Diffusion-Limited Component of Reactions Catalyzed by *Bacillus cereus* β-Lactamase I†

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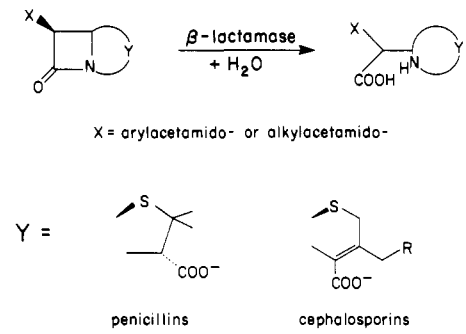
ABSTRACT: The *Bacillus cereus* β-lactamase I catalyzes the hydrolysis of a wide variety of penicillins and cephalosporins with values of k_{cat}/K_m varying over several orders of magnitude. The values of this parameter for the most reactive of these compounds, benzylpenicillin, I, and furylacryloylpenicillin, II (k_{cat}/K_m = 2.43 × 10^7 M^{-1} s^{-1} and 2.35 × 10^7 M^{-1} s^{-1}, respectively, at pH 7.0 in potassium phosphate buffer containing 0.17 M KCl, I_c = 0.63, 25 °C) are decreased markedly by increasing viscosity in sucrose- or glycerol-containing buffers. The relative sensitivities to viscosity of k_{cat}/K_m values for I and for cephaloridine, III, were found to be virtually unchanged at pH 3.8 from those observed at pH 7.0. The differential effects of viscosity on the reactive vs. the sluggish [e.g., cephalothin (IV), k_{cat}/K_m = 1 × 10^4 M^{-1} s^{-1}] substrates support the contention that the rates of reaction of the former with the enzyme are in part diffusion controlled. Quantitative analysis gives values for the association rate constants, k_1^0, of 7.6 × 10^7 M^{-1} s^{-1}, 4 × 10^7 M^{-1} s^{-1}, and 1.1 × 10^7 M^{-1} s^{-1} for I, II, and III, respectively. As both reactive and sluggish substrates associate with the active site of the enzyme with relatively similar rate constants, the variation in k_{cat}/K_m values is primarily due to the variation in the partition ratios, k_{-1}^0/k_2, for the ES complex, which are 2.3, 0.77, and 30 for I, II, and III, respectively. The preceding analysis is based on direct application of the Stokes–Einstein diffusion law to enzyme kinetics. The range of applicability of this law to the diffusion of substrate size molecules and the mechanics of diffusion of ionic species through viscous solutions of sucrose vs. polymers are explored.

The widespread occurrence of β-lactamases in clinically important pathogenic bacteria (Sykes & Matthew, 1976) is limiting the efficacy of penicillin- and cephalosporin-based chemotherapy (Mandell & Sande, 1980). This fact has provided a stimulus for the substantial experimental progress that has been made in the last few years in the understanding of the underlying mechanisms of action of β-lactamases from a number of Gram-positive (Knott-Hunziker et al., 1979; Cohen & Pratt, 1980; Cartwright & Coulson, 1980) and Gram-negative (Fisher et al., 1980, 1981; Knott-Hunziker et al., 1982a,b) bacteria.

There are at least three fundamentally different classes of β-lactamases, which inactivate penicillins and the related cephalosporins by catalyzing the hydrolysis of the β-lactam (Scheme I). Class A (Ambler, 1980) consists of the four β-lactamases from *Escherichia coli*, *Staphylococcus aureus*, *Bacillus licheniformis*, and *Bacillus cereus*, which show considerable amino acid sequence homology and share some mechanistic features. The gene for the class A enzyme from *E. coli* is plasmid encoded, and hence this enzyme (RTEM β-lactamase) is present in a wide variety of Gram-negative bacteria (Matthew & Hedges, 1976). The sole known member of class B (Ambler, 1980) is β-lactamase II of *B. cereus*, which is distinguished by a requirement for Zn^{2+} and lacks sequence homology to enzymes of the other classes. The chromosomally encoded β-lactamases of *E. coli* K12 (ampC β-lactamase) and *Pseudomonas aeruginosa* share sequence homology distinct from that characterizing class A enzymes and have been designated as class C (Jaurin & Grundström, 1981). There are many other β-lactamases that have not been classified on a molecular level since sufficient amino acid sequence data and mechanistic information are lacking (Ambler, 1980).

Scheme I



Some of these β-lactamases have been organized in an alternative classification system (Richmond & Sykes, 1973) that is based on criteria other than amino acid sequence.

Evidence has been accumulating that favors the central catalytic role of a serine in a highly conserved region of class A β-lactamases. Serine-70 [in the numbering system of Ambler (1979, 1980)] of *B. cereus* β-lactamase I is covalently modified by the irreversible inhibitor β-bromopenicillanate (Knott-Hunziker et al., 1979; Cohen & Pratt, 1980). Similar inhibitors covalently modify the corresponding serine in the homologous enzymes from *E. coli* (Fisher et al., 1981) and *S. aureus* (Cartwright & Coulson, 1980). Replacement of the conserved serine by threonine in the class A *E. coli* β-lactamase results in total loss of activity (Dalbadie-McFarland et al., 1982). Class C β-lactamases also contain a serine residue in a conserved sequence that is acylated by poor substrates for these enzymes (Knott-Hunziker et al., 1982a). The intermediacy of an acyl-enzyme in hydrolysis has been demonstrated for the class A *E. coli* β-lactamase with cefoxitin, a very poor substrate for that enzyme (Fisher et al., 1980). The covalent linkage between the cefoxitin and the *E. coli* β-lactamase has characteristics of an aliphatic ester, consistent with the assignment as an acylserine (Fisher et al., 1980). *B. cereus* β-lactamase I does not appear to bind cefoxitin at all (L. Hardy, unpublished observations).

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The intermediacy of an acyl-enzyme for chymotrypsin-catalyzed reactions of amides and esters has been proven by nucleophilic partitioning of the intermediate (Fastrez & Fersht, 1973b). It has not been possible to demonstrate an acyl-enzyme by this means with class A β-lactamases and normal substrates (Abraham & Waley, 1979; Anderson & Pratt, 1981; Brenner & Knowles, 1981). However, it is possible to reactivate, at least partially, the RTEM β-lactamase that has been covalently modified by substrate analogues such as clavulanate (Charnas et al., 1978) or olivanate derivatives (Easton & Knowles, 1982) by treatment of the inactivated enzyme with hydroxylamine. Partitioning of an acyl-enzyme between alcohol and water was recently demonstrated for class C β-lactamases (Knott-Hunziker et al., 1982b).

The magnitude of the second-order rate constants for the reactions of benzylpenicillin (I) and certain other substrates with class A β-lactamases ($k_{cat}/K_m > 10^7$ M^{-1} s^{-1} for I) from several sources [e.g., *B. cereus*, Abraham & Waley (1979), and *E. coli*, Fisher et al. (1980)] has led to suggestions that the rates of these reactions might be at least partially diffusion limited. Recent experience with the viscosity variation method for investigation of diffusive limitations of chymotrypsin-catalyzed reactions (Brouwer & Kirsch, 1982) has facilitated the present extension to the reactions of β-lactamase. The stability of *B. cereus* β-lactamase I in the presence of high concentrations of organic cosolvents (Csányi et al., 1971; Abraham & Waley, 1979) made the enzyme attractive for this purpose. The importance of using less reactive substrates, whose rates of reaction with the enzyme under study are thought not to be diffusion controlled, as controls for non-specific effects on the structure of the enzyme has been emphasized previously (Brouwer & Kirsch, 1982). We have therefore used substrates that exhibit a wide range of values for k_{cat}/K_m with *B. cereus* β-lactamase I, none of which exhibit the biphasic kinetics seen with hysteresis-inducing penicillins, i.e., those which lead to a time-dependent change in the enzyme kinetics (Citri et al., 1976).

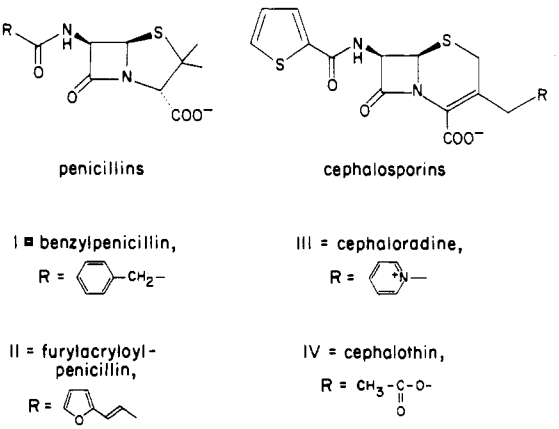
The second paper in this series (Hardy & Kirsch, 1984) reports an investigation of the pH dependences and solvent D_2O kinetic isotope effects on *B. cereus* β-lactamase I catalyzed reactions. That study, together with the results of the viscosity variation experiments reported here, provides a well-defined kinetic mechanism for the enzyme. The third paper (Hardy et al., 1984) analyzes the anomalous pH dependences that are exhibited by certain α-substituted benzylpenicillins and that suggest the presence of a basic anionic residue at the active site of β-lactamase.

Materials and Methods

Materials. *B. cereus* 569/H, a constitutive overproducer of β-lactamase I (Kogut et al., 1956), was purchased from the National Collection of Industrial Bacteria (NCIB strain no. 8933) and grown initially on plates of nutrient agar (Difco). A single colony was picked that produced large amounts of extracellular β-lactamase activity and was used for preparation of the enzyme. (This was crucial for high yields. After repeated culture the bacteria produced lower amounts of enzyme, perhaps due to spontaneous loss of the hyperconstitutive trait.) Large-scale cultures (ca. 10 L) of *B. cereus* were grown by using the casamino acids/citrate medium described by Miller et al. (1965) with $ZnSO_4$ omitted since the Zn^{2+} requiring β-lactamase II (Davies et al., 1974) was not needed.

β-Lactamase I was purified essentially as described by Davies et al. (1974), except that the enzyme was not lyophilized at any stage but was concentrated when necessary by ultrafiltration using Amicon UM10 membranes. The purified enzyme was stored frozen in plastic vials after exhaustive dialysis against 10^{-4} M EDTA (pH 7). The usual preparation of enzyme had a specific activity of 2.5×10^3 mol of benzylpenicillin (I) hydrolyzed per s per mol of enzyme at pH 7, 30 °C, when a value of 2.77×10^4 was used as the molar absorbency of *B. cereus* β-lactamase I (Kiener et al., 1980). All other kinetic measurements were made at 25 °C, where k_{cat} for I is 1.7×10^3 s^{-1} (pH 7).

The following substrates were studied:



The sodium salt of I was purchased from Sigma Chemical Co., and III and the sodium salt of IV were gifts from the Lilly Research Laboratories. The procedure used to prepare the triethylammonium salt of II was similar to that described by Durkin et al. (1977), except that 1 full equiv of triethylamine was employed in the mixed anhydride synthesis. The recrystallized salt of II had mp 106–108 °C dec [lit. mp 107–110 °C (Durkin et al., 1977)]. Sucrose and glycerol (AR grades) were purchased from Mallinckrodt. Ficoll type 400, approximate molecular weight 4×10^6, was obtained from Sigma. Glass-distilled water was used in all kinetic experiments described here and in the two following papers (Hardy & Kirsch, 1984; Hardy et al., 1984).

Methods. Buffer solutions of 0.2 M potassium phosphate at ionic strength (I_c) = ca. 0.6 M (with KCl) were prepared by mixing a solution containing 0.2 M $K_2HPO_4 \cdot 3H_2O$ and 0.1 M KCl with a solution containing 0.2 M KH_2PO_4 and 0.3 M KCl until the desired pH was reached. Buffer solutions of potassium phosphate/pyrophosphate were prepared by mixing a solution containing a concentration of KH_2PO_4 equal to the desired ionic strength with one containing 10-fold more dilute $K_4P_2O_7$. Equal concentrations of KCl were present in the phosphate and pyrophosphate solutions, when required. Potassium acetate/sulfate buffers at I_c = ca. 0.2 M were prepared by mixing 0.2 M potassium acetate with 0.2 M potassium acetate containing 0.2 M H_2SO_4 to give pH 3.8.

Buffers containing sucrose, ficoll, or glycerol were prepared by mixing two solutions as described above to reach the desired pH, each one containing the desired concentration of the viscosogenic agent. For the sucrose- or ficoll-containing solutions that were buffered with 0.2 M potassium phosphate at I_c = ca. 0.6 (with KCl), each component solution was prepared by dissolving the required amount of viscosogen in a previously prepared solution containing 0.2 M $K_2HPO_4 \cdot 3H_2O$ and 0.1 M KCl or 0.2 M KH_2PO_4 and 0.3 M KCl. For the glycerol-containing solution buffered with 0.2 M potassium phosphate at I_c = ca. 0.6 (with KCl) and the sucrose-containing solutions that were buffered with phosphate/pyrophosphate or acetate/sulfate, each component solution was prepared by dissolving the required amounts of salts and viscosogenic agents in water. The concentrations of viscosogens

in the resulting solutions were known in percent (w/v); the reported concentrations of sucrose were calculated as percent (w/w) by dividing by the densities of the buffers containing it. All buffer solutions were filtered through sintered-glass funnels prior to use.

The relative viscosities η_{rel} ($=\eta/\eta^0$) of sucrose-containing buffer solutions were calculated from the densities of the solutions and measurements with an Ostwald viscometer at 25 °C by using corresponding buffer solutions containing 0% sucrose as references (Tinoco et al., 1978). Spectral and pH measurements were made as described by Brouwer & Kirsch (1982). The wavelengths at which the hydrolyses of substrates were followed and the corresponding values for the changes in molar absorbency (SE) accompanying these hydrolyses in purely aqueous buffers were as follows: I, 232 nm, 1012 (10); II, 335 nm, 2675 (32); III, 255 nm, 9209 (27); IV, 262 nm, 7703 (72). The values of the molar absorbency change for I, III, and IV are independent of pH over the range studied in this and the following paper (Hardy & Kirsch, 1984). The value reported here of the pH-dependent molar absorbency change for hydrolysis of II is the average of determinations at pH 6.8 and 7.0. Slight increases were observed in the values of the molar absorbency changes for the hydrolysis of some substrates in solutions containing viscosogenic agents. The largest of these are less than 5% of the values in purely aqueous buffer and were compensated for (where statistically significant) in calculations of k_{cat} and K_m.

Reactions were initiated by addition of 10–25 μL of an ice-cooled solution of *B. cereus* β-lactamase I (in aqueous buffer) to ca. 2 mL of the temperature-equilibrated reaction mixture. The latter was prepared by mixing 25–50 μL of an ice-cooled solution of substrate into 2.0 mL of buffer solution in a quartz cuvette. Substrate solutions were prepared fresh daily in aqueous phosphate buffer (pH 6.8–7.2). At the end of reactions utilizing high enzyme concentrations (ca. 70 nM or greater), thorough rinsing of cuvettes with 50% aqueous ethanol containing ca. 1 M HCl was found to be essential to remove all traces of β-lactamase I, which adsorbs to glass (Kogut et al., 1956). Initial substrate concentrations were at least twice the K_m values in most runs from which the values of both k_{cat} and K_m were to be obtained. The experimental progress curves in these cases were fitted to the integrated from of the Michaelis–Menten equation with a computer program written by Dr. C. B. Sawyer (Rosenberg & Kirsch, 1979). This analytical method has been shown to be valid for *B. cereus* β-lactamase I (Samuni, 1975). The reaction catalyzed is essentially irreversible, and the products are not effective inhibitors of the enzyme. The K_I reported for benzylpenicilloate (hydrolyzed I) is 40 mM at pH 6.8 (Kiener & Waley, 1978). The calculated values of K_m and k_{cat} for progress curves of I at pH 3.8 were found to be insensitive to initial substrate concentrations between 1 and 4 mM under the present reaction conditions, indicating no significant product inhibition at this lower pH as well. Initial substrate concentrations were at least 10-fold lower than the values of K_m (Hardy & Kirsch, 1984) in runs from which only the values of k_{cat}/K_m were to be determined. The experimental progress curves of these pseudo-first-order reactions were analyzed by nonlinear least-squares regression to the pseudo-first-order rate equation.

The stability of β-lactamase I was assessed by incubating 0.2 μM enzyme at 25 °C in buffer solutions in quartz cuvettes. At timed intervals, 50-μL aliquots were removed and assayed by addition to 2.0 mL of 1.4 mM I in 0.2 M potassium phosphate buffer, pH 7.0, 25 °C. Initial rate measurements were made by following decreases in absorbance at 240 nm,

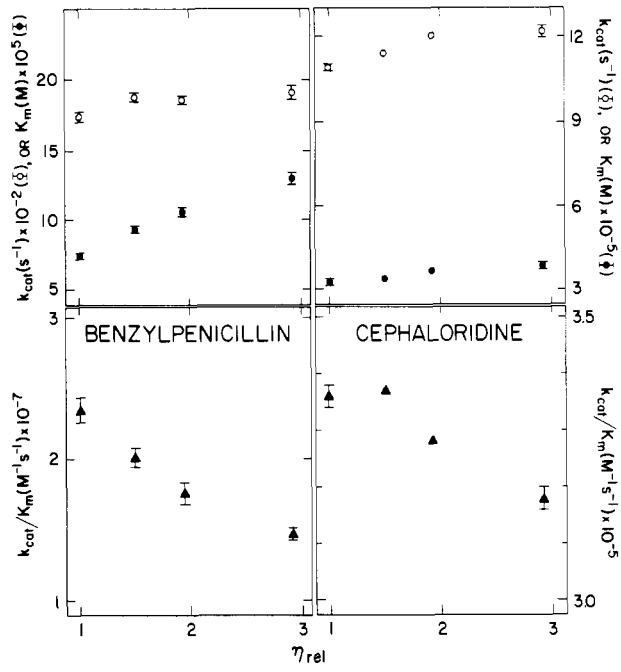


FIGURE 1: Effect of relative viscosity (η_{rel}) on (○) k_{cat}, (●) K_m, and (▲) k_{cat}/K_m for the *B. cereus* β-lactamase I catalyzed hydrolyses of (left) benzylpenicillin (I) and (right) cephaloridine (III), in 0.2 M potassium phosphate buffers, apparent pH 7.0, 0.17 M KCl, I_c = 0.63, 25 °C. The values of the kinetic parameters for I represent the means (±SE) of three or four determinations using [β-lactamase] = 0.735 nM and [I] = 0.20 mM. The values for III are derived from single or duplicate determinations (range indicated for the latter) using [β-lactamase] = 74.3 nM and [III] = 95 μM. Note that the ordinate scale expansion factors differ for the two substrates.

at which wavelength the hydrolysis of I shows a change in molar absorbency of 560. The substrate was saturating in these assays since under these conditions the K_m for I is ca. 44 μM (Hardy & Kirsch, 1984). The enzyme was found to be completely stable for at least 40 min in potassium acetate/sulfate buffer at pH 3.8 containing 0% and 29% (w/w) sucrose and in potassium phosphate/pyrophosphate at pH 7.0 and pH 9.0.

Results

The effects of viscosity in sucrose-containing buffers on the kinetic parameters for the *B. cereus* β-lactamase I catalyzed hydrolysis of I and III are compared in Figure 1. The values of k_{cat} for both substrates and of K_m for III increase slightly with increasing viscosity at pH 7, but the significantly larger increase observed in the values of K_m for I results in a much larger decrease in the relative values of k_{cat}/K_m for this substrate than is observed for III.

The kinetic parameters for I show similar pH dependences in KCl-free buffers containing 0% and 28% (w/w) sucrose (Figure 2). Similarly, no significant perturbations by sucrose in the pH dependences of the kinetic parameters for I are observed between pH 6.5 and pH 7.5 in the presence of KCl (0.22–0.13 M) at I_c = 0.6–0.7 (data not shown). The significantly higher values of K_m for I in Figure 1 vs. those in Figure 2, i.e., 75 vs. 44 μM at pH 7, are due to differences in KCl concentration and I_c. Similar decreases in the value of k_{cat}/K_m are observed as η_{rel} increases from 1.0 to ca. 2.9 under both sets of conditions. Raising the concentrations of the buffer components at pH 6 from I_c = 0.05 to I_c = 0.4 increases K_m by 38% (Table I). However, substitution of KCl for buffer components at constant I_c causes further substantial increases in K_m, indicating that Cl^- is a specific inhibitor. This effect is seen more clearly at pH 8 where K_m is independent

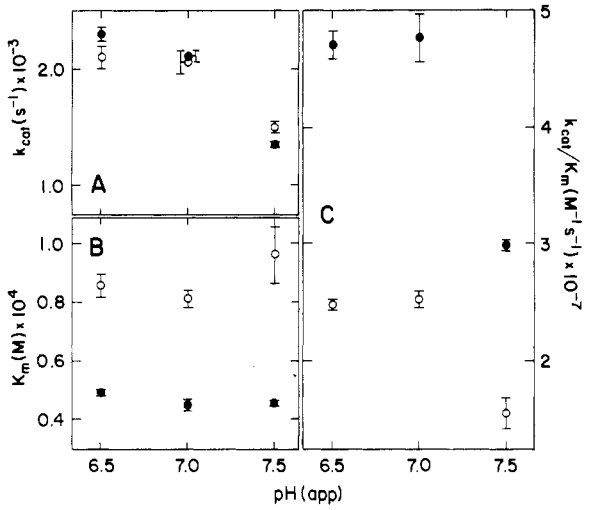


FIGURE 2: Effect of sucrose, (●) 0% and (○) 28.5% (w/w), on the apparent pH dependence of (A) k_{cat}, (B) K_m, and (C) k_{cat}/K_m for the *B. cereus* β-lactamase I catalyzed hydrolysis of benzylpenicillin (I) in potassium phosphate/pyrophosphate buffers, I_c = 0.2, 25 °C. Each point is the mean (±SE) of two to six determinations using [β-lactamase] = 1.02 nM and [I] = 0.22 mM.

Table I: Effects of Buffer and KCl Concentrations on the Kinetic Parameters for the *B. cereus* β-Lactamase I Catalyzed Hydrolysis of Benzylpenicillin (I)[a]

buffer composition,[b] [KH_2PO_4], [$K_4P_2O_7$] (M)	ionic strength, I_c	[KCl] (M)	$K_m \times 10^5$ (M) (range/2)	$k_{cat} \times 10^{-3}$ (s^{-1}) (range/2)
		pH 6.0		
0.050, 0.005	0.05	0.0	5.01 (±0.07)	1.51 (±0.02)
0.200, 0.020	0.20	0.0	5.67 (±0.26)	1.57 (±0.02)
0.400, 0.040	0.40	0.0	6.94 (±0.10)	1.52 (±0.02)
0.200, 0.020	0.40	0.20	8.79 (±0.11)	1.53 (±0.07)
0.050, 0.005	0.40	0.35	10.5 (±1.7)	1.62 (±0.14)
		pH 8.0		
0.050, 0.005	0.05	0.0	5.36 (±0.34)	1.20 (±0.07)
0.200, 0.020	0.20	0.0	5.07 (±0.89)	1.22 (±0.06)
0.400, 0.040	0.40	0.0	5.17 (±0.67)	1.24 (±0.03)
0.200, 0.020	0.40	0.20	6.78 (±0.20)	1.23 (±0.01)
0.050, 0.005	0.40	0.35	8.11 (±0.19)	1.24 (±0.10)

[a] 25 °C; [β-lactamase] = 0.735 nM; [I] = 0.23 mM. Each value is the mean of two determinations. [b] Buffer solutions were prepared by mixing a solution of KH_2PO_4 with one of $K_4P_2O_7$ of the indicated concentrations to give the desired pH. KCl where present was added to each component of the buffer.

Table II: Effect of Relative Viscosity (η_{rel}) on the Kinetic Parameters for the *B. cereus* β-Lactamase I Catalyzed Hydrolysis of Cephaloridine (III) in Sucrose-Containing, Chloride-Free Buffers[a]

η_{rel} (% sucrose, w/w)	k_{cat} (s^{-1}) (SE)	$K_m \times 10^5$ (M) (SE)	$k_{cat}/K_m \times 10^{-5}$ (M^{-1} s^{-1}) (SE)
1.0	12.2	2.96	4.11
(0)	(0.3)	(0.04)	(0.07)
2.86	12.7	3.26	3.89
(28.5)	(0.1)	(0.04)	(0.02)

[a] In potassium phosphate/pyrophosphate buffers; apparent pH 7.0; I_c = 0.2; 25 °C. Each value is the mean of six determinations using [β-lactamase] = 0.15 μM and [III] = 123 μM.

Table III: Effect of Relative Viscosity (η_{rel}) on k_{cat}/K_m for the *B. cereus* β-Lactamase I Catalyzed Hydrolyses of Benzylpenicillin (I) and Cephaloridine (III) in Sucrose-Containing Buffers at Apparent pH 3.8[a]

η_{rel} (% sucrose, w/w)	$k_{cat}/K_m \times 10^{-4}$ (M^{-1} s^{-1}) (SE) I[b]	III[c]
1.0	133.2	0.480
(0)	(2.5)	(0.005)
2.87	77.1	0.445
(28.8)	(2.5)	(0.005)

[a] In potassium acetate/sulfate buffers; I_c = ca. 0.2; 25 °C. Each value is the mean of three determinations. [b] [β-Lactamase] = 10.4 nM; [I] = 112 μM. [c] [β-Lactamase] = 1.09 μM; [III] = 34 μM.

Table IV: Effects of Ficoll and Glycerol as Viscosogenic Agents on the Kinetic Parameters for the *B. cereus* β-Lactamase I Catalyzed Hydrolysis of Benzylpenicillin (I)[a]

η_{rel}	$k_{cat} \times 10^{-3}$ (s^{-1}) (SE)	$K_m \times 10^5$ (M) (SE)	$k_{cat}/K_m \times 10^{-7}$ (M^{-1} s^{-1}) (SE)
	In Ficoll-Containing Buffers		
1.0	1.75	7.24	2.43
(0)[b]	(0.03)	(0.12)	(0.02)
1.4[c]	1.85	7.14	2.59
(3)	(0.05)	0.16	(0.01)
1.75	1.85	7.70	2.40
(6)	(0.04)	(0.09)	(0.06)
2.2	1.83	7.90	2.37
(8)	(0.09)	(0.09)	(0.10)
	In Glycerol-Containing Buffers		
1.0	1.74	7.87	2.22
(0)[d]	(0.02)	(0.09)	(0.01)
3.2[e]	1.50	22.9	0.655
(40)	(0.03)	(0.3)	(0.003)

[a] Experimental conditions are described in the legend to Figure 1. [b] Percent ficoll (w/v). [c] Values of η_{rel} for equivalent percent (w/w) ficoll as reported by Brouwer & Kirsch (1982). [d] Percent glycerol (w/v). [e] Estimated by linear interpolation of data from Chemical Rubber Co. (1972).

of I_c in the absence of KCl but increases where KCl is substituted for buffer at constant I_c. The effect of buffer concentration on K_m at pH 6 may be due to the accompanying change in I_c, since, at constant I_c and constant pH or pD, similar values of K_m are obtained in potassium phosphate/pyrophosphate buffers as in potassium acetate/sulfate buffers (Hardy & Kirsch, 1984; Hardy et al., 1984).

The kinetic parameters of III exhibit only a slight viscosity dependence in the absence of Cl^- (Table II), similar to that observed in the presence of Cl^- (Figure 1, right). Likewise, the values of k_{cat}/K_m at pH 3.8 for I and III (Table III) exhibit dependences on viscosity similar to those recorded for these substrates in the neutral pH range.

The effects of glycerol and ficoll as added viscosogens on the kinetic parameters for I are shown in Table IV. Qualitatively the effect of viscosity in glycerol-containing buffer is similar to that observed with sucrose, except that in 40% (w/v) glycerol the value of k_{cat} is decreased 14% whereas 32% (w/v) sucrose barely affects the observed value of k_{cat}. Ficoll has only slight effects on the values of the kinetic parameters, compared to those observed in sucrose-containing solutions of similar viscosity.

Table V: Effect of Relative Viscosity (η_{rel}) on the Kinetic Parameters for the *B. cereus* β-Lactamase I Catalyzed Hydrolyses of Furylacryloylpenicillin (II) and Cephalothin (IV) in Sucrose-Containing Buffers[a]

η_{rel} (% sucrose, w/w)	k_{cat} (s^{-1}) (range/2)	$K_m \times 10^5$ (M) (range/2)	$k_{cat}/K_m \times 10^{-5}$ (M^{-1} s^{-1}) (range/2)
	II[b]		
1.0 (0)	715 (±37)	3.04 (±0.06)	235 (±8)
1.49 (13.0)	675	3.93	172
1.93 (21.6)	661	4.72	140
2.92 (28.0)	649 (±8)	5.92 (±0.02)	110 (±1)
	IV[c]		
1.0 (0)	0.557 (±0.008)	5.19 (±0.07)	0.107 (±0.001)
2.92 (28.0)	0.517 (±0.007)	4.73 (±0.11)	0.109 (±0.001)

[a] In 0.2 M potassium phosphate buffers; apparent pH 7.0; 0.17 M KCl; $I_c = 0.63$; 25 °C. Each value for which a range is reported is the average of duplicate determinations. The other figures are the results of single determinations from progress curves at each sucrose concentration. [b] [β-Lactamase] = 0.735 nM; [II] = 0.16 mM. [c] [β-Lactamase] = 74.3 nM; [IV] = 98 μM.

Scheme II

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_2} E + P$$

Table V documents the effects of viscosity on the kinetic parameters for the *B. cereus* β-lactamase I catalyzed hydrolyses of compounds II and IV in sucrose-containing buffers. Increasing sucrose concentrations result in slightly lower values of k_{cat} for both of these substrates. However, the value of k_{cat}/K_m for IV obtained in 28% (w/w) sucrose is not significantly different from that measured in the absence of sucrose, whereas the value of k_{cat}/K_m for II shows the most marked dependence on η_{rel} observed in this study.

Discussion

Evaluation of Microscopic Rate Constants from Viscosity Variation Experiments. The parameter k_{cat}/K_m, the second-order rate constant for the reaction of enzyme with substrate, reflects the rate constants of all processes up to and including the first irreversible step of the reaction. The sensitivity of the value of k_{cat}/K_m to viscosity can provide a quantitative measure of the degree to which the rate of the first irreversible step in an enzyme-catalyzed reaction is controlled by diffusion (Loo & Erman, 1977; Nakatani & Dunford, 1979; Brouwer & Kirsch, 1982). Likewise, the viscosity dependence of k_{cat}, the value of which is determined by the rate constants for the slowest steps during enzymatic turnover, may measure the extent to which the rate of that process is limited by diffusion of product from the EP complex.

Evaluation of the diffusion-limited component of k_{cat}/K_m can be made by plotting the reciprocal of this parameter vs. relative viscosity, η_{rel}, according to eq 1 (Loo & Erman, 1977).

$$\frac{K_m}{k_{cat}} = \frac{1}{k_1^0}\eta_{rel} + \frac{k_{-1}^0}{k_1^0 k_2} \quad (1)$$

Such a plot yields the rate constant for enzyme–substrate association (k_1^0; cf. Scheme II) as the reciprocal of the slope, and the partition ratio of the Michaelis complex (k_{-1}^0/k_2) as k_1^0 multiplied by the ordinate intercept. The superscripts refer to the reaction at $\eta_{rel} = 1.0$, i.e., in the reference solution.

Limitations of the Stokes–Einstein Relationship. Equation 1 is based on the Stokes–Einstein relationship (Brouwer & Kirsch, 1982)

$$D = \frac{k_B T}{6\pi r}\frac{1}{\eta} \quad (2)$$

where k_B is the Boltzman constant and D and r are the diffusion coefficient and mean radius, respectively, of the species diffusing through a solvent of viscosity η. The relationship described by eq 2 was formulated (Einstein, 1905) to describe the translational diffusive motion of noninteracting spheres in a continuous medium, i.e., in a solvent whose particles are much smaller than the diffusing object. The accuracy of eq 2 has been verified for 91-nm polystyrene latex spheres in water–glycerol and water–sorbitol (Phillies, 1981) as well as for some systems where the size of the diffusing species approaches but is still larger than the components of the solvent. For example, the Stokes–Einstein relation between D and η holds well over nearly 5 orders of magnitude of η_{rel} for di-*tert*-butyl nitroxide diffusing in ethylene glycol, chloroform, water, and *n*-pentane (Berner & Kivelson, 1979).

Investigations carried out at η_{rel} values as high as 10^4 with small molecules diffusing in various pure and mixed solvents do show deviations from the Stokes–Einstein relationship (Evans et al., 1979, 1981; Davis et al., 1980). A full discussion of these papers is beyond the scope of the present work, but the results of these studies were found to be accommodated by an empirical correlation between D and η^{-q} (eq 3; Davis

$$D = AT/\eta^q \quad (3)$$

et al., 1980) where A is an empirical constant highly correlated with q, T is the temperature in K, and q, the exponential parameter, is a function of the radius r (in nm) of the diffusing species (Davis et al., 1980):

$$q = (-0.1296\ \text{nm}/r) + 1.166 \quad (4)$$

Although eq 4 gives q approaching 1.166 for very large species, Davis et al. (1980) list no values greater than 1, and it seems likely that for large molecules, eq 3 will reduce to the simple reciprocal relationship between D and the first power of η.

Equations 3 and 4 have been successfully used (Hasinoff & Chisthi, 1982) to analyze diffusive limitations in the binding of molecular oxygen and carbon monoxide to myoglobin. The value of q predicted by eq 3 and 4 for these small species is close to 0.5, and indeed the rate constants for the associations with myoglobin depend on approximately the square root of η_{rel} over 4 orders of magnitude in η_{rel} (Hasinoff & Chisthi, 1982; Beece et al., 1980).

The consequence of these considerations for the present study is that values of k_1^0 and k_{-1}^0/k_2 calculated from eq 1 will be overestimated to the extent that diffusion rates of substrates do not depend directly on the reciprocal of viscosity. Employing eq 3 to derive an expression analogous to eq 1 (Brouwer & Kirsch, 1982) leads to

$$D = \frac{1}{k_1^0}\eta_{rel}^q + \frac{k_{-1}^0}{k_1^0 k_2} \quad (5)$$

It seems unlikely that the value of q for penicillins and cephalosporins is as low as 0.5, since quantitative analysis of the data for II based on this figure gave a negative value for the E·S partition ratio k_{-1}^0/k_2. A more reasonable estimate of q can be obtained with eq 4, using a value of r estimated from the lattice parameters of crystalline penicillins and cephalosporins. From the X-ray diffraction studies of the

structure of sodium salt of I (Clark et al., 1949), the calculated value of r equals 0.46 nm, and for the hydrochloride monohydrate of III (Sweet & Dahl, 1970) r equals 0.50 nm. Using these values in eq 4 gives $q = 0.9$ for I and III.

Most of the values of k_1^0 and k_{-1}^0/k_2 calculated from the data reported here using eq 1 are ca. 20% higher than are obtained by using eq 5 with $q = 0.9$. The values of k_{-1}^0/k_2 for substrates I and II are 35–60% higher. Since the calculated values of k_{-1}^0/k_2 vary over a 40-fold range by using *either* eq 1 or eq 5, the *relative* values of the partition ratios are not affected by an amount much more than the estimated experimental errors when eq 5 is used rather than eq 1; therefore, eq 1 has been used throughout in the analyses discussed below.

Diffusion of Ionic and Neutral Species in Polymeric Solutions. The previous study on the sensitivity of the rates of reaction of chymotrypsin with esters of *N*-acyl-L-tryptophan to η_{rel} showed nearly identical dependencies with either sucrose or ficoll as the viscosogenic cosolute (Brouwer & Kirsch, 1982); thus, the present observed independence of the values of k_{cat}/K_m to η_{rel} in ficoll-containing buffers (Table IV) was unanticipated. The explanation for this difference may lie in the fact that the chymotrypsin substrates are hydrophobic neutral molecules, whereas the penicillins and cephalosporins are charged species. The diffusion rates of ions are retarded in aqueous solutions in proportion to the volume fraction of cosolute rather than to the reciprocal of macroscopic viscosity as shown by Stokes & Weeks (1964), who compared conductances of ions in solutions of sucrose and in ficoll. The increase in relative viscosity per volume fraction of added cosolute is much smaller for small molecules such as sucrose or glycerol than it is for polymers. For example, a solution of 4.93% ficoll has $\eta_{rel} = 2.1$ for a volume fraction of 0.03, while a 20% sucrose solution has $\eta_{rel} = 1.9$ but a volume fraction of 0.14 (Stokes & Weeks, 1964). Phillips et al. (1977) report similar findings for the self-diffusion coefficient of sodium ion in solutions of polyethylene glycol compared with ethylene glycol. The same principle also applies to the diffusion of small hydrophilic diffusing species such as glycolamide and acetamide (Biancheria & Kegeles, 1957). Some of the theories offered in explanation of these and similar results have recently been reviewed (Muhr & Blanshard, 1982).

Influence of Cosolute-Mediated Changes in Dielectric Constant on Diffusion-Controlled Reaction Rates. The theoretical value for the rate of association of a charged ligand with the active site of an enzyme has been discussed recently by Nolte et al. (1980) and is given by

$$k_1 = \frac{2\pi N}{1000}(D_E + D_L)d_{E\cdot L}\phi_1 \quad (6)$$

where

$$\phi_1 = \psi(\exp(\psi) - 1)^{-1} \quad (7)$$

and

$$\psi = Z_E Z_L e_0^2/(4\pi\epsilon_0\epsilon kT d_{E\cdot L}) \quad (8)$$

The definitions of the quantities in the equations together with numerical values valid under conditions very close to those used here are given by Nolte et al. (1980). The variable under present consideration is the dielectric constant, ϵ, which decreases from a value of 78.5 in water at 25 °C to 70.9 in a 30% sucrose/water solution (Åkerlöf, 1932).

Assuming the substrate to have a charge of 1− and the active site to have a charge of 6+, 2+, or 1+; the *increase* in the diffusion-controlled rate constant engendered by the above *decrease* in ϵ is 12%, 7%, or 4%, respectively. This consideration, even in an extreme case, introduces a relatively minor correction to the values of k_1^0 and k_{-1}^0/k_2 derived from the viscosity variation experiments. The slight dependence of the value of k_{cat}/K_m on I_c (Table II) is consistent with a small net charge at the active site of the enzyme (Tinoco et al., 1978).

Table VI: Values of the Association Rate Constants (k_1^0) and Partition Ratios (k_{-1}^0/k_2) for *B. cereus* β-Lactamase I Catalyzed Hydrolyses[a]

substrate (conditions)	no. of determn[b]	$10^{-6}k_1$ (M^{-1} s^{-1}) (SE)	k_{-1}^0/k_2 (SE)	EH/E_t[c]
I (pH 3.8)	6	3.4 (0.2)	1.6 (0.2)	0.017
I (pH 6.5)	8	57 (12)	1.2 (1.1)	0.89
I (pH 7.0)	12	97 (9)	1.2 (0.3)	0.93
I (pH 7.5)	4	80 (25)	1.1 (0.7)	0.88
I (+KCl, pH 7.0)	14	76 (6)	2.3 (0.2)	
II (+KCl, pH 7.0)	6	40 (2)	0.77 (0.13)	
III (pH 3.8)	6	0.12 (0.02)	23 (4)	0.017
III (pH 7.0)	12	14 (4)	32 (9)	0.93
III (+KCl, pH 7.0)	6	11 (2)	30 (5)	

[a] From nonlinear least-squares regression on eq 1 of the data, of which the mean values are given in Figures 1 and 3 and Tables II, III, and V. [b] Number of determinations of k_{cat}/K_m used in calculating the value of k_1^0 and k_{-1}^0/k_2. [c] Fraction of β-lactamase I in the active form, as calculated from the pK_a values of the free enzyme (Hardy & Kirsch, 1984).

Application to β-Lactamase. The values of k_{cat}/K_m for the *B. cereus* β-lactamase I catalyzed reactions investigated in the present study vary from $>10^7$ M^{-1} s^{-1} (for I and II) to 10^4 M^{-1} s^{-1} (for IV). The former values approach the diffusion-controlled limit for reaction of a protein with a ligand of molecular weight less than 500 (Sõlc & Stockmayer, 1973) and hence should be somewhat sensitive to viscosity. The latter value, being far below the diffusion-controlled limit, is expected to be insensitive to viscosity and thus provides a control for gross changes induced in the structure of the enzyme by the cosolute. It is further to be expected that the value of k_{cat}, usually measuring a first-order chemical process, would be relatively independent of η_{rel} for all substrates in the absence of either a cosolvent induced change in the structure of the enzyme or limitations due to rate-limiting dissociation of the EP complex. These expectations are largely borne out experimentally. The values of k_{cat} are altered by no more than 10% if at all in buffers containing up to 28% (w/w) sucrose (η_{rel} = ca. 2.9). In contrast, the values of k_{cat}/K_m decrease significantly with increased viscosity for substrates whose rate constants approach the diffusion-controlled limit (I and II) but only slightly or not at all for substrates whose rate constants are far below this limit (III and IV).

The effects of added sucrose on the value of k_{cat}/K_m are not due to shifts in the pH profile for this parameter (cf. Figure 2). This is an important point, since experiments with 6-aminopenicillanate showed that the pH profile of k_{cat}/K_m for that substrate is greatly perturbed by added sucrose (Hardy, 1983). This latter perturbation is probably due to an effect of changing solvent polarity on a substrate pK_a, which in turn might effect changes in the pH profile of k_{cat}/K_m [cf. Hardy et al. (1984)].

The data in Figures 1 and 2 and in Tables II–IV were analyzed by nonlinear least-squares regression on eq 1, and

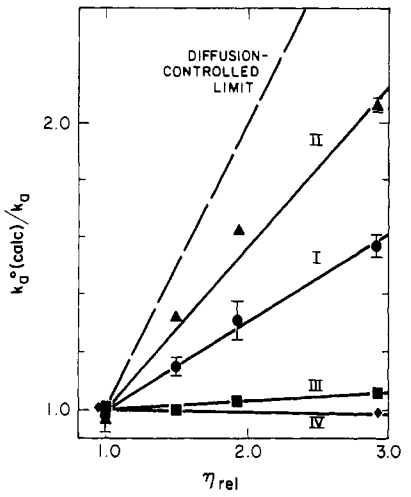


FIGURE 3: Plots of k_a^0(calcd)/k_a vs. η_{rel} for the reactions of *B. cereus* β-lactamase I with various substrates in sucrose-containing buffers (0.2 M potassium phosphate, apparent pH 7.0, 0.17 M KCl, I_c = 0.63, 25 °C): benzylpenicillin [I (●)], furylacryloylpenicillin [II (▲)], cephaloridine [III (■)] and cephalothin [IV (♦)]. The solid lines are drawn by using the least-squares values of k_1^0 and k_{-1}^0/k_2 calculated from eq 1 and plotted according to eq 9. The dashed line with slope = 1 is drawn for a completely diffusion-controlled rate of reaction.

the values of k_1^0 and k_{-1}^0/k_2 so obtained are given in Table VI. Because the values of k_{cat}/K_m range over 3 orders of magnitude, a graphic comparison of the effects of viscosity on the reaction rates of the different substrates is best visualized from a normalized plot according to eq 9 (Brouwer & Kirsch, 1982)

$$\frac{k_a^0(\text{calcd})}{k_a} = \frac{P}{1+P} + \frac{1}{1+P}\eta_{rel} \qquad (9)$$

where k_a is the observed value of k_{cat}/K_m and k_a^0(calcd) was calculated by inserting the least-square values of k_1^0 and P ($=k_{-1}^0/k_2$) from Table VI into eq 1 with η_{rel} = 1.0. Figure 3 is such a plot according to eq 9 of the data collected at pH 7.0 in the presence of 0.17 M KCl (I_c = 0.63). The value of k_a^0(calcd) for IV, 1.08 × 10^4 M^{-1} s^{-1}, is the average experimental value for this substrate. The reaction rates of I and II approach the diffusion-controlled limit (dashed line in Figure 3), that of III is slightly diffusion controlled, and that of IV is not significantly affected by viscosity. The ratios of k_{cat}/K_m values to k_1^0 values reveal that, under these conditions, the rates of reaction of *B. cereus* β-lactamase I with substrates I, II, and III are 34%, 59%, and ca. 3% diffusion controlled, respectively.

The values of the association rate constants, k_1^0, for the penicillins I and II are greater than that for the cephalosporin III at pH 7 (Table VI). This difference may reflect a preference for binding of penicillin rather than cephalosporin at the active site of *B. cereus* β-lactamase I. Alternatively, an anionic substrate (such as I or II) may bind more rapidly than a neutral zwitterion (such as III) due to charge–charge interactions. The apparent variation in k_1^0 for I and III as a function of pH is due to the pH dependence of the fraction of enzyme that is in the proper ionization state (EH/E_t) to bind substrate productively (Hardy & Kirsch, 1984). Values of k_1^0 divided by EH/E_t agree within a factor of 3 for a given substrate. The addition of KCl decreases the value of k_1^0 for I and III, at least in part because Cl^- behaves as a competitive inhibitor of β-lactamase I catalysis (Table I).

The values of the partition ratios are, within experimental error, independent of pH for I and III. This is consistent with the idea that a single protonic state of *B. cereus* β-lactamase I binds substrate productively (Hardy & Kirsch, 1984).

Registry No. I, 61-33-6; II, 28812-95-5; III, 50-59-9; IV, 153-61-7; β-lactamase I, 9001-74-5.

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pH Dependence and Solvent Deuterium Oxide Kinetic Isotope Effects on *Bacillus cereus* β-Lactamase I Catalyzed Reactions†

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ABSTRACT: The solvent kinetic isotope effects (SKIE's) on k_{cat} (^{D}V) and on k_{cat}/K_m [$^{D}(V/K)$] were determined for the *Bacillus cereus* β-lactamase I catalyzed hydrolysis of five substrates that have values of k_{cat}/K_m varying over the range (0.014-46.3) × 10^6 M^{-1} s^{-1} and of k_{cat} between 0.5 and 2019 s^{-1}. The variation of $^{D}(V/K)$ was only from 1.06 to 1.25 among these compounds and that in ^{D}V was from 1.50 to 2.16. These results require that $^{D}k_1$, the SKIE on the enzyme-substrate association rate constant, and $^{D}(k_{-1}/k_2)$, that on the partition ratio of the ES complex, both be near 1. The larger SKIE observed on ^{D}V requires that an exchangeable proton be in flight for either or both the acylation and the deacylation reaction. The pH dependence of the values of k_{cat}/K_m for three substrates shows identical pK_as of 5.5 and 8.4. This identity combined with the fact that only one of these three substrates is kinetically "sticky" proves that the substrates can combine productively with only one protonic form of the enzyme. There is considerable substrate variation in the pK_a values of k_{cat} observed vs. pH profiles; the inflection points for all substrates studied are at pH values more extreme than are observed in the pH profiles for k_{cat}/K_m.

The β-lactamase I of *Bacillus cereus* 569/H is one of a class of β-lactamases that show extensive amino acid sequence homology (Ambler, 1980). These enzymes also share some mechanistic features including the central role of an acylserine residue as a covalent catalytic intermediate similar to that observed with the serine proteases, such as chymotrypsin (Kraut, 1977). The essential evidence favoring the acyl-enzyme hypothesis for reactions catalyzed by β-lactamases of classes A and C is reviewed in the introduction to the first paper of this series (Hardy & Kirsch, 1984). The results of stopped-flow studies with the class A β-lactamase of *Staphylococcus aureus* have been interpreted both in terms of a single step [results of S. Halford, discussed by Thatcher (1975)] and of multiple steps (Anderson & Pratt, 1981). Only

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