Kinetics of Papain-Catalyzed Hydrolyses of Neutral Substrates*

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ABSTRACT: The effect of pH on the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester (BCME) and *p*-nitrophenyl benzyloxycarbonylglycinate (*p*-nitrophenyl Z-glycinate) have been determined by a spectrophotometric method. k_{cat}/K_m(app) for the hydrolysis of BCME was dependent on prototropic groups on the free enzyme of pK = 4.23 and 8.50 at 25° and ionic strength of 0.30. This dependence on pH in the acid region is essentially the result of the dependence of k_{cat} on a prototropic group as K_m(app) is nearly independent of pH in this region. In the acid region, k_{cat}/K_m(app) and $k_{cat}(=k_3)$ for the hydrolysis of *p*-nitrophenyl Z-glycinate are dependent on prototropic groups with pK values of 4.23 and 3.85, respectively. No evidence was found for the involvement of a prototropic group of pK = 7 in the hydrolysis of either substrate. The pK for the ionization of α-*N*-benzoyl-L-citrulline was determined to be 3.49–3.57 and 3.57–3.60 by two methods at 25° and at ionic strengths of 0.30 and 0.03, respectively.

It has been shown that the hydrolysis of a substrate by papain probably proceeds *via* the formation of an acyl-enzyme intermediate as shown in eq 1 (Kimmel and Smith, 1960; Bender and Brubacher, 1964, 1966; Lowe and Williams, 1964, 1965; Whitaker and Bender, 1965; Kirsch and Igelström, 1966; Brubacher and Bender, 1966), where ES is the enzyme–substrate complex, K_s its equilibrium constant, ES′ the acyl-enzyme, and P_1 and P_2 are the alcohol and acid portions of an ester substrate, respectively. The data of the present paper will be analyzed on this premise.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} ES' \xrightarrow[H_2O]{k_3} E + P_2 \quad (1)$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\quad + \quad P_1$$

The relationship between the usual Michaelis–Menten parameters and those of eq 1 can be seen by comparison of eq 1 and 2. These relationships are expressed in eq 3 and 4.

$$E + S \overset{K_m(app)}{\rightleftharpoons} E\cdot S \xrightarrow{k_{cat}} E + P_1 + P_2 \quad (2)$$

$$K_m(app) = [k_3/(k_2 + k_3)]K_s \quad (3)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (4)$$

There is disagreement concerning the effect of pH on k_{cat}, k_{cat}/K_m(app), and k_3 of papain-catalyzed reactions in the acid region. The reported effects of pH on k_{cat} range from an increase in k_{cat} with increase in pH with dependence on a prototropic group of pK between 3.5 and 4 (Whitaker and Bender, 1965; Bender and Brubacher, 1966), no effect of pH on k_{cat} (Smith and Parker, 1958;[1] Sluyterman, 1964; Kirsch and Katchalski 1965; Cohen and Petra, 1967), to a 3.5-fold increase in k_{cat} at pH 3.8 as compared with pH 4.2 and above for the papain-catalyzed hydrolysis of *N*-benzoylglycine ethyl ester (BGEE).[2] The effect of pH on k_{cat}/K_m(app) $=k_2/K_s$) which, in the absence of other perturbing factors, is a measure of the prototropic groups on the *free* enzyme essential for its activity (Peller and Alberty, 1959), gives a bell-shaped curve with pK values near 4 and 8.5 for all substrates tested (Whitaker and Bender, 1965; Bender and Brubacher, 1966) except one (Cohen and Petra, 1967). The deacylation rate constant (k_3) is dependent on a prototropic group of pK = 4.69 in the hydrolysis of *trans*-cinnamoyl-papain (Brubacher and Bender, 1966) and has been calculated to be dependent on a prototropic group with pK between 3.5 and 4 for several other substrates (Whitaker and Bender, 1965; Bender and Brubacher, 1966). On the other hand, k_3 for the hydrolysis of benzoyl-L-citrulline methyl ester (BCME) by papain was reported to be independent of pH in the acid region and to show a partial dependence on a prototropic group of pK = 7 (Cohen and Petra, 1967).

One of the difficulties in interpreting the meaning of the effect of pH on the various kinetic parameters is that, with the exception of BGEE and *N*-benzoylglycinamide (BGA), the substrates have all been charged. In order to eliminate this problem Cohen

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[1] According to a communication to Sluyterman (1964), the data reported were not corrected for incomplete ionization of α-*N*-benzoyl-L-arginine at low pH.

[2] Abbreviations used: BCME, α-*N*-benzoyl-L-citrulline methyl ester; BC, α-*N*-benzoyl-L-citrulline; BGEE, benzoylglycine ethyl ester; BAA, α-*N*-benzoyl-L-argininamide; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; Z, benzyloxycarbonyl.

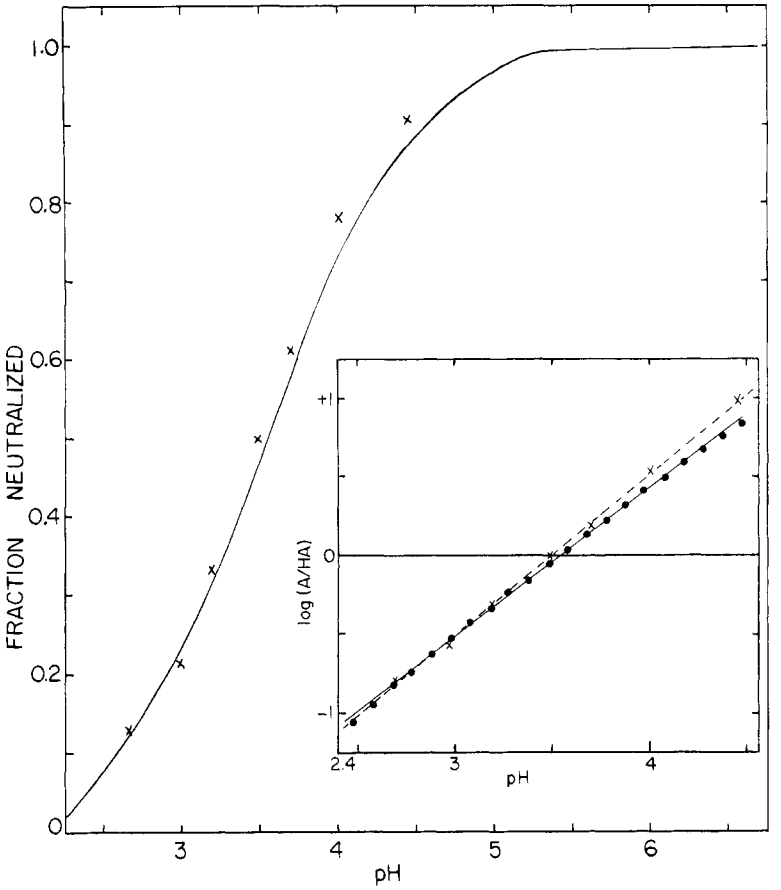

FIGURE 1: Determination of pK of α-N-benzoyl-L-citrulline. The methods used were (X) spectrophotometric and (●) titration at 25° and ionic strength of 0.300.

and Petra (1967) used BCME which is essentially isosteric with α-N-benzoyl-L-arginine ethyl ester (BAEE). Their results are extremely interesting in that they found not only k_{cat} but also k_{cat}/K_m(app) to be independent of pH in the acid region. They also reported kinetic evidence for the involvement of a group of pK = 7 in k_{cat} and k_3. In these two latter respects, their data are unique with respect to all other reported results. Because of the importance of these observations to an understanding of the mechanism of action of papain and other similar enzymes, we felt their results should be confirmed. In this paper we report not only the effect of pH on k_{cat}/K_m(app) for BCME but also the effect of pH on k_{cat}, k_3, K_m(app) and k_{cat}/K_m(app) for another neutral substrate, p-nitrophenyl benzyloxycarbonylglycinate (p-nitrophenyl Z-glycinate).

Materials and Methods

Materials. Papain (two-times crystallized, lot PAP 6JB from Worthington Biochemical Corp.) was crystallized as the mercuripapain derivative essentially as described by Brubacher and Bender (1966). Mercuripapain was activated just before use with 4-methylbenzenethiol by the procedure of Soejima and Shimura (1961) except the activation was performed out in the atmosphere and with 1.0×10^{-4} M Versene present. The normality of papain stock solutions was determined using BAEE and the correlation between rate assay with BAEE and titration assay with p-nitrophenyl Z-L-tyrosinate reported by Bender *et al.* (1966a,b). Protein concentration of papain solutions was determined at 280 mμ using ϵ_{280} 51,100 (Bender *et al.*, 1966a,b). The mercuripapain, on reactivation, was 72.7% active material.

p-Nitrophenyl Z-glycinate (lot C111B-77, Sigma Chemical Co.) was prepared as a 1.00×10^{-2} M stock solution in acetonitrile (Eastman). Benzoyl-L-citrulline (BC) and BCME were prepared by the procedure of Cohen and Petra (1967) and had melting points of 178.5–179 and 148–149.5°, respectively. BCME was prepared as a stock solution of 5.00×10^{-2} M in acetonitrile. The buffers contained 0.05 M each acetic acid, phosphoric acid, and boric acid, 1×10^{-4} M Versene, and sufficient KCl to give a final ionic strength of 0.300. The solutions were adjusted to the desired pH with NaOH.

pK of Benzoyl-L-citrulline. The pK was determined both by spectrophotometric and titration methods. In the spectrophotometric method, the absorbance

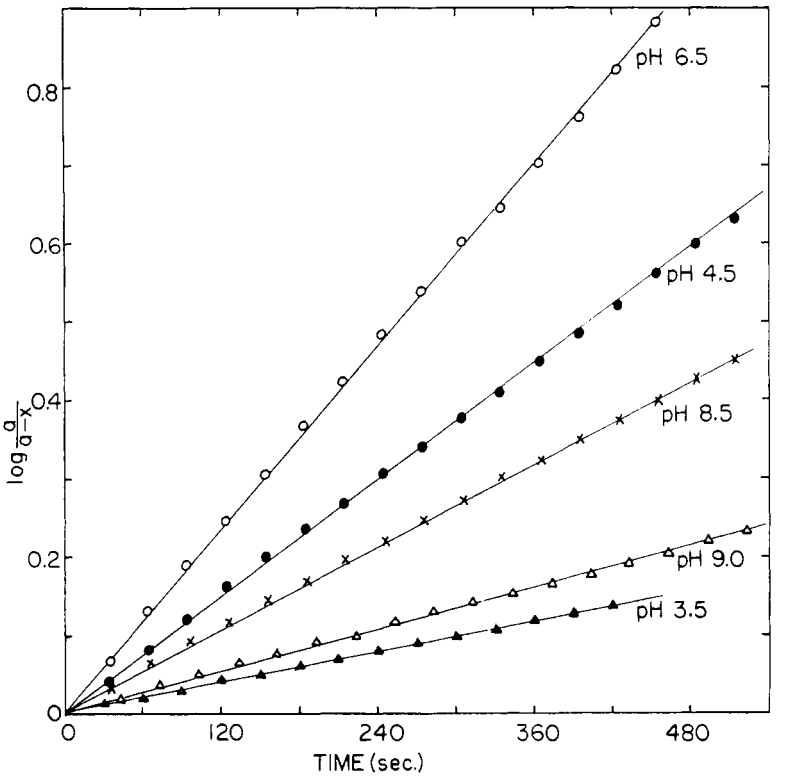

FIGURE 2: Effect of pH on rate of papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester. The conditions were E_{act}, 2.46×10^{-6} M; S_0, 8.06×10^{-4} M; 25.0 ± 0.1°; ionic strength of 0.300; 1.6% acetonitrile.

at 253 mμ of 4.85×10^{-4} M solutions of BC in 0.05 M acetate buffers of various pH values was determined using 4.85×10^{-4} BC in 0.1 M HCl as the blank. The Δε at maximum ionization was determined in 0.05 M phosphate buffer (pH 7.0) to be 1.14×10^3 M^{-1} cm^{-1}. The pH of the solutions was determined using both a Beckman Model G and a Radiometer 25 pH meter standardized against 0.05 M potassium acid-phthalate (pH 4.01 at 25°) and Beckman 3581 standard buffer (pH 7.02) at 25°. The p*K* was also determined by titration of BC with NaOH in a Radiometer titrator (Titrator 11, Titragraph SBR 2c, pH meter 25, buret SBU1a). The titration equipment was checked by use of acetic acid which was found to have a p*K* of 4.74 ± 0.02 (five determinations) at 25°. The ionic strength of the solutions in both methods was adjusted to either 0.03 or 0.30 with KCl.

Kinetic Measurements. Reactions were followed spectrophotometrically in a Beckman DB recording spectrophotometer thermostated at 25.0 ± 0.1°. Wavelengths used were 257 mμ for BCME and 340 mμ for *p*-nitrophenyl Z-glycinate at pH 7.0 and below and 440 mμ above pH 7.0. Reactions were performed as follows. Buffer (3.00 ml in reaction cuvet, 3.05 ml in blank cuvet) was allowed to reach thermal equilibration in the spectrophotometer. Then, papain solution (usually 50 μl) was added to the reaction cuvet and the absorbance was determined at 280 mμ. (This was possible only in the case of BCME as substrate because the amount of enzyme required for the *p*-nitrophenyl Z-glycinate reaction was too small to be measured in this way. In this case the enzyme concentration was calculated from the volume of stock enzyme used.) Then 50 μl of substrate was added to the blank and then rapidly to the reaction cuvet. In the alkaline pH region where nonenzymatic hydrolysis was detectable, the substrate was added to both cuvets simultaneously by two operators.

Recording was started either 10 or 15 sec after addition of substrate and all reactions were followed to completion. By this method it was possible to determine Δε at the pH of each reaction except for *p*-nitrophenyl Z-glycinate at pH 8.5 and above. In this case, Δε was determined independently on a completely hydrolyzed aliquot of substrate read against buffer. For BCME, the reactions followed good first-order kinetics (with respect to substrate) at all pH values. Since $v_0 = k_{cat}E_0(S)/K_m(app) = k_1E_0(S)$ at $S_0 \ll K_m(app)$, the pseudo-first-order reaction rate constant $k_1 = k_{cat}/K_m(app)$. For *p*-nitrophenyl Z-glycinate, k_{cat} was determined from the initial rates where $S_0 \gg K_m(app)$ and k_{cat} and $K_m(app)$ were determined from Lineweaver–Burk (1934) plots using data from each complete reaction (Bender *et al.*, 1964). The data were analyzed by means of a computer. All reactions were done at least in duplicate or triplicate.

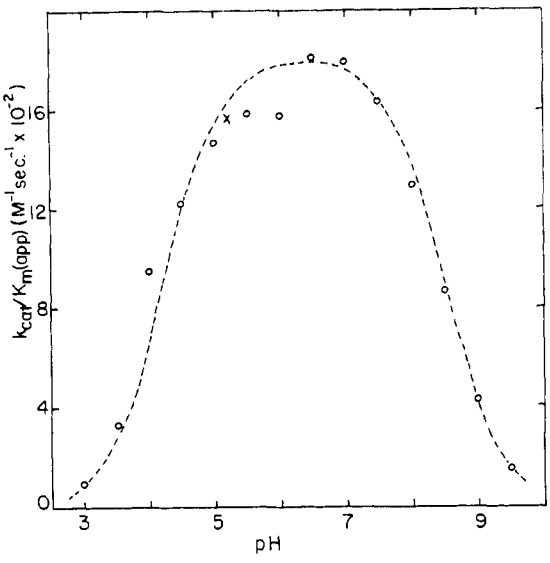

FIGURE 3: Effect of pH on k_{cat}/K_m(app) for papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester. The conditions were as in Figure 2 except data indicated by X were determined in 0.05 M acetate buffer, μ of 0.038. The dashed line is a theoretical one calculated for pK_1 and pK_2 of 4.23 and 8.50 and (k_{cat}/K_m(app)) lim of 18.08 $\times$ 10^2 M^{-1} sec^{-1}.

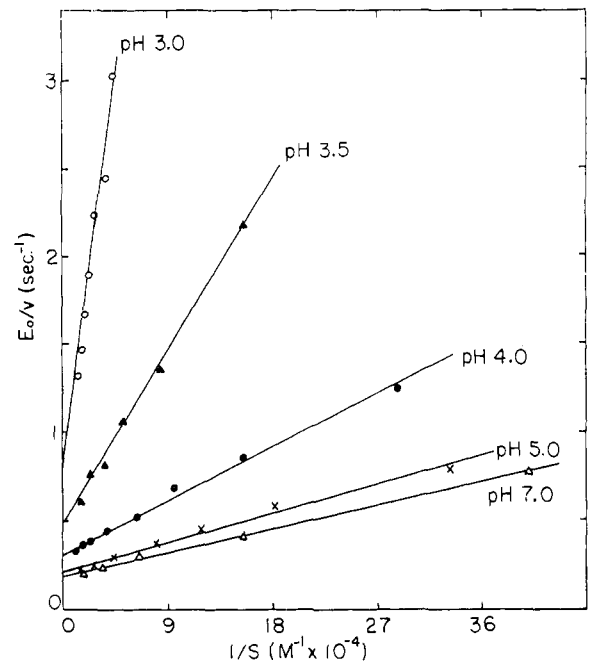

FIGURE 4: Lineweaver–Burk plots of data for papain-catalyzed hydrolysis of *p*-nitrophenyl Z-glycinate. The data were obtained from complete reaction progress curves. The conditions were: S_0, 1.54–1.61 $\times$ 10^{-4} M; E_{act}, 6.00 $\times$ 10^{-7} M at pH 3.00 and 3.50, 2.14 $\times$ 10^{-7} M at pH 4.00, 1.08 $\times$ 10^{-7} M at pH 5.00, and 1.35 $\times$ 10^{-7} M at pH 7.00; 25.0 $\pm$ 0.1°; ionic strength of 0.300; 1.6% acetonitrile.

Results

pK of Benzoyl-L-citrulline. The results of the determination of the p*K* of BC at an ionic strength of 0.300 are shown in Figure 1. In the insert of Figure 1 the p*K* value is given at the point where the line crosses at log (A/HA) $\approx$ 0. The determination of p*K* was also carried out at an ionic strength of 0.030. The p*K* values found were: spectrophotometric method, 3.49 $\pm$ 0.02 and 3.57 $\pm$ 0.07; titration method, 3.57 $\pm$ 0.06 and 3.60 $\pm$ 0.06 at ionic strengths of 0.300 and 0.030, respectively. The titration values were slightly higher than the spectrophotometrically determined values because of a slight deviation in the titration curves toward a higher p*K* value as we approached complete neutralization of the acid (Figure 1). The p*K* values reported are the average individual p*K* values calculated from the ratio of acid to salt at a given pH. In the case of the titration data, calculations were made at 0.1 pH intervals.

Papain-Catalyzed Hydrolysis of Benzoyl-L-citrulline Methyl Ester. The initial substrate concentration was chosen to be sufficiently less than K_m(app) so that first-order reaction rates, with respect to substrate concentration, would be observed as shown in Figure 2. Under these conditions, the reactions can be followed readily in a spectrophotometer and the observed velocity (v_0) = E_0k_1(S) = $k_{cat}E_0$(S)/K_m(app). Therefore, the ratio of k_{cat}/K_m(app) ($=k_2/K_s$) can be determined readily as a function of pH. The pH dependence of this ratio permits a determination of the prototropic groups on the *free* enzyme (Peller and Alberty, 1959) which are important for its activity in binding the substrate (K_s) and in rate of formation of the acyl-enzyme (k_2). The results of such a series of experiments at various pH values are shown in Table I and Figure 3. It will be seen that a bell-shaped curve is obtained. The dashed line in Figure 3 is a theoretically calculated

TABLE I: Effect of pH on the Papain-Catalyzed Hydrolysis of α-*N*-Benzoyl-L-citrulline Methyl Ester.[a]

pH	k_{cat}/K_m(app) (M^{-1} sec^{-1}) $\times$ 10^{-2}	pH	k_{cat}/K_m(app) (M^{-1} sec^{-1}) $\times$ 10^{-2}
3.00	0.967	6.50	18.19 $\pm$ 0.74
3.50	3.28 $\pm$ 0.13	7.00	17.98 $\pm$ 1.13
4.00	9.44 $\pm$ 0.68	7.50	16.36 $\pm$ 0.47
4.50	12.22 $\pm$ 1.00	8.00	12.92 $\pm$ 0.11
5.00	14.68 $\pm$ 0.51	8.50	8.68 $\pm$ 0.34
5.50	15.90 $\pm$ 0.99	9.00	4.29 $\pm$ 0.06
6.00	15.82 $\pm$ 0.84	9.50	2.16 $\pm$ 0.23

[a] Conditions were S_0 = 8.02 $\times$ 10^{-4} M, E_{act} = 2.46 $\times$ 10^{-6} M, temperature = 25.0°, and ionic strength = 0.30.

TABLE II: Kinetic Parameters of Papain-Catalyzed Hydrolysis of *p*-Nitrophenyl Z-Glycinate.[a]

pH	Initial Rate	Complete Reaction		
	k_{cat} (sec^{-1})	k_{cat} (sec^{-1})	K_m(app) (M × 10^6)	k_{cat}/K_m(app) (M^{-1} sec^{-1} × 10^{-5})
3.00	0.910 ± 0.027	1.18 ± 0.12	56.0 ± 9.2	0.211
3.50	1.90 ± 0.02	2.04 ± 0.01	20.3 ± 1.1	1.00
4.00	3.20 ± 0.04	3.19 ± 0.24	11.2 ± 2.2	2.85
4.50	3.82 ± 0.29	4.03 ± 0.11	9.90 ± 1.12	4.07
5.00	4.81 ± 0.25	4.62 ± 0.11	8.53 ± 1.38	5.42
5.50	5.20 ± 0.07	4.85 ± 0.20	5.81 ± 0.45	8.34
6.00	5.15 ± 0.12	5.16 ± 0.50	8.29 ± 0.56	6.24
6.50	5.09 ± 0.16	5.11 ± 0.39	7.44 ± 1.17	6.89
7.00	5.33 ± 0.11	5.34 ± 0.50	8.90 ± 1.57	6.00
7.50	5.21 ± 0.10	4.95 ± 0.13	8.52 ± 2.73	5.81
8.00	5.10 ± 0.17	5.77 ± 1.04[b]	20.6 ± 9.8[b]	2.80[b]
8.50	5.24 ± 0.17			
9.00	5.26 ± 0.03			
9.50	5.08 ± 0.12			

[a] Determined at 25.0°, 1.54–1.61 × 10^{-4} M initial substrate concentration, 1.08–6.00 × 10^{-7} M active papain concentration; 1.6% acetonitrile. [b] Values less accurate because of uncertainty in correction for nonenzymatic hydrolysis of substrate.

line based on pK_1 and pK_2 of 4.23 and 8.50 and (k_{cat}/K_m(app))lim of 1808 M^{-1} sec^{-1}. The value of (k_{cat}/K_m(app))lim is in excellent agreement with the average value of k_{cat}/K_m(app) of 1788 M^{-1} sec^{-1} in the pH range of 6–8 as determined by Cohen and Petra (1967) by Lineweaver–Burk analysis of data obtained with initial substrate concentrations of the order of K_m(app).

Papain-Catalyzed Hydrolysis of p-Nitrophenyl Z-Glycinate. The initial concentration was chosen such that it was much greater than K_m(app). Under these conditions, the initial rate gives a direct determination of k_{cat}. If there are no perturbing effects such as product inhibition or instability of the enzyme, k_{cat} and K_m(app) can be calculated from data taken from the complete reaction curve (Bender *et al.*, 1964) and treated in the usual fashion by the method of Lineweaver and Burk (1934) as shown in Figure 4. Whenever such treatment is valid, k_{cat}, as determined by the two methods, should be in agreement. That such is the case in the present situation is shown by the data of Table II. k_{cat} was found to be dependent upon a single prototropic group of pK = 3.85 in the pH range of 3.0–9.5. In the pH range of 5.5–9.5, k_{cat} is a constant with no indication of dependence on a group with pK of 7 (Figure 5). K_m(app) for *p*-nitrophenyl Z-glycinate was found to have the same type of pH dependence (up to 8.0) (Table II) as for the charged substrates, BAA and BAEE (Whitaker and Bender, 1965). The ratio of k_{cat}/K_m(app) shows a dependence in the acid region on a pK of 4.23 (Figure 6). k_{cat} and K_m(app) were not calculated from the complete reaction curves at pH values above 8.0 because of the uncertainties involved in correcting for nonenzymatic hydrolysis of the substrate which, at pH 9.5, was more than 50% of the observed enzymatic rate at the enzyme concentration used. Therefore, the theoretical curve drawn, based on a pK of 8.50 for BCME, should be taken only as an expected curve above pH 7.50.

Discussion

The effect of pH on k_{cat}/K_m(app) for the papain-catalyzed hydrolysis of BCME is found to be in agreement with the results obtained with all other substrates, both neutral and charged, of papain (Whitaker and Bender, 1965; Bender and Brubacher, 1966) except Z-glycylglycine (Smith *et al.*, 1958). k_{cat}/K_m(app) for the latter substrate increases at low pH presumably due to the protonation of the carboxyl group of the substrate. That the results with BCME are in agreement with those obtained for other substrates is reassuring since the effect of pH on k_{cat}/K_m(app) ($= k_2/K_s$), in the absence of any perturbing effects produced by ionization of prototropic groups on the substrate (as with Z-glycylglycine), is expected to reveal the prototropic groups on the *free* enzyme essential for its activity (Peller and Alberty, 1959). If such is the case and there is no reason to believe otherwise, then the effect of pH on k_{cat}/K_m(app) must always be the same for the hydrolysis of all substrates by a particular enzyme, regardless of the number of intermediates formed in the process. Whenever one finds evidence to the contrary, he should scrutinize his method carefully for possible sources of error.

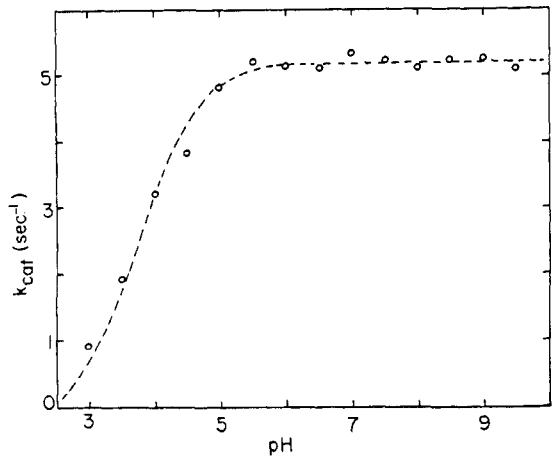

FIGURE 5: Effect of pH on k_{cat} for papain-catalyzed hydrolysis of *p*-nitrophenyl Z-glycinate. The k_{cat} values were determined from initial reaction rates. Conditions were as in Figure 4. The dashed line is a theoretical one calculated for pK of 3.85 and k_{cat}(lim) of 5.20 sec^{-1}.

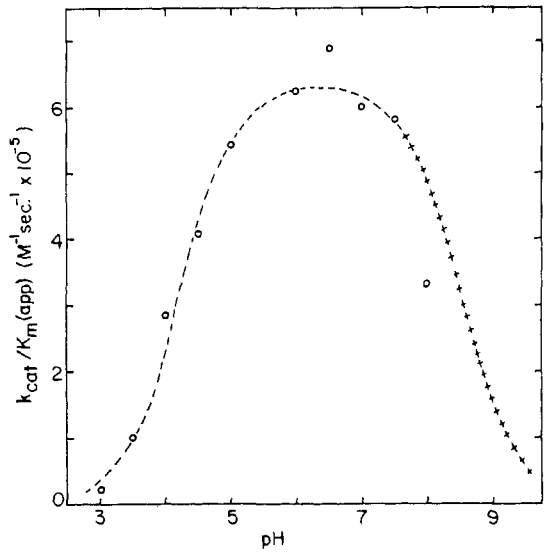

FIGURE 6: Effect of pH on k_{cat}/K_m(app) for papain-catalyzed hydrolysis of *p*-nitrophenyl Z-glycinate. The k_{cat} and K_m(app) values were calculated from Lineweaver–Burk analysis of complete reaction progress curves. Conditions were as in Figure 4. The dashed line is a theoretical one calculated for pK_1 and pK_2 of 4.23 and 8.50 and (k_{cat}/K_m(app))lim of 6.35 × 10^5 M^{-1} sec^{-1}. The theoretical curve above pH 7.5 (indicated by +) should be considered as an expected curve only.

If K_m(app) is essentially constant in the pH region of 3–8 for the papain-catalyzed hydrolysis of BCME as shown by Cohen and Petra (1967) then the effect of pH on k_{cat}/K_m(app) must reflect the effect of pH on k_{cat}. There are no compelling reasons why the K_m(app) values for BCME reported by Cohen and Petra are not correct. Qualitatively, they are in agreement with the values reported for BAEE (Whitaker and Bender, 1965) and BGEE (Sluyterman, 1964). In addition, K_m(app) for the neutral substrates, *p*-nitrophenyl α-*N*-acetyl-DL-tryptophanate (Bender and Brubacher, 1966) and *p*-nitrophenyl Z-glycinate (present report), are independent of pH from 4.0 to 8.0. When k_{cat} is calculated from the present data (k_{cat}/K_m(app)) by use of the K_m(app) values reported by Cohen and Petra (1967), it is found to be dependent in the acid region on a pK near 4 and to appear to be dependent in the alkaline region on a pK near 8.5. The data do not indicate the involvement of a group with pK near 7. The observed dependence of k_{cat} on a prototropic group of pK near 4 for hydrolysis of BCME in the acid region is in agreement with that found for BAEE and BAA (Whitaker and Bender, 1965), the *p*-nitrophenyl esters of Z-lysine, α-*N*-formyl-Z-lysine, and α-*N*-acetyl-L-tryptophan, and benzyl Z-lysinate (Bender and Brubacher, 1966) and *p*-nitrophenyl Z-glycinate (present report). On the other hand, k_{cat} values for the hydrolysis of BAEE (Smith and Parker, 1958; Sluyterman, 1964) and BGEE (Sluyterman, 1964; Kirsch and Katchalski, 1965) have been reported to be independent of pH in the acid region.

It is reasonable to assume that k_{cat} for the hydrolysis of *p*-nitrophenyl Z-glycinate is really k_3, the deacylation rate constant. Bender and Brubacher (1966) have shown that k_2 is $\gg 20k_3$ for the hydrolysis of *p*-nitrophenyl Z-lysinate by papain. The absence of a pH effect on k_{cat} for hydrolysis of *p*-nitrophenyl Z-glycinate in the alkaline region up to pH 9.5 also supports this assumption. The dependence of k_{cat} ($=k_3$) on a prototropic group of pK = 3.85 is in agreement with that found for a number of substrates (see above). Evidence that this prototropic group is probably a carboxyl group on papain has been reviewed (Kimmel and Smith, 1960; Whitaker and Bender, 1965; Bender and Brubacher, 1966). There is no evidence for the dependence of k_{cat} ($=k_3$) on a prototropic group of pK = 7 for the hydrolysis of *p*-nitrophenyl Z-glycinate. The value of 5.20 sec^{-1} for k_{cat}(lim) for hydrolysis of *p*-nitrophenyl Z-glycinate determined in this report is higher than the value of 2.73 sec^{-1} reported by Kirsch and Igelström (1966). This is the result of different methods of determining the enzyme concentration. The values of K_m(app) are in agreement with those of Kirsch and Igelström at pH 6.8 for this substrate.

The discrepancy between the results reported here and those of Cohen and Petra (1967) for the papain-catalyzed hydrolysis of BCME in the acid region can be resolved by use of the correct pK value for the ionization of the product, benzoyl-L-citrulline. In the spectrophotometric method used in this study, it was not necessary to know the pK of BC since Δε was determined for each reaction by allowing it to go to completion. However, it is essential to know the pK of BC in the pH-Stat method used by Cohen and Petra. When their results are corrected from a pK of 4.16 (their reported value) to a pK of 3.57 for BC, k_{cat}/

K_m(app), k_{cat}, k_2, and k_3 are all dependent in the acid region on a prototropic group (or groups) with a pK near 4.

Their results in the acid region are then in agreement with the results reported here. We believe the pK of 3.57 (μ = 0.03) for ionization of BC as determined by two methods to be correct. From a comparison of the pK_1 values of citrulline (pK_1 = 2.43) and arginine (pK_1 = 2.18) (Greenstein and Winitz, 1961) and α-*N*-benzoyl-L-arginine (pK = 3.24 at μ = 0.30 and 25°, Whitaker and Bender, 1965), one would predict that the pK value of α-*N*-benzoyl-L-citrulline would be 3.49 at 25° and μ = 0.30. This is precisely the value found by the spectrophotometric method.

We must conclude, therefore, that the pH *vs.* k_{cat}/K_m(app) profiles for the hydrolysis of all substrates by papain are probably bell-shaped curves because of dependency upon prototropic groups of pK's of ~4 and ~8.5 on the free enzyme, that k_{cat} values for hydrolysis of all substrates by papain are probably dependent upon a prototropic group with apparent pK values near 4 in the acid region,[3] and that k_3 values for deacylation of all acyl-papain intermediates are probably dependent on a single prototropic group of pK between 3 and 4. Furthermore, there are no available kinetic data, and for that matter no really compelling data obtained by other means, to indicate that a histidine residue is involved in the activity of papain.

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References

Bender, M. L., Begué-Cantón, M. L., Blakeley, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kézdy, F. J., Killheffer, J. V., Jr., Marshall, T. H., Miller, C. G., Roeske, R. W., and Stoops, J. K. (1966a,b), *J. Am. Chem. Soc. 88*, 5890.

Bender, M. L., and Brubacher, L. J. (1964), *J. Am. Chem. Soc. 86*, 5333.

Bender, M. L., and Brubacher, L. J. (1966), *J. Am. Chem. Soc. 88*, 5880.

Bender, M. L., Clement, G. E., Gunter, C. R., and Kézdy, F. J. (1964), *J. Am. Chem. Soc. 86*, 3697.

Bender, M. L., Gibian, M. J., and Whelan, D. J. (1966 a,b), *Proc. Natl. Acad. Sci. U. S. 56*, 833.

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

Cohen, W., and Petra, P. H. (1967), *Biochemistry 6*, 1047.

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, Vol. I, New York, N. Y., Wiley, p 487.

Himoe, A., Parks, P. C., and Hess, G. P. (1966), *J. Biol. Chem. 242*, 919.

Kimmel, J. R., and Smith, E. L. (1960), *Enzymes 4*, 133.

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

Kirsch, J. F., and Katchalski, E. (1965), *Biochemistry 4*, 884.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Lowe, G., and Williams, A. (1964), *Proc. Chem. Soc.*, 140.

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

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

Sluyterman, L. Ae. (1964), *Biochim. Biophys. Acta 85*, 305.

Smith, E. L., Chavré, V. J., and Parker, M. J. (1958), *J. Biol. Chem. 230*, 283.

Smith, E. L., and Parker, M. J. (1958), *J. Biol. Chem. 233*, 1387.

Soejima, M., and Shimura, K. (1961), *J. Biochem. (Tokyo) 49*, 260.

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

[3] Although there is no evidence yet available to indicate that the alkaline pH dependence of k_2/K_s reflects the effect of pH on K_s, and not on k_2, one should be cautious because of recent reports that K_s, and not k_2, is affected by pH in the case of α-chymotrypsin-catalyzed hydrolyses (Bender *et al.*, 1966a,b; Himoe *et al.*, 1966).