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Cephalosporinase and Penicillinase Activities of a β-Lactamase from *Pseudomonas pyocyanea*

By L. D. SABATH, M. JAGO and E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford

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1. *Pseudomonas pyocyanea* N.C.T.C. 8203 produces a β-lactamase that is inducible by high concentrations of benzylpenicillin or cephalosporin C. Methicillin appeared to be a relatively poor inducer, but this could be attributed in part to its ability to mask the enzyme produced. Much of the enzyme is normally cell-bound. 2. No evidence was obtained that the crude enzyme preparation consisted of more than one β-lactamase and the preparation appeared to contain no significant amount of benzylpenicillin amidase or of an acetyl esterase. 3. The maximum rate of hydrolysis of cephalosporin C and several other derivatives of 7-aminocephalosporanic acid by the crude enzyme was more than five times that of benzylpenicillin. Methicillin, cloxacillin, 6-aminopenicillanic acid and 7-aminocephalosporanic acid were resistant to hydrolysis, and methicillin and cloxacillin were powerful competitive inhibitors of the action of the enzyme on easily hydrolysable substrates. 4. Cephalosporin C, cephalothin and cephaloridine yielded 2 equiv. of acid/mole on enzymic hydrolysis, and deacetylcephalorsporin C yielded 1 equiv./mole. Evidence was obtained that the opening of the β-lactam ring of cephalosporin C and cephalothin is accompanied by the spontaneous expulsion of an acetoxy group and that of cephaloridine by the expulsion of pyridine. 5. A marked decrease in the minimum inhibitory concentration of benzylpenicillin and several hydrolysable derivatives of 7-aminocephalosporanic acid was observed when the size of the inoculum was decreased. This suggested that the production of a β-lactamase contributed to the factors responsible for the very high resistance of *Ps. pyocyanea* to these substances. It was therefore concluded that the latter might show synergism with the enzyme inhibitors, methicillin and cloxacillin, against this organism.

β-Lactamases (EC 3.5.2.6) from several Gram-positive bacteria, including strains of *Bacillus cereus* and *Staphylococcus aureus*, show much greater activity against benzylpenicillin than against cephalosporin C or most other derivatives of 7-ACA* (Crompton, Jago, Crawford, Newton & Abraham, 1962; Richmond, 1963). Nevertheless, crude culture filtrates of *B. cereus* 569 catalysed the hydrolysis of cephalosporin C at a considerably higher rate, relative to that of benzylpenicillin, than did purified penicillinase from the same organism, and the β-lactamase responsible for this hydrolysis was described as a cephalosporinase (Abraham & Newton, 1956). Fleming, Goldner & Glass (1963) reported that enzymes with a predominantly cephalosporinase activity are produced by some Gram-negative bacilli and that enzymes with a predominantly penicillinase activity are produced by others.

* Abbreviations: 7-ACA, 7-aminocephalosporanic acid; 6-APA, 6-aminopenicillanic acid.

In addition to a β-lactam ring, cephalosporin C contains an acetoxy group that is susceptible to hydrolysis by acetyl esterases (Jeffery, Abraham & Newton, 1961; Demain, Walton, Newkirk & Miller, 1963; O'Callaghan & Muggleton, 1963). The δ-(D-α-aminoadipoyl) side chain of cephalosporin C is not removed by acylases (or amidases; EC 3.5.1.11) that catalyse the hydrolysis of the phenylacetyl side chain of benzylpenicillin and of cephaloram (7-phenylacetamidocephalosporanic acid) (Huang, Seto & Shull, 1963). Susceptibility to these acylases appears to be determined by the nature of the *N*-acyl part of the molecule and not by that of the nucleus (Cole, 1964; Kaufmann & Bauer, 1964).

Jago, Migliacci & Abraham (1963) reported briefly that a crude preparation of an inducible enzyme from *Pseudomonas pyocyanea* (N.C.T.C. 8203, erroneously called N.C.T.C. 8230 by Jago *et al.* 1963) showed greater β-lactamase activity with cephalosporin C as substrate than with benzylpenicillin. More detailed studies of this cephalosporinase are described below.

METHODS

Enzyme preparations

Acetyl esterase. This was prepared from Jaffa orange peel according to a modification (Jeffery *et al.* 1961) of the procedure of Jansen, Jang & MacDonnell (1947). The enzyme solution obtained catalysed the hydrolysis of the *O*-acetyl group of cephalosporin C (10mM) at a rate that resulted in the production of 83·2 μequiv. of acid/ml. of enzyme solution/hr. at pH 7·25 and 30°.

Cephalosporinase. The crude cephalosporinase preparation was the supernatant obtained after centrifugation (1700*g* for 10min.) of cultures of *Ps. pyocyanea* N.C.T.C. 8203 and was prepared as follows. An overnight culture of the organism, grown in Oxoid nutrient broth (pH 7) at 37°, was used as an inoculum (10%, v/v) for the same broth distributed in 10ml. portions in inverted T-tubes (capacity 40ml.). The tubes were swung in a water bath at 35° until the extinction [measured in 1cm. cells with a Spekker absorptiometer (Adam Hilger Ltd.) with a neutral grey filter (H508)] reached 0·6–0·7. At this point an inducer was added, and incubation was continued for 3hr. Unless otherwise stated the inducer was benzylpenicillin (final concn. 10mg./ml.). 8-Hydroxyquinoline was then added (final concn. 1mM) and the culture kept at 4° overnight before removal of the cells by centrifugation. The supernatant is referred to below as 'crude enzyme solution'.

In some cases the crude enzyme solution (pH 7·2–7·4) was subjected to dialysis in cellophan tubing against running tap water overnight before use. In other cases cells in the whole culture were disrupted either by ultrasonic treatment at 4° for 3 or 4·5min. (MSE ultrasonic disintegrator, 60w, 20kcyc./sec.), or by freeze-pressing once at −20° in an X-press (from AB Biox, Box 235, Nacka 2, Sweden) and the resulting fluid was used as the enyme solution. The activities of these fluids were compared with those of corresponding control supernatants, or whole cultures, or with that of a suspension in water of the sediment obtained by centrifugation of disrupted cells for 1hr. at 8700*g* in a refrigerated centrifuge (Spinco model L).

Effect of methicillin on induction of cephalosporinase. In some experiments methicillin (final concn. 0·01–10mg./ml.) was added together with the inducer (benzylpenicillin, 5 or 10mg./ml.) to cultures of *Ps. pyocyanea* to determine whether methicillin would inhibit enzyme induction. Otherwise the conditions were as in studies of induction without methicillin. Solutions were assayed for β-lactamase activity both before and after dialysis for 17hr. or more in cellophan tubing against running tap water (to remove methicillin before assay).

Filtration through Sephadex. In some cases culture supernatants (10ml.) from experiments in which the inducer was methicillin (0·1 and 10mg./ml.) or benzylpenicillin were added to a column (40cm. × 2cm. diam.) of Sephadex G-25 (30g.) and elution was effected with 20mM-sodium acetate buffer, pH 7·6. Fractions (5ml.) were collected every 5min. Cephalosporinase activity emerged in fractions 6–9 and methicillin (when present), together with other products of low molecular weight, in fractions 21–29.

In one experiment a solution of the crude benzylpenicillin-induced enzyme (culture supernatant) was incubated with methicillin (0·1mg./ml.) at 35° for 1·5hr. A sample (10ml.) of the resulting solution was filtered through a column of Sephadex G-25, as described above, to remove the methicillin.

Substrates and inhibitors. Cephalosporin C, cephalothin [7-(2-thienylacetamido)cephalosporanic acid], 6-APA and 7-ACA were from Eli Lilly and Co., Indianapolis, Ind., U.S.A.; cloxacillin [6-(5-methyl-3-*O*-chlorophenylisoxazole-4-carboxamido)penicillanic acid] and ampicillin {6-[D(−)-α-aminophenylacetamido]penicillanic acid} were from Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey; cephaloridine {7-[(2-thienyl)acetamido]-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine} was from Glaxo Research Laboratories Ltd., Greenford, Middlesex; colistin (colomycin) was from Pharmax Ltd., Thames Road, Grayford, Dartford, Kent. Di-isopropyl phosphorofluoridate (DFP) was obtained from Dr D. R. Davis of the Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts.; *p*-nitrophenyl acetate was from British Drug Houses Ltd., Poole, Dorset. 7-(2,6-Dimethoxybenzamido)cephalosporanic acid was prepared from 7-ACA by the method used by Doyle *et al.* (1962) for the preparation of methicillin [6-(2,6-dimethoxybenzamido)penicillanic acid] from 6-APA.

Benzylpenicillin, methicillin, cephalosporin C_A (pyridine), cephaloram (7-phenylacetamidocephalosporanic acid), deacetylcephalosporin C, cephalosporin C_c (deacetylcephalosporin C lactone), cephalosporin N (penicillin N), phenethicillin, phenoxymethylpenicillin, D- and L-α-phenoxypropylpenicillin, triacetin and diacetylfluorescein were as described by Crompton *et al.* (1962).

[^{14}C]*Benzylpenicillin.* This was prepared as follows. [1-^{14}C]Phenylacetic acid (0·1mc, 0·6mg.; from The Radiochemical Centre, Amersham, Bucks.) in diethyl ether (0·5ml.) was added to unlabelled phenylacetic acid (204mg.) in a 25ml. pear-shaped flask. The ether was rapidly removed in an air stream and thionyl chloride (284mg.) was added. The flask, fitted with a reflux condenser and $CaCl_2$ tube, was warmed briefly to bring the mixture into solution, left at room temperature overnight, and finally heated in a boiling-water bath for 20min. The mixture was then cooled and excess of thionyl chloride removed *in vacuo* (water pump). The remaining [1-^{14}C]phenylacetyl chloride was dissolved in 0·5ml. of acetone.

6-APA (270mg.) was dissolved in a solution (5ml.) of $NaHCO_3$ (520mg.). The stirred solution was cooled in ice and the solution of [1-^{14}C]phenylacetyl chloride in acetone was added during 1min. The mixture was stirred for 20min. and stirring then continued for a further 30min. with the ice bath removed. The resulting solution was extracted twice with isopentan-2-one (1·25ml.). The aqueous phase was cooled in ice, stirred with cold isopentan-2-one (2·5ml.), and 6N-HCl added until the pH of the aqueous phase fell to 2·1. The mixture was centrifuged and the clear upper phase removed and dried (anhydrous Na_2SO_4). To the dry solution was added 0·2vol. of 50% (w/v) potassium 2-ethylhexanoate in butan-1-ol (Gourevitch *et al.* 1962). The resulting thick precipitate of crystalline potassium [^{14}C]benzylpenicillin was filtered off, washed with a little acetone and dried (310mg.).

Measurements of enzymic hydrolysis

β-Lactamase activity of enzyme from Ps. pyocyanea. The manometric method (Henry & Housewright, 1947; Pollock, 1952) was used for assay of material from Sephadex

columns, for the determination of Michaelis constants for cephalothin, cephaloridine and cephaloram respectively, for studies on enzyme stability and inhibition, for the determination of the energy of activation of the hydrolysis of cephalosporin C, and for a survey of the susceptibility of different derivatives of 6-APA and 7-ACA to enzymic hydrolysis.

The potentiometric titration method (Wise & Twigg, 1950) was used for the determination of Michaelis constants for benzylpenicillin and cephalosporin C, pH–activity curves, and equiv. of acid/mole of antibiotic liberated on hydrolysis of benzylpenicillin, cephalosporin C, cephalothin, cephaloridine and deacetylcephalosporin C. Determinations were made with a pH-stat (type TTT1b automatic titrator in conjunction with a type SBR2C Titrograph and type SBU1 syringe burette from Radiometer, Copenhagen, Denmark). By this method the hydrolysis of benzylpenicillin could be followed at concentrations as low as 70 μM.

An iodometric method (Perret, 1954) was used to follow the effect of the enzyme on 6-APA. The effect of the enzyme on 7-ACA was estimated from measurement of changes in extinction at 260 mμ.

Maximum rate of hydrolysis in the presence of an excess of substrate ($V_{max.}$) and Michaelis constants (K_m) were estimated from data plotted by the method of Eadie (1942) or of Lineweaver & Burk (1934). The quotient K_i/K_m, where K_i is the dissociation constant of the enzyme–inhibitor complex, was obtained from data plotted in the manner described by Hunter & Downes (1945). Energies of activation (E) were calculated from plots of log $V_{max.}$ (in arbitrary units) at 20°, 30° and 40° against $1/T$. The slopes of the resulting lines give $-E/2{\cdot}303\ R$.

Acetyl-esterase activity. This was estimated by measurement of the hydrolysis of triacetin (0·215 M) in a Warburg respirometer at pH 7·0 in the presence of 8·6 mM-$NaHCO_3$ with N_2+CO_2 (95:5, v/v) in the gas phase, and by spectrophotometric measurement of the hydrolysis of *p*-nitrophenyl acetate (Hartley & Kilby, 1952). Qualitative observations were made with diacetylfluorescein as substrate as described by Byrde & Fielding (1955) (in which a bright-green fluorescence in ultraviolet light is seen when fluorescein is liberated).

Paper electrophoresis of products of hydrolysis of benzylpenicillin. Hydrolysis of 8 mg. of benzylpenicillin (10 mM) by 1 ml. of the enzyme from *Ps. pyocyanea* was allowed to reach completion in a pH-stat at pH 7·0 and 30°. Samples of the solution (containing products derived from 10 or 20 μg. of the penicillin) were subjected to electrophoresis at 12·3 and 70·2 v/cm., for 2–5 hr. and 20–45 min. respectively, on Whatman no. 1 paper in pyridine–acetate (50 mM with respect to acetate) at pH 4·5 and in collidine–acetate (62 mM) at pH 7·0 (at 12·3 v/cm. only). Spots were located by spraying the dried paper with starch–iodine (10 ml. of 10 mM-I_2 in 3 mM-KI, 9 ml. of aqueous 2% starch and 1 ml. of M-Na_2HPO_4–M-KH_2PO_4 buffer, pH 7·0) with ninhydrin (1·28 g. of ninhydrin and 1·28 ml. of collidine, in 800 ml. of butan-1-ol), or $AgNO_3$ [10 ml. of aq. 10% (w/v) $AgNO_3$, 10 ml. of aq. NH_3 (sp.gr. 0·88) and 80 ml. of methanol]. 6-APA and 7-ACA were located on bioautographs [after phenylacetylation by spraying with M-pyridine in aq. 50% (v/v) acetone, and then with a solution of phenylacetyl chloride (0·1 ml. in 8 ml. of acetone)] for which the paper was applied to the surface of a plate of nutrient agar seeded with *S. aureus* (Oxford strain, N.C.T.C. 6571). In experiments in which [^{14}C]benzylpenicillin was used, radioactive spots were located by direct counting (scaler 1800; Isotopes Development Ltd., Reading, Berks.) and radioautography, and their positions compared with those of spots located in other sprays.

Samples (about 20 μg.) of benzylpenicilloate, and of the product of alkaline hydrolysis of 6-APA, prepared by alkaline hydrolysis of benzylpenicillin and 6-APA respectively at pH 11·0 and 30° in the pH-stat and subsequent adjustment of the pH of the solution to 7·0, were subjected to electrophoresis for comparative purposes in most experiments.

Changes in ultraviolet-absorption spectra during enzymic hydrolysis. Each derivative of 7-ACA (1 mg.) was mixed with 0·2 ml. of the solution of the crude enzyme from *Ps. pyocyanea* in a total volume of 2 ml. and the reaction allowed to proceed at 30° and at pH 7·0 in the pH-stat until no additional 10 m N-NaOH was required to maintain the pH. For measurements of extinction samples were diluted with water (before and after treatment with enzyme) to give a concentration corresponding to 0·05 mg. of the original substrate/ml. Similar measurements were made at various times when the reaction was carried out at 20° in solutions of 33 mM-Sörensen phosphate buffer with pH values of 4·0, 7·0 and 9·0 respectively. Extinctions were measured with appropriate blank solutions containing enzyme and buffer or enzyme alone.

An aqueous solution of benzylpenicillin (25 mg./ml.) in 0·3 M-sodium–potassium phosphate buffer, pH 7·0, was kept at 20–23° and its absorption spectrum between 220 and 360 mμ measured at intervals. Similar measurements were made with a solution of benzylpenicillin mixed with the crude enzyme from *Ps. pyocyanea* (1 ml. of enzyme, 75 mg. of benzylpenicillin in 1 ml. of water and 1 ml. of M-sodium–potassium phosphate buffer, pH 7·0). Frequent measurements were made at 322 mμ, at which benzylpenicillenic acid shows $\epsilon_{max.}$

Determination of minimum inhibitory concentration

Serial twofold dilutions of antibiotics in Oxoid nutrient broth (0·5 ml. amounts) were inoculated with 1 drop (containing about 10^4 organisms, by colony counts) of a 1:40 dilution of an overnight culture of *Ps. pyocyanea*. After incubation overnight at 37° the tubes were examined and the lowest antibiotic concentration to show virtually complete inhibition of growth was considered to be the minimum inhibitory concentration, although observations were made at 48 and 72 hr. also. Other determinations were made similarly, but with inocula containing from 10^6 to between 1 and 10 organisms in 0·5 ml., which were added to 0·5 ml. quantities of broth containing antibiotic.

RESULTS

Enzyme induction

Effect of cell disruption on the apparent concentration of enzyme. With control cultures to which no inducer was added no cephalosporinase or penicillinase activity was detected in the culture fluid and none was detected after rupture of the cells by

Table 1. *Effect of cell disruption on the amount of penicillinase and cephalosporinase activity detected in cultures of* Ps. pyocyanea *N.C.T.C. 8203 after induction*

In each case the inducer was added to a rapidly growing culture at 35° (for details see the Methods section). Induction was terminated after 3hr. by the addition of 8-hydroxyquinoline. Enzyme activities were assayed as described in Table 2. Preparations of disrupted cells were produced by ultrasonic treatment for 3min.

Concn. of inducer (benzylpenicillin) (mg./ml.)	β-Lactamase activity (μl. of CO_2/ml. of enzyme/hr.)					
	Of supernatant fluids		Of whole culture		Of disrupted culture	
	Penicillinase	Cephalosporinase	Penicillinase	Cephalosporinase	Penicillinase	Cephalosporinase
0	<10	<10	<10	<10	<10	<10
0·01	<10	<10	<10	<10	<10	42
0·1	<10	12	<10	18	24	252
1·0	60	264	60	336	252	3120
10·0	370	2040	420	3120	492	3720

ultrasonic treatment or by freeze-pressing in an X-press.

When enzyme was induced with benzylpenicillin in concentrations ranging from 0·01 to 10mg./ml. the whole culture showed a β-lactamase activity between 1·0 and 1·5 times that of the supernatant fluid. However, ultrasonically treated cultures (and also cultures whose cells had been disrupted in the X-press) from experiments with low inducer concentrations (0·01–1·0mg. of benzylpenicillin/ml.) had about 10 times as much cephalosporinase activity as the supernatant fluids or whole cultures, whereas with higher inducer concentrations (10mg./ml.) there was relatively little difference between the activities of supernatant, whole cultures or sonicated cultures (Table 1). During enzyme induction with low concentrations of inducer there was marked increase in the extinction of the cultures, indicating growth. At higher inducer concentrations (10mg./ml. or more) there was little change in extinction (Fig. 1), and it appeared that either no substantial growth occurred, or that growth was balanced by lysis.

Effect of different inducers on the concentration of enzyme in culture supernatants and ultrasonic extracts. Table 2 shows the penicillinase and/or cephalosporinase activity detected in supernatants from cultures of *Ps. pyocyanea* when different substances were used as inducers. Benzylpenicillin appeared to induce more enzyme activity than any of the other compounds tested, but a high concentration (5mg./ml.) was required for maximum induction and this was still so in the presence of small amounts of colistin, an antibiotic that had been reported to be identical with polymyxin E (Wilkinson, 1963) and that may therefore damage the cytoplasmic membrane of *Ps. pyocyanea* (Newton, 1956). Cephalosporin C was also an inducer and 6-APA showed considerable inducing activity at a concentration of 1mg./ml.

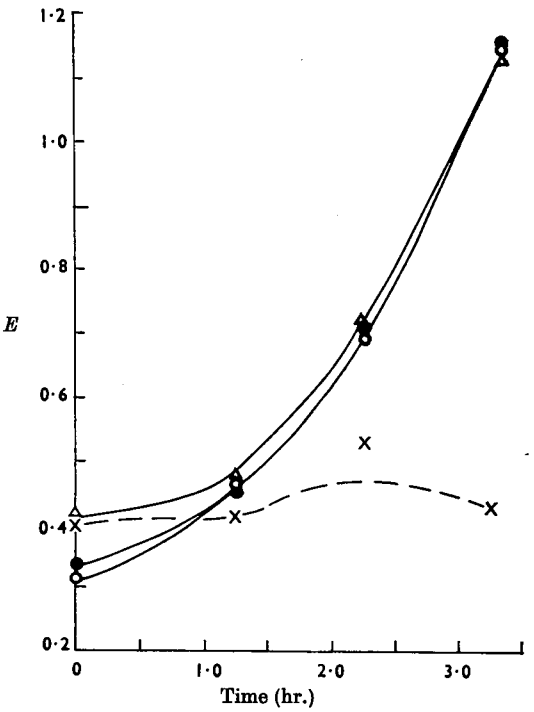


Fig. 1. Changes in extinction of cultures of *Ps. pyocyanea* N.C.T.C. 8203 during induction of β-lactamase with the following concentrations (mg./ml.) of benzylpenicillin: ●, 0·01; ○, 0·1; △, 1·0; ×, 10. The inducer was added at zero time.

Fig. 2 shows that the amount of enzyme that appeared in the dialysed supernatant fluid during induction with benzylpenicillin increased for 2hr. and that there was no significant change in the cephalosporinase activity/penicillinase activity ratio. Undialysed samples showed relatively smaller

Table 2. *Amounts of penicillinase and cephalosporinase activity detected in culture supernatants of* Ps. pyocyanea *N.C.T.C. 8203 after induction*

Enzyme was induced as described in Table 1. Supernatants obtained after induction with cephalosporin C were dialysed against running tap water for 18hr. before assay. After induction with benzylpenicillin no loss in activity was caused by dialysis under similar conditions. Penicillinase activity was assayed manometrically with 2·8mM-benzylpenicillin as substrate and cephalosporinase activity with 2·1mM-cephalosporin C. Under these conditions the rate of hydrolysis of benzylpenicillin did not differ significantly from $V_{max.}$ and that of cephalosporin C was approx. 0·66 $V_{max.}$ (see Table 4). Complete hydrolysis yielded 1·0mole of CO_2/mole of benzylpenicillin, but 1·7moles of CO_2/mole of cephalosporin C. —, Not determined.

Inducer		Apparent enzyme activity of supernatant (μl. of CO_2/ml. of enzyme/hr.)	
Substance	Concn. (mg./ml.)	Penicillinase	Cephalosporinase
Benzylpenicillin	80	<10	<10
	40	156	480
	20	276	2400
	10	228	3000
	10*	240	—
	5	252	4400
	1	—	740
	1*	<10	—
	0·1	<10	12
	0·1*	<10	—
Methicillin	40	<10	—
	10	<10	<10
	0·1	<10	<10
6-APA	1	210	—
	0·1–0·001	<10	—
Cephalosporin C	16	72	420
	8	126	960
	4	78	720
	2	42	300
	1	30	180
Cephaloram	5	24	—
	0·1–0·001	<10	—

* Plus colistin (0·01mg./ml.)

amounts of cephalosporinase at 0·5 and 1hr. than did later samples.

When methicillin (0·1–40mg./ml.) was used in place of benzylpenicillin as an inducer, untreated culture supernatants and ultrasonic treatment failed to show cephalosporinase activity (Tables 2 and 3), but two procedures were found to uncover activity: (1) dialysis against running tap water; (2) passage through a column of Sephadex G-25.

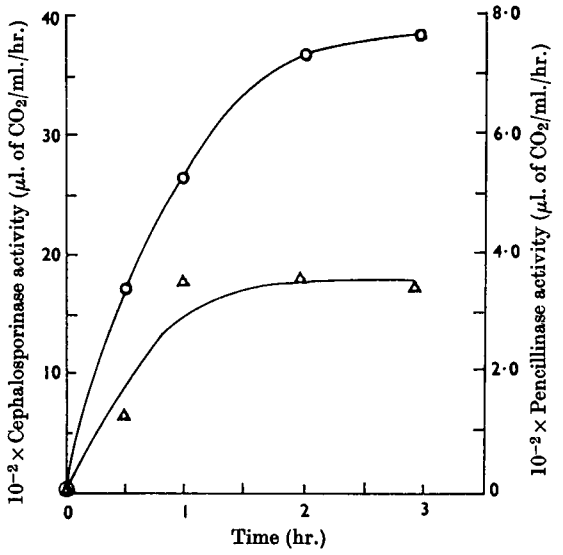


Fig. 2. Appearance of cephalosporinase activity (○) and penicillinase activity (△) in culture supernatants of *Ps. pyocyanea* N.C.T.C. 8203 after the addition of inducer. Enzyme was induced with benzylpenicillin (10mg./ml.) at 35° and induction stopped at different times by the addition of oxine. Samples were dialysed for 18hr. against running tap water before assay with benzylpenicillin (1mg./ml.) or cephalosporin C (1mg./ml.) as substrates.

The fractions from the column that contained cephalosporinase showed no antistaphylococcal activity (hole-plate assay) and therefore apparently contained less than 3μg. of free methicillin/ml., but the latter was found in later fractions with no enzymic activity.

Supernatant fluids containing cephalosporinase (benzylpenicillin-induced) were kept for 1·5hr. at 35° in the presence of 0·1mg. of methicillin/ml. In the presence of this concentration of methicillin 99% of the original enzymic activity was undetectable, but dialysis for 17hr. or passage through Sephadex G-25 led to the recovery of much of the activity (Table 3). When methicillin in high concentration (1–10mg./ml.) was used as the inducer no β-lactamase was detected after dialysis for 17hr., but after dialysis for 54hr. considerable enzyme activity was revealed after ultrasonic treatment. However, the maximum amount of enzyme whose presence could be demonstrated with methicillin as an inducer was less than that obtained when the inducer was benzylpenicillin.

Induction by benzylpenicillin in the presence of methicillin. In the presence of methicillin (0·01–1·0mg./ml.) there was no significant inhibition of the induction of cephalosporinase by benzylpenicillin (10mg./ml.), as judged from the activity of culture supernatants tested after dialysis for 18hr.

Table 3. *Effect of dialysis and passage through Sephadex G-25 on the apparent cephalosporinase activity of supernatants and ultrasonically treated preparations from cultures of* Ps. pyocyanea *N.C.T.C.* 8203

Enzyme induction, dialysis and desalting on Sephadex G-25 were carried out as described in the Methods section. Cephalosporinase activity was measured manometrically with cephalosporin C (2·1 mM) as substrate. In one case (*a*) the supernatant was incubated for 90 min. at 35° with methicillin (0·1 mg./ml.) before dialysis or passage through Sephadex G-25. In another (*b*) the culture was ultrasonically treated for 4·5 min. and the whole suspension dialysed before test.

Inducer			Apparent cephalosporinase activity (μl. of CO_2/ml. of enzyme/hr.)				
				After dialysis			After
Substance	Concn. (mg./ml.)	Further treatment	Original supernatant	For 17 hr.	For 54 hr.	For 100 hr.	Sephadex G-25*
Benzylpenicillin	10	—	3540	3540			2964
	10	*a*	48	1152			2674
Methicillin	10	—	< 10	< 10			14
	1·0	—	< 10	< 10			
	0·1	—	< 10	25			39
	10	*b*	—	< 10	825	428	
	5	*b*	—	< 10	315	320	
	1·0	*b*	—	< 10	120	180	
	0·5	*b*	—	54	42	38	
	0·1	*b*	—	66	42	< 10	

* Calculated on the assumption that the total activity of the effluent was present in a volume equal to that of the supernatant added to the column.

Behaviour of different substrates and inhibitors

Rates of hydrolysis. With solutions of the crude enzyme from *Ps. pyocyanea* the optimum pH for enzymic activity with either benzylpenicillin or cephalosporin C as substrate was between 8·0 and 8·5. The energies of activation with these two substrates differed, being $3·3 \pm 1·1$ kcal./mole with benzylpenicillin and $8·5 \pm 1·6$ kcal./mole with cephalosporin C.

Table 4 indicates that much greater values of $V_{max.}$ are obtained with cephalosporin C, and probably also with cephalothin [7-(2-thienylacetamido)-cephalosporanic acid] or cephaloram (7-phenylacetamidocephalosporanic acid), as substrate than with benzylpenicillin. On the other hand, K_m was markedly lower with benzylpenicillin than with cephaloram or cephalosporin C. At arbitrary substrate concentrations (1–70 mM) most derivatives of 7-ACA that were tested were hydrolysed much more rapidly than derivatives of 6-APA. The only derivative of 7-ACA tested that was hydrolysed only poorly (if at all) by the enzyme was 7-(2,6-dimethoxybenzamido)cephalosporanic acid, which has the *N*-acyl side chain of methicillin coupled with the 7-ACA nucleus. However, methicillin itself and cloxacillin were very poorly hydrolysed and a ten-fold concentration of the crude enzyme solution was necessary before the rate of hydrolysis could be determined manometrically. Similarly, both 7-ACA and 6-APA were resistant to hydrolysis.

Competitive inhibition. Methicillin showed a high affinity for the enzyme (Table 4) and was a powerful competitive inhibitor of the action of the enzyme on cephalosporin C, cephaloram, cephalothin and benzylpenicillin (Table 5). Cloxacillin was an even more powerful inhibitor than methicillin, whereas 7-ACA and 6-APA were weaker competitive inhibitors. The degree of inhibition of penicillinase produced at 30° by methicillin was no greater when the inhibitor was added 20, 10 or 2 min. before the substrate than when substrate and inhibitor were added simultaneously to crude enzyme solution (1 ml.) to give final concentrations of 2·8 and 0·15 mM respectively in a total volume of 3 ml.

Products of enzymic hydrolysis

Benzylpenicillin. With benzylpenicillin as substrate, hydrolysis by the crude enzyme solution came to an end, in both potentiometric and manometric assays, when 0·95–1·0 equiv. of acid had been liberated. Electrophoresis of the product at pH 7·0 revealed one spot in a position corresponding to D-benzylpenicilloate (Fig. 3) but failed to reveal a spot (on ninhydrin, silver nitrate or starch–iodine spray) that corresponded in position to 6-APA or to the product of alkaline hydrolysis (pH 11) of 6-APA, or that gave a zone of inhibition on a bioautograph against a penicillin-sensitive strain of *S. aureus* (N.C.T.C. 6571) after phenylacetylation. Electrometric titration revealed no

Table 4. *Behaviour of derivatives of 6-APA and 7-ACA as substrates of crude cephalosporinase from* Ps. pyocyanea *N.C.T.C.* 8203

Unless otherwise stated rates of hydrolysis were determined manometrically at 30°. The results of non-manometric measurements are expressed in terms of μl. of CO_2/ml. of enzyme solution/hr. on the assumption that hydrolysis of 1 μmole of substrate corresponds to 22·4 μl. of CO_2. Rates of hydrolysis relative to that of benzylpenicillin with the same enzyme preparation are given in terms of the moles of substrate hydrolysed/unit time at stated substrate concentrations. With cephalosporin C the number of moles hydrolysed is taken as moles of CO_2/1·7, and with cephaloram, cephaloridine and cephalothin as moles of CO_2/2·0 (see the text). At the concentrations used the rate of hydrolysis was virtually $V_{max.}$ for benzylpenicillin and probably greater than 0·6 $V_{max.}$ for most of the other substrates. Values for K_i were derived from data in Table 5.

Substrate	K_m (M)	K_i (M)	$V_{max.}$ (μmole/ml. enzyme/hr.)	Rate of hydrolysis (relative to benzylpenicillin)	Rate of hydrolysis (μl. of CO_2/ml. of enzyme/hr.)	At concn. of substrate (mM)
6-APA*				<1	<10	1·3
Benzylpenicillin	$1·3\times10^{-5}$	$1·3\times10^{-5}$	19·2§	100	349	2·8
Methicillin		$1·7\times10^{-7}$		<1	6	2·4
Cloxacillin		$2·6\times10^{-8}$		<1	7	3·6
α-Phenoxyethylpenicillin				<1	<10	4·4
Phenoxymethylpenicillin				76	171	2·7
D-α-Phenoxypropylpenicillin				<1	<10	4·2
L-α-Phenoxypropylpenicillin				10·4	30	4·2
Ampicillin				14	30	2·7
Cephalosporin N				12·5	36	1·1
7-ACA†				<1	<10	1·0
Cephalosporin C	1×10^{-3}		147§	614	3406	2·1
Cephaloram	1×10^{-3}			573	4050	2·4
Cephalothin	$4·5\times10^{-4}$			420	4680	2·14
Cephalosporin C_A (pyridine)				133	306	6·6
Cephaloridine	$1·3\times10^{-4}$			700	3225	4·6
Cephalosporin C_c				133	384	4·3
Deacetylcephalosporin C				120	580	3·9
7-(2,6-Dimethoxybenzoyl)-cephalosporanic acid				<1	<10	3·7
Triacetin				<1	<10	215
p-Nitrophenyl acetate‡				3	12·7	3·0

* Rate determined by the iodometric method of Perrett (1954).
† Estimate based on measurements of extinction at 260 mμ.
‡ Rate determined spectrophotometrically at 20°.
§ Measurements made with the same batch of enzyme.

Table 5. *Behaviour of derivatives of 6-APA and 7-ACA as inhibitors of crude cephalosporinase from* Ps. pyocyanea *N.C.T.C.* 8203

Values of K_i/K_m were determined as described in the Methods section. Benzylpenicilloate (2·7 mM) showed no inhibitory action with benzylpenicillin or cephalosporin C as substrate.

Inhibitor	Substrate	K_i/K_m
Methicillin	Benzylpenicillin	$1·3\times10^{-2}$
	Cephalosporin C	$1·6\times10^{-4}$
	Cephaloram	$1·6\times10^{-4}$
	Cephalothin	$4·2\times10^{-4}$
Cloxacillin	Cephalosporin C	$2·3\times10^{-5}$
	Benzylpenicillin	$2·2\times10^{-3}$
Benzylpenicillin	Cephalosporin C	4×10^{-2}
6-APA	Cephalosporin C	3×10^{-1}
7-ACA	Cephalosporin C	5×10^{-1}

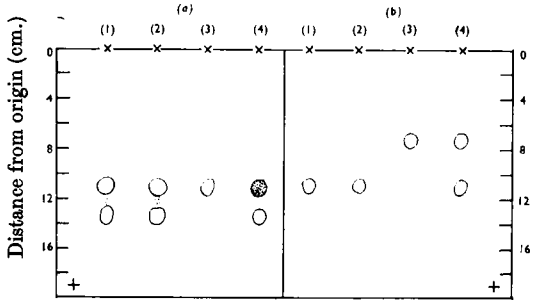


Fig. 3. Electrophoresis on paper (13 v/cm.) in (*a*) pyridine–acetate buffer, pH 4·5, for 4·5 hr. and (*b*) collidine–acetate buffer, pH 7·0, for 3 hr. of products of: (1) hydrolysis of benzylpenicillin by crude enzyme from *Ps. pyocyanea* N.C.T.C. 8203; (2) hydrolysis of benzylpenicillin at pH 11·0; (3) hydrolysis of 6-APA at pH 11·0; (4) mixture of (1) and (3). Spots were revealed by a starch–iodine spray (see the Methods section).

group with pK in the region of 8·2, corresponding to the amino group of the product of alkaline hydrolysis of 6-APA.

On electrophoresis at pH 4·5 the product of enzymic hydrolysis yielded two spots after starch–iodine and ninhydrin spray (as did the product of alkaline hydrolysis), one of which had the same location as the product of alkaline hydrolysis of 6-APA (Fig. 3). But both of the compounds responsible for these spots contained the side chain of benzylpenicillin, since they were radioactive when derived from [^{14}C]benzylpenicillin labelled in C-1 of its phenylacetyl group.

A solution containing benzylpenicillin (70mM) in 0·33M-sodium–potassium phosphate buffer and enough crude system to catalyse complete hydrolysis of the penicillin in 24hr. at 22° showed an increase in extinction at 322mμ within 2·5–3·5min. after the beginning of the reaction, and a subsequent decrease over 14hr. Benzylpenicillenic acid shows $\lambda_{max.}$ 332mμ (Peck & Folkers, 1949). However, the largest amount of penicillenic acid that could have been present when the measured extinction was maximal corresponded to only 0·004% of the initial substrate. A control solution of benzylpenicillin (70mM), in the same buffer but containing no enzyme, showed a gradual increase in extinction at 322mμ that reached a maximum in 8 days, at which time 0·24% of the starting material could have been present as benzylpenicillenic acid.

Cephalosporin C, deacetylcephalosporin C, cephalothin and cephaloridine. With a sample of cephalosporin C (sodium salt) that had been recrystallized from 83% (v/v) ethanol the amount of acid liberated by the crude enzyme from *Ps. pyocyanea* corresponded to 1·66–1·72equiv./mole on the assumption that the sodium salt used was pure. Samples of cephalosporin C of only 40–50% purity (as estimated from their extinction at 260mμ) yielded proportionately less acid. Distillation by freeze-drying of the solution of the reaction products, whose pH had previously been adjusted to 2·75–2·9 with toluene-*p*-sulphonic acid (from which volatile acid had been previously removed by steam-distillation), yielded a volatile acid that titrated potentiometrically like acetic acid (pK 4·7) and that accounted for 43–45% of the total acid produced on enzymic hydrolysis. (In control experiments about 90% of known amounts of acetate were recovered as acetic acid by the distillation method used.) Cephalothin yielded 2·0equiv. of acid/mole, nearly half of which was recovered as acetic acid, on enzymic hydrolysis. In contrast, deacetylcephalosporin C yielded 1equiv. of acid/mole.

Reaction of the citrus acetyl-esterase preparation (0·5ml.) with cephalosporin C (sodium salt) (10mg.) in a total volume of 2ml. yielded 0·87equiv. of acid/mole. The subsequent addition of 0·5ml. of a solution of the crude enzyme from *Ps. pyocyanea* yielded an additional 0·79equiv. of acid/mole, bringing the total acid liberated to 1·66equiv./mole. The addition of 1ml. of citrus acetyl esterase to a vessel in which a reaction with the enzyme from *Ps. pyocyanea* and cephalosporin C (10mg.) had reached completion (1·71equiv. of acid/mole) resulted in no further liberation of acid.

Cephaloridine yielded 2·0equiv. of acid/mole on enzymic hydrolysis. When the pH of the resulting solution was adjusted to 8·1 and the solution then freeze-dried the distillate contained a compound whose ultraviolet-absorption spectrum was identical with that of pyridine. The recovery of volatile product in the distillate corresponded to 0·67mole of pyridine/mole of cephaloridine.

Deacetylcephalosporin C lactone. Enzymic hydrolysis of this substance yielded one main product, which migrated slightly further than glutamic acid towards the anode on paper electrophoresis at pH 4·5 (deacetylcephalosporin C lactone shows no net charge under these conditions). The product showed an R_F that was slightly higher than that of deacetylcephalosporin C lactone and close to that of glutamic acid on chromatography on paper in butan-1-ol–acetic acid–water (4:1:4, by vol.).

Changes in ultraviolet-absorption spectra associated with enzymic hydrolysis

Hydrolysis of cephalosporin C and deacetylcephalosporin C by the enzyme from *Ps. pyocyanea* was accompanied by similar changes in ultraviolet-absorption spectra, the original $\epsilon_{max.}$ at 260mμ being replaced by a new $\epsilon_{max.}$ at 230mμ (Fig. 4). Measurements in 33mM-Sörensen phosphate buffer, pH 4, pH 7 and pH 9, at 20° disclosed that the peak at 260mμ disappeared more slowly as the pH was lowered. The maximum at 230mμ was due to a chromophoric grouping that was unstable in aqueous solution at pH 4, pH 7 or pH 9, and disappeared within 17hr. at 20° (Fig. 4).

In contrast with the changes in the ultraviolet-absorption spectra of these compounds the absorption maximum of deacetylcephalosporin C lactone at 257mμ was replaced by a new maximum of lower extinction at 265mμ on enzymic hydrolysis. Under similar conditions the enzyme produced no significant change in the ultraviolet-absorption spectrum of 7-ACA.

Enzyme stability during hydrolysis of benzylpenicillin and cephalosporin C

When successive 0·2mg. amounts of benzylpenicillin were added to 0·6ml. of a solution of the crude enzyme from *Ps. pyocyanea* in the pH-stat at 30° (total volume 4ml.), each addition being made as

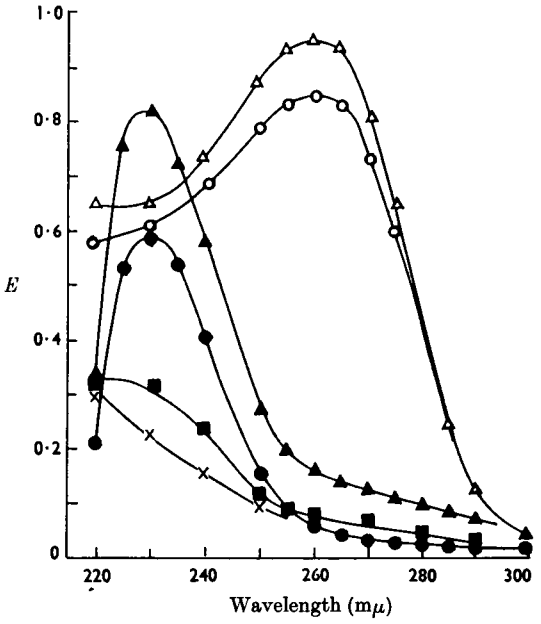


Fig. 4. Ultraviolet-absorption spectra of solutions of cephalosporin C (sodium salt) and deacetylcephalosporin C (sodium salt) (0·05mg./ml.) and the products of their hydrolysis by enzyme from *Ps. pyocyanea* N.C.T.C. 8203. ○, Cephalosporin C; ●, immediately after hydrolysis; ×, 7hr. later. △, Deacetylcephalosporin C; ▲, immediately after hydrolysis; ■, 7hr. later.

soon as hydrolysis of the preceding amount was complete (approx. 4min.), there was no decline in the rates of hydrolysis for about 2hr. The product of enzymic hydrolysis of benzylpenicillin, separated from the enzyme by dialysis of the mixture against distilled water and isolated from the diffusate by freeze-drying, exerted no inhibitory effect on the enzyme at a concentration of 23mM.

In contrast, the rates of hydrolysis of cephalosporin C in a similar experiment with 0·2ml. of enzyme were distinctly lower with each successive sample (0·25mg.). After hydrolysis of seven such samples only 48% of the original enzyme activity was detectable, as opposed to 100% when benzylpenicillin was the substrate. The results of further experiments with larger amounts of cephalosporin C and enzyme are shown in Table 6. When the solution was tested after dialysis the loss of cephalosporinase activity was not significantly different from the loss of penicillinase activity (Table 6).

The unidentified product or products of the reaction of this enzyme with cephalosporin C were separated from the enzyme by dialysis through cellophan tubing against distilled water at 20° for 18hr. and concentrated by freeze-drying. This material

Table 6. *Change in activity of β-lactamase from* Ps. pyocyanea *N.C.T.C. 8203 on reaction with cephalosporin C*

Cephalosporin C (sodium salt) (60mg.) in 1ml. of water was added to 3ml. of crude enzyme and the reaction allowed to reach completion at 30° and pH7·0 in the pH-stat. A further 60mg. of cephalosporin C in 1ml. of water was then added and the reaction allowed to reach completion under similar conditions. The total time of the two reactions was 26min. A sample of the solution was then dialysed for 18hr. against running tap water. Penicillinase and cephalosporinase activities were determined as described in the Methods section, and are expressed in arbitrary units. Dialysis of crude enzyme obtained by induction with benzylpenicillin resulted in no significant change in activity. The values for enzymic activities are the means of those from two similar experiments.

Enzyme solution	Cephalosporinase activity	Penicillinase activity
Culture supernatant	100	100
After reaction with cephalosporin C	44	37
After reaction with cephalosporin C and dialysis	62	59

was a weak inhibitor of the hydrolysis of cephalosporin C by fresh enzyme from *Ps. pyocyanea*, but the data obtained from Lineweaver–Burk plots were not sufficiently consistent to permit a decision as to whether the inhibition was competitive, non-competitive, or mixed.

Relationship of cephalosporinase activity to penicillinase activity

Several attempts to bring about a selective loss of the cephalosporinase or penicillinase activity of the crude enzyme preparation were unsuccessful. When a solution of the latter was kept for 1hr. at 50° and pH7·0, or at 20° and pH4·0 or pH10·0, there was little loss of either form of activity. After 1hr. at 100° (pH7·0) there was a complete loss of both activities. The decrease in activity that occurred during hydrolysis of cephalosporin C (see above) was accompanied by a similar decrease in activity against benzylpenicillin (Table 6).

Although acetate was liberated from the acetoxy group of cephalosporin C during the reaction of the latter with the crude enzyme preparation from *Ps. pyocyanea*, this preparation appeared to have very little acetyl-esterase activity. No hydrolysis of triacetin was observed and the rate of hydrolysis of *p*-nitrophenyl acetate was only 3% of that of benzylpenicillin at a similar molar concentration (Table 4). A qualitative test with diacetylfluorescein was only very weakly positive. Moreover, the hydrolysis of

Table 7. *Effect of di-isopropyl phosphorofluoridate on crude cephalosporinase from* Ps. pyocyanea *N.C.T.C.* 8203 *and citrus acetyl esterase*

Enzyme activities were determined manometrically at 30° (for details see the Methods section) with cephalosporin C (2·1 mM), benzylpenicillin (2·8 mM) or triacetin (215 mM) as substrate. With acetyl esterase 0·5 ml. of enzyme solution was used in each Warburg vessel. A 0·5 ml. sample of the cephalosporinase culture supernatant was used with benzylpenicillin as substrate and 0·1 ml. with cephalosporin C as substrate. Values for inhibition by di-isopropyl phosphorofluoridate (DFP) give the decrease in the rate of hydrolysis as a percentage of the corresponding rate in the absence of di-isopropyl phosphorofluoridate. After the di-isopropyl phosphorofluoridate (25 μl. of a solution of appropriate concentration in propan-2-ol) had been added to the enzyme solutions the mixtures were kept at 30° for about 20 min. before the addition of substrate. The control solutions contained the same amount of propan-2-ol. In the absence of di-isopropyl phosphorofluoridate the initial rates of hydrolysis with the acetyl esterase (μl./ of CO_2/ml./hr.) were 216 and 96 for triacetin and cephalosporin C respectively. With the cephalosporinase the rates were 2220 and 204 for cephalosporin C and benzylpenicillin respectively.

	Percentage inhibition of acetyl-esterase activity		Percentage inhibition of crude cephalosporinase activity	
Concn. of DFP (mM)	Against triacetin	Against cephalosporin C	Against benzylpenicillin	Against cephalosporin C
0·003	—	100	—	3
0·03	84	100	9	3
0·3	100	50*	0	2
3·0	100	100	12	22

* The reason for this low value is not clear.

cephalosporin C was only slightly inhibited by di-isopropyl phosphorofluoridate in a concentration of 3 mM (the inhibition being not significantly greater than that of the hydrolysis of the β-lactam ring of benzylpenicillin), whereas di-isopropyl phosphorofluoridate completely inhibited the action of citrus acetyl esterase on cephalosporin C at a concentration of 3 μM (Table 7).

Relation between inoculum size and minimum inhibitory concentration

As measured by the tube dilution method with inocula of 10^6 and 10^4 cells/tube, the minimum inhibitory concentration of benzylpenicillin for *Ps. pyocyanea* 8203 was found to be 8 mg./ml., whereas that of cephalosporin C was 4 mg./ml. Decrease of the inoculum to 1–10 cells/tube lowered the minimum inhibitory concentrations by 16–32-fold.

DISCUSSION

The finding that the β-lactamase activity of disrupted cells was much greater than that of whole cultures or of supernatants when benzylpenicillin was used as an inducer in concentrations varying from 0·01 to 1·0 mg./ml. indicates that much of the inducible enzyme in *Ps. pyocyanea* is normally cell-bound and not readily accessible to either benzylpenicillin or cephalosporin C as a substrate. Whether undamaged cells yield any significant quantity of extracellular enzyme is not certain from the present experiments. The large amount of enzyme found in culture supernatants after induction with 10 mg. of benzylpenicillin/ml. may have been liberated from cells damaged by the penicillin. Nevertheless, if the β-lactamase activity of disrupted cells is taken as a measure of the total enzyme produced, the concentration of benzylpenicillin required for maximum induction of a β-lactamase in this organism is higher by four orders of magnitude than that required with *B. cereus*. As with *S. aureus*, the high concentrations of benzylpenicillin required may reflect, at least in part, the destruction of this inducer by the enzyme. But the low activity that methicillin appeared to show as an inducer of the β-lactamase from *Ps. pyocyanea*, despite its resistance to hydrolysis by the enzyme, contrasts with the behaviour of this substance as an inducer in *S. aureus*. Methicillin has been reported to be a relatively poor inducer of β-lactamase in *Proteus morgani* and *Proteus vulgaris* (Ayliffe, 1963), but the possible masking of the enzyme by the inducer does not appear to have been considered. In the present experiments prolonged dialysis was required to uncover the enzyme induced by methicillin in high concentration and the maximum amount of enzyme synthesized may have been more than that detected.

The failure of methicillin to inhibit induction by benzylpenicillin suggests that it does not compete with the latter for the induction centre as effectively as it does for the active centre of the enzyme. It may therefore have a relatively poor affinity for the induction centre (in contrast with its high affinity for the active centre of the enzyme), or fail to gain access to the induction site, which is believed to be situated, at least in *B. cereus*, on or in the cytoplasmic membrane (Duerksen, 1964). The feeble antibacterial action of methicillin against *Ps. pyocyanea* might be accounted for by its failure to gain access to an 'antibacterial centre' that appears to be situated, at least in *S. aureus*, on the cytoplasmic membrane (Cooper, 1954). But there is no good reason to believe, as yet, that the induction centre and the antibacterial centre are identical.

With one possible exception, the results reported above are consistent with the view that the cephalosporinase and penicillinase activities of crude

enzyme preparation from *Ps. pyocyanea* are associated with a single inducible β-lactamase. With benzylpenicillin as an inducer, the appearance of both forms of activity follows a similar time-course when samples are dialysed before assay. The observation that the cephalosporinase activity of undialysed samples appeared to increase after the production of penicillinase had stopped may be accounted for by the enzymic hydrolysis of the benzylpenicillin inducer, which had previously acted as a competitive inhibitor during assay of the enzyme against cephalosporin C. No selective loss of cephalosporinase or penicillinase activity was observed under any of the conditions tried. Only one set of findings was not obviously accounted for by the assumption that both the cephalosporinase and penicillinase activities were due to the same molecule: with both benzylpenicillin and cephalosporin C as inducers, even in concentrations less than those required for maximal induction, the cephalosporinase/penicillinase activity ratio of culture supernatants from different experiments showed greater variation than could readily be attributed to experimental error. The reason for this is not clear.

At the temperature used in the present work for most measurements of enzymic activity (30°) the maximum rates of hydrolysis of cephalosporin C and 7-phenylacetamidocephalosporanic acid (cephaloram) were several-fold higher than that of benzylpenicillin. This contrasts with the relatively high resistance of these cephalosporins to hydrolysis by β-lactamases from *B. cereus* and *S. aureus*, which is reflected in values of $V_{max.}$ several orders of magnitude lower than that for benzylpenicillin (Crompton *et al.* 1962). The energy of activation with the enzyme from *Ps. pyocyanea* for the hydrolysis of cephalosporin C (8·5 ± 1·6kcal./mole) is considerably greater than that for benzylpenicillin (3·3 ± 1·1kcal./mole), and thus the ratio of $V_{max.}$ for cephalosporin C to $V_{max.}$ for benzylpenicillin at 37° is 1·29-fold that at 30° and 1·52-fold that at 10°. The energy of activation for benzylpenicillin is similar to that found for this substrate with β-lactamases from several other Gram-negative bacteria and lower than that with β-lactamases from Gram-positive bacteria (Smith & Hamilton-Miller, 1963).

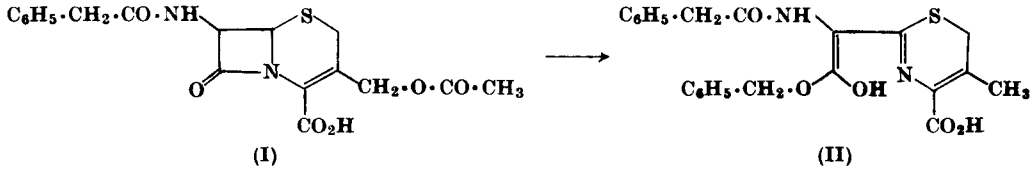
However, the high values of $V_{max.}$ for cephalosporin C and cephaloram, relative to $V_{max.}$ for benzylpenicillin, are associated with Michaelis constants that are considerably higher for the cephalosporins ($1·0 \times 10^{-3}$M) than for the penicillin ($1·3 \times 10^{-5}$M). Thus, at low substrate concentrations, e.g. 0·1mM or less, the calculated rates of hydrolysis of the cephalosporins at 30° will be lower than that of benzylpenicillin.

The hydrolysis of benzylpenicillin in the presence of the crude enzyme from *Ps. pyocyanea* at pH7·0 resulted in the liberation of 1equiv. of acid/mole. This would be expected if the only reaction that occurred resulted in the opening of the β-lactam ring with the formation of benzylpenicilloate. But if the crude enzyme had contained a benzylpenicillin amidase as well as a β-lactamase only 1equiv. of acid/mole would have been liberated at pH7·0, because opening of the β-lactam ring of 6-APA is accompanied by an increase in the pK_a of the amino group from 4·9 to 8·2. Several other tests for the presence of an amidase would fail to distinguish a mixture of an amidase and β-lactamase from a β-lactamase alone (Table 8). However, the failure to find 6-APA or the product of hydrolysis of the latter after the product of the enzymic reaction had been subjected to electrophoresis on paper and the failure to demonstrate any liberation of [1-^{14}C]phenylacetic acid from benzylpenicillin labelled with ^{14}C in its phenylacetyl side chain indicated that no significant amount of a benzylpenicillin amidase was present in the enzyme preparation. Nevertheless, the enzymic hydrolysis of benzylpenicillin resulted in the formation of at least two products, which could be resolved by electrophoresis on paper at pH4·5. A probable explanation of this finding is that the two products represent different diastereoisomers of benzylpenicilloate. The α-diastereoisomer, which is formed from benzylpenicillin in alkaline solution (Mozingo & Folkers, 1949), is known to undergo mutarotation in aqueous solution at pH7·5 (Levine, 1960). It seems possible that differences in the facility with which intramolecular hydrogen bonding occurs between the NH group (pK_a about 5) and CO_2H group of various diastereo-

Table 8. *Behaviour of benzylpenicillin on hydrolysis by a β-lactamase, a penicillin amidase, or a mixture of the two enzymes*

Details are given in the text.

Test for nature of hydrolysis — Enzyme ...	β-Lactamase	Amidase	β-Lactamase+amidase
Acid (equiv./mole) produced at pH7	1·0	1·0	1·0
Reduction of I_2 by product	+	−	+
Loss of antibacterial activity	+	+	+
Regain of activity after phenylacetylation	−	+	−
Spot (starch–iodine spray) in position of product of hydrolysis of 6-APA after electrophoresis at:			
pH4·5	+	−	+
pH7·0	−	−	+



(I) (II)

isomers are reflected in differences in mobility at pH 4·5. Rapson & Bird (1963) concluded from the results of electrometric titrations that two compounds, having groups with pK_a values of 5·3 and 4·8 respectively, could be formed from benzylpenicillin in alkaline solution. They suggested that the first of these compounds was a penicilloate and the second a penamaldate. However, penamaldates show high extinction at 282mμ (Trenner, 1949) and no compound with this property was formed during the present experiments.

Levine (1960) suggested that the gradual conversion of benzylpenicillin into D-benzylpenicilloate in aqueous solution at pH 7·4 and 37° might involve the slow formation of D-benzylpenicillenic acid and the rapid conversion of the latter into a mixture of penicilloate diastereoisomers. However, the half-life reported for benzylpenicillenic acid under these conditions (6·5min.) is too long for the compound to function as an intermediate in the enzymic hydrolysis unless both its formation and decomposition were enzyme-catalysed. Measurements of extinction at 322mμ during the action of the enzyme from *Ps. pyocyanea* on benzylpenicillin revealed that penicillenic acid was not present in more than trace amounts. But this does not exclude the possibility that it occurs as an enzyme-bound intermediate.

The enzymic hydrolysis of cephalosporin C and of cephalothin by the crude enzyme from *Ps. pyocyanea* was accompanied by the loss of a characteristic absorption band ($\lambda_{max.}$ 260mμ) and the production of 2equiv. of acid/mole: 1equiv. could be attributed to the opening of the β-lactam ring and the other to the liberation of acetic acid from the acetoxy group in these compounds. However, no significant amount of an acetyl esterase was detected in the enzyme preparation, the liberation of a second equivalent of acid was not inhibited by di-isopropyl phosphorofluoridate and the course of hydrolysis gave no indication that two reactions with different kinetics were involved. It is therefore proposed that a spontaneous expulsion of acetate from the acetoxy group occurs during the enzyme-catalysed opening of the β-lactam ring. Eggers, Emerson, Kane & Lowe (1963) reported that 7-phenylacetamidocephalosporanic acid (I) yielded a 6*H*-1,3-thiazine (II) when treated with sodium benzyl oxide in benzyl alcohol. They suggested that the reaction represented a concerted process in which extended fragmentation, involving the expulsion of acetate, was accompanied by prototropic rearrangement. But no product was formed, on enzymic hydrolysis of cephalosporin C, with an ultraviolet-absorption spectrum corresponding to a thiazine of this type. The unstable product of the enzymic reaction with $\lambda_{max.}$ 230mμ remains to be characterized. The expulsion of acetate from an acetoxy group does not appear to be involved in the formation of this product, since a compound with $\lambda_{max.}$ 230mμ was produced on hydrolysis of deacetylcephalosporin C. Moreover, the opening of the β-lactam ring of cephaloridine is apparently accompanied by the spontaneous expulsion of pyridine. However, the lactone of deacetylcephalosporin C yields one relatively stable product on hydrolysis with the enzyme from *Ps. pyocyanea*, as it does on hydrolysis with dilute alkali (Abraham & Newton, 1961).

The nature of the *N*-acyl side chain of derivatives of 7-ACA and 6-APA has a striking influence on the rate of hydrolysis of the derivative by the enzyme from *Ps. pyocyanea* and on its affinity for the enzyme.

The effect of a given change in side chain seems to be similar in the two series of compounds. It is different from the effect on the behaviour of derivatives of 6-APA to β-lactamases from *B. cereus* and *S. aureus*, but shows similarities to that observed with β-lactamases from several other Gram-negative bacteria (Hamilton-Miller, 1963; Sutherland & Batchelor, 1964; Hamilton-Miller & Smith, 1964). The attachment of an appropriate side chain to 6-APA or 7-ACA appears to be necessary for hydrolysis of the β-lactam ring by the enzyme from *Ps. pyocyanea* to occur at a significant rate, although 6-APA and 7-ACA themselves presumably combine with the active centre of the enzyme since they behave as competitive inhibitors of the hydrolysis of cephalosporin C. But methicillin, which has very low affinity for the β-lactamase from *S. aureus*, as judged by its failure to inhibit the hydrolysis of benzylpenicillin, is a much more powerful competitive inhibitor than 6-APA of the hydrolysis of benzylpenicillin and cephalosporin C by the enzyme from *Ps. pyocyanea*. Although it has a high affinity for this enzyme methicillin is resistant to hydrolysis,

as is also the corresponding 2,6-dimethoxybenzoyl derivative of 7-ACA. It appears that the dimethoxybenzoyl side chain facilitates the attachment of derivatives of 6-APA and 7-ACA to the active centre of the enzyme but that it may block the approach to the β-lactam carbonyl group of a nucleophilic grouping in the centre which initiates lactam hydrolysis. Similar considerations apply to cloxacillin. In contrast, a change in side chain from δ-(D-α-aminoadipoyl), in cephalosporin C, to phenylacetyl, in cephaloram, had relatively little effect on K_m or on $V_{max.}$.

Since the calculated quotient K_i (for benzylpenicillin)/K_m (for cephalosporin C) was of the same order as the ratio of the K_m values for benzylpenicillin and cephalosporin C (or cephaloram) respectively, the reciprocals of the K_m values may represent, approximately, the affinities of the corresponding substrates for the enzyme. The finding that the affinity of benzylpenicillin as an inhibitor was similar to its affinity as a substrate supports the view that a single enzyme is responsible for the hydrolysis of benzylpenicillin and cephalosporin C. If this is so, derivatives of 6-APA are more firmly attached to the active centre of this enzyme than are corresponding derivatives of 7-ACA, but are less readily hydrolysed at 30°. The gradual loss of activity of the enzyme during the hydrolysis of cephalosporin C (which does not occur during the hydrolysis of benzylpenicillin) raises the question whether combination with the cephalosporin is accompanied by a change in the region of the active centre to a less effective or less stable conformation.

The very high minimum inhibitory concentrations (4–8mg./ml.) for benzylpenicillin and cephalosporin C with large inocula of *Ps. pyocyanea* N.C.T.C. 8203 are almost certainly due in part to the β-lactamase that this organism produces, because a decrease of inoculum size results in a marked decrease in the minimum inhibitory concentration and because the growth did not occur at concentrations approaching these very high levels but only after most of the antibiotic had been hydrolysed (Sabath & Abraham, 1964). However, the possibility that a mechanism other than one involving a β-lactamase contributes to the resistance of this organism to benzylpenicillin and cephalosporin C has clearly not been excluded.

The finding that cloxacillin and methicillin showed only a very low activity against *Ps. pyocyanea* (minimum inhibitory concentration 4–8mg./ml.), although they were powerful competitive inhibitors of the hydrolysis of several derivatives of 6-APA and 7-ACA, suggested that they might act synergistically with the latter against this organism. The results of experiments that showed that synergism did in fact occur have been reported by Sabath & Abraham (1964).

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