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# Purification and Properties of Two Extracellular β-Lactamases from *Bacillus cereus* 569/H

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1. When *Bacillus cereus* 569/H was grown in a casamino acid (casein-hydrolysate) medium containing zinc sulphate rapid production of extracellular β-lactamase II preceded that of β-lactamase I. 2. β-Lactamase I was separated from β-lactamase II by fractional precipitation with ammonium sulphate. 3. β-Lactamase I was purified by a process involving chromatography on Celite and DEAE-cellulose and β-lactamase II by chromatography on DEAE-cellulose after denaturation of β-lactamase I by heat. Both enzymes were obtained in crystalline form. 4. β-Lactamase II prepared in this way appeared to have a higher molecular weight than β-lactamase I and required $Zn^{2+}$ as a cofactor for both cephalosporinase and penicillinase activities.

The penicillinase (β-lactamase) produced by *Bacillus cereus* 569 was found by Pollock (1950) to be inducible. From this inducible strain Kogut, Pollock & Tridgell (1956) isolated a constitutive mutant that was termed 569/H.

Pollock & Torriani (1953) obtained a preparation of penicillinase that was later believed to be nearly 50% pure from the culture fluid of *B. cereus* 569 grown in a peptone medium. Kogut *et al.* (1956) purified penicillinase from strains 569 and 569/H respectively. They grew the organisms on a casamino acid (casein-hydrolysate) medium and made use of the finding that the penicillinase could be adsorbed on, and eluted from, powdered glass. From both strains preparations of penicillinase were obtained that behaved as though they were largely homogeneous in the ultracentrifuge (Hall & Ogston, 1956) and yielded crystalline material. One preparation from strain 569/H did not differ significantly in specific activity, solubility in ammonium sulphate, electrophoretic mobility, sedimentation coefficient or diffusion coefficient from a preparation from strain 569. Both enzymes were reported to have molecular weights of about 31000. They resembled each other closely in amino acid composition and, in common with β-lactamases from a number of other organisms, appeared to contain no significant amount of cysteine (Citri & Pollock, 1966).

Experiments in which cephalosporin C, as well as benzylpenicillin, was used as a substrate indicated that culture fluids of *B. cereus* 569 and 569/H contained at least two β-lactamases, which could be distinguished by their enzymic activity and thermostability (Abraham & Newton, 1956; Crompton, Jago, Crawford, Newton & Abraham, 1962). One of these enzymes, unlike the highly purified penicillinase obtained by Kogut *et al.* (1956), catalysed the opening of the β-lactam ring of cephalosporin C and was referred to as a cephalosporinase. Other differences between the enzymes with penicillinase and cephalosporinase activities respectively were subsequently discovered (Sabath & Abraham, 1965). The penicillinase from *B. cereus* 569 and 569/H appears to require no cofactor, but the cephalosporinase was shown to be a $Zn^{2+}$-requiring enzyme (Sabath & Abraham, 1966). Sabath & Finland (1968) reported that activation of the cephalosporinase by $Zn^{2+}$ was prevented by prior treatment with certain thiol reagents. They suggested that zinc was bound to a thiol group in this enzyme and that the latter differed from the penicillinase from *B. cereus* 569 by at least one amino acid (cysteine).

Kuwabara & Abraham (1967) reported briefly that they had obtained both the extracellular penicillinase and the extracellular cephalosporinase from *B. cereus* 569/H in a highly purified form. Although the penicillinase showed virtually no cephalosporinase activity with cephalosporin C or most of the other cephalosporins tested as substrate, the cephalosporinase was active against a wide range of both cephalosporins and penicillins. It was therefore proposed to term the penicillinase β-lactamase I and the cephalosporinase β-lactamase II. The present paper describes methods for the purification of β-lactamase I and β-lactamase II and some of the properties of these enzymes.

## METHODS

### General

*Substrates and inhibitors.* Benzylpenicillin was from Glaxo Research Laboratories Ltd., Greenford, Middx., U.K. Other penicillins were from the Beecham Research Laboratory Ltd., Brockham Park, Betchworth, Surrey, U.K., The Distillers Co. (Biochemicals) Ltd., Speke, Liverpool, U.K., and Boots Pure Drug Co. Ltd., Nottingham, Notts., U.K. Cephalosporins were from Eli Lilly and Co., Indianapolis, Ind., U.S.A., and from Glaxo Research Laboratories. *N*-Ethylmaleimide and iodoacetic acid were from British Drug Houses Ltd., Poole, Dorset, U.K.

*Determination of protein.* Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) as a standard. Protein N was calculated on the assumption that the protein measured contained 15.8% N. In some cases determination of protein and of nucleic acid were made by the spectrophotometric method of Warburg & Christian (1941).

*Assay of β-lactamase activity.* The unit of activity is the amount of enzyme required to hydrolyse 1 μmol of substrate in 60min at pH7.0 (Pollock & Torriani, 1953). Unless otherwise stated measurements were made at 30°C and units were calculated from rates of hydrolysis corresponding to $V_{max.}$.

Assays were carried out with a pH-stat (type SBR2/SBU1 titrigraph in connexion with type TT1 automatic titrator). The reaction mixture contained 0.7ml of 1% (w/v) gelatin, pH7.0, and 5μl of enzyme solution suitably diluted with 1% (w/v) gelatin. This mixture was diluted with water (for assay of penicillinase activity) or water plus 0.7ml of 1mM-$ZnSO_4$ (for assay of cephalosporinase activity) to 1.8ml. A solution (0.2ml) of benzylpenicillin or cephalosporin C substrate (each 15mg/ml, pH7.0) was added and 20mM-NaOH was used to maintain the pH at 7.0 until the reaction was complete. With β-lactamase I the substrate concentration used was sufficient for the rate of hydrolysis to be virtually $V_{max.}$. With β-lactamase II a Lineweaver–Burk plot was used to determine $V_{max.}$ from the measured velocity.

Some assays were carried out manometrically in the manner described by Pollock (1952) and by Crompton *et al.* (1962), except that in the determination of cephalosporinase activity 0.45ml of 2mM-$ZnSO_4$ was added to the main compartment of the Warburg vessel in place of the same amount of water.

Activities were calculated on the assumption that 1equiv. of acid was liberated on hydrolysis of 1mol of benzylpenicillin, but that 2 equiv. was liberated from 1mol of cephalosporin C (Sabath & Abraham, 1966).

*Buffers used in the purification of β-lactamases.* In the purification of the β-lactamases two buffers (B and BZ) were used. Stock buffer B (0.1M with respect to tris) was made by addition of a solution of sodium acetate–acetic acid (pH5.0, 50mM with respect to acetate) to about 0.3M-tris–HCl buffer, pH8.0 (containing 0.1mol of tris), until the pH, unless otherwise stated, was 7.0, and dilution of the solution to 1 litre with water. Stock buffer BZ, used in the purification of β-lactamase II, was the same as buffer B except that it contained 1mM-$ZnSO_4$. Diluted buffers B and BZ (10mM and 1mM with respect to tris) were also used. In diluted buffer BZ the final concentration of $ZnSO_4$ was always 1mM.

### Production of β-lactamase I and β-lactamase II

*Spore suspension of* B. cereus 569/*H*. An S-agar plate containing Andrade indicator was seeded with a culture of *B. cereus* 569/H suitably diluted to give discrete colonies, and a colony that was a strong producer of penicillinase was selected by the method described by Kogut *et al.* (1956). A culture from this colony was grown on a nutrient agar slope and washed with sterile water into about 80ml of sterile S broth (Pollock, 1953) in a 500ml Erlenmeyer flask. The flask was incubated for 40h at 35°C on a rotary shaker (5cm throw) at 160rev./min. It was then heated for 1h at 60°C and the spores were collected by centrifugation. The spores were washed once with sterile water (about 25ml) and finally resuspended in 20ml of water. This stock spore suspension was stored at 4°C.

*Laboratory-scale production of β-lactamase.* About 80ml of S broth was inoculated with 0.1ml of stock spore suspension in a 500ml Erlenmeyer flask and the culture grown at 35°C on the rotary shaker for 6h. This culture was used as an inoculum for the production culture.

Production cultures were grown in 500ml and 1 litre Erlenmeyer flasks containing 100ml and 200ml respectively of a modification of the CH/C medium of Miller, Bach & Markus (1965). Solution A of the CH/C medium, without antifoam, was added to each flask, and 1M-$ZnSO_4$ then added to give a final concentration of 1mM. The resulting solution was steam-sterilized. Solutions B and C of the CH/C medium were mixed, Seitz-filtered and added aseptically. A 2% (v/v) inoculum was added and the flasks were incubated for 24h at 35°C on the rotary shaker. The cultures were spun at 1500*g*, and the supernatant was collected and stored (if not used immediately) at 4°C. It contained about 3000 units of penicillinase activity/ml and 100 units of cephalosporinase activity/ml.

*Larger-scale production of β-lactamase.* This was kindly carried out by Mr A. R. Whitaker at the Microbiological Research Establishment, Porton, Wilts., U.K., in 140 litre culture vessels similar to the 100 litre vessels described by Elsworth, Miller, Whitaker, Kitching & Sayer (1968). The 140 litre culture was seeded from a 20 litre culture (Elsworth, Williams & Harris-Smith, 1957). The medium (pH7.2) was similar to that used in the laboratory-scale production except that antifoam Silicone A was added and $ZnSO_4$ was omitted in some cases in which the culture fluid was to be used only for the purification of β-lactamase I. The aeration rate was 140 litres/min and the stirring rate was 520rev./min. During the growth of the culture the pH was kept below 7.5 by addition of an aqueous solution of citric acid (500g/l) (Elsworth & Stockwell, 1968).

Cultures were harvested after 10h, the cells were removed by centrifugation and the supernatant was cooled to 4°C. The penicillinase activities of most of the supernatants used varied from 3150 to 1200 units/ml, although later experiments showed that activities of more than 11000 units/ml could be obtained. The penicillinase activity/cephalosporinase activity quotient varied from

15 to 35 with cultures to which $ZnSO_4$ had been added. The protein content of a typical supernatant fluid was 56 μg/ml.

## RESULTS

### *Production of extracellular β-lactamase I and β-lactamase II*

Analysis of samples taken at intervals from a 142 litre culture grown in a medium containing 1 mM-zinc sulphate showed that the rate of increase of β-lactamase II reached its maximum when the culture was in its most rapid phase of growth. The rate of increase of β-lactamase I became maximal later when the rate of growth of the culture had begun to decline. The maximum concentration of β-lactamase I in the culture fluid was reached 2h after that of β-lactamase II, and when the concentration of the latter had fallen substantially from its peak value (Fig. 1).

When zinc sulphate was not added to the casamino acid culture medium used in the present work there was a substantial decrease in the amount of β-lactamase II found in the supernatants from cultures harvested after 10h. Thus the concentration of β-lactamase II in the supernatant used in the experiment summarized in Table 3 was four times that used in the experiment summarized in Table 2.

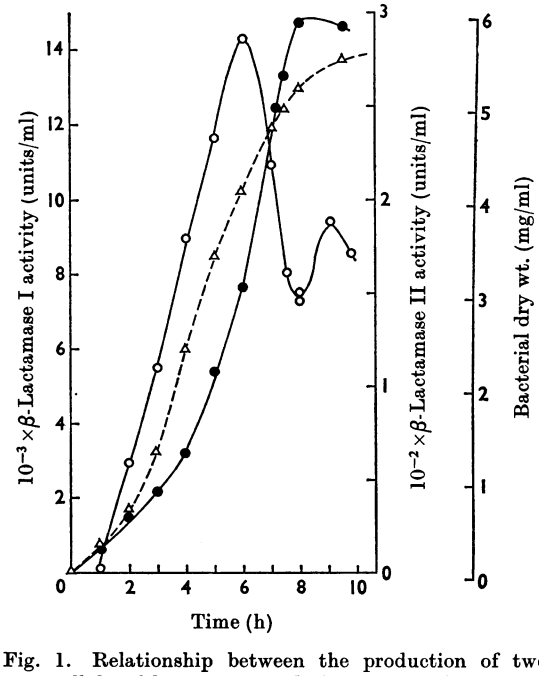



Fig. 1. Relationship between the production of two extracellular β-lactamases and the growth of *B. cereus* 569/H. Measurements were made of the rate of hydrolysis of cephalosporin C and benzylpenicillin respectively by samples of culture supernatant. ○, β-Lactamase II activity; ●, β-lactamase I activity; △, bacterial dry weight (mg/ml).

### *Preliminary experiments on the separation of β-lactamase I and β-lactamase II*

Earlier work had shown that the cephalosporinase (β-lactamase II) activity of culture supernatants was lost when penicillinase (β-lactamase I) was purified by adsorption on, and elution from, powdered glass or cellulose phosphate (Sabath & Abraham, 1965). Although crude enzyme preparations had been obtained in which the cephalosporinase activity/penicillinase activity quotient was much higher than it was in the original culture supernatant, the procedure involved the selective inactivation of the penicillinase activity by heat (Crompton *et al.* 1962). An attempt was therefore made to separate β-lactamase I and β-lactamase II by processes in which both enzymic activities would be retained.

The separation of β-lactamase I from β-lactamase II by fractional precipitation with ammonium sulphate is summarized in Table 1. The culture supernatant used was from a laboratory-scale culture. The pH of the supernatant was adjusted to 7.0 with 1 M-hydrochloric acid and all subsequent operations were carried out at 4°C. Precipitations were carried out by the slow addition of solid ammonium sulphate to gently stirred solutions and the precipitates were separated by centrifugation at 14000*g* for 15min.

The precipitate obtained by saturation with ammonium sulphate at stage 2 (Table 1) was dissolved in a small volume of 1 mM-buffer B and dialysed against 10 mM-buffer BZ for 18h. The resulting solution (43ml), which contained virtually all the penicillinase activity of the culture fluid supernatant, was clarified by centrifugation. About 70% of the β-lactamase I activity was precipitated when the solution was brought to 73% saturation with ammonium sulphate, but virtually all the β-lactamase II activity remained in the supernatant (stage 3). β-Lactamase II, together with the remaining β-lactamase I, was precipitated when the supernatant was saturated with ammonium sulphate. The material in this precipitate that dissolved on dialysis against 10 mM-buffer BZ contained β-lactamase II but no significant amount of β-lactamase I (stage 4*b*). The insoluble material, which dissolved in 0.1 M-buffer BZ, contained a mixture of β-lactamase I and β-lactamase II.

### *Purification of β-lactamase I and β-lactamase II*

*β-Lactamase I.* Purification of the β-lactamase I in 152 litres of supernatant from a culture of *B.*

Table 1. *Fractional precipitation of extracellular β-lactamase I and β-lactamase II with ammonium sulphate*

For details of the stages of separation see the text.

| Stage | Material from | Total vol. (ml) | Total protein (mg) | Protein recovered (%) | $\frac{E_{280}}{E_{260}}$ | $10^{-4}$ × Total units | | Units recovered (%) | | $10^{-4}$ × Sp. activity (units/mg of protein N) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | β-Lactamase I | β-Lactamase II | β-Lactamase I | β-Lactamase II | β-Lactamase I | β-Lactamase II |
| 1 | Culture supernatant | 850 | 45 | 100 | 0.825 | 268 | 8.50 | 100 | 100 | 37.6 | 1.19 |
| 2 | Precipitate on 100% saturation with $(NH_4)_2SO_4$ | 43 | 37.6 | 84 | 0.982 | 271 | 8.42 | 101 | 99 | 45.5 | 1.41 |
| 3 | Precipitate on 73% saturation with $(NH_4)_2SO_4$ of product from stage 2 | 6 | 15.6 | 34 | 1.25 | 138 | — | 51 | — | 55.9 | — |
| 4 | Precipitate on 100% saturation with $(NH_4)_2SO_4$ of supernatant from stage 3 | | | | | | | (72) | | | |
| | (*a*) Undissolved material after dialysis | 4 | 6.0 | 13 | — | 56.2 | 1.70 | 21 | 20 (105) | 59.2 | 1.79 |
| | (*b*) Solution after dialysis | 9 | 8.6 | 19 | 1.60 | — | 7.24 | — | 85 | — | 5.32 |

Table 2. *Purification of extracellular β-lactamase I from* B. cereus *569/H*

The culture supernatant was from a culture grown in a medium to which no $ZnSO_4$ was added. The values given for total units are those obtained with benzylpenicillin as substrate. The relative values for β-lactamase I and β-lactamase II activities were determined with benzylpenicillin and cephalosporin C respectively as substrates.

| Stage | Material from | Total vol. (ml) | $10^{-6}$ × Total units | Units recovered (%) | Total protein (mg) | Protein recovery (%) | $10^{-6}$ × Sp. activity (units/mg of protein N) | $\frac{\text{β-Lactamase I}}{\text{β-Lactamase II}}$ activity ratio | $\frac{E_{280}}{E_{260}}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Culture supernatant | 152000 | 479 | 100 | 8500 | 100 | 0.356 | 126 | 0.76 |
| 2 | Precipitate on 96.6% saturation with $(NH_4)_2SO_4$ | 850 | 428 | 89.4 | 5790 | 68.1 | 0.468 | 482 | 0.91 |
| 3 | Eluate from Celite 545 pH gradient | 390 | 364 | 75.9 | 1400 | 16.5 | 1.65 | 5980 | 1.34 |
| 4 | Eluate from first DEAE-cellulose column | 170 | 314 | 65.5 | 680 | 8.0 | 2.92 | 12500 | 1.84 |
| 5 | Eluate from second DEAE-cellulose column (NaCl gradient) | 100 | 287 | 59.9 | 510 | 6.0 | 3.56 | | 2.00 |
| 6 | Acetone treatment | 132 | 246 | 51.3 | 430 | 5.1 | 3.59 | | — |
| 7 | First crystallization | 10 | 74 | 15.4 | 87 | 1.0 | 5.42 | | -- |
| 8 | Second crystallization | — | — | — | — | — | 5.42 | | -- |

*cereus* 569/H grown in the absence of added zinc sulphate is summarized in Table 2. The pH of the supernatant was adjusted to 7.0 with 1M-hydrochloric acid and the fluid cooled to 4°C. Precipitations with ammonium sulphate were carried out as in the preliminary experiments on the separation of β-lactamase I and β-lactamase II.

The precipitate obtained at stage 2 (Table 2) was dissolved in a small volume (about 5ml/l of culture fluid) of 10mM-buffer B. The resulting solution was dialysed against the same buffer for 18h and filtered through three layers of Whatman no. 1 paper. Assay of the filtrate showed that more than two-thirds of the β-lactamase II activity of the original culture supernatant was lost at this stage.

The pH of the solution from stage 2 was adjusted to 4.5 with 1M-hydrochloric acid. The solution was then mixed with Celite 545 (about 1g/3000 units of β-lactamase I) and the mixture left at 4°C for 2h (Miller *et al.* 1965). After addition of 1mM-hydrochloric acid (3ml/g of Celite) the mixture was stirred for 4h and then poured into a column (40cm × 7cm diam.). The column was allowed to pack under nitrogen (5lb/in²), when a clear yellow liquid emerged, and then washed with water until the effluent became colourless. Elution was carried out with 0.1M-buffer containing 1M-sodium chloride and 0.1M-sodium citrate and having pH8.3. Fractions (each about 25ml) were collected. The active material emerged in fractions with pH rising from 7.0 to 7.9 and reached its maximum when the pH was 7.4 (fractions 65 and 66). The active fractions (57–76) were combined, the solution was saturated with ammonium sulphate, and the precipitate was dialysed against 10mM-buffer B overnight. Assay of the resulting solution showed that less than 10% of the β-lactamase II activity that remained at stage 2 was eluted with β-lactamase I from the column and that the specific activity of the β-lactamase I had increased 3.5-fold (Table 2, stage 3).

The material from stage 3 (390ml) was divided into four equal parts and each chromatographed on a column (100cm × 2.5cm diam.) of DEAE-cellulose (Whatman DE-11) that had been equilibrated with 1mM-buffer B at 4°C. Elution was carried out with 1mM-buffer B containing 1M-sodium chloride and fractions (5ml) were collected every 5min. The combined active fractions (13–21 from each column) contained β-lactamase I that was virtually free from β-lactamase II and nucleic acid (Table 2, stage 4). The specific activity of β-lactamase I increased about 1.8-fold at this stage.

Further chromatography on DEAE-cellulose was carried out in a sodium chloride gradient. The enzyme in the solution from stage 4 was precipitated by saturation with ammonium sulphate, and the precipitate was dissolved in about 50ml of 10mM-buffer B and dialysed overnight against 1mM-buffer B. The resulting solution (105ml) was divided into seven equal portions and each chromatographed on a column (60cm × 1.5cm diam.). Elution was carried out in a sodium chloride gradient obtained by use of 500ml of 1mM-buffer B in a mixing chamber with a magnetic stirrer and an equal volume of the same buffer containing 0.1M-sodium chloride in a reservoir. Fractions (each 3.8ml) were collected every 5min. Virtually all the activity was found in fractions 18–23 with a peak of activity in fraction 19. Elution of enzyme activity in most of the active band paralleled the elution of protein as determined from extinction values at 280nm and occurred when the concentration of sodium chloride was still relatively low (Fig. 2). The enzyme in the combined active fractions was precipitated by the addition of ammonium sulphate to saturation and the precipitate dialysed for 12h against 10mM-buffer B. The specific activity of the β-lactamase I in the resulting solution (stage 5) was about 1.2 times that at stage 4.

Acetone (30%, v/v, at −10°C) was added to the solution from stage 5 (100ml at 4°C) in an attempt to induce crystallization. The mixture was stirred gently for 45min at 4°C and then centrifuged. The precipitate contained most of the enzyme activity but was amorphous. It was dissolved in about 10ml of 10mM-buffer B (stage 6).

Crystallization of the enzyme took place on dialysis of the solution from stage 5 against either water or buffer B (pH5.6) of low ionic strength (0.1mM). After 6–12h a white cloud of material with a silvery sheen began to appear in the solution. After 18h the crystals were centrifuged down and washed once with a small volume of water (Table 2, stage 7). The crystals were sparingly soluble in water but soluble in salt solutions, such as buffer B (0.01M or more concentrated) or 50% saturated ammonium sulphate. They were hexagonal in shape, about 60μm long and 20μm wide (Plate 1). They were thin, but exhibited birefringence at the edges. Recrystallization was achieved repeatedly by dialysis against water, or a solution of low ionic strength, as in stage 7. The specific activity and the shape and size of the crystals did not change significantly on recrystallization (stage 8). The crystals were collected by centrifugation and dissolved in 50%-saturated ammonium sulphate, pH7.0, in 10mM-buffer B. When kept in this condition at 2–4°C and at a concentration of 15mg/ml, β-lactamase I was stable for months.

*β-Lactamase II.* The purification of β-lactamase II is summarized in Table 3. The enzyme in solution in buffer BZ obtained at stage 2 (825ml) was reprecipitated by saturation with ammonium sulphate, and the precipitate was dissolved in

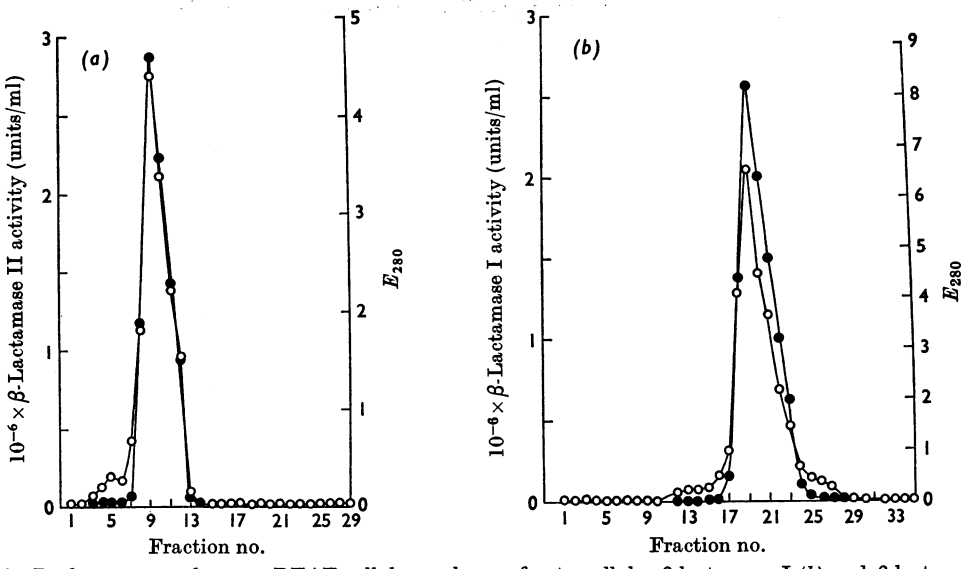



Fig. 2. Rechromatography on a DEAE-cellulose column of extracellular $\beta$-lactamase I (*b*) and $\beta$-lactamase II (*a*) from *B. cereus* 569/H. ●, Units of $\beta$-lactamase I activity with benzylpenicillin as substrate and units of $\beta$-lactamase II activity with cephalosporin C as substrate; ○, $E_{280}$. The preparations of $\beta$-lactamase I and $\beta$-lactamase II used contained 94.5mg and 72.5mg of protein respectively. For details see the text.

10mM-buffer BZ (300ml) and dialysed against the same buffer for 18h. The resulting solution was kept in a water bath at 65°C for 30min with gentle stirring and then cooled in an ice bath. A precipitate that formed during heating was removed by centrifugation. The $\beta$-lactamase I/$\beta$-lactamase II activity ratio of the supernatant was 0.01. Thus the heat treatment removed almost all of the $\beta$-lactamase I (stage 3).

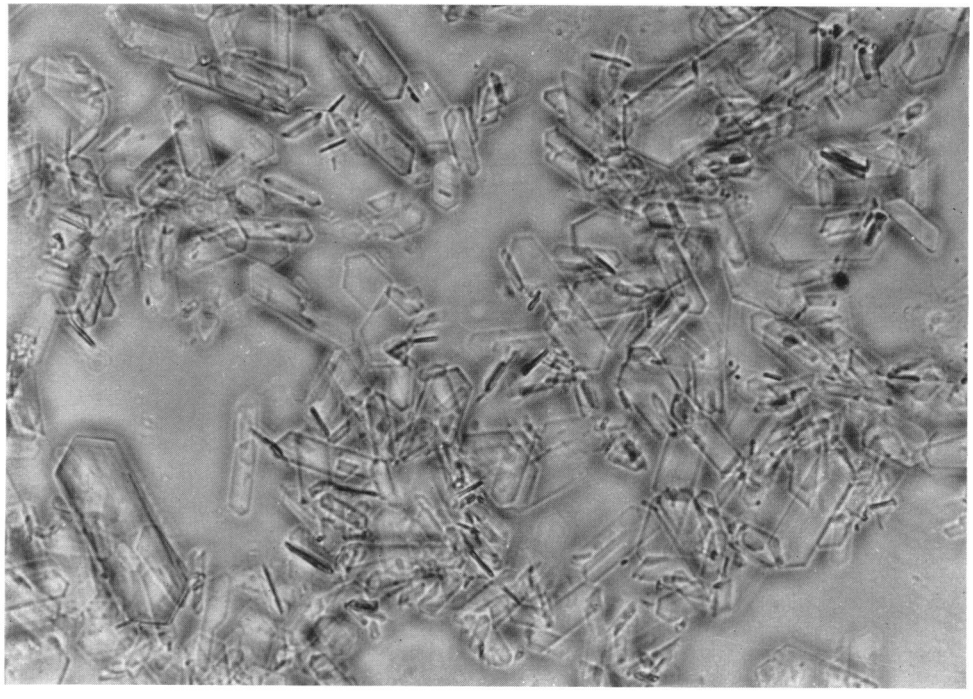
The enzyme in the solution from stage 3 was precipitated by 96.6% saturation with ammonium sulphate and the precipitate dialysed overnight against 1mM-buffer BZ. The resulting solution (17.2ml) was divided into two equal parts and each chromatographed at room temperature on a column (100cm × 2.5cm diam.) of DEAE-cellulose, elution being carried out with buffer BZ containing 1M-sodium chloride. Fractions (2.5ml) were collected at 5min intervals. There were two main protein peaks. The first was associated with $\beta$-lactamase II (fractions 5–10) and the second (fractions 12–17) with $\beta$-lactamase I of very low specific activity (204 units/mg of protein N). The second peak may have contained protein that had been denatured during the heat treatment. The fractions containing $\beta$-lactamase II activity showed no detectable $\beta$-lactamase I activity (stage 4).

The solution from stage 4 was brought to 96.6% saturation with ammonium sulphate. Centrifugation gave a heavy protein precipitate covered with a thin whitish layer of lighter material. The total precipitate was dissolved in the smallest amount of 10mM-buffer BZ and the solution filtered through Whatman no. 2 paper. Cold redistilled acetone (22.8ml, i.e. 2.5ml/ml of enzyme solution) was added to the filtrate (9.1ml) in a separating funnel. Two liquid phases separated with a thin layer containing solid material at the interface. The upper acetone-rich layer was filtered through paper (Whatman no. 2) and the filtrate dialysed against 1mM-buffer BZ for 24h with several changes of buffer in the outer vessel. The non-diffusible material was precipitated by 96.6% saturation with ammonium sulphate and the precipitate dialysed against 0.1mM-buffer BZ overnight.

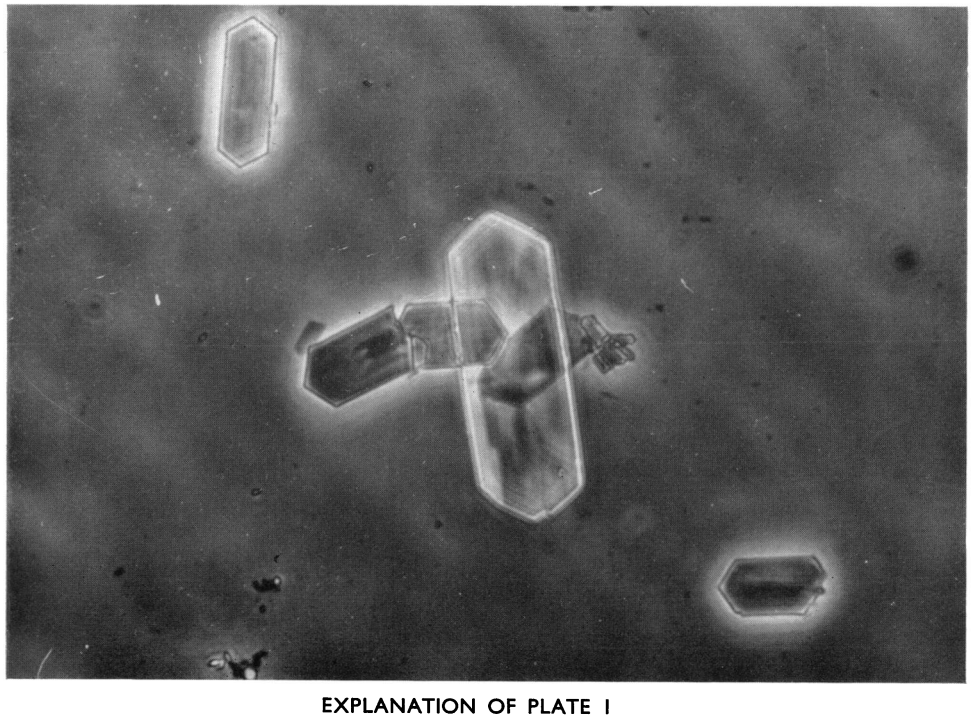
The resulting solution (32ml) was divided into two equal parts and each chromatographed on a column (60cm × 1cm diam.) of DEAE-cellulose as described for the second column used in the purification of $\beta$-lactamase I, except that buffer BZ was employed and the column was run at room temperature. Fractions (5ml) were collected every 5min. The $\beta$-lactamase II was eluted at an even lower concentration of sodium chloride than was $\beta$-lactamase I under similar conditions. In each case nearly all of the active material was found in fractions 8–12 (Fig. 2). Its specific activity (Table 3, stage 5) indicated that the acetone treatment followed by chromatography had resulted in an eightfold purification. Since relatively little inactive protein was eluted from the column the acetone treatment may have been mainly responsible for the large increase in specific activity.

The active material in tubes 8–12 of stage 5

(*a*)



(*b*)



EXPLANATION OF PLATE 1

Crystals of extracellular β-lactamase I from *B. cereus* 569/H. The photographs were taken in phase contrast. (*a*) Magnification × 300; (*b*) selective enlargement of three of the larger crystals in a preparation similar to that shown in (*a*).



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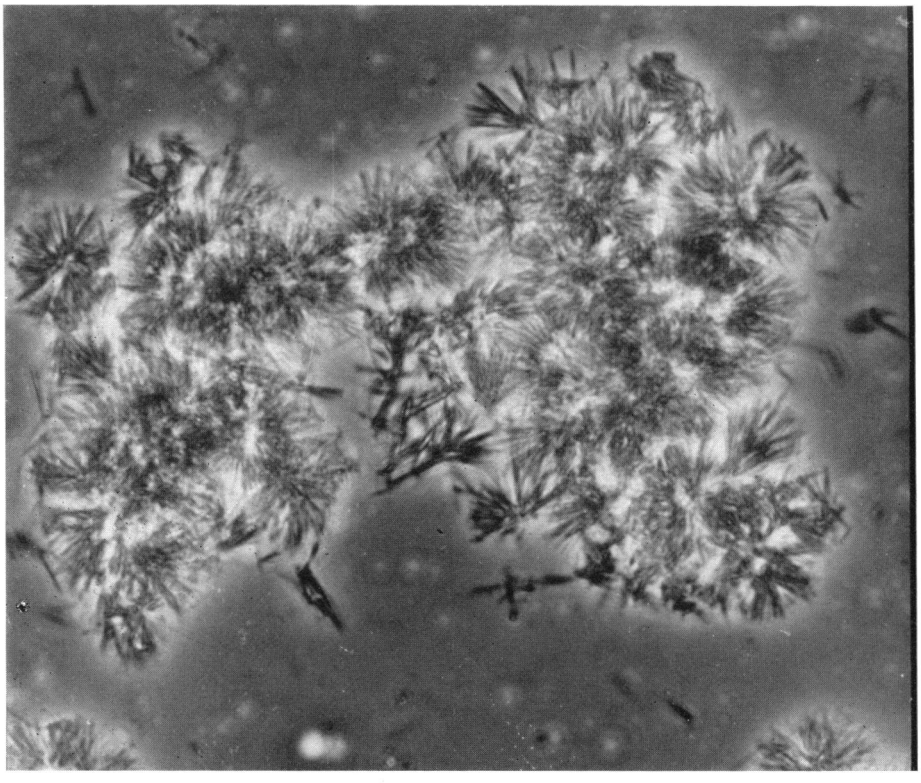
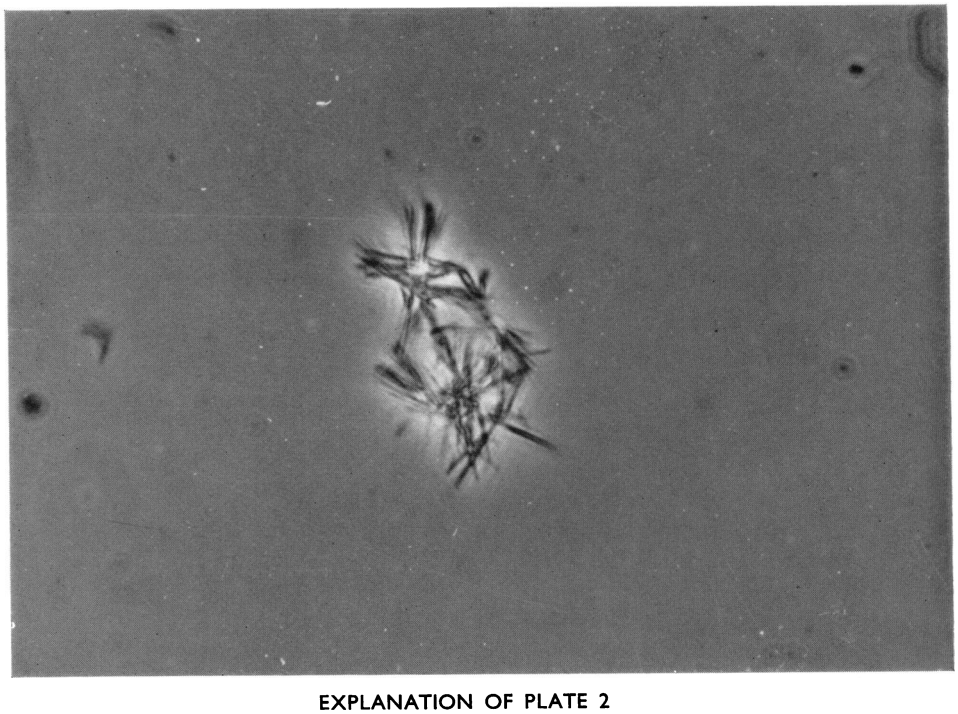
(*a*)



(*b*)



## EXPLANATION OF PLATE 2

Crystalline extracellular β-lactamase II from *B. cereus* 569/H. (*a*) Once crystallized (magnification × 300); (*b*) magnification of (*a*).

Table 3. *Purification of extracellular β-lactamase II from* B. cereus *569/H*

The culture supernatant was obtained from a culture grown in a medium containing 1.0 mM-$ZnSO_4$. The values given for units of β-lactamase II activity are those with cephalosporin C as substrate. From stage 3 onwards β-lactamase I activity (benzylpenicillin as substrate) was determined after β-lactamase II had been inactivated by removal of $Zn^{2+}$ by dialysis against a citrate buffer (for details see the text).

| Stage | Material from | Total vol. (ml) | $10^{-6}\times$ Total units | Units recovered (%) | Total protein (mg) | Protein recovery (%) | $10^{-6}\times$ Sp. activity (units/mg of protein N) | β-Lactamase I / β-Lactamase II activity ratio | $\frac{E_{280}}{E_{260}}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Culture supernatant | 145000 | 9.7 | 100 | 7830 | 100 | 0.0078 | 24.4 | 0.72 |
| 2 | Precipitate on 96.6% saturation with $(NH_4)_2SO_4$ | 825 | 9.1 | 93.8 | 5640 | 72.0 | 0.01 | 30.0 | 0.88 |
| 3 | Heat treatment | 51 | 8.4 | 86.6 | 242 | 3.1 | 0.22 | 0.01 | 0.92 |
| 4 | Eluate from first DEAE-cellulose column | 27 | 7.9 | 81.4 | 172 | 2.2 | 0.29 | <0.01 | 1.31 |
| 5 | Eluate from second DEAE-cellulose column (NaCl gradient) | 2.3 | 4.9 | 50.5 | 12.5 | 0.16 | 2.45 | <0.01 | 1.83 |
| 6 | First crystallization | 1.0 | 2.4 | 25.0 | 6.2 | 0.08 | 2.47 | <0.01 | 2.00 |
| 7 | Second crystallization | — | — | — | — | — | 2.59 | <0.01 | 2.08 |

crystallized spontaneously when the solution was kept at room temperature for 6h (Table 2, stage 6). The crystals formed as regular rosettes, 20–30 μm in diameter (Plate 2). Recrystallization was carried out in essentially the same manner as in stage 7 of the purification of β-lactamase I except that the solution contained 1 μM-zinc sulphate. The recrystallization of β-lactamase II was much slower than that of β-lactamase I and some of the best crystals obtained took 72h to form. They were plates, 60 μm in length and 30 μm in width but somewhat irregular in shape, and exhibited birefringence at the edges, as did those of β-lactamase I. The crystalline material (stage 7) was sparingly soluble in water, but soluble in salt solutions.

Although partially purified β-lactamase II was stable when freeze-dried from buffer BZ at a neutral pH, the crystalline enzyme lost all its activity under similar conditions. For storage the crystals were dissolved in buffer BZ (at least 15mg/ml) and the solution was brought to 65% saturation with ammonium sulphate. The resulting suspension was stored at 4°C.

### *Properties of β-lactamase I and β-lactamase II*

*Molecular weights.* The results of ultracentrifugal analysis of crystalline preparations of β-lactamase I and β-lactamase II are discussed by Lloyd & Peacocke (1970). Both enzyme preparations showed only one major component, but the molecular weight of that of the β-lactamase II preparation was significantly higher than that of the β-lactamase I, the calculated values being 35600 and 27800 respectively. The preparations behaved as though they were homogeneous except that each contained a small amount of material whose molecular weight was more than six times that of the major component. The amounts of these contaminants (or aggregates of the main components) in the preparations of β-lactamase I and β-lactamase II were about 1.5 and 3.5% respectively.

*Filtration on Sephadex G-75* . The behaviour on a column of Sephadex G-75 of a mixture of β-lactamase I and β-lactamase II that had previously been separated by fractional precipitation with ammonium sulphate is shown in Fig. 3. A portion (2ml) of the solution of β-lactamase I (Table 1, stage 3) was mixed with an equal volume of the solution of β-lactamase II (Table 1, stage 4*b*). The enzymes were precipitated by saturation with ammonium sulphate and the precipitate was dialysed against 10mM-buffer BZ. The resulting solution was added to the column and elution was carried out with the same buffer. Although the resolution of the two enzymes on the column was incomplete the early active fractions showed only β-lactamase

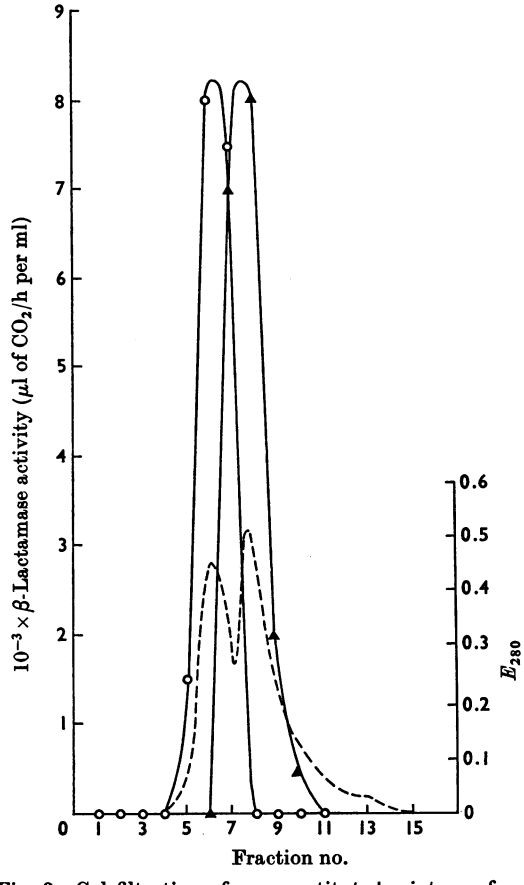



Fig. 3. Gel filtration of a reconstituted mixture of preparations of extracellular $\beta$-lactamase I (5.2mg of protein) and $\beta$-lactamase II (6.4mg of protein) on a column of Sephadex G-75 (40cm × 1cm diam.). ▲, $\beta$-Lactamase I activity; ○, $\beta$-lactamase II activity; ---, protein content ($E_{280}$). Fractions (about 2.25ml) were collected every 5min. The void volume of the column ($V_0$) was about 13.5ml.

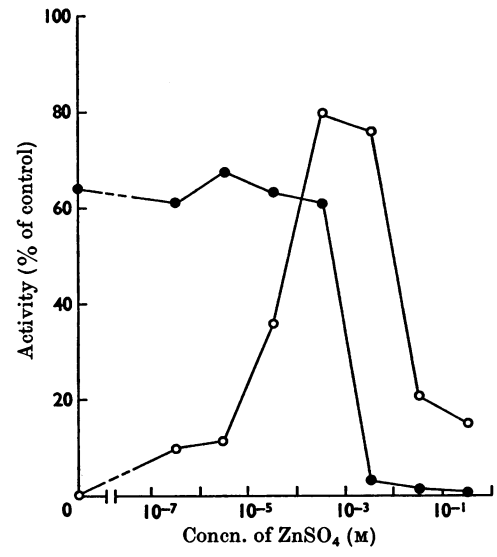



Fig. 4. Effect of concentration of $Zn^{2+}$ on the activities of extracellular $\beta$-lactamase I and $\beta$-lactamase II from *B. cereus* 569/H. $Zn^{2+}$ was removed from $\beta$-lactamase II by dialysis (for details see the text) and $\beta$-lactamase I was treated in a similar manner. ●, Rate of hydrolysis of benzylpenicillin by $\beta$-lactamase I in the presence of increasing concentrations of added $ZnSO_4$; ○, rate of hydrolysis of cephalosporin C by $\beta$-lactamase II under similar conditions.

II activity and the late ones only $\beta$-lactamase I activity.

*Effect of* $Zn^{2+}$ *on enzymic activity of* $\beta$*-lactamase I and* $\beta$*-lactamase II.* When a highly purified preparation of $\beta$-lactamase I was assayed in the presence of increasing amounts of zinc sulphate its activity showed no significant change until the concentration of $Zn^{2+}$ reached 0.33mM. In the presence of 3.3mM-$Zn^{2+}$ the activity fell to less than 5% of its original value.

A solution of purified $\beta$-lactamase II in buffer BZ was inactivated by dialysis at 4°C for 6h against 5mM-sodium citrate buffer, pH4.5, containing 1M-sodium chloride and subsequent dialysis overnight against 0.1M-buffer B, pH7.0. The resulting solution showed no activity with cephalosporin C, benzylpenicillin or methicillin as substrate. Activity with cephalosporin C as substrate was detected when zinc sulphate was added to give a concentration of 0.33$\mu$M-$Zn^{2+}$ in the test system and the activity reached a maximum (at least 80% of its original value) when the concentration of $Zn^{2+}$ was raised to between 0.33mM and 3.3mM. At higher concentrations of $Zn^{2+}$ the activity decreased rapidly (Fig. 4). When $Zn^{2+}$ (3mM) was added to a mixture of cephalosporin C and $Zn^{2+}$-free $\beta$-lactamase II in a vessel connected to the pH-stat no reaction was observed for about 1min. After this lag hydrolysis suddenly reached its maximum rate. The restoration of activity by addition of $Zn^{2+}$ with benzylpenicillin or methicillin as substrate paralleled closely that found with cephalosporin C as substrate.

## DISCUSSION

The $\beta$-lactamase I described in this paper is undoubtedly the penicillinase from *B. cereus* 569/H that was purified by Kogut *et al.* (1956), although some of its properties are apparently not identical with those reported for the latter. The two products resemble each other in their solubility

in ammonium sulphate, their ease of adsorption on and elution from powdered glass or Celite and their substrate profiles (Abraham & Newton, 1956; Crompton *et al.* 1962). The specific activity assigned to the most active preparation of β-lactamase I ($5.4 \times 10^6$ units/mg of protein N) was higher than that reported by Kogut *et al.* (1956) for their penicillinase ($2.3 \times 10^6$ units/mg of protein N), but this may reflect partly the presence of somewhat more denatured material in the latter and the fact that determinations of protein N were made by different methods.

Comparison of some of the properties of β-lactamase II described here with those of β-lactamase I indicate that the differences between these two enzymes are not merely the result of different conformations in a single protein.

Crompton *et al.* (1962) reported that the cephalosporinase activity of culture supernatants of *B. cereus* 569 decreased, relative to their penicillinase activity, with the age of the culture, and that the rapid formation of penicillinase continued after that of cephalosporinase had declined. The results shown in Fig. 1 confirm these observations. But, even in the early phase of the culture, 4h after inoculation, the amount of β-lactamase II produced was less than 10% of that of β-lactamase I. The decrease in the amount of β-lactamase II in the supernatant between 6 and 8h corresponded to only about 5% of the β-lactamase I formed during this interval. Thus these results do not suggest that β-lactamase II is a precursor of β-lactamase I. The reason why the maximum β-lactamase I activity shown in Fig. 1 is much higher than that in most of the culture supernatants used earlier for purification was not determined, but the latter may have been harvested when β-lactamase I activity was not at its peak. Whether the differences between the curves shown for β-lactamase I and β-lactamase II in Fig. 1 are associated with a differential sensitivity of the two β-lactamases to extracellular hydrolytic enzymes remains to be ascertained.

In *B. cereus* 569 the formation of both β-lactamase I and β-lactamase II is induced by a variety of penicillins and cephalosporins and the relative amounts of the two enzymes formed appear to be independent of the nature of the inducer (Crompton *et al.* 1962). β-Lactamase II was virtually excluded from Sephadex G-75 and emerged from a column of the latter in a volume of eluate close to the void volume, whereas β-lactamase I was slightly retarded. This behaviour is consistent with the finding of Lloyd & Peacocke (1970) that the former enzyme has a higher molecular weight than the latter. Thus the possibility must be considered that β-lactamase II has a major fragment in common with β-lactamase I, but also an additional component. If this is so, the additional component must influence the active centre of the common fragment. β-Lactamase II, in contrast with β-lactamase I, shows no activity with benzylpenicillin as substrate in the absence of $Zn^{2+}$; but the addition of $Zn^{2+}$ to β-lactamase I does not endow it with β-lactamase II activity. The metal-ion cofactor requirement of β-lactamase II for enzymic activity has a considerable degree of specificity (Sabath & Abraham, 1966; Sabath & Finland, 1968). $Zn^{2+}$ cannot be replaced by a variety of metal ions in similar concentrations, including $Co^{2+}$, although the latter is known to be able to activate several other $Zn^{2+}$-requiring enzymes (Coleman & Vallee, 1960; Vallee & Williams, 1968).

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## REFERENCES

Abraham, E. P. & Newton, G. G. F. (1956). *Biochem. J.* **63**, 628.
Citri, N. & Pollock, M. R. (1966). *Adv. Enzymol.* **28**, 281.
Coleman, J. E. & Vallee, B. L. (1960). *J. biol. Chem.* **235**, 390.
Crompton, B., Jago, M., Crawford, K., Newton, G. G. F. & Abraham, E. P. (1962). *Biochem. J.* **83**, 52.
Elsworth, R., Miller, G. A., Whitaker, A. R., Kitching, D. & Sayer, P. D. (1968). *J. appl. Chem.* **17**, 157.
Elsworth, R. & Stockwell, F. T. E. (1968). *Process Biochem.* **3**, 15.
Elsworth, R., Williams, V. & Harris-Smith, R. (1957). *J. appl. Chem.* **7**, 261.
Hall, J. R. & Ogston, A. G. (1956). *Biochem. J.* **62**, 401.
Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
Kuwabara, S. & Abraham, E. P. (1967). *Biochem. J.* **103**, 27 c.
Lloyd, P. H. & Peacocke, A. R. (1970). *Biochem. J.* **118**, 463.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Miller, G., Bach, G. & Markus, Z. (1965). *Biotechnol. & Bioengng*, **7**, 517.
Pollock, M. R. (1950). *Brit. J. exp. Path.* **31**, 739.
Pollock, M. R. (1952). *Brit. J. exp. Path.* **33**, 587.
Pollock, M. R. (1953). *J. gen. Microbiol.* **8**, 186.
Pollock, M. R. & Torriani, A.-M. (1953). *C. r. hebd. Séanc. Acad. Sci., Paris*, **237**, 276.
Sabath, L. D. & Abraham, E. P. (1965). *Proc. 5th Interscience Conf. Antimicrobial Agents and Chemotherapy*, p. 392.
Sabath, L. D. & Abraham, E. P. (1966). *Biochem. J.* **98**, 11 c.
Sabath, L. D. & Finland, M. (1968). *J. Bact.* **95**, 1513.
Vallee, B. L. & Williams, R. J. P. (1968). *Chem. in Br.* **4**, 397.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.