The Mechanism of Liberation of Penicillinase from *Bacillus subtilis*

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SUMMARY

The effects of various factors on the release of penicillinase from apparently undamaged cells of a growing culture of *Bacillus subtilis* were investigated. The enzyme was not eluted from the cells by treating them with high concentrations of salt. Its liberation did not take place at all at 0°, and was nearly completely inhibited at pH values below 6.0, whereas chloramphenicol, at concentrations sufficient to cause complete cessation of growth, caused only partial inhibition of enzyme release. The penicillinase-releasing action of extracts containing heat-labile 'autolytic' factors from older cells of the same organism could not be dissociated from their damaging effect on the cell, as indicated by concomitant release of the normally intracellular α -glucosidase. It is concluded that normal penicillinase liberation is controlled by enzymic reactions, as yet unidentified, involving detachment of the enzyme from structures superficially located in the cell envelope.

INTRODUCTION

In the first two papers of this series some evidence was presented to suggest that the liberation of penicillinase from a growing culture of *Bacillus subtilis* 6346 may involve a release of the enzyme from the superficial layers of apparently intact cells. The present paper records the results of a direct investigation of the liberation process itself, with a view to characterizing it and obtaining further information on its nature. Minor degrees of cell damage, insufficient to cause measurable lysis, were detected by following the leakage into the medium of an 'intracellular' α -glucosidase whose synthesis was induced in the cells, during the period when penicillinase was being formed, by the addition of a small quantity of maltose (Pollock, 1961*b*).

'Normal' or 'physiological' penicillinase liberation is defined here as the penicillinase release which occurs in untreated cultures in the absence of cell damage sufficient to cause leakage of α -glucosidase greater than the irreducible minimum of up to 5% that was always liable to take place. On this criterion, the conditions under investigation in many experiments caused significant cell damage, and in such cases it could only be concluded that the associated increase in penicillinase liberation, which nearly always occurred, was 'unphysiological'. Unfortunately this took place all too frequently, and in most of the other experiments the factors being studied had no detectable effect. Our conclusions have therefore been limited, extending only to an indication that 'normal' penicillinase liberation is not a matter only of physico-chemical factors, but involves more complicated processes having an enzymic basis.

METHODS

The strain (6346) of *Bacillus subtilis* used, the techniques employed, the definitions adopted and the general approach to the problem have been described and discussed previously (Pollock 1961*b*). It should be particularly noted that the standard 'zero' reference time (0 hr.) from which all time periods are measured was taken as the point when the population density of growing cultures reached the equivalent of 0.1 mg. bacterial dry wt./ml. Unless stated otherwise this has also been the time when cells were induced to form penicillinase and α -glucosidase.

RESULTS

Effect of pH value

When the casein hydrolysate (CH) medium was supplemented with 2% (w/v) glucose, the organisms were found never to release more than a small fraction of their total penicillinase. Even after 6 hr. such glucose-grown organisms had liberated only 15% of their total penicillinase, in contrast to organisms grown in CH medium without glucose, where 40% or more of the enzyme was released into medium during the same period (see Pollock, 1961*b*). This effect was finally shown to be due mainly, if not entirely, to the lower pH value of the culture, which usually fell from 7.0 to 5.0–5.5 in glucose-containing cultures.

It was confirmed that the low amounts of extracellular penicillinase were not due to selective inactivation of exo-enzyme under the conditions associated with glucose-grown cultures. Concentrated dialysed supernatant fluid from a penicillin-induced culture at a final concentration of 13.5 units penicillinase/ml. was added to a 3 hr. uninduced culture of *Bacillus subtilis* growing in CH medium + 2% (w/v) glucose. Incubation continued for a further 2 hr. caused no significant loss of activity.

In the experiment summarized in Table 1 a culture grown in CH medium + 2% (w/v) glucose was split into two portions at 3 hr., and both portions incubated in large tubes at 35°, aerated by a flow of air through a sparger at 500 ml./min. In one portion the pH value was adjusted to pH 7.8 and kept constant thereafter for the 2 hr. of the experiment by an automatic titrator (Radiometer, Titrator TTTI, V. A. Howe and Co. Ltd., 46 Pembroke Road, W. 11) delivering minute quantities of N-NaOH into the culture continuously under the control of a pH meter. The total volume of NaOH thus added never increased the volume of the cultures by more than 3.5% and was ignored in comparing the results. A complementary experiment was also done without glucose in the medium, one of the two sister cultures being kept at pH 5.2 by automatic delivery of N-HCl while the control culture was left to drift towards alkalinity without adjustment. It can be seen that several times less enzyme was liberated in cultures at the lower pH values, regardless of whether or not glucose was present.

Effect of chloramphenicol

Addition of 40 μ g. chloramphenicol/ml. to a culture of *Bacillus subtilis* 6346 inhibited growth (opacity increase) and penicillinase production almost completely. The effect on enzyme liberation appeared at first to be variable. To obtain higher enzyme titres and more reliable results, some cultures were induced with cephalosporin C (1 μ g./ml.) and it was at once clear that significant enzyme release occurred

Table 1. *Effect of pH value on the liberation of penicillinase from penicillin-induced Bacillus subtilis 6346 growing in CH medium*

Conditions	Penicillinase activities (units/ml.)							
	pH value						Released during 3-5 hr. period	
	At 3 hr. before adjustment of (B)	At 5 hr.	At 3 hr.		At 5 hr.		Amount	% of total
			Total	Sn*	Total	Sn		
	Exp. 1. In presence of 2 % (w/v) glucose							
(A) No special control of pH value	5.2	5.4	30.96	2.92	31.2	3.8	0.88	2.8
(B) pH Adjusted to pH 7.8 at 3 hr. and thereafter kept constant	5.2	7.8	31.60	3.4	30.0	11.2	7.8	25.5
	Exp. 2. Without added glucose							
(A) No special control of pH value	6.8	7.0	28.40	6.2	30.56	10.8	4.6	15.8
(B) Adjusted to pH 5.2 at 3 hr. and thereafter kept constant	6.8	5.2	28.76	6.0	27.76	7.6	1.6	5.6

* Sn = supernatant fluid.

in the presence of chloramphenicol; but no quantitative comparison with untreated cultures was possible because the total enzyme production was so much less than in the control. Repeated experiments with benzylpenicillin-induced cultures finally showed that chloramphenicol did in fact cause a significant and consistent inhibition of liberation of 30-50%; even in cultures where the slight difference in pH value from the control (due to growth inhibition and subsequently decreased metabolic drift towards alkalinity that is always shown by *B. subtilis* when growing in CH medium) was obliterated by the automatically titrating pH regulator, adjusted so that the pH value of the control culture was kept identical with that of the chloramphenicol-treated culture (measured independently with a second pH meter), as shown in Table 2. Neither earlier addition of chloramphenicol (namely at 2 hr.

Table 2. *Effect of chloramphenicol on the liberation of penicillinase from penicillin-induced Bacillus subtilis 6346 in CH medium*

Conditions	Penicillinase activities (units/ml.)							
	pH						Released into medium during 3-5 hr. period	
	At 3 hr.	At 5 hr.	At 3 hr.		At 5 hr.		Amount	% of total
			Total	Sn*	Total	Sn		
Control (no addition)	7.0	7.65	17.6	3.6	21.2	6.8	3.2	15
Chloramphenicol 40 µg./ml. added at 3 hr.	7.0	7.65	—	—	20.4	5.2	1.6	7.8

* Sn = supernatant fluid.

instead of 3 hr.) nor raising its concentration to 100 $\mu\text{g./ml.}$ caused any significant change in the proportion of enzyme released.

Effect of temperature

Figure 1 shows the effect of temperature on penicillinase release from a culture previously induced with 0.25 mg. maltose/ml. and cephalosporin C (1 $\mu\text{g./ml.}$) treated with chloramphenicol (40 $\mu\text{g./ml.}$) at 3 hr. and immediately distributed in three lots in water baths maintained at 0°, 21° and 35°, respectively. The proportions of α -glucosidase released after 2½ hr. incubation at these three temperatures were found to be 36.3, 4.1 and 5.6%, respectively. The cold treatment obviously

Fig. 1. The effect of temperature on the liberation in the presence of chloramphenicol of penicillinase from 3-hr. cells of *Bacillus subtilis* 6346, previously grown at 35° after induction with cephalosporin C (10 $\mu\text{g./ml.}$). 35° (circles: ●, ○); 21° (triangles: ▲, △); 0° (squares: ■, □); enzyme released into medium (open symbols: ○, △, □); total enzyme (filled-in symbols: ●, ▲, ■). Arrow indicates point of addition of chloramphenicol 40 $\mu\text{g./ml.}$

caused considerable cell damage, without, however, leading to penicillinase liberation, as long as the temperature was kept at 0°. When, however, the culture was returned to 35°, even brief cold treatment (e.g. 5 min. at 0°) resulted in substantial subsequent 'damage-release' of penicillinase, as shown in the first paper in this series (Pollock, 1961*b*; Fig. 5). In another experiment it was found that the penicillinase liberation increased further with temperatures above 35°; but at 45° this was always associated with considerable cell damage and could not therefore be regarded as physiological.

The rather striking dissociation between release of α -glucosidase and penicillinase at 0° in the experiment illustrated in Fig. 1 supports the original conclusion (Kushner & Pollock, 1961) that cell-bound penicillinase is normally fixed to particulate cell structure and does not exist 'free' in the cytoplasm (as appears to be the case with

α -glucosidase), and shows further that both the normal 'physiological' mechanism for the release of penicillinase and the secondary 'artefactual' release process, which under all other conditions so far investigated comes into effect following cell damage, are inoperative at 0° and therefore more likely to be dependent on some enzymic process in the cell rather than on diffusion or other purely physico-chemical factors.

Table 3. *Failure of high concentrations of salts to elute penicillinase from penicillin-induced Bacillus subtilis 6346 after 2 hr. treatment at 0°*

Treatment	α -Glucosidase (as μ mole <i>p</i> -nitrophenol/ml./hr.)			Penicillinase (units/ml.)		
	Total	In Sn	% in Sn*	Total	In Sn	% in Sn
Potassium phosphate (pH 7.0)						
10 ⁻³ M	3130	2030	65	17.0	0.48	2.8
5 × 10 ⁻² M	3130	2500	80	17.8	0.68	3.8
2 × 10 ⁻¹ M	3130	2500	80	16.1	0.76	4.7
Ammonium sulphate (pH 7.0)						
5 × 10 ⁻² M	3130	2630	84	16.6	0.56	3.4
2 × 10 ⁻¹ M	2970	2630	89	17.0	1.12	6.6

* Sn = supernatant fluid.

Effect of high salt concentrations

A 3 hr. culture of *Bacillus subtilis* 6346, previously induced with penicillin and maltose, was cooled to 0° and the organism resuspended in solutions of potassium phosphate and ammonium sulphate (pH 7.0) at concentrations of up to 0.2M for 2 hr. (at 0°, instead of 35°, to avoid 'damage-release'). Table 3 shows that negligible quantities of penicillinase (>7%) were released by this treatment, which would be expected to elute a substantial fraction of enzyme, were the normal liberation process due to a desorption of protein bound to the cell by simple ionic linkages. This experiment shows the profound cell damage that occurred as a result of this treatment which led to the release of up to 90% of the α -glucosidase. It is also a further indication of the different manner in which the penicillinase is bound to the cells of *B. subtilis* as compared with *B. cereus*, where analogous salt treatment eluted nearly all the cell-bound β -penicillinase within a few minutes (Pollock & Kramer, 1958).

Possible role of enzymes

Effect of enzyme inhibitors. With 3 hr.-cultures of *Bacillus subtilis* 6346 growing in CH medium many of the substances and factors tested (1.6 × 10⁻³M-*p*-chloromercuribenzoate; 0.05–1.0% (w/v) sodium deoxycholate; 8.3 × 10⁻⁴M-8-hydroxyquinoline) increased penicillinase liberation but rapidly led to cell damage (α -glucosidase leak) so that their action on physiological release could not be assessed. Other substances added (5 × 10⁻³M-NaN₃; 10⁻³M-2:4-dinitrophenol; CuSO₄, 100 μ g./ml.; 0.02M sodium succinate; mercaptoethanol, 0.5 mg./ml.; carbamylcholine, 0.1 mg./ml.; histamine phosphate, 0.1 mg./ml.) had no detectable effect. Ethylenediamine tetra-acetic acid (10⁻³M), in the presence of chloramphenicol (40 μ g/ml.), had a slight inhibitory effect on penicillinase liberation (up to 40% from cells of a cephalosporin C-induced culture in some experiments), but this was not consistently repeatable. A similar

variable but even slighter inhibitory effect was shown by low doses of ultraviolet radiation in the absence of chloramphenicol. High concentrations of Mg^{++} (0.05M- $MgSO_4$) which had been shown to have a very striking inhibitory effect on the release of the enzyme from membrane preparations or damaged cell suspensions (Kushner & Pollock, 1961) had no action whatever in cultures growing normally in CH medium.

Proteinases. The specific penicillinase-liberating action of proteinases on sucrose-treated suspensions of *Bacillus subtilis* 6346 (Kushner & Pollock, 1961) and the possibly enzymic nature of the liberation process suggested that this might normally involve the action of some proteolytic enzyme or esterase, either by direct detachment of the enzyme by hydrolysis of a peptide bond which linked it to some surface structure, or by destruction (solubilization) of a protein responsible for its fixation. Attempts to inhibit the hypothetical esterase involved, by addition of diisopropyl-fluorophosphate (DFP) and heparin (0.1 %) were unsuccessful. Concentrations of DFP up to a nearly saturated solution (addition of 1 drop of pure DFP in 10 ml. of culture, repeated twice during the 2 hr. experiment) had no significant effect on liberation of penicillinase from cephalosporin C-induced cells of a culture previously treated with chloramphenicol (40 $\mu g./ml.$).

Table 4. *The effect of crude dialysed extracts of organisms from old Bacillus subtilis 6346 cultures on release of α -glucosidase and penicillinase from cells of a maltose- and penicillin-induced homologous B. subtilis culture in casein hydrolysate (CH) medium*

Time	Volume (ml.) of extract* added (at 3 hr.) to 10 ml. culture	Bacterial suspension concentration (equiv. mg. dry wt./ml.)	α -Glucosidase (m μ mole <i>p</i> -nitrophenol/ ml./hr.)			Penicillinase units/ml.)		
			Total	In Sn†	% in Sn	Total	In Sn	% in Sn
3 hr. (before addition of extract)	—	0.85	4310	47	1.1	21.6	4.8	22.2
5½ hr.	0	2.10	4130	65	1.6	20.8	7.6	36.5
	0.023	2.25	4000	108	2.7	21.6	8.3	38.5
	0.067	2.44	4500	338	7.5	21.2	9.5	44.8
	0.2	2.10	4810	1275	26.5	22.0	12.2	55.3
	0.5	1.04	4900	3090	63	20.4	15.6	76.5
	1.8	0.37	5100	4560	89.5	21.6	13.3	61.7

* The extract was diluted so that 1.0 ml. contained material originally derived from the organisms of 10 ml. of a 24 hr. culture. Further details, see text.

† Sn = supernatant fluid.

Crude cell extracts. Extracts were prepared from organisms of an uninduced culture of *Bacillus subtilis* 6346, by resuspending the organisms at ten times their original concentration in $10^{-2}M$ -potassium phosphate buffer (pH 7.0) and crushing them in a Hughes press at -30° . The supernatant fluid after centrifugation of the disrupted suspension at 20,000 *g* for 20 min. was dialysed against $10^{-3}M$ -potassium phosphate (pH 7.0) and concentrated by freeze-drying. These extracts had enzyme-like properties which appeared to be similar to those of the 'autolysin' of *B. subtilis* strain H described by Nomura & Hosoda (1956). When added to cultures of *Bacillus subtilis* 6346 in CH medium the extracts consistently caused cell damage, the extent of

which depended upon the quantity of extract and the age of the culture from which it was prepared. Severe damage resulted in cell lysis. Less severe damage was shown by leakage of α -glucosidase and increase in penicillinase liberation; but it was not possible to dissociate these two effects by diluting the extract. A typical experiment is illustrated in Table 4 where increasing quantities of an extract prepared from a 24 hr. culture were added to 10 ml. samples of a 3 hr.-culture previously induced with maltose and penicillin. The effects on opacity increase and release of the two induced enzymes into the medium were followed during incubation for a further $2\frac{1}{2}$ hr. It can be seen: (a) only the highest concentration of extract caused obvious cell lysis (decrease in suspension opacity) although $1/27$ of this concentration caused a significant α -glucosidase leak of 7.5 %; (b) liberation of penicillinase was increased by addition of extract very roughly in proportion to the extent of α -glucosidase release; there was no concentration of extract which stimulated penicillinase release without significantly increasing the release of α -glucosidase above the basal value of 1.6 % shown by the control culture. Extracts from the organisms of young cultures were very much less active in causing cell damage than extracts from organisms of older cultures. For instance, the degree of cell damage, as measured by % α -glucosidase leakage, produced by extract of a given dry weight from organisms of 3 hr.- or 6 hr.-cultures, could be evoked by approximately one-fiftieth of that quantity of extract prepared from 20 hr.-cultures. The autolytic factor or factors concerned were completely inactivated by boiling for 30 min. It is clear that the factor(s) was being produced in quantity only in old cultures, where some slight activity was also demonstrable in concentrated dialysed supernatant fluid as well as in the organisms themselves.

In addition, extracts of organisms from 20 to 24 hr. cultures were found to contain proteinase activity, whose action in liquefying gelatin and destroying α -glucosidase was almost completely inhibited by 5×10^{-3} M-DFP. For this reason this proteinase activity can be almost certainly rejected as participating in normal penicillinase liberation or in the 'damage-release' effect of the cell extracts described above, because these processes are not affected by DFP. It is possible that some sort of lysozyme-like enzyme, known to be formed by certain strains of *Bacillus subtilis* (Richmond, 1959 *a, b*) might be partly responsible for the lytic action of these extracts; but it is rather unlikely on *a priori* grounds that such an enzyme plays a part in normal penicillinase liberation. In any case, egg-white lysozyme by itself had a completely different action: at concentrations of 1 μ g./ml. and above, it caused rapid lysis of 3-hr. cultures, whereas at 0.4 μ g./ml. and below there was no detectable effect whatever (no growth inhibition, no leak of α -glucosidase, no stimulation of penicillinase liberation). All further attempts, by graded ammonium sulphate precipitation, zone electrophoresis and column fractionation, to isolate a specific penicillinase-liberating factor from these extracts, by separation from other material which might be interfering with its action or causing cell damage, have so far failed.

DISCUSSION

The problem of exo-enzyme liberation from bacteria has been approached in the absence of any real knowledge about the nature of the structural barriers or physical or chemical entities which may obstruct free diffusion of penicillinase from a cell after the enzyme is formed. It may well be that *Bacillus subtilis* 6346 penicillinase

is a special case and that the mechanism of its liberation is unrelated to the mechanism acting on other exo-enzymes (e.g. α -amylase from the same strain) where little or no enzymic activity is found to be associated with the cell under any conditions (Dr R. A. Darrow; personal communication) and where, therefore, the liberation process may be linked with the formation of the enzymes (either *de novo* or from an enzymically inactive high molecular weight precursor).

The classical concept of the bacterial cell as consisting of cytoplasm surrounded by a selectively impermeable membrane enveloped by a relatively permeable cell wall (see Salton, 1960; McQuillen, 1960) rather implied that the essential process involved in enzyme release must be its passage through the cytoplasmic membrane. Such a picture may be mistaken. The cytoplasmic membrane has chemical and anatomical reality and there is good reason for believing it to be mainly responsible for the selective permeability of the bacterial cell. But little is known about how exo-enzymes are formed and nothing about the site of their synthesis. It is possible that the process of specific polypeptide chain production which leads to their formation occurs outside the main permeability barrier of the cell. In any case, the classical picture of a continuous and undifferentiated cytoplasmic membrane may be a gross oversimplification. In *Bacillus subtilis* itself there are now indications of a sort of rudimentary endoplasmic reticulum (Glauert, Brieger & Allen, 1961) which might play a part in enzyme liberation. Finally, the barrier to large molecules offered by the cell wall is quite unknown, though Mitchell & Moyle (1959) and Mitchell (1961) have produced evidence which suggests that in *Staphylococcus aureus* and *Escherichia coli* it may not be negligible.

The present work permits only tentative and limited conclusions; but they may be useful in forming a basis for further studies. Assuming that the population of organisms in the culture is homogeneous with respect to enzyme release it can be accepted that at least a fair proportion of the penicillinase originally bound to cells of *Bacillus subtilis* is released into the medium by a process not involving marked cell damage. The complete inhibition of liberation at 0°, the zero order kinetics, the partial inhibition by chloramphenicol and the failure to elute enzyme from the cells by high concentrations of salts, all suggest that the process may be enzymic rather than physico-chemical. Chloramphenicol would be expected to inhibit the formation but not the functioning of such an enzyme system and would therefore cause only partial inhibition of the processes governed by the enzyme; this was what was observed. But it must be admitted that the failure to increase the inhibition of enzyme release by adding chloramphenicol earlier on during cultivation, when the cell content of the hypothetical enzymes responsible for liberation might be expected to be lower, does not support this interpretation. Conclusive evidence about the nature of the enzyme or enzymes involved has not been obtained.

Cell extracts of organisms from old cultures of the homologous strain of *Bacillus subtilis* 6346 increased the rate of penicillinase release from organisms from 3-hr. cultures. The main question is, what part, if any, substances present in these extracts may play in normal physiological liberation of the enzyme. But this stimulatory effect on penicillinase release was always associated, *pari passu*, with cell damage, as indicated by a leakage of α -glucosidase which did not occur in cultures which were spontaneously liberating penicillinase in the normal way. It did not prove possible, by a variety of techniques, to dissociate the two effects. Such

'autolytic' extracts might be expected to contain many different enzymes; but the only activity present which was specifically identified was that of a DFP-sensitive proteinase. This could be rejected as a possible agent for normal penicillinase liberation because of the latter's insusceptibility to inhibition by DFP. This negative finding does not completely exclude the possibility that a proteinase of some sort plays a part in penicillinase liberation, since DFP-insensitive examples of this kind of enzyme (e.g. cathepsin B) have been reported (see Fruton, 1960). In any case, the possibility of some specific penicillinase-liberating factor being present in these extracts cannot be excluded simply by negative results which, it might still be argued, may only reflect the difficulty of reproducing an enzymic effect which is suspected of occurring somewhere within the cell envelope, by adding the enzyme artificially from the outside. The great decrease in liberation of enzyme at a pH value (5.5) at which most cell enzymes might be expected to function poorly, is also consistent with the hypothesis that the phenomenon has an enzymic basis.

Although gross cell damage appears to be excluded, it can always be argued that minor degrees of damage (e.g. the initial steps of a chain of metabolic events finally leading to observable damage and lysis) are, nevertheless, an essential preliminary to exo-enzyme release. This possibility, which is really the essence of the view put forward by Nomura, Hosoda & Yoshikawa (1958) is made less likely by the insusceptibility to the inhibitory effect of high Mg^{++} concentration, and the absence of concomitant α -glucosidase release which characterize 'physiological' liberation of penicillinase and thus distinguish it qualitatively from 'damage-release'.

It might, perhaps, be preferable to consider the possibility that liberation of penicillinase is a reflexion of some relatively non-specific process of change in the biochemical, chemical and physical properties of the cell envelope, affecting the extents to which several other substances, as well as penicillinase, were bound to it. Many bacterial exo-enzymes appear during the later stages of batch growth of *Bacillus* sp. (see Pollock, 1961*a*). The reason for this is not yet understood. It is striking, for instance, to note that the period in a batch culture when penicillinase is being liberated corresponds closely to that during which α -amylase is appearing in the medium. It would be useful, therefore, to know whether generalized changes do occur in the cell wall and other superficial cell structures over this period.

It seems clear that in *Bacillus subtilis* 6346 cell-bound penicillinase is normally fixed to solid cell structure, possibly in part to the cytoplasmic membrane itself. Although only a proportion ($> 50\%$) of this cell-bound penicillinase was conclusively shown by antiserum neutralization experiments (Kushner & Pollock, 1961) to be on the outside of the membrane, the results as a whole are consistent with it all being there. It is possible that the membrane itself may be the site of its formation. If this be so, the mechanism of exo-enzyme release may involve detachment of enzyme from some superficial structure (which the penicillinase-releasing effect of trypsin suggests may be protein in nature, Kushner & Pollock, 1961), followed by its passage through the cell wall. This appears, at least for the moment, to be a useful working hypothesis to guide further studies.

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