The Measurement of the Liberation of Penicillinase from *Bacillus subtilis*

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SUMMARY

The formation of penicillinase, induced in a growing culture of *Bacillus subtilis* 6346 by a single addition of benzylpenicillin, spontaneously ceases after about 3 hr. The enzyme continues, however, to be released into the medium, thus permitting the process of its liberation to be studied independently from its production. By using the escape into the medium of a normally intracellular, maltose-inducible α -glucosidase as a more sensitive indicator of cell damage than direct measurement of lysis, it was concluded that at least 40 % of the penicillinase is liberated from the cells without gross disorganization of their structure.

INTRODUCTION

The phenomenon in which certain enzymes are released from apparently undamaged cells of a growing bacterial culture has not so far been the subject of much systematic experimental investigation, the one notable exception being that of the extracellular α -amylase of *Bacillus subtilis*, studied by Nomura, Hosoda & Yoshikawa (1958). In a recent review (Pollock, 1961*b*) some of the principles and definitions involved in studying the problem were evaluated and the difficulties emphasized. There has been considerable scepticism about the reality of exo-enzymes as physiological entities, the view sometimes being expressed that they appear in the extracellular environment only to the extent that the cells are lysed, or damaged to a degree that is incompatible with normal growth and metabolism. However unlikely this view may appear to be in any specific instance, it is not one which can be dismissed without critical experimental evidence to the contrary.

For the work to be described here the penicillinase system of *Bacillus subtilis* NCTC 6346 was chosen for the following reasons.

- (1) The enzyme is stable and can be simply and accurately assayed.
- (2) It is inducible by benzylpenicillin and other closely related compounds, as with the penicillinase of *Bacillus cereus* (Pollock, 1953), and therefore its production can be brought under close control.
- (3) On the reasonable assumption that its mode of production does not differ fundamentally from that of *Bacillus cereus* penicillinase, it could be concluded, on the basis of work done on the latter system (Pollock & Kramer, 1958), that appearance of enzymic activity corresponds to the biosynthesis of the relevant protein molecule itself. No substrate-accessibility factors or high molecular weight precursors appear to be involved to an extent which might complicate interpretation of results based on the direct measurement of enzymic activities.

(4) Unlike the situation in *Bacillus cereus*, however, where 85 to 95 % of the penicillinase is extracellular under all conditions so far investigated (Pollock, 1956), the *B. subtilis* enzyme is mainly bound to the cells during the early stages of growth, only later being liberated into the medium. There were some grounds, therefore, for hoping that it might be possible to dissociate liberation of the enzyme from its production—an essential prerequisite for a proper study of the liberation phenomenon itself.

Most bacterial exo-enzymes, including the α -amylase of *Bacillus subtilis* 6346 itself, appear to exist almost exclusively in the medium (Pollock, 1961*b*); in other words, they are liberated as soon as they are formed. This is obviously not an ideal situation for the purpose of studying enzyme liberation distinct from enzyme formation. Although, therefore, the *B. subtilis* penicillinase system may be atypical, it seemed to offer an exceptional opportunity for studying one aspect of the phenomenon of enzyme liberation. Our objective has, correspondingly, been limited: that of attempting to understand the mechanism by which the cell-bound fraction of *B. subtilis* penicillinase is liberated into the surrounding medium. In order to do this, the problem has been divided into three parts.

(1) Measurement and control of cell lysis of gross cell damage and assessment of its possible role in the liberation process (this article). (2) Attempts to identify the location of the cell-bound fraction of the enzyme (Kushner & Pollock, 1961). (3) Observations on the 'normal' enzyme liberation process itself (Pollock, 1961*a*). It has been possible to show, by means of the studies reported in this and the following two papers, that the liberation of penicillinase occurs with little or no cellular damage, under conditions dissociated from cell growth and multiplication, and involves detachment of the enzyme from the superficial layers of the cell by a process which probably has an enzymic basis.

METHODS

Organism. *Bacillus subtilis* NCTC 6346 was chosen for reasons outlined above and because it is also a good producer of α -amylase whose production and liberation are forming the subject of a parallel study.

Medium. The basal medium (CH) for all experiments contained 1 % Casamino acids (Difco), 0.02M-KH₂PO₄ adjusted to pH 7.2 with NaOH; 1.7×10^{-3} M-MgSO₄ + 0.1 ml. 'stock iron' solution (mixture of 0.156 % ferrous ammonium sulphate + 0.168 % citric acid per 100 ml.) + 0.1 ml. trace metals mixture (= 'oligodynamic solution' as used by Pollock & Kramer, 1958) per 100 ml.

Growth conditions. Unless otherwise stated, cultures were incubated by shaking aerobically at 35°. After inoculation the previous night with a few drops of a standard spore suspension (3×10^8 viable spores/ml.), cultures were first incubated without shaking for about 16 hr. and then transferred in the morning to a shaker at 35° in a conical flask containing not more than 1/5 its volume of medium, and grown under fully aerobic conditions during the whole of the experiment.

Enzyme induction. Penicillinase was usually induced by the addition of 0.06 μ g. (= 0.1 unit)/ml. benzylpenicillin (Glaxo 'Crystapen'). In some experiments, where larger amounts of penicillinase were required, 1 μ g. or 10 μ g./ml. of the non-metabolizable inducer, cephalosporin C, was used instead. α -Glucosidase was induced by

the addition of 0.25 mg. maltose (BDH 'Analar')/ml. Unless specifically stated otherwise, both enzymes were induced when the culture density reached the equivalent of 0.1 mg. bacterial dry wt./ml. and this point has been used as a standard zero reference time in all experiments—e.g. '3 hr. cells' means cells from a culture in CH medium 3 hr. after the point where an opacity equiv. 0.1 mg. dry wt./ml. was reached.

Enzyme assays. Samples (usually 5 ml.) were removed from the culture when required and cooled rapidly in ice. Two to three ml. were then spun at 20,000 *g* in the cold room for 10 min. and the supernatant fluid removed for assay of released enzyme. One drop of a 2 mg./ml. solution of egg-white lysozyme (Armour Labs.) was added to the remainder of the sample which was incubated at 35° until lysis was complete (2–5 min.) and then kept at 0° until assay of total enzyme. This procedure prevented any further production of enzyme and allowed full expression of α -glucosidase activity which in intact cells assayed at only about 40 % of the activity found after lysis or other methods (e.g. toluene treatment) of disrupting cell structure. Neither toluene nor lysozyme had any effect on penicillinase activity.

Penicillinase was assayed at 30° either manometrically on 1.0 ml. samples (Henry & Housewright, 1947) at pH 7.0, or iodometrically at pH 6.5 (Perret, 1954), the latter method being preferred under conditions of high buffer concentration or at pH values removed from neutrality. As used in this work, the manometric method gave approximately 25 % lower values, partly because of the CO₂ retention due to phosphate buffer in the medium. No correction was made for this, since it could be assumed that it was the same for all samples; comparisons were of course confined to results obtained by the same method, and conclusions were not in any way affected by the discrepancy. When dialysed samples of lysozyme-lysed, penicillin-induced cultures were assayed for penicillinase activity by both methods, the manometric technique still gave results 15 % lower than the iodometric method. The agreement is reasonable, and excludes the possibility of there being any significant penicillin-amidase activity present which might theoretically give misleadingly high results in the manometric assay technique for penicillinase (= penicillin β -lactamase). Penicillin-amidase is now known to be formed by certain bacterial species (Rolinson *et al.* 1960) though it has not been reported as occurring in the *Bacillus* genus. Its presence might possibly contribute to the release of CO₂ from penicillin and bicarbonate in the manometric assay for penicillinase, but would not interfere with the iodometric assay since the products of its action on benzylpenicillin (phenylacetic acid and 6-aminopenicillanic acid) do not react with iodine. If penicillin-amidase were present in significant quantity any discrepancy that might result between the iodometric and manometric assays of penicillinase would be in the opposite direction from that which was actually observed.

One ml. of 1 % (w/v) gelatin was originally included in the manometer cups for protection of the enzyme against possible loss or inactivation (see Pollock & Perret, 1951), but this was discontinued when it was discovered that *Bacillus subtilis* penicillinase is much more stable than the *B. cereus* enzyme and is not absorbed by glass to any significant extent (Kushner, 1960). All results are expressed in units of enzyme/ml. (as defined by Pollock & Torriani, 1953).

α -Glucosidase was assayed by measuring the absorption at 420 m μ (in a Unicam spectrophotometer) of the *p*-nitrophenol liberated by the enzyme after hydrolysis

of *p*-nitrophenol- α -D-glucoside, in comparison with standard concentrations of *p*-nitrophenol prepared by dissolving 0.1 g. in 500 ml. water and making suitable dilutions in 0.05M-sodium phosphate buffer (pH 7.0) to 5.0 ml. lots of which was added 1 ml. of M-K₂CO₃. One ml. samples of enzyme (diluted, when necessary, with water) were mixed with 3.5 ml. of 0.05M-sodium phosphate buffer (pH 7.0) in a 35° water bath and the reaction started by the addition of 0.5 ml. of a 1.0 mg./ml. solution of the substrate. The reaction was stopped by the addition of 1.0 ml. M-K₂CO₃ after sufficient colour had developed. Where necessary, the reaction mixture was clarified by centrifugation at 3000 rev./min. for a few minutes. Blanks, prepared by incubating the ingredients under similar conditions (1 ml. of water being substituted for the enzyme samples) were used for comparison in all experiments. It was shown that under these conditions the reaction was linear for at least 60 min. incubation and that the rate was proportional to the enzyme concentration, as long as no more than 80 % of the substrate was hydrolysed. Activities are expressed as the quantity of *p*-nitrophenol formed/hr./ml. of original enzyme preparation. The *p*-nitrophenol- α -D-glucoside was prepared according to the method of Aizawa (1939), and gave a m.p. of 210°.

β -Galactosidase was assayed at 35° and pH 7.0 by the method of Lederberg (1950) with *o*-nitrophenol- β -D-galactoside as substrate, activities being expressed as the quantity of *o*-nitrophenol produced/hr./ml. original enzyme preparation.

Opacities of bacterial suspensions were measured in a Hilger Spekker absorptiometer with a neutral H 508 filter and expressed in terms of the equivalent bacterial dry weight as read off on a standard calibration curve prepared with suspensions of *Bacillus subtilis* of known dry weight values.

Total protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) against a bovine serum albumin standard, after precipitation with 5 % (w/v) trichloroacetic acid (TCA) at 90° for 20 min. and subsequent washing of the precipitate twice with 5 % TCA before dissolving in N-NaOH.

'260 $m\mu$ absorption' was measured directly in a Unicam spectrophotometer after making suitable dilutions in 10⁻²M-KH₂PO₄ (pH 7.0) solution.

RESULTS

Kinetics of release

Figure 1 shows the production and release of penicillinase from *Bacillus subtilis* 6346 growing in CH medium after induction with 0.06 μ g. benzylpenicillin/ml. at 0.1 mg. bacterial dry wt./ml. About 3 hr. after induction, formation of the enzyme practically ceased (quite unlike the situation with *B. cereus* where penicillinase synthesis continues apparently indefinitely), whereas cells went on growing and release of the enzyme persisted until, after overnight incubation, it had all been liberated into the medium. This means that, subsequent to a point 3 hr. after induction, liberation is spontaneously dissociated from production. Nearly all investigations on the liberation process were therefore confined to the first 2-3 hr. of this period. Cessation of penicillinase production at the '3 hr. point' was shown to be due to an induction failure, since on further addition of benzylpenicillin to '3 hr.' cells, production recommenced, and when an inducer, such as cephalosporin C, which is not detectably hydrolysed by the enzyme, was added at the normal induction time

(0 hr.) production persisted for as long as the organisms were growing. The liberation phase, however, could be shown to be not markedly influenced either by the absolute rate of penicillinase formation or the interval elapsed following addition of the inducer. For instance, in the experiment summarized in Table 1 there was perhaps a significantly lower proportion of penicillinase liberated at 4 and 5½ hr. by cells induced at 2½ hr. as compared with cells induced with the same concentration of penicillin at 0 hr.; but in a culture induced at 4 hr. the percentage release only 1½ hr. later was as high as that shown by cells induced 5½ hr. previously. Table 1 shows also that the proportion of extracellular penicillinase in cultures induced with cephalosporin C was not significantly different from that in penicillin-induced cultures where there might be only one-eighth as much total enzyme present.

Fig. 1. Production and liberation of penicillin-induced penicillinase by cells of *Bacillus subtilis* 6346 growing in casein hydrolysate. Total penicillinase (—●—●—); penicillinase liberated from cells into medium (—○—○—); cell-bound penicillinase (—▲—▲—); growth opacity (—+—+—).

The release of the enzyme, therefore, seemed to be related to the physiological state of the culture rather than to the rate or stage of enzyme induction. Moreover, when relatively 'young' (2 hr.) cephalosporin C-induced cells were treated with 40 µg. chloramphenicol/ml. and incubated in the supernatant fluid from an 'old' (5½ hr.) uninduced culture (after readjusting to pH 7.0 with N-HCl and supplementing with a further addition of 1% (w/v) Casamino acids), they were found to liberate the same quantity of penicillinase into the medium as did the cells of a similarly induced and chloramphenicol-treated control culture incubated in fresh medium. It would appear, therefore, that release of the enzyme must depend upon changes in the cells themselves and not factors produced or liberated in the medium.

State of released enzyme. The penicillinase released during the 3–6 hr. period appeared to be entirely in a soluble state. The titre (3.08 units/ml.) of induced enzyme activity in the supernatant fluid of a sample from a 5½ hr. culture centrifuged for 30 min. at 105,000 *g* was not significantly different from that (2.94 units/ml.) in the supernatant fluid of an sample spun for only 15 min. at 5000 *g*. Removal of cells by an entirely different method (filtration through an ‘Oxoid’ membrane) left nearly the same activity (2.60 units/ml.) in the cell-free filtrate.

The control and measurement of cell lysis and gross cell damage

Cells of *Bacillus subtilis* are notoriously prone to undergo lysis (see Nomura *et al.* 1958; Nomura & Hosoda, 1956), the metabolic basis of which is not known. Gross lysis, involving obvious diminution in opacity of bacterial suspensions, was frequently observed during incubation (*a*) under conditions of suboptimal aeration, (*b*) with limiting nutrients, (*c*) after cold ‘shock’ at 0°, (*d*) in the presence of various growth inhibitors. It always led to an increase in the release of penicillinase. Less severe and less obvious ‘interference’ with cultures, insufficient to cause an obvious decrease in suspension opacity, was often found to be enough to allow leakage of enzymes otherwise found fixed to growing cells, and this, too, was usually associated with increased liberation of penicillinase. On *a priori* grounds, such enzyme release might therefore not unreasonably be regarded as an indication of cell damage: i.e. ‘unphysiological’. Thus, it was first necessary to develop a method for measuring relatively small degrees of cell damage and to find a means of preventing it. The ideal tool would seem to be what we have called an intracellular ‘marker’, whose presence in the extracellular medium should indicate the extent of damage under all experimental conditions. To be satisfactory, such a ‘marker’ should have the following properties. (1) It should *normally* (i.e. under conditions of growth where there are no *a priori* grounds for believing that cell damage occurs) be entirely ‘cell-bound’ (i.e. associated with the deposit on centrifugation of a culture at a speed at which all the organisms are spun down). (2) It should be rapidly, and if possible completely, released into the medium by procedures known to cause cell lysis and/or disruption or disintegration of the cell ‘envelope’ (which, for the purposes of this work, is taken to mean the cell wall plus cytoplasmic membranes plus any other intimately associated structures or material). (3) It should be stable when present in the medium of a normally growing culture of the organism being investigated. (4) It should be capable of being easily, rapidly and accurately estimated.

The first marker tested, deoxyribonucleic acid, proved to be extremely rapidly broken down by cells in a growing culture and was therefore discarded. The second choice, a rather feeble, constitutive β -galactosidase, appeared during preliminary studies to be fairly satisfactory, although there was some loss (20–30 %) of activity during incubation with a growing culture over the 3 hr. period being studied. It was eventually discarded after the discovery that although this enzyme was almost completely released into the medium on cell disruption, less drastic damage to the cytoplasmic membrane (e.g. by incubation of cells with 4 % butanol) allowed only a relatively slow escape from the cell. It was therefore conjectured (and afterwards confirmed) that minor degrees of damage were being missed.

The marker finally chosen was α -glucosidase. This proved to be reasonably

satisfactory and its activity, in whole cultures and in the medium alone, was assayed in every experiment relating directly to the phenomenon of penicillinase liberation. The enzyme has the additional advantage of being inducible (by maltose). This meant that it was possible to adjust conditions such that its production by the cells was restricted to the period when they were forming penicillinase. Thus, unless there was a non-uniform and different distribution of the two enzymes amongst the cells of a heterogeneous population it could be more reasonably assumed that any release of α -glucosidase after the 3 hr. point (when penicillinase production largely ceased) reflected the condition of the same cells as those which were liberating penicillinase.

Fig. 2. The effect of different initial concentrations of maltose on the induction of α -glucosidase in a penicillin-induced culture of *Bacillus subtilis* 6346 growing in casein hydrolysate. The maltose was added at the same time as benzylpenicillin ($0.06 \mu\text{g./ml.}$), when bacterial suspension opacity reached the equivalent of $0.1 \text{ mg. dry wt./ml.}$ Initial maltose concentration of 2 mg./ml. (—●—●—); 1 mg./ml. (—+—+—); 0.5 mg./ml. (—▲—▲—); 0.25 mg./ml. (—■—■—); 0.1 mg./ml. (—○—○—).

Properties of the α -glucosidase system

Induction. The effect of different concentrations of maltose, added at the same time as the penicillin, on α -glucosidase formation, is illustrated in Fig. 2. It can be seen that at an initial concentration of $0.5 \text{ mg. maltose/ml.}$ and below there was little further production of the enzyme after 3 hr., due to exhaustion of the substrate. In order, therefore, to obtain reasonable α -glucosidase titres and at the same time to ensure that formation of the enzyme ceased at 3 hr. (i.e. at about the same time as penicillinase production faded out) an initial maltose concentration of

0.25 mg./ml. was chosen for all experiments. Addition of this concentration of maltose had no detectable effect either on cell growth or on penicillinase induction and liberation.

Assay. As mentioned above, the enzyme is partially cryptic, and lysis by lysozyme was used to allow full expression of activity. With a soluble preparation of the enzyme obtained from the supernatant fluid of a suspension of maltose-induced cells crushed at -30° in the Hughes's press, the pH value for optimum activity was found to be between 6.5 and 6.75 in 0.1 M-sodium phosphate buffers. The enzyme was stable during incubation for 2 hr. at 35° in 0.1 M-phosphate buffers from pH 6.0 to 8.2.

Fig. 3. Release of α -glucosidase ($\bigcirc$ — $\bigcirc$ —), β -galactosidase ($\triangle$ — $\triangle$ —), penicillinase ($\bullet$ — $\bullet$ —), total protein ($+$ — $+$ —) and material absorbing at $260\text{ m}\mu$ ($\square$ — $\square$ —) from cells of suspensions of *Bacillus subtilis* 6346, previously induced with maltose and benzylpenicillin, following treatment: (a) with $100\text{ }\mu\text{g.}$ lysozyme/ml. + 0.05 M-MgSO_4 at 35° ; (b) with 4% butanol at 35° ; (c) by sonication at 0 – 10° . In all experiments, the first samples were taken immediately after beginning of treatment. (For further technical details, see text.)

Rate and extent of release from disrupted cells. α -Glucosidase was more rapidly released from cells, after disruption or damage by a variety of procedures, than any other cell constituent measured. Figures 3a, b and c show the release of α -glucosidase, β -galactosidase, penicillinase, total protein and $260\text{ m}\mu$ -absorbing substances from sedimentable material ($20,000\text{ g.}$, 15 min.) after treatment of 3 hr. *Bacillus subtilis* cells (doubly induced with maltose and benzylpenicillin): (a) with lysozyme ($100\text{ }\mu\text{g./ml.}$ + 0.05 M-MgSO_4 + $0.01\text{ M-KH}_2\text{PO}_4$ buffer pH 7.0), after centrifugation and resuspension to $\frac{1}{2}$ vol. at 35° ; (b) with 4% butanol added directly to the culture at 35° ; (c) by sonication at 25 Kc/min. at 0 – 10° (after centrifugation of the culture and resuspension in fresh medium at $13.0\text{ mg. dry wt./ml.}$). In all experiments samples were removed at the times indicated, the '0 min.' sample being taken immediately after the beginning of treatment, cooled to 0° in an iced bath and thereafter centrifuged at 2° as soon as possible.

Table 1. *Effect of time and type of induction on the release of penicillinase from cells of a Bacillus subtilis culture in casein hydrolysate*

Time of induction* (hr.)	Penicillinase activities (units/ml.) after following times											
	2½ hr.			3 hr.			4 hr.			5 hr.		
	Total	Sn† released	% released	Total	Sn released	% released	Total	Sn released	% released	Total	Sn released	% released
Benzylpenicillin (0.06 µg./ml.)	11.4	2.4	21	.	.	.	14.2	4.2	30	.	.	.
	.	.	.	.	.	.	8.4	1.43	17	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.
Cephalosporin C (10 µg./ml.)	50.5	9.6	19	.	.	.	.	.	.	.	.	.
	.	.	.	110	28.6	26	125	41	33	148	52	35
	.	.	.	.	.	.	.	.	.	.	.	.

* Measured from point when optical density of culture reached the equivalent of 0.1 mg. dry bacterial wt./ml.

† Sn = supernatant fluid.

The '100 % release' value for comparison was obtained by assaying a measured sample of cells resuspended to the same cell density in buffer without Mg^{++} , after addition of 100 μg . lysozyme/ml. and incubation at 35° to obtain full lysis.

After crushing a 13.0 mg. dry wt./ml. suspension of cells in 0.01 M-sodium phosphate (pH 7.0) + 0.05 M- $MgSO_4$ in a Hughes's Press at -30°, 88 % of the enzyme was found to be released into the fraction unsedimented by centrifugation at 20,000 g at 2° for 15 min.

An analogous, dissociated release of up to 90 % of the α -glucosidase, with no more than 5 % release of penicillinase occurred when cells were treated at 0°, especially in the presence of salts (see Pollock, 1961*a*).

Stability in culture medium. An α -glucosidase preparation was obtained from the supernatant fluid of a 3 hr. maltose-induced culture of *Bacillus subtilis* lysed by subjection to 30 min. incubation at 35° without aeration. This was added at a dilution of 1/8 to a 3 hr. culture in CH medium, previously induced for penicillinase but not for α -glucosidase, growing at 35°, exactly under the conditions used for studying penicillinase release. The enzyme in the supernatant fluid was assayed at the beginning of the experiment and after 2½ hr. further incubation (see Table 2). The spontaneous release of α -glucosidase from cells of a maltose-induced sister culture, incubated under similar conditions, but without the addition of the enzyme to the medium, was followed in the same experiment.

The α -glucosidase was found to be inactivated by certain proteinases: trypsin (Armour Labs.); crystalline *Bacillus subtilis* N proteinase (Nagase and Co. Ltd.) and a concentrated supernatant fluid from older cultures of *B. subtilis* 6346 itself, known to have proteolytic activity. This proteolytic destruction was partially prevented by 10^{-4} M-diisopropylfluorophosphate (DFP) and almost completely (90 %) inhibited by 0.1 % heparin (Roche Products Ltd., 110 i.u./mg.). It seemed therefore important to know in this experiment whether the addition of heparin and other proteinase inhibitors had any effect both on the amount of α -glucosidase activity found in the supernatant fluids of cultures which were likely to be releasing the enzyme spontaneously, and on the stability of the enzyme added to cultures not forming it. None of the added substances had any significant effect on increase in bacterial suspension opacity. The results (Table 2) indicate in both experiments that, whereas soybean trypsin inhibitor (Worthington Biochem. Corporation) had no 'protective' effect, the activity in the supernatant fluid of cultures without either heparin or DFP was up to 10 % lower than in the others, therefore a very slight degree of inactivation may have occurred. Clearly, however, this was not enough to invalidate the use of the enzyme as a satisfactorily stable 'marker' in this study.

Results with α -glucosidase as 'marker'

'Damage-release'. By taking the release of preformed α -glucosidase into the medium as a criterion of cell damage, it was soon apparent that *Bacillus subtilis* cells were even more susceptible to harm than had previously been suspected. For instance, even removal of a culture from the shaker and allowing the flask to stand on the bench for a few minutes (such as might sometimes be necessary for sampling, etc.) could allow the subsequent release (after the culture was returned to fully aerated conditions) of up to 40 % of its α -glucosidase within 2 hr. without any significant decrease in optical density of the suspension. A similar degree of what

Table 2. *Stability of α -glucosidase in the culture medium of Bacillus subtilis growing in casein hydrolysate and the effect of certain proteinase inhibitors*

Culture	Additions (at 3 hr.)	α -Glucosidase activities (m μ mole <i>p</i> -nitrophenol formed/ml./hr.)			
		3 hr.		5½ hr.	
		Total	Sn*	Total	Sn
Induced with penicillin and maltose	Control (no additions)	2500	69	2660	86.5
	+ trypsin inhibitor 1 mg./ml.	.	.	.	86.5
	+ 0.1 % (w/v) heparin	.	.	.	110
	+ 3×10^{-4} M DFP	.	.	.	103
	+ Sat. soln. DFP	.	.	.	114
Induced with penicillin, but not maltose; α -glucosidase preparation added at 3 hr.	Control	353	255	.	285
	+ trypsin inhibitor, 1 mg./ml.	.	.	.	284
	+ 0.1 % heparin	.	.	.	304
	+ 3×10^{-4} M-DFP	.	.	.	297
	+ Sat. soln. DFP	.	.	.	316

For full details, see text.

Sn = supernatant fluid.

might be called 'damage-release' of α -glucosidase resulted from a single centrifugation of cells and resuspension in 0.01 M-sodium phosphate buffer, or even in the same medium.

More pronouncedly rough handling of the cells and longer periods of anaerobiosis led, as might be expected, to obvious lysis. But even the smaller degree of α -glucosidase leakage that resulted from minimal interference with standard conditions of culture was usually reflected (as long as it amounted to at least 20 % of the total) by a detectable lag in growth, although no lysis might be apparent, thus justifying the conclusion that escape of this enzyme is 'unphysiological'. Figure 4 shows the effect on α -glucosidase release of 20 min. semi-anaerobic treatment (removal from the shaking apparatus and left static at the same temperature) of a 3 hr. culture subsequently incubated with full aeration, in comparison with a control sister culture maintained under conditions of full aeration all the time. An even more striking example of cell-damage, caused by only 5 min. treatment of a 3 hr. culture at 0° (removal from 35° bath and incubation, with continued shaking in an ice bath), is illustrated in Fig. 5. This cold 'shock' led to the release of nearly 30 % of the total α -glucosidase during the first 30 min. of the ensuing incubation at 35°; and this was associated with (a) a parallel 'damage-release' of about the same proportion of penicillinase, over and above the normal penicillinase liberation, and (b) a 20 min. lag in growth, as measured by increase in optical density.

'Physiological' release. As long as proper care was taken to avoid mechanical and metabolic damage to the cells, the α -glucosidase released from cells of a growing *Bacillus subtilis* culture during the usual 0–5½ hr. period was found never to exceed 5 % of the total, whereas anything up to 50 % of the penicillinase was liberated during the same period. In a series of six similar experiments covering the 0–5½ hr. period a mean α -glucosidase release of 3.4 % was associated with a mean penicillinase release of 36 % of the total. At an earlier stage (0 to 3 hr. period) the figures were

1.2 and 20.5 %, respectively. Figure 6 illustrates a typical experiment. It was not possible to follow the subsequent release of the rest of the cell-bound penicillinase under conditions where damage-release could be excluded.

Fig. 4. The effect of transient semi-anaerobic treatment of a maltose-induced culture of *Bacillus subtilis* in casein hydrolysate on leakage of α -glucosidase from cells into the medium during subsequent aerobic incubation. Total α -glucosidase of control culture (—●—●—); total α -glucosidase of culture after 20 min. semi-anaerobic treatment (—■—■—); α -glucosidase released into medium from control culture (—○—○—); α -glucosidase released into medium from culture after 20 min. semi-anaerobic treatment (—□—□—); 20 min. period of semi-anaerobic treatment to 'test' culture (removal from shaking apparatus for 20 min.) indicated by ———.

DISCUSSION

The fact that cell damage in *Bacillus subtilis* leading to release of α -glucosidase, associated with increased liberation of penicillinase, could take place after apparently slight interference with the normal conditions used for growing and handling the organisms, emphasized the importance of careful control of all techniques. Apart from the dangers of transient deprivation of O_2 already mentioned, it was also found that a high proportion of substances, subsequently tested for their possible effect on penicillinase liberation, promoted release of α -glucosidase (see Pollock, 1961*a*). It seemed clear that any release of α -glucosidase above the irreducible minimum of up to 5 % observed even under 'normal' conditions, would have to be regarded as indicating presumptive cell damage (probably to the cytoplasmic membrane) and that any associated increase in penicillinase liberation must be considered unphysiological and therefore, according to our criterion, an artefact. Once the conditions which caused this α -glucosidase leakage were known, the greatest care was taken to avoid them (e.g. sampling was carried out without removing culture flasks from the shaker). This 'damage-release' appears to be a

Fig. 5. Effect of transient treatment (5 min.) at 0° of a penicillin- and maltose-induced culture of *Bacillus subtilis* growing in casein hydrolysate at 35° , on leakage of α -glucosidase, and associated 'damage-release' of penicillinase from cells into the medium. Total enzyme (square symbols: $\blacksquare$, $\square$); enzyme in culture supernatant (circular symbols: $\bullet$, $\circ$); growth (triangular symbols: $\blacktriangle$, $\triangle$); control culture (filled-in symbols: $\blacksquare$, $\bullet$, $\blacktriangle$); culture treated 5 min. at 0° , at point indicated by arrow (open symbols: $\square$, $\circ$, $\triangle$). (a) α -glucosidase; (b) penicillinase; (c) growth (optical density).

Fig. 6. Liberation of penicillinase and α -glucosidase (used as 'intracellular marker') from apparently undamaged cells of a penicillin- and maltose-induced culture of *Bacillus subtilis* growing in casein hydrolysate. Total penicillinase ($\text{---}\bullet\text{---}$); penicillinase liberated into medium ($\text{---}\circ\text{---}$); total α -glucosidase ($\text{---}\blacksquare\text{---}$); α -glucosidase liberated into medium ($\text{---}\square\text{---}$).

secondary phenomenon, associated with autolytic processes which are initiated by cell damage and inhibited by the higher concentrations (0.05 M) of Mg^{++} (see Pollock, 1961*a*), which are known to assist in maintaining the integrity of the cytoplasmic membrane and other cell structures (Weibull, 1956). It now seems probable that the mechanism of the increased penicillinase release which occurs in association with the conditions that lead to α -glucosidase release is quite different from the normal liberation which occurs in cultures of apparently undamaged organisms.

It can, of course, be argued that, had an intracellular 'marker' more sensitive than α -glucosidase been available, some evidence of cell damage might have been disclosed during the period when what has been referred to as 'physiological' liberation of penicillinase was occurring. There is no formally satisfactory answer to this objection. A great deal must depend upon what is accepted as a definition of cell 'damage'. Clearly, organisms releasing an enzyme into the medium at a high rate must in some way be different from organisms of the same culture which liberate the same enzyme much more slowly. In this connexion, it is unfortunate that it proved to be impossible to do accurate viable counts on the *Bacillus subtilis* strain used in this study, since a comparison of these with total counts might have provided a completely independent assessment of what many people would readily accept as cell 'damage'. The ratios of viable counts to bacterial suspension opacity, however, even in samples from the same culture, were extremely variable and usually very low; no confidence whatever could be placed in their significance. Under the circumstances, however, it seems not unreasonable to assume that a good indication of cell damage is provided by the measurement of α -glucosidase release. It is therefore fair to conclude that a high proportion (up to 40 %) of the cell-bound penicillinase of *B. subtilis* is liberated from the organisms by a process not involving lysis of cells or gross interference with their physiological functions.

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