Adaptation of Populations of *Bacillus cereus* to Tetracycline

ROBERT H. CONNAMACHER,[1] H. GEORGE MANDEL, AND FRED E. HAHN

Department of Pharmacology, The George Washington University School of Medicine, Washington, D.C. 20005, and Department of Molecular Biology, Walter Reed Army Institute of Research, Washington, D.C.

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SUMMARY

After initial inhibition by tetracycline at concentrations from 10^{-6} to 10^{-5} M, populations of *Bacillus cereus* in a casein hydrolyzate-salts medium resumed exponential growth. Sensitivity to the antibiotic was reestablished by the bacteria upon continued growth in the absence of tetracycline; restoring the antibiotic to such sensitive cultures resulted in a second emergence of resistance. Tetracycline was not significantly inactivated by the bacteria.

B. cereus accumulated tetracycline, and maximal intracellular concentrations were attained within approximately 20 min. The organisms escaped from the action of the drug by eliminating tetracycline progressively until the intracellular concentration of the antibiotic was no longer sufficient to sustain growth inhibition. The rate of egress of tetracycline from *B. cereus* was linear. Increasing the Mg^{2+} or Ca^{2+} concentrations or the pH of the medium resulted in the accumulation of lower levels of tetracycline by the cells but was without influence on the rate of egress of the drug.

B. cereus did not eliminate tetracycline at 2° or in media that either were devoid of amino acids or contained arsenite (10^{-3} M).

Ribosomal 30 S subunits from adapted or nonadapted *B. cereus* bound similar quantities of tetracycline-7-^{3}H upon centrifugation into sucrose gradients.

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INTRODUCTION

Two general hypotheses have been considered as explanations of the emergence of drug resistance in microorganisms: (a) The *mutation selection* hypothesis assumes the occurrence of spontaneous genetic changes, especially chromosomal mutations to drug resistance, in individual microbial cells *before contact* with the respective drug, followed by selective propagation of the descendants of such mutants in the presence of the drug. (1). (b) The *phenotypic adaptation* hypothesis assumes that resistance arises throughout entire microbial populations *upon contact* with a drug as the result of drug-induced adaptive modifications.

Previous studies have shown that acquired resistance to tetracyclines has a genetic basis. Northrop (2) has demonstrated the presence of spontaneous oxytetracycline-resistant mutants in cultures of *Bacillus megaterium*, and oxytetracycline- or tetracycline-sensitive bacteria can acquire resistance from resistant organisms by conjugation (1), transduction (3, 4) or R-factor transfer (5). We report the first example of phenotypic adaptation to tetracycline by an entire bacterial population upon contact with the antibiotic.

Genetically determined resistance of *Escherichia coli* to tetracyclines results from a reduced permeability of these organisms to the drugs so that only low concentrations of tetracyclines are taken up by such bacteria (6–10). While tetracycline-adapted *B. cereus* also contained antibiotic concentrations that were insufficient to sustain growth inhibition, this condition was caused by the release of previously accumulated tetracycline from the cells.

MATERIALS AND METHODS

Materials. Tetracycline, chlortetracycline, tetracycline-7-^{3}H (7 μC/μmole), and chlortetracycline-^{14}C were generously provided by Lederle Laboratories. Additional tetracycline-7-^{3}H (27 μC/μmole) was purchased from New England Nuclear Corporation. Oxytetracycline was donated by Pfizer Laboratories.

Bacillus cereus 569 or 569H were maintained on agar slants and stored at 4°. Cultures were grown at 37° by shaking in air in a Difco casamino acids-salts medium (11) supplemented with 3×10^{-6} M manganous sulfate. Growth was determined turbidimetrically at 540 mμ in a Beckman DU spectrophotometer. The turbidity of the culture was found to be directly related to the dry weight of the cells in the presence or absence of tetracycline. Similar observations have been made in experiments with other drugs (12, 13). In some experiments, the cells were grown in a brain-heart infusion broth medium (Difco) pH 7.4.

Membrane filtration technique. The uptake of tetracycline-7-^{3}H into exponentially growing *B. cereus* was measured by membrane filtration (14). A suspension of *B. cereus* was treated with 6×10^{-6} M tetracycline-7-^{3}H. Immediately and at intervals thereafter, 2-ml aliquots of the suspension were removed, quickly added to 2 ml of 0.14 M NaCl at 0°, and the bacteria were collected on Schleicher and Schuell B-6 membrane filters. The cells were washed once with 5 ml 0.14 M NaCl, the filters were glued to planchets, and the radioactivity was counted in a Nuclear-Chicago windowless gas-flow counter, using natural gas. Corrections were required for the adsorption of tetracycline-^{3}H to the filter and for self-absorption of tritium:

1. Approximately 600 counts per minute were detected on the membranes after filtration of 2 ml of medium containing 6×10^{-6} M tetracycline-7-^{3}H. This radioactivity was not removed by up to 4 washes of the filter. When unlabeled bacteria were then filtered, using such a radioactive filter, some of the radioactivity was quenched. For example, the bacterial residue from 2 ml of a culture at an OD_{540} of 0.2 reduced the radioactivity by 65%. The radioactivity bound to the filter was therefore measured following filtration of samples of bacterial suspensions at various turbidities. This amount of radioactivity was subtracted from the total counts in the subsequent experiments.

2. To account for the self-absorption of radioactivity by the tritium-labeled bacteria, different volumes of a bacterial suspension containing tetracycline-7-^{3}H-labeled cells were filtered and counted. The self-absorption correction was calculated for samples of varying turbidities, and in subsequent experiments radioactivity of filtered labeled bacteria was corrected for samples of infinite thickness.

Since the two corrections were in opposite directions, the final corrected values were usually within 10% of the values obtained experimentally.

Binding of tetracycline by ribosomes. Drug-sensitive or resistant *B. cereus* were suspended in 2.5 volumes (wet weight) of 10^{-2} M Tris buffer, pH 7.8, containing 10^{-2} M magnesium acetate, 6×10^{-2} M potassium chloride, and 6×10^{-3} M 2-mercaptoethanol (15). The suspension was sonicated at 0° for 10 minutes in a MSE ultrasonicator. The ribosomes from the extracts of sensitive and resistant cells were isolated by centrifugation in a Spinco Model L ultracentrifuge at 105,000 *g* for 90 min. One to two milligrams of ribosomes were placed on top of a 5 ml 5–20% sucrose gradient (pH 7.8, 10^{-4} M Mg^{2+}, 10^{-2} M Tris, and 6×10^{-2} M KCl). The ribosomes were centrifuged for 105 min in a Spinco SW 39 rotor and 0.2-ml fractions were collected by needle

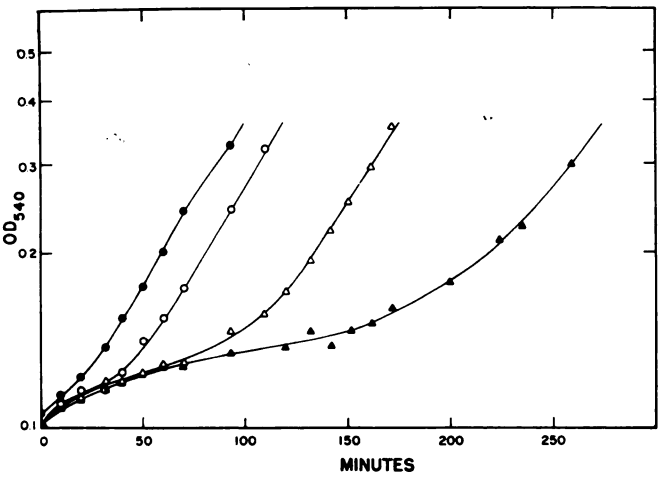

FIG. 1. *Growth measured turbidimetrically at 540 mμ of Bacillus cereus 569H*

Growth in casein hydrolyzate-salts medium in the presence of different concentrations of tetracycline: ●, 0; ○, 2×10^{-6} M; △, 6×10^{-6} M; ▲, 20×10^{-6} M.

puncture. Each fraction was diluted to 1.1 ml with water, the OD_{260} of each fraction was determined, and the radioactivity of each entire fraction was counted in 10 ml of Bray's solution in a Nuclear-Chicago liquid scintillation counter.

Partition experiments. To determine the partition of tetracycline between bacterial cells and the medium, *Bacillus cereus* was grown to an OD_{540} of approximately 0.45 and harvested by centrifugation at 10,000 *g*. The bacterial pellet was weighed (wet weight) and resuspended in a weight of medium equal to that of the pellet and containing 2×10^{-4} M tetracycline-7-^{3}H. Immediately thereafter, and after 20 min of incubation by shaking at 37°, aliquots of the suspension were centrifuged in an International clinical centrifuge for 3 min at maximum velocity, and four 0.01-ml samples of medium were plated onto planchets. Four 0.02 aliquots of the bacterial suspension, corresponding to 10 mg of cells, were each diluted with saline to a volume of 16 ml, and quadruplicate samples of 4 ml filtered through membrane filters. The filters were washed three times with saline, dried, and attached to planchets. The partition ratios, i.e., ratio of counts per minute in 10 mg of bacteria to those in 0.01 ml medium, were calculated in four experiments, each in quadruplicate.

Mg^{2+} and Ca^{2+} assays. Solutions containing these metals were assayed chelometrically following ashing in a 1:1 solution of concentrated nitric and 70% perchloric acids.[2]

RESULTS

Interconversion of Tetracycline Resistance and Sensitivity of B. cereus

Growth of *Bacillus cereus* strains 569 or 569H was strongly inhibited by 2×10^{-6} M tetracycline (Fig. 1). However, after periods of time that were approximately proportional to the antibiotic concentration, the cultures resumed exponential growth at rates approaching that of antibiotic-free cultures. Resistance to tetracycline concentrations higher than 2×10^{-5} M did not develop, and cultures that had become resistant to 6×10^{-6} M tetracycline remained susceptible to antibiotic concentrations higher than 5×10^{-5} M.

Populations of *B. cereus* that had developed resistance to 6×10^{-6} M tetracycline regained sensitivity if grown in a tetracycline-free medium. For example

[2] The cooperation of Dr. Marie Cassidy and Mrs. Saramma Thomas, Department of Physiology, The George Washington University School of Medicine, in carrying out these determinations is gratefully acknowledged.

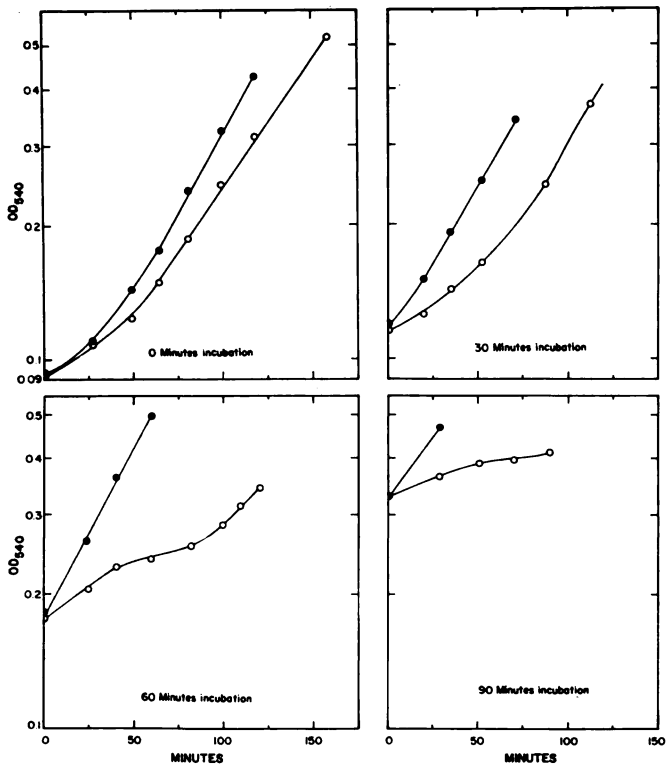

FIG. 2. *Return of tetracycline sensitivity to phenotypically drug-resistant Bacillus cereus*

Cells made resistant to 6×10^{-6} M tetracycline were resuspended and incubated in drug-free medium. At 0, 30, 60, and 90 min after resuspension, culture samples were removed and regrown in the presence (○) and absence (●) of 2×10^{-5} M tetracycline.

(Fig. 2), resistant cultures, when incubated for 0, 30, 60, or 90 min in drug-free medium, lost their resistance progressively until full sensitivity had been restored. The 60 min frame of Fig. 2 also shows that the newly sensitive cells reacquired resistance to tetracycline.

The escape of the cultures from inhibition by tetracycline was not the result of a metabolic inactivation or degradation of the antibiotic. Growth media in which *B. cereus* had adapted to tetracycline remained inhibitory to fresh inocula of sensitive bacteria, and heavy suspensions of resistant or sensitive *B. cereus*, incubated for 60 min with tetracycline-7-^{3}H (6×10^{-6} M), converted only 10% of the labeled drug into one unidentified product with an R_F upon paper chromatography (16) greater than that of tetracycline, while 90% of the radioactivity moved with tetracycline.

Accumulation and Elimination of Tetracycline by B. cereus

When a culture of *B. cereus* 569H was allowed to develop resistance to tetracycline-7-^{3}H (6×10^{-6} M) and the radioactivity associated with the bacterial cells was determined periodically by the membrane filter technique, results represented in Figs. 3 and 4 were obtained. The top curves show that radiotetracycline was rapidly accumulated by the microorganisms to concentrations that, in separate partition experiments after 20 min of incubation, reached 9.18 ± 2.49 times the drug concentration in the medium. This accumulation was followed after 20–30 min by an efflux of tetracycline at a linear rate. The arrows in Figs. 3 and 4 indicate the times and bacterial tetracycline concentrations at which the populations resumed normal

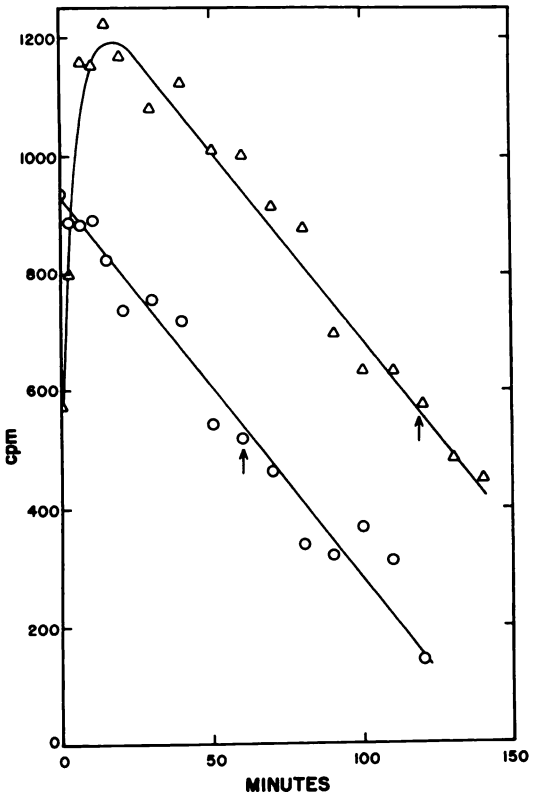

FIG. 3. *Effect of divalent cations on tetracycline-7-^{3}H content of Bacillus cereus*

The cells were grown in the presence of 6×10^{-6} M tetracycline-7-^{3}H, and 2-ml aliquots of the suspension were filtered through membrane filters. Radioactivity was determined in a windowless gas flow counter and corrected for tritium absorption and adsorption of drug to the filter. (△) Casein hydrolyzate-salts medium from which Mg^{2+} was deleted; (○) complete casein hydrolyzate-salts medium containing 6×10^{-3} M Mg^{2+}; arrow indicates time of return of exponential growth.

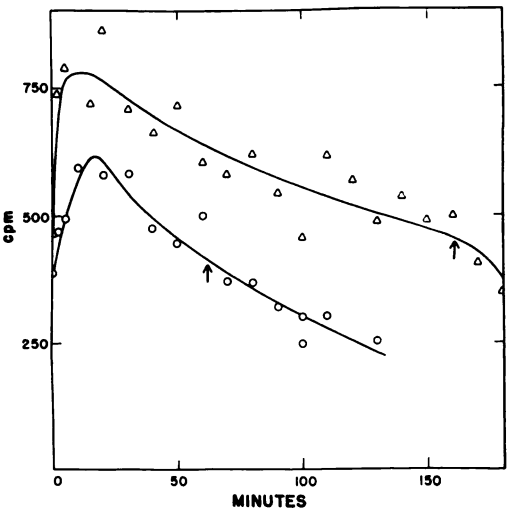

FIG. 4. *The uptake into and loss of radioactivity from Bacillus cereus as a function of pH*

Bacteria were grown with 6×10^{-6} M tetracycline-7-^{3}H in casein hydrolyzate medium at (○) pH 7.3 and (△) pH 6.7. See Fig. 3 for experimental details. Return of exponential growth at arrow.

growth. These results suggest that growth was resumed by the bacterial populations when the intracellular level of tetracycline fell below a critical growth-inhibitory concentration.

When the content of radioactivity was measured in bacteria already adapted to the radioactive drug and then treated with additional tetracycline-7-^{3}H, only subinhibitory concentrations of tetracycline were observed in the cells (Fig. 5).

In experiments in which adaptation to higher concentrations (2×10^{-5} M and above) of tetracycline-7-^{3}H was tested, the bacteria not only failed to develop resistance but also failed to eliminate the drug. Cultures of *B. cereus* were sensitive to 6×10^{-6} M chlortetracycline but did not develop resistance to the drug at that concentration. Under these conditions the antibiotic entered the bacteria but was not eliminated from them (Fig. 6). Exposure to 2×10^{-6} M chlortetracycline resulted in the development of resistance similar to that observed with 6×10^{-6} M tetracycline. On the other hand, resistance to oxytetracycline developed more readily than that to tetracycline when both drugs were present at 6×10^{-6} M.

Factors Influencing the Accumulation and Elimination of Tetracycline

When *B. cereus* was grown in brain-heart infusion broth (5×10^{-4} M Mg^{2+} in contrast to 6×10^{-3} M Mg^{2+} in casein hydrolyzate-salts medium) resistance to tetracycline did not develop. Supplementation of Mg^{2+} to the cultures either prior to the addition of the antibiotic or simultaneously as a tetracycline-Mg^{2+} mixture allowed for the development of resistance by the bacteria.

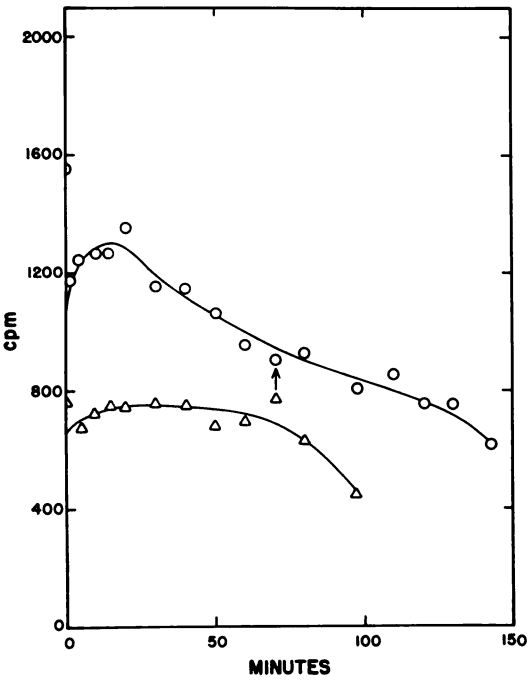

FIG. 5. *Uptake of radioactivity into normal cells* (○) *and into cells made resistant to the radioactive drug* (△) *by incubation in casein hydrolyzate medium containing* 6×10^{-6} M *tetracycline-7-*^3H

Return of exponential growth at arrow.

Figure 7 indicates that the periods of time required for adaptation to the antibiotic were inversely proportional to the Mg^{2+} concentration in the medium. Analogous results were obtained with graded concentrations of Ca^{2+} up to its limit of solubility.

The accumulation of tetracycline by *B. cereus* also was a function of the Mg^{2+} concentration. When the casein hydrolyzate-salts medium contained the usual Mg^{2+} concentration (6×10^{-3} M), less of the antibiotic entered the cell than when Mg^{2+}

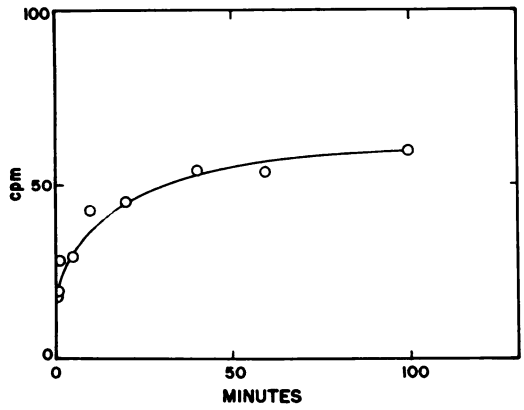

FIG. 6. *The uptake of radioactivity into Bacillus cereus grown in casein hydrolyzate-salts medium in the presence of* 6×10^{-6} M *chlortetracycline-*^{14}C.

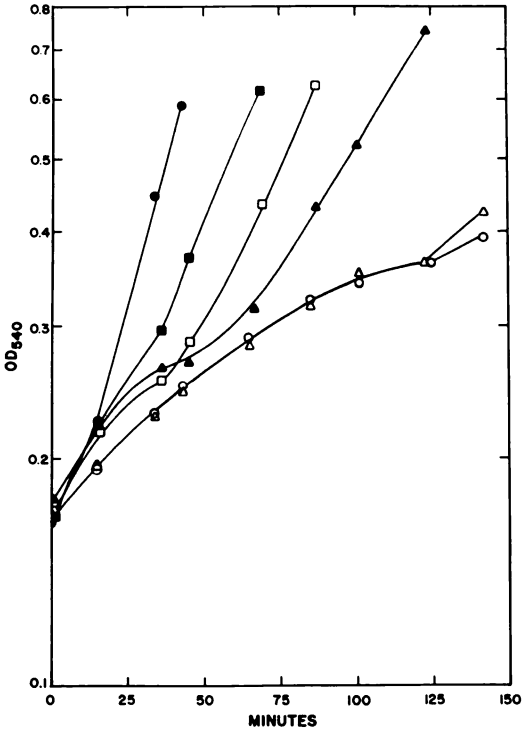

FIG. 7. *Failure of recovery of growth by Bacillus cereus grown in the presence of* 6×10^{-6} M *tetracycline in brain-heart infusion broth unless additional* Mg^{2+} *was supplemented*

●, Control; tetracycline cultures, additional Mg^{2+}: ○, 0; △, 6×10^{-3} M; ▲, 12×10^{-3} M; □, 19×10^{-3} M; ■, 25×10^{-3} M.

had been deleted from the medium (Fig. 3, lower curve). Since the rate of efflux was not altered by increasing concentrations of Mg^{2+}, the critical concentration at which exponential growth resumed therefore was attained earlier.

More antibiotic entered the cells at pH 6.7 than at pH 7.3, and the recovery of the culture from the effects of the antibiotic was delayed (Fig. 4).

While the rate and level of accumulation

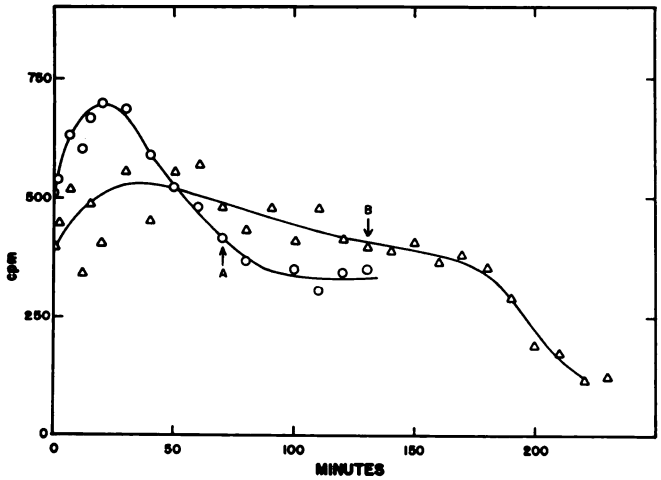

FIG. 8. *Inhibition of tetracycline-7-^{3}H influx and efflux at lower temperatures*

Culture (○) grown at 37°. Culture (△) grown at 2°, then warmed to 37° at arrow *B*. Recovery of exponential growth at arrow *A*.

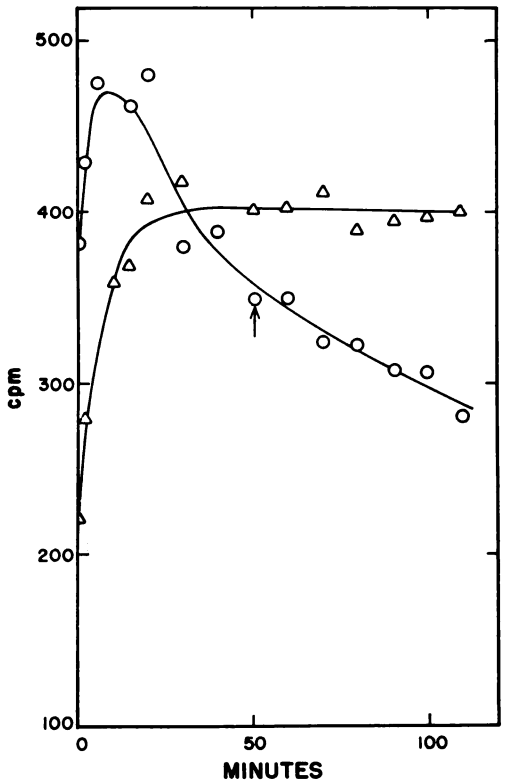

FIG. 9. *Inhibition of tetracycline-7-^{3}H influx and efflux by 10^{-3} M arsenite*

Tetracycline control, 6×10^{-6} M, (○); arsenite culture, (△). Recovery of exponential growth at arrow.

of tetracycline-7-^{3}H by *B. cereus* were somewhat lower at 2° than at 37°, virtually no tetracycline was eliminated from the bacteria at 2° (Fig. 8). When the incubation temperature was raised to 37° egress

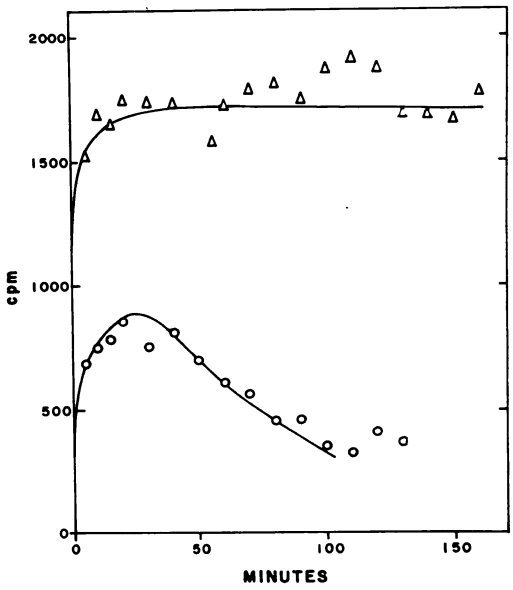

FIG. 10. *The effect of amino acids on the uptake and efflux of 6×10^{-6} M tetracycline-7-^{3}H in Bacillus cereus*

Normal casein hydrolyzate-salts medium (○); Medium in which the 10 g/l casein hydrolyzate had been replaced by 10 g/l glucose (△).

of tetracycline was again observed. The temperature dependency of the elimination process suggests that enzymic processes are involved. This is also supported by the effect of arsenite on the accumulation and elimination of tetracycline by *B. cereus*. Figure 9 shows that arsenite (10^{-3} M) reduced the maximal level of accumulated tetracycline by 20% and decreased the rate of accumulation. The egress of the antibiotic, however, was completely inhibited. Finally, the bacteria also failed to eliminate tetracycline when they were suspended in a rest medium containing glucose and inorganic ions but no casein hydrolyzate (Fig. 10).

Tetracycline Binding to Ribosomes from Sensitive and Resistant Cells

To determine whether development of resistance to tetracycline involved an altered affinity for the drug by ribosomes, binding of tetracycline-7-^{3}H to the 30 S ribosomes (17) from dialyzed extracts from resistant and sensitive *B. cereus* was studied (Fig. 11). No inhibition of binding was detected.

DISCUSSION

In this paper we have reported an example of phenotypic adaptation of bacteria to the action of an antibiotic which inhibits protein biosynthesis. We have shown that populations of *B. cereus* were sensitive to tetracycline when accumulating growth-inhibitory concentrations of this antibiotic. Such cells became resistant to tetracycline by progressively eliminating the antibiotic until the remaining intracellular tetracycline concentrations were no longer sufficient to maintain growth inhibition. Since tetracycline resistance is exhibited by the entire test population and since the time within which it occurs or resistance can be lost and reinduced is so short, the mutation-selection hypothesis cannot explain our observations.

The amino acid requirement for the tetracycline efflux suggests that the elimination process depends on biosynthetic events in tetracycline-inhibited cells. Since the linear egress of tetracycline occurred when protein was not being synthesized (18, 19),

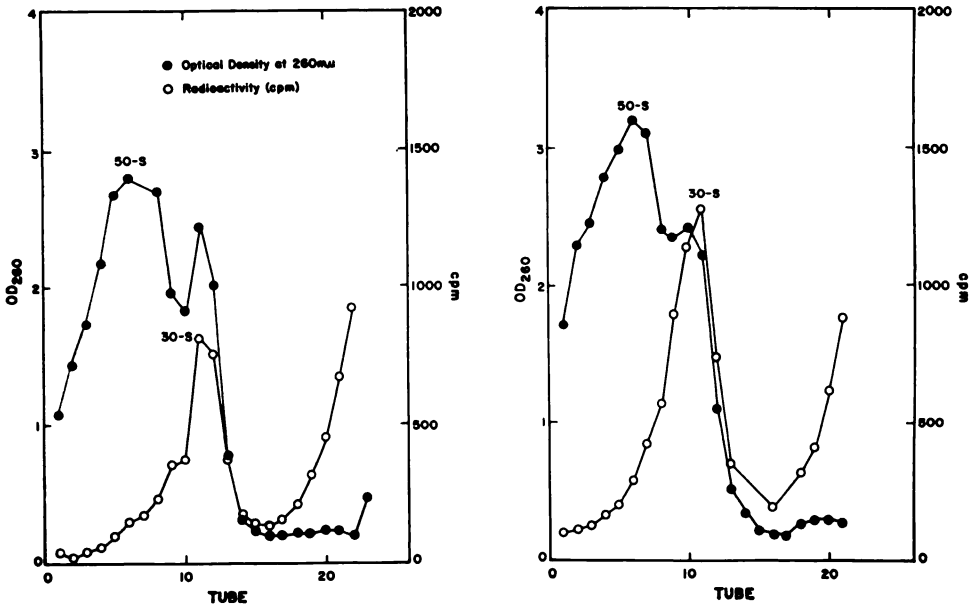

FIG. 11. *Elution patterns of sucrose gradients of ribosomes from tetracycline-sensitive and -resistant Bacillus cereus dialyzed in the presence of tetracycline-7-^{3}H*

Details described under Methods. The OD_{260} (●) and radioactivity (○) of each sample are plotted against the fraction number. Left, ribosomes from sensitive cells; right, ribosomes from resistant cells.

the elimination of tetracycline was not related to cellular protein biosynthesis.

The uptake of tetracycline by *B. cereus* was limited in time and extent and was too rapid to allow meaningful kinetic observations. It was suppressed only in part at 2° or in the presence of arsenite, suggesting that enzymic reactions were only partly involved. This is in marked contrast to the complete failure of tetracycline elimination under the same conditions or in the absence of amino acids.

The ability of *B. cereus* to become resistant to tetracycline extends to other members of the tetracycline family, such as chlortetracycline and oxytetracycline, although differences in potency have been observed. *Bacillus cereus* also becomes resistant to trivalent arsenic ions in a manner that appears analogous to the tetracycline adaptation (20). If these adaptations are interrelated, then the effects reported here may be a general method by which this *Bacillus* species is able to survive in a toxic environment.

ACKNOWLEDGMENTS

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