Characteristics of Penicillinase Release by Washed Cells of *Bacillus licheniformis*

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Saline-washed cells of *Bacillus licheniformis* strain 749/C (constitutive for penicillinase) were able to release exopenicillinase in the presence of concentrations of chloramphenicol that prevented protein synthesis completely. The release reaction was strongly pH-dependent, occurring at a faster rate at alkaline pH in anionic or cationic buffers than at neutral pH. A strongly pH-dependent release reaction was noted in growing cells also. The reaction in washed cells can be stopped completely by changing the pH to 6.0. Within 30 min at pH 9.0, about 55% of the cell-bound penicillinase was released; thereafter, release continued at a greatly reduced rate. Suspensions of washed cells retained their capacity to release penicillinase at pH 9.0 for 90 min. Penicillinase released at pH 9.0 from either cells or protoplasts was not readsorbed over a 60-min period after changing the pH to 6.0. The release reaction was strongly temperature-dependent. We examined the effect of a large number of metabolic inhibitors and other compounds on the pH-dependent release phenomenon. Quinacrine hydrochloride, chloroquine diphosphate, and chlorpromazine hydrochloride reduced secretion substantially at 10⁻⁴ M. Deoxycholate and Triton X-100 were active at 10⁻⁴ M, but tungstate, arsenate, and molybdate had small effects at 10⁻³ M. The rate of exopenicillinase release at pH 9.0 from fully stabilized protoplasts was one-half that of intact cells. Protoplasts lysed in hypotonic media or detergents showed even greater reduction in releasing activity. Penicillinase released from washed cells at pH 7.5 or 9.0 appeared to be derived from the periplasmic tubule and vesicle fraction that was released by protoplast formation.

Pollock (23) indicated that a prerequisite for the study of enzyme secretion was to dissociate enzyme synthesis from liberation of the enzyme. This was achieved by using the penicillinase of *Bacillus licheniformis* (23). By providing a small amount of inducer (benzylpenicillin), a burst of penicillinase synthesis was obtained in a growing culture followed by a period of greatly reduced synthesis in which accumulated cell-bound penicillinase continued to be released as exoenzyme. A study was made of factors affecting this release process (24). In kinetic terms, it may be said that Pollock had demonstrated a rate-limiting reaction between enzyme synthesis and secretion. Subsequently, Lampen (19) has shown that a similar release occurs from washed cells.

Pollock (23) attached considerable importance to distinguishing between enzyme secretion and damage release; subsequently, Lampen (18) indicated that exoenzyme could be unequivocally differentiated from any preparation of cell-bound enzyme by an electrophoretic criterion.

Both Kushner and Pollock (17) and Lampen (18) have expressed the view that the cell-bound enzyme may be covalently bound to a membrane protein and that the release reaction may involve hydrolysis of a covalent bond. Lampen (18) showed that conventional methods of dissociating noncovalently bound proteins [i.e., detergents, urea (7.2 M), ethylenediaminetetraacetate (EDTA), butyl alcohol, high salt concentrations, and ultrasound] failed to liberate exopenicillinase, whereas trypsin treatment converted the bound form to exopenicillinase.

A study of the nature of exopenicillinase release from washed cells of *B. licheniformis* strain 749/C is presented in this communication.

**MATERIALS AND METHODS**

Organisms, inocula, and media. *B. licheniformis* strain 749/C (constitutive for penicillinase) was employed for all studies in this communication (7). Inocula were prepared as described previously (10). CH/S medium buffered to pH 5.5, 6.5, or 7.5 was used as indicated previously (10); 0.05 M phosphate was employed for pH 5.5 medium to give greater buffering capacity. Maltose, separately sterilized (2
mg/ml, final concentration), was added to growth medium to induce α-glucosidase (23).

Bacterial growth was expressed in dry weight equivalents of opacity, and was determined as described previously (27).

Penicillinase was assayed by the method of Sargent (27) and α-glucosidase by the method of Pollock (23). Protein was assayed by the method of Lowry et al. (21).

**Washed cell experiments**. Cells from cultures 3 to 5 hr old [grown on (pH 6.5) CH/S medium, unless otherwise stated] were spun down, washed once in 0.85% NaCl at room temperature (26 C), and suspended to give a dry weight equivalent of 1 mg/ml in 0.85% NaCl. Samples (1 ml) were added to test solutions in 50-ml Erlenmeyer flasks to give a final volume of 10 ml. Final cell concentrations were 0.1 mg/ml. Most test systems contained 0.01 M (pH 9.0) borate-NaOH buffer, 0.001 M magnesium chloride, and 50 μg of chloramphenicol per ml (BMC). Flasks were incubated with gentle rotatory shaking at 30 C. In certain instances, the pH 9.0 borate buffer was replaced by 0.01 M (pH 7.5) tris(hydroxymethyl)-aminomethane (Tris)-chloride (TM) or by 0.01 M (pH 6.5) sodium phosphate buffer (PMC). All treatments of washed cells were for 30 min.

**Gel filtration**. Exopencillinase was distinguished from membrane-bound forms by its ability to penetrate Sephadex G-75. Samples (1 ml) containing 0.5 ml of Blue Dextran 2000 and 0.5 ml of enzyme preparation were applied to columns (1 by 22 cm) equilibrated in 0.1 M NaCl. The columns were eluted with the same solvent, and fractions before and including the blue band were discarded. The 10 ml, eluted subsequently, was collected and assayed for exopencillinase. Recoveries of about 100% were obtained with test samples of exopencillinase.

Accurate estimates of molecular weight have been made with longer columns (46.5 by 2 cm) by the method of Andrews (1). The solvent system described above was used. The column was calibrated with Blue Dextran 2000, ovalbumin (molecular weight 45,000), soybean trypsin inhibitor (molecular weight 21,400), and horse heart cytochrome c (molecular weight 12,400); the molecular weights are from Andrews (1). The elution volume of each protein was determined separately, 3-mg quantities being applied to the column together with Blue Dextran. The column was eluted at room temperature, and fractions of about 2 ml were collected with a drop counter. Optical density was recorded at 230 nm. Elution volumes were calculated as the volume between addition of sample and the peak protein concentration.

**Protoplast formation**. Protoplasts were prepared in a medium containing 0.75 M sucrose, 0.02 M (pH 6.5) sodium phosphate buffer, 0.001 M magnesium chloride, and 100 μg of lysozyme per ml (PM). Samples were incubated with gentle shaking at 30 C for 20 min and were then centrifuged at 12,000 × g for 15 min. The sediment contains protoplasts (FI) and the supernatant fraction penicillinase-containing particles (FP; 27).

**Chemicals**. Soybean trypsin inhibitor and egg albumin were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Horse heart cytochrome c, chloroquine, and dextran sulfate (molecular weight 2,000,000) were obtained from Sigma Chemical Co., St. Louis, Mo. Phenylmethylsulfonylfluoride (PMSF), heparin, Triton X-100, and spermine were obtained from Calbiochem, Los Angeles, Calif. Ouabain, quinacrine dihydrochloride (also known as aetrin) and 1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride chloromethyl ketone (TLCK) were obtained from Mann Research Laboratories, Amobarbital (also known as Amytal) was obtained from K & K Laboratories, Jamaica, N.Y. Chlorpromazine hydrochloride was obtained from Smith, Kline & French Laboratories, Philadelphia, Pa. p-Nitrophenyl-α-D-glucoside was obtained from General Biochemicals Corp., Chagrin Falls, Ohio.

**RESULTS**

**Effects of pH on penicillinase distribution during growth**. The effect of the pH of the growth medium on the distribution of penicillinase is illustrated in Fig. 1. A plot of total penicillinase per milliliter against the dry weight of cells present indicates that the specific activity of cells at a comparable stage in growth is constant irrespective of pH over the range 5.5 to 7.5. The doubling times of the cells at pH 5.5, 6.5, and 7.5 were 1.8, 1.3, and 1.2 hr, respectively, during the period of logarithmic growth.

In cultures starting from small inocula (as in these experiments), average specific activity varied from 5,200 to 7,500 units per mg (dry weight). These are higher than the values given in the literature (3,000 to 5,000 units per mg), although we have found that the latter are typical values with large inocula (7).

The fraction of total enzyme secreted, however, varied markedly over this pH range. At about 0.1 mg of cells per ml, enzyme in the medium represents about 13, 23, and 36% of the total at pH 5.5, 6.5, and 7.5, respectively; at a later stage in growth, the amount in the medium rises to about 20, 33, and 50% at 0.5 mg/ml (Fig. 1).

While the differential rate of synthesis was the same at all pH levels tested, the release was clearly pH-dependent, with penicillinase accumulating in the cells at lower pH. Previous studies have indicated that no free exoenzyme is present in the cell and that it is predominantly membrane-bound (17, 18).

**Penicillinase released from washed cells**. When cells grown at pH 6.5 were transferred to pH 9.0, under conditions in which protein synthesis is abolished (chloramphenicol, 40 μg/ml), they released about 55% of their cell-bound penicillinase in 30 min (Fig. 2), whereas at pH 6.5 release was very slow. Clearly the release reaction can be stopped more or less completely by a pH change;
released penicillinase is not readorsorbed within 30 min of the transition to lower pH.

This latter point was demonstrated more rigorously with protoplasts as well as cell suspensions. Protoplasts (1 ml) from washed cells were suspended in 8 ml of BMC for 30 min (Fig. 2). The pH was then changed to pH 6.0 with 1 ml of 1 M potassium dihydrogen phosphate, and the incubation at 30 C was continued for 60 min. At 30 and 60 min after adding the phosphate, the amount of exoenzyme remaining in the preparation was determined with small columns of Sephadex G-75. No decrease in the amount of exoenzyme was observed with protoplasts or intact cells. A control containing protoplasts or cells, incubated at 30 C with potassium dihydrogen phosphate, added prior to pH 9.0 buffer, failed to release exoenzyme.

Although the time course of penicillinase release at pH 9.0 was not completely linear for 30 min, we chose this time interval for studies of factors affecting the release process because the data obtained at this point were more reliable. The amount of penicillinase released in 30 min may vary between 40 and 60% of the cell-bound level in different experiments, although differences of 10% between treatment and control are significant.

The elution pattern on a large Sephadex G-75 column of penicillinase release at pH 9.0 is illustrated in Fig. 3. Practically all the enzyme penetrates the gel and is eluted in a single, apparently homogeneous peak with ratio of Ve/Vo (1) of 1.75. Partially purified exopenicillinase prepared by the method of Pollock (25) gives a ratio of 1.72. The molecular weights of exopenicillinase and penicillinase released by pH 9.0 treatment were calculated to be 23,800 and 22,700, respectively; a difference that is probably not significant.

To estimate damage release of other proteins, we have measured release of α-glucosidase (23) and total protein. Unfortunately, under these conditions (pH 9.0) released α-glucosidase was partially inactivated. The level of cell-bound α-
glucosidase, however, decreased by only 5% during 2 hr of incubation at pH 9.0.

The amount of protein (material reactive with the Lowry reagent) released at pH 9.0 was extremely small and amounted to less than 5 µg/ml (21). Specific activities of penicillinase released have been in the range of 50 to 150 units of protein per µg, which represents 15 to 40% pure enzyme [calculated from Pollock's figure for specific activity, 325 units/µg (25)]. This value for the purity of the released enzyme is almost certainly an underestimate because smaller polypeptides sensitive to the Lowry reagent are probably released as well.

In most subsequent experiments, the amount of exopenicillinase in a sample was determined with small columns of Sephadex G-75. Unless otherwise stated, penicillinase released at pH 9.0 was predominantly (> 80%) exopenicillinase.

Stability of release reaction. If cells are incubated in saline at 30 C for 90 min, they remain capable of releasing 55% of their cell-bound penicillinase as exoenzyme in 30 min at pH 9.0. A small amount of penicillinase is released in saline, but the sum of this and the penicillinase subsequently released in 30 min at pH 9.0 amount to about 55% of the cell-bound penicillinase. Therefore, the release process is stable; this may indicate that the reason why penicillinase release in washed cells declined so sharply after 30 min at pH 9.0 (Fig. 2) is that part of the cell-bound penicillinase is not available to the release process (Table 3).

Effect of pH on penicillinase release. The effect of pH in Tris and borate buffer is illustrated in Fig. 4. Penicillinase release is evidently favored by alkaline pH both in cationic and anionic buffers. There is no evidence for a reduced rate of penicillinase release at high pH.

Effect of temperature. Penicillinase liberation was very slow at 0 C. If cells are washed in ice-cold saline, the subsequent rate of release of penicillinase at 30 C is depressed, although ultimately the same amount of enzyme is liberated (Fig. 2).

Effect of metabolic inhibitors and other substances on penicillinase release. To determine the effect of a variety of compounds on penicillinase release, washed cell suspensions were incubated for 30 min in 0.01 M (pH 9.0) borate-NaOH buffer containing 50 µg of chloramphenicol per ml, and the various additions were also adjusted to pH 9.0. In each case, the amount of penicillinase liberated was determined and compared with the control with no additions.

There have been reports that a variety of organic ions affect release of penicillinase from washed cells of Staphylococcus aureus (4, 5) and of α-amylase from B. subtilis (29). We examined the effect of borate, citrate, phosphate, Tris, glycine, α-glycerophosphate, and succinate on our system in 0.1 M concentrations. In each case, the amount of penicillinase released was within 10% of the control without additions.

FIG. 3. Elution pattern on Sephadex G-75 of penicillinase released from B. licheniformis at pH 9.0. Washed cells (10 ml) were added to 40 ml of BMC to give a final cell concentration of 2 mg/ml. Cells were removed by centrifugation after 30 min. The supernatant fluid was fractionated on a Sephadex G-75 column (46.5 by 2 cm). Elution volumes of markers and sample are designated by (a) Blue Dextran 2000; (b) partially purified exopenicillinase; (c) penicillinase released from washed cells by pH 9.0 treatment; (d) soybean trypsin inhibitor.

FIG. 4. pH dependence of penicillinase release from washed cells. Samples (1 ml) of washed cells in 0.85% NaCl were added to flasks containing 9-ml quantities of 0.1 x Tris (□) or 0.1 x boric acid (○), adjusted to pH between 7 and 10 with NCl and NaOH, respectively. Samples were incubated for 30 min at 30 C, and then total and supernatant penicillinase was determined. The pH shown was the average of pH before and after treatment.
Inhibitors of protein synthesis, energy metabolism, and other enzyme systems were tested. Preliminary experiments showed that chloramphenicol had no effect on the phenomenon (Table 1), thus all other inhibitors were tested in the presence of chloramphenicol to prevent penicillinase synthesis during the experiment. Many well-known metabolic inhibitors, including inhibitors of respiration and energy metabolism were inactive, with a few provocative exceptions. Quinacrine, chloroquine, and chlorpromazine were fairly potent inhibitors at $10^{-4}$ M. Deoxycholate and Triton X-100 were less active in this range, and tungstate, molybdate, and arsenate caused slight inhibition at very high concentrations, although fluoride was ineffective. Mercaptoethanol, glutathione, and spermine had a slight stimulatory effect at pH 9.0, but there was no stimulation of release at pH 6.5. Dextran sulfate gave some inhibition at 5 mg/ml, which is comparable to the observation of Coles and Gross (4, 5); heparin, another macroanion was without significant effect.

To characterize the quinacrine-induced inhibition of penicillinase release, we attempted to overcome it by treating the cells with certain agents at a concentration of $10^{-4}$ M before adding buffer or inhibitor. Subsequently BMC buffer and quinacrine ($10^{-4}$ M final concentration) were added, and the cells were incubated for 30 min. Quinacrine alone gave approximately 40% inhibition of penicillinase release. Adenosine triphosphate, diphosphate and monophosphate, flavine adenine dinucleotide, flavine mononucleotide, riboflavin, spermine, diethylbarbiturate, and amobarbital were unable to overcome this inhibition.

**Release from protoplast preparations.** Exoenzyme release was examined in osmotically stabilized protoplast preparations, in protoplast preparations lysed in water or detergents, and in cell fractions (Table 2). The amount of penicillinase released by untreated protoplast preparations at pH 9.0 in 30 min was approximately one-half of that released by intact cells. Protoplasts (lysed in water) release one-third of that released by intact cells in the same time interval, but lysis by detergents abolishes exoenzyme release almost completely (19). We have also tested the cell fractions described by Sargent, Ghosh, and Lampen (27). Purified protoplasts (FII), although containing about 40% of the cell-bound penicillinase, release very little exoenzymic penicillinase, but the particle fraction (FI) containing the penicillinase secretory apparatus releases more exoenzyme. However, the sum of the exoenzyme produced by purified protoplasts (FII) and particle fraction (FI) is only 32% of that released by the whole cell in 30 min and only 59% of that from the stabilized protoplast preparation.

**Location of penicillinase released from washed cells.** We have shown that the cell-bound penicillinase is located both in a periplasmic particle fraction and in the plasma membrane (2). Operationally, these were distinguished by protoplast formation, one fraction being released and the other remaining tightly bound to the protoplast. It was of interest, therefore, to demonstrate whether one of these pools was depleted prefer-
Table 2. Release of penicillinase from protoplasts, lysed protoplasts, and cell fractions

<table>
<thead>
<tr>
<th>Prep</th>
<th>Penicillinase (at 30 min)</th>
<th>Exoenzyme released in 30 min (units/ml)</th>
<th>Exoenzyme released in 30 min (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells in BMC</td>
<td>395</td>
<td>187</td>
<td>47.3</td>
</tr>
<tr>
<td>Protoplasts in sucrose-BMC (stabilized)</td>
<td>395</td>
<td>91</td>
<td>23.0</td>
</tr>
<tr>
<td>Protoplasts in BMC (lysed)</td>
<td>390</td>
<td>56</td>
<td>14.4</td>
</tr>
<tr>
<td>Protoplasts in sucrose-BMC plus 0.1% deoxycholate (lysed)</td>
<td>360</td>
<td>21</td>
<td>6.0</td>
</tr>
<tr>
<td>Protoplasts in sucrose-BMC plus 0.1% Triton X-100 (lysed)</td>
<td>360</td>
<td>23</td>
<td>6.4</td>
</tr>
<tr>
<td>Particle fraction (FI) in sucrose-BMC (stabilized)</td>
<td>232</td>
<td>43</td>
<td>18.5</td>
</tr>
<tr>
<td>Purified protoplasts (FII) in sucrose-BMC (stabilized)</td>
<td>153</td>
<td>9</td>
<td>5.9</td>
</tr>
<tr>
<td>Sum of FI and FII</td>
<td>385</td>
<td>52</td>
<td>13.5</td>
</tr>
</tbody>
</table>

* Suspensions of washed cells in 0.85% NaCl and of protoplasts in PM were prepared to give final densities equivalent to 1 mg (dry weight) of cells/ml. Portions (1 ml) were added to 9 ml of solutions indicated. The cell fractions FI and FII, previously described (27), were tested under equivalent conditions. After 30 min at 30°C, samples were centrifuged, and exoenicillinase in the supernatant fraction was determined with the short Sephadex G-75 columns.

During the pH-dependent release from washed cells, the particle fraction was, in fact, preferentially depleted both at pH 9.0 (BMC) and at pH 7.5 (TMC), with relatively little penicillinase release from the plasma membrane (Table 3).

**Discussion**

The experiments of Pollock (24) indicated that, in the secretion of penicillinase by *B. licheniformis*, there was a rate-limiting reaction that caused penicillinase to accumulate in the cell. Lampe (19) subsequently showed that exopenicillinase and cell-bound penicillinase are identical kinetically, but the latter, apparently, is composed of exopenicillinase bound to a membrane component. The rate-limiting reaction, therefore, involves release from the membrane. One aspect of this is revealed by our studies of growing cultures of *B. licheniformis* at pH levels between 5.5 and 7.5. The specific activity of growing cultures of *B. licheniformis* (units of penicillinase per milligram, dry weight) is constant within the pH range 5.5 to 7.5. The proportion of penicillinase found in the medium, however, is strongly dependent on the pH of the medium (Fig. 1), indicating that there is a pH-dependent release step (24) which, when conditions are unsuitable for operation of the release mechanism, results in penicillinase accumulating in the cell.

To characterize this rate-limiting step, we examined release of exopenicillinase from washed nongrowing cells. When cells grown at neutral pH are incubated at a higher pH, a large proportion of their penicillinase is released very rapidly. The release is specific (i.e., very little intracellular enzyme is released); the released protein can contain 20 to 50% penicillinase, and the enzyme is the exoenzyme. Release is strongly pH-dependent and is markedly affected by temperature. This particular effect is complex; cells washed in cold saline and subsequently incubated at 30°C showed a reduced rate of secretion, suggesting that the low temperature treatment causes some kind of disorganization of the release system.

Cells incubated in 0.85% NaCl for 90 min have the same capacity to release penicillinase as they have when they are freshly prepared, although, in all cases, the rate drops sharply after 50% of the cell-bound penicillinase has been released. This may indicate that part of the cell-bound penicillinase cannot be removed by the process, a point of view that is supported by studies of the origin of the penicillinase released (Table 3). The reaction appears to be irreversible, but can be stopped at any moment by adjusting the pH to 6.0 (Fig. 2).

To implicate an enzyme in penicillinase secretion, as suggested by Pollock (24), we examined a wide range of metabolic inhibitors (Table 1), including specific inhibitors of protein synthesis, respiration, proteolytic and sulphydryl enzymes, and others. There is a reasonable possibility that these potential inhibitors will reach the site of penicillinase release, since the cell-bound enzyme is readily accessible to its substrate and, at least in part, to antipenicillinase antisera (17). The phosphatase inhibitors, tungstate, molybdate, and arsenate, cause slight inhibition of release at high concentrations; mercaptoethanol, glutathione, and spermine were mildly stimulatory at pH 9.0, but were ineffective at pH 6.5. These effects are probably of little consequence, but we have found three fairly potent inhibitors, quinacrine, chlorpromazine, and chloroquine. The three compounds are structurally similar; all three are tertiary amines with predominantly...
hydrophobic aromatic groups containing a single chlorine atom. They appear to have many biochemical properties in common. The literature concerning them is large, and their effects in biological systems seem to be as numerous. Thus, quinacrine and chlorpromazine are known to inhibit many flavine-containing enzymes and adenosine triphosphatase activities (3, 6, 8, 11-14, 16, 20, 22); but we have been unable to overcome this inhibition with flavine nucleotides and riboflavin or adenosine phosphates even in concentrations 10 times as great (as might have be expected if the release mechanism involved a flavine or adenosine triphosphatase). The three compounds are also known to inhibit other enzymes (9, 13, 15, 32). In addition, they have a large number of pharmacological effects that resulted in their use as anti-inflammatory agents, sedatives, local anesthetics, and for reversal of carbon tetrachloride poisoning. A number of investigators now interpret these effects in terms of one basic effect, i.e., stabilization of membranes. Thus, it is known that chloroquine and chlorpromazine stabilize lysosomes (11, 33), chlorpromazine stabilizes the erythrocyte at low osmotic pressures (28, 31), and chlorpromazine and quinine (a homologue of quinacrine) block nerve impulse transmission (11).

Several investigators have studied the capacity of chlorpromazine to penetrate monomolecular films of lipids (2, 30, 34), and it is clear that it raises the lateral pressure of a film, lowers the surface tension, and increases the charge on the surface (2).

With such a multiplicity of possible actions for the three inhibitors, no sound conclusions concerning the nature of the penicillinase release process can be reached, although a number of attractive possibilities are clearly indicated.

In certain respects, the phenomenon of release of penicillinase from washed cells that we have observed is similar to that observed by Coles and Gross (4, 5) in S. aureus (i.e., release is strongly pH- and temperature-dependent). There are, however, some marked differences. Thus, in S. aureus, enzyme release is markedly biphasic and appears to occur only in anionic buffers, and it is stimulated strongly by certain anionic polymers. In contrast, the release reaction in B. licheniformis is not biphasic, it occurs equally in anionic and cationic buffers (Fig. 4), and other organic ions of small or large molecules do not affect the reaction. Coles and Gross (4, 5) concluded that, in the first reaction, penicillinase is displaced from the cell surface by anions by virtue of their charge and, in the second step, organic ions are thought to activate an enzyme involved in penicillinase secretion.

Tsara and his co-workers (29) claim that glycine has a marked inhibitory effect on exoamylase production in B. subtilis as a result of inhibition of membrane synthesis. We were unable to demonstrate this phenomenon in our system.

Our data can be interpreted in two ways. (i) Penicillinase release may involve the enzymatic rupture of a covalent bond between penicillinase and a membrane component perhaps at a penicillinase-phosphate bond or a peptide bond. (ii) Penicillinase may be maintained by noncovalent bonds in the membrane in a transitory state before release, and during secretion penicillinase molecules separate from the structure.

At present no critical evidence is available distinguishing the two possibilities. The effects of inhibitors can be regarded in the former case to
be caused by inhibiton of the release factor and in the latter to result from stabilization of the membrane-penicillinase bonds. The effect of pH can be explained if the releasing enzyme has a high pH optimum or, in the latter case, if high pH values favor solubilization. The isoelectric point of penicillinase is about pH 6.0, a pH at which enzyme release from washed cells is slow. The decreased releasing activity in protoplast preparations can be explained in the former case if the release factor is partially soluble or is less effectively oriented to its substrate, the cell-bound enzyme. In the latter case, it can be argued that the periplasmic vesicles (27) assume a more stable form after release from the cell.

The data of Lampen (18) favor the view that penicillinase is covalently bound to the membrane, in which case the first alternative must be accepted. However, the data cannot be regarded as conclusive because they are essentially of a negative type. The final and perhaps most important consideration regarding the phenomenon of pH-dependent release of penicillinase from washed cells is its relevance to the secretion phenomenon in growing cells. This problem is being studied in our laboratory, and no clear answer is yet available. One fact, however, is clear; we are dealing with a system in which a membrane-bound enzyme is converted to its exoform under moderately well-defined conditions, and, whether it is physiologically significant or not (and it appears to be fairly specific), these conditions must have an important bearing on the forces maintaining the enzyme in the bound state at pH 6.0.

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LITERATURE CITED