimulation of synthesis. When bleomycin-stimulated DNA synthesis is examined in such a strain in the presence of ATP, we again find an initial stimulation followed by a plateau of synthesis (Fig. 7). In contrast, the results of Reiter (23) suggest that polymerase II of *Bacillus subtilis* is stimulated by exposure to phleomycin before toluene treatment.

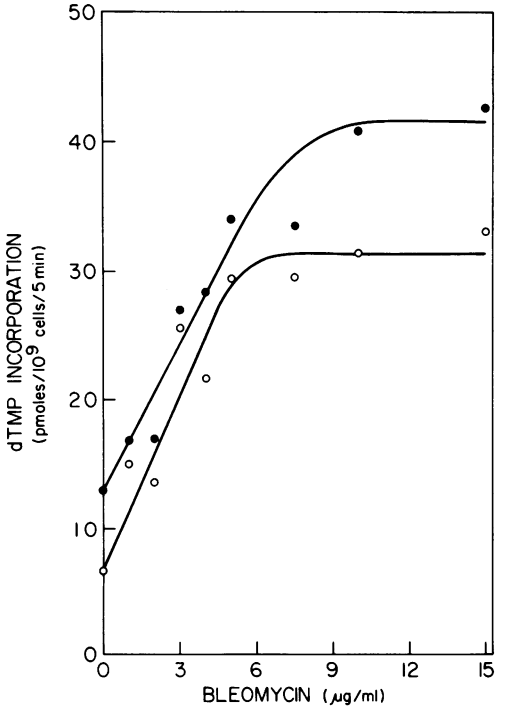

FIG. 6. *Effect of bleomycin on DNA synthesis in polA$_{ex1}$ toluene-treated cells at 32 and 42 C. Toluene-treated cells of strain 5064, were prepared and assayed as described in the text, except that incubations were done at 32 and 42 C for 5 min. Symbols: (●) incubated at 32 C and (○) incubated at 42 C.*

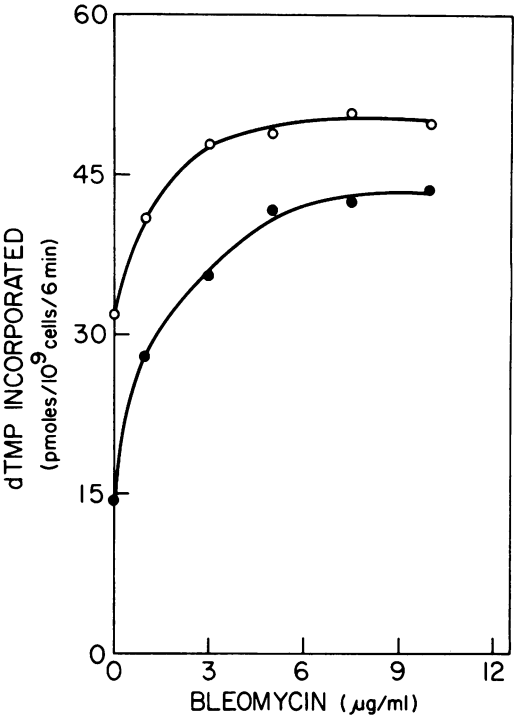

FIG. 7. *Effect of bleomycin on DNA synthesis in polB100 toluene-treated cells. Toluene-treated cells of strain HMS85 were prepared and assayed as described in the text. Symbols: (●) 0 mM ATP and (○) 1.3 mM ATP. Incubation time was 6 min.*

Effect of bleomycin on DNA synthesis by purified DNA polymerase I and II. An active DNA polymerase I appears to be required for bleomycin to induce additional DNA synthesis in toluene-treated cells. Since this suggests the possibility of a direct effect on the enzyme, we examined the effect of bleomycin on the purified DNA synthesizing enzymes, DNA polymerase I and II. Bleomycin has no effect on the amount of DNA synthesis that occurs with either enzyme when activated salmon sperm DNA is used as substrate (Table 2). Attempts to convert unactivated salmon DNA to a good template by incubation with bleomycin were unsuccessful and gave no increase in activity.

DISCUSSION

When bleomycin is added to a growing culture, there is a decrease in the rate and extent of the growth of the cells (Fig. 1), the decrease becoming greater as larger amounts of bleomycin are added. Umezawa et al. (32) have suggested that the ability of bleomycin to cause

strand scissions may be associated with its antibiotic effect. The speed with which the arrest of growth occurs suggests that a second round of replication is prevented.

This system appears to offer a good basis for studying the effects of bleomycin on DNA metabolism, even if such effects are not the primary cause of lethality. The response of DNA polymerase I to bleomycin activity may or may not represent a significant in vivo repair response. Our results reported here do indicate that a specific response can be elicited in vitro, however. It seems unlikely that the response reported here is of importance in the antibiotic effect because *polA*$^-$ mutants are no more sensitive in vivo than *polA*$^+$ strains.

Since the evidence suggests that bleomycin causes DNA strand scissions, our conclusion that the drug stimulates repair synthesis seems reasonable. In one scheme, the drug would generate a break in the phosphodiester backbone and DNA polymerase I would bind at the nick. As the concentration of bleomycin in the reaction mixture is increased, more nicks would be formed, thus leading to an increase in DNA synthesis. Eventually, a bleomycin concentration would be reached that would saturate the nick-translating system and no increase in synthesis would occur at higher drug concentrations. Given the ability of bleomycin to nick double-stranded DNA, the drug should inhibit replication, but the results in Fig. 3 indicate it does not. We interpret our results presented in Fig. 2 as bleomycin-stimulated repair activity superimposed on a background of replication induced by ATP. This interpretation is supported by the results of the isopycnic centrifugation experiment.

We interpret the results of experiments with toluene-treated *dnaB* cells to indicate that bleomycin does not stimulate DNA synthesis, which is thermolabile in the mutant at the restrictive temperature (43 C). Hence, the DNA polymerase I response stimulated by bleomycin is independent of replication since it occurs at both 32 and 42 C.

When toluene-treated *polA*$^-$ cells are incubated with bleomycin, there is no stimulation of DNA synthesis (Fig. 5). This result indicates that an active DNA polymerase I is required for bleomycin to stimulate DNA synthesis in the absence of ATP. However, in the absence of ATP, bleomycin does not stimulate synthesis dependent on either DNA polymerase II or III. Cells conditionally defective in the 5′ → 3′ exonuclease activity of DNA polymerase I exhibit bleomycin stimulation of DNA synthesis at both the permissive and restrictive temperatures. Since bleomycin appears to produce nicking of the DNA, the nucleotide incorporation we observed could easily be explained by nick translation. The lack of an exonuclease requirement would seem to argue against this possibility. It seems more likely that the incorporation results from a specific repair response.

When bleomycin is added to a reaction mixture with *polA*$^-$ cells, increasing bleomycin concentration has no effect on the amount of DNA synthesis (Fig. 5). The bleomycin-resistant, ATP-dependent DNA synthesis seen best in *polA*$^-$ strains could be an ATP-stimulated repair functioning at its maximum extent or replication that is resistant to the inhibitory effects of bleomycin. The apparent DNA strand-breaking ability of bleomycin suggests that replication should be sensitive to bleomycin. However, the results of the density gradient experiment (Fig. 3) argue that replication is preserved unaltered by the presence of bleomycin. This result again suggests that a rapid repair of the DNA is occurring. Replication, as defined by isopycnic analysis, proceeds at the same rate in the presence and absence of bleomycin. This was determined by density analysis at various times during incubation.

TABLE 2. *Effect of bleomycin on activity of DNA polymerases I and II*

Bleomycin concn (μg/ml)	Extent of DNA synthesis (pmol)[a]	
	DNA polymerase I	DNA polymerase II
0	657	254
6	675	262
30		268
80	675	

[a] DNA polymerase I was prepared by the method of Jovin et al. (13), and 1.4 U of the enzyme was added to the reaction mixture. DNA polymerase II was prepared by the method of Moses and Richardson (19), and 4 U of the enzyme was used in each assay. The assays were as listed in the text.

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