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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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the latter interpretation is consistent with an acyl-enzyme intermediate in β-lactamase catalysis, but the results of Halford's study do not disprove the acyl-enzyme hypothesis.

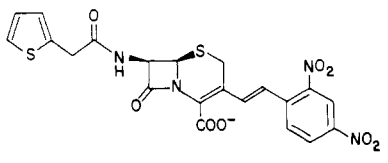
The deacylation of acyl chymotrypsins, typically monitored as k_{cat} for an ester substrate, exhibit solvent D_2O kinetic isotope effects (SKIE's)[1] of 2–3 (Bender et al., 1964). Somewhat smaller SKIE's are recorded for the acylation of chymotrypsin (Bender et al., 1964; Hubbard & Kirsch, 1972). In contrast, there have been two reports of no significant SKIE's on reactions catalyzed by the β-lactamases from *S. aureus* [work of S. Halford, reported by Thatcher (1975)] and *Escherichia coli* (Brenner & Knowles, 1981). The results reported in the present study of *B. cereus* β-lactamase I catalyzed reactions represent the first observations of significant SKIE's of β-lactamase catalyzed reactions.

The pH dependences of the kinetic parameters for the *B. cereus* β-lactamase I catalyzed hydrolyses of benzylpenicillin (I) and ampicillin have been studied previously (Waley, 1975) and led to the suggestion that $K_m = K_s$ for I. In view of the partial diffusion-control component of k_{cat}/K_m for the reaction of I with this β-lactamase (Hardy & Kirsch, 1984), this conclusion is now untenable. For this reason, as well as to facilitate the interpretation of the kinetic isotope effect data, the pH dependence of k_{cat} and k_{cat}/K_m for *B. cereus* β-lactamase I with substrate I has been reinvestigated and extended to include other less reactive substrates.

Materials and Methods

The enzyme preparation and general assay procedures are described in the previous paper (Hardy & Kirsch, 1984). Assay volumes of ca. 1 mL were used in the present work in order to conserve D_2O-containing buffers. The volumes of H_2O-containing stock substrate and enzyme solutions added to D_2O reaction mixtures were 5% or less of the total. No corrections have been made for resulting dilutions of deuterium. The same reagent stock solutions were used for runs in H_2O and D_2O, which were done alternately. For a series of runs in which pH (pD) was varied, a palindromic sequence of variation was employed, so that the first pH (pD) values used were also used last. Possible errors due to loss of activity in stock solutions of enzyme (stored on ice or at 4 °C) were thereby monitored and randomized. Such losses were, however, almost never observed on the time scale (48 h or less) required to determine a complete pH (pD) rate profile for a single substrate. The enzyme is stable at the assay temperature (25 °C) for at least 40 min at pH 3.8, 7.0, and 9.0. Identical kinetics were observed when stock solutions of enzyme prepared in H_2O and D_2O were used.

Benzylpenicillin (I), furylacryloylpenicillin (II), cephaloridine (III), and cephalothin (IV) are described in the previous paper (Hardy & Kirsch, 1984). Nitrocefin (V) was used as



Nitrocefin, V

an additional substrate in the present investigation. V (also referred to as cephalosporin 87/312) has had great utility in the rapid and sensitive detection of β-lactamase activity (Matthew & Harris, 1976) since its color changes from yellow to red upon hydrolysis (O'Callaghan et al., 1972). Users should be aware of a report that V is a carcinogen in laboratory animals (personal communication to A. L. Fink from BBL Microbiology Systems). Nitrocefin (V) should be handled with caution until estimates of its mutagenic potency become available. (D. Levin from the laboratory of B. N. Ames of this department has determined V and/or a metabolite of V to be a weak mutagen in *Salmonella typhimurium*.)

The free acid form of V was provided by Glaxo Research, Ltd. Since this material is not very water soluble, a stock solution of 18.8 mM was prepared by dissolving the free acid in a mixture of equal volumes of 0.08 M K_2HPO_4 (in H_2O) and acetonitrile (Burdick & Jackson Laboratories). The reaction mixtures for kinetic runs with V thus contained 0.5–1.2% (v/v) of acetonitrile, which was shown in control experiments with other substrates not to affect the activity of the enzyme.

The hydrolysis of V was monitored at 640 nm, where the change in molar absorbency accompanying hydrolysis is −822 (±22). The hydrolysis of III was followed at 280 nm, where the change in molar absorbency upon hydrolysis is 4953 (±18). The indicated limits are standard errors (SE's) from multiple determinations with a single stock solution of the indicated substrate. Isotope effects on the values of the absorbency changes accompanying hydrolysis were 5% or less for all substrates studied.

Kinetic parameters were calculated by fitting the absorbance changes for individual runs measured over the entire time course of the reaction to an integrated form of the Michaelis–Menten equation (Rosenberg & Kirsch, 1979). The lengths of the time courses were kept short by use of appropriate enzyme concentrations in order to minimize nonenzymatic hydrolyses of substrates at the extremes of pH. For example, the runs with I at pH 3.8 were completed in less than 3 min, during which time the first-order nonenzymatic hydrolysis of this penicillin would have caused less than a 2% decrease from its initial concentration [calculated from the rate constant for acid hydrolysis at 30 °C (Yamana et al., 1977)]. Cephalosporins are more acid stable in the pH range investigated (Yamana et al., 1974). Measurements of absorbance changes in controls (reaction mixtures lacking enzyme) confirmed that nonenzymatic hydrolyses and rearrangements were negligible on the time scale of the enzymatic reactions.

The buffers used for most of this work were potassium acetate/sulfate, $I_c \sim 0.2$ (pH ≤5.6, pD ≤6.4) and potassium phosphate/pyrophosphate, $I_c = 0.2$ (pH ≥5.6, pD ≥6.0). Potassium acetate/sulfate buffers were prepared by mixing 0.2 M potassium acetate with 0.2 M potassium acetate containing 0.2 M H_2SO_4 to give the desired pH. Potassium phosphate/pyrophosphate buffers were prepared by mixing 0.2 M KH_2PO_4 with 0.02 M $K_4P_2O_7$ to give the desired pH. All buffer solutions were filtered through sintered glass funnels prior to use.

Buffer solutions in D_2O were prepared similarly to those in H_2O. Potassium acetate or potassium pyrophosphate solutions in D_2O were prepared by addition of the anhydrous salt to 99.75% D_2O (purchased from Bio-Rad Laboratories and glass distilled). D_2SO_4 (0.2 M) was prepared by addition of 1.1 mL of 98% D_2SO_4 (99.5% D, Aldrich Chemical Co.) per 100 mL of final solution. In a typical preparation of 0.2 M KD_2PO_4, 10 mmol of KH_2PO_4 was dissolved in 10 mL of D_2O and the frozen solution lyophilized. The dry partially exchanged salt was redissolved in 10 mL of D_2O and the frozen solution lyophilized. The dry KD_2PO_4 was finally dissolved in D_2O and transferred quantitatively to a volumetric flask, and the solution was made to 50 mL with D_2O.

[1] Abbreviation: SKIE, solvent D_2O kinetic isotope effect.

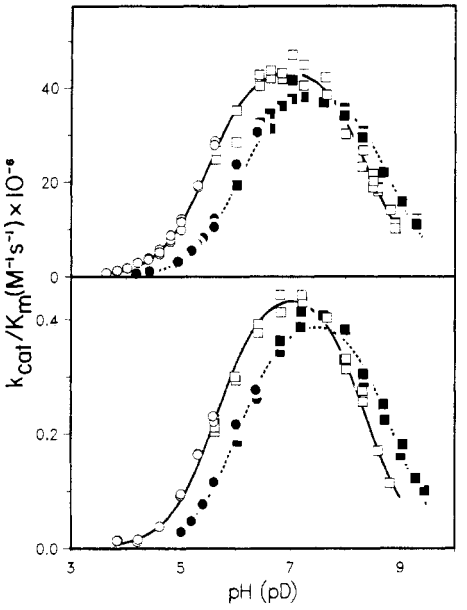


FIGURE 1: pH (pD) dependence of k_{cat}/K_m for the *B. cereus* β-lactamase I catalyzed hydrolyses of I (A) and III (B) in H_2O (open symbols) and D_2O (solid symbols) in potassium acetate/sulfate (circles) and potassium phosphate/pyrophosphate (squares) buffers, I_c = 0.2, 25 °C. Each point is the value calculated from the time course of a single run. The solid lines are the theoretical curves calculated for H_2O from eq 1 by using the pK_a values listed in Table I; the broken lines are the theoretical curves for D_2O. Component concentrations were as follows: (A) [β-lactamase] = 1.4–7.1 nM and [I] = 0.22–1.1 mM; (B) [β-lactamase] = 0.15–0.36 μM and [III] = 0.09–0.34 mM.

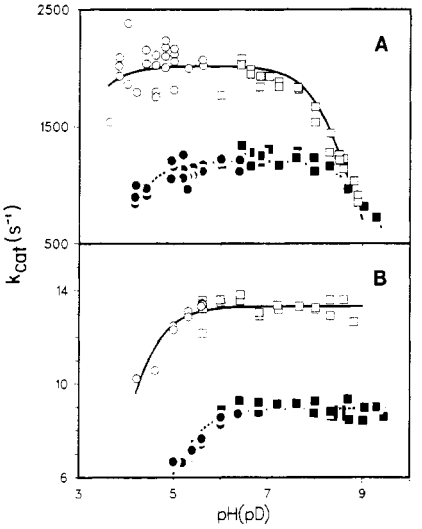


FIGURE 2: pH (pD) dependence of k_{cat} for the *B. cereus* β-lactamase I catalyzed hydrolyses of I (A) and III (B) in H_2O (open symbols) and D_2O (solid symbols). Experimental conditions are given in the legend to Figure 1. The solid (H_2O) and (D_2O) broken lines are the theoretical curves calculated from (A) eq 1 and (B) eq 2 by using the pK_a values listed in Table I.

The pH (pD) of buffers, and of all reaction mixtures at the end of kinetic runs, were measured by using a Radiometer Model pHM64 meter and a GK2421c electrode. The values of pD were recorded as pH meter readings + 0.4 (Glasoe & Long, 1960; Covington et al., 1968).

Results

The effects of variation of pH and pD on k_{cat}/K_m values for I and III are shown in Figure 1. The values of k_{cat} for I and III are plotted as functions of pH and pD in Figure 2. The apparent pK_a values obtained from nonlinear least-squares regression analyses of the data in Figures 1 and 2, and of similar data (not shown) obtained with V, are listed in Table I. The data were fit to eq 1 to obtain all of the pK_a values

$$k_{obsd} = \frac{k_{lim}}{1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}} \quad (1)$$

$$k_{obsd} = \frac{k_{lim}}{1 + 10^{pK_{a1}-pH}} \quad (2)$$

listed in Table I, with the exception of those obtained from the pH and pD dependences of k_{cat} for III, which were obtained by fitting the data in Figure 2B to eq 2.

Table I: Apparent pK_a Values for Reactions of *B. cereus* β-Lactamase I in H_2O and D_2O[a]

substrate	k_{cat}/K_m pK_{a1} (SE) H_2O	k_{cat}/K_m pK_{a1} (SE) D_2O	k_{cat}/K_m pK_{a2} (SE) H_2O	k_{cat}/K_m pK_{a2} (SE) D_2O	k_{cat} pK_{a1} (SE) H_2O	k_{cat} pK_{a1} (SE) D_2O	k_{cat} pK_{a2} (SE) H_2O	k_{cat} pK_{a2} (SE) D_2O
I[b]	5.49 (0.02)	6.06 (0.05)	8.39 (0.02)	8.80 (0.05)	2.6 (0.3)	3.8 (0.1)	8.7 (0.1)	9.4 (0.1)
III[b]	5.66 (0.03)	6.07 (0.03)	8.36 (0.04)	8.84 (0.04)	3.8 (0.1)	4.7 (0.1)		
V[c]	5.50 (0.07)	5.93 (0.14)	8.42 (0.05)	8.88 (0.08)	5.0 (0.1)	5.4 (0.1)	9.5 (0.1)	10.1 (0.3)

[a] Calculated by nonlinear least-squares regression to eq 1, except for the pK_as for the k_{cat} values for III, which were obtained from eq 2. [b] From data shown in Figures 1 and 2. [c] Experimental conditions for the pH and pD profiles ov V were identical with those given in legend of Figure 1, except [β-lactamase] = 25–75 nM and [V] = 0.17–0.59 mM. The pK_a values were calculated from 18 runs between pH 5.61 and pH 8.80 and 17 runs between pD 6.41 and pD 9.28.

The kinetic parameters for V were measured over only a limited pH (pD) range (Table I, footnote *c*); therefore, it is not certain how well the kinetic data, especially the k_{cat} values, for V are modeled by eq 1. It was not possible to extend the pH profile for V to lower values of pH due to its insolubility below pH 5.6, presumably due to protonation of this substrate's carboxylate group. If so, this implies an unexpectedly high value of the pK_a for the carboxylate of V. Cephalosporins normally have pK_a values between 1.6 and 3 in H_2O (Stedman et al., 1964; Yamana & Tsuji, 1976),[2] and one might have expected the value for V to be at the low end of this range due to the inductive effect of the dinitrostyryl group.

The values of k_{cat} in H_2O for I are so imprecise at low pH that the sum of squared residuals and the calculated value of pK_{a2} were insignificantly affected by deleting pK_{a1} from the expression for the pH dependence (i.e., from eq 1). However, it is evident from the data obtained in D_2O that an acidic pK_a is present in the pD profile of k_{cat}. For this reason, the data in H_2O were also fit to eq 1.

The values of both pK_{a1} and pK_{a2} from the pH dependences of k_{cat}/K_m are 0.4–0.5 unit higher in D_2O than in H_2O. Isotope effects of greater magnitude were observed on the apparent pK_a values from the pH dependence of k_{cat} (see Table I). All of the isotope effects observed on pK_a values are (within experimental error) typical for weak acids (Bunton & Shiner, 1961).

The values of k_{cat} and k_{cat}/K_m in H_2O given in Table II for I, III, and V are the limiting values of these quantities calculated by the regression analyses of the pH profiles for these three substrates. Likewise, the values of ^DV[3] and $^D(V/K)$[4]

[2] Higher values for cephalosporin pK_as (3–5) recorded in the review of Hou & Poole (1971) come from work done by the group at Lilly Laboratories, which routinely performs these titrations in 66% dimethylformamide/H_2O [cf. Spencer et al. (1966)].

[3] (k_{cat} in H_2O)/(k_{cat} in D_2O).

Table II: Kinetic Parameters and Solvent Deuterium Kinetic Isotope Effects for *B. cereus* β-Lactamase I

	$k_{cat}(H_2O)$ (s^{-1}) (SE)	$10^{-6} \times k_{cat}/K_m(H_2O)$ (M^{-1} s^{-1}) (SE)	$^DV^a$ (SE)	$^D(V/K)^b$ (SE)
I[c]	2019	46.3	1.67	1.12
	(26)	(0.6)	(0.03)	(0.03)
II[d,e]	758	35.4	2.06	1.06
	(25)	(3.6)	(0.06)	(0.11)
III[c]	13.3	0.470	1.50	1.12
	(0.1)	(0.010)	(0.07)	(0.03)
IV	0.52[f]	0.0139[d,g]	2.16	1.25[d,g]
	(0.01)	(0.0001)	(0.07)	(0.03)
V[c]	33.3	0.827	1.53	1.09
	(0.4)	(0.022)	(0.02)	(0.06)

[a] $(k_{cat}$ in $H_2O)/(k_{cat}$ in $D_2O)$. [b] $(k_{cat}/K_m$ in $H_2O)/(k_{cat}/K_m$ in $D_2O)$. [c] Calculated from the limiting values of the rate constants obtained by regression analyses of pH and pD profiles; experimental conditions are given in the legend to Figure 1 and Table II, footnote *c*. [d] Calculated from the mean values of the parameters from two runs in H_2O (pH 6.8) and two runs in D_2O (pD 6.8), 25 mM potassium phosphate, 25 °C. [e] [β-Lactamase] = 1.5 nM; [II] = 97 μM. [f] Calculated as the mean of 15 determinations in H_2O between pH 5.6 and pH 8.9; the value of k_{cat} in D_2O used to calculate ^DV is the mean of 12 determinations between pD 6.2 and pD 7.7. The runs were done at 25 °C in a variety of buffers: 25 mM potassium phosphate; 20 mM potassium phosphate plus ca. 0.95 M KCl, I_c = 1.0; potassium phosphate/pyrophosphate, I_c = 0.2; potassium acetate/sulfate, I_c = ca. 0.2. [β-Lactamase] = 0.074–0.75 μM; [IV] = 0.09–0.24 mM. [g] [β-Lactamase] = 0.75 μM; [IV] = 0.13 mM.

given in Table II for I, III, and V were obtained by using the limiting values of k_{cat} and k_{cat}/K_m, respectively, obtained from the regression analyses of the pH and pD profiles. The values of the kinetic parameters and isotope effects given in Table II for II were calculated from data collected at pH 6.8 and pD 6.8, as were the values of k_{cat}/K_m (H_2O) and $^D(V/K)$ given in Table II for IV. The value of k_{cat} for IV is, within the limits of experimental error, independent of pH between pH 5.6 and pH 8.9. The values of $k_{cat}(H_2O)$ and ^DV reported in Table II for IV were calculated from multiple determinations in H_2O between pH 5.6 and pH 8.9 and in D_2O between pD 6.2 and pD 7.7.

Standards errors in the values of ^DV and $^D(V/K)$ in Table II were propagated by using eq 3.

$$\frac{\text{SE of } [(k \text{ in } H_2O)/(k \text{ in } D_2O)]}{(k \text{ in } H_2O)/(k \text{ in } D_2O)} = \left[\sum\left(\frac{\text{SE of } k}{k}\right)^2\right]^{1/2} \quad (3)$$

Discussion

pH Dependence of k_{cat}/K_m. The classical kinetic model accommodating the typical bell-shaped curve describing the pH dependence of k_{cat}/K_m for a single substrate/product enzymatically catalyzed reaction is shown in Scheme I (solid lines) in which a nonionizing substrate combines productively with a single protonic form of the enzyme. The model is described algebraically by eq 4:

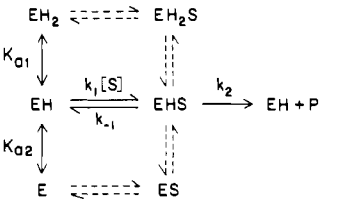
$$\frac{k_{cat}}{K_m} = \frac{(k_a)_{lim}}{1 + H/K_{a1} + K_{a2}/H} \quad (4)$$

$$(k_a)_{lim} = \frac{k_1 k_2}{k_{-1} + k_2} \quad (4a)$$

where $(k_a)_{lim}$ is the pH-independent second-order rate constant for reaction of the substrate with EH, H is the hydronium ion

[4] $(k_{cat}/K_m$ in $H_2O)/(k_{cat}/K_m$ in $D_2O)$.

Scheme I



concentration, and K_{a1} and K_{a2} are the ionization constants for enzymic groups that must be unprotonated and protonated, respectively, for productive binding to occur. Substrate ionizations are relevant when there are ionic forms of the substrate that differ in affinity for binding to EH, more than one of which are present in kinetically significant quantities at pH values in the range of pK_{a1} and pK_{a2} (Hardy et al., 1984). When only a single ionic form of the substrate binds productively, substrate ionization is treated analytically by multiplying the right side of eq 4 by an expression for the fraction of substrate in the reactive state, as a function of pH and substrate pK_a (s).

The common practice of assigning free enzyme pK_a values from the pH dependence of k_{cat}/K_m is predicated on two assumptions (Peller & Alberty, 1959; Knowles, 1976). They are (a) that proton transfers to and from free enzyme and substrate are much faster than formation and breakdown of the enzyme–substrate complex and (b) that productive binding is allowed only to a single protonic state of the enzyme. Assumption a, the "proton transfer equilibria approximation", is probably a reasonable one for most enzymes (Cornish-Bowden, 1976), although its validity has been questioned (Ottolenghi, 1971) and exceptions may exist under certain circumstances [e.g., carbonic anhydrase (Silverman & Tu, 1975)]. Assumption b, the "single path hypothesis", generally requires justification, since it is not rigorously correct for several well-studied enzymes [e.g., pepsin (Knowles et al., 1969) and chymotrypsin (Fersht & Requena, 1971a; Renard & Fersht, 1973)]. However, for substrates that are relatively nonsticky,[5] assumption b is not essential for the treatment of Peller & Alberty (1959) to be a good approximation. With independent knowledge of the kinetic stickiness of a substrate and of the true pK_a values of the enzyme, a reasonable estimate of the applicability of assumption b can be made. Equation 5 gives the apparent value of pK_{a1} where the dashed pathway in Scheme I is of kinetic significance (Cleland, 1977).

$$pK_{a1}(\text{apparent}) = pK_{a1}(\text{free enzyme}) - \log(1 + k_2/k_{-1}) \quad (5)$$

An analogous equation can be written for pK_{a2}:

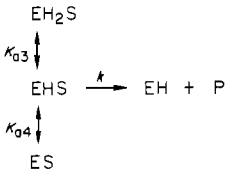
$$pK_{a2}(\text{apparent}) = pK_{a2}(\text{free enzyme}) + \log(1 + k_2/k_{-1}) \quad (6)$$

A particularly elegant example of the above analysis of the pH dependence of k_{cat}/K_m is provided by the investigation of δ-chymotrypsin by Renard & Fersht (1973), who used the perturbations of pK_a values to obtain estimates of the kinetic stickiness of very reactive substrates for which assumption b does not hold.

Nearly identical pK_a values were obtained from the k_{cat}/K_m vs. pH and pD profiles of I, III, and IV with *B. cereus* β-lactamase I (Table I). Since I is quite sticky [k_2/k_{-1} = 0.8

[5] A sticky substrate is one that dissociates from the active site of an enzyme at a rate comparable to or slower than the rate at which the ES complex goes on to form product (Cleland, 1977). In terms of the mechanism shown in Scheme I, the stickiness of a substrate is proportional to the ratio k_2/k_{-1}.

Scheme II



(Hardy & Kirsch, 1984)] relative to III and probably IV (k_2/k_{-1} = 0.03 for III), the perturbations in apparent pK_a values predicted by eq 5 and 6 are not observed. We may, therefore, conclude that the reaction pathway is adequately described by the solid lines in Scheme I, which means that either the EHS complex cannot accept or lose a proton at a kinetically significant rate or EH_2 and E are not in mobile equilibrium with their corresponding productive substrate complexes. Nonproductive binding might effect the pH dependence of k_{cat} but not of k_{cat}/K_m (Fastrez & Fersht, 1973).

The only ionizable moieties on I and III are the carboxylic acids of pK_a = 2.7 (Hou & Poole, 1971) and 1.7 (Yamana & Tsuji, 1976). The pK_a for the carboxyl group of V is not known, although the insolubility of V below pH 5.6 suggests that the pK_a may be considerably higher than in I or III. Hence, the pK_a values (5.55 and 8.4 in H_2O at 25 °C, I_c = 0.2) characterizing the pH dependence of k_{cat}/K_m for *B. cereus* β-lactamase I represent intrinsic ionization constants for prototropic groups on the free enzyme that are essential for productive binding of substrates.

pH Dependence of k_{cat}. The pH dependence of k_{cat}, for an enzymatic reaction mechanism having a single catalytic step, reflects ionizations of essential groups in the Michaelis complex (e.g., Scheme II). The mechanism shown in Scheme II is quantitatively described by eq 7:

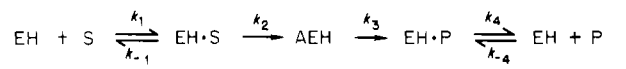
$$k_{cat} = \frac{k}{1 + H/K_{a3} + K_{a4}/H} \quad (7)$$

where k is the pH-independent rate constant for the single rate-determining step for catalytic turnover and K_{a3} and K_{a4} are the ionization constants for essential groups in the Michaelis complex. [This treatment assumes the absence of nonproductive binding of substrate to enzyme forms that are in the wrong protonation state (Fastrez & Fersht, 1973).] To the extent that substrate binding changes the environment at the enzymic active site, the pK_a values of groups essential to catalysis as well as to binding will be different in the Michaelis complex and in the free enzyme. Different substrates might be expected to perturb these pK_a values by differing amounts. To accommodate a more realistic multistep mechanism, the simple model of Scheme II must be expanded for β-lactamase. In the special case where all protonic forms of the enzyme–substrate complexes are in rapid equilibrium, a pK_a value calculated from the observed variation of k_{cat} as a function of pH will be the kinetically weighted mean of the pK_a values of all the catalytic intermediates (Fersht & Requena, 1971b), including in this case a probable acyl-enzyme (see below).

The apparent pK_a values characterizing the pH dependence of k_{cat} for *B. cereus* β-lactamase I catalyzed hydrolyses of I, III, V, and probably IV are all shifted outward with respect to the pK_a values of the free enzyme (Table I), with pK_{a3} being lower and pK_{a4} higher. If there is a pK_{a4} for III, it must be well above pH 9. There is considerable variation in these pK_a values for the various substrates, for which an adequate explanation is presently not available [see Hardy et al. (1984) for further discussion].

While there is agreement in the main of the values of k_{cat} and K_m as well as their pH dependence for the *B. cereus* β-lactamase I catalyzed hydrolysis of I with those reported earlier by Waley (1975) under slightly different conditions, important differences leading to some alternative conclusions do exist. The most prominent of these are the differences in pK_{a1} values from the pH profiles of k_{cat}/K_m, 5.49 (Table I) vs. 4.85 (Waley, 1975), and of k_{cat}, ca. 2.6 (Table I) vs. 4.85 (Waley, 1975). Thus, our observations indicate that K_m for this substrate is pH *dependent*. Further, the viscosity dependence of k_{cat}/K_m (Hardy & Kirsch, 1984) argues against the earlier conclusion that $K_m = K_s$ for this substrate (Waley, 1975).

Scheme III



The small difference in temperature [25 °C, this work vs. 30 °C (Waley, 1975)] seems to be an unlikely explanation for the differences observed in the value of pK_{a1} from k_{cat}/K_m, particularly in view of the probable assignment of pK_{a1} to a carboxyl group (Abraham & Waley, 1979) for which $\Delta H_{ionization} \simeq 0$ (Greenstein & Winitz, 1961). More significant perhaps is the presence of 0.3 M chloride in the earlier assay conditions (Waley, 1975). This ion is a competitive inhibitor of the enzyme (Hardy & Kirsch, 1984) and its K_I may be pH dependent. The reason for the larger difference between the pK_{a1} value of k_{cat} found by Waley (1975) and that reported here is elusive. The observed pH profile of k_{cat} for I (Figure 1A) is, however, consistent with the findings of Durkin et al. (1977a), who saw maximal activity of purified *B. cereus* β-lactamase between pH 5.5 and pH 6.0, with nearly 80% of maximal activity persisting at pH 4.0. The assay conditions used by Durkin et al. (1977a) were not stated.

In other studies with purified β-lactamase I from *B. cereus*, V has been variously reported not to be a substrate (Durkin et al., 1977b) or to be hydrolyzed by this enzyme more rapidly than I (Abraham & Waley, 1979). The results reported in Table II show that V is indeed a substrate for *B. cereus* β-lactamase I, albeit with a much lower value of k_{cat}.

Solvent D_2O *Kinetic Isotope Effects*. The differences between the values of ^DV and $^D(V/K)$ are consistent with the growing body of evidence [summarized by Hardy & Kirsch (1984)] that an acyl-enzyme intermediate (AEH) is central to the catalytic function of β-lactamases (Scheme III).

The general expression for $^D(V/K)$ is given by

$$^D(V/K) = \frac{k_{1,H_2O}(1 + k_{-1,D_2O}/k_{2,D_2O})}{k_{1,D_2O}(1 + k_{-1,H_2O}/k_{2,H_2O})} \quad (8)$$

where k_{i,H_2O} and k_{i,D_2O} (i = −1, 1, and 2) represent the microscopic rate constants for the reaction conducted in the indicated solvent. Although one might have expected the values of k_1 and k_{-1}, the rate constants for association and dissociation, respectively, to be slightly affected by the higher viscosity of D_2O, which is 1.24-fold greater than that of H_2O at 25 °C (Arnett & McKelvey, 1969), the intrinsic SKIE's on k_1 and k_{-1}/k_2 must be near unity since the value of $^D(V/K)$ is about 1 for *all* substrates reported here, which range widely in kinetic stickiness [i.e., values of $k_{-1,H_2O}/k_{2,H_2O}$ vary from $\gg$30 to <1 (Hardy & Kirsch, 1984)]. It is impossible to explain the observed SKIE's (Table II) via a mechanism having a single catalytic step subsequent to substrate binding unless there is a significant SKIE on the ratio $(k_{-1} + k_2)/k_1$ (Kurz & Frieden, 1983; D. A. Julin and J. F. Kirsch, unpublished experiments) whose magnitude is inversely proportional to ^DV. While this implausible circumstance cannot be ruled out, the evidence already extant for an acyl-enzyme

militates for an interpretation of the SKIE data in terms of a two-step mechanism.

The expression for k_{cat} in terms of the two-step model is given by eq 9:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad (9)$$

One or both of the rate constants for acylation (k_2) and deacylation (k_3) must be limited at least partially by the rate of one or more proton transfers, since ^{D}V is significantly greater than 1. Acylation of chymotrypsin, another serine hydrolase, by the specific ester substrate *N*-acetyltryptophan ethyl ester gives $^{D}(V/K)$ near unity (Bender et al., 1964), indicating that for this substrate the SKIE on k_2 is closer to unity than it is on k_3. Deacylation of an acylchymotrypsin proceeds with an SKIE between 2 and 3 (Bender & Kezdy, 1975). Further analysis of the SKIE's on β-lactamase reactions in terms of intrinsic isotope effects on either k_2 or k_3 is unwarranted in the absence of a value for the SKIE on K_s, which would allow calculation of intrinsic SKIE's on k_{-1} and on k_2 and hence on k_3.

Product dissociation, shown as a separate step in Scheme III for completeness, cannot be rate determining for the overall reaction since k_{cat} is little affected by the viscosity of the solvent (Hardy & Kirsch, 1984); therefore, $k_4 \gg k_2k_3/(k_2 + k_3)$. A similar conclusion has been drawn by Abraham & Waley (1979), who have calculated that k_4 cannot be rate determining from the value of K_I for product and a plausible estimate for its association rate constant, k_{-4}.

The values of ^{D}V between 1.5 and 2 reported here for *B. cereus* β-lactamase contrast with the results obtained by Halford using the *S. aureus* enzyme, which shows no SKIE on the rate of hydrolysis of compound V [Halford's results described by Thatcher (1975)]. Brenner & Knowles (1981) also observed no SKIE on the rate of *E. coli* β-lactamase catalyzed hydrolysis of penicillinate sulfone.

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Registry No. I, 61-33-6; II, 28812-95-5; III, 50-59-9; IV, 153-61-7; V, 41906-86-9; β-lactamase I, 9001-74-5.

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