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MECHANISM OF ACTION OF BLEOMYCIN—I
BACTERIAL GROWTH STUDIES*

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Abstract—Experiments have been carried out demonstrating the inhibition of *Bacillus subtilis* with bleomycin. The antibiotic was highly inhibitory to non-proliferating cells as well as growing cells. A recombination-deficient (rec$^-$) strain of *B. subtilis* behaved differently in the presence of bleomycin than did the respective wild type organism. The wild type cells were able to recover from lower levels of bleomycin while the rec$^-$ strain was not. A bleomycin-resistant strain of *B. subtilis* was isolated and found to be cross-resistant to mitomycin C but not to daunomycin.

BLEOMYCIN is a complex antibiotic which contains 13 separable but apparently similar glycopeptides and has been shown by Japanese workers[1] to have significant antitumor activity. The primary component of clinical grade bleomycin, bleomycin A_2, has been shown after hydrolysis to be composed of several unusual amino acids and sugars.[2–5]

Suzuki *et al.*[6] have investigated the effects of bleomycin on *Escherichia coli* and various lines of tumor cells. The antibiotic appeared to inhibit the synthesis of DNA as measured by [^{3}H]thymidine incorporation and, in some systems such as Ehrlich ascites cells, [^{14}C]leucine incorporation was affected as well. Bleomycin appeared to inhibit cell division at lower concentrations than were required for an effect on DNA synthesis. This suggests that interference with DNA synthesis may not be the primary mechanism of action of bleomycin.

Evidence has been presented demonstrating an interaction *in vitro* between bleomycin A_2 and DNA.[7] This component of the antibiotic was shown to decrease the thermal transition midpoint of DNA[7] and also to give rise to single strand scissions.[8] Experiments carried out with growing bacterial and mammalian cells suggested that DNA strand scission could also occur *in vivo*.[8] The mechanism underlying these observations as well as their significance is unknown.

In this report we present a series of experiments which were designed to provide evidence for the acceptance or rejection of the hypothesis that the primary mode of action of bleomycin is the modification of DNA. In view of the unavailibility of purified bleomycin A_2 at the outset of this investigation a study was initiated of the effects of clinical grade bleomycin on bacteria. It was felt that information thus obtained would relate more closely to the clinical use of the drug and serve as a suitable prerequisite to mammalian studies.

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MATERIALS AND METHODS

Bacillus subtilis 168 was grown in a minimal medium previously described.[9] Growth was carried out in 250-ml nephelometer flasks (19 × 130 mm sidearms) with incubation at 37° in a New Brunswick (G-77) water bath shaker. Absorbancy measurements were made on a Bausch & Lomb Spectronic 20 colorimeter at 420 nm.

For short-term growth studies, as previously descriebd,[10] cells were grown to an absorbancy of approximately 0·35 and divided into portions of 10–15 ml. The desired additions (drug, etc.) were made and incubation was continued for 2–3 hr. Growth was monitored by absorbancy readings at appropriate time intervals. Viable cell counts were determined as previously described.[9] The methods used in the colorimetric determinations of the levels of DNA, RNA and protein in growing cells have been described elsewhere.[9]

The isolation of a bleomycin-resistant strain of 168, 168 Bleo^R, was accomplished by increasing stepwise the concentration of the drug in minimal medium. An aliquot of the culture was plated onto minimal agar and a single colony was picked and checked for bleomycin resistance.

A recombination-deficient strain of *B. subtilis*, GSY 1025 (trp C2, met B4, rec A1), and the corresponding wild type strain, GSY 1026 (trp C2, met B4) were provided by Dr. J. A. Hoch. Clinical grade bleomycin hydrochloride was obtained from Bristol Laboratories. Purified bleomycin A_2 was a generous gift of S. Itoh, Nippon Kayaku Co., Ltd.

RESULTS AND DISCUSSION

Growth of *B. subtilis* 168 was markedly inhibited by bleomycin as shown in Fig. 1. Viability of the cells, as measured by their ability to form colonies on a complete medium, decreased before an inhibitory effect was apparent in the absorbance measurements. Colorimetric analysis of the levels of DNA, RNA and protein in the cells at various times during incubation with the drug (Fig. 2) showed a consistent correlation with the growth curves, with the possible exception of DNA. At 25 μg/ml bleomycin, the level of DNA did not drop as much as those of RNA and protein. Since analyses were carried out on washed cells, there was no interference by the products of those cells which had lysed in the medium. The same phenomenon was observed in the incorporation of labeled thymidine and uridine into acid-insoluble material. Although experiments of this type can be misleading, it appears that RNA synthesis *in vivo* may be impaired to a greater degree, or sooner than DNA replication. Inhibition of protein synthesis could then result from the effect on RNA synthesis.

One approach to investigating the mechanism of drug action is a study of drug-resistant bacteria. A bleomycin-resistant strain of *B. subtilis* 168, 168 Bleo^R, was isolated as described in Materials and Methods. This strain was resistant to bleomycin at concentrations as high as 50 μg/ml, although there was slight inhibition at this level (Fig. 3A). The wild type, strain 168, is sensitive to levels much lower than this. Graph B of Fig. 3 indicates that 168 Bleo^R is cross-resistant to mitomycin C, which has been shown[14] to alter DNA through the formation of cross-links. Daunomycin, on the other hand, has been shown to interact with DNA by intercalation (non-covalent interaction).[15] The bleomycin-resistant strain displayed slight, if any, resistance to daunomycin (Graph C). The resistant properties of this strain were found to be stable in the absence of drug. These observations could reflect similarity in the actions

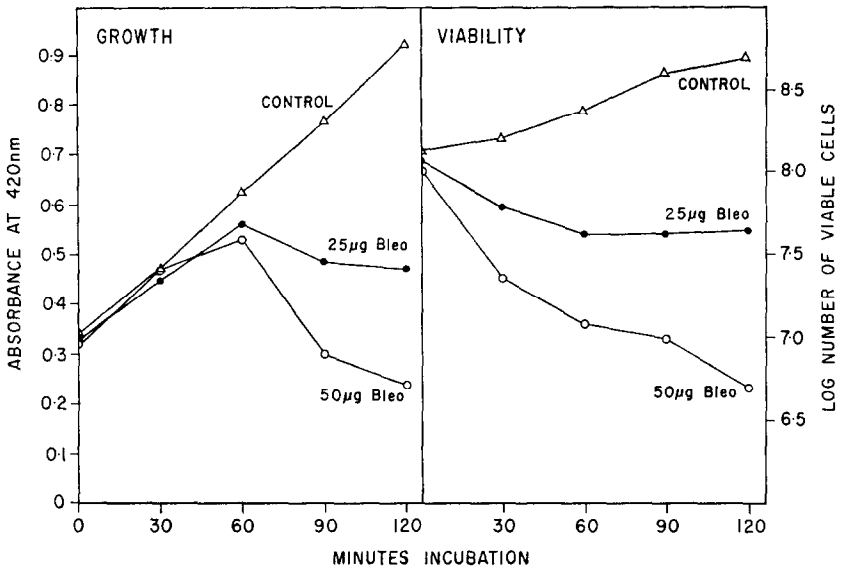

FIG. 1. Effect of bleomycin on growth and viability of *B. subtilis*. Concentrations refer to micrograms of bleomycin per milliliter. Incubation was carried out at 37° with shaking. Growth was measured by absorbance at 420 nm and viability was determined by plating appropriate dilutions on a complete medium as described previously.[9]

of bleomycin and mitomycin C. It is also possible that the resistance of 168 $Bleo^R$ results from altered permeability. However, this seems unlikely in view of the marked structural difference between bleomycin and mitomycin C. The same reasoning would apply to the possibility of drug degradation as a resistance mechanism. It is also of interest to note the amount of time required before inhibitory effects on the cultures were apparent. In the cases of bleomycin and mitomycin C at least 30 min was required before a decrease in absorbance occurred. Daunomycin, on the other hand, appeared to have an immediate effect. This difference could reflect either entrance of the drugs into the cells or a time requirement for the drug to act.

If bleomycin inhibits the cells by means of a chemical mechanism (DNA strand scission) as suggested by Suzuki *et al.*[8] it should be lethal to resting or non-proliferating cells. By plating such cells, after washing them free of drug, on a complete medium one can determine the numbers of surviving cells. Figure 4 shows that cells deprived of a required amino acid, thus in a non-proliferating state, are highly sensitive to bleomycin but are insensitive to 5-fluorouracil (FU) under the same conditions. This observation would be consistent with a mechanism of action which involved a chemical alteration of any of the essential cellular macromolecules which does not occur with FU. An argument which can be advanced in opposition to this experiment is that there may be enough residual bleomycin inside the cells after washing to be lethal when growth is resumed on the plates. The only direct way to establish this question would be a measurement of the amount of drug in the cells. Such an experiment has not been executed due to the lack of a sensitive and accurate assay for bleomycin.

If DNA strand scission is the lethal action of bleomycin, it is conceivable that one could detect a difference in sensitivity to bleomycin between a wild type cell and one

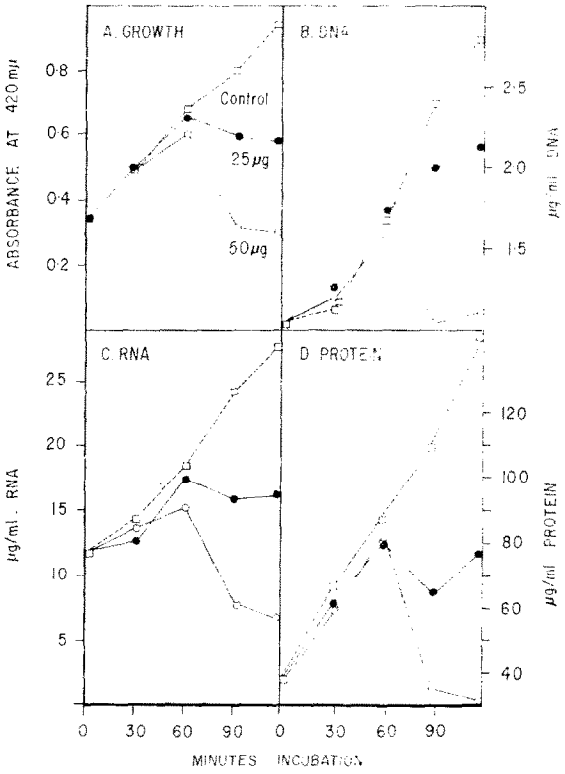

FIG. 2. Effect of bleomycin on levels of DNA (B), RNA (C) and protein (D) in growing *B. subtilis*. Minimal medium containing the appropriate additions was inoculated as described in Materials and Methods and dispensed into nephelometer flasks (50 ml/flask). Incubation was carried out as described in the text. At the designated intervals, one flask from each set was removed and the contents were centrifuged. The cells were resuspended in 10 ml of cold deionized water, placed in ice and concentrated trichloroacetic acid (TCA) was added to 5%. The samples were allowed to stand overnight at 4°, and the precipitate was harvested by centrifugation and washed twice with cold 5% TCA. The pellet was resuspended in 4 ml of 5% TCA, heated at 98° for 30 min, cooled in an ice bath for 30 min and centrifuged. The pellets were dissolved in 0·2 N NaOH by heating in boiling water for 5–10 min and were assayed for protein by the method of Lowry *et al.*[11] The supernatant fractions were assayed for RNA by a modified orcinol reaction[12] with ribose as the standard. DNA was determined by the method of Burton,[13] using calf thymus DNA as the standard. Amounts of protein, RNA and DNA are given in micrograms per milliliter of medium. Numbers below the curves refer to micrograms of bleomycin per milliliter of growth medium.

that is recombination-deficient. A recombination-deficient (rec$^-$) bacterium is unable to carry out the process of genetic recombination which presumably involves an enzyme-catalyzed breakage and reunion of DNA strands. The strain employed here, 1025, has been shown to be radiation sensitive and deficient in transformation.* Thus it is possible that a rec$^-$ strain would be more sensitive to bleomycin, particularly low levels, by virtue of its presumed inability to repair strand breaks. Figure 5 compares with the effect of several concentrations of bleomycin on two strains of *B. subtilis*, 1025 (rec$^-$) and 1026 (rec$^+$). The rec$^-$ strain appeared at first to be slightly

* J. A. Hoch, personal communication.

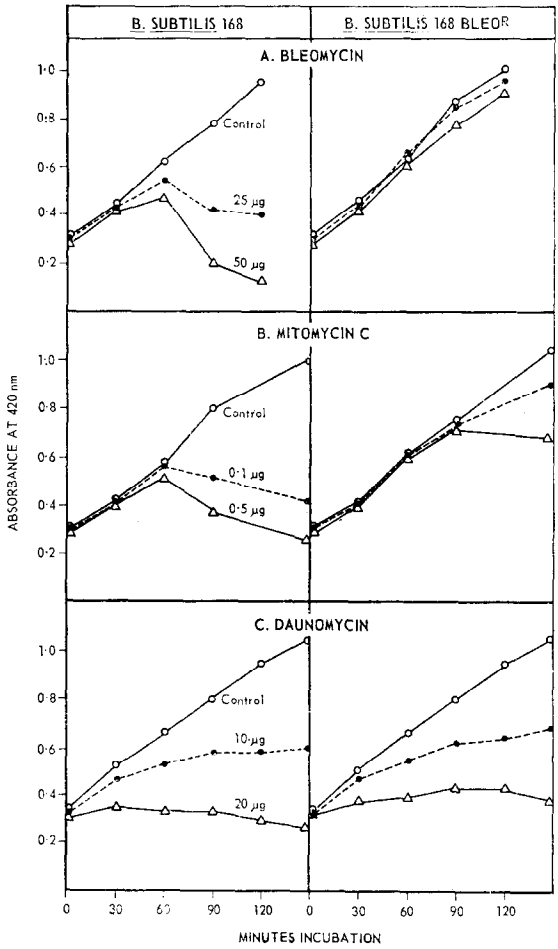

FIG. 3. Effects of bleomycin (A), mitomycin C (B) and daunomycin (C) on *B. subtilis* 168 and 168 Bleo^R. Growth curves were carried out as described for Fig. 1. The concentrations refer to micrograms per milliliter of the indicated drug. Symbols for graph A: (○) control; (●) 25 μg/ml bleomycin; (△) 50 μg/ml bleomycin. Symbols for graph B: (○) control; (●) 0·1 μg/ml mitomycin C; (△) 0·5 μg/ml mitomycin C. Symbols for graph C: (○) control; (●) 10 μg/ml daunomycin; (△) 20 μg/ml daunomycin.

more resistant to bleomycin in that cell lysis started somewhat later than in the rec$^+$. Later in the growth curves, however, it was apparent that at the lower drug concentrations, the rec$^+$ strain began to recover from the effect of bleomycin but the rec$^-$ did not, even at the lower levels of drug. This effect was reproduced several times. In Fig. 6 the numbers of viable cells in the cultures showed a similar phenomenon. This could be due to the inability of the rec$^-$ strain to repair strand breaks. These data, although not conclusive, suggest that the lethality of bleomycin could indeed result from DNA strand scission. If DNA were not involved, there should have been no difference in response to bleomycin between the two strains.

Suzuki *et al.*[8] have reported experiments in which growing *E. coli* cells, previously labeled with [^{14}C]thymine, were incubated with bleomycin. The DNA was extracted

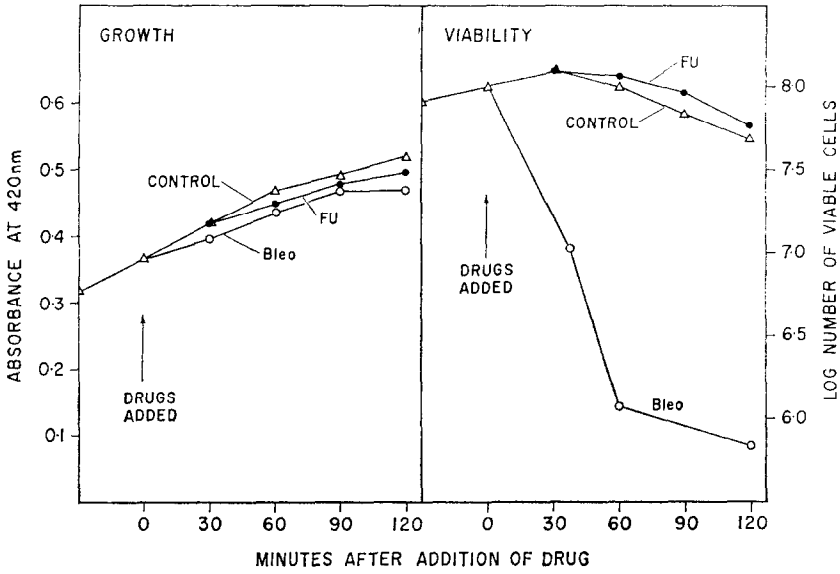

FIG. 4. Effect of bleomycin on non-proliferating *B. subtilis*. A culture of strain 168 was grown to early log phase, harvested on a millipore filter, washed and resuspended in the same medium lacking tryptophan which is required by strain 168. After 30 min of incubation to utilize any endogenous tryptophan, the desired additions were made and incubation continued. Samples were withdrawn and treated as described to obtain the viable counts. Treatment of the cultures in this way has been shown to result in cell populations whose levels of DNA, RNA and protein remain essentially constant.[16] The levels of bleomycin and fluorouracil (FU) were 50 μg/ml and 10 μg/ml respectively.

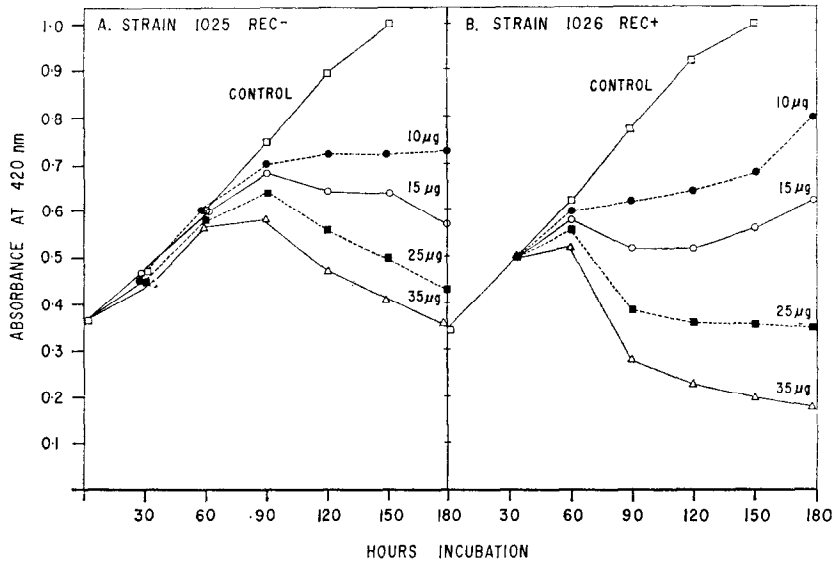

FIG. 5. Effect of bleomycin on growth of a recombination-deficient strain of *B. subtilis*. Strains GSY 1025 (recombination-deficient) and GSY 1026 (recombination-normal) were grown in the presence of varying levels of bleomycin, as described for Fig. 1. Concentrations refer to micrograms per milliliter of bleomycin.

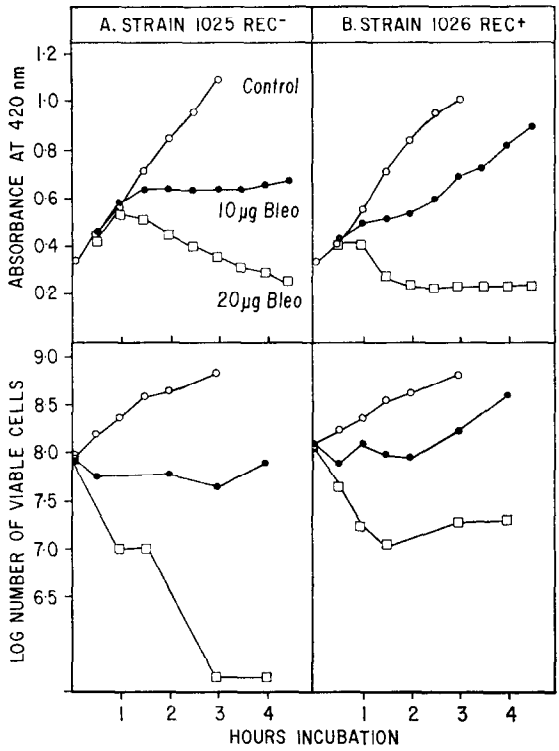

FIG. 6. Effect of bleomycin on growth and viability of *B. subtilis* strains GSY 1025 and GSY 1026. The experiment was carried out as described for Fig. 1. Symbols: (○) control; (●) 10 μg/ml bleomycin; (□) 20 μg/ml bleomycin.

and analyzed (profile of ^{14}C counts) for single strand breaks by centrifugation through alkaline sucrose gradients. The DNA from those cells exposed to bleomycin was slower sedimenting than DNA from cells trested similarly without bleomycin, suggesting single strand breaks. These investigators did not show control experiments to rule out degradation of DNA by residual bleomycin during manipulation of the cells and extracts. We have executed a similar experiment with *B. subtilis* and have included a control, the bleomycin-resistant strain, to make the result more meaningful. Figure 7 shows that bleomycin does indeed result in strand scission in *B. subtilis* 168 DNA. The DNA from the control cells went nearly to the bottom of the tube, while that from cells treated with drug gave a broad peak in the gradient. DNA from the bleomycin-treated, bleomycin-resistant cells, however, was also degraded, although to a lesser extent. DNA from untreated bleomycin-resistant cells (not shown) behaves the same as that from untreated cells of strain 168. This experiment was repeated several times with manipulations carried out as quickly as possible and with the lysozyme incubation as short as 5 min. An additional control was also carried out (not shown) in which bleomycin was added to a culture after the incubation period and then immediately washed out. DNA from these cells also appeared to be degraded. The DNA from cells harvested during recovery from bleomycin treatment also appeared to be degraded. The same results were obtained when the phenol extraction step was

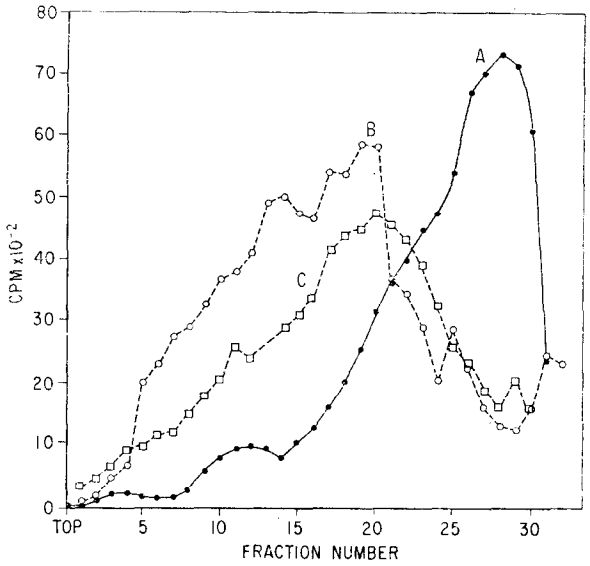

FIG. 7. Effect of bleomycin on DNA of growing *B. subtilis* 168 (A,B) and Bleo^R (C). Cells were grown to an absorbance of 0·5 in minimal medium containing 2 μc/ml [^{3}H]thymidine and were then harvested, washed and resuspended in minimal medium containing (A) no additions, (B) and (C) 50 μg/ml bleomycin. These cultures were incubated in the usual manner at 37° for 30 min, harvested, washed twice with cold saline containing 5×10^{-3} M EDTA, and resuspended in 0·5 ml of the same. Lysozyme, 100 μg, was added and the mixture was incubated 30 min at 37° followed by the addition of 0·1 ml of sodium dodecyl sulfate and 0·1 ml of 1 N NaOH. The mixture was extracted with an equal volume of phenol. Samples, 0·3 ml, were then applied to 5 ml of 5–20% alkaline sucrose gradients and centrifuged for 3·5 hr at 40,000 rev/min in an SW 50·1 rotor at 4°. The gradients were fractionated with the aid of a Buchler Densi-flow. Fractions of about 0·15 ml were collected, mixed with 2 ml of cold 5% TCA and the resulting precipitates collected on S & S glass fiber filters, washed several times, dried and counted in a toluene-based scintillation fluid containing 0·01% 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene and 0·4% 2,5-diphenyloxazole.

omitted. Thus it appears that the apparent scission of DNA strands observed in this type of experiment occurs in part during manipulation of the cells and extracts after removal of the antibiotic. Although the growth experiments described here suggest that it may be possible to observe alterations in the DNA from bleomycin-treated cells, it will be necessary to find an agent which quickly and effectively inhibits the action of the antibiotic in order to do really meaningful experiments of this kind.

Since most of the work concerning the effects of bleomycin on DNA *in vitro* has been carried out with bleomycin A_2, we have included Fig. 8, which compares the effect of this component with that of clinical grade bleomycin on the growth of *B. subtilis* 168 (A) and 168 Bleo^R (B). Bleomycin A_2 appears to be somewhat more active than the mixture in inhibiting growth of strain 168. Strain 168 Bleo^R was, however, equally resistant to both forms of the drug at the levels tested. This suggests that bleomycin A_2 may be the primary active component of bleomycin.

Although there are reports in the literature concerning the effect of bleomycin on DNA, there are few data which actually link these observations with the lethality of the agent. Barranco and Humphrey[17] have observed, in Chinese hamster ovary cells, that mitosis and the G_2 phase are those periods of the growth cycle most sensitive to

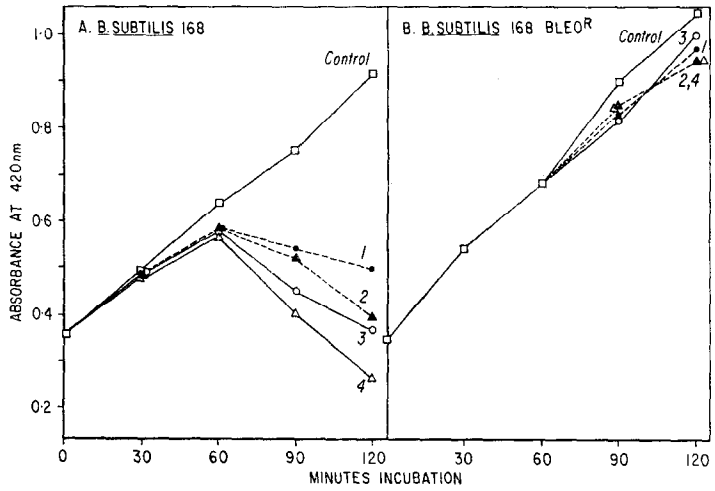

FIG. 8. Relative effectiveness of bleomycin A_2 and bleomycin. Growth curves of strains 168 and 168 Bleo^R were carried out as described for Fig. 1 with one control flask for each strain (□). The levels of bleomycin used were 20 μg/ml (●, curve 1) and 30 μg/ml (▲, curve 2). The levels of bleomycin A_2 were 20 μg/ml (○, curve 3) and 30 μg/ml (△, curve 4).

bleomycin. This is similar to their observations with actinomycin D[18] which is a potent inhibitor of RNA synthesis by virtue of its binding with DNA.[19] The variety of growth experiments presented here provides considerable, although indirect, evidence substantiating the notion that the primary mechanism of action of bleomycin may indeed be its interaction with DNA. These observations and their implications can be summarized as follows.

(1) Bleomycin inhibits DNA, RNA and protein synthesis in growing cells with possibly an earlier effect on RNA and protein synthesis than on DNA synthesis. These observations are somewhat contradictory to the results of Suzuki *et al.*[6] These investigators have indicated that in *E. coli*, Ehrlich carcinoma and HeLa cells DNA synthesis was affected more markedly than RNA or protein. It is unclear, however, whether they removed the cells from the medium before analysis or merely analyzed an aliquot of the culture (cells plus medium and cell debris). This could influence the result significantly.

(2) A bleomycin-resistant strain was isolated and found to be cross-resistant to mitomycin C but not to daunomycin. Although one can envision several possible explanations for this observation, it could suggest a similarity in action between bleomycin and mitomycin C (chemical alteration of DNA). Although the latter has been shown to form cross-links in DNA while bleomycin apparently causes strand scission, it is conceivable that resistance to either could result from the acquisition of a more efficient DNA repair mechanism.

(3) A recombination-deficient strain of *B. subtilis* was found to be more sensitive to bleomycin than its wild type counterpart. These data again emphasize a role of DNA in the action of bleomycin.

Although the experiments described here do not establish the primary mechanism of action of bleomycin, they are highly suggestive that DNA is involved and have

formed a basis for further, more definitive experiments. In another report[20] we describe the inhibition of DNA-dependent RNA polymerase by bleomycin which is the result of the agent's interaction with DNA. Thus, cell death could result from either or both of the following: (a) sufficient strand scission (or other interaction with DNA) to interfere with DNA replication, or (b) interference with transcription resulting in synthesis of incomplete RNA's. However, it should be kept in mind that clinical grade bleomycin is a mixture of several (apparently similar) glycopeptides whose distribution, modes of action, etc., could differ significantly. Thus, in order to assess truly the mechanism *in vivo*, it will be necessary to consider all components. Experiments are currently in progress with purified bleomycin A_2.

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