Cryoenzymology of β-Lactamases†

Steven J. Cartwright and Stephen G. Waley*
Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.
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ABSTRACT: The cryoenzymology of several different β-lactamases has been investigated. Particular attention has been paid to the experimental pitfalls of the technique. These include such factors as false bursts at the start of the reaction, instability of the enzymes during turnover, and K_m values so high that little of the enzyme is present as a complex. Many of the difficulties in cryoenzymology stem from the use of organic cryosolvents. A novel "salt" cryosolvent has been tested: ammonium acetate solutions can be used down to about −60 °C. The enzymes examined are readily soluble, and stable, in this solvent. Nevertheless, out of 17 β-lactamase β-lactam systems, only 4 proved suitable for detailed investigation. In two of these, the hydrolysis of nitrocefin or 7-(thienyl-2-acetamido)-3-[[2-[[4-(dimethylamino)phenyl]azo]pyridinio]-methyl]cephem-4-carboxylic acid (PADAC), by β-lactamase I from *Bacillus cereus*, substrate was converted into product at a slow enough rate (at −60 or −55 °C, respectively) for it to be possible to do successive scans during the course of the reaction. The spectra were those of substrate and product, and no intermediate was detected. The results argue against the accumulation of intermediate acyl-enzyme. The hydrolysis of PADAC by the P99 β-lactamase from *Enterobacter cloacae* again showed spectra characteristic of substrate and product, and there was, moreover, a break in the Arrhenius plot; it is possible that a conformational change is (at least partially) rate-determining. The hydrolysis of dinitrophenylpenicillin by the P99 β-lactamase did show features suggesting the accumulation of acyl-enzyme. Spectroscopic changes were complex, but low-temperature exclusion chromatography (under various conditions) provided evidence for a covalently labeled enzyme.

Cryoenzymology holds out high hopes of studying intermediates in enzyme reactions. Intermediates can be characterized spectroscopically, provided that they accumulate during the course of the reaction. The most powerful technique is the combination of cryoenzymology and crystallography (Douzou & Petsko, 1984), followed by the combination of cryoenzymology and nuclear magnetic resonance (Lazarus et al., 1982; Mackenzie et al., 1984). But by far the most widespread method of scrutiny in cryoenzymology is absorption spectroscopy (Fink & Cartwright, 1981), and indeed this is a useful prelude to any more demanding method.

The first necessity in the cryoenzymological study of intermediates is that they should accumulate. Thus a relatively low K_m is important. For example, the accumulation of a second intermediate (Y) (e.g., an acyl-enzyme) in the three-step mechanism

$$E + S \rightleftharpoons X \xrightarrow{k_2} Y \xrightarrow{k_3} E + P$$

depends on both K_m and k_3/k_2 and may readily be shown to be

$$[Y]/[E]_{total} = 1/[(1 + K_m/[S])(1 + k_3/k_2)]$$

This holds for steady-state conditions and will be an upper limit if the concentration of enzyme becomes comparable with that of the substrate. Unfortunately, it is all too common for K_m to be raised, often greatly, in organic cryosolvents (Fink & Cartwright, 1981; Douzou, 1986). Indeed, the use of such solvents as 50% dimethyl sulfoxide or 70% methanol has introduced a number of problems, many of which are illustrated in the work reported here. Thus the present paper is to some extent an exploration of the pitfalls in cryoenzymology. We thought it useful to present an explicit and detailed account of the factors that make a system suitable for detailed study by cryoenzymology. The disadvantages of organic cryosolvents can often be avoided by the use of salt solutions, and we describe a novel system that can be used down to about −60 °C.

The enzymes investigated in the present work are β-lactamases, mechanistically interesting and clinically important enzymes (Cartwright & Waley, 1983; Coulson, 1985; Frere & Joris, 1985; Knowles, 1985). The present work may help to pave the way for the crystallographic cryoenzymology of β-lactamases; the electron-density map of β-lactamase I from *Bacillus cereus* at 2.5-Å resolution is being interpreted (Samraoui et al., 1986), and the P99 β-lactamase from *Enterobacter cloacae* is also being studied crystallographically (Charlier et al., 1983). These are enzymes containing an active-site serine that react by an acyl-enzyme mechanism (Coulson, 1985; Bicknell & Waley, 1985a), first shown for a related β-lactamase (Fisher et al., 1980). The zinc β-lactamase II from *B. cereus* has already been studied by cryoenzymology (Bicknell & Waley, 1985b) and by scanning stopped-flow spectroscopy (Bicknell et al., 1987).

The first part of Results lists the considerations necessary for a low-temperature study in the order that they would be encountered. The detailed study of four systems is described.

Materials and Methods

Materials. β-Lactamases I and II from *B. cereus* 569/H/9 were as previously described (Baldwin et al., 1980). β-Lactamase from *E. cloacae* P99 (henceforth referred to simply as P99) (Marshall et al., 1972) and the constitutive β-lactamase from *Pseudomonas aeruginosa* 1822/S/H (henceforth referred to as the *Pseudomonas* enzyme) (Sabath et al., 1965; Flett et al., 1976; Berks et al., 1982) were purified by affinity chromatography (Cartwright & Waley, 1984).

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Nitrocefin (O'Callaghan et al., 1972) was from Becton Dickinson UK Ltd. (Oxford, UK); it had λ_{max} = 386 nm, ϵ = 17 500 M^{-1} cm^{-1} and λ_{max} = 482 nm, ϵ = 17 400 M^{-1} cm^{-1} for substrate and product, respectively. Benzylpenicillin and cephalosporin C were gifts from Glaxo Laboratories (Greenford, Middx., UK). PADAC[1] (Schindler & Huber, 1980) was a gift from Hoechst AG (Frankfurt am Main, Germany); substrate and product had λ_{max} = 573 nm, ϵ = 57 750 M^{-1} cm^{-1} and λ_{max} = 466 nm, ϵ = 36 100 M^{-1} cm^{-1}, respectively. Imipenem was a gift from Merck Sharp and Dohme Research Laboratories (Rahway, NJ); substrate and product had λ_{max} = 299 nm, ϵ = 9679 M^{-1} cm^{-1} and λ_{max} = 270 nm (shoulder), ϵ = 1400 M^{-1} cm^{-1}, respectively. 6-(Dansylamino)penicillanic acid was prepared as previously described (Cartwright & Fink, 1982): λ_{max} = 540 nm (fluorescence, after excitation at 345 nm). The fluorescence intensity of the product was 25% greater than that of the substrate. (Dinitrophenyl)penicillanic acid was prepared according to the method of Schneider and de Weck (1966); substrate and product had λ_{max} = 354 nm, ϵ = 20 000 M^{-1} cm^{-1} and λ_{max} = 359 nm, ϵ = 20 400 M^{-1} cm^{-1}, respectively, λ_{max} = 455 nm (fluorescence after excitation at 300 nm), and the fluorescence of the product had λ_{max} = 460 nm and intensity 72% that of the substrate. Solvents were of gold-label grade from Aldrich Chemical Co. (Gillingham, Dorset, UK).

Methods. Cryosolvents containing organic solvents were prepared as described by Fink and Geeves (1979). The composition of solvents was by volume. The apparent protonic activity (pH*) in the aqueous organic solvents was the reading on a glass electrode at 0 °C. The pH below 0 °C was estimated by extrapolation from plots of pH* against temperature over the range 0–30 °C. These are the procedures recommended by Douzou et al. (1976). Unless otherwise stated in the text, cryosolvents containing organic solvents were made up with triethanolamine hydrochloride and sodium chloride to pH* 8 (0 °C) and a final concentration of 20 and 80 mM, respectively. Cryosolvents at pH* 4.25 employed chloroacetate rather than triethanolamine.

Ammonium acetate cryosolvents were prepared as follows: a saturated solution was made at 4 °C; dilutions to 50% saturated and 75% saturated (freezing points −40 and −60 °C, respectively) were then prepared (e.g., by taking 50 mL of the saturated solution and making up to 100 mL) and used as is. The apparent pH of these cryosolvents is 7.6 (0 °C) but can be reduced to 6 with glacial acetic acid or raised to 9.6 with 30% (w/v) ammonia. All experiments in this study were performed at pH 7.6 (0 °C).

Stock solutions of all substrates were prepared in 50% ethylene glycol, 20 mM triethanolamine, and 80 mM NaCl, pH* 8 (0 °C), and stored at −20 °C. Stock solutions of enzyme were prepared in 50% ethylene glycol for use in organic solvents or 50% saturated ammonium acetate for use in ammonium acetate cryosolvents. No loss in activity at 0 °C was observed in any case, but stocks were kept at −20 °C.

β-Lactamase concentrations were determined on the basis of A_{280} (β-lactamase I ϵ_{mM} = 30, β-lactamase II ϵ_{mM} = 22, P99 ϵ_{mM} = 54.21, *Pseudomonas* ϵ_{mM} = 69.8).

Determination of Steady-State Parameters and Observation of Intermediates. Progress curves for the hydrolysis of β-lactams and spectra of intermediates were measured with a Cary 219 spectrophotometer modified for low-temperature work. Specially constructed cell blocks through which cold ethanol was continuously pumped by a Neslab Endocal ULT 80 cryostat gave cell temperatures as low as −75 ± 0.2 °C; insulation was achieved using Armaflux. All optical surfaces were continuously flushed with dry nitrogen; cell temperature was monitored with a BAT-12 thermocouple. Reactions were carried out in 1-mL cuvettes rather than 3-mL cuvettes since fluctuations in temperature on mixing were found to be less; stirring was performed manually with a thin squared plastic rod. Where necessary (because of high substrate and/or product absorbance), detection of intermediates was carried out in 3-mL cuvettes with inserts giving a path length of 0.1 cm.

Scanning of intermediates was performed with the wavelength program facilities of the Cary 219 spectrophotometer. Fluorescence measurements and scanning of intermediates were carried out in a modified Farrand MK1 spectrofluorimeter; as with the Cary 219, a specially modified cell block was continuously circulated with cold ethanol from a Neslab Endocal ULT80 cryostat, and all optical faces were continuously flushed with dry nitrogen.

Progress curves were analyzed by a modification of the intersection method (Schonheyder, 1952; Cartwright, 1979), which has the advantage of being able to indicate quickly whether inactivation or changes in kinetic parameters are occurring during the course of these reactions. From the integrated form of the Michaelis–Menten equation

$$t = yA_0/V_{max} - (K_m/V_{max}) \ln (1 - y)$$

where t = time, y = fractional extent of reaction, and A_0 = initial substrate concentration. t/yA_0 is plotted against $-t/\ln (1 - y)$ for each value of y. The intersection point gives apparent values of $1/V_{max}$ and K_m/V_{max}. Selwyn's test (Selwyn, 1965) was routinely used to check for inactivation during hydrolysis. Where K_m was high (>2 mM), initial rates were used; generally such high K_m values indicated the substrate was not suitable for cryoenzymological studies.

The hydrolysis of substrates was monitored at wavelengths that were appropriate for the experiments; extinction coefficients of hydrolysis were corrected for the effect of temperature, solvent, and pH.

Low-Temperature Gel Filtration. An all glass jacketed column with a bed volume of Sephadex G50 (coarse) of 8.5 cm × 1 cm was used. The temperature of the column was maintained to within ±0.2 °C by the rapid circulation of cold ethanol from a Neslab Endocal ULT80 cryostat. The glass jacket was externally insulated with plastic tubing of diameter 1.1 cm greater than the external diameter of the column (1.6 cm). Through this dead space dry nitrogen was flushed, which allowed viewing of the resin/buffer interface in order to place the enzyme and substrate on top of the resin.

The column was run at −20.8 °C. The flow rate with 50% ammonium acetate was set at 1.2 mL/min; this meant the time taken for the elution of the void volume was about 3 min.

Reactions were initiated on top of the resin by adding precooled enzyme (200 μL) in 50% ammonium acetate to precooled substrate (100 μL) in 50% ammonium acetate to give concentrations of both 300 μM. Stirring was carried out with a long thin hypodermic syringe, which was also used to introduce the solutions. After incubation for the required time, the reaction mixture was run into the resin followed by an equal volume of 50% saturated ammonium acetate followed by sufficient solvent to run the column. Fractions were collected at room temperature. The length of the column was such that the enzyme alone (P99, 300 μM) eluted with a peak at fraction 6 (0.82-mL fractions) and dnpAPA alone (300 μM)

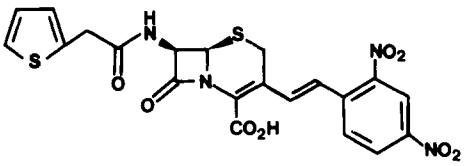
[1] Abbreviations: dnsAPA, 6-[[5-(dimethylamino)naphthalene-1-sulfonyl]amino]penicillanic acid; dnpAPA, 6-[(2,4-dinitrophenyl)-amino]penicillanic acid; PADAC, 7-(thienyl-2-acetamido)-3-[[2-[[4-(dimethylamino)phenyl]azo]pyridinio]methyl]cephem-4-carboxylic acid.

at fraction 11; overlap was negligible. Dead volume between the bottom of the resin and the fraction collector was about 1 mL.

Where appropriate, cloxacillin (20 μL) was added to the reaction mixture [from a stock freshly made in 50% saturated ammonium acetate by adding 100 mM cloxacillin in 20 mM triethanolamine and 80 mM NaCl, pH 8, at 4 °C (10 μL) to 100% saturated ammonium acetate (10 μL) at 4 °C] to give a final concentration of 3.125 mM.

Results

Choice of Substrate. The usual method of following the conversion of substrate into product in cryoenzymology is spectroscopic. The hope is that an intermediate will be spectroscopically distinct, a hope more likely to be realized if there is a marked difference (say, >10 nm in λ_{max}) between substrate and product. Moreover, changes in the ultraviolet are more sensitive to temperature changes and to incomplete mixing, and so colored substrates were preferred. Nitrocefin (I) and PADAC (II), which are cephalosporins, show marked



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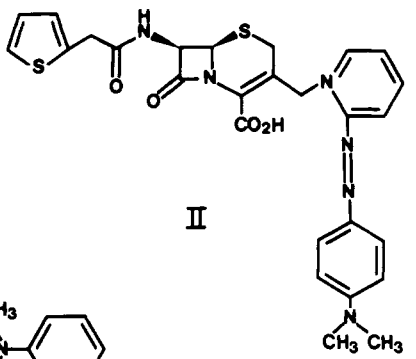

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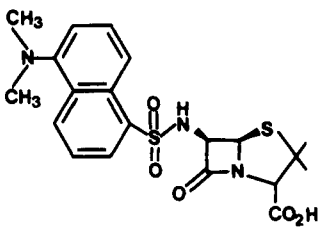

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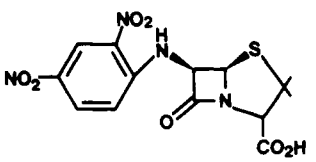

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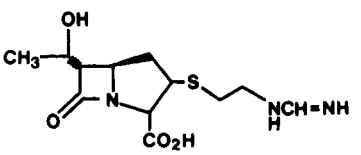

V

changes in the visible region. dnsAPA (III) and dnpAPA (IV) are penicillins; the former contains a strong fluorophore, and the latter is both fluorescent and shows changes (unfortunately, relatively small ones) in the visible region on hydrolysis. Imipenem (V), a carbapenem, shows a large change in the UV on hydrolysis.

Table I: Physical Properties of Selected Solvents

solvent	freezing point (°C)	UV cutoff (nm)	viscosity[a] (cP)	time taken for bubbles to disperse[b] (min)
ethylene glycol–methanol–H_2O (25:45:30 by vol)	<−70	230	80	5
ethylene glycol–methanol–H_2O (40:20:40 by vol)	−70	230	195	11
70% (v/v) methanol	−85	230	25	1.7
60% (v/v) ethylene glycol	−69	230	420	28
50% (v/v) dimethyl sulfoxide	−60	260	800[c]	50
5 M NaCl	−20	230		
75% saturated ammonium acetate[d]	−60	240	80[c]	5

[a] At −50 °C. [b] From measurements of A_{400} at −50 °C, after mixing for 1 min. [c] Approximate estimate from time taken for bubbles to disperse. [d] Prepared by dilution of solution saturated at 4 °C.

Table II: Solubilities of Enzymes and Substrates in Cryosolvents

	approx solubility (μM) for enzymes			
solvent[a]	β-lactamase I	β-lactamase II	*Ps.*[b]	P99[c]
50% ethylene glycol		2000	500	1000
ethylene glycol–methanol–H_2O (25:45:30)	350			
70% methanol	5	200		
50% dimethyl sulfoxide			5	
75% saturated ammonium acetate	>5000	>5000	>5000	>5000

	approx solubility (μM) for substrates			
solvent[a]	nitrocefin	PADAC	dnpAPA	dnsAPA
aqueous	200	800	$>10^4$	$>10^4$
50% saturated ammonium acetate	100	400	4000	1000
ethylene glycol–methanol–H_2O (25:45:30)	10^4	10^4	$>10^4$	$>10^4$

[a] Compositions are by volume. Organic cryosolvents were 20 mM in triethanolamine hydrochloride and 80 mM in NaCl, pH* 8 at 0 °C. Ammonium acetate solutions are expressed with respect to a solution saturated at 4 °C. [b] β-Lactamase from *Pseudomonas aeruginosa* 1822SH. [c] β-Lactamase from *Enterobacter cloacae*.

Choice of Solvent. Cryosolvents must form mixtures with water that have low freezing points and are not too viscous. As an example, at −60 °C in 45% (v/v) methanol and 25% (v/v) ethylene glycol, mixing takes 1–2 min; it takes 5 min for the temperature of the solution to return to its original value (there is a temperature jump of about 5 °C on mixing), and it takes 10 min for bubbles introduced during mixing to disperse. Thus the first 12 min of reaction are lost at this temperature. A practical guide to the viscosity is provided by the time taken for bubbles to disperse (Table I). Artifacts arising from high viscosities and inefficient mixing have on occasion been misinterpreted as intermediates (Markeley et al., 1981).

Both the substrate and the enzyme must be sufficiently soluble at low temperatures to detect an intermediate. The solubility of certain substrates is given in Table II; K_m is also relevant here. The K_m is commonly raised (often exponentially) in organic cryosolvents (Fink & Cartwright, 1981); this

Table III: Kinetic Parameters for β-Lactamases in Cryosolvents and Water

enzyme[b]	substrate	solvent[c]	K_m[a] (μM) solvent	K_m water	k_{cat} (min^{-1}) solvent	k_{cat} water
Ps.	PADAC	NH_4OAc	550	550	196	2680
Ps.	nitrocefin	Me_2SO	1860	35	3970	17400
Ps.	dnpAPA	NH_4OAc (50%)	78	10	1.5	1.0
Ps.	dnsAPA	NaCl	0.1	0.1	1.0	0.23
Ps.	dnpAPA	EgMe2	5000	10	2.0	1.0
Ps.	ceph C[d]	Me_2SO	531	158	316	2700
Ps.	imipenem	EgMe2	29	25	0.5	0.1
Ps.	pen G[e]	Me_2SO	300	3.3	656	1490
P99	PADAC	NH_4OAc	1350	300	2000	3070
P99	nitrocefin	NH_4OAc	370	150	35200	30800
P99	dnpAPA	NH_4OAc (50%)	46	11	0.1	0.23
P99	dnpAPA	EgOH	1000	11	4.0	0.23
βI	PADAC	EgMe	80	3	67	44
βI	PADAC	NH_4OAc	30	3	100	44
βI	nitrocefin	EgMe	100	20	200	200
βI	dnsAPA	MeOH	1750	210	4370	14400
βI	pen G	MeOH	700	43	8475	27000
βII	PADAC	EgMe	>5000	221	10500	4000
βII	PADAC	NH_4OAc	200	221	322	4000

[a] At 0 °C, in 20 mM triethanolamine hydrochloride and 80 mM NaCl, pH 8, for solvents other than NH_4OAc, which has apparent pH 7.6. [b] Names of β-lactamases as in Table II. [c] NH_4OAc is 75% saturated ammonium acetate (unless stated otherwise), Me_2SO is 50% dimethyl sulfoxide, EgMe is ethylene glycol–methanol–water (25:45:30), EgMe2 is ethylene glycol–methanol–water (25:25:50), MeOH is 70% methanol, EgOH is 50% ethylene glycol, and NaCl is 5 M NaCl. [d] Cephalosporin C. [e] Benzylpenicillin.

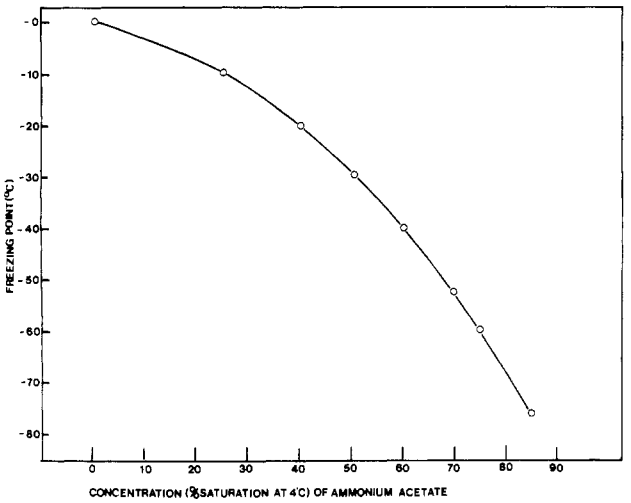

FIGURE 1: Plot of freezing point (°C) against concentration of ammonium acetate expressed as percentage saturation at 4 °C.

has the disadvantage that high concentrations of reactants have to be used to attain measurable concentrations of complex. Even then the complex may be hard to detect because of high background absorbance of substrate and instrumental limitations. A cryosolvent that does not raise K_m is therefore highly desirable. A selection of the values found for K_m are given in Table III, from which it can be seen that ammonium acetate has the advantage that K_m is not raised greatly.

Ammonium Acetate Solutions as Cryosolvents. The freezing points of ammonium acetate solutions are shown in Figure 1, plotted against the percentage saturation at 4 °C. The advantages of ammonium acetate solutions as cryosolvents are (i) liquid down to −60 °C with a viscosity apparently comparable with that of ethylene glycol–methanol–water (25:45:30); (ii) high solubility of the β-lactamases (Table II); (iii) high stability of the β-lactamases (Table IV); (iv) K_m not greatly raised (Table III); and (v) k_{cat} not greatly altered (Table III). Disadvantages are (i) limited pH range accessible (pH 6–9.7, measured at 30 °C) and (ii) low solubility of some substrates at low temperatures (Table II).

It is worth noting that penicillins and cephalosporins that are generally highly susceptible to nucleophilic attack nevertheless remain quite stable in ammonium acetate solutions at or below 0 °C for long periods.

Table IV: Stability of β-Lactamases during Turnover[a]

substrate	solvent	inactivation during turnover	no inactivation during turnover
nitrocefin	70% MeOH	βII,[c] *Ps.*, P99	βI
	EgOH–MeOH–H_2O[b]	*Ps.*, P99	βI, βII
	75% saturated NH_4OAc	*Ps.*	P99
	aqueous	*Ps.*	
PADAC	EgOH–MeOH–H_2O[b]	*Ps.*, P99, βII	βI
	75% saturated NH_4OAc	βII	*Ps.*, P99, βI
	aqueous	βII	
dnsAPA	EgOH–MeOH–H_2O[b]	*Ps.*, P99	βI
	50% saturated NH_4OAc	*Ps.*	P99
	5M NaCl		*Ps.*
dnpAPA	EgOH–MeOH–H_2O[d]	P99	*Ps.*
	50% ethylene glycol		P99
	50% saturated NH_4OAc		*Ps.*, P99

[a] Progress curves tested by the method of Selwyn (1965) at 0 °C in 20 mM triethanolamine hydrochloride, pH* 8, containing 80 mM NaCl, for the organic cryosolvents, and apparent pH 7.6 for ammonium acetate solutions. [b] Ethylene glycol–methanol–water (25:45:30 by vol). [c] βI and βII are β-lactamase I and β-lactamase II from *B. cereus*, P99 is P99 β-lactamase from *Enterobacter cloacae*, and *Ps.* is the β-lactamase from *Pseudomonas aeruginosa*. [d] Ethylene glycol–methanol–water (25:25:50).

Stability of β-Lactamases during Turnover. The stability of enzymes in cryosolvents is a major problem; although the enzyme alone may be stable, it is often found that inactivation accompanies turnover. The use of Selwyn's (1965) test is therefore essential. The results (Table IV) show that the aqueous ammonium acetate systems allowed turnover to proceed without inactivation in seven out of nine instances, whereas for the ethylene glycol–methanol systems the corresponding values were five out of thirteen. More extensive data (not cited) suggest that the order of stability of the enzyme–substrate systems decreases from aqueous ammonium acetate or NaCl through aqueous ethylene glycol and ethylene–methanol–water to aqueous methanol.

Possibility of Reaching Temperature at Which Reaction Is Halted. Clearly k_{cat} must be low enough for spectroscopic scrutiny (and exclusion chromatography) to see whether in-

Table V: Effects of Temperature and Solvent on k_{cat} and of Temperature on K_m

β-lactamase[b]	substrate[b]	solvent[b]	k_{cat}[a] (min^{-1}) aqueous	k_{cat}[a] (min^{-1}) solvent	E[c] (kcal/mol) (for k_{cat})	T_{halt}[d]	K_m[e] (mM)	E[c] (kcal/mol) (for K_m)
Ps.	PADAC	NH_4OAc	2680	196	8.6 (8)	−95	1000	10.3
Ps.	pen G	Me_2SO	1487	656	3.3/11.3 (3.3/11)[h]	−92		
Ps.	nitrocefin	Me_2SO	80000[f]		7.2/16 (7/16)[h]	−80		
Ps.	ceph C	Me_2SO	2700	316	10/16 (10/16.3)	−70		
Ps.	dnpAPA	NH_4OAc	1.0	1.5	12 (13)	−46	8.4	9.12
Ps.	dnpAPA	EgMe2	1.0	2.0	12.5 (13)	−42		
Ps.	dnsAPA	NaCl	0.23	1.0	13 (13)	−39		
Ps.	imipenem	EgMe2	0.1	0.5	14.5 (14.5)	−23	0.63	18.3
P99	PADAC	NH_4OAc	3070	2000	10/33[h]	−46	0.05[i]	
P99	dnpAPA	NH_4OAc	0.1	0.23	16.4	−16	0.070[g]	
βI	nitrocefin	EgMe	200	200	8.7/16.3[h] (8.8/16.1)	−65	0.1[g]	
βI	PADAC	EgMe	44	67	16 (16)	−60	0.39	3.3
βI	dnsAPA	EgMe	14400	4320	11.5	−100		
βI	pen G	MeOH	27000	8475	11.3 (9.5)	−105		

[a] k_{cat} at 0 °C in 20 mM triethanolamine hydrochloride, pH 8, containing 80 mM NaCl (aqueous) or in cryosolvent at 0 °C, pH* 8 or pH* 7.6 for ammonium acetate. [b] Names of β-lactamases and solvents given in Table III. [c] Apparent energy of activation in cryosolvent; figures in parentheses are for aqueous solution. [d] Temperature (0 °C) (calculated) at which k_{cat} = 1 h^{-1}. [e] Calculated K_m at temperature at which k_{cat} = 1 h^{-1}. [f] At 30 °C. [g] K_m unaffected by temperature. [h] Breaks in Arrhenius plots: *Ps.* pen G 8.7 °C (aqueous) 0 °C (solvent); *Ps.* nitrocefin 8.7 °C (aqueous) 0 °C (solvent); *Ps.* ceph C 9 °C (aqueous) 0 °C (solvent); P99 PADAC 5.6 °C (solvent); βI nitrocefin 16 °C (aqueous) 9 °C (solvent). [i] Curved plot.

Table VI: Suitability of β-Lactamases for Detailed Investigation by Cryoenzymology

β-lactamase	β-lactam	inactivation during turnover: organic cryosolvents[a]	inactivation during turnover: salt cryosolvents[b]	T_{halt}[c] too low	K_m too high	suitable for detailed investigation
Ps.	nitrocefin	yes	yes			no
Ps.	PADAC	yes	no	yes	yes	no
Ps.	dnsAPA	yes	yes	yes[d]		no
Ps.	dnpAPA	no	no		yes	no
Ps.	imipenem	no			yes	no
P99	nitrocefin	yes	no		*e*	no
P99	PADAC	yes	no	no	no	yes
P99	dnsAPA				yes[h]	no
P99	dnpAPA	yes	no		no[f]	yes
βI	nitrocefin	no	(no)	no	no	yes
βI	PADAC	no	(no)	no	no	yes
βI	dnsAPA	no		yes	yes	no
βI	dnpAPA	no		yes	yes[i]	no
βII	PADAC	yes[g]	yes[g]			no
βII	dnsAPA			yes	yes[j]	no
βII	dnpAPA				yes[k]	no
βII	imipenem				yes[l]	no

[a] Principally ethylene glycol–methanol–water (25:45:30 by vol). [b] Ammonium acetate (75% saturated). [c] Calculated temperature at which k_{cat} = 1 h^{-1}. [d] In 5 M NaCl. [e] Solubility of substrate too low and k_{cat} too high (60 000 min^{-1} at 30 °C aqueous). [f] K_m is too high in organic solvents but not in ammonium acetate. [g] Cephalosporins with leaving groups at C3 commonly inactivate β-lactamase II. [h] 1 mM. [i] 30 mM. [j] 3.3 mM. [k] 2.3 mM. [l] K_m = 2 mM.

termediates have accumulated; a value of 1 h^{-1} has been taken as low enough here. Whether this value of k_{cat} is obtained at temperatures that can be reached depends both on the value of k_{cat} at 0 °C in cryosolvent and on the activation energy (Table V). The former is too high for β-lactamase I and dnsAPA, and the latter is too low for the *Pseudomonas* β-lactamase and PADAC. The value of k_{cat} is affected by the presence of cryosolvents: both increases and decreases were observed (Table V). A decrease in k_{cat} can of course be a help, as long as it does not reflect a change in the catalytic pathway. An increase in k_{cat}, however, will shift unfavorably the temperature at which k_{cat} becomes 1 h^{-1}. For instance, if E_a = 13 kcal/mol, then k_{cat} decreases 2.13-fold per 10 °C (at ordinary temperatures); a 4.4-fold increase in k_{cat} due to solvent therefore represents a 20 °C "loss" in temperature reduction. For the *Pseudomonas* β-lactamase and dnsAPA (Table V) this means that a low enough temperature to scan for intermediates cannot be reached in 5 M NaCl, which turned out to be the only solvent in which inactivation did not occur.

Effect of Temperature on K_m. Although the effect of organic solvents on K_m has received much attention, the effect of temperature on K_m has not. Not infrequently, large increases in K_m occurred (Table V), and these precluded the accumulation of intermediate at temperatures where k_{cat} was 1 h^{-1}. Arrhenius plots for K_m were linear (Figure 2); the large positive slopes for the *Pseudomonas* β-lactamase reactions are noteworthy. For this enzyme, acyl-enzyme accumulates (Knott-Hunziker et al., 1982) in the hydrolysis of benzylpenicillin or cloxacillin. If the same applies to the hydrolysis of dnpAPA, PADAC, and imipenem, then it may be shown that the slope in Figure 2 is given by $-\Delta H + E_2 - E_3$; here ΔH is the change in enthalpy on forming ES, and E_2 and E_3 are the apparent activation energies for acylation and deacylation, respectively. Here, to account for the positive slope, $\Delta H < 0$, or $E_2 > E_3$ or, quite probably, both. A further point is that the low activation energy (3.3 kcal/mol) for the hydrolysis of benzylpenicillin at ordinary temperatures (Sabath et al., 1965; Table V) presumably indicates that a conformational equilibrium precedes the bond-breaking step, and so the change in enthalpy denoted by ΔH may cover both binding and isomerization.

Choice of Systems To Investigate in Detail. Some 17 systems were initially investigated, but only 4 were suitable for further work (Table VI). Apparently rather few enzymes

Table VII: Intermediates in the Hydrolysis of dnpAPA by P99 β-Lactamase in 50% Saturated Ammonium Acetate at −20.8 °C

	λ_{max} (nm)	ϵ_M (M^{-1} cm^{-1})	fluorescence λ_{max} (nm) (emission)	fluorescence int[a]	extent of labeling[b] (mol·mol)
substrate	354	14680	415	127	
product	355.5	14680	407	93	
during reaction phase					
immediate	354	15620	400	1500	0.83, 0.25,[c] 0.15[d]
30 min	354	14680	400	793	0.82, 0.8[c]
240 min	359	15947	400	793	0.3

[a] Relative (aqueous solution = 100) fluorescence intensity; excitation was at 300 nm. [b] dnp moiety coeluting with enzyme (estimated from A_{359}) after exclusion chromatography after reaction for time given by phase. [c] Cloxacillin (20 μL of 50 mM solution) was added immediately after mixing enzyme and substrate (immediate) or 30 min later (30-min phase); in both cases, exclusion chromatography was then carried out at once. [d] Cloxacillin (20 μL of 50 mM solution) was added to the enzyme before the substrate.

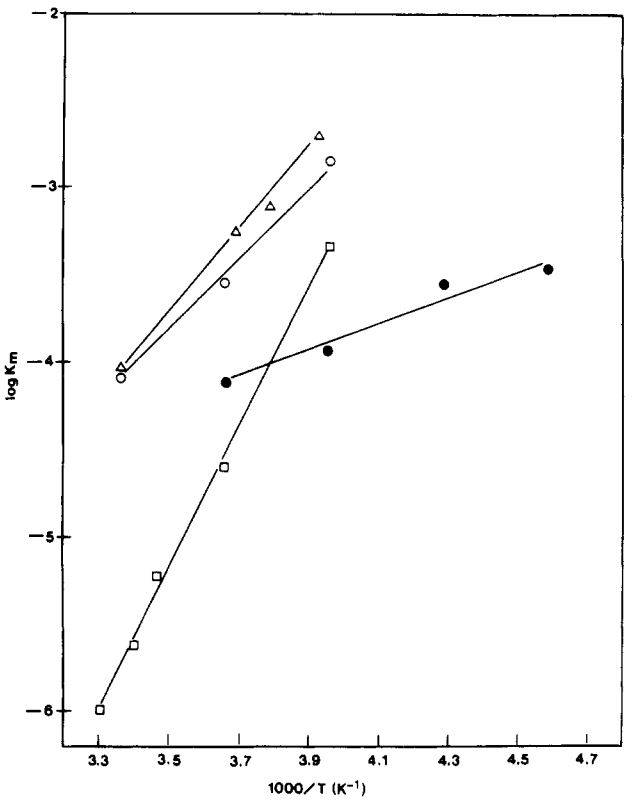

FIGURE 2: Arrhenius plot of K_m for the hydrolysis of PADAC by β-lactamase I in ethylene glycol–methanol–water (25:45:30) (EgMe) (●) and by the *Pseudomonas* β-lactamase in 75% saturated ammonium acetate (NH_4OAc) (△) and for the hydrolysis of dnpAPA in NH_4OAc) (○) and imipenem in ethylene glycol–methanol–water (25:25:50) (EgMe2) (□) by the *Pseudomonas* β-lactamase.

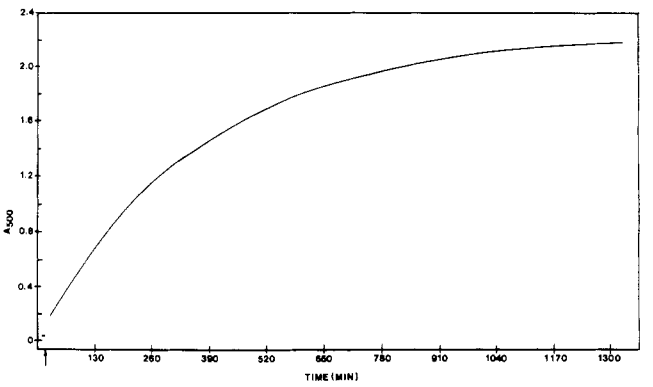

FIGURE 3: Progress curve for the hydrolysis of 130 μM nitrocefin by 12 μM β-lactamase I at −60 °C. The solvent was ethylene glycol–methanol–aqueous buffer (25:45:30, by vol), the buffer being 20 mM triethanolamine hydrochloride, 80 mM NaCl, pH* 8. A_{500} was measured with path length of 1 cm; $\Delta\epsilon_M$ = 16460 M^{-1} cm^{-1}. Here, and in later figures, the time of addition of enzyme is shown by an arrow.

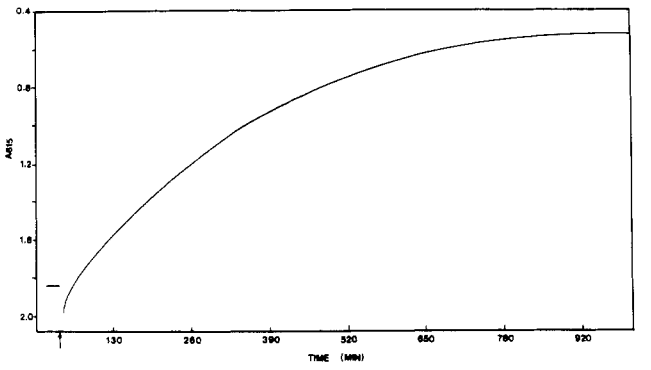

FIGURE 4: Progress curve for the hydrolysis of 450 μM PADAC by 46.7 μM β-lactamase I at −55.2 °C. The solvent was as in Figure 3. A_{615} was measured with path length of 1 cm; $\Delta\epsilon_M$ = 2800 M^{-1} cm^{-1}.

turn out to be suitable cases for detailed treatment by cryoenzymology, at least if β-lactamases are typical; this contrasts with an earlier view (Fink & Cartwright, 1981). The four examples shown as suitable in Table VII are now described individually.

β-Lactamase I and Nitrocefin. In 25:45:30 ethylene glycol–methanol–water at −60 °C and pH* 8, both k_{cat} (0.033 min^{-1}) and K_m (100 μM) were low enough to make detection of an intermediate feasible. The results, however, of successive scans (every 5.7 min) on a mixture of 89 μM β-lactamase I and 93 μM nitrocefin were to show clear conversion of substrate into product with an isosbestic point at 431 nm (the position expected for a mixture of substrate and product). The enzyme-bound substrate is apparently spectroscopically indistinguishable from free substrate, as was also found for β-lactamase II and nitrocefin (Bicknell & Waley, 1985b). An acyl-enzyme would be expected to be closer to product spectroscopically; furthermore, a progress curve showed no genuine burst (Figure 3) (that seen was due to mixing, see above).

Difference spectra with nitrocefin in both cuvettes were also used. With 975 μM nitrocefin (0.1-cm path length) and 210 μM β-lactamase I (in the sample cuvette only) there were again no changes other than the conversion of nitrocefin into product, with an isosbestic point at 431 nm. The same held true at pH* 4.25 (where k_{cat} = 0.014 min^{-} and K_m = 200 μM). This evidence suggests that acyl-enzyme does not accumulate and that the rate-limiting step is opening of the β-lactam ring.

β-Lactamase I and PADAC. The same cryosolvent was used to investigate β-lactamase I (350 μM) and PADAC (350 μM) at −55.2 °C; successive scans (every 7.5 min) showed clear conversion of substrate into product with the expected isosbestic point of 498 nm. When the concentration of PADAC was doubled, there was again no evidence of an intermediate, and the same was also true at pH* 4.25. At lower

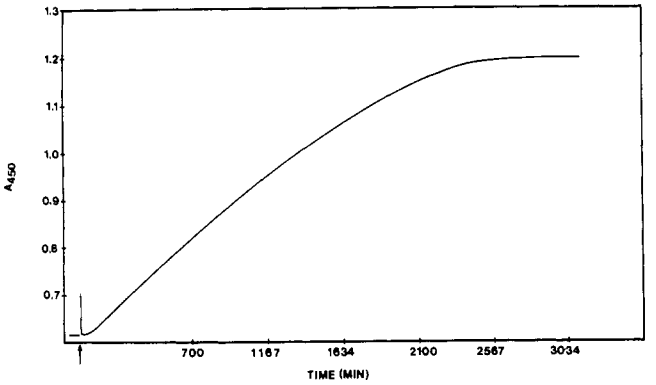

FIGURE 5: Progress curve for the hydrolysis of 785 μM dnpAPA by 46.1 μM P99 β-lactamase at −20.8 °C in 50% saturated ammonium acetate. A_{450} was measured with a path length of 1 cm; $\Delta\epsilon_M$ = 732 M^{-1} cm^{-1}. The initial burst and the following lag are explained in the text.

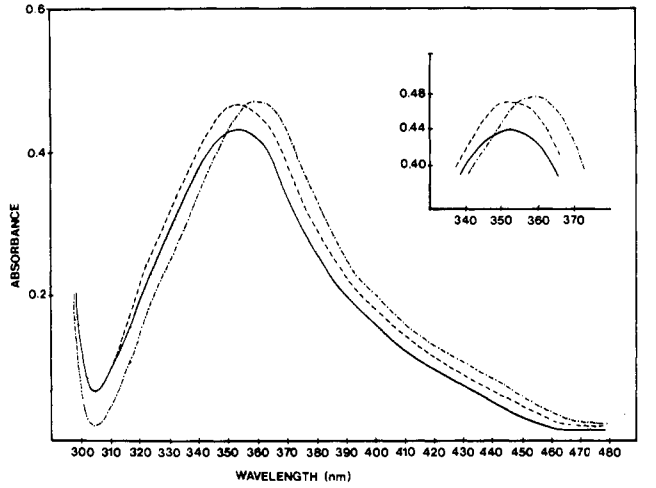

FIGURE 6: Successive scans for the hydrolysis of 300 μM dnpAPA by 300 μM β-lactamase P99 in 50% saturated ammonium acetate at −20.8 °C. Apart from the scan of substrate alone (—), scans are immediately after mixing (---), 30 min later (—), and 240 min later (-·-). Inset shows λ_{max} of intermediates. Path length was 0.1 cm.

pH* values the enzyme is unstable. Higher concentrations of substrate were beyond the capabilities of the instrument. As before, a progress curve (k_{cat} = 0.038 min^{-1}, K_m = 328 μM) showed no burst (Figure 4) (the apparent lag is due to mixing), and again we conclude that acyl-enzyme formation (or some prior step) is rate-limiting.

P99 β-Lactamase and PADAC. Both k_{cat} and K_m decrease markedly with temperature, and the large value, 33 kcal/mol, for the energy of activation below −5.6 °C suggests that a change in the conformation of the enzyme is playing a part in the reaction, as if partial unfolding accompanied turnover. Successive scans of a reaction mixture in 75% saturated ammonium acetate containing 300 μM P99 β-lactamase and 300 μM PADAC at −45 °C again showed clear conversion of substrate into product, with the usual isosbestic point at 498 nm. Under these conditions, k_{cat} = 0.0215 min^{-1} and K_m = 50 μM. The same conclusion as above can be drawn.

P99 β-Lactamase and dnpAPA. This reaction showed a number of unexpected (and, to some extent, unexplained) features, but did provide evidence for an intermediate, probably the acyl-enzyme. The progress curve at −20.8 °C in 50% saturated ammonium acetate (followed at 450 nm, Figure 5) was consistent with steady-state kinetics, with $k_{cat} = 8.7 \times 10^{-3}$ min^{-1} and K_m = 80 μM; the product was not an inhibitor, there was no inactivation by Selwyn's (1965) test, and the same kinetic parameters were obtained from progress curves with differing initial substrate concentrations.

Successive scans of a reaction mixture containing both P99 β-lactamase and dnpAPA at 300 μM showed there to be several changes (Figure 6). There was an immediate increase in absorbance, followed by a decrease (a return to the absorbance of substrate on its own) within 30 min and then an increase in absorbance together with a shift in λ_{max} over the next 240 min. The spectral characteristics at the various stages are given in Table VII. These successive phases are evident in experiments in which A_{359} was followed as a function of time (Figure 7). There were three phases: (i) immediate (i.e., within the time of mixing), (ii) 30 min, and (iii) 240 min, where the times refer to the approximate duration of the phases. When the reaction was followed by fluorescence, only the immediate and 30-min phases were seen.

The product can bind to the enzyme; when dnpAPA was hydrolyzed by catalytic amounts of β-lactamase I, the subsequent addition of P99 β-lactamase brought about an increase of A_{359}. This binding is not at the active site, however, as product does not inhibit. We suspect that substrate may also be able to bind at this second site and that this may be the cause of the increase in absorbance in the immediate phase but that other changes also took place rapidly.

Exclusion chromatography on Sephadex G50 at −20.8 °C was used to see whether the dnp moiety coeluted with the enzyme at the three temporal phases (Table VII). After 240 min, little did; the reaction was essentially complete, as expected from progress curves. In a separate experiment, P99 β-lactamase and dnpAPA hydrolyzed with β-lactamase I were mixed and subjected to exclusion chromatography, and again little dnp moiety coeluted with P99 β-lactamase. The fact that the dnp moiety did coelute with β-lactamase in the immediate phase (Table VII) suggests the intervention of a complex more

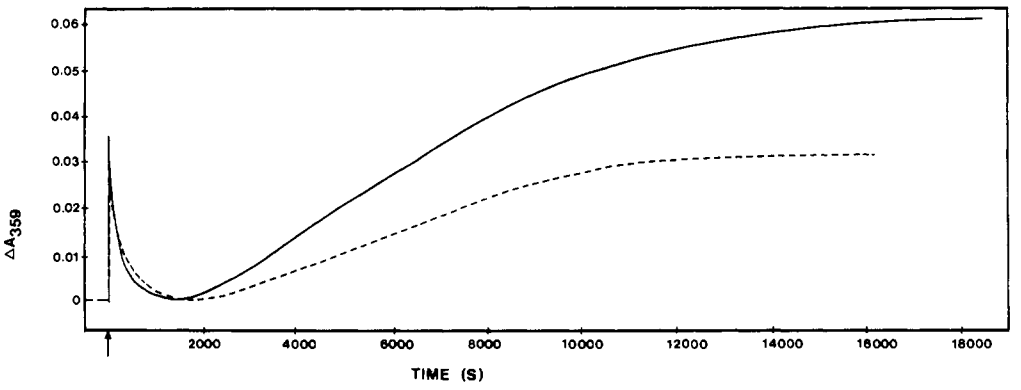

FIGURE 7: Spectral changes at 359 nm in the hydrolysis of dnpAPA by P99 β-lactamase. The conditions were as in Figure 6 (---) or with 452 μM substrate (—); the latter trace shows higher absorbance immediately after mixing and finally. Path length was 0.1 cm.

stable than a noncovalent enzyme–substrate complex, even in the early phase. Attempts to characterize the linkage by denaturation in situ were unsuccessful. Ammonium acetate protected P99 β-lactamase against denaturation even at room temperature, and most denaturants (e.g., urea, guanidinium chloride, sodium dodecyl sulfate, calcium chloride, lithium bromide, sodium salicylate) were poorly soluble in ammonium acetate solutions. There is another approach, however; a tightly binding slowly hydrolyzed inhibitor (or inhibitory substrate) could also be used. 6β-Iodopenicillanic acid reacts with P99 β-lactamase (De Meester et al., 1986), but neither this nor the penam sulfone YTR 830 (Hall et al., 1985) inactivated the enzyme rapidly enough in ammonium acetate at −21 °C. Cloxacillin, however, was much more effective [k_{cat} = 0.01 min^{-1}, K_m = 9 nM at 30 °C (Joris et al., 1984)]. Even low (100 μM) concentrations caused immediate inactivation of P99 β-lactamase at 21 °C. When exclusion chromatography was carried out after treatment of the β-lactamase/dnpAPA mixture with cloxacillin, only the enzyme in the 30-min phase now had bound dnp moiety (Table VII). This suggests that the decrease in A_{359} during the 30-min phase is signaling formation of acyl-enzyme (Figure 7). The subsequent rise in A_{359} during the 240-min phase then reveals the rate-limiting hydrolysis of the acyl-enzyme.

Discussion

Pitfalls in Cryoenzymology. An unplanned outcome of our investigations on the cryoenzymology of β-lactamases over the past few years has been a rather thorough exploration of the pitfalls. These have mostly been described above and include the instability of the enzymes, the solubility of the substrates or the enzymes (or both), and unfavorable kinetic parameters. The disadvantages in practice of a high K_m and the need to test the stability of the enzyme by Selwyn's (1965) method have perhaps not been adequately emphasized previously. The use of colored substrates that show marked spectroscopic changes has been stressed because these offer the best hope of detecting and characterizing intermediates. Thus the "standard" substrate for β-lactamases, benzylpenicillin, has λ_{max} for the change on hydrolysis at 232 nm (Waley, 1974) and is thus unsuitable for spectroscopic characterization of an acyl-enzyme intermediate. Although cephalosporin C shows a marked change in A_{260} on hydrolysis of the β-lactam ring (Abraham & Newton, 1956), the 3′-leaving group (Faraci & Pratt, 1985) brings in the complications of a branched pathway.

Many of the difficulties in cryoenzymology have arisen from the use of organic cosolvents. The altered properties of enzymes in these solvents, e.g., trypsin in dimethyl sulfoxide, may lead to bursts now regarded as due to conformational changes but previously ascribed to the intervention of tetrahedral intermediates (Compton & Fink, 1984). The break (marked change of slope) in the Arrhenius plot of k_{cat} for P99 β-lactamase and PADAC also seems likely to be due to a conformational change, of a rather unusual nature, judging by the high apparent energy of activation. Our discovery that aqueous ammonium acetate can be used as a cryosolvent down to at least −60 °C should be widely useful; aqueous ammonium acetate is free from many of the disadvantages of organic cryosolvents and, in particular, proteins are both stable and soluble in it.

Intermediates in β-Lactamase Action. There is now considerable evidence for intermediates, including acyl-enzymes, in the action of different β-lactamases [Bicknell et al. (1985) and Coulson (1985) and references cited therein]. Class A β-lactamases (Ambler, 1980) form a relatively diverse group, and examples are known both of acyl-enzymes accumulating and of the opposite (Fisher et al., 1980; Faraci & Pratt, 1985; Anderson & Pratt, 1983; Bicknell & Waley, 1985a). In some instances, covalently modified enzymes have been isolated (Kiener et al., 1980; Cartwright & Fink, 1982). The results described above with β-lactamase I (a class A enzyme) were reasonably clear-cut: there was no evidence for the accumulation of a spectroscopically distinct acyl-enzyme (or other intermediate) with either nitrocefin or PADAC. These are rather different substrates: PADAC loses the group at C3 on hydrolysis and so is a true cephalosporin; nitrocefin has no leaving group and behaves more like a penicillin with several class A β-lactamases in that k_{cat} is higher than it is when typical cephalosporins are substrates. If we may assume that an acyl-enzyme would hardly have had an absorption spectrum indistinguishable from those of substrate and product, then the evidence points to acylation being the rate-determining step in βI catalysis, at least for these two substrates.

With class C β-lactamases (Jaurin & Grundström, 1981), in contrast to the class A enzymes, acyl-enzyme accumulation appears to be the rule (Knott-Hunziker et al., 1982; Joris et al., 1984). Moreover, the class C β-lactamases closely resemble each other in amino acid sequence and kinetics. Nevertheless, P99 β-lactamase (a class C enzyme) and PADAC again showed only substrate and product in successive scans at −45 °C. The nonlinear Arrhenius plot has been referred to, and it may be that as well as a conformational change there is also a change in rate-determining step, from deacylation at ordinary temperatures to acylation at low temperatures.

The reaction of P99 β-lactamase with a penicillin, dnpAPA, *did* provide evidence for an intermediate acyl-enzyme. One of the strengths of cryoenzymology is the possibility of isolating an intermediate, and exclusion chromatography at 21 °C was used to isolate labeled enzyme and to show that the enzyme was still labeled even in the presence of a powerfully competing inhibitory substrate. Detailed kinetic study of this reaction was precluded by the complexities arising from nonspecific binding of product and (probably) substrate.

The β-lactamases that are not serine enzymes are zinc enzymes, as far as we know. In the branched-pathway mechanism of the zinc enzyme, β-lactamase II, the intermediates are noncovalent rather than acyl-enzymes. The conclusions from cryoenzymology in organic cosolvents (Bicknell & Waley, 1985b) have, in this instance, been confirmed by rapid-scanning spectroscopy in aqueous solution (Bicknell et al., 1987).

Further work on the cryoenzymology of β-lactamases would be helped if there were substrates available that had good solubility in ammonium acetate solutions at low temperatures and showed a marked change in absorbance on hydrolysis. The work reported here provides further evidence that cryoenzymology is a powerful technique, but one fraught with pitfalls ("pitfallible").

Registry No. I, 41906-86-9; II, 77449-91-3; III, 81416-73-1; IV, 1976-41-6; V, 64221-86-9; ceph C, 61-24-5; pen G, 61-33-6; EgOH, 107-21-1; Me_2SO, 67-68-5; MeOH, 67-56-1; NH_4OAc, 631-61-8; NaCl, 7647-14-5; β-lactamase I, 9001-74-5; β-lactamase II, 9012-26-4; β-lactamase, 9073-60-3.

References

Abraham, E. P., & Newton, G. G. F. (1956) *Biochem. J. 63*, 628–634.

Ambler, R. P. (1980) *Philos. Trans. R. Soc. London, B 289*, 321–331.

Anderson, E. G., & Pratt, R. F. (1983) *J. Biol. Chem. 258*, 13120–13126.

Baldwin, G. S., Edwards, G. F. St. L., Kiener, P. A., Tully, M. J., Waley, S. G., & Abraham, E. P. (1980) *Biochem. J. 191*, 111–116.

Berks, M., Redhead, K., & Abraham, E. P. (1982) *J. Gen. Microbiol. 128*, 155–159.

Bicknell, R., & Waley, S. G. (1985a) *Biochem. J. 231*, 83–88.

Bicknell, R., & Waley, S. G. (1985b) *Biochemistry 24*, 6876–6887.

Bicknell, R. B., Cartwright, S. J., Emanuel, E. L., Knight, G. C., & Waley, S. G. (1985) *Recent Advances in the Chemistry of β-Lactam Antibiotics* (Brown, A. J., & Roberts, S. M., Eds.) pp 280–292, Royal Society of Chemistry, London.

Bicknell, R., Schäffer, A., Waley, S. G., & Auld, D. S. (1987) *Biochemistry* (in press).

Cartwright, S. J. (1979) Ph.D. Thesis, University of Edinburgh, Edinburgh, Scotland.

Cartwright, S, J., & Fink, A. L. (1982) *FEBS Lett. 137*, 186–188.

Cartwright, S. J., & Waley, S. G. (1983) *Med. Res. Rev. 3(4)*, 341–382.

Cartwright, S. J., & Waley, S. G. (1984) *Biochem. J. 221*, 505–512.

Charlier, P., Dideberg, O., Frere, J. M., Moews, P. C., & Knox, J. R. (1983) *J. Mol. Biol. 171*, 237–238.

Compton, P. D., & Fink, A. L. (1984) *Biochemistry 23*, 2989–2994.

Coulson, A. F. W. (1985) *Biotechnol. Genet. Eng. Rev. 3*, 219–253.

De Meester, F., Frere, J. M., Waley, S. G., Cartwright, S. J., Virden, R., & Lindberg, F. (1986) *Biochem. J. 239*, 575–580.

Douzou, P. (1986) *Cryobiology 23*, 38–47.

Douzou, P., & Petsko, G. A. (1984) *Adv. Protein Chem. 36*, 245–361.

Douzou, P., Hui Bon Hoa, G., Maurel, P., & Travers, F. (1976) *Handbook of Biochemistry and Molecular Biology* (Fasman, G., Ed.) Chemical Rubber Co., Cleveland, OH.

Faraci, W. S., & Pratt, R. F. (1985) *Biochemistry 24*, 903–910.

Fink, A. L., & Geeves, M. A. (1979) *Methods Enzymol. 63*, 336–370.

Fink, A. L., & Cartwright, S. J. (1981) *CRC Crit. Rev. Biochem. 11*, 145–207.

Fisher, J., Belasco, J. G., Khosla, S., & Knowles, J. R. (1980) *Biochemistry 19*, 2895–2901.

Flett, F., Curtis, N. A. C., & Richmond, M. H. (1976) *J. Bacteriol. 127*, 1585–1586.

Frere, J.-M., & Joris, B. (1985) *CRC Crit. Rev. Microbiol. 11*, 299–396.

Hall, T. W., Maiti, S. N., Micetich, R. G., Spevak, P., Yamabe, S., Ishida, N., Kajitani, M., Tanaka, M., & Yamasaki, T. (1985) *Recent Advances in the Chemistry of β-Lactam Antibiotics* (Brown, A. G., & Roberts, S. M., Eds.) pp 242–254, Royal Society of Chemistry, London.

Jaurin, B., & Grundström, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4897–4901.

Joris, B., Dusant, J., Frere, J.-M., van Beemen, J., Emanuel, E. L., Petursson, S., Gagnon, J., & Waley, S. G. (1984) *Biochem. J. 223*, 271–274.

Kiener, P. A., Knott-Hunziker, V., Petursson, S., & Waley, S. G. (1980) *Eur. J. Biochem. 109*, 575–580.

Knott-Hunziker, V., Petursson, S., Waley, S. G., Jaurin, B., & Grundström, T. (1982) *Biochem. J. 207*, 315–322.

Knowles, J. R. (1985) *Acc. Chem. Res. 18*, 97–104.

Lazarus, R. A., DeBrosse, C. W., & Benkovic, S. J. (1982) *J. Am. Chem. Soc. 104*, 6869–6871.

Mackenzie, N. E., Malthouse, J. P. G., & Scott, A. I. (1984) *Biochem. J. 219*, 437–444.

Markeley, A. L., Travers, F., & Balny, C. (1981) *Eur. J. Biochem. 120*, 477–485.

Marshall, M. J., Ross, G. W., Chanter, K. V., & Harris, A. M. (1972) *Appl. Microbiol. 23*, 765–769.

O'Callaghan, C. H., Morris, A., Kirby, S. M., & Shingler, A. H. (1972) *Antimicrob. Agents Chemother. 1*, 283–288.

Sabath, L. D., Jago, M., & Abraham, E. P. (1965) *Biochem. J. 96*, 739–752.

Samraoui, B., Sutton, B. J., Todd, R. J., Artymiuk, P. J., Waley, S. G., & Phillips, D. C. (1986) *Nature (London) 320*, 378–380.

Schindler, P., & Huber, G. (1980) in *Enzyme Inhibitors* (Brodbeck, U., Ed.) pp 169–176, Verlag Chemie, Weinheim.

Schneider, C. H., & de Weck, A. L. (1966) *Helv. Chim. Acta 49*, 1707–1714.

Schonheyder, F. (1952) *Biochem. J. 50*, 378–384.

Selwyn, M. J. (1965) *Biochim. Biophys. Acta 105*, 193–195.

Waley, S. G. (1974) *Biochem. J. 139*, 789–790.