The Location of Cell-bound Penicillinase in *Bacillus subtilis*

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SUMMARY

Before penicillinase is released from a penicillin-induced culture of *Bacillus subtilis* it accumulates and up to 85 % is found to be bound to the cells. This cell-bound penicillinase is not free in the cytoplasm since it cannot be released in a soluble state simply by rupture of the cell envelope. It appears to be attached to the cell in a fairly superficial location since up to 40 % is accessible to antiserum and up to 98 % can be released, by trypsin in the presence of hypertonic sucrose, from apparently intact cells.

INTRODUCTION

Most people who have considered the problem of exoenzyme liberation from living cells appear either explicitly or implicitly to suppose that the process must involve the passage of the enzyme from 'inside' the cell (where it is assumed to be formed) across the cell membrane to the extracellular environment. With bacteria, there is no direct evidence that this is so. One essential step in the study of the liberation process is clearly to determine the location of the enzyme whilst it is still associated with the cell. Until that is known, the experimental approach to the problem of its release may be disorientated. In addition, exact knowledge of the location of the cell-bound enzyme might help to solve the question of the site of its formation, a problem which is only just becoming susceptible to experimental attack. With most bacterial extracellular enzyme systems the proportion of activity found fixed to the cells is so low as to be barely detectable (see Pollock, 1961*b*). The penicillinase of *Bacillus subtilis*, however, is a striking exception to the rule, and for this reason is particularly susceptible to analysis in relation to the problem of enzyme liberation.

METHODS

The organism, medium and general techniques used and definitions adopted were identical with those described in detail in the preceding paper (Pollock, 1961*b*). Standard 'zero' reference time (0 hr.) from which all time periods were measured, was taken to be the point when the concentration of growing cultures reached the equivalent of 0.1 mg. bacterial dry wt./ml. Unless stated otherwise, this also was the time when cells were induced to form penicillinase and α -glucosidase.

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RESULTS

Role of the cell wall

Unlike the situation with *Bacillus cereus* (Sheinin, 1959), no evidence was obtained that a significant proportion of the cell-bound penicillinase of *B. subtilis* was attached to the cell by adsorption to the cell wall. Preparations of isolated cell walls (Cummins & Harris, 1956), Mickle-disrupted cells and intact cells from an uninduced 3 hr. culture of *B. subtilis* strain 6346 grown in casein hydrolysate (CH) medium, resuspended at equiv. 2 mg. dry wt./ml. in a concentrated supernatant fluid containing 100 units penicillinase/ml. from a penicillin-induced culture, in the presence of 0.01 M-KH₂PO₄ (pH 7.0) at 35° for 1 hr., were not found to have caused more than a 15% decrease in the penicillinase activity of the supernatant fluid after centrifugation, when compared with the total activity before centrifugation (Miss E. Janczura, private communication). In another experiment, three 4.0 ml. samples of a 3 hr. uninduced culture in casein hydrolysate medium (CH) were adjusted to pH 6.0, 7.0 and 8.0, respectively, and shaken at 35° after addition of 1 ml. of a concentrated supernatant fluid from a penicillin-induced culture, giving a final concentration of 50 units/penicillinase/ml. After 2½ hr., removal of the cells by centrifugation for 20 min. at 20,000 g did not result in any detectable decrease in penicillinase activity. This observation that *B. subtilis* penicillinase, unlike the *B. cereus* enzyme, was neither adsorbed to intact cells nor to isolated cell walls, may be related to the fact that, again in contrast to *B. cereus* penicillinase, it is not appreciably adsorbed to powdered glass (Kogut, Pollock & Tridgell, 1956; Kushner, 1960).

Effect of repeated cell washing

A 3 hr. penicillin-induced culture was centrifuged in the cold-room at 2° and the organisms resuspended to equiv. 2 mg. bacterial dry wt./ml. in ice-cold 0.02 M-KH₂PO₄ (pH 7.0). After removing a sample for assay (27.6 units penicillinase/ml.) the suspension was re-centrifuged, and the organisms washed four times successively in 10 ml. lots of iced buffer, being finally resuspended in the same volume of buffer and reassayed (28.2 units penicillinase/ml.). It is obvious that the enzyme was not removed by repeated washing.

Effect of cell disruption

Hughes's press. A suspension of standard 3 hr. penicillin-induced organisms at a concentration equiv. 20 mg. bacterial dry wt./ml. in iced 0.01 M-KH₂PO₄ buffer, (pH 7.0) + 0.05 M-MgSO₄ was crushed in a Hughes's press at -30°. When the crushed preparation was thawed and homogenized to obtain an evenly dispersed suspension, 43% of the total penicillinase activity was found in the supernatant fluid after centrifugation for 10 min. at 20,000 g.

Lysozyme treatment. 'Spheroplasts' (see McQuillen, 1960), prepared by incubating organisms with 100 µg. lysozyme/ml. at 35° in the presence of 0.3 M-sucrose or 20% (w/v) polyethylene glycol appeared, microscopically, to be stable, but were found to leak the normally cell-bound α-glucosidase (induced in the cells as an intracellular 'marker'; see Pollock, 1961*b*) into the medium and released a variable proportion of penicillinase. The addition of high concentrations of Mg⁺⁺ (0.05 M-MgSO₄) decreased the rate of enzyme release during lysozyme treatment, and the

Table 1. *Liberation of penicillinase and α -glucosidase from disrupted spheroplasts of penicillin—and maltose-induced Bacillus subtilis 6346*

Treatment	Description	Penicillinase		α -Glucosidase	
		Units/ml.	% released to medium after treatment	μ mole <i>p</i> -nitrophenol ml./hr.	% released to medium after treatment
Resuspended organisms from 3 hr. culture	Whole organisms	16.80	.	5.70	.
Samples of whole organisms treated at 35° with 100 μ g. lysozyme/ml. + 0.3 M-sucrose for (a) 2 min., (b) 5 min., followed by centrifugation and resuspension in 0.05 M-MgSO ₄	Spheroplasts, prepared by lysozyme treatment for:				
	(a) 2 min.	17.12	(-2)	2.42	57.5
	(b) 5 min.	14.32	14	1.95	65
'Spheroplasts', allowed to lyse in 0.05 M-MgSO ₄ , centrifuged and supernatant fluid retained	Supernatant fluid from disrupted spheroplasts, previously prepared by lysozyme treatment for:				
	(a) 2 min.	0.34	2	2.24	93
	(b) 5 min.	0.50	3.5	1.92	96.5

For full experimental details see text.

spherical form was only slowly attained, although resuspension in dilute buffer solution (0.01 M-KH₂PO₄, pH 7.0, + 0.05 M-MgSO₄) without sucrose caused immediate lysis. This stabilizing effect appeared to be mainly on the cell membranes which disintegrated only very slowly as long as the Mg⁺⁺ concentration was kept high (as found with *Bacillus megaterium* by Weibull, 1956). The use of Mg⁺⁺ thus allowed the preparation of fairly stable 'membranes' (i.e. resuspended, lysed spheroplasts) which retained most of the penicillinase activity of the intact organisms, although nearly all the α -glucosidase had escaped into the medium during the lysozyme treatment and subsequent lysis in hypotonic buffer solution. Table 1 summarizes the results of an experiment which demonstrated this. A 3 hr. culture (90 ml.) induced with maltose and penicillin was centrifuged and the organisms resuspended at 35° in 45 ml. 0.01 M-KH₂PO₄ (pH 7.0) + 0.05 M-MgSO₄ and 0.3 M-sucrose. Lysozyme (100 μ g./ml.) was then added and 18 ml. samples removed after incubation for 2 min. and 5 min., cooled as rapidly as possible and centrifuged at 2° for 10 min. at 20,000 *g*. The deposits were resuspended to the same volume in iced buffer + 0.05 M-MgSO₄ and homogenized rapidly (2 min.) in a Potter-Elvehjem homogenizer, the temperature being kept as low as possible. Samples of these 'disrupted spheroplasts' were centrifuged for 10 min. at 20,000 *g* and the penicillinase and α -glucosidase activities remaining in the supernatant fluid compared with total activities before centrifugation and with the activities of a sample of the original cell suspension treated with lysozyme in the absence of sucrose or Mg⁺⁺. The results showed that (a) practically all the α -glucosidase was liberated when a lysozyme-treated preparation

of cells was lysed by resuspension in hypotonic medium, and that more than 50 % escaped even before the sucrose was removed, whereas (b) barely significant amounts of penicillinase were released after only 2 min. lysozyme treatment and very little even after 5 min. As shown previously (Pollock, 1961*b*), longer incubation resulted in further penicillinase liberation by processes which were considered to be secondary autolysis.

Table 2. *Liberation of penicillinase from disrupted 'spheroplasts' of Bacillus subtilis 6346*

	Total penicillinase (units/ml.)	Penicillinase released after incubation at 35° (units/ml.)	% released
Expt. 1: 90 min. incubation			
Control	13.6	7.8	58
+ MgSO ₄ 0.05 M	13.6	2.8	21
+ <i>p</i> -Chloromercuribenzoate 2.5×10^{-3} M	12.8	5.4	42
+ heparin 1 mg./ml.	14.4	8.0	56
+ diisopropylfluorophosphate 2×10^{-3} M	14.4	8.8	60
Expt. 2: 0.05 M-MgSO ₄ added to all tubes; incubation for 10 min.			
Control	17.8	4.4	25
+ trypsin, 1 mg./ml.	19.0	17.6	93
Expt. 3: 0.05 M-MgSO ₄ added to all tubes; incubation for 10 min.			
Control	32.4	7.1	22
+ trypsin, 1 mg./ml.	31.0	17.5	57
+ ribonuclease 1 mg./ml.	33.2	7.5	23
+ butanol 1 %	32.4	7.8	24
+ sodium deoxycholate 1 %	32.4	28.3	88

For full experimental details, see text.

This autolytic-type of penicillinase release from 'disrupted spheroplasts' (prepared as described above) was studied further in an experiment summarized in Table 2 where the effects of various reagents on the process were investigated. The following points may be seen. (a) Trypsin, at 1 mg./ml., rapidly liberated a large (though rather variable) proportion of the penicillinase even in the presence of 0.05 M-Mg⁺⁺. Sodium deoxycholate (10 mg./ml.) had a similar effect. (b) The 'autolytic' release was inhibited by Mg⁺⁺ (0.05 M) and by *p*-chloromercuribenzoate (2.5×10^{-3} M) but not by diisopropylfluorophosphate (DFP) or heparin. The question of how closely related this 'autolytic' liberation phenomenon may be to normal (physiological) release of enzyme in growing cultures is considered later.

Combined effect of trypsin and hypertonic sucrose

Trypsin alone (at least up to 1 mg./ml.) had no detectable action on growth or enzyme liberation from cultures of *Bacillus subtilis* 6346 in CH medium, and little effect on release of penicillinase or α -glucosidase from cells of a 3 hr. penicillin- and maltose-induced culture resuspended at 35° in phosphate buffer. But (see Table 2) it rapidly liberated the enzyme from a 'membrane' preparation (disrupted 'spheroplasts'). Sucrose alone (0.9 M) completely inhibited growth and caused a variable degree of cell damage in similarly prepared suspensions of induced cells (as indicated

by α -glucosidase release into the medium) but had no effect on penicillinase liberation, at least during incubation for 30 min. at 35°. Treatment with trypsin and sucrose together, however, led to considerable release of cell-bound penicillinase within about 20 min. The extent of this effect, however, varied from day to day, maximal penicillinase release in 30 min. varying between the extremes of 45 and 98 % in different experiments. α -Glucosidase release also varied, but not in a fashion apparently correlated with the extent of penicillinase release. In the most striking experiments, a 98 % release of penicillinase was associated with only a 5 % release of α -glucosidase. Other typical experiments are shown in Table 3 and Fig. 1.

Fig. 1. The release of α -glucosidase (—○—○—) and penicillinase (—●—●—) from maltose- and penicillin-induced cells of *Bacillus subtilis* 6346 resuspended in 0.01 M-sodium phosphate (pH 8.0) at 35° in the presence of 0.9 M-sucrose + trypsin 1 mg./ml.

Table 3. Release of penicillinase from *Bacillus subtilis* 6343 by treatment with sucrose and trypsin for 30 min. at 35°

	Penicillinase units/ml.		Amount released into medium (%)
	Total	In supernatant fluid	
Control	19.8	1.8	9.1
+sucrose 0.9 M	18.5	1.2	6.5
+trypsin, 1 mg./ml.	19.6	3.4	17.3
+0.9 M-sucrose and trypsin 1 mg./ml.	18.9	13.1	69.5

For full experimental details, see text.

The full procedure for the above experiment was as follows. A 3 hr. culture of *Bacillus subtilis* 6346 induced with penicillin and with maltose was centrifuged, the organisms resuspended in different mixtures in 0.01 M-KH₂PO₄ (pH 8.0) + 0.05 M-MgSO₄, with or without 0.9 M-sucrose and with or without trypsin 1 mg./ml. and all mixtures incubated aerobically at 35°. Samples were cooled rapidly, a few ml.

retained for total penicillinase assay and the remainder centrifuged at 20,000 *g* for 10 min. in the cold. The supernatant fluids were assayed for penicillinase activity, and the deposits resuspended to volume in a buffer mixture containing 0.02M- KH_2PO_4 (pH 7.0) and 0.1% heparin (to inhibit the further action of trypsin on α -glucosidase as it is released) and 100 μg . lysozyme/ml., incubated at 35° until lysis was complete and then assayed for α -glucosidase activity as soon as possible. The proportion of α -glucosidase released was calculated from the difference between the activities of the lysed deposits and those of the sample of original cell suspension lysed directly with lysozyme under the same conditions but with Mg^{++} , sucrose or trypsin. Whatever degree of penicillinase release was achieved, it was usually maximal after 20 min. incubation (although this particular feature does not happen to be well illustrated by the example shown in Fig. 1). This indicated that the inconstant results were due to variation either in the proportion of cells completely insusceptible to the action of trypsin and sucrose or in the extent to which the cell-bound penicillinase had reached a location on the cells accessible to trypsin.

Trypsin had no effect on penicillinase, but rapidly inactivated the α -glucosidase as soon as it was liberated from the cell, though not before. The problem of assaying cell-bound α -glucosidase in the presence of trypsin was complicated by the need to lyse the cells with lysozyme in order to allow full expression of activity, after which the enzyme was rapidly destroyed by the trypsin. It had, however, been found that the inactivation of α -glucosidase by trypsin could be almost completely prevented by 0.1% heparin (see Pollock, 1961*b*) which was therefore added as soon as samples were taken. In this way, it could be shown that as long as α -glucosidase remained within the cells, it was almost completely protected from the proteolytic action of trypsin. This suggested that the trypsin had not actually penetrated the cell membrane during the time it was releasing the penicillinase and must therefore be acting relatively superficially. At concentrations of sucrose below 0.9M (e.g. at 0.3M) the extent of penicillinase release by trypsin was relatively slight. It therefore seemed possible that liberation by trypsin was dependent on some degree of plasmolysis, perhaps involving separation of cell membrane and cell wall associated with surface damage, which allowed access of trypsin to certain structures (presumably fully exposed to the action of trypsin in the preparation of disrupted 'spheroplasts' described above) which were vital for the retention of penicillinase.

When 0.9M-sucrose was replaced by 4% (w/v) polyethylene glycol (mean M.W. 400) which should be osmotically equivalent, no release of penicillinase occurred in the presence of trypsin. It is not, however, known whether polyethylene glycol can penetrate the cell wall. If this does not occur and the cell wall is not completely rigid, the cell may simply shrink as a whole and no plasmolysis involving separation of membrane and cell wall would take place. Whatever the nature of the bonds responsible for this binding of penicillinase, it seems likely that the trypsin could not have penetrated the barrier of the cytoplasmic membrane and that the bulk of the penicillinase, therefore, is probably bound on the outer surface of the membrane, or even more superficially.

No direct evidence, however, could be obtained on the mechanism of action of sucrose; neither observation by phase contrast nor electron microscope studies revealed any convincing difference between cells treated and untreated with 0.9M-sucrose, with or without trypsin. Amongst other enzymes tested because of their

ability to attack substrates which possibly form part of the superficial cell structure, the effect of trypsin was shared only by another proteolytic enzyme (*Bacillus subtilis* proteinase itself; Nagase and Co. Ltd., Itachibori-minamidori 1-chome, Osaka, Japan). Bovine pancreatic ribonuclease (Armour Pharmaceutical Company Ltd., Eastbourne), *Clostridium welchii* lecithinase (kindly given by Dr Marjorie Macfarlane of the Lister Institute of Preventive Medicine, Chelsea Bridge Road, S.W. 1), a pancreatic lipase preparation (Mann Research Laboratory, 136, Liberty Street, New York 6, N.Y.) and a microbial lipase preparation (Mann Research Laboratory, 136, Liberty Street, New York 6, N.Y.), all at a concentration of 1 mg./ml., tested in the presence of 0.9 M-sucrose, were without effect. The action of lysozyme has already been mentioned and discussed.

Experiments with anti-penicillinase serum

Specific neutralization by anti-enzyme serum has been used on several occasions as evidence for the localization of cell-bound enzyme on the cell surface (Sevag, Newcomb & Mill, 1954; Pollock, 1956*a*). The value of the method depends upon the assumption that rabbit γ -globulin cannot penetrate to 'within the cell' (i.e. pass through the cytoplasmic membrane), for which there is some experimental evidence in yeast (Krebs & Wright, 1951; Pasternak, Sevag & Miller, 1954). The extent to which it may or may not be able to pass through the cell wall is not known. Obviously, therefore, only positive enzyme neutralization tests can be satisfactorily interpreted. A negative result might mean that the enzyme was within the cytoplasmic membrane, or outside the membrane but protected by the cell wall from combination with antibody.

An antiserum against *Bacillus subtilis* 749 exopenicillinase prepared in rabbits was found to give 78% neutralization of the heterologous exopenicillinase from *B. subtilis* 6346 (Kushner, 1960). Cells from induced cultures of *B. subtilis* 6346 were centrifuged at times between 3 and 6 hr. after induction, resuspended to 1/3 the original volume in 0.01 M-KH₂PO₄ (pH 7.0) containing 8×10^{-4} M-8-hydroxyquinoline to prevent further enzyme synthesis. Two lots of 0.5 ml. of this suspension were put into each of two Warburg flasks, one containing 0.2 ml. water and the other 0.2 ml. of anti-749 penicillinase serum (equivalent to approximately twice the quantity necessary for maximal neutralization of the enzyme). The flasks were left at room temperature for 20 min. Then 1.0 ml. of a mixture containing 1% (w/v) gelatin + 8×10^{-4} M-8-hydroxyquinoline, bicarbonate and penicillin was added as before, and the penicillinase activities measured. The results, giving '% enzyme in combination with antiserum' are calculated from the % difference in enzyme assay with and without antiserum, after allowing for the 22% residual activity of the enzyme + antibody complex consistently demonstrable, even in the presence of a gross antibody excess. It was found that the addition of an equivalent quantity of lysed or intact organisms from an uninduced culture had no significant effect on the neutralization of soluble enzyme by antisera, and that therefore the presence of cells was unlikely *per se* to affect the reaction between enzyme and antibody. Control experiments showed that negligible quantities of enzyme were liberated during the period required for allowing the antiserum to neutralize the enzyme and subsequent assay.

When the % cell-bound penicillinase combining with antiserum was measured in the period 3–5 hr. after induction, it was found, despite somewhat variable results,

that there was usually 30–40 % demonstrable at 3 hr. (after which there is little further production of enzyme in penicillin-induced cultures; Pollock, 1961*b*), but that after a further 2 hr. growth the proportion had fallen to 0–15 % (Fig. 2). By 5–7 hr., up to 60 % of the total enzyme formed had been liberated. The fact that the proportion of cell-bound enzyme which combined with antiserum tended to fall from 3 hr. onward suggested that it was this moiety which was first released. Calculations in individual experiments showed that the amount of enzyme released was sufficient to account for the decrease in antibody-combinable enzyme. These results also show, however, that enzyme release can take place from cells in which little or no neutralizable enzyme is detectable; this suggests that though enzyme molecules in the antibody-combinable state may be preferentially released, such a state is not necessarily a preliminary one to release from the cell.

Fig. 2. A scatter diagram summarizing all results obtained in a series of experiments showing the decrease in the proportion of cell-bound *Bacillus subtilis* penicillinase combinable with anti-exopenicillinase serum, during the 2–7 hr. period following the point when the opacity of a culture growing in casein hydrolysate reached the equivalent of 0.1 mg. bacterial dry wt./ml. (being also the time when enzyme formation was induced with penicillin).

The proportion of antiserum-combinable cell-bound enzyme at 3 hr. after induction was not altered by conditions which increased the total amount of enzyme formed. In one experiment, where the culture had been induced with cephalosporin C (10 μ g./ml.) and the total quantity of penicillinase produced was nearly five times as great as that of a sister culture induced with penicillin, the above proportions were not significantly different (30 and 32 %, respectively). It should be recalled that increasing the total enzyme formed, by induction with cephalosporin C, did not influence the extent to which it was liberated into the medium (Pollock, 1961*b*). After overnight incubation of the culture, all the enzyme was liberated into the medium and was then found to be neutralized by antiserum to maximal extent, as with enzyme liberated in earlier stages of growth. It is thus clear that the cell-bound penicillinase has no immunologically distinct fraction similar to the ' γ -penicillinase' possessed by *Bacillus cereus* (Pollock, 1956*b*).

When 3 hr. cells of *Bacillus subtilis* 6346 were disrupted with lysozyme, not all the penicillinase was thereby immediately made accessible to antiserum, though the proportion rose from 40 to 60 % (means of 6 experiments). A similar increase was observed when 3 hr. cells were crushed in a Hughes's press. When 5 hr.-cells, which had already released most of their antiserum-accessible enzyme to the medium, were treated with lysozyme, the proportion increased from 11.5 to 53 % (means of 2 experiments). When lysed or crushed organisms were incubated overnight at 35°, the proportion further increased to 70–90 %, possibly through an autolytic destruction of bonds which prevented access of antibody to the relevant part of the enzyme molecule.

DISCUSSION

These results show that the cell-bound fraction of *Bacillus subtilis* 6346 penicillinase is largely, if not entirely, bound to insoluble cell structures, sedimentable by 15 min. centrifugation at 20,000 g. This fact, and the visible appearance of the cell debris following treatment with lysozyme in the presence of high concentrations of Mg^{++} , suggest that the enzyme is probably bound to structures forming the cell envelope. At least 30 % of the cell-bound fraction is fixed superficially to the cell, being accessible to combination with antiserum. The results with antiserum also suggest that it is this superficially-bound fraction of enzyme which is selectively released during the 3–6 hr. period during which the liberation process has been studied.

The action of trypsin in liberating a high proportion of cell-bound penicillinase after treatment of the cells with hypertonic sucrose, but apparently without penetrating the cytoplasmic membrane, suggests that the penicillinase was probably released from structures outside the main permeability barrier of the cell. This effect appears to be analogous to the action of trypsin in promoting the release of the 'M' protein from streptococci (Elliott, 1945). With streptococci, no preconditioning with sucrose or other adjuvant is necessary. The M protein appears to be bound directly or indirectly to cell wall material since it is not normally liberated into the medium in significant amounts except in old cultures, whereas it is not found attached to protoplasts which, nevertheless, appear still capable of forming it (Freimer, Krause & McCarty, 1959). A proportion of this protein must likewise be superficially located since it can be shown to react with antiserum in undamaged cells. On the whole it would appear that *B. subtilis* 6346 penicillinase may be fixed to the cells in a similar manner, but perhaps rather more deeply buried than is the M protein.

The results as a whole are consistent with the hypothesis that cell-bound penicillinase is attached to the outside of the cytoplasmic membrane, and may actually be formed at its surface. It is possible that at least a portion may be bound in, or closely associated with, the cell wall. Nor is it excluded that part might be in a soluble state, sandwiched between the cell membrane and an impermeable or poorly permeable cell wall (e.g. in the so-called 'periplasm' postulated by Mitchell, 1961). But it is difficult to understand, if this be so, how most of the α -glucosidase is released from the cells under conditions (e.g. incubation at 0°; Pollock, 1961*c*; or after brief lysozyme treatment with high Mg^{++} concentrations) which do not allow escape of penicillinase. The only evidence possibly in favour of the enzyme being in a 'periplasm' is the rapid release of over 40 % of the penicillinase after crushing in a Hughes's press. However, in order to obtain evenly dispersed suspensions of

disrupted cell material by this technique (without which, of course, no comparison of particle-bound and soluble enzyme is valid) it is necessary to thaw out the frozen crushed suspension for several minutes and homogenize it thoroughly before centrifugation. It is possible that this may be time enough for secondary autolytic processes to cause a considerable amount of release of penicillinase from cell fragments. In any case, it seems at the moment reasonable to approach the problem of 'normal' release of *Bacillus subtilis* 6346 penicillinase on the basis of a working hypothesis that it may involve the detachment of the enzyme from structures on the surface of, or outside, the main permeability barrier of the cell, rather than passage through the barrier itself.

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