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Vesicle Penicillinase of *Bacillus licheniformis*: Existence of Periplasmic-Releasing Factor(s)

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In earlier studies of the membrane-bound penicillinase of *Bacillus licheniformis* 749/C, the enzyme present in the vesicles that were released during protoplast formation and the enzyme retained in the plasma membrane of protoplasts appeared to differ (i) in their behavior on gel permeation chromatography in the presence or absence of deoxycholate and (ii) in their tendency to convert to the hydrophilic exoenzyme (Sargent and Lampen, 1970). We have now shown that these vesicle preparations contain a soluble, heat-sensitive enzyme(s) that is released along with the vesicles during protoplast formation. The enzyme will convert the vesicle penicillinase to a form that resembles exopenicillinase, and this conversion can be inhibited by deoxycholate under certain circumstances. Sedimentation of such vesicle preparations at 100,000 × *g* produces vesicles which contain penicillinase that behaves as the plasma membrane enzyme obtained from protoplasts. Exopenicillinases released by growing cells at pH 6.5 and by washed cells or protoplasts at pH 9.0 have the same NH_2-terminal residues (lysine and some glutamic acid); in addition, the various release systems show a parallel sensitivity to inhibition by deoxycholate, quinacrine, chloroquine, and *o*-phenanthroline. The formation of exopenicillinase (by cleavage of the membrane-bound enzyme) may well be dependent on the action of the releasing enzyme.

Bacillus licheniformis 749/C produces penicillinase (EC 3.5.2.6, penicillin amido-β-lactamhydrolase) as an extracellular hydrophilic enzyme (9) and as a hydrophobic cell-bound form(s) that is located on the outer surface of the plasma membrane of the protoplast (8) and is also associated with vesicular membrane components ("periplasmic vesicles") believed to arise from mesosomes (12). Although penicillinase appears to occur in three distinct locations, there is only a single structural gene for the enzyme (4).

Sargent and Lampen (15) converted exponential-phase cells to protoplasts with lysozyme, and solubilized penicillinase from the plasma membrane of the protoplast and from the vesicle fraction using 0.1% sodium deoxycholate (DOC) in conjunction with 0.05 M pyrophosphate buffer at pH 9.0 (DOC-PP). The extracts from the two fractions were compared by gel permeation chromatography. They reported that the two enzymes eluted with an apparent molecular weight of 45,000 on Bio-Gel A-5M in the presence of deoxycholate and suggested that both enzymes bind large amounts of the detergent. Contrasting behavior was observed in the absence of detergent. The plasma membrane enzyme apparently aggregated and eluted with a molecular weight of approximately 600,000; however, the crude vesicle penicillinase eluted identically with exoenzyme (which does not bind detergent [5]). Explanation of this anomalous behavior was further complicated by the demonstration that a crude preparation (DOC-PP extract) of plasma membrane enzyme, incubated with 25% KH_2PO_4 at pH 9.0, would subsequently chromatograph in large part as the exoenzyme, i.e., with a molecular weight of 24,000 in the presence and absence of detergent. The apparent molecular weight of exopenicillinase in deoxycholate-pyrophosphate buffer (pH 9.0) or in 0.05 M NaCl is 24,000 (5); in taurodeoxycholate-pyrophosphate (pH 6.5) it is 29,000 (16), which is the value expected on the basis of the amino acid sequence (2). The reason for the anomalous molecular weight at alkaline pH is unknown.

The differences in behavior of hydrophobic penicillinase preparations from the two locations were interpreted as resulting from confor-

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mational variations; the plasma membrane enzyme was thought to have the most hydrophobic conformation, and the vesicle enzyme was considered to be an intermediate in the conversion to the hydrophilic exopenicillinase. This concept led to the view that the formation and release of exoenzyme was the result of conformation shifts (14, 15) and, since production of exoenzyme occurs more rapidly at pH 9.0 than at pH 6.5 (14), these changes were thought to be facilitated by the more alkaline pH.

The membrane penicillinase of strain 749/C (isolated mainly from the plasma membrane) was subsequently shown to be a phospholipoprotein which has an NH_2-terminal sequence of 24 amino acids and a covalently bound phosphatidylserine that are not present in the exoenzyme (18, 19). This made it unlikely that the differences among the various forms of penicillinase were primarily the result of conformational variation. We therefore reexamined the nature of the vesicle enzyme and the process of exoenzyme release.

MATERIALS AND METHODS

Organism and growth conditions. *B. licheniformis* 749/C, a magnoconstitutive mutant of the penicillinase-inducible strain 749 (9), was maintained on slants of sporulation medium (13). Cultures were grown at pH 6.5 in casein hydrolysate-salts medium (12) from an initial cell density of 0.05 mg/ml to a final density of 0.7 mg/ml. Growth was measured as milligrams of cells (dry weight equivalent) per milliliter as described by Sargent and Lampen (15).

Preparation of mesosomal vesicles. Cultures in exponential growth were harvested at a cell density of 0.7 mg/ml; the ratio of cell-bound penicillinase to exoenzyme was usually maximal at this time. Cells were washed once with physiological saline and suspended at 15 mg/ml in a medium consisting of 0.75 M sucrose, 0.02 M potassium phosphate (pH 6.5), 0.001 M $MgCl_2$, lysozyme (100 μg/ml), and chloramphenicol (40 μg/ml) for 30 min at 30°C. Formation of protoplasts was monitored by phase-contrast microscopy.

The protoplasts were sedimented at 12,000 × *g*, and the supernatant fluid was decanted and spun at 20,000 × *g* for 20 min to remove any remaining protoplasts and large membrane fragments. The supernatant fluid was then spun at 100,000 × *g* for 2 h at 2°C to pellet the vesicles. Electron microscopy of negatively stained material showed that this pellet consisted mostly of vesicles. The release of vesicles (apparently by mesosomal eversion) during protoplast formation is relatively specific, in that the vesicle fraction contains about 15% of the total cell protein and 50 to 60% of the penicillinase but only 1 to 2% of the membrane nicotinamide adenine dinucleotide-dehydrogenase and 5% of the soluble cytoplasmic α-glucosidase (12).

Vesicles were also prepared as described by Sargent and Lampen (15). In this method, the protoplast suspension was centrifuged at 12,000 × *g* for 1 h at 2°C, and the supernatant fluid was applied to a column (2 by 40 cm) of fine-grade Sephadex G-25, equilibrated in 0.05 M NaCl, to remove the sucrose and salts of the protoplasting medium. The void volume fractions were pooled and concentrated by lyophilization.

Soluble periplasmic macromolecules. After sedimentation of the vesicles at 100,000 × *g* as described above, the supernatant fluid was applied to a column (2 by 40 cm) of fine-grade Sephadex G-25 equilibrated in 0.05 M NaCl. The void volume fractions, containing the soluble periplasmic macromolecules, were pooled and concentrated by lyophilization.

Gel permeation chromatography. This was performed at 4°C using columns (2 by 40 cm) of Bio-Gel A-5M equilibrated either in 0.05 M sodium pyrophosphate (pH 9.0) containing 0.1% deoxycholate (DOC-PP), or in 0.05 M NaCl. Flow rates of approximately 20 ml/h were maintained. Recovery of penicillinase was between 70 and 85% in all experiments.

Separation of exo- and membrane penicillinases. The method is based on the fact that the hydrophobic membrane enzyme forms stable complexes with detergent but the exoenzyme does not (5). If an anionic detergent is used, such as taurodeoxycholate, the complex binds very tightly to diethylaminoethyl-Sephadex at neutral pH, whereas the exoenzyme is loosely bound and is readily eluted with 0.5 M NaCl. The membrane enzyme can then be eluted with a neutral detergent (Triton X-100) and 0.5 M NaCl. The neutral detergent presumably replaces the anionic detergent in the complex, thus reducing sharply the affinity of the complex for the ion exchange resin.

In the usual procedure, samples (not exceeding 6,000 U of penicillinase) in 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 0.1% taurodeoxycholate were applied to a column (0.5 by 10 cm) of diethylaminoethyl-Sephadex A-50 equilibrated in the same buffer. The column was washed with 2 column volumes of the buffer, and the hydrophilic penicillinase was then eluted with 2 column volumes of the buffer containing 0.5 M NaCl. Finally, the hydrophobic penicillinase was removed with 2 column volumes of 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 0.1% Triton X-100 and 0.5 M NaCl. The fractions eluted with the three buffer mixtures were assayed for penicillinase activity (10; Table 1).

NH_2-terminal amino acid analysis. The dansyl (1-dimethylaminonaphthalene-5-sulfonyl)chloride procedure of Gray (7) was used for the identification of the NH_2-terminal amino acid of the hydrophilic penicillinases released (i) during exponential-phase growth of cells at pH 6.5 to 7.0, (ii) by washed cells suspended in pyrophosphate buffer (pH 9.0), and (iii) by protoplasts in osmotically supported medium at pH 9.0. The released enzymes were purified by the method of Pollock (9). At least 10 nmol of protein was used for dansylation, which was carried out at pH 9.0 either for 3 h at 37°C or overnight at 28°C. The dansyl derivatives of protein constituent amino acids were separated by thin-layer chromatography

TABLE 1. *Separation of exo- and membrane penicillinases by DEAE-Sephadex*[a]

Penicillinase (units on column)	Units of penicillinase eluted with:			% Recovery	
	Tris-TDC	Tris-TDC-NaCl	Tris-Triton-NaCl	Exopenicillinase	PM or Ves
Exo (4,000)	25	3,900	-	97	-
PM (4,590)	0	0	4,445	-	96
Ves (4,600)	0	14	4,490	-	97
PM (2,250) and Exo (2,000)	10	1,900	2,210	95	98
Ves (2,275) and Exo (2,000)	0	1,900	2,115	99	93

[a] The procedure was as given in Materials and Methods, using a maximum sample volume of 1.0 ml. The diethylaminoethyl (DEAE)-Sephadex A-50 column was eluted successfully with 0.05 M Tris-hydrochloride buffer (pH 7.5) containing (i) 0.1% sodium taurodeoxycholate (Tris-TDC), (ii) 0.1% taurodeoxycholate and 0.5 M NaCl (Tris-TDC-NaCl), and (iii) 0.1% Triton X-100 and 0.5 M NaCl (Tris-Triton-NaCl).

on Silica Gel G plates (Analtech Inc., Newark, Del.) (6) with chloroform-(*t*-amyl)alcohol-acetic acid (70:30:3, vol/vol), or chloroform-ethanol-acetic acid (38:4:3, vol/vol). Both solvent systems contained 0.05% 2-mercaptoethanol to reduce destruction of the dansyl amino acids on silica.

Penicillinase assay. Penicillinase activity was measured by the method of Sargent (10). One unit of enzyme hydrolyzes 1 μmol of benzylpenicillin per h at 30°C. Assay buffer consisted of 0.01 M KH_2PO_4 (pH 7.0) containing 0.1% sodium taurodeoxycholate.

Chemicals. Potassium penicillin G was obtained from Eli Lilly & Co., Indianapolis, Ind.; sodium deoxycholate, chloroquine diphosphate, lysozyme, *o*-phenanthroline, and the dansyl amino acid standards from Sigma Chemical Co., St. Louis, Mo.; taurodeoxycholate from Calbiochem, La Jolla, Calif.; dansyl chloride from Pierce Chemical Co., Rockford, Ill.; quinacrine hydrochloride from Schwarz/Mann, Orangeburg, N.Y.; and ovalbumin from Worthington Biochemicals Corp., Freehold, N.J. Exopenicillinase was purified by the method of Pollock (9). Plasma membrane penicillinase was a gift of S. Yamamoto of this Institute.

RESULTS

Behavior of crude vesicle penicillinase preparations upon gel permeation chromatography. To determine why the hydrophobic penicillinase in the vesicle preparations of Sargent and Lampen (15) was readily converted to a hydrophilic form, we decided to reexamine the phenomenon in more purified vesicles. Cells from an exponential-phase culture of *B. licheniformis* 749/C were converted to protoplasts, and samples of the released vesicles were collected by centrifugation at 100,000 × *g* as described in Materials and Methods. For comparison, vesicles were also prepared by gel permeation on Sephadex G-25 (to remove salts and sucrose) as previously done by Sargent and Lampen (15). Penicillinase was solubilized from the two preparations with 0.1% deoxycholate in 0.05 M sodium pyrophosphate at pH 9.0 (DOC-PP) and chromatographed on a column of Bio-Gel A-5M equilibrated with DOC-PP (Fig. 1A). Under these conditions, the enzymes extracted from the two preparations both eluted with an apparent molecular weight of approximately

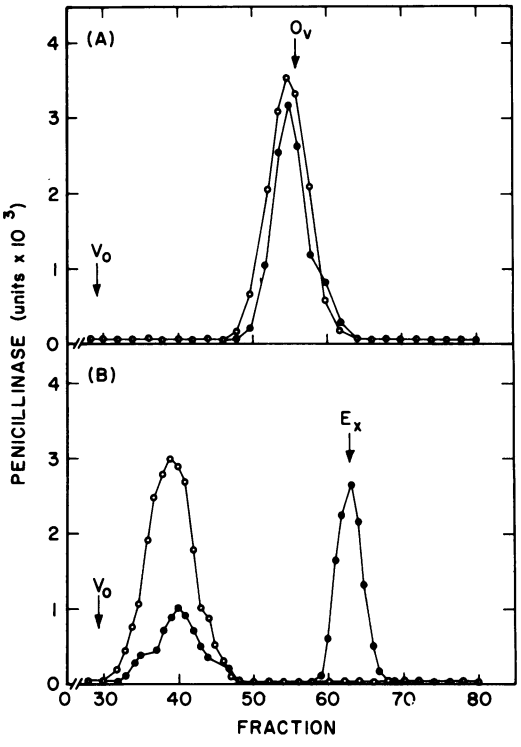

FIG. 1. *Gel filtration behavior of two types of crude vesicle penicillinase preparations in the presence or absence of detergent. Vesicle penicillinase prepared by gel filtration on Sephadex G-25 (●) or by centrifugation at 100,000 × g (○) was extracted with 0.1% deoxycholate-0.05 M sodium pyrophosphate buffer (DOC-PP) at pH 9.0 and applied to a column of Bio-Gel A-5M. The column was equilibrated in and eluted with DOC-PP (A) or with 0.05 M NaCl (B). Abbreviations: Ex, Elution volume of exopenicillinase (m_R, 29,000); Ov, elution volume of ovalbumin (M_R, 43,500). Controls with purified plasma membrane enzyme are not shown; results are essentially identical with those for the 100,000 × g pellet* (○).

45,000. The results differed sharply, however, when the two DOC-PP extracts were chromatographed in the absence of DOC (Fig. 1B). As previously reported (15), most of the enzyme from vesicles prepared by gel permeation now chromatographed as exoenzyme; this was true even when the material in the peak was rechromatographed in DOC-PP, suggesting that it had been irreversibly converted to exoenzyme, possibly during the chromatography in 0.05 M NaCl. In contrast, the enzyme from the vesicles prepared by ultracentrifugation behaved as the plasma membrane penicillinase and eluted with an apparent molecular weight of 600,000. Apparently the anomalous conversion to exoenzyme occurred only in the preparation that still contained the soluble macromolecules released during protoplasting. Accordingly, the possible ability of these macromolecules to catalyze the observed conversion was examined.

Treatment of vesicle penicillinase with soluble periplasmic macromolecules. The soluble periplasmic macromolecules from approximately 100 mg of cells were added to 3.0 ml of DOC-PP, and the solution was divided into three equal portions. The amount of non-sedimentable penicillinase in the macromolecule fraction was measured by applying one portion directly to a column of Bio-Gel A-5M equilibrated in 0.05 M NaCl. The exopenicillinase content was usually quite low (Fig. 2). The second portion was incubated for 30 min at 30°C with vesicles (prepared by centrifugation) from 30 mg of original cells, and the mixture was then chromatographed in the absence of detergent. The third portion was boiled before adding to a similar vesicle preparation. The elution profiles (Fig. 2) show that the hydrophobic vesicle penicillinase was converted to a hydrophilic form resembling exoenzyme by a heat-labile factor(s) in the periplasmic macromolecule fraction. Since conversion did not take place in the presence of DOC-PP (Fig. 1A), the previously observed cleavage (Fig. 1B) must have occurred during the subsequent chromatography in 0.05 M NaCl.

To characterize further the inhibitory effect of deoxycholate on the conversion reaction, a vesicle penicillinase sample prepared by gel permeation and solubilized in DOC-PP was incubated in pyrophosphate buffer (pH 9.0) for 30 min at 30°C in the absence of deoxycholate. The mixture was then supplemented with 0.1% deoxycholate and chromatographed in DOC-PP buffer (Fig. 3). Most of the enzyme had been converted to the hydrophilic form; however, if 0.1% deoxycholate was present during the initial incubation, conversion was prevented.

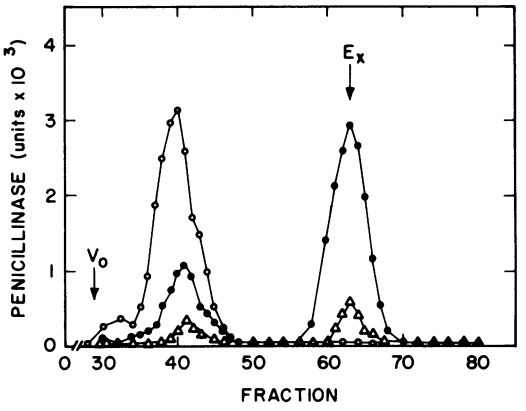

FIG. 2. *Conversion of crude vesicle penicillinase (100,000 × g vesicle pellet) to an exoenzyme-like form by incubation with the soluble periplasmic macromolecules. Mixtures containing vesicle penicillinase (DOC-PP extract) incubated with the soluble macromolecules (●), vesicle enzyme incubated with soluble macromolecules which had been heated to 100°C (○), or only the soluble periplasmic macromolecules (△) were applied to a column of Bio-Gel A-5M equilibrated in 0.05 M NaCl and were eluted with 0.05 M NaCl. Ex, Elution volume of exopenicillinase.*

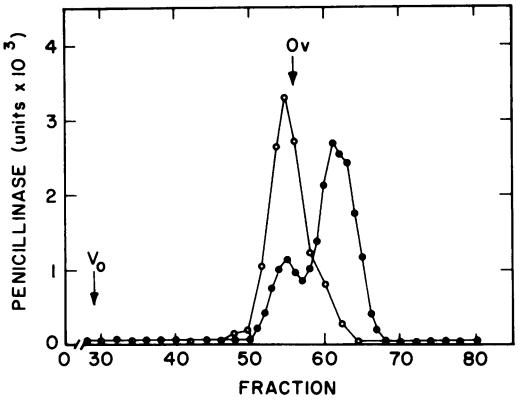

FIG. 3. *Effect of deoxycholate on the conversion of crude vesicle penicillinase at pH 9.0 to a hydrophilic form. Vesicle penicillinase prepared by gel filtration on Sephadex G-25 was incubated 30 min at 30°C in 0.05 M sodium pyrophosphate (pH 9.0) in the absence (●) or the presence (○) of 0.1% deoxycholate. The preparations were then applied to a column of Bio-Gel A-5M equilibrated in DOC-PP buffer and eluted with the same buffer. Ov, Elution volume of ovalbumin.*

Effect of high salt. Crude plasma membrane penicillinase solubilized in DOC-PP was converted to a hydrophilic form during incubation in the presence of 25% potassium phosphate at pH 9.0, presumably because the deoxycholate precipitates at about 15% potassium phosphate,

thus eliminating its inhibitory effect (14). We therefore conclude that the plasma membrane must have contained some penicillinase-releasing enzyme. Similarly, the penicillinase extracted with DOC-PP from vesicles collected by sedimentation was converted to exoenzyme in the presence of 25% potassium phosphate, pH 9.0, but not in its absence (data not presented in detail).

NH_2-terminal analysis of the hydrophilic penicillinase released by washed cells and protoplasts at pH 9.0. Sargent et al. (11) found that saline-washed cells incubated at pH 9.0 in the presence of 50 μg of chloramphenicol per ml, to block protein synthesis, released a penicillinase that resembled exoenzyme. This enzyme appeared to be derived mostly from the periplasmic vesicles that would be released during conversion of such cells to protoplasts. It was not determined if the enzyme had the same NH_2-terminal amino acid as authentic exopenicillinase, i.e., the enzyme released during exponential-phase growth at pH 6 to 8.

Cells from an exponential-phase culture (pH 6.5 to 7.0) were harvested and washed with physiological saline at 28°C. They were then suspended at a final density of 20 mg/ml in 0.01 M sodium borate (pH 9.0) containing 0.001 M magnesium chloride and 40 μg of chloramphenicol per ml and incubated for 30 min at 30°C with gentle rotatory shaking. The enzyme released was purified by the method of Pollock (9). In addition, protoplasts prepared from exponential-phase cells (pH 6.5 to 6.7), as described in Materials and Methods, were suspended at 15 mg/ml in a medium consisting of 0.05 M borate (pH 9.0), 0.75 M sucrose, and 40 μg of chloramphenicol per ml and incubated for 30 min at 30°C. The protoplasts were then sedimented at 15,000 $\times$ g for 20 min, and the supernatant fluid containing the exo-like enzyme was decanted. After the addition of 0.05 M KH_2PO_4 to this supernatant, the pH was adjusted to 4.9 with acetic acid, and the enzyme was then purified (9).

NH_2-terminal amino acid analysis of the enzyme released from washed cells and protoplasts and of a sample of authentic exopenicillinase purified from an exponential-phase culture (pH 6.5 to 6.7) gave lysine as the main NH_2-terminal residue in all three cases. In addition, NH_2-terminal glutamic acid was detected in authentic exopenicillinase (in agreement with Ambler and Meadway [2]) and in the enzyme released from washed cells and protoplasts at pH 9.0.

Effect of inhibitors on release. Exopenicillinase release by washed cells, protoplasts, and vesicles at pH 9.0 was examined for sensitivity to a variety of enzyme inhibitors. Chloroquine, quinacrine, *o*-phenanthroline, and deoxycholate all inhibited release (Table 2), with quinacrine and chloroquinine being the most effective (11). Quinacrine will also inhibit release by an exponential-phase culture at pH 6.5 to 6.7 (about 50% inhibition at 10^{-4} M; S. Yamamoto, personal communication). Deoxycholate at 10^{-3} M strongly inhibited release by washed cells; 0.1% or 2.4 $\times$ 10^{-3} M was used in the gel permeation experiments with vesicle extracts in which inhibition by deoxycholate was demonstrated (Fig. 2 and 3; text).

DISCUSSION

Previous observations on the nature of the penicillinase associated with the mesosome-derived vesicles from *B. licheniformis* 749/C suggested that it was notably different from the plasma membrane penicillinase (14, 15). The findings presented here show that the method of preparing the crude vesicle enzyme was an important factor in this distinction and that the vesicle enzyme (i) is, in fact, quite similar to the plasma membrane penicillinase, a phospholipoprotein, (18, 19) but (ii) can be modified by a factor present in some vesicle preparations. When the vesicles released during protoplasting were sedimented at 100,000 $\times$ g to remove the soluble periplasmic macromolecules and the penicillinase was solubilized by DOC-PP, the enzyme behaved as plasma membrane enzyme. Both plasma membrane and vesicle enzymes appeared to have molecular weights of 45,000 upon gel permeation in the presence of deoxycholate and of approximately 600,000 in the absence of detergent. The capacity of these cell-bound hydrophobic forms to bind detergent (5, 18) and to aggregate in its absence distinguishes them from the hydrophilic exoenzyme.

The conversion of vesicle penicillinase to a hydrophilic form at pH 9.0, as originally observed (15), requires a soluble, heat-sensitive factor(s) that is released during protoplast formation (Fig. 2). Gel permeation chromatography at pH 9.0 in the presence of the soluble periplasmic macromolecules brought about the conversion, and heating the soluble macromolecule fraction to 100°C destroyed that activity. Sargent and Lampen's vesicle preparations consisted essentially of the void volume pool from a column of Sephadex G-25 (15) and contained the active factor which can be effectively removed by sedimenting the vesicles at 100,000 $\times$ g (Fig. 1B). Consequently, the penicillinase extracted from sedimented pellets and that obtained from the protoplast membrane behaved identically

TABLE 2. *Sensitivity of exopenicillinase release to various inhibitors*

Inhibitor	Final concn	Exopenicillinase released (% of control value)[a]		
		Washed cells[b]	Protoplasts[c]	Vesicle penicillinase[d]
None	—[e]	100	100	100
None, chloramphenicol omitted	—	100	100	100
Chloroquine diphosphate	10^{-4} M	69	77	—
	10^{-3} M	14	25	—
Quinacrine-hydrochloride	10^{-4} M	43	66	57
	5×10^{-4} M	16	35	46
Ethylenediaminetetraacetic acid	10^{-3} M	113	94	—
o-Phenanthroline	10^{-4} M	98	—	55
	5×10^{-4} M	69	60	—
a,a-Dipyridyl	10^{-3} M	92	94	—
Soybean trypsin inhibitor	0.2 mg/ml	98	94	92
Sodium deoxycholate	10^{-4} M	102	—	
	10^{-3} M	24	—	

[a] Released exopenicillinase was isolated by the diethylaminoethyl-Sephadex procedure (Materials and Methods) and assayed as in (10).

[b] Cells from a 6-h culture (0.7 mg/ml) in casein hydrolysate-salt medium were washed in physiological saline and suspended at 1.0 mg/ml in physiological saline. Samples (1.0 ml) were added to 9.0 ml of a test solution consisting of 0.05 M borate buffer (pH 9.0), 0.001 M $MgCl_2$, 40 μg of chloramphenicol per ml, and the inhibitor. The mixtures were incubated at 30°C for 30 min. At the end of this period the cells were sedimented, and the supernatant fluid was assayed for exoenzyme. In the absence of inhibitor, the cells released about 50% of their bound penicillinase as exoenzyme during the 30-min period.

[c] Protoplasts prepared from exponential-phase cells, as described in Materials and Methods, were suspended at 1 mg/ml in 0.75 M sucrose, 0.001 M $MgCl_2$, 0.02 M KH_2PO_4 buffer (pH 6.0), and 40 μg of chloramphenicol per ml. One milliliter of this suspension was added to 9 ml of the test solution (see *b*), which also contained 0.75 M sucrose, and incubated for 30 min. Protoplasts were then sedimented, and the supernatant fluid was assayed for exoenzyme. In the absence of inhibitor, the protoplasts released about 30% of their bound penicillinase.

[d] Vesicles (6,000 units of bound penicillinase) prepared by sedimentation at 100,000 × *g* were incubated at 30°C for 30 min with the soluble periplasmic macromolecules from 60 to 100 mg of original cells in a volume of 0.5 ml of 0.05 M borate (pH 9.0), 0.001 M $MgCl_2$, 40 μg of chloramphenicol per ml, and the inhibitor. After this period samples were assayed for exoenzyme. About 45% of the vesicle penicillinase was converted to exoenzyme in the absence of inhibitor. See text for discussion of the results for 10^{-3} and 10^{-4} M sodium deoxycholate.

[e] —, Not tested.

TABLE 2—*Cont.*

on Bio-Gel A-5M in the presence or absence of detergent.

The observation that the conversion of the vesicle penicillinase to a hydrophilic form was inhibited in the presence of 0.1% deoxycholate (Fig. 3) explains why the crude (Sephadex G-25) vesicle preparations were converted only during the gel permeation chromatography in 0.05 M NaCl (Fig. 1; 15). Under these circumstances the deoxycholate micelles in the extract would disaggregate, and deoxycholate would probably be removed from the enzyme-containing fractions much more rapidly than would the releasing enzyme (molecular weight, 21,500 [1]). Similarly, when DOC-PP extracts of vesicle pellets (or plasma membrane [14]) were stripped of the bulk of their deoxycholate by the addition of 25% potassium phosphate (pH 9.0), hydrophilic penicillinase was formed during subsequent incubation. Purified plasma membrane penicillinase is not cleaved (to yield exoenzyme) under these incubation conditions (T. Sawai and J. O. Lampen, unpublished data). Thus the extracts of both the plasma membrane fraction and the vesicle pellet must have contained some of the releasing enzyme, but the levels were probably too low to produce a detectable conversion during gel permeation in the absence of detergent (Fig. 1B), since under these conditions the soluble releasing enzyme would soon become separated from the 600,000-molecular-weight aggregate of the hydrophobic penicillinase and other membrane proteins. Additionally, these two sets of experiments demonstrate that the inhibition of the release reaction by deoxycholate is reversible.

Tests carried out with the purified releasing enzyme (1) have revealed that taurodeoxycholate (0.01 to 0.03%) prevents the release of penicillinase from the vesicle fraction but does not inhibit the hydrolysis of casein. The existence of membrane penicillinase-deoxycholate complexes may well be the basis for this apparent difference in sensitivity; deoxycholate probably does not interact with the releasing enzyme, but instead converts the substrate (membrane penicillinase) to a form not cleaved by the enzyme.

The releasing enzyme could be detected in the culture fluid during stationary phase but not during exponential growth (L. J. Traficante

and J. O. Lampen, unpublished data), although it is present in exponential-phase cells and almost all of it is released in a soluble form when the cells are converted to protoplasts. The exopenicillinase released at pH 9.0 by a washed cell suspension was derived mostly from the membrane fraction that would be expelled as vesicles during conversion of such organisms to protoplasts and not from the fraction that would remain with the protoplasts (11). These observations suggest that the releasing enzyme is initially localized in or near the mesosomal portion of the cell membrane. The situation is more complex, however, in that the small amount of releasing enzyme which is retained by the protoplast can eventually convert about half of the membrane-bound penicillinase to exoenzyme at pH 8.5 to 9.5 but not at pH 6.0 to 6.5, even though the purified releasing enzyme is relatively active throughout the pH range of 6 to 9 (1). The basis for this apparent compartmentation in the cell surface of *B. licheniformis* is yet unclear. It may be related to the finding of Collins (4) that penicillinase is synthesized by these organisms in discrete packets (up to 5 per cell) and thus possibly at only a few sites on the cell membrane.

Trypsin treatment of total *B. licheniformis* 749/C cell-bound penicillinase (which includes both vesicle and plasma membrane fractions) produces a single hydrophilic enzyme that differs from the exoenzyme only by the absence of the NH_2-terminal lysine residue (2). Consequently, the major (hydrophilic) portion of the plasma membrane and vesicle penicillinases must be identical. The evidence presented here shows that the two larger hydrophobic forms are also very similar and may be identical. In fact, there is no longer any indication that they are chemically different (L. J. Traficante, Ph.D. thesis, Rutgers University, New Brunswick, N.J., 1975).

The hydrophilic penicillinases released at pH 9.0 by washed cells and by protoplasts were shown to have lysine as their main NH_2-terminal residue, as does the exopenicillinase produced during growth at pH 6 to 8. Some material with an NH_2-terminal glutamic acid was also present in all preparations. In addition, there is a general parallelism in sensitivity to inhibitors of penicillinase release by washed cells and protoplasts and by a mixture of vesicles and the releasing enzyme (Table 2) (and, at least for quinacrine, by a culture growing at pH 6.5 to 6.7). Thus the mechanism of release under these differing conditions is probably similar. On the basis of the available data, the liberation of penicillinase could well take place by means of the releasing enzyme here demonstrated.

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