The acyl-enzyme mechanism of β -lactamase action

The evidence for class C β -lactamases

Vroni KNOTT-HUNZIKER,* Sigthor PETURSSON,* Stephen G. WALEY,* Bengt Jaurin† and Thomas GRUNDSTRÖM†

*Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K., and †Department of Microbiology, University of Umeå, S-90187 Umeå, Sweden

(Received 10 May 1982/Accepted 12 July 1982)

Methanol or ethanol can replace water in the action of certain chromosomal β -lactamases on benzylpenicillin: the products are α -methyl or α -ethyl benzylpenicilloate. The β -lactamases were from a mutant of *Pseudomonas aeruginosa* 18S that produces the enzyme constitutively [Flett, Curtis & Richmond (1976) *J. Bacteriol.* **127**, 1585–1586; Berks, Redhead & Abraham (1982) *J. Gen. Microbiol.* **128**, 155–159] and from *Escherichia coli* K12 (the *ampC* β -lactamase) [Lindström, Boman & Steele (1970) *J. Bacteriol.* **101**, 218–231]. The variation of the rates of alcoholysis and hydrolysis with concentration of alcohol show that the rate-determining step is breakdown of an intermediate. This intermediate is likely to be the acyl-enzyme. The esters, α -methyl or α -ethyl benzylpenicilloate, are themselves substrates for the *Pseudomonas* β -lactamase, benzylpenicilloic acid being formed. Thus this β -lactamase can be an esterase. The kinetics for the hydrolysis of cloxacillin by the *Pseudomonas* β -lactamase are consistent with the acyl-enzyme, formed by acylation of serine-80, being an intermediate in the overall hydrolysis.

β -Lactamases are efficient and diverse enzymes that present challenges both to the clinician and to the biochemist. They play a large (and sometimes overwhelming) part in antibiotic resistance. Classification based on the amino acid sequences of the enzymes has led to the recognition of three distinct classes, called A, B and C (Ambler, 1980; Jaurin & Grundström, 1981). Members of class A are now regarded as serine enzymes (Knott-Hunziker *et al.*, 1979, 1980b; Cohen & Pratt, 1980; Fisher *et al.*, 1981; Waley, 1981b). Two chromosomally encoded β -lactamases have been assigned to class C: the enzyme specified by the *ampC* gene of *Escherichia coli* K12 (Lindström *et al.*, 1970; Jaurin & Grundström, 1981) and the enzyme from *Pseudomonas aeruginosa* (Sabath *et al.*, 1965; Flett *et al.*, 1976; Berks *et al.*, 1982). These are referred to below as the *ampC* β -lactamase and the *Pseudomonas* β -lactamase respectively. We have used a substrate with a low k_{cat} (cloxacillin) to show that these, also, seem to be serine enzymes, albeit unrelated to the class A serine enzymes (Knott-Hunziker *et al.*, 1980a, 1982). Kinetic evidence is now presented for the role of the acyl-enzyme from cloxacillin as an

intermediate. We also show that class C β -lactamases, unlike the class A enzymes, can utilize alcohols as a substitute for water; the kinetics suggest that the acyl-enzyme accumulates during the hydrolysis of a good substrate. Moreover, the *Pseudomonas* β -lactamases can catalyse the hydrolysis of penicilloyl esters to penicilloic acids, i.e. the enzyme can function as an esterase. This is the first time that a β -lactamase has been reliably shown to catalyse the hydrolysis of any compound other than a β -lactam. Thus the structural differences between class A and class C β -lactamases give rise to significant kinetic differences.

Materials and methods

Materials

The β -lactamases from *Pseudomonas aeruginosa* and *E. coli* were prepared as described previously (Knott-Hunziker *et al.*, 1980a; Jaurin & Grundström, 1981). α -Methyl and α -ethyl benzylpenicilloate were prepared from benzylpenicillin, essentially as described by Busson *et al.* (1976). α -Methyl benzylpenicilloate had m.p. 129–131°C [Busson *et al.* (1976) give 125.5–127°C], and α -ethyl benzylpenicilloate, crystallized as the benzylammonium salt, had m.p. 138–139°C [Mozingo & Folkers

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

(1949) give m.p. 140–141°C]. The n.m.r. spectra were consistent with the structures.

α -Methyl 6-(3-*o*-chlorophenyl-5-methylisoxazole-4-carboxyamido)penicilloate was similarly prepared from cloxacillin, and had m.p. 80–85°C (dehydrates) and $[\alpha]_{589}^{20} +45.5^\circ$ (*c* 1 in methanol). The n.m.r. spectrum recorded on a Bruker 300 MHz instrument in C^2HCl_3 with tetramethylsilane as an internal standard showed δ (p.p.m.) 1.23 and 1.54 [3H, S–C(CH₃)₂], 2.78 (3H, s, isoxazole–5–CH₃), 3.46 (1H, s, H-3), 3.71 (3H, s, OCH₃), 4.66 (1H, dd, $J_{H-6, H-5}$ 4.9 Hz, $J_{H-6, NH}$ 7.5 Hz, H-6), 4.87 (1H, d, $J_{H-5, H-6}$ 4.9 Hz, H-5), 5.2 (2H, broad, 4–NH₂⁺), 6.15 (1H, d, $J_{NH, H-6}$ 7.5 Hz, amide NH) and 7.44–7.57 (4H, m, aromatic) (Found: C, 46.2; N, 7.8; C₂₀H₂₂ClN₃O₆S·3H₂O requires C, 46.0; N, 8.1%; partial loss of water of crystallization, and decomposition, were encountered on attempted drying; analysis was by Dr. F. B. Strauss, Oxford).

Methods

H.p.l.c. was performed with a Waters Associates system (6000A solvent delivery system and U6K injector) on a column (25 cm × 0.46 cm diam.) filled with Shandon ODS-Hypersil C18 (5 μ m); u.v. detection at 225 nm in a 10 mm-pathlength cell was used, with a Pye–Unicam LC-UV detector. The mobile phase was 20% (v/v) acetonitrile in 25 mM-sodium phosphate buffer, pH 7, and the flow rate was 1 ml/min. The hydrolysis of benzylpenicillin (10 mM) was performed in 0.1 M-sodium phosphate buffer, pH 7 (or 0.2 M-sodium acetate buffer, pH 5.5), in the presence of ethanol or methanol at 30°C; the concentration of β -lactamase was in the range 5–50 nM. Portions (10 μ l) of the reaction mixture were added to 1 ml volumes of the chromatographic mobile phase, at 4°C (this effectively quenches the reaction), and then 100 μ l samples were taken for chromatography. The relative amounts of benzylpenicillin and the penicilloates were estimated by cutting out and weighing the peaks, the total amount injected being taken as 10 nmol. A given amount of each compound (e.g. 5 nmol) gave a peak of approximately the same area. The hydrolysis of α -ethyl benzylpenicilloate and α -methyl benzylpenicilloate was measured similarly.

Results

Alcoholysis of β -lactams by class C β -lactamases

The products of the action of the *Pseudomonas* β -lactamase on benzylpenicillin (Scheme 2, compound 1) in the presence of ethanol, analysed by h.p.l.c. were benzylpenicilloate (Scheme 2, compound 2) and α -ethyl benzylpenicilloate (Scheme 2, compound 3, R = Et) (Fig. 1). Similarly, in the presence of methanol, α -methyl benzylpenicilloate (Scheme 2, compound 3, R = Me) was formed, both

Fig. 1. H.p.l.c. of products from the action of the *Pseudomonas* β -lactamase on benzylpenicillin in the presence of 10% (v/v) ethanol

The conditions were as described in the Materials and methods section. The reaction was performed at pH 7. (a) shows the initial chromatogram (benzylpenicillin), (b) shows an intermediate time (30 min), and (c) shows the final chromatogram; virtually all the benzylpenicillin has been consumed and the products are the benzylpenicilloic acid (eluted earlier) and the α -ethyl benzylpenicilloate, eluted after benzylpenicillin.

Fig. 2. H.p.l.c. of products from the action of the class C β -lactamases on benzylpenicillin in the presence of 10% (v/v) methanol

The conditions were as described in the Materials and methods section. The reaction was performed at pH 7. The concentration of benzylpenicillin was 10 mM, and the concentrations of the *Pseudomonas* and *ampC* β -lactamases were 21 nM and 27 nM respectively. (a) shows the products from the *Pseudomonas* enzyme (after reaction for 42 min), and (b) shows the products from the *ampC* β -lactamase (after reaction for 63 min). Reaction is virtually complete in both cases. The amount of material applied to the column corresponds to the digestion of 10 nmol of benzylpenicillin. The peak eluted first is benzylpenicilloic acid and the one eluted second is α -methyl benzylpenicilloate.

with the *Pseudomonas* β -lactamase and with the *ampC* β -lactamase (Fig. 2). Neither β -lactamase I nor β -lactamase II from *Bacillus cereus*, representative class A and class B enzymes, gave detectable amounts of ester.

In partition experiments, it is important to measure the rates of formation of individual products, and so h.p.l.c. was used to measure k_{cat} for the production of α -ethyl benzylpenicilloate (k_{p}) and of benzylpenicilloic acid (k_{s}) as well as for the loss of benzylpenicillin ($k_{-\text{s}}$). The increase in $k_{-\text{s}}$ with

Fig. 3. Variation of k_{cat} with concentration of ethanol in the action of the *Pseudomonas* β -lactamase on benzylpenicillin

The reaction was performed at pH 7 with 10 mM-benzylpenicillin as described in the Materials and methods section. The measured k_{cat} for the formation of α -ethyl benzylpenicilloate (O), called k_{p} in eqn. (2), is directly proportional to the concentration of ethanol. The measured k_{cat} for the disappearance of benzylpenicillin (Δ), referred to as $k_{-\text{s}}$ in eqn. (1), increases linearly with the concentration of ethanol; the k_{cat} for the formation of benzylpenicilloic acid ($\square$), referred to as k_{p} in eqn. (2), is independent of the concentration of ethanol.

Scheme 1

concentration of ethanol for the action of the *Pseudomonas* β -lactamase on benzylpenicillin was linear (Fig. 3). The value of k_{p} (benzylpenicilloic acid) was independent of the concentration of ethanol, and the value of k_{Q} was directly proportional to the concentration of ethanol (Fig. 3). Product was thus formed in the rate-determining step, and an intermediate, taken to be the acyl-enzyme, must accumulate (see Scheme 1). The same

pattern was obtained with methanol instead of ethanol (Fig. 4) for the *Pseudomonas* and the *ampC* β -lactamases and at pH 5.5 as well as at pH 7.

The simplest mechanism is set out in Scheme 1.

Fig. 4. Variation of k_{cat} with concentration of methanol in the action of the *Pseudomonas* β -lactamase on benzylpenicillin

The reaction was performed at pH 7, with 10 mM-benzylpenicillin, as described in the Materials and methods section. Formation of α -methyl benzylpenicilloate (O), loss of benzylpenicillin (Δ) and formation of benzylpenicilloic acid ($\square$) are shown. The pattern is similar to that in Fig. 3.

where E and S refer to enzyme and substrate, ES is the (non-covalent) enzyme-substrate complex, X is the second intermediate taken to be the acyl-enzyme, P and Q refer to benzylpenicilloic acid and α -methyl or α -ethyl benzylpenicilloate and ROH refers to methanol or ethanol. It may be shown that, if $k_{+2} \gg k_{-2}$, k_{+3} , $k_{+4}[\text{ROH}]$, then the k_{cat} for the disappearance of benzylpenicillin is:

$$k_{-S} = k_{+3} + k_{+4}[\text{ROH}] \quad (1)$$

This obtains for the initial rate under steady-state conditions when the concentration of substrate is large enough. The values given below (Table 2) show that, at the concentration of benzylpenicillin (10 mM) used in these experiments, the rate approaches closely to V_{max} . Similarly, for the appearance of benzylpenicilloic acid (k_p) and ester (k_q) the equations are:

$$k_p = k_{+3} \quad (2)$$

$$k_q = k_{+4}[\text{ROH}] \quad (3)$$

At the concentrations of enzyme used in these experiments (approx. 20 nM), hydrolysis of the esters (see below) is negligible.

The values in Table 1 (where the ranges of concentration of ethanol and methanol were 0.88–3.58 M and 0.6–5 M respectively for the *Pseudomonas* enzyme, and the range of concentration of methanol for the *ampC* enzyme was 2.5–5 M) show that ethanol is approximately half as reactive as methanol. Water (55 M) is approximately twice as reactive as 1 M-methanol for the penicilloyl- (*Pseudomonas*) enzyme, and about 9 times as reactive for the penicilloyl- (*ampC*) enzyme. The value of the ratio k_{+3}/k_{+4} seems to be characteristic of the enzyme, but more or less independent of the substrate, and had approximately the same value at pH 5.5 as at pH 7. As far as can be told, the condition $k_{+2} \gg k_{-2}$, k_{+3} , $k_{+4}[\text{ROH}]$ holds, since if k_{+2} were comparable with (say) $k_{+4}[\text{ROH}]$ then k_q would no longer be directly proportional to the concentration of methanol. It was not possible to use

Table 1. Rate constants for alcoholysis catalysed by class C β -lactamases

The reactions were performed with 10 mM substrate in 0.1 M-sodium phosphate buffer, pH 7, or 0.2 M-sodium acetate buffer, pH 5.5, at 30°C in the presence of (a) ethanol or (b) methanol. The range of concentrations of ethanol was 0.88–3.58 M, and the range of concentrations of methanol was 0.6–5 M for the *Pseudomonas* β -lactamase and 2.5–5 M for the *ampC* β -lactamase. The reaction mixtures were analysed by h.p.l.c. as described in the text. The values of k_{+3} and k_{+4} were obtained from eqns. (1)–(3) in the text.

	Substrate	Enzyme	pH	k_{+3} (min ⁻¹)	k_{+4} (M ⁻¹ ·min ⁻¹)	k_{+3}/k_{+4} (M)
(a) Ethanol present	Benzylpenicillin	<i>Pseudomonas</i>	7	3740	1050	3.55
(b) Methanol present	Benzylpenicillin	<i>Pseudomonas</i>	7	3600	2100	1.8
	Cloxacillin	<i>Pseudomonas</i>	7	1.5	1.25	1.4
	Benzylpenicillin	<i>Pseudomonas</i>	5.5	2100	1250	1.7
	Benzylpenicillin	<i>ampC</i>	7	4800	550	8.8
	Benzylpenicillin	<i>ampC</i>	5.5	3100	340	9.2

Table 2. Kinetic parameters for the action of the *ampC* and *Pseudomonas* β -lactamases on benzylpenicillin in the presence of methanol

The initial rate of change in A_{232} for reactions in 0.1 M-sodium phosphate buffer pH 7, at 30°C was measured by the chord method (Waley, 1981a). The concentration of benzylpenicillin was varied over the range 7 μ M–2 mM and the concentration of enzyme was in the range 5–50 nM. Values for the kinetic parameters were obtained with the program of Cornish-Bowden based on the method of Cornish-Bowden & Eisenthal (1978).

Concn. of methanol [M]	<i>Pseudomonas</i> β -lactamase			<i>ampC</i> β -lactamase		
	$10^{-3} \times k_{\text{cat}}$ (min ⁻¹)	K_m (μ M)	k_{cat}/K_m (μ M ⁻¹ ·min ⁻¹)	$10^{-3} \times k_{\text{cat}}$ (min ⁻¹)	K_m (μ M)	k_{cat}/K_m (μ M ⁻¹ ·min ⁻¹)
0	4.5	11	420	5.7	10	570
1.25	10	52	200	6.9	22	310
2.49	17	130	140	7.1	24	300
3.74	20	190	110	7.9	50	160
5.01	27	540	49	9.2	98	94

Fig. 5. Variation of k_{cat}/K_m with concentration of methanol in the action of the β -lactamases on benzylpenicillin

The hydrolysis of benzylpenicillin was measured by the change in A_{232} (Waley, 1974), and K_m/k_{cat} values were obtained from the initial rates. ○, *Pseudomonas* β -lactamase; Δ, *ampC* β -lactamase.

Table 3. Hydrolysis of α -methyl benzylpenicilloate by the *Pseudomonas* β -lactamase

The hydrolysis of 10 mM- α -methyl benzylpenicilloate was performed in 100 mM-sodium phosphate buffer, pH 7, at 30°C. The reaction was followed by h.p.l.c.; the details are as described in the Materials and methods section.

Concn. of enzyme (μ M)	Initial rate (μ M/min)
1.6	88
4	200
8	420

The experiments so far described were conducted at high concentrations of substrate. We come now to experiments in which the concentration of substrate was altered. The variation of k_{cat}/K_m and K_m with concentration of methanol was determined from initial-rate measurements by the spectrophotometric assay (Waley, 1974). The change in A_{232} was the same, to within 5%, in the presence or in the absence of 5 M-methanol. Since, in the action of the *Pseudomonas* enzyme on benzylpenicillin, the methyl ester represents about 80% of the products, the absorbance change reflects opening of the β -lactam ring, and is approximately the same whether the acceptor is water or an alcohol. The effect of methanol was pronounced (Table 2). The value of k_{cat}/K_m decreased with increasing concentration of methanol (Fig. 5), and the organic-solvent effect, which swamped the effect of nucleophilic partitioning, was comparable with that found for reactions catalysed by chymotrypsin (Maurel, 1978). The magnitude of the effect, which perhaps suggests an appreciable contribution from hydrophobic interactions in binding was similar for both enzymes (Fig. 5).

The action of the *Pseudomonas* β -lactamase on

higher concentrations of methanol than 5 M because the enzyme was no longer stable. Nor have we so far succeeded in detecting partitioning with a more reactive nucleophile (e.g. NH_3 , NH_2OH), perhaps partly because most such compounds react readily with penicillins.

Fig. 6. Hydrolysis of cloxacillin by the *Pseudomonas* β -lactamase

Cloxacillin (5 mM) was digested with *Pseudomonas* β -lactamase (72 μ M) in 0.1 M-sodium phosphate buffer, pH 7, at 30°C. The products were analysed by h.p.l.c., 10 μ l portions being diluted with 50 vol. of 75% (v/v) acetonitrile, and passed through a C₁₈ Sep-Pak cartridge (Waters Associates), which was then washed with 0.5 ml of 75% (v/v) acetonitrile. The combined filtrate and washings were diluted with 2 vol. of 25 mM-sodium phosphate buffer, pH 7, and 100 μ l portions were taken for h.p.l.c. under the conditions described in the Materials and methods section. Concentrations of cloxacillin (O) and of the corresponding penicilloic acid (Δ) are shown. The right-hand part of the Figure ($\blacktriangle$) shows the hydrolysis of a second portion of substrate, added after 70 min.

cephalosporin C in the presence of methanol gave results that paralleled those obtained with benzylpenicillin. The instability of the products (Hamilton-Miller *et al.*, 1970) disfavours cephalosporin C for detailed study.

Esters of penicilloic acid are substrates of the Pseudomonas β -lactamase

The fact that α -methyl benzylpenicilloate was a product from the *Pseudomonas* enzyme and benzyl-

penicillin in the presence of methanol suggested that the ester itself might be a substrate. This proved to be so. The initial rate of hydrolysis (which was approx. 1% of the rate for benzylpenicillin) was directly proportional to the concentration of enzyme (Table 3), showing that the rate of non-enzymic hydrolysis was negligible under these conditions, as expected from the data of Frère (1981). Moreover, there was no detectable hydrolysis by β -lactamase I (in 100 min at 30°C, 7.5 μ M-enzyme), nor by β -lactamase II (in 100 min at 30°C, 7.5 μ M-enzyme).

mase II (in 120 min at 30°C, 6 μ M-enzyme) (results not shown). α -Ethyl benzylpenicilloate was also a substrate of the *Pseudomonas* β -lactamase, albeit a poorer one; the initial rate of hydrolysis was about one-fifth of that of the methyl ester. The route of hydrolysis of the ester via the acyl-enzyme is depicted in Scheme 2.

Cloxacillin as a substrate of the Pseudomonas β -lactamase

Although cloxacillin has in the past been regarded as merely an inert inhibitor of the *Pseudomonas* β -lactamase, it is in fact a substrate (Fig. 6). The value of k_{cat} (at 30°C, pH 7) was $1.5 \pm 0.2 \text{ min}^{-1}$ [from five determinations, confidence limit obtained as Dean & Dixon (1951)]. When a second portion of substrate was added at the end of the reaction, this was hydrolysed at essentially the same rate (Fig. 6), showing that the enzyme was not inactivated by the cloxacillin. The K_m for cloxacillin is too low to be measured directly, but a reliable value can be obtained by the use of cloxacillin as a competing substrate; the value was 17 nM [cf. 14 nM obtained by Berks *et al.* (1982) under slightly different conditions], so the value for k_{cat}/K_m is about one-quarter of that for benzylpenicillin. This would seem to justify regarding cloxacillin as a specific substrate.

Fig. 7. Regain of activity of *Pseudomonas* β -lactamase after incubation with cloxacillin

Cloxacillin (20.4 μ M) and *Pseudomonas* β -lactamase (1.4 μ M) were incubated together for 18 min, and then a 50 μ l portion was added to 1.05 ml of 2.19 mM-benzylpenicillin in 0.1 M-sodium phosphate buffer, pH 7, containing 0.45 M-NaCl, at 30°C, and A_{232} was monitored (light-path 3 mm).

The rate of hydrolysis of cloxacillin was also monitored as follows. Cloxacillin and the *Pseudomonas* β -lactamase were incubated together, and portions of the mixture were withdrawn and diluted for assay with benzylpenicillin as substrate (Fig. 7). The increase in slope (regain of activity) was first-order, and the rate constant ($1.5 \pm 0.3 \text{ min}^{-1}$) [median, 95% confidence limits, Dean & Dixon (1951)] was the same as the k_{cat} obtained for hydrolysis. The regain of activity is assigned to deacylation, as it is known that the acyl-enzyme is formed under these conditions (Knott-Hunziker *et al.*, 1980a). The presence of methanol (10–20%, v/v) increased the rate of action of the *Pseudomonas* β -lactamase on cloxacillin (Table 1), and the corresponding methyl ester was formed. The methyl ester was identified by comparison with an authentic specimen obtained by base-catalysed methanolysis of cloxacillin. The value of k_{+3}/k_{+4} was 1.4 M, similar to the value for benzylpenicillin and the *Pseudomonas* β -lactamase (Table 1).

Discussion

Acyl-enzyme mechanism for β -lactamases

Class A β -lactamases are now generally regarded as proceeding by an acyl-enzyme mechanism, although there is still scanty evidence for the intervention of an acyl-enzyme in the hydrolysis of a good substrate. All experiments to detect nucleophilic partitioning have proved fruitless (Brenner *et al.*, 1981; Anderson & Pratt, 1981; S. G. Waley, unpublished work). The isolation of a 'covalent compound' under conditions in which the putative acyl-enzyme unfolds is not, in itself, sufficient evidence for an intermediate (Kiener *et al.*, 1980; Cartwright & Fink, 1982). Conversely, the acyl-enzyme from cefoxitin, known to be an intermediate (Fisher *et al.*, 1980), is more prone to proteolysis (presumably a sign of unfolding) than is free enzyme (Citri & Zyk, 1982).

It is quite otherwise with class C β -lactamases, where, as described above, an intermediate accumulates during the hydrolysis of a good substrate (benzylpenicillin). The fortunate circumstance of nucleophilic partitioning makes the case for the intervention of an acyl-enzyme quite strong. This case is supported by the isolation of the acyl-enzyme from a specific substrate with an exceptionally low k_{cat} . Finally, a class C β -lactamase can function as an esterase. Thus the analogy with the behaviour of well-characterized serine proteinases is here quite close. The class C β -lactamases appear to be unrelated to the D-alanine carboxypeptidases (Jaurin & Grundström, 1981), nor is there any evidence for relatedness to proteinases.

In structural terms, the acyl-enzyme is derived from the α -carboxy group of penicilloic acid and the

Scheme 3. Classification of β -lactamases

hydroxy group of serine-80 (Scheme 2). It turns out that it is the hydrolysis of this ester, rather than of the β -lactam ring, that is the rate-determining step in the hydrolysis of benzylpenicillin by the class C β -lactamases studied in the present work. This is perhaps not unexpected, since β -lactams are rather more reactive than esters. Whether the same holds for class A β -lactamases remains uncertain.

Molecular classification of β -lactamases

Provisionally, the classification of β -lactamases based on structure and mechanism may be set out as shown in Scheme 3. At the moment, the sole representative of class B is β -lactamase II from *Bacillus cereus* (Sabath & Abraham, 1966); there is no evidence for (or against) an acyl-enzyme mechanism (Hill *et al.*, 1980). The diversity of β -lactamases, a fact unpleasantly evident to the clinician, suggests that further classes will be discovered. An example of the relevance of the molecular classification is that class C β -lactamases are not effectively inactivated by clavulanic acid (Reading & Cole, 1977).

Class C β -lactamases are commonly referred to as cephalosporinases. This is something of a misnomer. Thus k_{cat}/K_m is 12 times greater for benzylpenicillin than for cephalosporin C for the *Pseudomonas* enzyme (Sabath *et al.*, 1965), and it is k_{cat}/K_m that determines specificity. The term cephalosporinase has come into use because the activity of the β -lactamase is usually measured with high concentrations of substrate.

The support of the Medical Research Council is gratefully acknowledged. This work was supported by grants from the Swedish Natural Science Research Council (Dnr 3373) and from the Swedish Medical Research Council (Dnr 5428). We thank Mr. N. Gascoyne and Mr. T. Beesley for expert assistance and Beecham Research Laboratories and Glaxo Research Laboratories for gifts. This paper is a contribution from the Oxford Enzyme Group.

References

- Ambler, R. P. (1980) *Philos. Trans. R. Soc. London Ser. B* **289**, 321–331
- Anderson, E. G. & Pratt, R. F. (1981) *J. Biol. Chem.* **256**, 11401–11404
- Berks, M., Redhead, K. & Abraham, E. P. (1982) *J. Gen. Microbiol.* **128**, 155–159
- Brenner, D. G., Charnas, R. L., Fisher, J., Kemal, C. & Knowles, J. R. (1981) in *β -Lactam Antibiotics* (Salton, M. R. J. & Shockmann, G. D., eds.), pp. 301–310. Academic Press, New York.
- Busson, R., Claes, P. J. & Vanderhaeghe, H. (1976) *J. Org. Chem.* **41**, 2556–2560
- Cartwright, S. J. & Fink, A. L. (1982) *FEBS Lett.* **137**, 186–188
- Citri, N. & Zyk, N. (1982) *Biochem. J.* **201**, 425–427
- Cohen, S. A. & Pratt, R. F. (1980) *Biochemistry* **19**, 3996–4003
- Cornish-Bowden, A. & Eisenthal, R. (1978) *Biochim. Biophys. Acta*, **523**, 268–272
- Dean, R. B. & Dixon, W. J. (1951) *Anal. Chem.* **23**, 636–638
- Fisher, J., Belasco, J. G., Khosla, S. & Knowles, J. R. (1980) *Biochemistry* **19**, 2895–2901
- Fisher, J., Charnas, R. L., Bradley, S. M. & Knowles, J. R. (1981) *Biochemistry* **20**, 2726–2731
- Flett, F., Curtis, N. A. C. & Richmond, M. H. (1976) *J. Bacteriol.* **127**, 1585–1586
- Frère, J.-M. (1981) *Biochem. Pharmacol.* **30**, 549–552
- Hamilton-Miller, J. M. T., Newton, G. G. F. & Abraham, E. P. (1970) *Biochem. J.* **116**, 371–384
- Hill, H. A. O., Sammes, P. G. & Waley, S. G. (1980) *Philos. Trans. R. Soc. London Ser. B* **289**, 333–344
- Jaurin, B. & Grundström, T. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4897–4901
- Kiener, P. A., Knott-Hunziker, V., Petursson, S. & Waley, S. G. (1980) *Eur. J. Biochem.* **109**, 575–580
- Knott-Hunziker, V., Waley, S. G., Orlek, B. S. & Sammes, P. G. (1979) *FEBS Lett.* **99**, 59–61
- Knott-Hunziker, V., Redhead, K., Petursson, S. & Waley, S. G. (1980a) *FEBS Lett.* **121**, 8–10
- Knott-Hunziker, V., Orlek, B. S., Sammes, P. G. & Waley, S. G. (1980b) *Biochem. J.* **187**, 797–802
- Knott-Hunziker, V., Petursson, S., Jayatilake, G. S., Waley, S. G., Jaurin, B. & Grundström, T. (1982) *Biochem. J.* **201**, 621–627
- Lindström, E. B., Boman, H. G. & Steele, B. B. (1970) *J. Bacteriol.* **101**, 218–231
- Maurel, P. (1978) *J. Biol. Chem.* **253**, 1677–1683
- Mozingo, R. & Folkers, K. (1949) in *The Chemistry of Penicillin* (Clarke, H. T., Johnson, J. R. & Robinson, R., eds.), pp. 535–656. Princeton University Press, Princeton
- Reading, C. & Cole, M. (1977) *Antimicrob. Agents Chemother.* **11**, 852–857
- Sabath, L. D. & Abraham, E. P. (1966) *Biochem. J.* **98**, 11c–13c
- Sabath, L. D., Jago, M. & Abraham, E. P. (1965) *Biochem. J.* **96**, 739–752
- Waley, S. G. (1974) *Biochem. J.* **139**, 789–790
- Waley, S. G. (1981a) *Biochem. J.* **193**, 1009–1012
- Waley, S. G. (1981b) *Chem. Ind. (London)* 131–134