The Cell-bound Penicillinase of *Bacillus cereus*

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SUMMARY: Only 30-50 % of the cell-bound penicillinase of *Bacillus cereus* NRRL 569 is neutralized by antiserum prepared against the exo-penicillinase. The un-neutralizable fraction is not decreased by cell disintegration which liberates a proportion of the cell-bound enzyme into solution. Absence of neutralization cannot therefore be explained by the existence of a mechanical barrier which prevents access of antibody; it has in fact been shown to be due to the presence of another type of penicillinase, not liberated from the cells, which is enzymically similar to, but immunologically and physico-chemically distinct from, the exo-enzyme. This cell-bound penicillinase has been separated from the exo-enzyme by fractional precipitation with ammonium sulphate. It is inducible by penicillin, like the exo-enzyme, and is present in cells of the constitutive mutant strain, 569/H.

The penicillinase of *Bacillus cereus* strain 569, like that of other strains of this species, is mainly exocellular. With liquid cultures in broth or casein hydrolysate only about 10 % of the total activity is found attached to the organisms after centrifugation. This proportion has not been found to vary markedly with conditions of culture, phase of growth or extent to which total penicillinase production is increased by induction with penicillin (see Pollock, 1952). It was thought that some information might be obtained on the localization of this cell-bound enzyme by studying the action of an anti-penicillinase serum. Krebs & Wright (1951) found that an antiserum to purified yeast triosephosphate dehydrogenase caused 90 % inhibition of activity of the isolated enzyme, although it had no effect on glucose fermentation in intact organisms; they concluded that antibody molecules could not penetrate the cell wall. Analogous findings were reported by Pasternak, Sevag & Miller (1951) with yeast hexokinase and carboxylase, neither of which was affected in intact organisms by treatment with antisera which neutralized the enzymes after extraction from the organisms. Sevag, Newcomb & Miller (1954) discovered that the cell-bound α -glycerophosphatase of dried yeast could be completely inhibited by a specific anti- α -glycerophosphatase serum and so concluded that the enzyme must be 'at or near the cell surface'. It seems to be a fair assumption that antibody molecules of γ -globulin cannot penetrate inside normal, intact microbial cells. Specific neutralization of the activity of cell-bound enzyme by an antiserum can therefore reasonably be taken as an indication that the enzyme is on the cell surface. In the work reported here it was found that only about 50 % of the cell-bound enzyme activity could be neutralized by antiserum which neutralized nearly completely the activity of the exo-enzyme (Pollock, 1956). Attempts to understand the reasons for this absence of neutralization led to the discovery of an immunologically and physico-chemically distinct type of penicillinase which was probably situated inside the cell wall.

METHODS

Organism and enzyme formation. *Bacillus cereus*, NRRL 569, was grown from a spore inoculum in 'S' peptone broth (Pollock & Perret, 1951) containing 1% (w/v) gelatin (to prevent enzyme inactivation), shaken at 35°. Without addition of penicillin only small amounts of enzyme were produced. In order to induce the formation of penicillinase, 1 unit benzylpenicillin/ml. was added at a concentration of organisms corresponding to 0.1 mg. dry weight organisms/ml. and incubation continued for up to 60 min. Oxine (8-hydroxyquinoline) at a concentration of 8.3×10^{-4} M was added to stop further enzyme formation, and all enzyme preparations (whether of soluble exo-enzyme, suspensions of whole organisms or disrupted organisms and extracts therefrom) were prepared, unless stated otherwise, in the presence of 8.3×10^{-4} M-oxine and 1% gelatin.

The mutant strain 569/H of *Bacillus cereus*, which produces penicillinase constitutively (subsequently referred to as being of a 'constitutive-penicillinase' type) was derived from strain 569 (see Kogut, Pollock & Tridgell, 1956). It was used for a few experiments and was grown in a similar manner, except that no additions of penicillin were necessary for maximal production of penicillinase.

Antiserum. A solution of γ -globulin was prepared from the serum of a rabbit immunized with purified induced exo-penicillinase from *Bacillus cereus*, strain 569 (Pollock, 1956).

Enzyme assay. Total enzyme was normally assayed manometrically at pH 7.0 by the method of Henry & Housewright (1947) using 1.0 ml. samples in oxine and gelatin. The 'unneutralizable' moiety is expressed as a percentage of the total activity remaining after addition of sufficient antibody to neutralize four equivalents of exo-enzyme. Now, when anti-penicillinase serum is added to exo-enzyme, there always remains from 2 to 4% of the activity unneutralized, even in gross excess of antibody. This was shown to be due to the residual enzymic activity of the enzyme-antibody complex (Pollock, 1956) and has nothing to do with the present problem except in so far as it decreases the accuracy of assay of the 'unneutralizable fraction' in a mixture. When the latter forms a low proportion of total activity, there may be considerable error in its determination. But the error becomes progressively less as the proportion of unneutralizable fraction rises ($> 8\%$ in a mixture of equal parts) and does not significantly affect the results reported here. An example may illustrate the technique used. Suppose a suspension of organisms or disintegrated cell debris is found to have a total enzyme activity of 25 units/ml. A sample (usually 2.0 ml.) is mixed with 0.5 ml. antiserum γ -globulin containing a total of 100 569-neutralization units of antibody. The mixture is assayed after 30 min. at room temperature and the activity expressed as a percentage of that of the untreated sample. The neutralization titre of the antibody preparation is measured by accurate assay of the activity which remains after mixing a constant amount of the 569 exo-enzyme with varying quantities of antiserum (Pollock, 1956), one neutralization unit being defined

as that amount of antibody which will neutralize 1 unit of penicillinase in the presence of excess enzyme.

For the determination of penicillinase activity at different pH values, the iodometric assay technique of Perret (1954) was used. For the determination of Michaelis constants, enzymic activity was measured by cup-plate bio-assay of the residual penicillin, with *Bacillus subtilis* I.C.I. strain as test organism (Humphrey & Lightbown, 1952) since the affinity of penicillinase for its substrate is so high that manometric assay of the reaction at concentrations of penicillin insufficient to saturate the enzyme is impossible. It was found that at an enzyme concentration of about 0.02 unit/ml., penicillin could be accurately assayed in the presence of enzyme provided that: (a) the reaction mixture was diluted at least tenfold in ice-cold phosphate buffer before sampling in the cups; (b) the penicillin in the cups was allowed to diffuse into the surrounding agar of the assay plate at 2° for at least 4 hr. before incubation. In this way reaction velocities could be measured by incubation for 60 min. at 30° in 1% gelatin with 0.01 M-phosphate (pH 7.0) at penicillin concentrations down to 10 units/ml., taking samples every 15 min. All penicillin samples were assayed in eight replicates. The maximum enzyme activity measured by this technique was found to be about 15% higher than that measured manometrically.

RESULTS

Fig. 1 shows the effect of adding increasing amounts of anti-penicillinase antibody to: (a) exo-enzyme (penicillin-induced culture supernatant fluid); (b) organisms from the same culture centrifuged and resuspended in oxine + gelatin; (c) organisms washed three times in oxine + gelatin; (d) organisms washed five times before resuspension. It can be seen that about 40% of the cell-bound enzyme in (b) was neutralized by antibody, and that most of this neutralizable, bound enzyme was only loosely bound to the organisms since it was removed by repeated washing, leaving the unneutralizable moiety still attached (d). The neutralization slope of that fraction of the cell-bound enzyme, which was easily removed by washing the organisms, was similar to that of the exo-enzyme, and shows that these two fractions of enzyme are immunologically identical or very closely related. Indeed, the process of washing off, which will occur at +2° in the presence of oxine, appears to be rather in the nature of mechanical removal, from absorption on the surface of the organisms, of 'mature' enzyme, otherwise indistinguishable from exo-enzyme. Addition of organisms from an uninduced culture had no effect on the neutralization by antibody of exo-enzyme (induced culture supernatant) and thus confirmed that the unneutralized cell-bound fraction was a distinct moiety which was for some reason unable to react with antibody. It seemed at first natural to attribute this absence of neutralization to the presence of some barrier, associated with intact cell structure, which allowed penicillin to reach the enzyme but prevented access of the relatively large molecules of immune rabbit γ -globulin.

However, disintegration of the organisms did not lead to increased neutrali-

zation of enzyme by antiserum. Table 1 summarizes the results of four experiments with previously induced cultures of *Bacillus cereus*, strain 569, in which 7 ml. of a thick suspension of washed organisms were crushed in the Hughes press at -30° , in the presence of 8.3×10^{-4} M-oxine and 1% (w/v) gelatin. Microscopic examination of cell material after such crushing revealed a mass of debris and partially disintegrated cells but no significant proportion of normal intact organisms. The total penicillinase activity after disintegration (A) was on an average about 8% less, and in no instance greater than that of unbroken

Fig. 1. Effect of antibody (prepared against exo-penicillinase) on penicillinase activity of supernatant fluid and cells of a penicillin-induced culture of *Bacillus cereus* 569. $\bullet$ — $\bullet$ — $\bullet$ —, supernatant fluid (= exo-penicillinase); $\circ$ — $\circ$ — $\circ$ —, unwashed organisms, resuspended after centrifugation; $\times$ — $\times$ — $\times$ —, 3 times washed organisms; $\triangle$ — $\triangle$ — $\triangle$ —, 5 times washed organisms.

organisms, so confirming that the intact cell structure offered no barrier to access of penicillin to the enzyme. Moreover, the preparation of disintegrated organisms showed the same proportion of unneutralizable activity as the intact organisms. The disintegrated organisms were then suspended in an equal volume of oxine+gelatin mixture and divided into two portions: one was centrifuged for 15 min. at 2000 *g* as soon as the ice crystals had melted, and the supernatant fluid (B) separated; the other portion was incubated at 35° for 10 min., to allow the mass to melt and the whole preparation to be homogenized by stirring, and was then centrifuged and the supernatant fluid (C) separated. It can be seen that the supernatant B contained on an average

about 25 % of the activity extracted in supernatant C, but the percentage of unneutralizable enzyme was in some instances disproportionately high. Altogether about 40 % of the total cell-bound activity and 20 % of the unneutralizable cell-bound activity was thus eventually liberated into solution, but the total amount of unneutralizable activity was never increased. Thus although a proportion of the cell-bound enzyme appeared to be 'intracellular' (in the sense that it was not detached from the organisms until they were broken), its inability to be neutralized by antiserum could not be explained by the presence of some solid barrier preventing access of antibody molecules. It seemed reasonable to suppose that a proportion, at least, of the unneutralizable penicillinase moiety consisted of some immunologically distinct type of molecule or molecular complex.

Table 1. *Total and unneutralizable penicillinase activities found in washed penicillin-induced Bacillus cereus 569 before and after disruption in the Hughes press and subsequent extraction into solution*

For full details see text. Results are expressed as: (a) total penicillinase activities in units per mg. dry weight of the bacterial cells from which the preparation was derived: (b) as percentage of this activity which is not neutralized by an antiserum to the exo-enzyme.

Expt.	Period of incubation after induction with penicillin (1 unit/ml.) (min.)	Soluble fraction from disrupted organisms							
		Intact organisms		(A) Disrupted organisms (total)		(B) Rapid extraction		(C) Extraction after incubation at 35° for 10 min.	
		(b)		(b)		(b)		(b)	
		(a) Total	Unneutraliz- able (%)	(a) Total	Unneutraliz- able (%)	(a) Total	Unneutraliz- able (%)	(a) Total	Unneutraliz- able (%)
1	40	—	—	5.22	52	0.54	94	2.10	51
2	40	7.15	58	6.80	57	1.47	79	—	—
3	60	17.1	66	15.6	65	2.21	71	7.95	54
4	75	20.5	72	18.5	69	2.54	64	9.38	51

For convenience, the penicillinase of *Bacillus cereus* has been divided empirically into different fractions based primarily on their ability to be neutralized by an antiserum prepared against the exo-enzyme and the degree to which they are associated with sedimentable solid cell material, as shown in Table 2. The α -fraction is simply the 'normal' exo-enzyme, liberated into the culture medium. It has been purified, and characterized in some detail (Kogut *et al.* 1956). The β -fraction, most of which can be removed from the organisms by repeated washing, is the neutralizable moiety of the cell-bound enzyme. It is immunologically indistinguishable from the α -fraction. The γ -fraction is that portion of the unneutralizable cell-bound activity which can be extracted into solution after disintegration of the organisms. The relative activities of the various fractions are indicated because they were found to be approximately constant in several experiments with induced cultures and did not differ markedly from those obtained before induction.

Table 2. *Distribution and properties of different penicillinase fractions obtained from penicillin-induced Bacillus cereus 569*

	Penicillinase activity (% of total)	Descriptive term
Exo-cellular	85-95	α -Penicillinase
Cell-bound:		
(a) Total	5-15	'Cell-bound' enzyme
(b) Neutralized by antiserum to exo-enzyme	1-8	β -Penicillinase
(c) Not neutralized by antiserum to exo-enzyme		
(i) Total	4-7	'Unneutralizable' fraction (normally all γ -penicillinase)
(ii) Extractable into solution	2-3	γ -Fraction (γ -penicillinase)

Fig. 2. Separation of γ -penicillinase from the neutralizable cell-bound penicillinase (β -fraction) by fractionation, in ammonium sulphate, of a preparation from an autolysate of washed penicillin-induced *Bacillus cereus*, 569. Plot of activities remaining in supernatant fluid after centrifuging off precipitate at different concentrations of ammonium sulphate. —●—●—, penicillinase activity neutralized by antiserum to exo-enzyme; --○--○--, penicillinase activity not neutralized by antiserum; ...×...×...×..., percentage of total activity not neutralized by antiserum.

Isolation of γ -penicillinase. Approximately 500 g. dry weight of *Bacillus cereus*, strain 569, from 320 l. induced culture grown in casein hydrolysate and used for preparation of purified induced-penicillinase (Kogut *et al.* 1956) was washed with 2 l. of 8.3×10^{-4} M-oxine in 0.01 M-phosphate (pH 7.0), resuspended in 2 l. oxine + phosphate and incubated for 16 hr. at 35°. This treatment allowed considerable autolysis of the organisms to occur. The cell debris was centrifuged down as far as possible and the supernatant fluid, containing a total of 2.5×10^6 units penicillinase (of which 23% was unneutralizable), was saturated with ammonium sulphate and the precipitate allowed to flocculate at 2° overnight.

The massive precipitate was filtered off, suspended in 100 ml. water and dialysed against 5×10^{-3} M-phosphate (pH 7.0). Most of the precipitate dissolved and, after spinning off insoluble matter, the supernatant fluid (150 ml.) was dialysed successively at 2° against increasing concentrations of ammonium sulphate at pH 6.0, starting at 0.50 saturation. After separating the precipitate that was formed at each concentration and assaying both the supernatant fluid and precipitate (dissolved in 0.1 M-phosphate; pH 7.0) for total and unneutralizable penicillinase activity, the supernatant fluid was dialysed against the next higher concentration of ammonium sulphate. This fractional precipitation was continued until a concentration of 0.90 saturated ammonium sulphate had been reached, at which point the enzyme remaining in solution was 99% unneutralizable by antiserum (see Fig. 2). The yield of γ -fraction, thus freed from the β -fraction, was only just over 5% of that present in the extract from autolysed organisms, but was enough to allow some investigation of its properties. The fact that it was possible to separate it almost completely from the 'normal' enzyme by a simple fractionation procedure, based on differences in solubility in ammonium sulphate, showed that it belonged to a distinct molecular kind of protein, which is henceforth referred to as ' γ -penicillinase'.

Properties of γ -penicillinase

The solution of γ -penicillinase so obtained was free from α - and β -fractions, but was likely to be contaminated with other proteins. Moreover, only very small quantities (about 5×10^4 units) were available so that direct investigation of its physico-chemical properties was not attempted.

Table 3. *Relative rates of hydrolysis of different penicillins by γ -penicillinase and exo-penicillinase of Bacillus cereus 569*

Enzyme	Relative activity on substrates (benzylpenicillin = 100)		
	Penicillin K	Cephalosporin N	Phenoxyethyl penicillin
Exo-penicillinase	75	61	130
γ -Penicillinase	81	72	154

Relative enzymic activities are means of duplicate experiments done at a substrate concentration of 10^{-3} M.

Enzymic action. Quantitative manometric estimation showed that 0.92 acid equivalents were produced from catalytic breakdown of benzylpenicillin by the γ -penicillinase, so that it is reasonable to suppose that penicilloic acid is the product of the reaction, as with the exo-enzyme. The relative rates of hydrolysis by γ -penicillinase of benzylpenicillin, penicillin K, phenoxyethylpenicillin and cephalosporin N did not differ significantly from those of the exo-enzyme (Table 3). Neither γ -penicillinase nor exo-penicillinase caused significant destruction of cephalosporin C (Dr E. P. Abraham; personal communication).

Michaelis constant. The initial velocities of penicillin destruction by purified exo-enzyme and by the γ -penicillinase preparation were both measured by the

bio-assay technique described in Methods, at initial penicillin concentrations of 12.5, 20 and 1000 units/ml. The results are plotted according to the method of Lineweaver & Burk (1934) and recorded in Fig. 3. It can be seen that there is no significant difference between α - and γ -penicillinase whose Michaelis constants are calculated to be 28.5 and 27.8 units/ml., respectively. By this method the constant for the exo-enzyme is nearly 25% lower than that obtained previously by a different technique (Manson, Pollock & Tridgell, 1954).

The enzyme activity at different pH values. The effect of hydrogen-ion concentration on the activity of the γ -penicillinase preparation is illustrated in Fig. 4, where values are plotted as a percentage of the maximum velocity obtained by using the iodometric assay and the same buffers as employed

Fig. 3. Lineweaver & Burk plot for determination of Michaelis constants of exo-penicillinase (—●—●—) and γ -penicillinase (—○—○—) of penicillin-induced *Bacillus cereus* 569. *S*, initial penicillin concentration (units/ml.). *V*, rate of destruction of penicillin (units/ml./hr.).

previously (Manson *et al.* 1954) for a similar curve with the exo-penicillinase (also recorded for comparison in Fig. 4). Although the (H^+) for optimum activity (pH 6.0) is the same for both enzymes, the activity of the γ -penicillinase decreased far more steeply on either side of the optimum value. The difference is clearly significant and seems unlikely to be due to impurities in the γ -penicillinase preparation, because no differences were detected between the pH/activity curves of purified exo-enzyme and a crude preparation of culture supernatant fluid. It should be noted that all the manometric assays of penicillinase have so far been done at pH 7.0, which is nearly the optimum for activity of the exo-enzyme, but would give values about 40% below the optimum for γ -penicillinase. Thus, at pH 6.0, the γ -enzyme activities would be 66% higher than those recorded here, while the activity of the exo-enzyme would remain unchanged.

Sensitivity to iodine. Penicilloic acid reacts specifically, and apparently stoichiometrically, with iodine to form a compound which does not produce a

blue colour with starch; this fact has been used as the basis for iodometric assay of penicillinase (Perret, 1954). A rapid approximate assay can be made by measuring the time required to decolorize a standard amount of iodine; and this can be done in the presence of iodine during the course of the reaction since the exo-enzyme is inactivated by the iodine relatively slowly under the conditions employed. γ -Penicillinase, when studied by this 'quick' iodine method, appeared to be enzymically completely inactive until it was discovered that the enzyme was destroyed almost instantaneously by the iodine. When the iodine was added later on, as an external indicator of the production of penicilloic acid, the enzymic hydrolysis of penicillin to penicilloic acid was

Fig. 4. Enzyme activity at different pH values for induced γ -penicillinase of *Bacillus cereus* 569, using a concentration of 6.7 units enzyme/ml. Results are plotted as percentage maximal activity (at pH 5.75). The dotted line shows the comparable curve obtained by Manson *et al.* (1954) for the induced exo-penicillinase from the same strain.

found to occur at the rate expected from manometric assay. This is a confirmation that penicilloic acid is the product of the reaction and shows that α - and γ -penicillinases differ markedly in their reactions with iodine.

Dr N. Citri (unpublished experiments) has found that, in suspensions of organisms from cultures grown under normal conditions, the fraction of penicillinase activity which is not neutralized by antiserum to the exo-enzyme corresponds approximately to that which is rapidly inactivated by iodine. It therefore seems reasonable to conclude that, under the conditions employed, all unneutralizable activity—whether extractable or firmly bound to cell debris—is due to γ -penicillinase, and not to enzyme of the α - β -immunological type buried behind some structural barrier which prevents access of antibody.

Immunological properties. The fact that γ -penicillinase activity was not neutralized by anti-569 exo-penicillinase antibody did not necessarily mean that it could not specifically combine with that antibody. Combination might occur, as happens in certain specific enzyme-antibody reactions, without

neutralization. However, addition of 10^4 units γ -penicillinase to 0.5 ml. antibody solution produced no precipitate, while an enzymically equivalent quantity of exo-enzyme gave an obvious precipitate with the same amount of antibody. Moreover, addition to purified exo-enzyme of an enzymically equivalent quantity of the γ -penicillinase preparation did not modify the neutralization slope of the exo-enzyme by antibody. In other words, even in the presence of excess enzyme, the γ -penicillinase did not interfere with the reaction between exo-enzyme and antibody. It is therefore reasonable to conclude that γ -penicillinase does not combine with antibody prepared against exo-penicillinase.

Table 4. *Attempt to separate γ -penicillinase and exo-penicillinase of Bacillus cereus 569 by differential ultra-centrifugation for 2 hr. at 104,000 g*

Expt.	Layer	Vol. (ml.)	Total activity (units/ml.)	Percentage of total activity unneutralizable by antiserum
1 'Natural' mixture	Before centrifugation	13.5	665	30
	After centrifugation:			
	Top	3.0	415	23
	Bottom	1.0	975	29
	Deposit	0.5	1120	37
2 Artificial mixture	Before centrifugation	13.5	1640	20
	After centrifugation:			
	Top	1.9	1600	20.5
	Middle	9.0	1880	19
	Bottom	0.9	2800	20

Differential ultracentrifugal analysis. It was thought possible that a partial separation of soluble β - and γ -fractions obtained from preparations of disintegrated organisms might be achieved by the centrifugation of a mixture. Two such experiments were done: (a) on a 'natural' mixture obtained during the course of separation of the γ -penicillinase from an autolysate of organisms; (b) on an artificial mixture of the fully separated γ -penicillinase and pure exo-enzyme obtained from another large-scale preparation. Samples (13.5 ml.) were spun in the preparative head of the Spinco ultracentrifuge for 2 hr., at 104,000 g. Immediately after the run, samples were carefully pipetted from the top, middle and bottom layers with the minimum of disturbance, and assayed for total and unneutralizable activity; the results are shown in Table 4. It can be seen that there was a considerable sedimentation of enzyme, as shown by the higher activities in the lower layers. However, although there is some suggestion that the γ -enzyme in the 'natural' mixture had sedimented rather more rapidly than the neutralizable fraction, there was no significant separation of the two types in the artificial mixture. Thus it seems unlikely that their molecular weights are widely different.

Attempts to transform γ -penicillinase

It seemed possible that γ -penicillinase might be either a natural intracellular precursor of the α - and β -fractions, or simply a complex formed by the β -fraction and some other substance present inside the organisms, this complex having biological and physico-chemical properties different from the β -enzyme. Attempts were therefore made to transform one type into the other both by *in vitro* and *in vivo* experiments. It was decided that loss in activity of the unneutralizable fraction associated with significant gain in activity of the neutralizable moiety or vice versa should be taken as *prima facie* evidence for interconversion. On this criterion, all results were negative. The following tests were done. *In vitro*: mixtures of approximately equal parts of neutralizable and unneutralizable penicillinase extracted from induced organisms were incubated alone, with trypsin and with deoxyribonuclease and ribonuclease for 1 hr. at 35°. No significant changes in the proportion of the two types occurred. *In vivo*: induced organisms were resuspended in fresh medium and the neutralizable and unneutralizable activity followed under conditions where total enzyme production was suppressed or markedly inhibited. These were: incubation at 35° (a) anaerobically in broth (argon bubbling through culture), (b) aerobically in broth with 20 μ g. chloramphenicol/ml., (c) aerobically in broth with 8.3×10^{-4} M-oxine, (d) aerobically in 0.1 M-glucose + 0.02 M-phosphate (pH 7.0) without added source of N. No decrease in activity of either the unneutralizable or neutralizable fractions took place.

Inducibility of γ -penicillinase

In un-induced cultures of *Bacillus cereus*, strain 569, about 50 % of cell-bound penicillinase was found to be unneutralizable by excess antibody (as in induced cultures); and a proportion (about 30 %) of the cell-bound unneutralizable basal enzyme could be extracted in a soluble form after the breaking of organisms in the Hughes press. It can therefore be concluded that γ -penicillinase, like exo-penicillinase, is present in a culture before induction with penicillin. After induction with 1 unit penicillin/ml., the unneutralizable cell-bound enzyme activity increased to constitute an approximately constant proportion (limits: 4.7–6.0 %) of the total enzyme (see Fig. 5). Similarly, after induction with a suboptimal concentration of penicillin (0.1 unit/ml.), unneutralizable enzyme was found to constitute the same constant proportion (limits: 4.7–6.9 %) of total penicillinase at all stages. It is quite clear, therefore, that γ -penicillinase formation is inducible by penicillin. Table 5 shows the quantities of total, of unneutralizable and of solubilized unneutralizable cell-bound penicillinase fractions found in cultures of un-induced organisms and in cultures harvested 40 min. after induction with 1 unit penicillin/ml. Even after this relatively short period of induction, there was an approximately 20-fold increase in all fractions, including the unneutralizable fraction which could be extracted in solubilized form. This increase is of the same order proportionally as the increase of exo-penicillinase under the same conditions.

Table 5 also shows that 83% of the cell-bound penicillinase activity of the constitutive-penicillinase *Bacillus cereus* mutant 569/H was not neutralizable by excess antiserum and that 45% of this unneutralizable fraction was extracted in a soluble form after disintegration of the organisms, as with strain 569. The 569 (induced) and the 569/H (constitutive) exo-penicillinase have

Fig. 5. Formation of the different fractions of penicillinase in a culture of *Bacillus cereus* 569 growing in 1% (w/v) gelatin broth following induction with 1 unit penicillin/ml. In order to follow enzyme production over a long period a sample of the culture was diluted with an equal volume of fresh medium every 40 min.; and results are expressed as total enzyme production in terms of the original culture (viz. activity/ml. $\times$ dilution factor). — $\triangle$ — $\triangle$ —, total penicillinase; --- $\bullet$ — $\bullet$ —, cell-bound penicillinase; $\cdots$ $\circ$ $\cdots$ $\circ$ $\cdots$, fraction of cell-bound penicillinase not neutralizable by antiserum to exo-enzyme.

Table 5. γ -Penicillinase:inducibility in *Bacillus cereus* 569 and its presence in *Bacillus cereus* 569/H (constitutive-penicillinase mutant strain)

Strain	Cell-bound penicillinase		
	Total	Unneutralizable by antiserum to exo-enzyme	Fraction of unneutralizable activity extractable into solution after cell disintegration (= γ -penicillinase)
			Penicillinase activity (units/mg. dry wt. organisms)
<i>Bacillus cereus</i> 569:			
Not induced	0.64	0.23	0.11
40 min. after induction with 1 unit penicillin/ml. at concentration of organisms corresponding to 0.3 mg. dry wt./ml.	13.10	6.10	2.60
<i>Bacillus cereus</i> 569/H (constitutive-penicillinase mutant strain)	167	137	61.7

been shown (Kogut *et al.* 1956) to be physico-chemically and immunologically indistinguishable. Thus, although no further studies have been made on the soluble unneutralizable penicillinase of strain 569/H, it seems probable that it is identical with the γ -penicillinase of strain 569; and that, in this respect as in all others investigated, strain 569/H behaves, in the absence of added penicillin, in the same manner as strain 569 after induction with penicillin. In contrast to strain 569/H, little, if any γ -penicillinase was found in the other constitutive-penicillinase strain of *Bacillus cereus*, 5/B (Pollock, Torriani & Tridgell, 1956; Sneath, 1955). Less than 7% of the total cell-bound activity of strain 5/B failed to react with antiserum.

*Test for the presence of γ -penicillinase in cell-free supernatant fluid
from a culture of Bacillus cereus*

γ -Penicillinase is not normally liberated to any significant extent into the medium. This was shown by an experiment in which 1.0 ml. of untreated supernatant fluid (containing 1550 units of enzyme) from a culture of the 'constitutive-penicillinase' *Bacillus cereus* mutant, strain 569/H, was mixed with a gross excess of antibody (0.25 ml. anti-569 α -penicillinase serum containing a total of 5500 neutralization units of antibody; see Pollock, 1956). The mixture was incubated overnight at 35°, and the supernatant fluid, after removal of the precipitate by centrifugation, was found to contain only 5.5 units, or 0.35% of the original penicillinase activity. The unneutralizable activity contained in the cells from 1.0 ml. of the same culture was found to be 86 units. It can safely be assumed that no significant quantity of γ -penicillinase was carried down with the precipitate formed between the α -enzyme and the anti- α -penicillinase antibody. This is clear from an experiment in which 1.0 ml. of the same culture supernatant referred to above (containing 1550 units of penicillinase) was mixed with 275 units of purified γ -enzyme and 0.1 ml. of antiserum (=2200 neutralization units: 50% more than that required to combine with all the α -enzyme present in the culture supernatant). After centrifuging down the precipitate which formed during 16 hr. of incubation at 35°, no loss of γ -penicillinase activity from the supernatant fluid was observed.

The 5.5 units of activity left after precipitation of the exo-enzyme with excess antibody might have been due either to the residual activity of a small amount of α -enzyme/antibody complex still remaining in solution (the more likely explanation) or to traces of γ -penicillinase itself. However, even assuming that it were all γ -penicillinase, it would only represent 6% of that found fixed to the organisms, thus indicating the maximum possible proportion liberated into the medium from a growing culture.

DISCUSSION

There are several other instances, analogous to that of the α - and γ -penicillinases of *Bacillus cereus*, where a protein formed by some tissue or population of cells occurs in two or more closely related, but physico-chemically or immunologically distinct, forms having apparently similar biological actions (pan-

creatic ribonucleases, Martin & Porter, 1951; heart lactic dehydrogenases, Neilands, 1952; beef insulins, Harfenist, 1953; beef chymotrypsins, Jacobsen, 1947; ox liver catalases, Brown, 1952). In none, however, is there any information on possible differences in localization of the fractions in relation to cell structure. Also, in general, the observed differences have been finer than those found between the two types of penicillinases described here. One of these penicillinases—the γ -enzyme—appears to be exclusively cell-bound. Indeed, since γ -penicillinase can only be obtained from organisms in a soluble form after breaking them in a Hughes press, it seems reasonable to consider it ‘intracellular’. This is, however, primarily an operational denotation. Its rapid appearance in the supernatant fluid, obtained by centrifuging the debris after disruption of the organisms, suggests that at least a proportion of the γ -enzyme exists in the cytoplasm, and is thus immediately liberated by damage to the cell wall. But the possibility that the enzyme is normally bound on the outside of the organisms and is freed into solution by mechanical disintegration of their supporting structure, though rather unlikely, has not been formally excluded.

γ -Penicillinase is distinguishable from α - and β -penicillinase by its immunological reactions, the shape of the pH/activity curve, its greater solubility in ammonium sulphate and its relatively rapid inactivation by iodine. This property of iodine-sensitivity has been used to show that the fraction of cell-bound penicillinase which is not neutralized by antibody to exo-enzyme is probably all γ -penicillinase.

Since it is possible to wash off from whole organisms most of the enzyme that is neutralized by antibody (β -fraction), it seems likely that in normal growing cultures the α -fraction is derived directly from the β -fraction. In other words, it is probable that penicillinase of the α - β immunological type first occurs in an active form as cell-bound enzyme, and that immediately or very shortly after formation it appears on the surface of the organisms in a position sufficiently exposed to allow combination with antibody. Shortly after that, one may suppose that it is liberated into the medium as normal exo-enzyme. In any case, it is reasonable to conclude that β -penicillinase is the normal precursor of α -penicillinase.

The relationship of γ -penicillinase to the other two fractions is less clear. Three of the most likely alternatives are illustrated in Fig. 6. All reactions have been assumed to be irreversible. In scheme 1, γ -penicillinase and the exo-enzyme are visualized as being formed by independent mechanisms. But this takes no account of the similarity in the substrate-combining groups (as indicated by identical Michaelis constants) or the constant proportion of unneutralizable to total penicillinase activity formed in un-induced cultures at all stages after induction with two different concentrations of penicillin. Both of these facts suggest that the specific mechanisms for the formation of the two types of enzyme may be closely related at one point in the sequence of necessary reactions.

Scheme 2 is not supported by the failure, hitherto, of all attempts to demonstrate conversion of unneutralizable penicillinase to neutralizable penicillinase.

Scheme 3, in which no interconversion of γ - and β -penicillinase would be expected, accounts for the similarities in properties and rates of production of the two types of enzymes by postulating the existence of a common specific penicillinase precursor (not essential to the other two schemes) for which there is already some indirect evidence (Pollock, 1953). However, although at the moment the facts appear to favour scheme 3, further information on the relative rates of synthesis of the different fractions under a wide variety of conditions is needed before deciding which hypothesis is the most probable.

Fig. 6. Three alternative schemes to illustrate the possible metabolic relationships between the α -, β - and γ -penicillinases of *Bacillus cereus* 569.

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