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Purification and Properties of Penicillinases from Two Strains of *Bacillus licheniformis*: a Chemical, Physicochemical and Physiological Comparison

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1. The penicillinases formed by penicillinase-constitutive mutant strains from two closely related varieties (749 and 6346) of *Bacillus licheniformis* have been isolated, characterized and compared. They are chemically, physicochemically and immunologically very similar, but differ enzymologically in absolute and relative activity on, and affinity for, different penicillins and cephalosporins. 2. The molecular weights of both types are approx. 23000. Neither enzyme contains any cyst(e)ine. However, in most other respects they show little resemblance to any of the other penicillinases so far isolated. 3. Their properties, whether isolated from cells (to which approx. 50% of the activity is normally bound) or from the culture supernatant, appear to be similar. However, the molecular weight of a preparation of enzyme from strain 749/C obtained from the culture supernatant was found to be significantly (over 20%) higher than that obtained from cells alone. 4. With benzylpenicillin, the enzyme from strain 749 has $V_{max.}$ approx. 6 times higher than that of the enzyme from strain 6346, but this difference is 'compensated' by its affinity being 6 times lower. Thus, at the very low biologically effective concentrations of penicillin met with under natural conditions, where neither type of enzyme is more than a fraction saturated with its substrate, the antibiotic is hydrolysed at the same rate by both. As expected, the penicillin-sensitivities of single cells from the two strains were found to be identical. 5. It is suggested that the concept of 'physiological efficiency' (defined as $V_{max.}$ divided by K_m), applied to enzymes acting naturally under conditions of poor saturation with their substrates, may be useful for expressing their biological function *in vivo*.

It has long been recognized that many species in the *Bacillus* genus are powerful producers of penicillinase (penicillin amidohydrolase; EC 3.5.2.6), and strains of *Bacillus subtilis* have often been included in this group (Abraham, 1951). Indeed, during the last few years two penicillinase-inducible strains (749 and NCTC 6346) of what, until recently, were referred to as *B. subtilis*, have been the subject of enzymological (Manson, Pollock & Tridgell, 1954), immunological (Kushner, 1960; Pollock, 1964) and physiological studies on penicillinase, its secretion and its induction (Kushner & Pollock, 1961; Pollock, 1961*a*,*b*, 1963). The discovery of an experimental system of genetic transformation and transduction in *B. subtilis* (Spizizen, 1958; Thorne, 1962) led to a renewed interest in the possibility of investigating the genetics of penicillinase, and a co-ordinated programme of research into the genetic and environmental control of penicillinase structure and synthesis, in the two strains referred to above, was recently initiated in this Laboratory.

Advances in taxonomical classification of the difficult *Bacillus* genus, taken together with results of preliminary bacteriophage-sensitivity tests and standard biochemical reactions, soon led to the conclusion (P. H. A. Sneath, personal communication) that strains 749 and 6346 would be more appropriately grouped (mainly on the basis of their ability to form gas on anaerobic reduction of nitrates) as varieties of *Bacillus licheniformis*. Moreover, as data on a wide range of *Bacillus* cultures (obtained from all parts of the world) accumulated, it became apparent that (*a*) no strain from among those classified, on primarily biochemical grounds, as belonging to *B. subtilis*, formed more than traces (if any) of a true penicillin β-lactamase, with or without induction, and (*b*) all the strains classified, on other grounds, as belonging to *B. licheniformis*, were penicillinase-inducible. Indeed, all the ten wild-type *B. licheniformis* strains so far tested were found to produce penicillinases, which, on the basis of their enzymological and

immunological properties, fell clearly into one or other of the two distinct 'brands' typified by the 749 and 6346 strains originally studied (P. H. A. Sneath, personal communication).

Isolation of these two types of penicillinase, from strains 749 and 6346, was undertaken partly to characterize and to try to understand the interesting, and partially complementary, enzymological differences shown by types of molecules of very similar chemical composition, and partly to provide the biochemical basis for interpreting the results of inter-strain transformations, involving the penicillinase genes, between these two strains, and to allow plausible speculations on their evolutionary origin and relationship.

MATERIALS AND METHODS

Organisms. Penicillinase-magno-constitutive mutants (749/C and 6346/C) of the penicillinase-inducible wild-type *B. licheniformis* strains 749 and 6346 respectively were isolated as described by Pollock (1963) and preserved as spores in watery suspensions that served as source inoculum for all cultures used in this work.

Medium. This was a casein hydrolysate containing a final concentration of 1% of Difco casamino acids, potassium phosphate, pH 7·2 (0·02 M), and 1 ml. of the following salt solution/l.: 25 g. of $MgSO_4,7H_2O$; 100 mg. of $FeSO_4,7H_2O$; 100 mg. of $ZnSO_4,7H_2O$; 10 mg. of $MnSO_4,4H_2O$; 1 mg. of $CuSO_4,5H_2O$; 0·2 mg. of $K_2Cr_2O_7$; dissolved in 100 ml. of water with a little conc. HCl. The whole medium (referred to below as CH/S medium) was then autoclaved at 115° for 20 min. In certain preparations 1% (final concn.) of glucose, autoclaved separately, was added.

Growth conditions. For small (10 l.) batches, ten 100 ml. lots of CH/S medium in 500 ml. conical flasks were each inoculated with 0·1 ml. of standard suspension of spores (3×10^8 viable organisms/ml.) and incubated at 35°, without shaking, overnight. The whole contents of each of these flasks was then inoculated into approx. 1 l. of medium in a 5 l. conical flask and shaken at full aeration at 35° for 6–8 hr., by when cell opacity had usually reached 1·0–1·5 mg. dry bacterial wt./ml. Where glucose was added, it was essential to keep the pH up to 7·0–7·6 by the intermittent addition of N-NaOH. In cultures without glucose the pH value did not usually increase above 7·8 and no adjustment was normally necessary.

For the single large (200 l.) batch so far prepared (with strain 749/C), the chain of inoculum cultures was initiated by seeding each of 15 Roux bottles (containing a total of 1·5 l. of peptone–agar) with a few drops of standard spore suspension and incubating for 12 hr. at 35°. The cells were washed off the agar early the next morning with a total of 400 ml. of CH/S medium and added to the main inoculum culture (10 l. of CH/S medium), which was grown in conical flasks exactly as for the main culture in smaller batches. After 4 hr. of shaking at 35°, the main inoculum culture had grown to a density of 0·7 mg. dry wt./ml. and was added to the fermenter in which the 200 l. of CH/S medium (pH 7·2) had been previously sterilized. This stainless-steel fermentation vat had a total capacity of 350 l., and an air flow of 6 l./min., with stirring at 190 rev./min. A check was kept on growth (measured by referring the extinction at 675 mμ to a standard curve relating it to dry weight of cells), enzyme titre and pH value throughout the run. Only towards the end, when the pH value had increased to 7·8, was a small adjustment made, by the addition of 200 ml. of 5 N-HCl to reduce it to 7·2. Growth started failing after 4½ hr. at the unusually low level of 0·55 mg. dry wt./ml., but, since the total enzyme produced (5040 units/ml.) was satisfactory, the run was terminated after 5 hr. and the culture fed out into a Sharples centrifuge for separation of the cells.

Penicillinase assays. Activity was normally assayed manometrically at pH 7·0 and 30° by the method of Henry & Housewright (1947), 1·0 ml. of 1·0% gelatin solution being added to the manometer cups before assay. However, with 6-aminopenicillanic acid as substrate and for all pH–activity curve measurements, the quantitative iodometric method of Perret (1954) was used. All dilutions of enzyme samples were made in 1% gelatin containing 8-hydroxyquinoline (0·84 mM). For the determination of affinity constants with substrates other than the cephalosporins, the micro-iodometric method of Novick (1962*b*) was employed. For rapid semi-quantitative assays of eluate fractions from chromatographic columns, a starch–iodine time–decolorization spot test based on that described by Perret (1954) was used. Activities are expressed as μmoles of substrate destroyed/hr. at pH 7·0 and 30°, which, if referable to benzylpenicillin, are expressed as units (Pollock & Torriani, 1953), without introducing adjustments to correct for the slightly higher (15%) activities found with the standard iodometric method or the rather lower results obtained with the micro-assay technique, as compared with manometric assays.

After enzymic hydrolysis of the cephalosporins, manometry indicated that about 30% more acid was released than would be expected from a simple opening of the β-lactam ring to produce the corresponding cephalosporoic acid. A similar discrepancy has been reported by Crompton, Jago, Crawford, Newton & Abraham (1962). It may possibly be related to the finding that the iodometric assay of cephalosporinase activity, based on an iodine equivalence of 4·0 atoms/mol. of cephalosporoic acid, reported by Fleming, Goldner & Glass (1963), gave values about 30% below those determined manometrically. This is in contrast with the corresponding discrepancy between the two methods with benzylpenicillin as substrate, which is in the other direction. The explanation of these findings is still uncertain. But it is possible that the cephalosporinase values reported in the present paper, which have been obtained manometrically, may have to be revised accordingly.

Amino acid analysis. About 5 mg. of each of the purified enzyme preparations were precipitated with acetone at −2° for 6 hr. and the precipitate, collected by centrifugation at −10°, was dissolved in water; HCl was then added to give a final concn. of 6 N and the sample hydrolysed for 16 hr. at 105° in a sealed tube. The HCl was then removed *in vacuo* over NaOH and the amino acids were estimated automatically on the Spinco analyser (Beckman Instrument Co., Palo Alto, Calif., U.S.A.; Spackman, Stein & Moore, 1958) by Dr S. Jacobs.

For cysteine analysis, 4·5 mg. were first oxidized with performic acid, the sample then being hydrolysed (as described by Pollock & Richmond, 1962) and cysteic acid

estimated on the Spinco analyser and compared against a known standard.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Starch-gel electrophoresis. This was done according to the method of Smithies (1955), with a bridge solution of 0·33 M-sodium borate buffer, pH 8·45, and run for 4–5 hr. at approx. 48 mA. The gel itself was prepared in 0·05 M-glycine–NaOH buffer, pH 8·9. Zones of enzymic activity were disclosed by soaking the gel (after electrophoresis) for 20–30 min. in a saturated aqueous solution of *N*-phenyl-1-naphthylamine-azo-*o*-carboxybenzene (British Drug Houses Ltd.), prepared by first making a saturated solution of the indicator in dimethylformamide, diluting threefold in aqueous 0·1 M-phosphate buffer, pH 7·0, and filtering; the dye was then drained off and the gel flooded with an aqueous solution of approx. 1·0 M-benzylpenicillin. For greater sensitivity the gel was sprayed directly with a solution of 0·15 M-benzylpenicillin in 0·016 N-I_2 and 0·06 M-KI in 0·05 M-phosphate buffer, pH 7·0.

Chemicals. Benzylpenicillin [6-(phenylacetamido)penicillanic acid (sodium salt)] was a commercial preparation obtained from Glaxo Laboratories Ltd., Greenford, Middlesex. Methicillin [6-(2,6-dimethoxybenzamido)penicillanic acid (sodium salt)] and 6-aminopenicillanic acid were gifts from Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey. Cephalosporin C [7-(D-5-amino-5-carboxyvaleramido)cephalosporanic acid] and benzylcephalosporin C [7-(phenylacetamido)cephalosporanic acid] were gifts from Glaxo Laboratories Ltd., Stoke Poges, Bucks.

Cellulose phosphate (Whatman powder P40) and DEAE-cellulose (Eastman Kodak Co., Rochester, N.Y., U.S.A.) were obtained commercially. Lysozyme (from egg white), ribonuclease and trypsin (all from Armour Pharmaceutical Co., Eastbourne, Sussex), and deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), were also commercial products.

Preparation of the acid form of cellulose phosphate. A known weight of the powder (which is normally in the NH_4^+ form) was washed with 0·2 N-HCl on a coarse sintered-glass filter, the material being allowed to soak in acid for 2–3 min. The powder was washed with distilled water, then with more HCl, the process being repeated until the filtrate was free from NH_3. A final wash with distilled water was followed by a wash through with 0·1 N-KOH until the filtrate became alkaline to bromothymol blue. The cellulose phosphate was then converted back into the H^+ form, after a preliminary washing with distilled water, by a second treatment with 0·2 N-HCl. It was finally washed with water before use.

Buffers. Phosphate buffers were prepared by adding N-NaOH to a solution of KH_2PO_4 at the appropriate concentration, in quantities sufficient to bring the pH to the required value. For pH–activity curves, McIlvaine buffers were prepared with Na_2HPO_4 and citric acid, as described by Britton (1942), and the final pH values checked with a pH-meter.

RESULTS

Purification procedures

B. licheniformis strain 749/C produces a total of approx. 4000, and strain 6346/C about 600, units of penicillinase/mg. dry wt. of cells. In cultures in CH/S medium nearly 50% of this is liberated into the medium (see Pollock, 1961*a*) by the time the cultures reach a cell density of 1·0 mg. dry wt./ml. In the presence of glucose, growth is slightly better, but the fall in pH leads to a greater retention of enzyme on the cells. Penicillinase can be isolated from the cells, from the culture supernatant or from the whole culture (after lysis). The penicillinase extracted artificially from the cells by the methods described in the present paper appears in most respects to be indistinguishable from the enzyme liberated spontaneously into the medium. No clear and consistent differences (with either strain) between the penicillinase present in crude culture supernatants and that of purified preparations have yet been disclosed with respect to (*a*) electrophoretic patterns on starch-gel electrophoresis, (*b*) substrate 'profile' (see below), (*c*) iodine-sensitivity, or (*d*) immunological reactivity (Pollock, 1964). A possible difference in molecular weight is discussed below.

The general isolation procedures were identical for both types of penicillinase, and differed only (as indicated) in the fractionations involving precipitation with ammonium sulphate, in which the enzyme from strain 749/C appeared at first to be more soluble than that from strain 6346/C. This difference, however, is only apparent if expressed in terms of enzymic activity, and probably does not reflect true differences in solubility of the two proteins.

Isolation from cells

This has been done only with strain 749/C. The cells from 10 l. of culture were separated from the medium by centrifugation at 5000 *g* for 20 min. and resuspended in 500 ml. of 0·01 M-phosphate buffer, pH 7·0. They were then lysed by incubation at 35° for 30 min. with lysozyme (50 μg./ml.), and deoxyribonuclease (5 μg./ml.) and ribonuclease (10 μg./ml.) were added to reduce the viscosity. The pH was adjusted to 8·0 with N-sodium hydroxide, and trypsin (20 μg./ml.) was added to free the penicillinase from its attachment to the cell envelope (Kushner & Pollock, 1961). Incubation at 35° was continued for 16 hr. This did not involve any significant loss in activity of the penicillinase, which is resistant to tryptic hydrolysis. The debris was spun off at 18000 *g* (for 30 min.) and discarded, 85–90% of the enzyme being liberated in a soluble state in the supernatant, which was then dialysed against 2 l. of 85% saturated ammonium sulphate solution (pH adjusted with sodium hydroxide to 6·5) at 2° for 24 hr. (In this, as in all subsequent cases, ammonium sulphate concentrations are expressed as percentage saturation at 0° after equilibrium is reached with

the sac contents.) The precipitate (containing only 25–30% of the penicillinase and a great deal of other protein) was discarded and the supernatant dialysed exhaustively against running tap water and then reduced in volume, by freeze-drying, to about 75 ml. This solution was dialysed against 0·01 M-tris buffer, pH 7·5, and added to a column (24 cm. long × 3·1 cm. diam.) of DEAE-cellulose, which was then washed through with 200 ml. of the same tris buffer. The enzyme was eluted from this column by a linear gradient of tris buffer, pH 7·5, running from 0·02 M to 0·25 M (mixing vol. 800 ml.), 25 ml. samples being collected and assayed. The maximum penicillinase activity usually appeared between fractions 19 and 24 inclusive after initiation of the gradient. Collection of the five fractions 'covering' the peak generally ensured recovery of 50–60% of the total enzyme put on the column (see Fig. 1), and yielded material having a mean specific activity of approx. 200 units/μg. of protein (i.e. about 60% pure, in relation to the final product).

Final purification was achieved by dialysis against a saturated solution of ammonium sulphate at pH 6·5, at an enzyme concentration of approx. 8×10^4 units/ml.; only the supernatant was retained, and this, after dialysis against mM-phosphate, pH 7·0, was concentrated by freeze-drying. The specific activity was 305 units/μg. of protein, and neither repeated fractionation with ammonium sulphate nor rechromatography on DEAE-cellulose, as before, yielded any preparation with an activity higher than 350 units/μg. The overall yield was 35%. The preparation is referred to below as 749/C_{cells}.

Isolation from whole culture

This has so far been used only for strain 6346/C, cultures of which are prone to spontaneous lysis. The general method was the same as that described for the isolation of enzyme from strain 749/C from cells alone, except that the cells were not separated, and the lysozyme, nucleases and trypsin were added directly to the whole culture. When lysis had already occurred spontaneously (as in the batch here described) the addition of lysozyme was omitted. The whole lysate was then concentrated in a van Heyningen (1949) still at 35° from 10 l. to 900 ml., dialysed against running tap water overnight and further concentrated to 100 ml., first again in the still and then in a Craig evaporator. It was then clarified by centrifuging, the deposit discarded and the supernatant dialysed against 85% saturated ammonium sulphate, pH 6·5, as in the previous procedure. Further steps in purification were identical until the final fractionation with ammonium sulphate, which was achieved by dialysis,

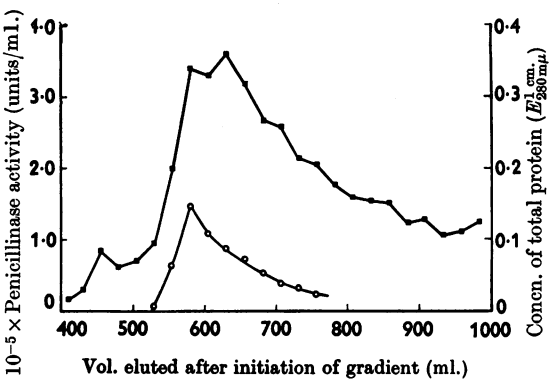

Fig. 1. Chromatographic elution of *B. licheniformis* strain 749 penicillinase (○) and total protein (■) from a DEAE-cellulose column with a linear gradient of tris buffer, pH 7·5. Full details are given in the text.

also at an enzyme concentration of approx. 8×10^4 units/ml., against 90% saturated ammonium sulphate, with retention of the precipitate only. This was dissolved in a few millilitres of water and then dialysed against mM-phosphate buffer, pH 7·0, being concentrated, as with the enzyme from strain 749/C, by freeze-drying. This preparation had a specific activity of 54 units/μg. of protein, which was not increased by repeated fractionation in ammonium sulphate. The final overall yield was 17%. The preparation is referred to below as 6346/C_{whole}.

Isolation from culture supernatant

This is by far the simplest and quickest method, with the additional advantage of having a starting material where the penicillinase accounts for up to 30% of the total protein present. It is, however, wasteful, since usually only rather less than half of the total enzyme produced is liberated from the cells into the medium. Attempts to increase this proportion are liable to be associated with cell damage and release of other proteins into the medium. This interferes with adsorption of penicillinase on the cellulose phosphate, an essential first step in the extraction process, and must be avoided, if possible.

The method has been applied so far only to the enzyme from *B. licheniformis* 749.

Adsorption on cellulose phosphate. The efficiency of adsorption of penicillinase from a culture supernatant seems to depend, among other things, on the amount of other proteins present. The quantity of cellulose phosphate (prepared in the H^+ form, as described in the Materials and Methods section) that was added has to be judged empirically with each

batch, the objective being to use the minimum amount compatible with a reasonably high percentage adsorption of the enzyme. The pH of the culture supernatant was first adjusted to 5·0 with acetic acid and enough cellulose phosphate suspension was added to give a final concentration of 2 g./l. After being stirred and then left for 5 min. the cellulose phosphate was spun off from a sample and the proportion of enzyme remaining in the supernatant assayed. Further lots of cellulose phosphate were added until about 90% of the penicillinase had been adsorbed. A final concentration of 10 g./l. may be needed (as in the large batch described below). The whole preparation was then allowed to stand overnight at 2°, when the cellulose phosphate had settled to allow most of the supernatant to be decanted or siphoned off. The remaining slurry was then poured directly into a suitably sized column, with a support and some glass wool at the bottom, to allow chromatographic elution of the penicillinase. The material in the column had to be evenly, but not too tightly, packed by gentle suction, and not allowed to dry. It was then washed through with 0·01 M-phosphate buffer, pH 5·0, and the penicillinase eluted by the addition of 0·2 M-phosphate buffer, pH 7·6, at the top, and recovered in a very small volume containing most of the proteins and about 90% of the enzyme originally adsorbed. Its passage down the column was indicated by a narrow yellowish band travelling down with the more concentrated phosphate.

It was essential to check, by rapid enzyme assays, which fractions needed to be retained; the front of the enzyme band was always very sharp; but a certain amount of 'tailing' was difficult to avoid. Essentially this chromatographic 'fractionation' is little more than a method for concentrating the enzyme into about 0·5% of the original volume.

The cellulose phosphate could be regenerated by washing the column with 0·2 M-phosphate buffer, pH 7·6, to remove all adsorbed protein, followed by thorough flooding through with water and final restoration to the H^+ form with 0·1 N-hydrochloric acid.

The penicillinase solution thus obtained contained usually only 25–30% of other protein impurities and could be finally purified by ammonium sulphate fractionation. When sufficient material was present in sufficiently high concentration, the best method was by a 'cut' between 90% and 100% saturated solutions, pH 6·5, at an enzyme concentration of about $1{\cdot}5 \times 10^5$ units/ml., retaining only that fraction soluble at 90% saturation and insoluble at 100% saturation. By these means an apparently pure preparation was obtained from the 200 l. batch, containing nearly 75% of the quantity adsorbed on the cellulose phosphate and 68% of what was present in the original culture supernatant. This is referred to below as $749/C_{Sn}$. The specific activity of different samples ranged between 320 and 358 units/μg. Attempts at further purification were made by dissolving about 400 mg. of the material in 0·01 M-phosphate, pH 7·0, at the relatively high concentration of 10 mg./ml., and dialysing at 2° against ammonium sulphate (pH 6·5) at a starting concentration corresponding to 60% saturation. Further quantities of solid ammonium sulphate were added each day, keeping the pH constant with sodium hydroxide, until a faint opalescence appeared (at approx. 80% saturation) in the enzyme solution. It was then left for

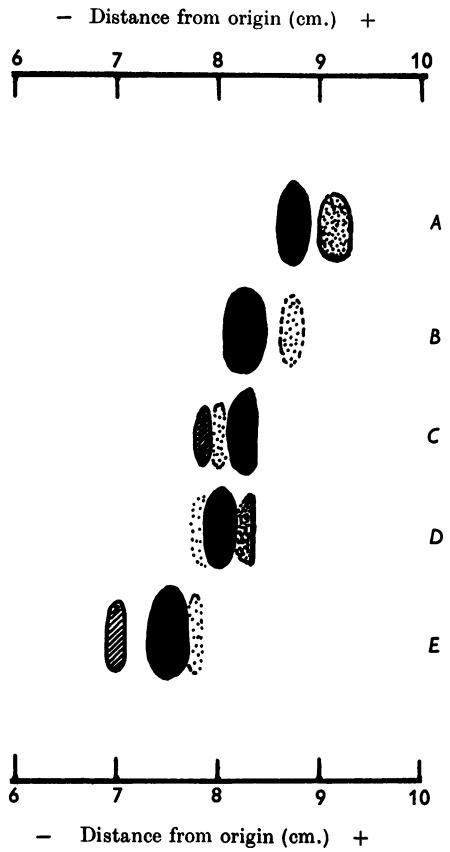

Fig. 2. Starch-gel electrophoretic patterns of different preparations of penicillinases from *B. licheniformis* strains 749/C and 6346/C (applied in enzymically equivalent amounts) after a run of 4½ hr. in 0·33 M-sodium borate buffer, pH 8·45. The patterns were developed by the addition of a concentrated solution of benzylpenicillin, after soaking in a saturated aqueous solution of *N*-phenyl-1-naphthylamine-azo-*o*-carboxybenzene. *A*, $6346/C_{whole}$ (purified); *B*, $6346/C_{Sn}$ (crude); *C*, $749/C_{cells}$ (purified); *D*, $749/C_{Sn}$ (purified); *E*, $749/C_{Sn}$ (crude). Full details are given in the text (Materials and Methods section).

about a week, after which a large precipitate appeared during 2–3 days. A portion (50 mg.) of this precipitate was spun down and dissolved in 0·01 M-phosphate buffer, pH 7·0. Its specific activity (330 units/μg.) was no higher than that of the mother preparation. Ultracentrifugal analysis of this material showed a single symmetrical peak with a sedimentation coefficient similar to that of the 749/C_{cells} preparation.

Properties

The characters of the two types of penicillinase have been compared mainly by studies on the purified 749/C_{cells} and 6346/C_{whole} preparations. Some comparisons have also been made between the 749/C_{cells} and 749/C_{Sn} preparations. These latter include comparisons of relative activities on different substrates and immunological (enzymic activity/antiserum concentration) curves that have not been reported because no significant differences were found.

Physicochemical properties (*Table* 1). (1) Ultracentrifuge analysis. Solutions of the 749/C_{cells}, 749/C_{Sn} and 6346/C_{whole} enzyme preparations at concentrations of 0·5–0·7% after dialysis against phosphate buffer, pH 7·0 and I 0·1, were run in the Spinco analytical ultracentrifuge for 165 min. at 59780 rev./min. All gave single symmetrical peaks. From analysis of patterns obtained at lower speeds molecular weights were calculated by Dr P. A. Charlwood, using the Archibald method or sedimentation-equilibrium method or both. In calculations, $\bar{v}$ was taken as 0·735; this value was based on amino acid composition.

(2) Boundary electrophoresis. This was done in the Perkin–Elmer Tiselius apparatus (micro-cells) with 749/C_{cells} and 6346/C_{whole} preparations in veronal buffer, pH 8·15 and I 0·1, at currents of 0·13 and 0·073 A respectively for 3½ hr. Both gave single symmetrical peaks, the penicillinase from strain 6346/C moving approx. 20% more rapidly than that from strain 749/C.

(3) Zone electrophoresis. This, done in starch gel at pH 8·45 (see the Materials and Methods section), likewise showed that the mobility of the penicillinase from strain 6346/C was slightly higher than that from strain 749/C. The pattern of multiple enzyme bands was also different, though there was some variation from batch to batch in relative intensities and in the apparent mobilities, there being typically two major bands with the preparation from strain 6346/C and three with that from strain 749/C (Fig. 2).

Chemical properties (*Table* 2). Table 2 gives the number of residues of different amino acids found in the three samples so far analysed. The tryptophan contents were not estimated and have been ignored in calculations of the proportions of the other amino acids present. No significant amount of cyst(e)ine was found in either of the two preparations tested.

Enzymological properties (*Table* 3). The relative and absolute specific activities at substrate saturation ($V_{max.}$) with five different substrates are summarized in Table 3, together with dissociation constants for benzylpenicillin and methicillin. In each case the relative specific activities on different substrates were not significantly different from those measured in dialysed concentrated preparations of the crude culture supernatant. It is therefore reasonable to conclude that they represent, for each strain, the substrate 'profile' of a single species of enzyme. The K_m for benzylpenicillin was

Table 1. *Comparison of physicochemical properties of penicillinases from* Bacillus licheniformis *strains* 749/*C and* 6346/*C*

Details are given in the text.

	6346/C_{whole}	749/C_{cells}	749/C_{Sn}
Electrophoretic mobility (cm.$^{-2}$/v/sec.) in veronal buffer, pH 8·15 and I 0·1			
(*a*) Ascending	$-4{\cdot}95\times10^{-5}$	$-4{\cdot}33\times10^{-5}$	
(*b*) Descending	$-4{\cdot}50\times10^{-5}$	$-3{\cdot}65\times10^{-5}$	
Sedimentation coefficient ($S_{20,w}$) (S)	2·63	2·70	2·66
Molecular weight			
(*a*) Archibald method	23200	22900	28100
(*b*) Sedimentation equilibrium			28200
Specific activity (μmoles of benzylpenicillin hydrolysed/hr. at 30° and pH 7·0)			
(*a*) per μg. of N	389	2180	2350
(*b*) per μg. of protein	54	305	330
Molecular activity (moles of benzylpenicillin hydrolysed/mole of enzyme/min. at 30° and pH 7·0)	$2{\cdot}10\times10^{4}$	$1{\cdot}18\times10^{5}$	$1{\cdot}08\times10^{5}$
$E^{1\,cm.}_{280m\mu}$ at 1 mg. of N/ml.	5·65	5·45	4·75

Table 2. *Comparison of amino acid analyses of penicillinases from* Bacillus licheniformis *strains 749/C and 6346/C*

Details are given in the text. The results are expressed as numbers of residues (to the nearest integer) per molecule.

	6346/C_{whole}	749/C_{cells}	749/C_{Sn}
Assumed mol.wt. ...	23000	23000	28000
Lys	19	22	30
Arg	12	12	19
His	1	1	1
Asp	28	31	35
Thr	18	16	18
Ser	10	8	8
Glu	24	21	25
Pro	9	10	10
Gly	12	11	14
Ala	19	21	24
Val	10	8	13
Met	5	4	2
Ile	9	9	13
Leu	21	22	25
Tyr	5	6	5
Phe	5	5	7
Cys	0*	—	0†

* Less than 1 mole/$2{\cdot}5 \times 10^5$ g.
† Less than 1 mole/$4{\cdot}5 \times 10^5$ g.

Table 3. *Comparison of enzymological and physiological properties of penicillinases from* Bacillus licheniformis *strains 749/C and 6346/C*

Details are given in the text.

	749/C	6346/C
Mean specific activities ($V_{max.}$)*		
Benzylpenicillin	325 (100)	54 (100)
Methicillin	1·5 (0·45)	0·54 (1·0)
6-Aminopenicillanic acid	16·2 (5·0)	7·0 (13)
Cephalosporin C	3·5 (1·1)	8·1 (15)
Benzylcephalosporin C	7·9 (2·4)	22·7 (42)
Dissociation constants		
Benzylpenicillin (K_m)	49 μM	9·5 μM
Methicillin (K_i)	0·93 μM	0·23 μM
Cephalosporin C (K_m)	< 50 μM	< 50 μM
Benzylcephalosporin C (K_m)	< 50 μM	< 50 μM
'Physiological efficiencies' ($V_{max.}/K_m$ or $V_{max.}/K_i$)†		
Benzylpenicillin	$6{\cdot}6 \times 10^6$	$5{\cdot}7 \times 10^6$
Methicillin	$1{\cdot}6 \times 10^6$	$2{\cdot}4 \times 10^6$
Benzylpenicillin resistance of relevant strain‡	0·10 unit/ml.	0·10 unit/ml.

* Expressed as μmoles of substrate hydrolysed/μg. of enzyme/hr. under conditions of enzyme saturation at 30° and pH 7·0; values relative to benzylpenicillin (= 100) are given in parentheses.
† See text for full definition.
‡ Minimum concn. inhibiting colony formation from at least 50% of cells inoculated in nutrient agar.

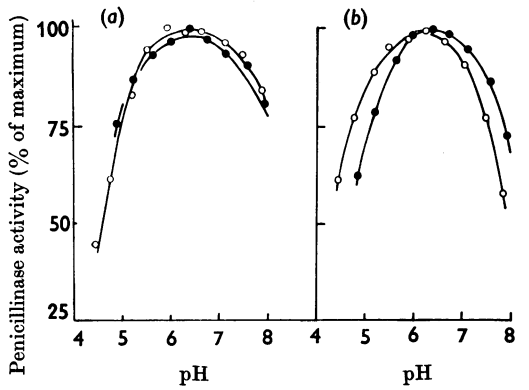

Fig. 3. pH–activity curves of purified penicillinases from *B. licheniformis* strains 749 (○) and 6346 (●) acting on (*a*) benzylpenicillin and (*b*) methicillin in McIlvaine buffers at 30°.

measured at four substrate concentrations (between 20 and 330 μM) and calculated graphically by the method of Lineweaver & Burk (1934). The affinity for methicillin is extremely high and was therefore measured as K_i against benzylpenicillin, with three concentrations (0·36, 0·72 and 1·44 mM) of methicillin and two (5 and 10 mM) of benzylpenicillin, and calculating the K_i/K_m ratio according to the method of Hunter & Downs (1945). The rates of hydrolysis of benzylpenicillin under these conditions indicated a strictly competitive inhibition by methicillin.

The relationships between activity and pH for both benzylpenicillin and methicillin are summarized in Fig. 3, from which it is clear that there are barely significant differences between the two types of enzymes and that the two substrates give approximately the same curves.

Other properties. Both these penicillinases are stable to prolonged dialysis and, indeed, to general manipulations of most kinds. The extent of their adsorption to glass and other non-specific surfaces is negligible except at very high dilutions. Enzymic activities were in both cases unaffected by treatment (for up to 5 hr. at 35° at pH 7·8) with trypsin (20 μg./ml.), and even after 2 days there was no more than a 10% decrease. Dialysis of a preparation, previously treated with trypsin (100 μg./ml.) at pH 7·8 for 2 hr. at 30°, against mM-sodium phosphate, pH 7·0, for 24 hr., did not cause any significant change in specific enzyme activity.

The sensitivities to iodine were tested at 0° at an iodine concentration of 3·8 mM (in the presence of 0·02 M-potassium iodide) in 0·02 M-phosphate buffer, pH 7·0, under conditions described by Garber & Citri (1962), with purified enzyme

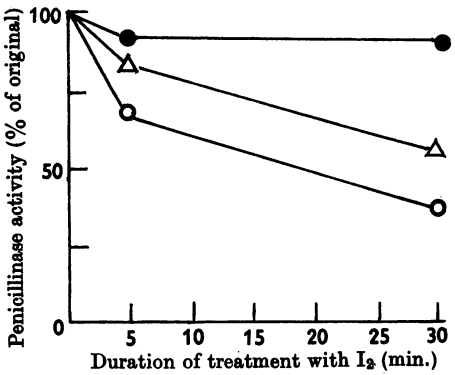

Fig. 4. Sensitivities of purified penicillinases from *B. licheniformis* strains 749/C and 6346/C to treatment with 3·8 mM-iodine at 0° and pH 7·0. ○, Enzyme from strain 749/C alone; ●, enzyme from strain 6346/C alone; △, mixture of equal activities of each enzyme. The total original activity in all experiments was 50 units/ml.

preparations at concentrations varying between 0·035 and 0·2 μg./ml. After 5 and 30 min., samples were titrated with 0·038 N-sodium thiosulphate to remove free iodine. Activities were then assayed iodometrically. Fig. 4 shows a big difference between the two types of penicillinase in sensitivity to iodine, that from strain 749 being over 50% inactivated in 30 min. whereas that from strain 6346 was almost completely resistant. Similar strain differences were found with crude culture supernatants. A mixture of equal activities of each type of enzyme gave inactivation rates intermediate between those of each separately. The difference is analogous to, though much less marked than, that shown between the α and γ forms of *Bacillus cereus* penicillinase that are believed to differ only in their tertiary structures (Citri, Garber & Sela, 1960).

DISCUSSION

Physically and chemically the differences between the penicillinases from *B. licheniformis* strains 749/C and 6346/C appear to be minimal. The recorded differences in amino acid analyses of the $749/C_{cells}$ and $6346/C_{whole}$ preparations are probably within the limits of accuracy of the method used, as applied to these single samples. It is not therefore possible to conclude to what extent, if any, the two enzymes differ in primary sequence. The total absence of cysteine is shared with all other penicillinases so far examined (Jacobs, 1956; Richmond, 1963), and, indeed, most other bacterial extracellular enzymes (Pollock & Richmond, 1962), lending further support to the hypothesis that this feature may have some physiological significance in the mechanism of enzyme liberation. The other amino acids, however, are present in proportions very different from those in the penicillinases from *Staphylococcus aureus* (Richmond, 1963) and *B. cereus* (Jacobs, 1956). The only features that may perhaps be shared to a certain extent by all three groups are the high content of basic (31–48 residues of arginine + histidine) and acidic (52–69 residues of aspartic acid + glutamic acid) amino acids.

The molecular weights of the two penicillinase preparations, $749/C_{cells}$ and $6346/C_{whole}$, from different strains of *B. licheniformis*, do not differ significantly, being by a clear margin the lowest of the penicillinases so far analysed.

However, the preparation from strain 749/C isolated from culture supernatant alone ($749/C_{sn}$) appears to have a molecular weight (measured by two different methods) significantly higher (20%) than that of the other two preparations, although its sedimentation coefficient is similar. The 'discrepancy' may be related to its being isolated without the trypsin treatment applied to the other two preparations, rather than to any physiological difference between the cell-bound and the naturally liberated enzyme moieties, which in other respects appear to be indistinguishable. The 'discrepancy' does not seem to be reflected by any clear differences in specific enzyme activities.

The electrophoretic mobilities of the enzymes from the two strains differ, at pH 8·5, by only 20%, and their behaviour during the fractionation procedures used in their isolation suggests that their solubilities in ammonium sulphate and their distribution coefficients between DEAE-cellulose and tris buffer are very similar. An immunological analysis (Pollock, 1964) strongly suggests that combination with antisera involves similar antigenic groups on the two types of penicillinase, even though it leads to different effects on enzymic activity.

Only in strictly enzymological properties are the two penicillinase strains clearly differentiated. Their specific activities on benzylpenicillin differ by a factor 6. This is surprising enough in itself for enzymes from the same species. It is even more surprising when the specific activity of one of the types (from strain 749) appears to be almost identical with that of the chemically, physicochemically and immunologically different penicillinases from two distinct varieties of *B. cereus* (Kogut, Pollock & Tridgell, 1956), a species that is taxonomically not closely related to *B. licheniformis*. However, the affinities of the two *B. licheniformis* types for the same substrate also differ by about sixfold, but in the opposite direction. This, of course, means that at very low concentrations of benzylpenicillin (below 10 μM) the two types of enzyme destroy the antibiotic at about the same rate.

Concept of 'physiological efficiency' of an enzyme

It is difficult to believe that this reciprocal relationship between $V_{max.}$ and K_m is coincidental. The very low, but antibiotically effective, penicillin concentrations at which the penicillinases are functionally equivalent are just those that the organisms that produce them might be expected to meet in their natural soil habitat, and against which they would presumably need to protect themselves.

There are indeed some reasons for supposing that many enzymes are normally working *in vivo* on steady-state concentrations of their substrates well below the K_m. This may often be true for soluble extracellular enzymes that can be studied *in vitro* under conditions presumably similar to those in which they normally function *in vivo*. The generalization cannot be so easily applied to enzymes of intermediary metabolism, where spatially orientated sequences of possibly compartmentalized systems preclude any reliable estimate of steady-state substrate concentrations in the immediate location of the enzyme concerned. Nevertheless, for comparative purposes, specific activities, measured, as they normally are, at enzyme saturation, may give misleading information about the physiological efficiency of an enzyme acting under natural conditions. It is therefore suggested that in certain cases the concept of physiological efficiency of an enzyme for any particular substrate might be usefully introduced for comparative characterization, and that it could be conveniently defined quantitatively as the specific activity (μmoles of substrate metabolized/μg. of enzyme protein/hr.) at enzyme saturation divided by the K_m (molarity) for any particular substrate.

Thus defined, the physiological efficiencies of the penicillinases from strains 749/C and 6346/C for benzylpenicillin hydrolysis are very similar, being $6{\cdot}6 \times 10^6$ and $5{\cdot}7 \times 10^6$ respectively. In analogous fashion, the relatively very low rate of methicillin hydrolysis is to a large extent 'compensated' by a very high affinity for this substrate. The physiological efficiencies of the two strains for methicillin (determined, in this case, by the use of K_i instead of K_m, though it is appreciated that the two values may not be identical) approximate not only one to another, but, by a relatively small margin, to those for benzylpenicillin.

With the knowledge that the differential rates of synthesis of these penicillinases by strains 749/C and 6346/C, on a weight basis, are approximately the same, it could be predicted that the resistances of individual cells to benzylpenicillin, resting as they do on the rates at which they are capable of destroying this compound at antibiotically effective concentrations, would be approximately the same. Penicillin-sensitivity tests on single organisms (either spores or vegetative cells) inoculated into nutrient agar (200–500/plate) containing different concentrations of benzylpenicillin gave identical 'end points' (minimum concentration giving at least 50% reduction in colony counts) of 0·10 unit/ml. This is in striking contrast with the situation with the two penicillinase-constitutive mutant strains 5/P and 5/B of *B. cereus* (Pollock, 1957), whose differential rates of production of penicillinase activity are approximately the same as those of *B. licheniformis* strains 6346/C and 749/C respectively. But the sensitivities of *B. cereus* strains 5/P and 5/B to benzylpenicillin (measured by the same plate test as that described above) differ by 30-fold, being 0·10 and 3·0 units/ml. respectively (Pollock, 1957). However, in contrast with *B. licheniformis* strains 6346/C and 749/C, which may be presumed to differ in their penicillinase-structural genes, *B. cereus* strains 5/P and 5/B appear to differ by mutations in the penicillinase-regulatory gene, since their different penicillinase activities are due to purely quantitative differences in rates of formation of the same type of enzyme. This is indicated by their identical 'neutralization slopes' with antipenicillinase antiserum, the same technique being employed as that used for demonstrating the identity of enzyme from *B. cereus* strain 5/B and that from its wild-type parent strain 5 (Pollock, 1957). The observed difference in their resistance to penicillin is not therefore surprising.

This reasoning might not, of course, apply so forcibly to crowded cell populations, where large amounts of penicillinase would be formed rapidly and high concentrations of a penicillin (more nearly sufficient for enzyme saturation) might be necessary to maintain the level high enough for a long enough time to be antibiotically effective.

Yet another situation is illustrated by staphylococcal penicillinase, which, in the strain first studied by Richmond (1963), was found to have an exceptionally high affinity (K_m 2·5 μM) for benzylpenicillin but an almost negligible affinity (K_m 0·03 M) for methicillin (Novick, 1962*a*). The values for $V_{max.}$ are 40 and 1·2 units/μg. respectively. Thus, on benzylpenicillin, its physiological efficiency ($1{\cdot}6 \times 10^7$) works out to be of the same order for the *B. cereus* and *B. licheniformis* penicillinases, whereas on methicillin it is only 40. The very high effectiveness of the latter in treating infections due to penicillinase-producing staphylococci is thus, as pointed out by Novick (1962*a*), readily explained.

In one other respect the two types of penicillinase show a striking difference. Both the relative and absolute rates of hydrolysis of the cephalosporins are much higher with the enzyme from strain 6346/C. Indeed, the rate of hydrolysis of benzylcephalosporin C by penicillinase from strain 6346 is nearly 50% that of benzylpenicillin, which should qualify

this enzyme to rank as a cephalosporinase. Unfortunately, it has not yet been possible to use the micro-assay method with the cephalosporins. Their K_m values have not therefore been measured, and the physiological efficiencies of the two penicillinases for these substrates cannot be compared.

There are few characters shared by the penicillinases so far isolated from the three different species, *B. cereus* (Kogut *et al.* 1956), *Staph. aureus* (Richmond, 1963) and *B. licheniformis*, although, within a species, different types of the enzyme obtained from distinct varieties are very closely related. Indeed, apart from the absence of cyst(e)ine and a molecular weight about 30000, neither of which is a very distinctive character, there is little similarity apart from their common ability in catalytic hydrolysis of the β-lactam bond in penicillins and cephalosporins, the property on which they have been defined and selected. Their similar physiological efficiencies on benzylpenicillin may indicate nothing more than a similar environmental threat from naturally produced penicillin against which their evolutionary momenta have been pitched.

These large interspecific differences are not, perhaps, surprising in view of the relatively distant taxonomic affinities of the three species concerned. Even *B. cereus* and *B. licheniformis*, though nominally in the same genus, would not be placed very near each other by those systematists who believe in the fundamental significance of differences in DNA base ratios (see Marmur, Falkow & Mandel, 1963).

Whether or not there are some important characters such as amino acid sequence runs shared by any or all of these penicillinases, which might indicate a common evolutionary origin, is something it is hoped to determine by further chemical, immunological and genetical analysis.

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