Purification and Properties of the Exopenicillinase from *Staphylococcus aureus*

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Resistance of *Staphylococcus aureus* to benzylpenicillin in clinical practice is usually caused by the production by the bacteria of an inducible penicillinase (EC 3.5.2.6) (Barber, 1962). The penicillinase of induced exponentially growing cultures of *S. aureus* is partitioned between the cells and the culture medium, the exact distribution varying from strain to strain and depending on the growth medium and the phase of growth (Swallow & Sneath, 1962; Novick, 1962*a*, 1963). In the strains used in the experiments described below about 60 % of the enzyme activity is cell-bound at the end of exponential growth.

All attempts to produce a soluble form of the cell-bound enzyme have failed so far. The enzyme is closely associated with some insoluble part of the bacterial cell, since the purest preparations are particulate (Saz, Lowery & Jackson, 1961; M. H. Richmond, unpublished work). No systematic attempts to purify the exocellular enzyme have been reported, although Eriksen & Hansen (1954) noted that, like the penicillinases of *Bacillus cereus* 569 and 5/B (Kogut, Pollock & Tridgell, 1956; Pollock, Torriani & Tridgell, 1956), the exo-enzyme is strongly adsorbed to sintered glass.

The present paper describes a method for purification of the exocellular penicillinase produced by *S. aureus*, together with a description of some properties of the purified enzyme. The staphylococcal exo-enzyme is distinct in its properties and amino acid composition from the exopenicillinases obtained from *B. cereus* 569 and 5/B (Kogut *et al.* 1956; Pollock *et al.* 1956) and from *Bacillus subtilis* 6346 and 749 (M. R. Pollock, unpublished work).

METHODS AND MATERIALS

Organisms and media. The purification procedure has been worked out with *S. aureus* strain PC1, a constitutive strain constructed in two steps from the penicillinase-negative strain *S. aureus* NCTC 8325 (Novick, 1963). First, the penicillinase gene from the inducible strain *S. aureus* 524 SC (Rogers, 1953) was transduced to strain 8325 by means of phage 53*α* to give a new inducible strain, *S. aureus* 8325-18. Strain 8325-18 was then treated with ethyl methanesulphonate (Loveless & Howarth, 1959; Novick, 1963) to give a constitutive mutant, *S. aureus* PC1. A further inducible strain, P2, has been used in certain experiments. It was selected after treatment of strain 8325-18 with ethyl methanesulphonate. When fully induced this strain produces about one-twentieth as much penicillinase activity as does the induced parent, but has a normal induction ratio (i.e. about 30). It has been identified as a mutant of 8325-18 producing normal amounts of a penicillinase protein of one-twentieth of the normal specific activity. The quantities of penicillinase produced by these strains under different cultural conditions are shown in Table 1.

All growth experiments were carried out in 1% CY medium (Novick, 1963), which has the following composition: sodium β -glycerophosphate, 0.12*M*; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 *mM*; trace-metal solution, 0.02 ml./l.; yeast extract (Difco), 1.0% (w/v); acid-hydrolysed casein (Difco), 1.0% (w/v); glucose, 0.8% (w/v). The trace-metal solution contained: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5% (w/v); $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% (w/v); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% (w/v); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.2% (w/v); conc. HCl, 10% (w/v). For solid medium, agar (Difco) (1.5%, w/v) was added to 1% CY medium and the β -glycerophosphate was omitted until after autoclaving.

Substituted cellulose. Cellulose phosphate and carboxymethylcellulose were Whatman products. Both were in powder form, cellulose phosphate being grade P40 and carboxymethylcellulose grade C70.

Table 1. *Formation of penicillinase by various strains of Staphylococcus aureus in the late exponential growth phase*

Experimental details are given in the text. The activities are expressed as units of enzyme/mg. dry wt. of bacteria.

Strain	Uninduced penicillinase activity		Induced penicillinase activity		Approx. induction ratio
	Total	Exocellular	Total	Exocellular	
524 SC	4-6	2-3	120-150	50-70	30
8325	0	0	0	0	—
8325-18	8-10	4-6	300	120	30
PC 1	450	200	500	250	1.1
P 2	0.5	0.2	15	5-6	30

Growth of organisms. Exocellular penicillinase was prepared from cultures of strain PC1 grown overnight in 1% CY medium. The medium (1 l. of medium/5 l. conical flask) was inoculated with about 10^8 organisms/ml. to ensure that the culture was in the early stationary phase after overnight growth. When harvested the culture density was usually about 5 mg. dry wt. of bacteria/ml., the level of exocellular enzyme activity about 1000–1200 units/ml., and the pH value of the culture about 5.7.

Enzyme assays. Penicillinase activities are expressed in units similar to those defined by Pollock & Torriani (1953) for *B. cereus* penicillinase: 1 unit $\equiv$ 1 μ mole of penicillin hydrolysed/hr. at 30° and pH 5.9. Penicillinase was assayed either iodometrically at pH 5.9 by the method of Perret (1954), as modified by Novick (1962*a*), or manometrically at pH 7.0 (Henry & Housewright, 1947). A conversion factor allowed comparison of values obtained at the two pH values. The hydrolysis of cephalosporin derivatives was determined manometrically at pH 7.0. Michaelis constants were determined by the microiodometric assay (Novick, 1962*b*).

Electrophoresis in a sucrose gradient. All experiments were carried out at pH 7.0 in $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4\text{--NaCl}$ buffer, 1.0 M (Miller & Golder, 1950) with the apparatus designed by Charlwood & Gordon (1958). Between 2 and 10 mg. of protein was used in various experiments.

Amino acid analysis. The purified enzyme preparation was precipitated with acetone at -2° for 6 hr. and the precipitate collected by centrifuging at -10° . The precipitate was dissolved in water, conc. HCl was added to give a final concentration of 6*N*, and the samples were hydrolysed for 16 hr. at 105° in a sealed tube. After hydrolysis the HCl was removed *in vacuo* over NaOH and the amino acid content of the hydrolysis determined with an automatic amino acid analyser (Beckman Spinco Amino Acid Analyser; Beckman Instrument Co., Palo Alto, Calif., U.S.A.) (Spackman, Stein & Moore, 1958).

Analysis for N-terminal amino acids was carried out on 5.0 mg. of pure enzyme by the method described by Porter (1957). This bis-DNP-lysine was identified chromatographically by comparison with authentic material in 2-methylbutan-2-ol-phthalate buffer, pH 6.0 (Blackburn & Lowther, 1951) and 1.5*M*- $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$ buffer, pH 6.0 (Smith, 1960). The method for oxidation with performic acid and analysis for cysteic acid has been described by Pollock & Richmond (1962).

Preparation of anti-(exopenicillinase) serum. Sandy-lop rabbits were each injected in alternate thighs at 3-day intervals with a total of four 0.2 ml. portions of a solution of purified exopenicillinase (approx. 5.0 mg. of enzyme protein/ml. of Freund's adjuvant). After 2 weeks each rabbit was injected intravenously with three successive 0.2 ml. portions and one 0.5 ml. portion of an alum-precipitated preparation of purified enzyme (approx. 5 mg./ml.) at 2-day intervals. The animals were killed 10 days after the last intravenous injection, and serum was prepared in the normal way. Enzyme-antibody precipitates obtained with this antiserum had enhanced enzyme activity. It was found that 1.0 ml. of serum was capable of precipitating 4.8×10^4 units of enzyme.

Titration of enzyme with antiserum. (a) Measurement of total enzyme activity. A series of flasks were prepared, each containing 1.0 ml. of a solution (100 units/ml.) of the enzyme to be tested. The appropriate quantity of antiserum

was then added to each flask and the enzyme-antiserum mixture incubated for 30 min. at room temperature. Control flasks contained antiserum but no enzyme. After 30 min. standard penicillin was added to the flasks and the activity of the enzyme-antiserum mixture assayed iodometrically in the normal manner. Enzyme was added to the control flasks after the addition of iodine.

(b) Measurement of activity in antiserum precipitates. About 100 units of enzyme were placed in a series of 10 ml. conical centrifuge tubes and the appropriate amount of antiserum was added. The mixture was then incubated for 30 min. at room temperature, and 1.0 ml. of normal rabbit serum (buffered to pH 8.4 with 0.1*M*-borate buffer) added. After mixing well, sufficient saturated ammonium sulphate (adjusted to pH 8.0 with ammonia) was added to bring the final ammonium sulphate concentration to 40% of saturation. The tubes were left at room temperature for 10 min. to allow the precipitates to form, and then centrifuged at 3000*g* for 15 min. to collect the precipitate. The supernatants were decanted and the precipitates dissolved in 1.0 ml. of 0.85% sodium chloride. The precipitation with ammonium sulphate was repeated and the precipitates were once more dissolved in 1.0 ml. of 0.85% sodium chloride. The enzyme activity of this preparation was assayed iodometrically by using 0.4 ml. for the test and 0.4 ml. as control.

RESULTS

Purification of exopenicillinase

The recoveries obtained at each stage of the purification are shown in Table 2.

Stage 1. Cellulose phosphate was added (4.5 g. of powder/ 10^6 units of enzyme activity) to the whole culture obtained after overnight growth. This preparation was stirred at room temperature for about 1 hr. and the cellulose phosphate separated from the bacteria by filtration through a sintered-glass funnel (porosity 1). More than 95% of the enzyme activity was adsorbed to the cellulose phosphate under these conditions. The enzyme remained active for weeks when stored on moist cellulose phosphate at 2°.

Stage 2. The cellulose phosphate was suspended in distilled water and poured as a column (dimensions unimportant). The column was washed with distilled water (500 ml./g. of cellulose phosphate), 0.02*M*-tris-hydrochloric acid buffer, pH 7.5 (200 ml./g. of cellulose phosphate), 0.1*M*-tris-hydrochloric acid buffer, pH 7.5 (200 ml./g. of cellulose phosphate), and the enzyme was eluted as a sharp band at the solvent front in 2*M*-tris-hydrochloric acid buffer, pH 7.5. Stages 1 plus 2 gave a water-clear solution of enzyme with a specific activity of about 2–3 units/ μ g., and achieved about 100-fold concentration of enzyme protein. The solution of enzyme in 2*M*-tris-hydrochloric acid buffer, pH 7.5, is stable for periods of weeks when stored at -2° .

Stage 3. When the enzyme solution in 2*M*-tris-hydrochloric acid buffer, pH 7.5, is dialysed to

Table 2. *Summary of the purification of staphylococcal exopenicillinase*

Experimental details are given in the text. The starting material was 4 l. of culture containing 970 units/ml. of supernatant (total, 3.88×10^6 units).

Stage no.	Procedure	Enzyme activity recovered (units)	Specific enzyme activity (units/ μ g. of enzyme protein)	Recovery (%)	
				Per stage	Overall
1	Adsorption to cellulose phosphate	3.66×10^6	—	94.3	94.3
2	Elution from cellulose phosphate with 2M-tris-HCl, pH 7.5	3.3×10^6	2.7	90.1	85.1
3	Adsorption to carboxymethylcellulose	3.0×10^6 (by difference)	—	90.9	77.3
4	Chromatography on carboxymethylcellulose	1.6×10^6	28	53.3	41.2
5	Centrifuging in sucrose gradient	1.47×10^6	35	91.8	37.9
6	Electrophoresis in sucrose gradient	0.85×10^6	38–40	57.8	23.2
7	Rechromatography on carboxymethylcellulose	0.52×10^6	40	61.1	14.2

equilibrium against distilled water or solutions of low ionic strength, precipitation of the enzyme occurs, and losses of enzyme may be heavy. To minimize losses, the enzyme solution in 2M-tris-hydrochloric acid buffer, pH 7.5, is dialysed in the presence of carboxymethylcellulose. Under these conditions the enzyme tends to adsorb on to the carboxymethylcellulose rather than to precipitate. Carboxymethylcellulose (4 g./ 10^6 units of enzyme) was added to the solution of enzyme in 2M-tris-hydrochloric acid buffer, pH 7.5, and the whole preparation dialysed at 2° against two batches of 100 vol. of distilled water. The carboxymethylcellulose was removed from the solution by filtration under gravity, washed with distilled water (500 ml./g. of carboxymethylcellulose) and collected.

Stage 4: Chromatography on carboxymethylcellulose. The carboxymethylcellulose collected in the previous stage was loaded on top of a carboxymethylcellulose column prepared as follows: fresh carboxymethylcellulose (2 g./ 10^6 units of enzyme already adsorbed in stage 3) was washed in 0.1M-sodium acetate buffer, pH 5.9, poured into a column (length:diameter ratio, 2:3), and washed with 500 ml. of 0.01M-sodium acetate buffer, pH 5.9. The carboxymethylcellulose containing the enzyme was then applied to the top of the column as a slurry in water and washed with about 500 ml. more of water, followed by a linear concentration gradient of sodium acetate buffer, pH 5.9. A typical example of the gradient used for a column 15 cm. long $\times$ 1.2 cm. diam. was as follows: in the concentrated buffer vessel, 200 ml. of 0.4M-sodium acetate buffer, pH 5.9; in the mixer, 200 ml. of 0.02M-sodium acetate buffer, pH 5.9. Fig. 1 shows a typical elution diagram obtained with this arrangement. The enzyme was eluted as a band at a buffer concentration of about 0.06M. The specific enzyme

Fig. 1. Chromatography of staphylococcal penicillinase on a column of carboxymethylcellulose. The elution gradient was a linear concentration gradient of sodium acetate-acetic acid buffer, pH 5.9. Experimental details are given in the text. ●, Enzyme activity; +, $E_{280}^{1\text{ cm}}$. Tubes 16–23 (inclusive) were pooled (see horizontal bar). Tube 1 was turbid.

activity of each fraction eluted from the column was determined, and fractions having a specific activity higher than 30 units/ μ g. of protein were pooled. This preparation was dialysed against 0.1M-sodium acetate buffer, pH 5.9, freeze-dried to about 3–5 ml. and redialysed against more 0.1M-sodium acetate buffer, pH 5.9. At this stage the preparation had a specific enzyme activity of about 30 units/ μ g.

Stages 3 and 4 are the least satisfactory steps in the purification procedure since they give a variable overall yield. In eleven experiments the minimum yield for stages 3 and 4 has been 19% (average 38%).

Stage 5: Centrifuging in a sucrose gradient. The preparation obtained at the end of stage 4 was loaded on top of a sucrose gradient for centrifuging in the Spinco model L centrifuge. The gradient was

made by pipetting 3.8 ml. each of 10% (w/v), 15% (w/v) and 20% (w/v) solutions of sucrose in 0.1 M-sodium acetate buffer, pH 5.9 (in that order), into a 13.0 ml. plastic tube, the denser sucrose fractions being delivered beneath the less dense by using a long catheter tube. The gradient was allowed to stand at room temperature for at least 8 hr. for the boundaries between the layers to diffuse, and then the sample to be centrifuged (1.0 ml. of preparation from stage 4 containing up to 30 mg. of protein) was carefully layered on top. The samples were centrifuged at room temperature for 16 hr. at 40 000 rev./min. in the Spinco no. 40 rotor. At the end of this period, the gradient was dripped out as about twenty separate fractions by puncturing the bottom of the plastic tube, and the specific activity of the enzyme in each fraction was determined. Fig. 2 shows the distribution of penicillinase in the gradient. Fractions containing enzyme of specific activity higher than 30 units/ μ g. were pooled and dialysed exhaustively against 0.1 M-disodium hydrogen phosphate-sodium dihydrogen phosphate buffer, pH 7.2, to remove the sucrose. The enzyme at this stage of purification had a specific enzyme activity of about 35 units/ μ g.

Stage 6: Electrophoresis in a sucrose gradient. The total volume of the enzyme preparation obtained at the end of stage 5 was adjusted to about 4.0 ml. and this was dialysed to equilibrium against the electrophoresis buffer (see the Methods and Materials section). The enzyme solution was then loaded into the sucrose-gradient electrophoresis apparatus and separated for 42 hr. at room temperature. The enzyme moved towards the cathode at this pH value, and a typical distribution of the enzyme in the gradient is shown in Fig. 3. Determinations of specific activity showed that the main enzyme peak was essentially homogeneous with a specific enzyme activity of about 40 units/ μ g. The samples with specific enzyme activity greater than 35 units/ μ g. were pooled, dialysed extensively to remove the sucrose, and concentrated by freeze-drying. The homogeneity of this material was checked by chromatography on a column of carboxymethylcellulose under the conditions set out in stage 4. About 95% of the enzyme activity was recovered in twelve fractions, which all had the same specific enzyme activity $\pm 15\%$. This material has been used for the experiments on the properties of the enzyme reported below.

Properties of exopenicillinase

Molecular weight. The molecular weight of the purified enzyme was found by the Archibald method (Schachman, 1959) to be $29\,600 \pm 5\%$, when a value of 0.746 for the partial specific volume, $\bar{v}$, was used in the calculation. This value for $\bar{v}$ was obtained from the amino acid analysis of the protein

Fig. 2. Distribution of staphylococcal penicillinase after centrifuging at 105 000 g for 16 hr. in a sucrose gradient. Nineteen samples were taken in all. Experimental details are given in the text. ●, Enzyme activity; +, $E_{280}^{1\text{ cm.}}$. Tubes 11-14 (inclusive) were pooled (see horizontal bar).

Fig. 3. Distribution of staphylococcal penicillinase after electrophoresis in a sucrose gradient at pH 7.0. Experimental details are given in the text. ●, Enzyme activity; +, $E_{280}^{1\text{ cm.}}$. Tubes 24-26 (inclusive) were pooled (see horizontal bar). Tube 1 always contained traces of enzyme arising by contamination on filling the apparatus (see Charlwood & Gordon, 1958). The broken line shows the extinction due to non-specific ultraviolet absorption in the sucrose of the gradient (determined from a dummy run).

(see below). The enzyme had $S_{20,w}$ of 2.62s at a concentration of 5.5 mg./ml. in 0.02M-disodium hydrogen phosphate-potassium dihydrogen phosphate buffer, pH 7.5, containing sodium chloride (0.05M). These values suggest an approximately spherical molecule.

Amino acid composition. Examination by a spectrophotometric method (Beaven & Holiday, 1952) showed that the tyrosine:tryptophan molar ratio in the enzyme molecule was 5.5. Total amino acid analyses were carried out on three separate batches of purified enzyme precipitated and hydrolysed as described in the Methods and Materials section (Table 3). The number of residues of each amino acid was calculated on the basis of a molecular weight of 29 600 and 2 residues of tryptophan/mole (inferred from the tyrosine:tryptophan ratio and an approximate estimate of the tyrosine content of the protein). Only one set of hydrolysis conditions was used, and the values obtained for serine and threonine may, therefore, be low by a factor of up to 10% (Wallenfels & Ahrens, 1960). The ammonia value comprises ammonia arising from amide groups of glutamine and asparagine, from tryptophan (which breaks down completely on acid hydrolysis) and traces from the decomposed serine and threonine. No breakdown products of cysteine were found under these conditions of hydrolysis, and examination of hydrolysates of the enzyme after oxidation with performic acid showed that the cysteic acid content of the purest penicillinase preparation corresponded

to about 1 mole of cysteic acid/65 000 g. of protein. This suggests that this enzyme, like the exopenicillinases from *B. cereus* 569 and 5/B, contains no cysteine and therefore no disulphide bridges (Pollock & Richmond, 1962). The inducible exopenicillinase synthesized by strain 524 SC was purified by the same method, and gave an amino acid analysis indistinguishable from that given by the constitutive enzyme when the inaccuracy of the method is taken into account (Table 3).

Analysis of two batches of constitutive enzyme for *N*-terminal amino acids, by using 1-fluoro-2,4-dinitrobenzene followed by acid hydrolysis, gave bis-DNP-lysine as the sole DNP-amino acid apart from ϵ -DNP-lysine. If the absence of *N*-terminal tryptophan is assumed, the enzyme contains only a single polypeptide chain. This result is consistent with the absence of disulphide bridges.

The protein is notable for the high abundance of lysine and aspartic acid, since these two amino acids account for about 41% of the weight of the protein. If the ammonia content of the hydrolysate is assumed to represent the maximum amount of amide ammonia in the molecule, a balance sheet of possible acid and basic groups at neutral pH values shows the protein to have a net basic charge. This agrees with the cathodic mobility shown on electrophoresis (see stage 6 of the purification).

Total nitrogen content. The nitrogen content of three carefully dried and weighed samples of purified enzyme (1.13, 0.53 and 0.48 mg.) was measured by the 1,2,3-indanetrione hydrate (ninhydrin) method (Jacobs, 1960, 1962). The nitrogen contents of the three samples (16.1, 16.3 and 16.7%) gave an average value of 16.4% by weight. The total nitrogen content of the protein calculated on the amino acid analysis is 16.9%. The lower value obtained in practice could be due to the presence of traces of water in the protein samples.

A solution containing 1.0 mg. of enzyme/ml. had an extinction at 280 m μ of 1.21. Thus the value for $E_{280}^{1\text{cm.}}/\text{mg. of N}$ is 7.38.

Substrate specificity and Michaelis constants. The purified enzyme was tested for its kinetics of hydrolysis of several penicillins (Table 4). In all cases the maximum velocity and the Michaelis constants were close to the values reported by Novick (1962a) with the crude exocellular enzyme. The purified enzyme also hydrolysed cephalosporin C at about 0.5–0.6% of the rate against benzylpenicillin. This property is discussed below, in relation to the 'cephalosporinase' activity of staphylococcal cultures. Gourevitch, Pursiano & Lein (1962) have reported that the cell-bound penicillinase of *S. aureus* is inhibited by methicillin. Tests with the purified exo-enzyme from strain PC1 showed that the enzyme degraded more than 85% of added methicillin (over a period of 90 min.). The addition

Table 3. *Amino acid composition of purified exopenicillinase from various strains of Staphylococcus aureus*

Experimental details are given in the text. All results are quoted as residues/mole.

Amino acid	Strain PC1 (constitutive)	Strain 524 SC (inducible)
Lys	44	44
NH ₃	(29)	(33)
Arg	4	5
His	2	3
Asp	42	43
Thr	13	14
Ser	19	20
Glu	17	20
Pro	10	10
Gly	13	15
Ala	20	19
Val	14	14
Met	2	2
Ileu	18	17
Leu	23	24
Tyr	12	12
Phe	7	7
Try	2	2
Mol. wt.	29 600	29 600
<i>N</i> -Terminal amino acid ...	Lys	?

of further methicillin at this point showed that the catalytic activity of the enzyme was unimpaired.

pH-activity curve. The pH-activity curve of the pure enzyme was determined over the range pH 4.0–9.0 (Fig. 4). The enzyme has an optimum at about pH 5.9 in 0.1 M-disodium hydrogen phosphate-sodium dihydrogen phosphate buffer, but similar activities were also achieved at pH 7.0 in 0.1 M-tris-hydrochloric acid buffer, whereas at pH 7.0 in phosphate buffer the enzyme activity was about 60 % of that achieved in tris-hydrochloric acid buffer. The enzyme is remarkable for the sharp fall in activity at alkaline pH values.

Adsorption to surfaces. The purified staphylococcal enzyme adsorbs strongly on to sintered or scratched glass, sand, bentonite and kieselguhr. Attempts to elute the enzyme in concentrated salt solutions at pH 8.5 (Kogut *et al.* 1956) or at low pH values achieved recoveries of 10 % or less. The staphylococcal enzyme is therefore more strongly bound to negatively charged particles than the exopenicillinases from *B. cereus* 569 and 5/B, and this is presumably associated with the predominance of basic groups in the staphylococcal enzyme at physiological pH values.

Inhibitors. The activity of the purified enzyme was unaffected by iodoacetic acid (1 mM), *p*-chloromercuribenzoate (1 mM) or mercuric chloride (1 mM). There was no evidence for an 'activated' histidine in the active centre of the enzyme since treatment with iodoacetic acid, under conditions in which carboxymethylate 'activated' histidine, had no effect on staphylococcal penicillinase (Grundlach, Stein & Moore, 1959). Enzyme activity was also unaffected by EDTA (sodium salt) (5 mM) or oxine (1 mM). Batchelor, Cameron-Wood, Chain &

Rolinson (1961) have reported that crude preparations of exocellular staphylococcal penicillinase can be potentiated by Mg^{2+} ions. Maximum stimulation occurred at a concentration of 0.01 M, but above this value inhibition occurred. The purified enzyme from strain PC1 was unaffected by the presence of magnesium sulphate up to a concentration of 0.01 M, and at a concentration of 0.05 M the enzyme was 10 % inhibited.

A solution of 1.0 mg. of staphylococcal penicillinase/ml. of 0.1 M-disodium hydrogen phosphate-potassium dihydrogen phosphate buffer, pH 7.5, was 30 % inactivated by treatment for 30 min. with 10 μ g. of trypsin/ml. at 30°. In this respect the staphylococcal exo-enzymes differed from the exopenicillinase from *B. subtilis*, which is almost completely resistant to the action of trypsin (Kushner & Pollock, 1961).

Ionic strength. Crude preparations of the enzyme (e.g. stage 2) are precipitated when the ionic strength of the solution falls below about 0.02. A precipitate formed in this way can be partially redissolved by increasing the ionic strength. Tris, NH_4^+ , Na^+ , K^+ , Mg^{2+} , SO_4^{2-} , Cl^- and HPO_4^{2-} ions seem equally effective. Recoveries of activity after precipitation and solution are usually 40–60 %. The

Table 4. *Kinetics of hydrolysis of various penicillin and cephalosporin derivatives by purified staphylococcal exopenicillinase*

Experimental details are given in the text. All compounds were measured at saturating concentrations of substrate except methicillin and methylphenylisoxazolylpenicillin, which were measured from the initial rate of hydrolysis of the substrate at a concentration of 0.01 M. V_{max} values are expressed as μ moles of substrate destroyed/mole of enzyme/min. at 30°.

Substrate	V_{max} (turnover no.)	K_m (μ M)
Benzylpenicillin	2×10^4	5
Phenoxyethylpenicillin	2×10^4	7
Phenoxyethylpenicillin	1.8×10^4	10
6-(2,6-Dimethoxybenzamido)- penicillanic acid (methicillin)	6×10^2	$\sim 10\ 000$
Methylphenylisoxazolyl- penicillin (BRL 1400)	1.4×10^3	$\sim 5\ 000$
6-Aminopenicillanic acid	2×10^3	—
Cephalosporin C	1×10^3	—
Benzylcephalosporin (cephalosporin G)	1×10^3	—

Fig. 4. pH-activity curve for purified staphylococcal penicillinase in: ●, 0.1 M-sodium acetate-acetic acid buffer; +, 0.1 M-sodium citrate buffer; ▲, 0.1 M- Na_2HPO_4 - KH_2PO_4 buffer; ■, 0.1 M-tris-HCl buffer. Experimental details are given in the text.

purified enzyme is not precipitated in this way, and it seems likely that there is another protein in the crude preparations which co-precipitates with the penicillinase in solutions of low ionic strength.

Inhibition by iodine plus potassium iodide. Separate samples of purified staphylococcal penicillinase were incubated for 10 min. at 30° in 0.1 M-disodium hydrogen phosphate-potassium dihydrogen phosphate buffer, pH 7.0, containing various concentrations of iodine plus potassium iodide. The residual enzyme activity found after this treatment was plotted logarithmically against the (iodine plus potassium iodide) concentration used (Fig. 5). Treatment of the enzyme for 10 min. with 0.01 N-iodine plus 0.04 M-potassium iodide caused a 94 % inhibition. The degree of inhibition obtained with iodine is approximately proportional to $\log [I_2 + KI]$ over a 100-fold concentration range. The sensitivity of the staphylococcal enzyme to iodine plus potassium iodide is in marked contrast with the enzyme from *B. cereus*, which is insensitive to iodine, at least when benzylpenicillin is used as substrate (Citri, 1958; Garber & Citri, 1962).

Fig. 5. Inhibition of purified staphylococcal penicillinase by iodine plus potassium iodide solution. Experimental details are given in the text.

'Cephalosporinase' activity of staphylococcal cultures

Exponentially growing cultures of the constitutive strain PC1 liberate into the growth medium an enzyme capable of hydrolysing the β -lactam bond of cephalosporin C. This activity is not measurable in uninduced inducible cultures, but appears in the growth medium simultaneously with penicillinase activity on induction either with a penicillin or cephalosporin C. Crompton, Jago, Crawford, Newton & Abraham (1962) have shown that cephalosporin C is a competitive inhibitor of *S. aureus* penicillinase when benzylpenicillin is used as substrate. This strongly suggests that a penicillinase with an active centre capable of reacting both with cephalosporin C and benzylpenicillin is produced by *S. aureus*. It is not clear, however, whether all of the cephalosporinase activity of the culture rests solely with the penicillinase molecule or whether there is another enzyme present, with a predominant cephalosporinase activity. This cephalosporinase, if it occurred, might be co-induced with the penicillinase.

Examination of exponentially growing cultures of strain PC1 showed that both the total and exocellular cephalosporinase activities were about 0.5 % of the equivalent penicillinase activity at a culture density of 3.2 mg. dry wt. of bacteria/ml. The relative cephalosporinase activity of purified enzyme was 0.53 % of the penicillinase activity, and this suggests that penicillinase is responsible for all of the cephalosporinase activity in strain PC1. Other evidence supports this conclusion: (1) strain PC1 is a mutant that synthesizes penicillinase constitutively and 'cephalosporinase' activity is also constitutive in this strain; (2) in a mutant (strain P2) that produces normal quantities of a protein with specific enzyme activity one-twentieth of normal, the cephalosporinase activity is decreased to one-seventeenth of the normal. It is virtually certain, therefore, that staphylococcal penicillinase has a subsidiary cephalosporinase action and that this is the only 'cephalosporinase' produced by these strains.

Immunological properties

Antiserum to the staphylococcal exopenicillinase precipitated the enzyme, but, unlike the exopenicillinase from *B. cereus*, this precipitated enzyme had a higher activity than the free enzyme in solution. Staphylococcal exopenicillinase was titrated with increasing quantities of antibody (constant antigen titration), and the enzyme activity of the total preparation was measured (Fig. 6). With increasing quantities of antiserum the activity in the preparation increased to a maximum of about four times that found in the absence of serum. At higher antibody concentra-

Fig. 6. Reaction of purified staphylococcal exopenicillinase with antipenicillinase serum. Experimental details are given in the text. ●, Effect on total activity in the preparation; +, quantity of enzyme precipitated. E, Equivalence point.

tions the level of enzyme activity sometimes decreased, but never lower than a net threefold increase over the value obtained for enzyme in the absence of antiserum. Analysis of the precipitation reaction by collecting the enzyme precipitated with the antiserum under these conditions (see the Methods and Materials section) shows (Fig. 6) that there is a linear relationship between the quantity of activity precipitated and the quantity of antiserum added. At the equivalence point (E in Fig. 6) the activity precipitated is equivalent to four times the enzyme activity measured without antiserum. This phenomenon of stimulation of an enzyme by antiserum is qualitatively similar to the behaviour of some anti-exopenicillinase preparations made by Dr M. R. Pollock against pure exopenicillinase from *B. subtilis* strain 749.

SUMMARY

1. A method for the purification of staphylococcal exopenicillinase is described.
2. The enzyme has a molecular weight of 29 600.
3. Analysis of the amino acid content of the pure enzyme revealed that the molecule contains no cysteine and has lysine as the *N*-terminal amino acid.
4. The purified exopenicillinase has sufficient 'cephalosporinase' activity to account for all the 'cephalosporinase' found in culture filtrates of *S. aureus* strains PC1, 524 and 8325-18.

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