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Wild-Type Variants of Exopenicillinase from *Staphylococcus aureus*

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1. Three variants of staphylococcal exopenicillinase (types A, B and C) can be distinguished on chemical, enzymological and immunological grounds. 2. Enzyme type A has a higher specific activity than that of type B, but has a similar combination affinity with anti-(exopenicillinase type A) serum. 3. Enzyme types A and C have a similar specific activity, but enzyme type C has a lower combination affinity for anti-(exopenicillinase type A) serum than has enzyme type A. 4. The sedimentation coefficients and amino acid analyses of the three enzyme types are similar. 5. All three enzyme types have small but significant differences in kinetics of action when hydrolysing benzylpenicillin, methicillin, cloxacillin and cephalosporin C. 6. Peptide maps, obtained from enzyme types A and C after digestion with trypsin, show that these two variants probably differ in the nature of only a very few amino acid residues. 7. Enzyme type B seems to be confined to staphylococci that are members of staphylococcal phage group II. Enzyme types A and C are produced by staphylococci that are members either of phage group I or III, but never group II. 8. The low specific enzyme activity and affinity of enzyme type B towards all penicillins tested suggest that this enzyme type has a lower 'efficiency' in hydrolysing penicillin and therefore in protecting bacteria from the action of penicillin. This could account for the low incidence among 'hospital staphylococci' of penicillin-resistant staphylococci that are members of phage group II.

Both *Bacillus cereus* and *Bacillus licheniformis* each produce at least two penicillinase (β-lactamase, EC 3.5.2.6) variants that may be distinguished on chemical, enzymological and immunological grounds (Pollock, 1960, 1964, 1965). The purification of the exopenicillinase from *Staphylococcus aureus* (Richmond, 1963*a*) and the preparation of specific sera against this protein have allowed a search to be made for exopenicillinase variants in *S. aureus*. So far three variants of staphylococcal exopenicillinase have been distinguished on the basis of enzymic and immunological tests, and these enzymes are referred to below as exopenicillinase types A, B and C. Examination of the properties of these enzymes has shown that enzyme types A and C have a similar specific enzyme activity, but that exopenicillinase type A has a higher combining affinity for anti-(exopenicillinase type A) serum than has enzyme type C. Enzyme type B, on the other hand, has a lower specific enzyme activity than those of enzyme types A and C but an affinity for anti-(exopenicillinase type A) serum similar to that of enzyme type A.

A survey of the distribution of the exopenicillinase types among some of the strains examined previously for quantitative penicillinase production (Richmond, Parker, Jevons & John, 1964) has shown that enzyme type B seems always to be associated with staphylococci that are members of phage group II, whereas enzyme types A and C are found both in phage groups I and III. In view of the low specific activity and high K_m of exopenicillinase type B and its distribution among phage group II strains, it is possible that the low incidence of penicillin-resistant group II strains among 'hospital staphylococci' (Anderson & Williams, 1956; Munch-Petersen & Boundy, 1962) is related to the relative inefficiency of the exopenicillinase associated with these strains.

METHODS AND MATERIALS

Organisms and media. The 58 strains used in these experiments were selected from the 186 examined for quantitative penicillinase production under standard conditions of induction (Richmond *et al.* 1964). Strain 524SC (Rogers, 1953) and strain PC1 (Novick, 1962*a*, 1963; Richmond, 1963*a*) were used as a source of enzyme type A, enzyme type B was prepared from strain 12865/62, and enzyme type C was prepared from strain 364/62 (the last-named two strains being obtained from the Cross-Infection Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W.9). All the cultures were grown for experimental purposes in 1% CY medium (Novick, 1962*a*).

Phage typing. All phage patterns were determined by the

Cross-Infection Reference Laboratory, Central Public Health Laboratory, London, N.W.9, by the method of Blair & Williams (1961).

Exopenicillinase preparations. Normally, the exopenicillinase preparations used for titration with antiserum were culture supernatants obtained by centrifuging off bacteria from cultures 3½ hr. after induction (see Richmond *et al.* 1964). The cultures usually contained about 2·0 mg. dry wt. of bacteria/ml. at this point. Culture supernatants (referred to below as 'crude enzyme') from group I or group III strains usually contained about 400–600 units of penicillinase activity/ml. Crude enzyme preparations from group II strains, however, rarely contained more than 70 units of penicillinase/ml.

'Purified enzyme' consisted of an enzyme preparation taken to the end of stage 2 of the purification procedure (Richmond, 1963*a*) and which had, in addition, been dialysed against 0·1 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0. Preparations of this sort from group I or group III strains contained at least 40000 units of penicillinase/ml. (about 1·0 mg. of enzyme protein/ml.); from group II strains the preparations contained about 5000 units/ml. (about 0·8 mg. of enzyme protein/ml.).

Antiserum preparations. Only antiserum prepared against purified enzyme for strain 524SC (enzyme type A) was used. It was prepared as described by Richmond (1963*a*).

Antiserum titrations. The reaction of enzyme with antiserum has been followed either (1) by measuring the total enzyme activity of the preparation in the presence of various amounts of antiserum, or (2) by measuring the activity of enzyme–antiserum precipitates. The procedures followed for both these titrations are given by Richmond (1963*a*) and all titrations were carried out with benzylpenicillin. The term 'combination titre' is used to measure the quantity of enzyme precipitated/ml. of antiserum in the presence of excess of enzymes and of 'carrier' serum to aid precipitation of the antigen–antibody complex with $(NH_4)_2SO_4$. Precipitation of enzyme–antibody complexes with 'carrier' gives a higher value for the titre of the serum than precipitation in the absence of 'carrier', and therefore the term 'combination titre' is preferred to 'precipitation titre' in describing these experiments.

Enzyme assays. Penicillinase activity is expressed in units similar to those defined by Pollock & Torriani (1953): 1 unit is that amount of enzyme which will hydrolyse 1 μmole of benzylpenicillin/hr. at pH5·9 and 30°. The enzyme was assayed iodometrically at pH 5·9 by the method of Perret (1954), as modified by Novick (1962*a*). 'Physiological efficiency' is measured as $K_m/V_{max.}$, as defined by Pollock (1964, 1965).

Determination of sedimentation coefficients. Sedimentation coefficients were determined by centrifugation of protein samples in a sucrose gradient in the presence of marker quantities of ^{131}I-labelled serum albumin ($S_{20,w}$ 4·2S) and ^{125}I-labelled lysozyme ($S_{20,w}$ 1·9S) (Charlwood, 1963). The gradient was made by pipetting 3·8 ml. each of 10% (w/v), 15% (w/v) and 20% (w/v) sucrose solutions in 0·1 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·1, to the bottom of a 13·0 ml. plastic tube, the denser layers being delivered below the less dense with a long catheter tube. The gradient was kept at room temperature for at least 8 hr. to allow the boundaries between the layers to diffuse, and 1·0 ml. of protein solution (containing about 20 μg. of protein in 0·1 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·1) layered carefully on top. The tubes were centrifuged for 16 hr. at 40000 rev./min. in the Spinco model L centrifuge (no. 40 rotor) at 18°. At the end of this period the bottom of the tube was punctured and the contents were allowed to drip out (Syzbalski, 1960) to form about 20 fractions. The penicillinase content of each tube was measured enzymatically after dilution, and the ^{125}I- and ^{131}I-labelled proteins were counted as described by Charlwood (1963).

The ^{125}I-labelled lysozyme and ^{131}I-labelled serum albumin were prepared by Dr Mary Hamilton, as described by McFarlane (1958).

Amino acid analysis. The purified enzyme preparation was precipitated with acetone at −2° for 6 hr. and the precipitate collected by centrifugation at −10°. The precipitate was then dissolved in water, conc. HCl was added to give a final concentration of 6 N and the samples were hydrolysed 16 hr. at 105° in sealed tubes. The amino acid content of the hydrolysate was determined on samples containing approx. 4 mg. of protein with an automatic amino acid analyser (Beckman Spinco Amino Acid Analyzer; Beckman Instrument Co., Palo Alto, Calif., U.S.A.) (Spackman, Stein & Moore, 1958).

Analysis for *N*-terminal amino acids was carried out on 3–5 mg. of pure enzyme after treatment with 1-fluoro-2,4-dinitrobenzene (Porter, 1957) or after reaction with KCNO (Stark & Smyth, 1963). Cysteine was estimated as cysteic acid after oxidation of the protein by the method described by Pollock & Richmond (1962).

Preparation of peptide maps. (*a*) Digestion with trypsin. Samples of purified enzyme (5–10 mg.) were dissolved in 1·0 ml. of 1% (w/v) NH_4HCO_3, pH 8·4, and trypsin solution was added to give a final trypsin content equal to 2% (by wt.) of the enzyme protein. The concentration of the trypsin solution was adjusted so that the required amount could be added in 0·05–0·1 ml. Digestion was carried out at 37° for 45 min. and the reaction stopped by freezing and freeze-drying. The freeze-dried hydrolysate was dissolved in a minimum of water.

(*b*) Separation of hydrolysate. A high-voltage electrophoresis apparatus of the 'cooled-plate' type (Werner & Westphal, 1955) was used. About 2·0 mg. of hydrolysate (in 25 μl. or less) was loaded on to paper that had already been soaked in electrophoresis buffer and allowed to equilibrate on the electrophoresis plate for 15 min. without the current flowing. Separation of the hydrolysate to form the peptide map was carried out first by electrophoresis in pyridine–acetic acid–*n*-butanol–water (1:1:3:35, by vol.), pH 4·7 (Dintzis, 1961), for 5 hr. at 30 v/cm. and 100 mA. After drying, additional paper was stapled to the top edge of the electrophoresis strip and the peptides were separated by ascending chromatography, overnight, in *n*-butanol–acetic acid–water (63:27:10, by vol.; upper phase) (Richmond, 1963*b*). The map was developed first by spraying with ninhydrin (0·2% in water-saturated *n*-butanol). Thereafter peptides containing tyrosine were detected by reaction with α-nitroso-β-naphthol (Thomas, 1944), and those containing arginine by the Sakaguchi reagent as described by Smith (1960).

Penicillins. Methicillin [6-(2,6-dimethoxybenzamido)-penicillanic acid]; oxacillin [6-(3-phenyl-5-methyl-4-isoxazolylamido)penicillanic acid] and cloxacillin [6-(3-*o*-chlorophenyl-5-methyl-4-isoxazolylamido) penicillanic acid] were gifts from Beecham Research Laboratories, Betchworth Park, Surrey. Quinacillin [6-(3-carboxy-2-quin-

oxalinylamido)penicillanic acid] (Richards, Housely & Spooner, 1963) was a gift from Boots Pure Drug Co., Nottingham. Cephalosporin C [7-(4-amino-4-carboxybutyl) cephalosporanic acid] was a gift from Glaxo Laboratories Ltd., Greenford, Middlesex.

RESULTS

Enzyme purification

All three enzymes were purified by the method described by Richmond (1963*a*), and Table 1 shows the purification and yield obtained at each stage. Enzyme type C proved reasonably easy to purify, but enzyme type B was difficult and recoveries were poor. It is not yet clear whether the poor recoveries of enzyme type B are due to the properties of the enzyme or the quantity of enzyme protein available. In all enzyme preparations, the material obtained at the end of stage 7 was homogeneous when examined in the ultracentrifuge. Enzyme type A had a specific activity close to 40 units/μg. of protein, whereas the highest specific activity of enzyme type C was 37 units/μg. Three batches of enzyme type B were purified, and specific enzyme activities of 5·8, 4·8 and 6·2 (mean 5·6) units/μg. of protein were found. In the immunological work described below, values of 40, 6·0 and 40 units/μg. of protein are used respectively in calculations involving the specific activities of enzyme types A, B and C.

Immunological properties

Total activity measurements. Preparations of purified exopenicillinase of each type were titrated with antiserum by measuring the total activities of the preparations in the presence of various amounts of serum. The results of this experiment have been plotted in terms of 'percentage of initial enzyme activity' against the 'quantity of antiserum added/μg. of enzyme protein' (Fig. 1). This method of plotting has the advantage that it discounts the appreciable difference in specific activity of enzymes types A and C on the one hand and that of enzymes type B on the other. Also, identical values for the equivalence point of different enzyme preparations in a plot of this type strongly suggests identity of the groups in the enzymes entering into reaction with the antiserum for a discussion of this point (see Pollock, 1964). Fig. 1 shows that addition of increasing quantities of serum to enzyme type A led to an increase in activity of the preparation until a point about 4-fold the activity of the initial preparation was reached. Thereafter the addition of more serum caused a decrease in activity, but it never fell below a net 3-fold stimulation. Enzyme type B was also stimulated by serum, but in this case the increase amounted only to about a net 1·5-fold. Enzyme type C was not stimulated at all, and in some experiments a neutralization of up to 10% of the initial activity was obtained. Examination of the preparations of enzymes type A and B showed that these enzymes have very similar equivalence points (E_A and E_B in Fig. 1). These results suggest that the same groups in enzyme types A and B are involved in the reaction with anti-(exopenicillinase type A) serum. It was not possible to determine an equivalence point with enzyme type C from an experiment of this kind since the antiserum had so little effect on the total activity of the preparation.

Table 1. *Summary of the purification of staphylococcal exopenicillinase types A, B and C*

Values for enzyme type A are taken from Richmond (1963*a*).

Stage no.	Procedure	Enzyme activity recovered (units)			Specific activity (units/μg. of enzyme protein)			Recovery overall (%)		
		Type A	Type B	Type C	Type A	Type B	Type C	Type A	Type B	Type C
	Initial preparation	$3·9\times10^6$	$3·5\times10^5$	$1·8\times10^6$				100	100	100
1	Adsorption on cellulose phosphate	$3·7\times10^6$	$3·2\times10^5$	$1·7\times10^6$				94·3	92·0	97·0
2	Elution from cellulose phosphate	$3·3\times10^6$	$2·5\times10^5$	$1·5\times10^6$	2·7	0·3	1·8	85·1	71·1	86·0
3	Adsorption on CM-cellulose (by difference)	$3·0\times10^6$	$2·0\times10^5$	$1·1\times10^6$	—	—	—	77·3	57·7	61·1
4	Chromatography on CM-cellulose	$1·6\times10^6$	$6·2\times10^4$	$5·6\times10^5$	28	2·1	24	41·2	17·8	31·1
5	Centrifugation in sucrose gradient	$1·47\times10^6$	$5·3\times10^4$	$5·2\times10^5$	35	3·8	31	37·9	15·1	28·7
6	Electrophoresis in sucrose gradient	$8·5\times10^5$	$3·5\times10^4$	$3·5\times10^5$	38	5·8	35	23·2	10·0	19·8
7	Rechromatography on CM-cellulose	$5·2\times10^5$	$1·5\times10^4$	$1·5\times10^5$	40	6·2	37	14·2	4·2	8·8

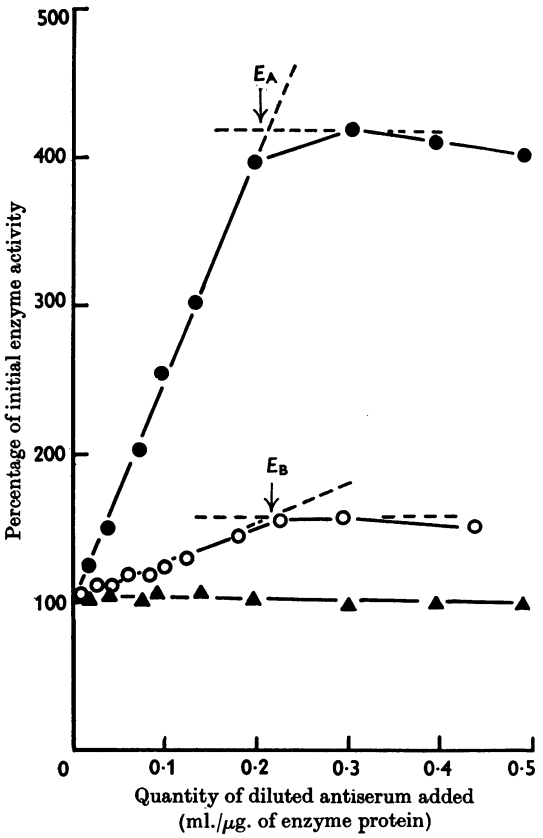

Fig. 1. Stimulation of enzyme types A (●), B (○) and C (▲) by anti-(exopenicillinase type A) serum. Initial enzyme activities were: enzyme type A, 25 units; enzyme type B, 10 units; enzyme type C, 25 units. The equivalence points for enzyme types A and B are denoted E_A and E_B respectively. Neat antiserum was diluted 1:10 with 0·9% NaCl before use.

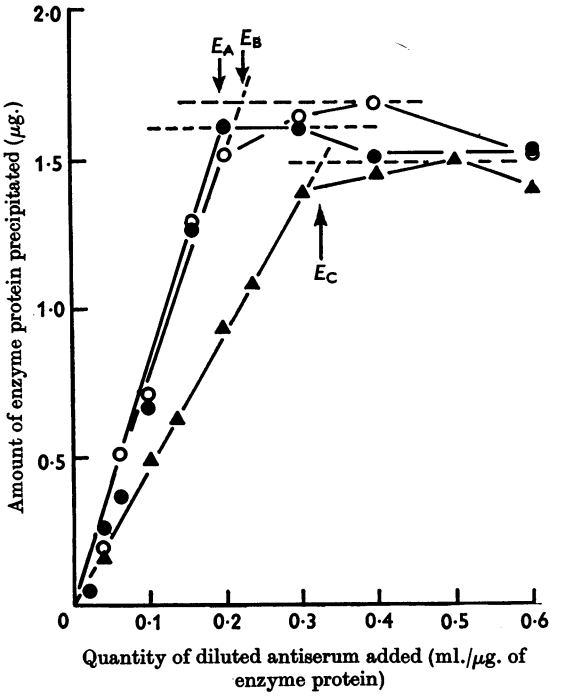

Fig. 2. Comparison of the precipitations of enzyme types A, B and C by anti-(exopenicillinase type A) serum. Carrier normal serum was used to collect the penicillinase–anti-penicillinase complex (see the Methods and Materials section). About 1·5 μg. of each enzyme type was used in the test. The equivalence points for enzyme types A (●), B (○) and C (▲) are denoted E_A, E_B and E_C respectively. Neat antiserum was diluted 1:10 with 0·9% NaCl before use.

Precipitated activity measurements. Previous experiments have shown that anti-(exopenicillinase type A) serum precipitates enzyme type A and that the precipitate has enhanced activity (Richmond, 1963*a*). A comparison of the reaction of anti-(exopenicillinase type A) serum with purified preparations of exopenicillinase types A, B and C is shown in Fig. 2, where the results are plotted as the 'amount of enzyme protein precipitated' against the 'quantity of antiserum added/μg. of enzyme protein'. The amounts of enzyme protein precipitated were calculated from the amounts of enzyme activity precipitated, taking into account the known specific activity of the enzymes, the 4-fold stimulation of enzyme type A and the 1·5-fold stimulation of enzyme type B. The combination titre (see the Methods and Materials section) of anti-(exopenicillinase type A) serum may be calculated from the slopes in Fig. 2. It has a value of about 8·3 μg. of enzyme protein/ml. of serum when measured with either enzyme type A or type B. Further, the equivalence points (E_A and E_B in Fig. 2) obtained with these two enzymes are very similar when plotted in this way. These results suggest that enzyme types A and B have an identical reaction as antigens with anti-(exopenicillinase type A) serum. However, the different specific activities and degrees of stimulation by serum of the two enzymes make the reaction of the enzymes with serum appear different when measured in terms of enzyme activity as opposed to enzyme protein.

Comparison of the properties of enzyme type C with those of enzymes types A and B shows that the combination titre obtained with this protein is 4·6 μg. of enzyme protein/ml. of serum, and that the equivalence point is 1·6-fold higher/unit weight of enzyme (Fig. 2). These results suggest that enzyme

type C has a similar specific enzyme activity to that of enzyme type A but a lower combination affinity for anti-(exopenicillinase type A) serum.

Precipitated activity measurements on mixtures of exopenicillinase types A and C. If enzyme type C has a lower affinity than has enzyme type A for antiserum, precipitation of mixtures of enzyme types A and C would not be expected to give a linear relationship between the quantity of enzyme precipitated and the amount of serum added. The precipitation of enzyme activity from a mixture containing 20 units of enzyme type A and 40 units of enzyme type C was measured by the addition of various quantities of antiserum. As predicted, the relationship between the quantity of antiserum added and the amount of enzyme activity precipitated was not linear in this experiment (Fig. 3), the effective combination titre at low antiserum quantities being about 4.5-fold that found at high antiserum concentrations and close to that characteristic of enzyme type A. Taken in conjunction with the lower combination titre found when enzyme type C was tested alone (Fig. 2) and the similar specific enzyme activity of enzyme types A and C (see above), these results confirm that anti-(exopencillinase type A) serum has a higher combining affinity towards enzyme type A than towards enzyme type C.

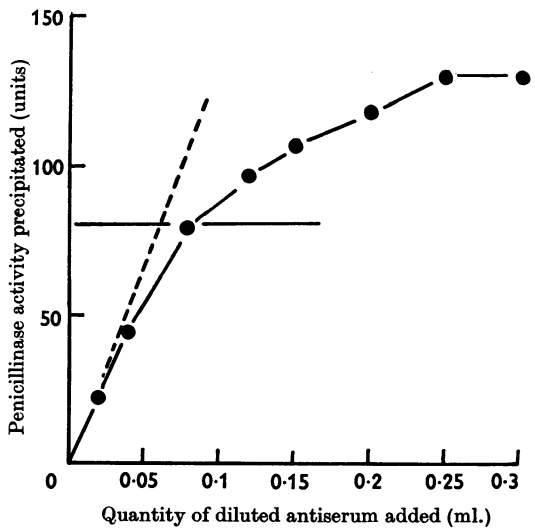

Fig. 3. Precipitation of a mixture of enzyme types A (20 units) and C (40 units) by anti-(exopenicillinase type A) serum. The broken line gives the slope expected for pure enzyme type A. The 20 units of enzyme type A activity are equivalent to 80 units after precipitation (see the horizontal bar). Neat serum was diluted 1:10 with 0·9% NaCl before use.

Chemical properties

Sedimentation coefficient. The sedimentation coefficient of the three enzyme types was compared by centrifugation in a sucrose gradient in the presence of ^{131}I-labelled bovine serum albumin and ^{125}I-labelled lysozyme as markers (Charlwood, 1963). This method gave a sedimentation coefficient about 2·5s for each protein type, whereas 2·62s had been obtained on examination of purified enzyme type A in the analytical ultracentrifuge (Richmond, 1963*a*). In view of the extremely similar amino acid analyses of these three protein types (see below), it seems likely that the molecular weight of all three staphylococcal exopenicillinases is close to the value of 29600±5% determined previously for enzyme type A by the Archibald method.

Amino acid composition. Samples of each exopenicillinase type were hydrolysed as described by Richmond (1963*a*) and their amino acid contents determined on an amino acid analyser. The results expressed in residues/mol. (assuming a mol. wt. 29600 and 2 tryptophan residues/mol.) are shown in Table 2. All three proteins have closely similar

Table 2. *Amino acid composition of staphylococcal exopenicillinase types A, B and C*

All results are given as residues/mol., assuming mol.wt. 29600. The results for enzyme type A are averages of seven analyses, those for enzyme type B the averages of two analyses, and those for enzyme type C the averages of two analyses. Ammonia values are subject to large errors.

	Amino acid composition		
Amino acid	Type A	Type B	Type C
Lys	42	43	45
NH_3	(30)	(28)	(32)
Arg	5	5	4*
His	2	2	3*
Asp	40	42	39
Thr	13	12	11
Ser	18	17	17
Glu	18	22*	18
Pro	10	11	10
Gly	13	16*	15*
Ala	19	21	19
Val	13	11	11
Met	2	2	2
Ile	17	15*	18
Leu	22	22	23
Tyr	11	9*	14*
Phe	7	7	7
Trp	2	?	?
Cys	0	0	0
N-Terminal amino acid ...	Lys	?	Lys

* Values outside the limit of accuracy of methods (see the text).

analyses. Since only one set of hydrolysis conditions has been used (see the Methods and Materials section) and since the quantities of enzyme types B and C available for hydrolysis were small, it is not possible to be absolutely certain that there are any differences in the amino acid composition of the three enzymes. However, if the values obtained for enzymes types B and C are referred to those obtained with enzyme type A (the average of seven analyses), the values marked with an asterisk are outside the limits of accuracy normally associated with this type of analysis (Spackman *et al.* 1958). The significance of these results is discussed below.

Qualitative analysis by treatment with fluorodinitrobenzene (Porter, 1957) has shown that enzyme type A has lysine as its *N*-terminal amino acid (Richmond, 1963*a*), and this result has been confirmed by the cyanate method for the quantitative determination of *N*-terminal amino acids (Stark & Smyth, 1963). Enzyme type A gave 81% of the theoretical yield of lysine (assuming a single peptide chain since cysteine is absent from the protein), and enzyme type C gave a 72% yield of the same amino acid contaminated with 4·7% of aspartic acid (again assuming 1 residue/mol. of protein). The *N*-terminal amino acid of enzyme type B has not yet been determined.

Peptide maps. Samples of enzyme types A and C were digested with trypsin, as described in the Methods and Materials section, and approx. 2 mg. of each of the resulting peptide mixtures was separated to form a peptide map. The overall pattern of peptides is clearly similar in the two hydrolysates (Fig. 4). In all, the map of enzyme type A contains four peptides not present in that of enzyme type C, and the map of enzyme type C contains four peptides not present in that of enzyme type A (see Fig. 4). The significance of these results is discussed below.

A peptide map of enzyme type B has shown that the general distribution of peptides is similar to that found for enzyme types A and C, but a detailed examination has not yet been carried out.

Enzymological properties

Substrate specificity and Michaelis constants. The relative rates of hydrolysis of a number of peni-

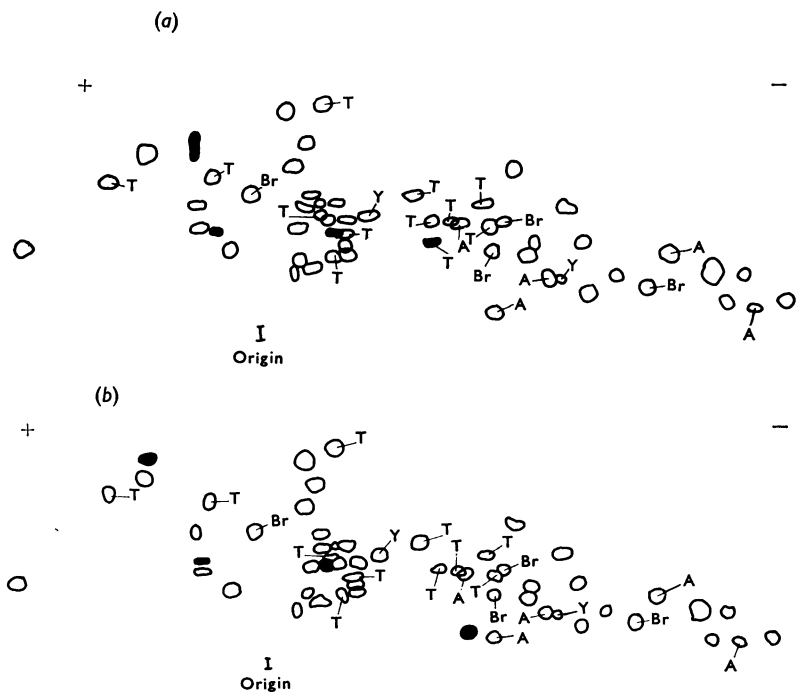

Fig. 4. Peptide maps of enzyme types A (*a*) and C (*b*) obtained after digestion with trypsin. Horizontal separation: electrophoresis at pH 4·7 (+, anode; −, cathode); vertical separation: chromatography in *n*-butanol–acetic acid–water. T, Tyrosine-containing peptide; A, arginine-containing peptide; Y, peptide giving an initial yellow colour with ninhydrin; Br, peptide giving brown colour with ninhydrin. Peptides not common to both maps are indicated as black spots on the tracings (see the text).

Table 3. *Rate of hydrolysis of various substrates by purified preparations of staphylococcal exopencillinase types A, B and C*

Values of 100 are taken arbitrarily as the rate of hydrolysis of benzylpenicillin by each enzyme type. The values in parentheses give the rates for enzyme type B relative to the value of 100 for enzyme type A acting on benzylpenicillin. The absolute rates of hydrolysis of benzylpenicillin (enzyme units/μg. of protein) were: for enzyme type A, 40; for enzyme type B, 5·6; for enzyme type C, 40. Values for oxacillin, cloxacillin and quinacillin are subject to large errors (see the text). Penicillin G, penicillin V and broxil were tested at saturating concentrations of substrates; the remaining substrates were tested at a concentration of 0·01 M.

Substrate	Relative rate of hydrolysis		
	Type A	Type B	Type C
Benzylpenicillin (penicillin G)	100	100 (15)	100
Phenoxymethylpenicillin (penicillin V)	100	100 (15)	100
Phenoxyethylpenicillin (broxil)	90	90 (13·5)	90
6-(2,6-Dimethoxybenzamido)penicillanic acid (methicillin)	1·5	1·5 (0·23)	0·6
6-(3-Phenyl-5-methyl-4-isoxazolylamido)penicillanic acid (oxacillin)	4·5	4·5 (0·7)	1·0
6-(3-*o*-Chlorophenyl-5-methyl-4-isoxazolylamido)penicillanic acid (cloxacillin)	3·5	3·5 (0·5)	1·0
6-(3-Carboxy-2-quinoxalinylamido)penicillanic acid (quinacillin)	4·5	4·5 (0·7)	1·0
7-(4-Amino-4-carboxybutyl)cephalosporanic acid (cephalosporin C)	0·5	0·5 (0·075)	1·2

Table 4. *Michaelis constants for purified preparations of staphylococcal exopenicillinase types A, B and C acting on various substrates*

Substrate	10^{-6} K_m (M)		
	Type A	Type B	Type C
Benzylpenicillin (penicillin G)	5	12	7·5
Phenoxymethylpenicillin (penicillin V)	7	14	10
Phenoxyethylpenicillin (broxil)	10	—	13
6-(2,6-Dimethoxybenzamido)penicillanic acid (methicillin, orbenin)	16000	14000	16000

cillins and cephalosporin C by the three types of staphylococcal penicillinase are shown in Table 3. As shown in Table 1, enzyme type B is only about 15% as active as enzyme types A and C against benzylpenicillin as substrate. However, if the initial rates of hydrolysis of benzylpenicillin by each enzyme type are taken as a reference point, then these enzymes have indistinguishable relative activities against benzylpenicillin, phenoxymethylpenicillin and phenoxyethylpenicillin. Enzyme types A and B hydrolyse methicillin about twice as fast as enzyme type C does, and enzyme type A also has a more rapid initial rate of hydrolysis of cloxacillin and quinacillin than has enzyme type C. In contrast, enzyme type C hydrolyses cephalosporin C about 2½ times as fast as enzyme type A does.

Measurement of the Michaelis constants for these enzymes against a number of substrates (Table 4) shows that enzyme type B has about a 5-fold lower affinity for benzylpenicillin and phenoxymethylpenicillin than those of enzyme types A and C. The measurement of K_m values for the 'new penicillins' is complicated, since these penicillins inhibit exocellular penicillinase irreversibly. The values obtained for methicillin as substrate are valid, since this penicillin only inhibits staphyloccocal exopenicillinase significantly at concentrations above 25 mM (M. H. Richmond & K. G. H. Dyke, unpublished work). Michaelis constants with methicillin as substrate may therefore be determined by using a range of substrate concentrations up to 20 mM without appreciably inhibiting the enzyme, but only so long as the initial rate of hydrolysis is measured over a short period. It is not, however, possible to obtain any meaningful K_m values for cloxacillin and quinacillin as substrates by normal methods, since both these compounds exert considerable inhibitory effect on exopenicillinase at concentrations as low as 0·2 mM. Michaelis constants with cephalosporin C as substrate could not be determined directly because this substrate saturated the enzyme at the minimum concentration that may be used to estimate cephalosporin C hydrolysis manometrically, and the micro-iodometric assay of penicillinase (Novick, 1962*b*) has not so far proved satisfactory for measuring 'cephalosporinase' activity.

Inhibition by iodine plus potassium iodide. Purified samples of the three enzyme types were tested for their sensitivity to iodine-plus-potassium iodide solution by the method used by Richmond (1963*a*).

Table 5. *Phage pattern and type of exopenicillinase synthesized by the 56 strains tested*

	Strain no.	Phage pattern	Enzyme type
Phage group I	3719	80	A
	3708	80/+	A
	3633	80/81	A
	3494	52A/79/80	A
	3394	80/81	A
	3393	(52/52A/80)	A
	3379	81	A
	3335	52/52A/80	A
	3291	81	A
	7182	(81)	A
	16449	52A/79	A
	16463	29/52A	A
	16467	52/52A/80/81	A
	3617	52/52A/80/81	C
	1035	(81)	C
	7183	(80/81)	C
Phage group II	1179	3A	B
	24661	3A	B
	49	3B/3C/55/71	B
	27138	71	B
	11023	55/71	B
	2528	55/71	B
	16461	71	B
	12848	71	B
	8891	3A/55	B
	8077	3B/3C/75	B
	17171	3A	B
	4061	55/71	B
Phage group III	3720	7/47/53/54/75/77/+	A
	3711	75/77/+	A
	3693	6/7/42E/47/53/54/75/77+	A
	3564	6/7/42E/47/53/54/75/83A/+	A
	3498	6/54/75	A
	3493	47/53/77/+	A
	3448	7/47/54/75	A
	3375	6/7/47/83/54/75/83A/+	A
	1581	75/77	A
	2861	(47)	A
	3659	(83A)	A
	4620	53/75/77	A
	6645	77	A
	8694	(7)	A
	6786	6/7/42E/47/53/54/75/77/83A/+	A
	1922	7/47/53/54/75/83A/+	
	4027	6/47/53/75	A
	4682	6/54/75	A
	5441	6/7/53/54/83A	A
	2832	(77)	C
	4061	7	C
	13477	(7/42E/54/81)	C
	13719	7/42E/54/81	C
	14186	(77)	C
	6789	53/77/83A	C
	364	53/83A	C
Miscellaneous group	8712	187	C
	8893	187	C

Approx. 50 μg. of purified enzyme was used for each test, and then diluted for assay. In each case the degree of inhibition is proportional to $\log[I_2 + KI]$ over a 100-fold concentration range and the enzymes show only small differences in their sensitivity to iodine-plus-potassium iodide solution. A 50% inhibition of enzyme type A is obtained in 10 min. at 30° in a solution containing iodine (0·3 mM) plus potassium iodide (1·2 mM). This concentration of iodine-plus-potassium iodide solution caused a 60% inhibition of enzyme types B and C.

Exopenicillinase type and staphylococcal phage pattern. Barber & Wildy (1958) recognized at least six variants of staphylococcal coagulase on immunological grounds. The three main types of enzyme were associated with staphylococci of phage group I, phage group II (omitting patterns containing type 3A) and phage group III (omitting patterns containing type 42E). The other three coagulase types were associated with staphylococci sensitive to phages 3A, 42E and 187 respectively. In view of this correlation between immunological variants and phage pattern, the distribution of exopenicillinase type was studied in relation to phage pattern in a number of staphylococcal strains. Table 5 shows the phage patterns together with the type of exopenicillinase synthesized by the 56 strains tested. The only clear correlation seems to concern organisms from phage group II, which all (12 strains) produced enzyme type B. Since no organisms from any other phage group produced enzyme type B, it seems likely that production of enzyme type B is confined to organisms from phage group II. Enzyme type C occurs both in phage group I and group III strains, although it is noticeable that enzyme type C is less common than enzyme type A among members both of phage group I and group III. In the strains examined so far, three out of 6 phage group I and seven out of 26 phage group III strains produced enzyme type C. In addition, both the strains sensitive to phage 187 produced enzyme type C, but this sample is not large enough to know how widely this correlation occurs.

Exopenicillinase from Staphylococcus albus

The taxonomic distinction between *S. albus* and *S. aureus* is ill-defined (Shaw, Stitt & Cowan, 1951); however, one commonly used classification of the two species (Mackie & McCartney, 1961) regards *S. aureus* as a pigmented coagulase-positive micrococcus, sensitive to one or more of the standard series of *S. aureus* phages (Williams, 1956), and *S. albus* as non-pigmented coagulase-negative and non-typable.

Within this classification *S. albus* is known to produce penicillinase (Rountree, 1956; Kjellander & Finland, 1963), and examination of a crude

preparation of the enzyme made by the method used for *S. aureus* penicillinase showed that the enzyme from *S. albus* was precipitated by antiserum to *S. aureus* exopenicillinase type A, and was similar in immunological properties to enzyme type C from *S. aureus*. The enzyme has not, however, been examined further, and, beyond this cross-reaction with antiserum, it is not possible to say how closely it is related to the exopenicillinase produced by 'true' *S. aureus*.

DISCUSSION

The main differences between the properties of the three variants of staphylococcal exopenicillinase detected so far are the relatively low specific activity of enzyme type B and the low affinity of enzyme type C for anti-(exopenicillinase type A) serum. Apart from these major differences, the three enzymes differ to a smaller degree in a number of characters, for example in their relative rates of hydrolysis of substrates and Michaelis constants (Tables 3 and 4). None of these properties, by itself, however, gives any indication as to whether the variation in properties of the three enzymes is due to differences in primary amino acid sequence or to changes in enzyme conformation. Evidence for a difference in primary sequence of the three proteins rests on the analyses of the relative abundance of amino acids (Table 2) and the peptide maps obtained with enzyme types A and C (Fig. 4). Although highly suggestive, these results cannot be regarded as conclusive. The amino acid analysis of enzyme type B differs from that of enzyme type A in having 22 glutamate, 16 glycine, 15 isoleucine and 9 tyrosine residues/mol. in enzyme type B, against 18 glutamate, 13 glycine, 17 isoleucine and 11 tyrosine residues/mol. in enzyme type A (Table 2). Similarly, enzyme type C has 4 arginine, 3 histidine, 15 glycine and 14 tyrosine residues/mol., against 5 arginine, 2 histidine, 13 glycine and 11 tyrosine residues/mol. in enzyme type A. Although these differences are outside the limits of inaccuracy normally associated with assays of this kind (Spackman *et al.* 1958), it is not absolutely certain that they represent differences in the primary sequence of the proteins. One reason for treating these analyses with caution is that the analysis of enzyme type C shows 4 arginine residues/mol., yet the peptide map of enzyme type C (Fig. 4*b*) shows five arginine-containing peptides. Further, the analyses in Table 2 are calculated on the assumption that the three enzyme variants have the same molecular weight. This may not be true since the methods used to determine molecular weight have an accuracy of at least $\pm 5\%$. The best evidence for a difference in primary structure of enzyme types A and C comes from the peptide maps. Here four peptides on the map of enzyme type A are not present in that of enzyme type B and vice versa. Nothing is known about the amino acid composition of these peptides except that two peptides from enzyme type C that are not present in the map of enzyme type A contain tyrosine, and this is probably associated with the relatively higher tyrosine content of enzyme type C (Table 2). On the basis of these experiments the three variants of staphylococcal exopenicillinase therefore probably differ to a small extent in primary sequence, although the evidence for this being the case is stronger for enzyme types A and C than for enzyme types A and B.

Significance of the different enzyme types in the emergence of penicillinase-resistant staphylococci

Virtually all the pencillin-resistant strains of staphylococci isolated from hospital infections owe their resistance to the production of penicillinase (Barber, 1957), and it therefore seems likely that one factor influencing the ability of staphylococci to survive in environments containing penicillin is the efficiency of their penicillinase in destroying the antibiotic. The 'efficiency' of penicillinase in this sense depends not only on the maximum velocity, $V_{max.}$, of hydrolysis of the substrate but also on the Michaelis constant, K_m, of the enzyme, and Pollock (1964) has suggested the term 'physiological efficiency' (defined as $K_m/V_{max.}$) to describe the efficiency of a penicillinase at destroying penicillin under physiological conditions. Calculation of the 'physiological efficiency' of the staphylococcal penicillinase variants (Table 6) from the results in Tables 3 and 4 shows that enzyme type B has about a 10-fold lower 'efficiency' than those of enzyme type A or C, and group II staphylococci (to which synthesis of enzyme type B is confined) should therefore be less able to protect themselves against the inhibitory action of penicillin. The group II organisms could offset the lower 'physiological efficiency' of their penicillinase by producing larger amounts of enzyme protein than group I or group III strains do. In fact, this does not seem to occur, since the 15 group II strains tested for quantitative enzyme production (Richmond *et al.* 1964) produced only 10–20% of the enzyme activity found on average with organisms from groups I and III, and, by taking into account the lower specific activity of enzyme type B, this means that group II organisms synthesize approximately the same amount of enzyme protein as organisms belonging to phage groups I and III. This argument suggests, therefore, that the predominance of members of phage groups I and III among the penicillin-resistant 'hospital staphylococci' (Anderson & Williams, 1957; Munch-Petersen & Boundy, 1962) may be associated with the higher 'enzymic efficiency' of

Table 6. *'Physiological efficiency' of staphylococcal exopenicillinase types A, B and C*

'Physiological efficiency' is defined as $K_m/V_{max.}$, where K_m is measured in moles/l. and V_{max} in enzyme units/mg. of protein N (Pollock, 1964).

Enzyme type	$V_{max.}$ (units/μg. of protein)	$V_{max.}$ (units/μg. of protein N)	K_m (M)	'Physiological efficiency'
A	40	$2{\cdot}5\times10^{5}$	5×10^{-6}	$2{\cdot}0\times10^{-11}$
B	5·6	$3{\cdot}5\times10^{4}$	$1{\cdot}2\times10^{-5}$	$3{\cdot}4\times10^{-10}$
C	37	$2{\cdot}3\times10^{5}$	$7{\cdot}5\times10^{-6}$	$3{\cdot}1\times10^{-11}$

their penicillinase than that of the enzyme type B synthesized by group II strains. The fact that the exocellular penicillinase is only part of the total penicillinase synthesized by the cell does not vitiate this argument, since the cell-bound enzyme of all staphylococcal cultures examined so far has a markedly lower $V_{max.}$ and affinity (and thus 'physiological efficiency') than those of the exocellular enzyme (Novick, 1962*a*; M. H. Richmond, unpublished work).

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