mechanism in which imidazole (from histidine) attacks the thiol ester to form a new acyl-enzyme (acyl-imidazole), followed by a general base catalyzed nucleophilic attack by a bound nucleophile (H_2O or added nucleophile) in which the general base is a group other than the nucleophilic imidazole. This possibility may be ruled out, however. If the first (nucleophilic) step is rate determining, deacylation would not show a kinetic dependence on the concentration of added nucleophile, contrary to experiment; if the second (general base catalyzed) step is rate determining, the intermediate (acyl-imidazole) should be observable, yet no such intermediate has been observed. Nucleophilic catalysis by imidazole may also be ruled out on the basis of the high reactivity of amine nucleophiles compared to alcohol nucleophiles towards *trans*-cinnamoyl-papain. If such catalysis occurred, the added nucleophiles would actually be attacking an acyl-imidazole, toward which amines and alcohols do not show such widely differing reactivities (*vide supra*).

The Kinetics and Mechanism of Papain-Catalyzed Hydrolyses[1]

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Abstract: The kinetics of the papain-catalyzed hydrolysis of the *p*-nitrophenyl, benzyl, and methyl esters of α-N-benzyloxycarbonyl-L-lysine have been studied as a function of pH at 25° and are consistent with the two-step mechanism involving an acyl-enzyme intermediate. The complex rate constant, k_{cat}, is dependent on an ionizable basic group of $pK_a = 3.5$; k_{cat}/K_m shows a bell-shaped pH dependence. The acylation and deacylation rate constants and the binding constants were determined for each ester. Differences in K_s suggest that the leaving group is involved in binding. In the hydrolyses of *p*-nitrophenyl ε-N-formyl-α-N-benzyloxycarbonyl-L-lysinate and *p*-nitrophenyl α-N-acetyl-L-tryptophanatate k_{cat} is dependent on ionizable basic groups of $pK_a = 3.96$ and 4.70, respectively. The rate of inhibition of papain by L-1-chloro-3-tosylamido-4-phenyl-2-butanone is dependent on an ionizable basic group of $pK_a = 8.28$, presumably the thiolate group of a cysteine residue. Amino acid analysis of the alkylated papain shows no loss of histidine. The mechanistic implications of these results and the results of other workers are discussed.

Several investigators have concluded that papain, like α-chymotrypsin, catalyzes hydrolytic reactions by the mechanism of eq 1.[2-5] The rate of disappearance of substrate, v, is given by eq 2, and eq 3 and 4 relate the complex constants k_{cat} and K_m to the rate constants of eq 1.[6,7]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (1)$$

$$v = k_{cat}(E)_0(S)/((S) + K_m) \quad (2)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (3)$$

$$K_m = k_3K_s/(k_2 + k_3); \; K_s = k_{-1}/k_1 \quad (4)$$

We have found that the *p*-nitrophenyl, benzyl, and methyl esters of α-N-benzyloxycarbonyl-L-lysine are the best substrates yet found for papain, and it was of interest to determine whether the kinetics of their hydrolyses are consistent with eq 1. The results are reported here along with some studies on the inhibition of papain by L-1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK).[8]

(1) This research was supported by grants from the National Institutes of Health.

(2) E. L. Smith, *J. Biol. Chem.*, **233**, 1392 (1958).

(3) J. R. Whitaker and M. L. Bender, *J. Am. Chem. Soc.*, **87**, 2728 (1965).

(4) G. Lowe and A. Williams, *Biochem. J.*, **96**, 199 (1965).

(5) J. F. Kirsch and M. Igelström, *Biochemistry*, **5**, 783 (1966).

(6) Except for k_{cat} the symbols used for the kinetic constants are those recommended by the International Union of Biochemistry, "Enzyme Nomenclature," Elsevier Publishing Co., Amsterdam, 1965, p 49.

(7) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(8) Abbreviations used in this paper are: TPCK, L-1-chloro-3-tosylamido-4-phenyl-2-butanone; Z, benzyloxycarbonyl substituted at the α-amino position of L-amino acids.

Experimental Section

Materials. Papain, buffer materials, and routine procedures have been described in the preceding paper.[9] The normality of papain stock solutions was determined using α-N-benzoyl-L-arginine ethyl ester as a secondary standard according to the published procedure.[10] The *p*-nitrophenyl,[11] benzyl,[12] and methyl,[12] esters of Z-lysine, and the *p*-nitrophenyl ester of α-N-acetyl-DL-tryptophan[13] have been described in previous papers from this laboratory. Stock solutions of the lysine substrates were made up in acetonitrile with 5–10% distilled water added to increase solubility. No spontaneous hydrolysis occurred in these solutions when stored at 2° for a period of several weeks. Stock solutions of *p*-nitrophenyl α-N-acetyl-DL-tryptophanate were prepared using anhydrous acetonitrile.

TPCK was purchased from the Cyclo Chemical Corp. and recrystallized from 95% ethanol–water by Dr. R. L. Blakeley; mp 106–107° (lit.[14] 102–103°).

Kinetic Measurements. Esters of Z-Lysine. Reactions were followed spectrophotometrically using the Cary Model 14 CM recording spectrophotometer. Wavelengths used were: methyl ester, 224 mμ ($\Delta\epsilon$ −73.0 at neutral pH); benzyl ester, 236 mμ ($\Delta\epsilon$ −38.9 at neutral pH); and *p*-nitrophenyl ester, 340 mμ below pH 7, 400 mμ above pH 7. In experiments with the benzyl and methyl esters, a 1.1 neutral density screen was placed in the reference light path when using the 0.0–2.0 slide wire, a 0.5 neutral density screen when using the 0.0–0.2 slide wire (benzyl ester only). Reactions were generally performed as follows. Substrate (50 μl)

(9) L. J. Brubacher and M. L. Bender, *J. Am. Chem. Soc.*, **88**, 5871 (1966).

(10) M. L. Bender, *et al.*, *ibid.*, **88**, 5890 (1966).

(11) M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, **19**, 161 (1965).

(12) M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.*, **87**, 4954 (1965).

(13) B. Zerner, R. P. M. Bond, and M. L. Bender, *ibid.*, **86**, 3674 (1964).

(14) G. Schoellman and E. Shaw, *Biochemistry*, **2**, 252 (1963).

was added to 3.00 ml of buffer solution in a 1.00-cm cell, spontaneous hydrolysis was observed for several seconds, and then the desired volume (usually 50 μl) of papain was added. (See Tables I, II, and III for $(S)_0$ and $(E)_0$.) At the lowest papain concentration (1×10^{-8} *M*) used in the *p*-nitrophenyl ester reactions some of the enzyme appeared to be rendered inactive essentially instantaneously upon addition to the cell. Hence these data were not used to determine k_{cat}.

All reactions were followed to completion. Thus it was possible to determine the $\Delta\epsilon$ at the pH of each reaction. The kinetic constants k_{cat} and K_m were determined from Lineweaver–Burk plots[15] using data from each complete reaction[16] with the following exceptions. K_m for the benzyl ester between pH 6 and 9 was determined as the substrate concentration at which the slope of the spectrophotometric trace was half the initial (zero-order) slope; k_{cat} values for the *p*-nitrophenyl ester below pH 7, and for the benzyl ester between pH 5 and 8, were determined from the zero-order part of the spectrophotometric trace where $(S)_0 \gg K_m$. No spontaneous hydrolysis was observed with the methyl and benzyl esters. Corrections for spontaneous hydrolysis of the *p*-nitrophenyl ester were made. The absence of significant product inhibition was demonstrated for each ester (see Results section).

p-Nitrophenyl α-N-Acetyl-DL-tryptophanate. The procedure for following the papain-catalyzed hydrolysis of this substrate was the same as for *p*-nitrophenyl Z-lysinate, except that both k_{cat} and K_m were determined from the Lineweaver–Burk plot.

Inactivation by TPCK. The rate of inactivation of papain by TPCK was followed by adding excess TPCK to papain, then withdrawing aliquots at various times and assaying for enzymatic activity toward *p*-nitrophenyl Z-lysinate with $(S)_0$ 75-fold greater than K_m. To 3.00 ml of the appropriate buffer at 25.0° was added 50 μl of 1.83×10^{-4} *M* papain. After 10–15 min 100 μl of 3.37×10^{-3} *M* TPCK in acetonitrile was added. A 50-μl aliquot of this solution was added to a 1.00-cm cell containing 3.00 ml of 0.05 *M* acetate buffer (μ = 0.038, pH 5.2), and the time noted. The change to low pH and the large dilution effectively quenches the reaction between papain and TPCK. Then 50 μl of a 9×10^{-3} *M* solution of *p*-nitrophenyl Z-lysinate in acetonitrile was added to the cell and the reaction followed for 50–100 sec at 340 mμ. The zero-order initial rate gives a measure of the remaining concentration of papain. Further aliquots were assayed at later times including an "infinity" assay. The disappearance of papain activity was found to be pseudo first order in papain.

Amino Acid Analysis of TPCK-Inactivated Papain. To 0.316 μmole of papain (which is 58.7% active by titration) in 20.7 ml was added 4.51 μmoles of TPCK in 0.7 ml of acetonitrile. After 100 min the papain activity was less than 1% of its initial value, and the solution was dialyzed for 44 hr at 4° with six changes of distilled water. Spectrophotometric analysis of the dialysates indicated that 3.9 ± 0.4 μmoles of TPCK was removed. At the end of the dialysis period the papain activity was 0.1% of its initial activity and 0.5% of the control. The solution was lyophilized, then hydrolyzed with 3.00 ml of triply distilled constant-boiling HCl for 22 hr at 111°. The hydrolysate was evaporated over P_4O_{10} and NaOH in a vacuum dessicator overnight. The residue was dissolved in 5.00 ml of pH 2.2 citrate buffer; 2 ml of this solution was analyzed on the Beckman Model 120 B amino acid analyzer for histidine and lysine. Another 1.00 ml was analyzed for the neutral and acidic amino acids.[17]

A papain control with no added TPCK and a TPCK-treated mercuripapain control were carried through the same procedure described above.

Results

Kinetics of Hydrolysis of *p*-Nitrophenyl, Benzyl, and Methyl Z-Lysinate. Some typical Lineweaver–Burk plots for each of the three esters are shown in Figure 1. The experimental values of k_{cat} and K_m for the *p*-nitrophenyl, benzyl, and methyl esters are compiled in Tables I, II, and III, respectively. The uncertainties

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(16) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kézdy, *ibid.*, **86**, 3697 (1964).
(17) Mrs. M. Gragg performed the amino acid analyses. Thanks are due to Drs. J. W. Holleman and W. R. Groskopf for assistance in this phase of the work.

Table I. Kinetic Constants of the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl Z-Lysinate[a]

Buffer	pH	k_{cat},[b] sec^{-1}	pH	K_m,[c] μM
0.1 *M* formate			3.18	11.5 ± 1.2[g]
	3.19	11.6 ± 0.3	3.19	10.8 ± 1.3[h]
	3.47	21.2 ± 0.6	3.47	6.67 ± 0.60
	3.76	30.1 ± 0.9	3.75	5.25 ± 0.57
	4.03	35.4 ± 1.1	4.02	2.87 ± 0.31
0.2 *M* acetate	3.98	33.0 ± 1.0	4.00	3.53 ± 0.37
	4.61	40.3 ± 1.2	4.64	2.68 ± 0.19
	5.38	45.3 ± 1.1[d]	5.40	1.96 ± 0.12
	5.61	44.1 ± 1.3	5.63	1.74 ± 0.31
0.067 *M* phosphate	5.41	44.0 ± 1.3	5.47	1.68 ± 0.15
	6.19	44.5 ± 1.8	6.21	1.71 ± 0.25
	6.95	42.5 ± 2.1	6.97	2.41 ± 0.20
	7.50	45.7 ± 2.7	7.53	2.16 ± 0.58[e]
			7.53	1.98 ± 0.22[f]
	8.03	38.9 ± 2.3	8.07	1.62 ± 0.31
0.03 *M* borate	7.90	41.0 ± 2.5	7.91	3.33 ± 0.46
	8.28	39.0 ± 2.3	8.29	4.92 ± 0.74

[a] 1.6% acetonitrile–water (v/v); μ = 0.200; 2×10^{-5} *M* ethylenediaminetetraacetic acid; 25.0 ± 0.1°. [b] $(S)_0$ was 1.4 to 3.0×10^{-4} *M*; enzyme concentration was 5.32 to 11.89×10^{-8} *M*. [c] $(S)_0$ was 1.4 to 3.0×10^{-5} *M*; enzyme concentration was 1.0 to 2.0×10^{-8} *M*. [d] Average of five determinations with standard deviation. [e] $(S)_0 = 1.43 \times 10^{-5}$ *M*. [f] $(S)_0 = 2.83 \times 10^{-5}$ *M*. [g] $(S)_0 = 3.04 \times 10^{-5}$ *M*. [h] $(S)_0 = 3.04 \times 10^{-4}$ *M*.

Table II. Kinetic Constants of the Papain-Catalyzed Hydrolysis of Benzyl Z-Lysinate[a]

Buffer	pH	k_{cat},[b] sec^{-1}	K_m, μM
0.1 *M* formate	3.15	11.9 ± 0.4	
	3.17		337 ± 33
	3.42	21.7 ± 0.7	
	3.47	22.2 ± 0.6	288 ± 25
	3.70	27.4 ± 0.8	
	3.77	29.3 ± 1.0	86 ± 12
	3.96	33.2 ± 1.0	
	4.01	36.9 ± 0.5	99 ± 6
0.2 *M* acetate	3.92	29.7 ± 0.9	
	3.96	33.3 ± 0.9	91 ± 11
	4.57	37.2 ± 1.1	
	4.57	36.0 ± 1.1	
	4.60	40.9 ± 0.4	31 ± 3
	5.32	40.1 ± 1.2	
	5.36		29 ± 10
	5.51	43.5 ± 1.3	
	5.58	44.0 ± 1.3	28 ± 8
0.067 *M* phosphate	6.00	43.2 ± 1.3	
	6.14	44.4 ± 1.3	26 ± 8
	6.92	44.5 ± 1.3	29 ± 8
	7.43	41.8 ± 1.5	36 ± 8
	7.46	42.4 ± 1.3	
	7.91	42.4 ± 1.3	28 ± 8
0.0125 *M* borate	7.87	39.3 ± 2.0	43 ± 10
	8.30	39.0 ± 1.9	
	8.30	39.0 ± 2.2	55 ± 16
	8.50	38.4 ± 1.3	64 ± 12
	8.74	38.2 ± 1.3	113 ± 25
	8.76	37.0 ± 1.4	74 ± 14
	9.18	36.3 ± 1.2	123 ± 25
	9.18[c]	38.0 ± 1.2	91 ± 17
	9.20[d]	39.2 ± 1.6	116 ± 16
	9.39	32.8 ± 0.9	129 ± 17
	9.40	31.2 ± 1.0[e]	
0.0038 *M* borate	9.08	33.0 ± 1.5	115 ± 13

[a] 1.6% acetonitrile–water (v/v); μ = 0.200; 2×10^{-5} *M* ethylenediaminetetraacetic acid; 25.0 ± 0.1°. Enzyme concentration was 1.4 to 14.6×10^{-7} *M*. $(S)_0$ was 1.39×10^{-3} *M* unless otherwise indicated. [b] Entries at pH less than 6 with no corresponding K_m value were with $(S_0) = 4.67 \times 10^{-3}$ *M*. [c] 3.62×10^{-3} *M* benzyl alcohol added. [d] $(S)_0 = 6.95 \times 10^{-4}$ *M*. [e] Substrate was added after enzyme had preincubated in the pH 9.40 buffer for 620 sec.

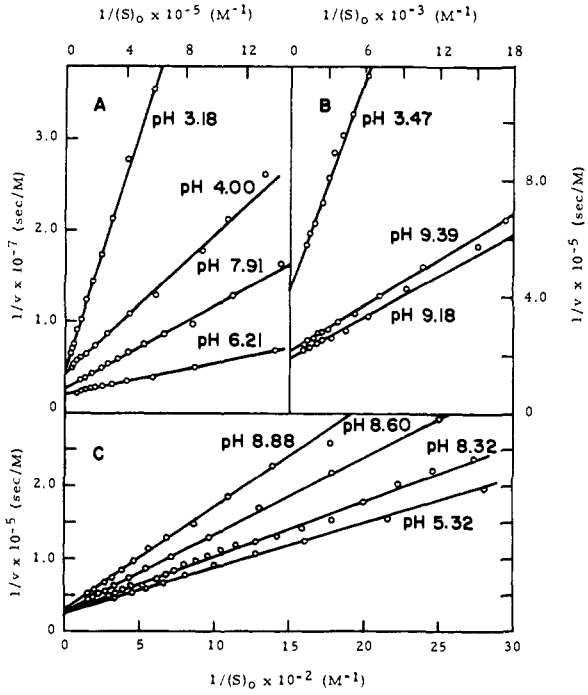

Figure 1. Some typical Lineweaver–Burk plots for the papain-catalyzed hydrolyses of Z-lysine esters; 1.6% acetonitrile–water (v/v), μ = 0.200, 25.0°. A, *p*-nitrophenyl ester; B, benzyl ester; C, methyl ester.

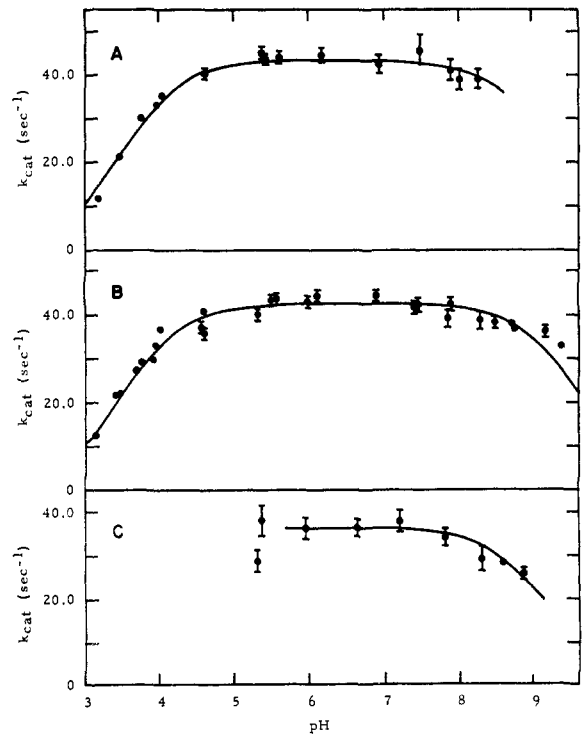

Figure 2. The effect of pH on k_{cat} for the papain-catalyzed hydrolyses of Z-lysine esters; 1.6% acetonitrile–water (v/v), μ = 0.200, 25.0°. A, *p*-nitrophenyl ester; B, benzyl ester; C, methyl ester. Theoretical curves are calculated from the values of k_2, k_3, and K_s in Table IV.

associated with these constants were estimated from the two extreme lines judged to be compatible with the data points in the Lineweaver–Burk plots or zero-order reactions. Five determinations of k_{cat} for the *p*-nitrophenyl ester at pH 5.38, some on different days with different enzyme solutions, gave the value[18] 45.3 ± 1.1 sec^{-1} indicating good reproducibility.

Table III. Kinetic Constants of the Papain-Catalyzed Hydrolysis of Methyl Z-Lysinate[a]

Buffer	pH	k_{cat}, sec^{-1}	K_m, m*M*
0.2 *M* acetate	5.32	34.7 ± 2.6	2.45 ± 0.34
	5.33	38.2 ± 3.8	3.04 ± 0.51
0.067 *M* phosphate	5.97	36.2 ± 2.7	2.25 ± 0.34
	6.65	36.4 ± 1.8	2.35 ± 0.35
	7.23	37.8 ± 2.4	2.83 ± 0.47
0.08 *M* borate	7.83	34.2 ± 2.0	2.70 ± 0.30
	8.32[b]	29.2 ± 2.7	2.95 ± 0.56
	8.60[c]	28.2 ± 0.7	3.99 ± 0.18
	8.88[d]	25.8 ± 1.4	4.86 ± 0.49

[a] 1.6% acetonitrile–water (v/v); μ = 0.200; 1.0 × 10^{-4} *M* ethylenediaminetetraacetic acid; 25.0 ± 0.1°; $(S)_0$ = 3.58 × 10^{-3} *M*; enzyme concentration was 1.14 to 1.32 × 10^{-6} *M*. [b] $(S)_0$ = 3.89 × 10^{-3} *M*. [c] $(S)_0$ = 7.11 × 10^{-3} *M*. [d] $(S)_0$ = 7.77 × 10^{-3} *M*.

All the reactions with the *p*-nitrophenyl and benzyl esters were performed in the presence of 2 × 10^{-5} *M* ethylenediaminetetraacetic acid so that small amounts of metal ion impurities would be complexed and prevented from inhibiting the enzyme which is at low concentration. The addition of more ethylenediaminetetraacetic acid up to 1.8 × 10^{-4} *M* had no effect on k_{cat} at pH 5.38 for the *p*-nitrophenyl ester. With the methyl ester 10^{-4} *M* ethylenediaminetetraacetic acid was used.

The absence of product inhibition in the case of the *p*-nitrophenyl ester was demonstrated in that, both at pH 3.18 and 7.53, two different initial substrate concentrations gave the same K_m within experimental error at each pH (see Table I). In the case of the benzyl ester the following comparisons from Table II indicate the absence of product inhibition. (a) In the formate buffers k_{cat} is the same whether 4.6 × 10^{-3} or 1.4 × 10^{-3} *M* substrate was used; (b) at pH 9.18 the addition of 3.62 × 10^{-3} *M* benzyl alcohol had no effect on k_{cat} or K_m; and (c) at pH 9.18 k_{cat} and K_m are the same within experimental error whether 0.70 × 10^{-3} or 1.4 × 10^{-3} *M* substrate was used. Since the methyl ester was studied at approximately the same level of concentration as the benzyl ester, it can be assumed that the acid product of the hydrolysis will not inhibit the reaction since it is identical in both cases. The alcohol product, methanol, is a much poorer nucleophile than benzyl alcohol[9] and thus should have no inhibitory effect at the concentration used.

The stability of papain over the pH region studied has been demonstrated by Whitaker and Bender.[3] In addition Table II shows that at pH 9.40 essentially the same value of k_{cat} is obtained for the benzyl ester whether the enzyme has been incubated at the pH of the reaction for 620 sec (the length of a typical reaction) or not.

The treatment of the data of Tables I, II, and III parallels that for α-N-benzoyl-L-arginin amide and

(18) This value may be used for purposes of titrating papain solutions using *p*-nitrophenyl Z-lysinate as an alternative to ethyl α-N-benzoyl-L-argininate as a secondary standard. The former has the advantages that (1) its high solubility and low K_m permit the assay to be made under saturation conditions thus avoiding the necessity of making a first-order plot, and (2) its high reactivity allows enzyme concentrations as low as 10^{-8} *M* to be assayed in less than 5 min.

Table IV. Limiting Values of Kinetic Constants and Corresponding pK Values for the Papain-Catalyzed Hydrolyses of the p-Nitrophenyl, Benzyl, and Methyl Esters of Z-Lysine[a]

Kinetic constant	Limiting value			pK_1; pK_2		
	PNPE	BE	ME	PNPE	BE	ME
k_{cat}, sec^{-1}	45.0 ± 1.1	43.5 ± 1.2	36.8 ± 0.9[c]	3.52; ...	3.50; ...	...; ...
$k_{cat}/K_m \times 10^{-5}$, M^{-1} sec^{-1}	260 ± 31[b]	16.0 ± 4.0	0.155 ± 0.011	4.30; 8.0[b]	4.35; 8.35	...; 8.53
k_3, sec^{-1}	45.9 ± 1.6[d]	45.0 ± 0.6[d]	46.0 ± 2.5[d]	3.33[e]; ...	3.30[e]; ...	...; ...
k_2, sec^{-1}	⩾860[f]	830 ± 340[f]	175 ± 54[g]	4.30;[h] 8.0[h]	4.35;[h] 8.35[h]	...; 8.53[h]
K_s, mM	⩾0.033[d]	0.52 ± 0.08[d]	10.7 ± 2.6[d]			

[a] 1.6% acetonitrile–water (v/v); μ = 0.200; 2 to 10 × 10^{-5} M ethylenediaminetetraacetic acid; 25.0 ± 0.1°. PNPE, BE, and ME are the p-nitrophenyl ester, benzyl ester, and methyl ester, respectively, of Z-lysine. [b] pK_2 for PNPE is only approximate. [c] Average of three values between pH 5.9 and 7.3. [d] Determined from a plot according to eq 8. [e] Calculated value, see text. [f] Calculated using eq 7 and the values of K_s and (k_{cat}/K_m)(lim). [g] Average of two values, one determined as in footnote f, the other determined from k_{cat}(lim) and k_3(lim) according to eq 9. [h] These pK values, which were determined for (k_{cat}/K_m), also apply to k_2 assuming that K_s is pH independent. The pK_1 values may be considered upper limits.[21]

ethyl ester[3] and much of the rationale will not be repeated here. The papain-catalyzed hydrolyses of the three Z-lysine esters clearly follow Michaelis–Menten kinetics. For reasons to be presented in the Discussion section it is assumed that the mechanism of eq 1 describes the reaction sequence more completely than the simple Michaelis–Menten scheme. The effects of pH on k_{cat}, k_{cat}/K_m, and K_m for the three substrates are shown in Figures 2, 3, and 4, respectively. Only a small supply of the methyl ester was available and it was studied only over the limited range shown. Spontaneous hydrolysis of the p-nitrophenyl ester precluded studies on it beyond pH 8.3.

The complex rate constant k_{cat} was found to be dependent on an ionizable basic group with a dissociation constant, K_1, for both the p-nitrophenyl and benzyl esters in the low pH region, as described by eq 5. The method of Brubacher and Kézdy[19] was used to determine the values of k_{cat}(lim) and pK_1 which are given in Table IV. At high pH k_{cat} begins to diminish but not sufficiently to permit the determination of an apparent pK_2 value. The ratio k_{cat}/K_m depends on an ionizable basic group of pK_1 at low pH and an ionizable acidic group of pK_2 at high pH as described by eq 6.

$$k_{cat} = k_{cat}(\text{lim})/(1 + [(H^+)/K_1]) \quad (5)$$

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)(\text{lim})}{1 + [(H^+)/K_1] + [K_2/(H^+)]} \quad (6)$$

The quantities (k_{cat}/K_m)(lim), pK_1, and pK_2 for all three esters were determined[19] where the data were available and are tabulated in Table IV. These pK_1 and pK_2 values represent the ionization of the free enzyme and

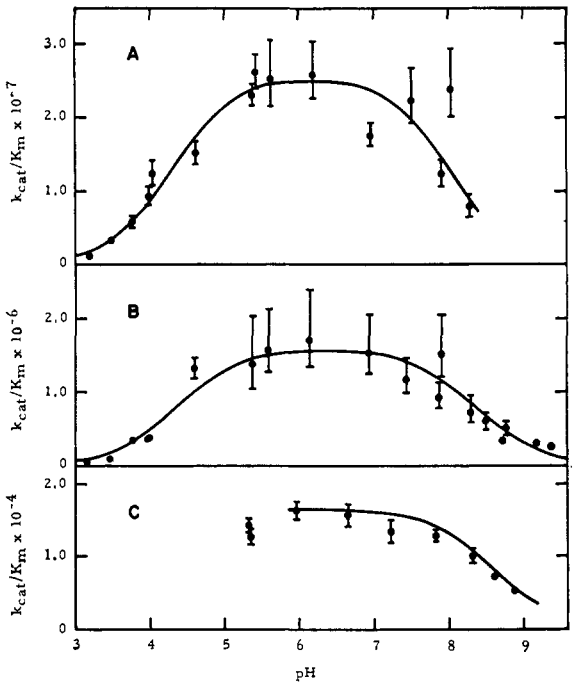

Figure 3. The effect of pH on k_{cat}/K_m for the papain-catalyzed hydrolyses of Z-lysine esters; 1.6% acetonitrile–water (v/v), μ = 0.200, 25.0°. A, p-nitrophenyl ester; B, benzyl ester; C, methyl ester. Theoretical curves are calculated from the values of k_2, k_3, and K_s in Table IV. Ordinate is in units of M^{-1} sec^{-1}.

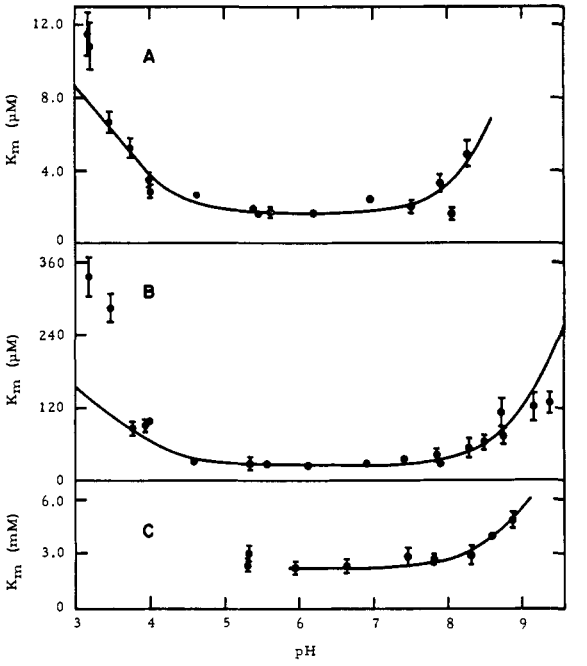

Figure 4. The effect of pH on K_m for the papain-catalyzed hydrolyses of Z-lysine esters; 1.6% acetonitrile–water (v/v), μ = 0.200, 25.0°. A, p-nitrophenyl ester; B, benzyl ester; C, methyl ester. Theoretical curves are calculated from the values of k_2, k_3, and K_s in Table IV.

(19) L. J. Brubacher and F. J. Kézdy, unpublished procedure utilizing a generalized plot of log (k/k(lim)) $vs.$ pH − pK.

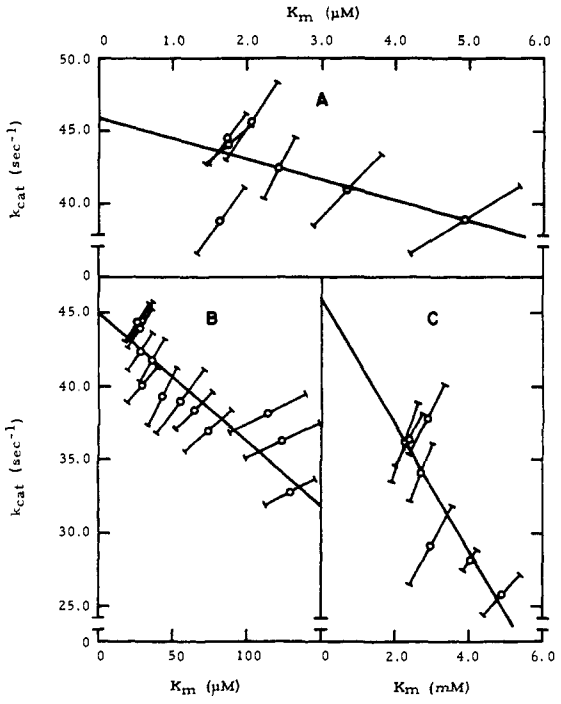

Figure 5. Plots of k_{cat} *vs.* K_m above pH 5.5 according to eq 8 for the papain-catalyzed hydrolyses of Z-lysine esters; 1.6% acetonitrile–water (v/v), μ = 0.200, 25.0°. A, *p*-nitrophenyl ester; B, benzyl ester; C, methyl ester.

thus should be identical for all substrates,[20] provided there is no pH-dependent interaction of positive substrate with noncatalytic groups on the enzyme which would affect K_s and k_2 differently. Such an effect, if present at all, is small in this case above pH 4 since pK_1 and pK_2 values do agree well with those for other substrates, both charged and uncharged.[3]

Equation 7 follows from the definitions of eq 3 and 4. If eq 4 and 7 are both solved for k_2 and then equated to eliminate k_2 and rearranged, eq 8 is obtained which can be used to obtain K_s and k_3 from the data of Tables I–III. It is assumed that k_3 and K_s are pH independent in the region above pH 5.5.[3] The positive charge in

$$k_{cat}/K_m = k_2/K_s \quad (7)$$

$$k_{cat} = k_3 - (k_3K_m/K_s) \quad (8)$$

these substrates apparently has little, if any, effect on k_{cat}(lim) or on K_m above pH 5 since the uncharged substrate *p*-nitrophenyl ε-N-formyl-Z-lysinate has essentially the same k_{cat}(lim) (32 sec^{-1}) and the same K_m (2.3 μM at pH 5.4) in its papain-catalyzed hydrolysis as the unformylated, charged, ester. Furthermore, since this work was done below pH 9.4, the amount of the uncharged species never becomes significant since the pK_a of the ε-ammonium group of lysine should be about 10.5. Thus it is assumed that K_s is constant at least above pH 5.

Plots of k_{cat} *vs.* K_m according to eq 8 are shown in Figure 5 for all three esters using the data above pH 5.5; the values of k_3 (y intercept) and K_s (x intercept) so obtained are tabulated in Table IV. The value of 33 μM for the K_s of the *p*-nitrophenyl ester is viewed as a lower limit because of the considerable scatter in Figure 5A. A value of K_s as high as 100 μM is consistent with the data for the *p*-nitrophenyl ester. The values of k_3 for all three esters show excellent agreement, as indeed they should if they describe the deacylation of the same acyl-enzyme, Z-lysyl-papain.

Values of k_2(lim) were calculated using eq 7 and values of (k_{cat}/K_m)(lim) and K_s from Table IV. For the methyl ester k_2(lim) by this method is 166 ± 52 sec^{-1}. An alternative method of calculating k_2(lim) for the methyl ester utilizes eq 9 which is a rearrrangement of eq 3 in the pH-independent region. By this method

$$1/k_2(\text{lim}) = 1/k_{cat}(\text{lim}) - 1/k_3(\text{lim}) \quad (9)$$

k_2(lim) for the methyl ester is 184 ± 73 sec^{-1}. The value listed in Table IV for the methyl ester is the average of the two results. The values of pK_1 and pK_2 shown in Table IV for k_2 are those obtained from the pH profiles for k_{cat}/K_m ($=k_2/K_s$). Since K_s is believed to be pH independent (at least above pH 5) the effect of pH on k_{cat}/K_m reflects the effect of pH on k_2.[21]

Finally the values of pK_1 in Table IV for the deacylation (k_3) step were calculated for the *p*-nitrophenyl and benzyl esters from the known values of k_3(lim), k_{cat}(lim), and k_2(lim) and the pK_1 values of the latter two constants. The pK_1 values for the deacylation step with the *p*-nitrophenyl and benzyl esters are ~0.2 pH unit lower than the pK_1 for k_{cat}.[21] The pK_1 values for k_{cat} (≃3.5) are considerably smaller than pK_1 for k_2 (≃4.3); thus k_{cat}, which is predominantly k_3 above pH 4.3, becomes influenced significantly by k_2 below pH 4. Thus the observed pK_1 for k_{cat} is an apparent pK and is actually a weighted average of the pK_1 values for k_2 and k_3.

These theoretical pH dependencies based on the constants in Table IV are shown as solid lines in Figures 2, 3, and 4, respectively. In general the data fit the theoretical curves very well, thus giving support to the validity of eq 1 for describing the reaction pathway. For the *p*-nitrophenyl and benzyl esters at low pH, K_m tends to be higher than the calculated value. This discrepancy could be due to an increase in K_s due to electrostatic repulsion between the positively charged lysine substrate and the highly positively charged enzyme. Assuming an average pK_a of 3.5 for the free carboxyl groups in papain, the net positive charge on the enzyme will increase considerably in the region below pH 4.

Kinetics of Hydrolysis of *p*-Nitrophenyl α-N-Acetyl-DL-tryptophanate. The hydrolysis of only half of the racemic ester, presumably the L isomer, is catalyzed by papain. The parameters k_{cat}/K_m, k_{cat}, and K_m, are plotted in Figure 6 as a function of pH. Some of the points in Figure 6 were obtained from Lineweaver–Burk plots of data from complete reactions, as was done above with the lysine esters, some from Lineweaver–Burk plots of initial rates at varying initial substrate concentrations. The good agreement between the two methods indicates that there is no product in-

(20) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, **81**, 5907 (1959).

(21) If the increase in K_m from pH 5 down to pH 3 for *p*-nitrophenyl and benzyl Z-lysinate is due wholly or in part to an increase in K_s (due possibly to the increasing repulsion of the positively charged substrate by the increasing global positive charge on the enzyme or to a perturbation of pK_1 of the enzyme:substrate complex to a lower value than the pK_1 of the free enzyme),[22] the true pK_1 for acylation would be lower than the pK_1 for k_{cat}/K_m. This in turn would make the calculated pK_1 for deacylation coincide more closely to the pK_1 for k_{cat}. Thus the difference of one pH unit between the pK_1 values for acylation and deacylation in Table IV represents an upper limit for this difference.

(22) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *J. Am. Chem. Soc.*, **86**, 3680 (1964).

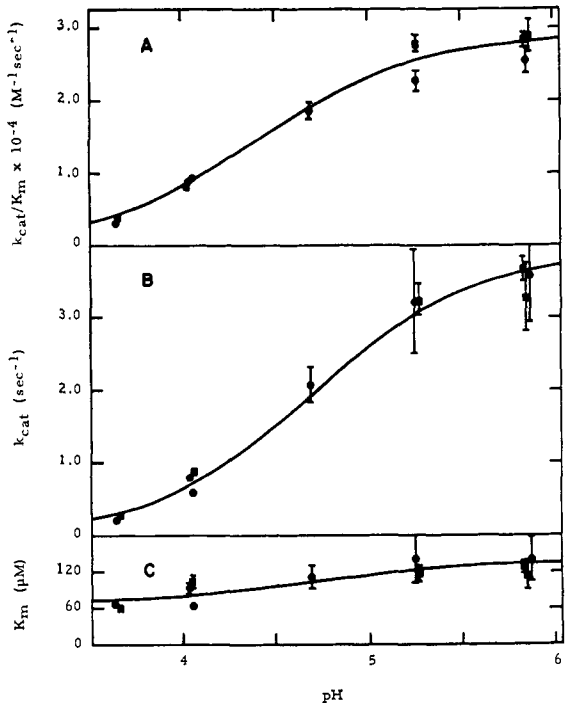

Figure 6. The effect of pH on (A) k_{cat}/K_m, (B) k_{cat}, and (C) K_m, in the papain-catalyzed hydrolysis of *p*-nitrophenyl α-N-acetyl-L-tryptophanate; 1.6% acetonitrile-water (v/v), μ = 0.500, 15.0°. ●, initial rates method; ■, total reaction method.

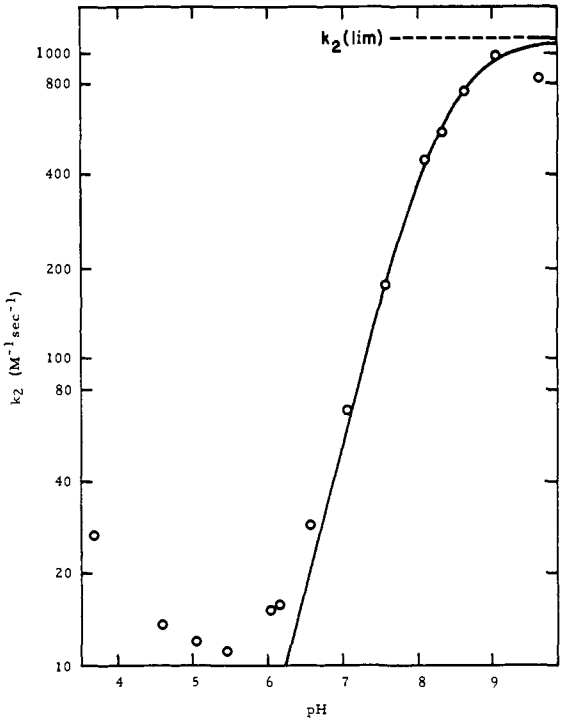

Figure 7. The effect of pH on the second-order rate constant for the inactivation of papain by TPCK; 3.2% acetonitrile-water (v/v), μ = 0.100, 25.0°. The theoretical (solid) curve is for dependency on a single ionizable basic group of pK_a = 8.28 and k_2(lim) = 1110 M^{-1} sec^{-1}.

hibition. It was also shown that there is no acetate buffer effect.

The pH dependencies of k_{cat}/K_m and k_{cat} were analyzed and the values of (k_{cat}/K_m)(lim) and k_{cat}(lim) along with their respective pK_1 values are recorded in Table V. The theoretical sigmoid curves in Figures

Table V. Limiting Values of the Kinetic Constants and Corresponding pK_1 Values in the Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl α-N-Acetyl-L-tryptophanate[a]

Constant	Limiting value	pK_1[b]
k_{cat}/K_m	2.90×10^4 M^{-1} sec^{-1}	4.40
k_{cat} (k_3)	3.90 sec^{-1}	4.70
K_m	138 μM[c]	

[a] 1.6% acetonitrile-water (v/v); μ = 0.500; 2×10^{-5} M ethylenediaminetetraacetic acid; 25.0 ± 0.1°. Enzyme concentration was 3.7 to 30.7×10^{-7} M; $(S)_0$ was 0.10 to 1.43×10^{-4} M (L isomer). [b] True pK_1 values for acylation and deacylation, respectively, reading down. [c] See text for details of determination.

6A and 6B were calculated from these values. Since *p*-nitrophenyl α-N-acetyl-L-tryptophanate shows a burst with papain at pH 3.2, $k_2 \gg k_3$.[10] Thus $k_{cat} \simeq k_3$ and the pK_1 of 4.70 observed for k_{cat} is the pK_1 for deacylation. Assuming that K_s is pH independent for this neutral substrate, the pK_1 of 4.40 for k_{cat}/K_m ($= k_2/K_s$) is actually the pK_1 for acylation. Since the acylation pK_1 is 0.30 unit smaller than the pK_1 for deacylation, the ratio k_3/k_2 will increase by a factor of 2 in going from pH 3 to 6. Thus it is possible to calculate the pH dependence of K_m ($= k_3K_s/k_2$) and a limiting value of K_m at pH >6, namely 138 μM.

Inactivation of Papain by TPCK. With TPCK in considerable excess, papain is inactivated according to pseudo-first-order kinetics. Table VI is a compilation of the pseudo-first-order rate constants, k_{obsd}, as a function of pH and TPCK concentration. At pH 6.16 three concentrations of TPCK up to the solubility limit of 2.05×10^{-4} M give essentially constant values of k_2, the second-order inactivation rate constant. The small decrease in k_2 at the highest inactivator concentration is considered to be within experimental error although it might also reflect some binding of TPCK to the enzyme.

Table VI. Kinetics of Inactivation of Papain by TPCK[a]

Buffer	pH	(TPCK) × 10^4, M	k_{obsd} × 10^3, sec^{-1}	k_2, M^{-1} sec^{-1}
0.05 M acetate	3.59	1.070	2.85	26.6
	4.60	1.070	1.46	13.6
	5.05	1.070	1.28	12.0
0.033 M phosphate	5.47	1.070	1.19	11.1
	6.03	1.070	1.61	15.1
	6.16	0.512	0.831[c]	16.2, 16.1[b]
	6.17	1.024	1.680[c]	16.4
	6.16	2.048	3.08[c]	15.0
	6.56	1.070	3.08	28.8
	7.05	0.535	3.63	67.9
	7.55	0.535	9.42	176.0
	8.08	0.535	24.0	448.0
0.0125 M borate	8.30	0.535	29.6	554.0
	8.61	0.535	40.4	755.0
	9.02	0.535	52.5	981.0
	9.60	0.535	44.5	831.0

[a] 3.2% acetonitrile-water (v/v); μ = 0.100; 1×10^{-4} M ethylenediamine tetraacetic acid; 25.0 ± 0.1°. Enzyme concentration was 2.90×10^{-6} M. [b] Second-order rate constant obtained directly from a second-order plot of the data. [c] Enzyme concentration was 3.44×10^{-6} M.

The effect of pH on k_2 is shown in Figure 7. The data above pH 7 show a limiting value for k_2 of 1110 M^{-1} sec^{-1}, and dependence upon an ionizable basic group

of $pK_a = 8.28$. This pK_a value is similar to the pK_2 values for acylation of papain by various substrates, a fact which suggests that the same group which is inactivated by TPCK is essential in the acylation step. In the inactivation reaction, however, the basic form is the reactive form whereas acylation is dependent upon the acidic form.

Amino acid analyses were made of TPCK-inactivated papain and are reported in Table VII. The amino acid composition in the final column was derived from

Table VII. Amino Acid Analysis of TPCK-Inactivated Papain[a]

	— Moles of amino acid per mole of papain —			
Amino acid	TPCK + papain	Papain, control	TPCK + mercuri-papain	Papain[b]
Lysine	8.2	8.2	8.2	9
Histidine	1.36	1.36	1.36	2
Ammonia	23.0	20.3	19.8	24
Aspartic acid	17.7	17.4		19
Threonine	6.7	7.0		7
Serine	10.7	10.9		12
Glutamic acid	18.6	18.6		18
Proline	10.0	9.2		10
Glycine	26.4	26.2		27
Alanine	13.4	13.2		13
Half-cystine	3.6	5.4		7
Valine	14.3	14.3		15
Isoleucine	8.5	8.3		10
Leucine	10.2	9.9		10
Tyrosine	17.2	17.3		19
Phenylalanine	3.6	3.6		4

[a] The data in the papain (control) column were calculated on the basis of the known amount of protein added to the amino acid analyzer column. The data in the other columns were corrected to the same recovery of added norleucine as in the control hydrolysate. The correction amounted to −3.5% in the TPCK + papain column. [b] Reference 23.

the tentative sequence reported by Smith's group,[23] and the corresponding molecular weight of 21,930 was used to calculate the number of moles of papain applied to the amino acid analyzer column. The papain was 58.7% active prior to treatment with TPCK. Thus the amino acid alkylated by TPCK should show a decrease of at least 0.587 mole of amino acid per mole protein relative to the control, if recovery in the latter is complete. Since only 68% of the histidine is recovered in the control reaction,[24] the TPCK-treated papain should yield 0.40 mole less histidine than the control if histidine is alkylated by TPCK. Thus histidine is clearly not the site of attack by TPCK. The direct analysis for cysteine is generally unreliable so that no firm conclusion can be drawn from the results for this amino acid. However, the process of elimination leaves cysteine as the most likely site of alkylation.

Williams[26] has also found that papain is inactivated by various alkylating agents similar to TPCK, but in no instance is histidine alkylated. It has been reported by Yu-Kum and Chen-Lu[27] that bromoacetate and iodoacetate alkylate a histidine residue in papain at pH 5 contrary to the results of Finkle and Smith[28] and Light[29] that only cysteine is alkylated at pH 8.5 and 5 under the same conditions. This discrepancy remains unresolved.

(23) A. Light, R. Frater, J. R. Kimmel, and E. L. Smith, *Proc. Natl. Acad. Sci. U. S.*, **52**, 1276 (1964).

(24) The low recovery of histidine in the papain control is characteristic of amino acid analyses of papain.[25]

(25) E. L. Smith, A. Stockell, and J. R. Kimmel, *J. Biol. Chem.*, **207**, 551 (1954).

(26) A. Williams, Doctoral Thesis, Oxford University, 1964.

(27) S. Yu-Kum and T. Chen-Lu, *Sci. Sinica*, **12**, 1845 (1963).

Discussion

Lowe and Williams have recently observed that a series of hippuric acid esters of differing reactivities toward hydroxide ion have essentially identical k_{cat} values in their papain-catalyzed hydrolyses.[4] Kirsch and Igelström have made similar observations in the papain-catalyzed hydrolyses of some Z-glycine esters.[5] Both groups of workers concluded on the basis of these results that a common acyl-enzyme intermediate is involved in each series of esters. Such a conclusion assumes, for a given series of esters with the same acyl group, that the relative k_2 values are the same as the relative k_{OH^-} values. However, the small (fivefold) difference between the k_2 values for the ethyl ester and the amide of α-N-benzoyl-L-arginine[3] indicates that this may not be so. First, the nucleophile in papain is a thiol group and may exhibit a different selectivity than hydroxide ion. Second, it is possible that, in addition to the intrinsic nucleophilicity of the nucleophile in papain, some other factor may have an influence on k_2. Such an additional factor might be binding of the leaving group, resulting in favorable or unfavorable orientation of the substrate reacting center depending on the particular way in which the leaving group is bound, which in turn depends on the nature of the leaving group. The 20-fold difference in the K_s values for the benzyl and methyl esters of Z-lysine (Table IV) strongly infers that the leaving group of the substrate is bound to a site on the enzyme. The K_s values of *n*-butyl hippurate ($K_s = 10.2$ mM) and hippuramide ($K_s = 202$ mM) also differ by 20-fold.[4] Since added nucleophiles are apparently bound to the acyl-enzyme,[9] it seems likely that the substrate leaving group is also bound (*i.e.*, K_s varies for different esters of the same acid) although the principle of microscopic reversibility requires this conclusion only if acylation is the reverse of deacylation. On the other hand, the correlation between k_{cat}/K_m and k_{OH^-} for esters of hippuric acid and Z-glycine has been taken as evidence against binding of the leaving group.[5] Further investigation of the acylation step is required to resolve these discordant conclusions.

The kinetics of the papain-catalyzed hydrolyses of the *p*-nitrophenyl, benzyl, and methyl esters of Z-lysine, the best substrates yet found for papain, are consistent with the two-step mechanism of eq 1 as noted in the Results section. The kinetic data for these esters were analyzed assuming that a chemical intermediate is involved, but without specifying the nature of the intermediate. That the resulting k_3 values are identical is strong evidence that a common intermediate or a common conformational change occurs with all three esters. Possible intermediates include an oxazolone[30] or an acyl-enzyme. However, an oxazolone intermediate seems unlikely in the present case since α-N-benzyloxycarbonylamino acids do not racemize readily and hence presumably do not form oxazolones readily.

(28) B. J. Finkle and E. L. Smith, *J. Biol. Chem.*, **230**, 669 (1958).

(29) A Light, *Biochem. Biophys. Res. Commun.*, **17**, 781 (1964).

(30) J. de Jersey, A. A. Kortt, and B. Zerner, *ibid.*, **23**, 745 (1966).

Since other acyl-papains have been observed[9,31] or implicated[10] (*vide infra*) the principle of simplicity of mechanism suggests that a common acyl-enzyme intermediate occurs in this case also, namely, Z-lysyl-papain. For α-chymotrypsin also, all the reliable evidence indicates a single mechanism of reaction for both specific and nonspecific substrates.

An important quantity in eq 1 is the magnitude of the ratio k_{-1}/k_2; if it is much greater than unity the initial adsorption step may be described by a true equilibrium constant $K_s = k_{-1}/k_1$, whereas, if it is equal to or less than unity, K_s is not a true equilibrium constant and is given by $(k_{-1} + k_2)/k_1$. For α-chymotrypsin there are several indications that the former situation holds.[32] Although it has been proposed that $k_2 \gg k_{-1}$ in the papain-catalyzed hydrolysis of α-N-benzoyl-L-argininamide,[33] the evidence is not very convincing especially since the substrate was probably impure.[34] A Hammett plot of log (k_{cat}/K_m) *vs.* σ for the papain-catalyzed hydrolysis of several substituted phenyl hippurates gives a linear relationship ($\rho = +1.2$).[4] This was interpreted to mean that $k_{cat}/K_m = k_2/K_s$ and hence that $k_2 \ll k_{-1}$. This argument suffers from the possibility that K_s as well as k_2 may vary for the different esters used, as noted above.

The best solution to the above problem is to measure k_{-1} directly. This has not been done for papain although the measurement of such a presumably fast rate constant is now experimentally possible.[35] In almost all enzyme–substrate complexes which have been studied, lower limits of 10^7 to 10^9 M^{-1} sec^{-1} are obtained for k_1.[35] There is no reason to believe that papain should behave differently. Thus if $k_1 = 10^8$ M^{-1} sec^{-1} and $K_s = 10^{-4}$ M for a papain-catalyzed reaction, then $k_{-1} = 10^4$ sec^{-1}; hence in most cases the assumption that $k_2 \ll k_{-1}$ is probably valid. The substrate most likely to be an exception to this generalization is *p*-nitrophenyl Z-lysinate for which $k_2 \geq 860$ sec^{-1} and $K_s \geq 3.3 \times 10^{-5}$ M (Table IV). Thus for k_{-1}/k_2 to be greater than 10, k_1 would have to be greater than 2.5×10^8 M^{-1} sec^{-1}.

The Acyl-Enzyme Intermediate. The evidence for an acyl-enzyme intermediate in certain papain-catalyzed reactions is convincing. It includes (1) the spectrophotometric observation of thionohippuryl-papain;[31,36] (2) the isolation and spectrophotometric observation of *trans*-cinnamoyl-papain;[9,37] (3) the "burst" of *p*-nitrophenol with the *p*-nitrophenyl ester substrates of Z-tyrosine and α-N-acetyl-DL-tryptophan;[10] (4) the equivalence of k_{cat} ($=k_3$, presumably) for a series of hippurate esters[4] and Z-glycine esters;[5] and (5) the equivalence of k_3 for three esters of Z-lysine (*vide supra*).

The acyl-enzyme hypothesis is consistent with the observation that papain catalyzes the exchange of ^{18}O from the carbonyl position of Z-amino acids to water and *vice versa*.[38] The ^{18}O label in the acid may be either protonated or unprotonated in the free acid (eq 10) and it will be lost to the solvent or retained in the acyl-enzyme, respectively, when acylation of the enzyme occurs. Since the two forms of the acid are in equilibrium essentially all the label will eventually be lost. The incorporation of solvent ^{18}O label occurs by the reverse reaction in eq 10. An unsuccessful attempt to

$$\mathrm{R\overset{\overset{^{18}O}{\|}}{C}OH} \rightleftharpoons \mathrm{R\overset{\overset{^{18}OH}{|}}{C}{=}O} \overset{E}{\rightleftharpoons} \mathrm{R\overset{\overset{^{18}OH}{|}}{C}{=}O{\cdot}E} \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} \mathrm{R\overset{\overset{O}{\|}}{C}E} + H_2{}^{18}O \quad (10)$$

observe an acyl-enzyme in the reaction of papain with the acid α-N-benzoyl-L-arginine at pH 3.2 was made under such conditions of enzyme and acid concentration as to indicate that k_{-3} is at least tenfold less than k_3.

It is also clear that acyl-papains are thiol esters as indicated by the high reactivity of *trans*-cinnamoyl-papain toward amine nucleophiles[9] and by the spectra of thionohippuryl-papain[31,36] and *trans*-cinnamoyl-papain.[9,37] In addition an acidic ionizable group of $pK_2 \sim 8.4$ is important in acylation and this is most probably the nucleophilic sulfhydryl group, which is known to be essential to papain activity.[39] The magnitude of pK_2 and its temperature dependence are consistent with this assignment.[33] Further support comes from the probable reaction of TPCK (and other alkylating agents[26]) with the sulfhydryl group, a reaction which depends on an ionizable basic group of $pK = 8.28$. A similar dependence has been observed for the reaction of chloroacetamide and iodoacetamide with the sulfhydryl group in ficin.[40]

The Nature of the Deacylation Step. In Table VIII are collected the known data pertaining to the deacylation of various acyl-papains. Values of pK_1, when observed, are as low as 3.3 for charged substrates and as high as 4.7 for uncharged aromatic substrates. Williams[26] has reported no decrease in k_{cat} ($=k_3$) at low pH for the papain-catalyzed reactions of hippuryl esters, and Kirsch and Katchalski have made similar observations with ethyl hippurate.[41] Smith's data on ethyl α-N-benzoyl-L-argininate, when corrected for the nonionization of the product acid at low pH, are also claimed to show no pH dependence down to pH 3.7.[42] This latter result is in direct contradiction to the results of Whitaker and Bender with the same substrate which indicate that at pH 3.6 (their lowest pH) k_3 is only one-third its maximum value.[3] This contradiction indicates that either the spectrophotometric method or the pH-Stat titration method gives unreliable results. No compelling reason can be given that either of these methods should be unreliable; however the sensitivity of the pH-Stat does decrease at low pH owing to the incomplete dissociation of the product acid, whereas with the spectrophotometer this is often not the case. Furthermore, it is possible that the enzyme might perturb the pK_a of the product acid to a lower value by preferentially binding the negatively charged carboxylate ion. (At low pH the enzyme surface is highly positively charged.) If this is so, the correction applied to the observed pH-Stat rate at a particular pH, based on a pK_a for the product acid measured in the

(31) G. Lowe and A. Williams, *Biochem. J.*, **96**, 189 (1965).
(32) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3671 (1964).
(33) A. Stockell and E. L. Smith, *J. Biol. Chem.*, **237**, 1 (1957).
(34) E. L. Smith and M. J. Parker, *ibid.*, **233**, 1387 (1958).
(35) M. Eigen and G. G. Hammes, *Advan. Enzymol.*, **25**, 1 (1963).
(36) G. Lowe and A. Williams, *Proc. Chem., Soc.*, 140 (1964).
(37) M. L. Bender and L. J. Brubacher, *J. Am. Chem. Soc.*, **86**, 5333 (1964).
(38) V. Grisaro and N. Sharon, *Biochim. Biophys. Acta*, **89**, 152 (1964).
(39) E. L. Smith and J. R. Kimmel, *Enzymes*, **4**, 134 (1960).
(40) M. R. Holloway, A. P. Mathias, and B. R. Rabin, *Biochim. Biophys. Acta*, **92**, 111 (1964).
(41) J. F. Kirsch and E. Katchalski, *Biochemistry*, **4**, 884 (1965).
(42) L. A. Ae. Sluyterman, *Biochim. Biophys. Acta*, **85**, 305 (1964).

Table VIII. Kinetic Constants of Deacylation of Various Acyl-Papains

Substrate	pH range	Temp, °C	k_3(lim),[g] sec^{-1}	pK_1	Method	Ref
trans-Cinnamoyl-papain	3.4–12.7	25	0.00368[a]	4.69	Spect	9
p-Nitrophenyl Z-lysinate	3.2–8.3	25	45.9[b]	3.33	Spect	This work
Benzyl Z-lysinate	3.15–9.4	25	45.0[b]	3.30	Spect	This work
Methyl Z-lysinate	5.3–8.9	25	46.0[b]	...	Spect	This work
p-Nitrophenyl ε-N-formyl-Z-lysinate	3.7–5.4	25	32.0[k]	3.96	Spect	This work
p-Nitrophenyl α-N-acetyl-L-tryptophanate	3.6–5.9	25	3.90[c]	4.70	Spect	This work
p-Nitrophenyl Z-tyrosinate	3.2	25	(3)[d]	...	Spect	10
Ethyl α-N-benzoyl-L-argininate	3.5–9.6	25	20.2	3.91	Spect	3
	3.8–8.5	25	9[f,h]	None[e]	pH-Stat	34
Ethyl hippurate	3.7–8.4	40	3.1[h,i]	None	pH-Stat	42
	4.2–8.7	38	3.3[h,i]	None	pH-Stat	41
Hippurate esters	6.0	35	2.7[h,i]	None[j]	pH-Stat	4, 26
Z-Glycine esters	6.8	25	2.4[h,i]		Spect	5

[a] Acyl-enzyme isolated and observed directly. [b] Obtained by a plot of high pH data according to eq 8. [c] Actually k_{cat}, but assumed to be k_3 since k_2 must be greater than k_3 to account for observed burst. [d] Calculated assuming pK_1 = 4.5 and knowing $k_3 = k_{cat} = 0.14$ sec^{-1} at pH 3.2. [e] Although a value of 3.5 was reported for the pK_1 for k_{cat}, when a correction is made for the nonionization of the product acid, k_{cat} becomes pH independent.[42] [f] Value for k_{cat}, and probably a mixture of k_2 and k_3 (ref 3). [g] Base on titrated enzyme unless otherwise indicated. [h] Enzyme not titrated. [i] Value for k_{cat}; probably equal to k_3. [j] Williams[26] reports no decrease in k_3 down to pH 3.8. [k] This value for k_{cat} is probably equal to k_3 by analogy to *p*-nitrophenyl Z-lysinate.

absence of enzyme, would give an erroneously high hydrolysis rate at that pH. In the spectrophotometric method this possible pitfall was avoided by measuring $\Delta\epsilon$ for each reaction.[3]

Dependence of the deacylation step on a group of pK_1 of 3.3 to 4.7 has now been observed for specific substrates, both charged (Z-lysine esters) and uncharged (*p*-nitrophenyl esters of α-N-acetyl-L-tryptophanate and ε-N-formyl-Z-lysinate), as well as for nonspecific substrates (*trans*-cinnamoylimidazole) (see Table VIII). Thus it is concluded that a basic group of p$K_1 \sim 4$ probably participates in the deacylation step of all papain-catalyzed reactions.

Two lines of evidence indicate that this basic group acts as a general basic catalyst and not as a nucleophilic catalyst in deacylation. (1) Large D_2O effects have been observed in the deacylation of α-N-benzoyl-L-argininyl-papain ($k_{3,H_2O}/k_{3,D_2O} = 2.75$)[3] and *trans*-cinnamoyl-papain ($k_{3,H_2O}/k_{3,D_2O} = 3.35$).[9] (2) The reactivity of amine nucleophiles in the deacylation of *trans*-cinnamoyl-papain is independent of pK_a.[9]

There is as yet no positive direct evidence for the identity of this general basic catalytic group. Since the pK_1 of ~4 is not significantly affected by temperature, and since it is typical of a free carboxylic acid group in an enzyme, it was suggested that a carboxylate group is essential in the deacylation step.[33] More recently a partially buried imidazole has been invoked[43] to explain the pK_1 of 4.69 observed in the deacylation of *trans*-cinnamoyl-papain, and, as evidence for this possibility, the alkylation of histidine by haloacetates was cited. However, the validity of the alleged reaction of haloacetates with histidine[27] is in doubt.[29] The inactivation of papain by TPCK was studied on the rationale that since a histidine in α-chymotrypsin is alkylated by TPCK,[14] a similar, catalytically important histidine in papain should also be attacked. In fact, histidine in papain is not attacked by TPCK at pH 5.2 whereas cysteine probably is. Thus histidine is probably not involved.

The strongest argument against carboxylate ion as the catalytic entity in deacylation comes from model studies of the intramolecular catalysis of the hydrolysis of thiol esters by carboxylate ion.[43] The rates of hydrolysis of the S-hippurylthioglycollate and S-ethylmonothiosuccinate anions are 10^5- to 10^6-fold slower than the deacylation rates of hippuryl-papain and α-N-benzoyl-L-argininyl-papain.[43] The data for the hydrolysis of S-hippurylthioglycollate anion show that imidazole catalysis ($k_{inter} = 4.5 \times 10^{-3}$ M^{-1} sec^{-1}) is 5000-fold better than carboxylate ion catalysis ($k_{intra} = 0.88 \times 10^{-5}$ sec^{-1}).[44] However, these model reactions are examples of intramolecular *nucleophilic* catalysis by the carboxylate group since this large difference in catalytic efficiency is characteristic of carboxylate and imidazole as nucleophiles,[46] whereas deacylation of the acyl-enzyme is *general base catalyzed*.[9] In general base catalyses, carboxylate ion is only *ca.* 100-fold less effective than imidazole. Thus these model studies are not strictly applicable to the deacylation reaction of acyl-papains and do not rule out carboxylate ion as the probable catalytic entity of p$K_1 \sim 4$.

Although acylation (as reflected in $k_{cat}/K_m = k_2/K_s$) is dependent on an ionizable group of p$K_2 \sim 8.4$, no such dependence is observed for the deacylation step of *trans*-cinnamoyl-papain, the only case where a pure deacylation reaction has been directly studied.[9] This same result is observed with *trans*-cinnamoyl-α-chymotrypsin.[47] For a reaction in which k_2(lim) $\gg$ k_3(lim), k_{cat} is observed to remain independent of pH at high pH until k_2 has decreased to the same magnitude as k_3, whereupon k_{cat} also decreases due to the influence of k_2. In papain-catalyzed reactions this has been observed in the case of ethyl α-N-benzoyl-L-argininate,[3] and in the current work the best example is benzyl Z-lysinate. Such behavior is an indirect indication that k_3 is pH independent at high pH.

It has been reported recently that there is no loss of ^{18}O label to the solvent in the papain-catalyzed hydrolysis of ethyl (carbonyl-^{18}O)hippurate.[41] In the hydrox-

(43) G. Lowe and A. Williams, *Biochem. J.*, **96**, 194 (1965).

(44) Intramolecular participation by a catalytic group is approximately equivalent to intermolecular catalysis with a catalyst concentration of 10 *M*.[45]

(45) M. L. Bender, *Chem. Rev.*, 60, 53 (1960).

(46) T. C. Bruice and R. Lapinski, *J. Am. Chem. Soc.*, 80, 2265 (1958).

(47) M. L. Bender, G. R. Schonbaum, and B. Zerner, *ibid.*, 84, 2562 (1962).

ide ion catalyzed hydrolysis of the same substrate the ^{18}O label is lost to the solvent at a rate $^1/_{14}$th that of hydrolysis indicating that in this case a tetrahedral intermediate is formed with two equivalent OH groups attached to the original carbonyl carbon atom.[48] The absence of detectable exchange in the papain-catalyzed reaction indicates that a symmetrical tetrahedral intermediate is not formed in either the acylation or deacylation reactions. It is possible, however, that a tetrahedral intermediate is formed in which the original carbonyl oxygen is bound to the enzyme in such a way that it cannot be lost to the solvent.

In summary, the deacylation step in papain-catalyzed reactions is catalyzed by a general basic group on the enzyme of $pK_a = 3.3$ to 4.7. This basic group is most likely the carboxylate ion of an aspartic or glutamic acid residue. The attacking nucleophile appears to be bound more or less strongly to the acyl-enzyme in a preequilibrium step, from which position it attacks the thiol ester bond of the acyl-enzyme.[9] A symmetrical tetrahedral intermediate is apparently not formed.

The Mechanism of Papain-Catalyzed Reactions. In postulating a mechanism for papain-catalyzed reactions it must be emphasized that our knowledge in this area is relatively limited. A useful approach to an understanding of the papain mechanism is to model investigations after those which have proved so fruitful in the study of similar enzymes, notably α-chymotrypsin, and many similarities between papain and α-chymotrypsin are now known. In addition to the probability that both enzymes follow the same acyl-enzyme pathway of eq 1, the following similarities may be noted. Both enzymes exhibit (1) a sigmoid pH dependence in the deacylation step;[9] (2) similar D_2O effects in the deacylation step; (3) reaction with various added nucleophiles in the deacylation step;[9] (4) the same relative reactivities of the *trans*-cinnamoyl-enzymes toward methanol and water;[9] (5) a similar perturbation of the absorption spectrum of the *trans*-cinnamoyl moiety in the *trans*-cinnamoyl-enzyme;[9] and (6) catalysis of ^{18}O exchange between free amino acids and water,[38,49] but no ^{18}O exchange during the course of ester hydrolysis.[41,50]

Some ways in which papain catalyses differ from α-chymotrypsin include, for papain, (1) the essentiality of of an SH group;[39] (2) the thiol ester nature of the acyl-enzyme;[9] (3) a lower pK_1 in both acylation and deacylation; (4) absence of inhibition by diisopropylphosphofluoridate;[51] (5) apparent binding of the leaving group in ester substrates and also of nucleophiles;[9] (6) only a small D_2O effect ($k_H/k_D = 1.35$) in the acylation step;[3] (7) inhibition by TPCK by reaction at a site other than histidine; and (8) observation of a pK_2 of $\sim$8.4 in acylation (but not in deacylation). Most of these differences may be attributed to different catalytic entities performing the same functions in both papain and α-chymotrypsin. Thus to a first approximation the mechanism postulated by Bender and Kézdy in eq 6 of ref 52 may be assumed to apply to papain as well, with two changes: the acyl-enzyme is a thiol ester in the case of papain and the general basic group is probably a carboxylate ion. Any further refinement of that mechanism as applied to papain is hardly justified at this time.

Acknowledgments. The authors thank Dr. Ferenc J. Kézdy for many helpful suggestions. L. J. B. also takes this opportunity to thank the Du Pont Company for a Teaching Assistant Award during the academic year 1962–1963.

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