Characteristics of Secretion of Penicillinase, Alkaline Phosphatase, and Nuclease by *Bacillus* Species

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The distribution of alkaline phosphatase and nuclease activity between cells and medium was examined in one strain of *Bacillus licheniformis* and four strains of *B. subtilis*. Over 95% of both activities was found in the medium of the *B. licheniformis* culture, but in the *B. subtilis* cultures the amount of enzyme activity found in the medium varied with the strain and the enzyme considered. *B. licheniformis* 749 and its penicillinase magnococonstitive mutant 749/C were grown in continuous culture with phosphorous as the growth-limiting factor, and the kinetics of penicillinase formation and secretion were examined. Nutrient arrest halted secretion (usually after a lag of about 30 min) in both the inducible and constitutive strains. Chloramphenicol did not eliminate secretion, but under certain circumstances reduced its rate. In the inducible strain treated with a low level of inducer, the rate of secretion was more affected by the rate of synthesis than by the level of cell-bound enzyme. During induction, the onset of accretion of cell-bound penicillinase and secretion of the exoenzyme were nearly simultaneous. It seems unlikely that a long-lived, membrane- or cell-bound intermediate is mandatory in the secretion of the three enzymes by *Bacillus* species. In the case of penicillinase secretion, there are at least two different phases. When penicillinase synthesis is proceeding rapidly, the rate of secretion is five to six times greater at equivalent concentrations of membrane-bound penicillinase than it is when penicillinase synthesis is reduced. The data require that any membrane-bound intermediate in the formation of exoenzyme be much shorter-lived in cells with a high rate of synthesis than in cells with a low rate. Either there are two separate routes for the secretion of penicillinase or the characteristics of the process vary substantially between the early stages and the declining phase of induction.

Lampen (9) has proposed that the secretion of penicillinase by *Bacillus licheniformis* takes place in the following way. Immediately after it is synthesized, the enzyme becomes bound to an intrusion of the cytoplasmic membrane; it remains membrane-bound for more than a cell doubling time, while growth, or some other process, moves it to the outer surface of the membrane. When the enzyme reaches the outer surface, a hydrolytic (possibly proteolytic) cleavage liberates it from the membrane in the form in which it will be found in the medium, and the liberated enzyme diffuses outward through the cell wall.

This proposed pathway rests, in part, on certain observed characteristics of penicillinase secretion in *Bacillus* species. First, most of the penicillinase produced during the first one to two cell doubling times after induction has been initiated is cell bound (4), and the cell-bound enzyme makes up 50 to 80% of the total enzyme, both in induced cultures and in cultures of constitutive mutants when the enzyme is being synthesized rapidly (8). Second, the cell-bound enzyme is largely, or totally, attached to the cell membrane (8, 10). Third, secretion of penicillinase occurs at the expense of cell-bound enzyme when synthesis of enzyme has been halted by exhaustion of induction (14), or by suspension of the synthesizing cells in media deficient in nitrogen (11) or an amino acid essential for growth (17). Fourth, the membrane-bound enzyme can be liberated from cells by trypsin (8) and from membrane fragments, either spontaneously under conditions similar to those in which the cell secretes enzyme, or by treatment with certain proteinases (11).

If the secretion of penicillinase is typical of the way in which exoenzymes are secreted by *Bacillus*, then these characteristics should also be observ-
able in the secretion of other exoenzymes by this genus. However, whereas nuclease secretion by B. subtilis is characterized by a high cell-medium ratio (1), the reverse is true for the secretion of alkaline phosphatase (2).

It was thus of interest to compare the secretion characteristics of all three of these enzymes in B. licheniformis in order to determine what aspects of the proposed scheme for penicillinase secretion might be incorporated in a general model for enzyme secretion by this genus.

MATERIALS AND METHODS

**Bacteria and their maintenance.** B. licheniformis 749 and its penicillinase-magnocoagulative mutant 749/C were originally obtained from M. R. Pollock, Department of Molecular Biology, University of Edinburgh. B. subtilis strains 15575, SB168, 15244, and 15819 were obtained from Ruth Gordon of the Institute of Microbiology.

Stock cultures were maintained on soil extract agar slants (6) at 4 C. Transfers were made directly from these slants to shake flasks.

**Continuous-culture apparatus and growth-limiting medium.** A continuous-culture apparatus similar to that described by Jannasch (7) was constructed. This type of apparatus utilizes hydrostatic pressure acting against a flow-limiting orifice to control the rate of addition of fresh medium to the growth chamber.

The growth chamber was a 1-liter Florence flask modified to permit overflow when the contained volume was 135 ml. The chamber was immersed in a water bath at 34 C and magnetically stirred.

Phosphorus was the growth-limiting nutrient to insure derepression of alkaline phosphatase. The limiting phosphorus (LP) medium contained (per liter): 0.05 mole of tris(hydroxymethyl)aminomethane (Tris) buffer, 0.1 mole of KCl, 0.0016 mole of MgSO 4 , 0.0044 mole of trisodium citrate, 0.0056 mole of maltose, 10 -2 mole of K 2 HPO 4 , and 1.0 g of Difco Peptone no. 3. The pH of the mixture was adjusted to 6.8 with HCl and 5.0 ml of a concentrated salt mixture was added per liter. The concentrate was 0.33 m in MgSO 4 , 0.018 m in MnSO 4 , 0.54 m in KCl, 0.0045 m in Na 2 (MoO 4 ), 0.013 m in FeSO 4 , 0.068 m in CaCl 2 , and 7.3 X 10 -4 m in ZnCl 2 . A small amount of concentrated HCl was used to aid solubility. The medium was autoclaved for 30 min at 121 C in 4-liter lots.

Phosphate analysis following acid hydrolysis of the medium (kindly performed by W. L. McLellan, Jr., of the Institute of Microbiology) showed it to contain 7.5 X 10 -4 moles/liter. Used in batch culture, this medium yields 50% as much growth of B. licheniformis in 12 hr as when excess phosphate (0.01 m) is added.

**Experimental procedure.** The growth chamber was seeded with 5.0 ml from a 16-hr shaken broth culture, and the dilution rate was adjusted to the desired level. The steady state was usually established in 3-days. On the 2nd and 3rd days, samples were taken for turbidity measurement and microscopic examination.

If the turbidity was constant between samplings and the cells were in short chains and uniform in size and shape, the experiment was performed. When the steady state was achieved, the pH of the medium was 6.8.

Samples of 2.0 ml were drawn from the growth chamber into the barrel of a syringe with a Swinney filtration attachment and were rapidly filtered. The filtrate was immediately placed in an ice bath, and the membrane (0.4-μ pore size) with the retained cells was placed in 2.0 ml of ice-cold 0.01 M Tris buffer, adjusted to pH 7.1 with HCl and containing 20 μg of 8-hydroxyquinoline and 100 μg of lysozyme per ml.

When all the samples in an experiment had been collected, the solutions containing the membranes were mechanically agitated for 15 to 30 sec to disperse the cells and then brought to 37 C for 15 min. Microscopic examination showed all the cells to be lysed by this treatment. All samples were frozen until analyzed.

**Enzyme assays.** Alkaline phosphatase was measured by the method of Stearns et al. (5) using p-nitrophenyl phosphatase as substrate.

Nuclease was measured as described by Chesbro, Stuart, and Burke (3), except that the glycine buffer was adjusted to pH 9.2 to make the assay comparable to that used by Birnboim (1), and reaction mixtures were quenched with 0.1 volume of 10% trichloroacetic acid and held in an ice bath until the 7% perchloric acid could be added to all samples simultaneously, thus simplifying handling large numbers of samples. α-Glucosidase was measured as described by Pollock (13).

Penicillinase was measured by a method developed by A. B. Pardee, Department of Biology, Princeton University (personal communication). A stock solution composed of 33 ml of water, 15 ml of 0.5 m sodium citrate (pH 7.0), and 30 ml of 3.5 X 10 -3 m I - in 2 ml KI was diluted 1:10 with distilled water to provide a working solution. The working solution (5.0 ml amounts in 1-cm quartz cuvettes) was brought to 25 C, and 0.001 to 0.050 ml of enzyme solution was added to one of a pair of such cuvettes. Both cuvettes were received 0.05 ml of solution containing 2,000 units of benzylpenicillin and were immediately placed in a double-beam spectrophotometer with a water-jacketed cell compartment at 25 C. The absorbance of the enzyme mixture was measured at 351 m, after 30 and 90 sec. Under these conditions, an optical density difference of 0.01 between the two measurements, corresponding to 0.014 units of penicillinase as determined by the iodometric method of Perret (12), was used as the unit of penicillinase activity (the equivalence of the two methods was established with the kind assistance of M. G. Sargent of the Institute of Microbiology). For the semiquantitative assay of penicillinase in column fractions, a rapid starch-iodine method (12) was used.

**Gel filtration and acrylamide gel electrophoresis.** Sephadex G-75 columns, held at 4 C and eluted with 0.1 M Tris buffer (pH 6.8), were used for gel filtration studies. They were calibrated with ovalbumin, trypsin, γ-chymotrypsinogen, and lysozyme as molecular-weight markers.

Acrylamide gel electrophoresis was performed with
7.5% gel, buffered at pH 8.9 with a commercially available buffer (LKB 3276-GB10 high-resolution buffer, 3.0 millimhos). Runs were for 4.5 hr with a gradient of 10 v/cm. Enzyme activity was located by placing on the gel slab a filter-paper strip saturated with the iodine-starch mixture described by Perret (12) plus 1,000 units of benzylpenicillin, and noting bands of decolorization.

RESULTS

Distribution of alkaline phosphatase and nuclease activity between cell-bound and free forms in Bacillus licheniformis. Both enzymes were produced by B. licheniformis 749/C in the LP medium in batch and continuous culture. In continuous cultures with doubling times of 5.0 and 7.5 hr, 95 and 93%, respectively, of the alkaline phosphatase activity was in the medium, as was over 90% of the nuclease activity.

To insure that this apparent distribution was not due to the action of a cellular inhibitor which limited the activity of cell-bound enzyme (which is the case for B. subtilis ribonuclease (18)), cell lysates and their corresponding culture filtrates were mixed in various proportions, and the activities of the mixtures were measured. The observed activities of the mixtures were the sums of the activities of the components in all cases. Thus, there was no overt evidence for a cell-bound inhibitor capable of interfering with the activity of either enzyme.

Although the distribution of alkaline phosphatase between cells and medium was the same as that reported for strain 60-009 of B. subtilis (2), the distribution of nuclease activity was not the same as that for strain 168-2 of B. subtilis, where about 65% of this enzyme has been reported to be cell-bound (1). In the 749/C strain of B. licheniformis studied here, less than 5% was cell-bound.

Consequently, four strains of B. subtilis were grown (with shaking) for 14 hr at 27 C as batch cultures in LP medium. Samples of the cultures were filtered and the distribution of the two enzyme activities determined. The results (Table 1) indicated that the ratio of cell-bound to free-form enzyme under these growth conditions varied with the strain studied and with the enzyme examined. The distribution of the two enzymes in the 15819 strain was particularly interesting, because the alkaline phosphatase was essentially cell-bound, whereas the bulk of the nuclease activity was in the medium.

Effect of nutrient arrest on exoenzyme production in B. licheniformis. In the model of penicillinase secretion (9) described earlier, synthesis and secretion of penicillinase are temporally separated events, and it has been observed (9, 14, 17) that secretion continues under conditions in which new penicillinase is not being synthesized.

In this case, the cell-bound form decreases in proportion to the appearance of exoenzyme. Thus, it was expected that, when nutritional arrest was imposed upon a continuous culture of the penicillinase-magnoconstitutive mutant 749/C of B. licheniformis growing in the LP medium, penicillinase secretion would continue at the expense of cell-bound enzyme, and we wished to know what the concurrent behavior of alkaline phosphatase and nuclease would be.

Consequently, a continuous culture of the mutant growing with a doubling time of 6 hr was placed in nutrient arrest, with sampling and assay for penicillinase, alkaline phosphatase, nuclease, and α-glucosidase [as an index to the behavior of cytoplasmic enzymes and as a criterion of the integrity of the arrested cells (13)]. The results are shown in Fig. 1.

Penicillinase secretion continued during the first 30 min after arrest was imposed. (In this experiment, the secretion was more rapid during this interval than it had been in the previous steady state, but in repetitions of the experiment it was closer to the prearrest rate.) Cell-bound enzyme was more nearly constant for the first 30 to 50 min postarrest, then it too increased. Thereafter, both activities were constant for 2 to 3 hr. Exopenicillinase then began to increase, while cell-bound enzyme was constant for 4.5 hr postarrest; then it also increased slowly (not shown in Fig. 1).

Nuclease activity remained constant for both extracellular and cell-bound forms for about 1.25 hr postarrest. Thereafter, both forms increased, with four to six times as much enzyme being secreted during the next 3.5 to 4.0 hr as was retained by the cells.

In four of six repetitions, alkaline phosphatase behaved as shown; the exoenzyme concentration increased during the first 30 min postarrest, then

<table>
<thead>
<tr>
<th>Strain</th>
<th>Alkaline phosphatase</th>
<th>Nuclease</th>
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<tbody>
<tr>
<td></td>
<td>Filterate</td>
<td>Cells</td>
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<tr>
<td></td>
<td>units/ml</td>
<td>units/ml</td>
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<tr>
<td>15819</td>
<td>0.90</td>
<td>8.30</td>
</tr>
<tr>
<td>15244</td>
<td>0.45</td>
<td>215</td>
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<tr>
<td>SB168</td>
<td>2.85</td>
<td>2.55</td>
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<tr>
<td>15575</td>
<td>2.40</td>
<td>0.80</td>
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* Grown 14 hr at 27 C.

Table 1. Distribution of nuclease and alkaline phosphatase activity in shaken batch cultures of Bacillus subtilis strains in a limited phosphorus medium.
became constant, while cell-bound enzyme was constant for 1 to 2 hr postarrest and thereafter increased slightly. In two of the six repetitions, cell-bound enzyme and exoenzyme concentrations remained constant throughout the experiment.

The cytoplasmic enzyme $\alpha$-glucosidase showed a small increase immediately after arrest, then remained constant for about 1.25 hr, increasing thereafter. No significant amount of $\alpha$-glucosidase was found in the medium through the experiment.

Overall, it was apparent that, during the initial postarrest period, the increase in exoenzyme was accompanied by continuing synthesis of the enzyme, since there was never a corresponding decrease in cell-bound enzyme. The increase in penicillinase during this first postarrest period is in agreement with the observation that penicillinase synthesis involves a long-lived messenger ribonucleic acid (15, 19), which thus could maintain penicillinase synthesis for an interval despite the unavailability of phosphorus (the growth-limiting factor) for synthesis of new messenger.

The second, static phase following arrest was in contradiction to the behavior anticipated from the secretion model, which predicts continuing secretion from the reservoir of cell-bound enzyme.

The third phase of renewed increase in both cell-bound and exoenzyme, signaling renewed synthesis, indicated that the cells had mobilized some intracellular phosphate. Alkaline phosphatase, nuclease, and $\alpha$-glucosidase all showed renewed synthesis during this phase; the culture turbidity rose, and the cells, which were in chains of 8 to 20, increased visibly in size.

A feature of penicillinase production in this third phase was that the start of the increase in exoenzyme preceded the start of the increase in cell-bound enzyme (not shown in Fig. 1) by about 2 hr.

**Effect of chloramphenicol on penicillinase secretion by the constitutive mutant.** To establish whether secretion of the cell-bound enzyme would continue in cells whose protein synthesis was interrupted by chloramphenicol, sufficient antibiotic to yield an initial concentration of 25 $\mu$g/ml was added to a continuous culture of strain 749/C growing with a doubling time of 7 hr.

Approximately 10 min after adding the antibiotic, both cell-bound enzyme and exoenzyme concentrations started to fall. The cell-bound enzyme decreased at a rate greater than that predicted for simple washout from the culture dilution rate, while the exoenzyme decreased at a rate less than that predicted for simple washout. Thus, there was evidence that the antibiotic halted synthesis without halting secretion.

**Kinetics of induction by a low level of penicillin.** Pollock (14) found that induction of a batch culture with low levels of penicillin resulted initially in the rapid appearance of cell-bound enzyme and a slower increase in secreted enzyme. In a culture growing with an approximate doubling time of 2 hr, the induction became exhausted after about 3 hr and enzyme synthesis practically ceased. How-
however, secretion still continued, while the level of cell-bound enzyme fell proportionately, clearly showing its conversion to the free form.

An experiment of this sort was performed with the inducible strain 749 of *B. licheniformis*. Sufficient benzylpenicillin to yield an initial concentration of 0.02 units/ml was added to a steady-state, continuous culture of the organism growing in LP medium with a doubling time of 5 hr. The subsequent course of penicillinase production during and after induction is shown in Fig. 2.

Although the growth conditions and medium were different from those employed by Pollock and the doubling time was more than twice as long, the time course of penicillinase induction and induction exhaustion was nearly identical to that reported by Pollock.

We also plotted these data with the method used by Collins (4), who noted that relatively little exoenzyme was produced in the first hours following induction and proposed, partly from this observation, that after its synthesis the enzyme was cell-bound for 1.6 doubling times before being secreted (1.6 doubling times would be 8 hr in the experiment shown in Fig. 2). As can be seen from the inset, Fig. 2, plotting in this manner obscures the fact that, although the bulk of enzyme synthesized immediately after induction is indeed cell-bound, cell-bound enzyme and exoenzyme appear concurrently.

The rate of change of both the cell-bound enzyme and exoenzyme concentrations became zero about 4 hr after the addition of inducer, and both concentrations were very nearly constant for approximately 1 hr; thus, the kinetics of secretion were particularly amenable to examination during this interval.

At any time, the rate of change in exopenicillinase concentration \( \text{(Pexo)} \) is:

\[ -\frac{d(Pwo)}{dt} + \frac{d(Psec)}{dt} = \frac{d(Pexo)}{dt} \quad (1) \]

where \( Psec \) is the amount of enzyme secreted per milliliter of culture and \( Pwo \) is the amount of enzyme washed out. Since, at the turnover, \( \frac{d(Pexo)}{dt} = 0 \) then, at this point:

\[ \frac{d(Psec)}{dt} = \frac{d(Pwo)}{dt} \quad (2) \]

Because the level of \( Pexo \) per unit volume of culture does not change significantly during the 1-hr interval bracketing the turnover point, the rate of washout is given by

\[ \frac{d(Pwo)}{dt} = -\frac{d(Pexo)}{dt} \]

where \( D \) is the dilution rate: and rearranging

\[ \frac{d(Pwo)}{dt} = -(D)(Pexo)dt \]

and integrating

\[ \Delta Pwo = -(D)(Pexo)\Delta t \quad (3) \]

Fig. 2. Penicillinase induction in a culture of *Bacillus licheniformis* 749 growing under phosphorus limitation at a dilution rate of 0.137 hr\(^{-1}\). At 0 hr, sufficient benzyl-penicillin was added to the growth chamber to yield an initial concentration of 0.02 units per ml. The inset shows the data for the first 8 hr replotted in the manner used by Collins (4). Symbols: ○, exopenicillinase; ●, cell-bound penicillinase, □, total penicillinase; solid lines, observed values; broken lines, values after 6 hr predicted by simple washout without further synthesis or secretion of penicillinase.
yields the amount of exoenzyme washed out during this interval. And since integration of equation 2 yields

\[ \Delta P_{\text{wo}} = \Delta P_{\text{sec}} \]

\( \Delta P_{\text{sec}} \), the amount of enzyme secreted during the turnover interval, has also been obtained. For Fig. 2, \( \Delta P_{\text{sec}} \) per hr is 27.4 units. It is now possible to examine the relationship of the concentration of cell-bound enzyme (Pcb) to the secretion process.

If the secretory apparatus is not rate-limiting but, instead, the secretion rate is dependent on the concentration of the membrane-bound precursor, then

\[ \frac{d(P_{\text{sec}})}{dt} = k_{\text{sec}}(P_{\text{cb}}) \]

During the turnover period, Pcb is also very nearly constant; thus, the same operations that yielded equation 3 can be applied, yielding

\[ \Delta P_{\text{sec}} = k_{\text{sec}}(P_{\text{cb}})(\Delta t) \]  \hspace{1cm} (4)

and, for the turnover interval where Pcb is 530 units, \( k_{\text{sec}} \) will be 0.047. If \( P_{\text{sec}} \) is dependent on Pcb, this value for the secretory constant should predict the value of \( P_{\text{sec}} \) at different times during the course of the induction.

At 8.5 hr after induction, when induction is largely exhausted but Pcb is large, Pexo is 170 and is essentially a linear function of time at this point, so that the \( P_{\text{wo}} \) per hour can be approximated from equation 3 as 22 units. From equation 1, \( \Delta P_{\text{sec}} = \Delta P_{\text{exo}} + \Delta P_{\text{wo}} \) and from Fig. 2, \( \Delta P_{\text{exo}} \) for this interval is -9 units per ml per hr; thus the observed \( \Delta P_{\text{sec}} \) is approximately 13 units per ml per hr. Pcb at 8.5 hr after induction is 410, consequently \( (P_{\text{eb}})(k_{\text{sec}}) \) yields a calculated value for \( \Delta P_{\text{sec}} \) of 19 units per ml per hr.

Pcb has the same value (410) at 2 hr in the ascending phase of the induction and repeating the calculations described above, \( \Delta P_{\text{exo}} \) is +58 per hr. \( \Delta P_{\text{wo}} \) is 15 per hr; thus, \( \Delta P_{\text{sec}} \) from the curve has the value 73 units per ml per hr, while \( (P_{\text{eb}})(k_{\text{sec}}) \) is again 19 per hr.

Thus, the \( k_{\text{sec}} \) of 0.047 calculated from the turnover point does not exactly describe the secretion on either limb of the curve, being somewhat too large to describe the behavior on the descending limb and much too small to describe the behavior on the ascending limb. Consequently, the secretion rate is not strictly dependent anywhere on the curve on Pcb, the concentration of membrane-bound penicillinase. The calculation made from the turnover point must either represent a composite of rate constants (i.e., more than one secretory process is involved and the proportion of these processes changes during induction with faster processes dominating in early induction), or a single process is involved whose rate changes with time, being most rapid early in induction.

Values of \( k_{\text{sec}} \) were derived for the secretion process in the magnon constituent mutant, where the steady-state equations 1 to 4 apply exactly, and found to vary from 0.047 to 0.075 with different dilution rates, suggesting that the slower secretory process or processes are predominant in the mutant.

After the 6th hr, the observed levels of cell-bound enzyme and exoenzyme were higher everywhere on the curves than the levels calculated for simple washout. Thus, both synthesis and secretion continued after induction exhaustion at levels approaching those in the preinduction state. Because of this continuing synthesis, the conversion of cell-bound enzyme to exoenzyme (which would have been indicated by the observed levels of cell-bound enzyme being lower than the levels calculated for simple washout) was not clearly evident here, as it was in Pollock's (14) experiments and in the experiments described in the next section.

Effects of nutrient arrest and chloramphenicol following induction of penicillinase. The secretion of cell-bound enzyme in the period when synthesis is decreasing was then examined for susceptibility to nutrient arrest and chloramphenicol (Fig. 3).

In the interval from 4 to 7.5 hr after adding the inducer, the exoenzyme concentration was essentially constant, indicating that it was being secreted at a rate close to the dilution rate of the culture. Cell-bound enzyme decreased at a rate less than that predicted for simple washout. Consequently, penicillinase was still being synthesized, although at a rate inadequate to maintain a constant level of cell-bound enzyme.

Nutrient arrest immediately halted detectable secretion of exoenzyme without the lag observed in the constitutive mutant (i.e., exoenzyme was being secreted at the rate of 73 units per hr in the prearrest period and, if secretion at this rate had continued during the arrest period, the level of exoenzyme would have risen 95 units by the end of the period). The level of cell-bound enzyme also remained constant, indicating that the synthesis of penicillinase had stopped and that there was no conversion to exoenzyme taking place during the arrest interval.

When nutrient flow was resumed, the rate of decrease of cell-bound enzyme was faster and that of exoenzyme slower than predicted by washout; thus, secretion had resumed and cell-bound enzyme was clearly being converted to exoenzyme. When chloramphenicol was then added to the
culture, there was little or no apparent effect upon secretion.

The effects of nutrient arrest and chloramphenicol addition were then examined during the early stages of induction (Fig. 4). Sample variance was greater, but essentially the same response to nutrient arrest was observed as had been observed with the magnoconstitutive mutant. Cell-bound penicillinase and exopenicillinase increased for approximately 40 min following arrest, thus differing from the behavior observed when nutrient arrest was imposed in the latter phases of induction (Fig. 3). Synthesis and secretion then stopped; with nutrient resumption, both started again.

Addition of chloramphenicol inhibited the formation of penicillinase but did not prevent its secretion. In other experiments, chloramphenicol did, however, reduce the secretion rate significantly.

Gel filtration and electrophoresis of exopenicillinase. Since Lampen (11) has shown that penicillinase release by cell damage can be recognized by the apparent high molecular weight of the exoenzyme (which in this circumstance is bound to cell fragments), it was desirable to demonstrate that the extracellular penicillinase obtained in the continuous-culture filtrates was of the proper molecular weight for secreted enzyme rather than enzyme released due to cell damage.

The supernatant fluid of a continuous culture of the magnoconstitutive mutant and the material precipitated from this supernatant fluid by saturation with ammonium sulfate were filtered through a column of Sephadex G-75. Over 95% of the penicillinase activity, both in the crude supernatant fluid and in the ammonium sulfate precipitate, appeared in a single peak. The apparent molecular weight of the enzyme was 27,000, consistent with what might be expected from normal secretion (16). Additionally, the penicillinase activity in culture filtrates of the inducible strain from both the early and late phases of induction exhibited the same elution volume from the gel.

The ammonium sulfate precipitate from the

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**Fig. 3. Effect of nutrient arrest and chloramphenicol upon cell-bound (○) and exopenicillinase (○) concentrations late in induction.** Bacillus licheniformis 749, growing under phosphorus limitation in continuous culture at a dilution rate of 0.122 hr⁻¹, was induced at 0 hr with benzylpenicillin at an initial concentration of 0.02 units per ml. At 7 hr 40 min, the nutrient flow to the growth chamber of the culture apparatus was stopped (first arrow). At 9 hr 5 min, the nutrient flow was restarted (second arrow). At 12 hr 50 min, sufficient chloramphenicol was added to the growth chamber to yield an initial concentration of 50 µg per ml (third arrow).

**Fig. 4. Effect of nutrient arrest and chloramphenicol upon cell-bound (○) and exopenicillinase (○) concentrations early in induction.** Bacillus licheniformis 749, growing under phosphorus limitation in continuous culture at a dilution rate of 0.122 hr⁻¹, was induced at 0 hr with benzylpenicillin at an initial concentration of 0.02 units per ml. At 1 hr 30 min, the nutrient flow to the growth chamber of the apparatus was stopped (first arrow). At 2 hr 45 min, the nutrient flow was restarted (second arrow). At 3 hr 35 min, sufficient chloramphenicol was added to the growth chamber to yield an initial concentration of 50 µg per ml (third arrow).
magnocinstitutive strain and the peak of activity obtained by gel filtration of this material were subjected to acrylamide gel electrophoresis concurrently with a preparation (kindly supplied by M. G. Sargent and B. K. Ghosh of the Institute of Microbiology) made from the magnocinstitutive mutant by the method of Pollock (16). The activity in both the ammonium sulfate precipitate and the gel filtrate migrated as a single band moving 4.25 cm toward the anode in 4.5 hr. The activity in the Pollock-type preparation yielded two bands of activity migrating 4.25 and 4.5 cm toward the anode.

The preparations thus were electrophoretically similar. The occurrence of more than one band in the Pollock-type preparation is characteristic of B. licheniformis penicillinase (16), and the lower activity of the preparations from the continuous culture available for analysis may have prevented detection of minor components.

**DISCUSSION**

Measurement of the amounts of cell-bound and exocellular penicillinase, alkaline phosphatase, and nuclease in B. licheniformis, and of alkaline phosphatase and nuclease in B. subtilis showed that alkaline phosphatase and nuclease, unlike penicillinase, do not exist in a long-lived, cell-bound form in B. licheniformis and that the level of the cell-bound form of these two enzymes varies from strain to strain in B. subtilis. Consequently, it is apparent that a long-lived, cell-bound intermediate is not mandatory in the secretion of all exoenzymes by Bacillus.

In the case of penicillinase, the evidence obtained in this study sustains the observation made by Pollock (14) that the long-lived, membrane-bound penicillinase can be the precursor of the exoenzyme when induction has become exhausted in the inducible strain and penicillinase synthesis has diminished greatly. A minimum, secretion-dependent half-life of the membrane-bound penicillinase for these conditions can be estimated from the experimental data described in Fig. 2. The secretion rate in the late phase of induction can be approximated as the product of the concentration of the cell-bound enzyme times a secretory rate constant $k_{sec}$ with a value of 0.035 hr$^{-1}$. (However, as noted above, a $k_{sec}$ derived from the assumption that the secretion rate is a function of the concentration of cell-bound enzyme throughout the entire course of the induction does not, in fact, closely describe the secretion rate at any time.) Since the doubling time in that experiment was 5 hr, the secretion-dependent half-life of the membrane-bound penicillinase in the late phase of induction was on the order of four cell doubling times.

In the early phase of induction, the secretion-dependent half-life of any membrane-bound intermediate was obviously much shorter. For instance, 48 min after adding inducer, the concentration of cell-bound enzyme was 100 units per ml. The secretion rate at that time and for the succeeding 1 hr was 55 units per ml per hr. The cell-bound enzyme at 48 min thus had a probable half-life of less than 1 hr (0.2 doubling times). Also, as would be expected if membrane-bound precursors to exoenzyme were turning over rapidly during the early phase of induction, the onset of accretion of cell-bound enzyme and secretion of exoenzyme occurred nearly simultaneously, as did the occurrence of the maximal accretion and secretion rates.

Consequently, a membrane-bound intermediate with a half-life that is an appreciable fraction of the cell doubling time is also not mandatory in the secretion of penicillinase.

Reconciliation between our results and Collins’ (4) estimate that the membrane-bound precursor to exopenicillinase has a mean residence time of 1.6 cell doublings may possibly lie in the fact that his published studies are based on only one growth rate, the maximum for the conditions he employed, and, thus, that the value he obtained might be found to vary with growth rate. Replotting our data on the appearance of cell-bound enzyme and exoenzyme in early induction in the manner used by Collins for his data masked the nearly simultaneous initiation of accretion and secretion. If the secretion rate is a function of both the rate of penicillinase synthesis and the level of membrane-bound enzyme, as suggested below, one can infer that secretion rates and residence times calculated from studies with the mutant are composites of the rates and residence times characteristic of several secretion stages or routes.

A feature of penicillinase secretion that emerges from these studies is the relatively close coupling of the rates of penicillinase synthesis and secretion. From the experiment described in Fig. 2, secretion is most rapid during and shortly after the period when penicillinase synthesis is most rapid, then it undergoes a five-to sixfold reduction in rate during the 30- to 60-min interval when the induction becomes exhausted and the rate of penicillinase synthesis declines rapidly and thereafter approaches a rate that is a function of the amount of cell-bound enzyme, as penicillinase synthesis falls to a low level.

There are, thus, two factors affecting the secretory rate: the rate of penicillinase synthesis and the level of membrane-bound enzyme. This could be inferred from the kinetic evidence, but it was directly observable in the experiments involving nutrient arrest and chloramphenicol. Nutrient
arrest halted secretion in the constitutive mutant and in the early phase of induction only after a 30- to 50-min lag, in parallel with the observed persistence of synthesis during this interval. However, nutrient arrest immediately halted detectable secretion late in induction, when penicillinase synthesis had fallen to low levels. Further, chloramphenicol had little or no effect upon the slow secretion rate late in induction, but reduced the rapid rate seen early in induction and the rate in the constitutive mutant, toward a level approximating the slow secretion rate.

Pollock (15) originally found that penicillinase synthesis showed resistance to actinomycin inhibition and interpreted this to indicate a long half-life for the penicillinase messenger ribonucleic acid. Yudkin (19) also reported that penicillinase synthesis was inhibited by actinomycin after a lag in the constitutive mutant 749/C, but was rapidly inhibited in the inducible parent strain 749. He concluded that penicillinase messenger ribonucleic acid has a shorter half-life in the inducible than in the constitutive strain. If the resistance of penicillinase synthesis to nutrient arrest we observed in the constitutive strain and in the inducible strain before induction exhaustion can be attributed to the relatively long half-life of the penicillinase messenger, then our results suggest that this half-life was comparable in both cases. In the case of the inducible strain after induction exhaustion, however, the rapid elimination of residual penicillinase synthesis by nutrient arrest could mean that the messenger had a shorter half-life.

It must be emphasized, however, that the reasoning just presented to explain the elimination by nutrient arrest of the secretion closely coupled to penicillinase synthesis provides no understanding of the manner in which the slow release of the cell-bound enzyme is halted. This release was not inhibited by chloramphenicol (which halted synthesis promptly) and does not appear to be dependent upon protein turnover. Since phosphorus is the growth-limiting factor in the medium, some process requiring phosphorus turnover may well be essential for secretion of the major pool of cell-bound penicillinase.

The observed secretion rate and apparent half-life of the membrane-bound intermediate thus represent composites, with synthetic rate being the dominant factor in early induction, when levels of cell-bound enzyme are relatively low, leading to an apparently short half-life of the membrane-bound intermediate. In late induction, and in the constitutive mutant, when the levels of membrane-bound enzyme are relatively high in proportion to synthetic rate, the level of membrane-bound enzyme determines the rate and leads to an apparently long half-life of the membrane-bound intermediate.

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LITERATURE CITED

in *Bacillus subtilis* and *Bacillus cereus*. Biochim. Biophys. Acta 76:80-93.


