Kinetics of the Hydrolysis of Synthetic Substrates by Pepsin and by Acetyl-Pepsin*

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ABSTRACT: The pH dependence of the kinetic parameters for the hydrolysis by pepsin of benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester (Z-His-Phe-Phe-OEt) at the Phe–Phe bond has been studied by means of an automatic ninhydrin method. With this cationic substrate, the values of K_M and k_{cat} are invariant over the pH range 1–3, whereas in the range 3–4.5 a drop in K_M and an increase in k_{cat} are observed. Treatment of pepsin with acetylimidazole yields an acetylated enzyme which hydrolyzes Z-His-Phe-Phe-OEt with a K_M value similar to that for untreated pepsin, but with a value of k_{cat} that is markedly increased; the pH dependence of these parameters is similar for the two enzyme preparations. The significance of these data is discussed, especially in relation to other studies on the pH dependence of pepsin action on synthetic substrates.

Most of the recent studies on the kinetics of pepsin action have involved the use of acetyl dipeptides (*e.g.*, Ac-Phe-Tyr)[1] of the type introduced by Baker (1951, 1954), who showed that these substrates are cleaved by pepsin more rapidly at pH 2 than at pH 4. The work of Silver *et al.* (1965), Jackson *et al.* (1965, 1966), Zeffren and Kaiser (1966, 1967), Lutsenko *et al.* (1967), and Denburg *et al.* (1968) has provided valuable data on the kinetic parameters in the peptic hydrolysis of such acetyl dipeptides, and has confirmed their optimal hydrolysis near pH 2. Inouye *et al.* (1966) called attention to the possibility that the diminished rate of cleavage of these substrates at pH 4, as compared to that at pH 2, might be a consequence of the inhibitory effect of a carboxylate group adjacent to the sensitive peptide bond, as the pK_a of such substrates is near 3.5. These authors compared the pH-dependence curves for the hydrolysis of Z-His-Phe-Phe-OEt and of Z-His-Phe-Phe at the Phe–Phe bond, and noted that the pH optimum shifted from about 4.5 (for the ester) to about 3 (for the acid), the latter compound being hydrolyzed more slowly at its pH optimum than was the ester. More recently, Zeffren and Kaiser (1967) also concluded, from their study of the hydrolysis of Ac-Phe-TyrBr$_2$, that the carboxylate group of the substrate (for which they reported pK_a = 4) may be repelled by an anionic group of the enzyme.

The recognition that the change in the state of ionization of the substrate may seriously complicate the interpretation of the pH dependence of the kinetic parameters in terms of pK_a values of catalytically important groups of pepsin has led to studies (Clement and Snyder, 1966; Lutsenko *et al.*, 1967; Denburg *et al.*, 1968) on the hydrolysis of uncharged substrates such as Ac-Phe-Tyr-OMe or Ac-Phe-Tyr-NH$_2$. Because of the sparing solubility of such neutral acyl dipeptide derivatives in water, these kinetic measurements have been conducted in the presence of organic solvents (dioxane, methanol, and ethanol). As shown by Tang (1965), and recently confirmed by Zeffren and Kaiser (1967), such solvents inhibit the action of pepsin on synthetic substrates.

From their values of k_{cat} and K_M for the hydrolysis of Ac-Phe-Tyr and Ac-Phe-Tyr-OEt over the pH range 1.5–5.5, Lutsenko *et al.* (1967) have inferred a pK_a of 3.2 for a catalytically important group in the free enzyme (from a plot of log (k_{cat}/K_M) *vs.* pH) and a pK_a near 4 for the enzyme–substrate complex (from a plot of log k_{cat} *vs.* pH).[2] Clement and Snyder (1966) have reported a bell-shaped curve of k_{cat} *vs.* pH (over the pH range 1–5), and have concluded that pepsin has two catalytically important groups, with pK_a values of 1.6 and 3.4. Zeffren and Kaiser (1967), from their study of the peptic hydrolysis of Ac-Phe-TyrBr$_2$ over the pH range 0.5–4.5, also have provided evidence for two catalytically important groups with pK_a values of 0.75 and 2.7 for the free enzyme and of 0.9 and 3.4 for the enzyme–substrate complex. Denburg *et al.* (1968) have concluded that, in the peptic hydrolysis of Ac-Phe-Tyr-NH$_2$, two catalytically important groups of pK_a = *ca.*

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Phe(NO$_2$), *p*-nitro-L-phenylalanyl; Pol, L-phenylalaninol; Pla, β-phenyl-L-lactyl; TyrBr$_2$, 3,5-dibromo-L-tyrosine; TyrI$_2$, 3,5-diiodo-L-tyrosine; Z, benzyloxycarbonyl; Ac, acetyl. The abbreviated designation of amino acid residues denotes the L form, except where otherwise indicated.

[2] The kinetic parameters mentioned in this paper are defined by the equation $v = V_{max}S/(K_M + S)$ for the process

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2$$

where v = initial velocity, $V_{max} = k_{cat} \times$ total enzyme concentration, S = initial substrate concentration, $K_M = (k_{cat} + k_{-1})/k_1$, and $K_S = k_{-1}/k_1$. For competitive inhibition, K_I denotes the dissociation constant of EI in the process $E + I \rightleftharpoons EI$.

1.4 and *ca.* 4.35 are involved. Although there is some uncertainty as regards the presumed catalytically important group of pK_a near 1, there is agreement in the literature that pepsin has a group of pK_a = *ca.* 3.5, whose protonation favors enzymic cleavage of acetyl dipeptides, of acetyl dipeptide esters, and of acetyl dipeptide amides.

As noted by Zeffren and Kaiser (1967), the usual interpretation of the pH dependence curves of k_{cat} and k_{cat}/K_M in terms of pK_a values of enzymic groups important for catalysis (Dixon and Webb, 1964) depends on the assumption that, over the pH range under study, either the state of ionization of the substrate is unchanged or all ionic forms interact with the enzyme to the same extent. Although the acetyl dipeptides do not meet this requirement, and additional assumptions are needed to interpret the data on their hydrolysis, the use of acetyl dipeptide esters or amides has been assumed to be free of complication, aside from their very slow hydrolysis by pepsin and the need to introduce organic solvents into the test solutions. The synthetic substrates recently developed in this laboratory (Inouye *et al.*, 1966; Inouye and Fruton, 1967) that have an imidazolium group (as in Z-His-Phe-Phe-OEt) or an ammonium group (as in Gly-Gly-Phe-Phe-OEt) are hydrolyzed much more rapidly at the Phe–Phe bond than is Ac-Phe-Tyr-OEt; also, these cationic compounds are sufficiently soluble in aqueous buffer solutions in the pH range 1–5 to obviate the need for the addition of organic solvents. Furthermore, these substrates may be considered to be nearly 100% in one ionic form over the pH range 1–5, as the pK_a of the imidazolium group of a histidine residue within a peptide chain is about 6.5 (Schneider, 1963) and as the α-ammonium group of peptides has its pK_a near 7.5. In view of the finding that the pH optimum for the peptic hydrolysis of such cationic substrates is near 4.5 (Inouye *et al.*, 1966), there is an apparent contradiction between the conclusion that, for the hydrolysis of a neutral substrate such as Ac-Phe-Tyr-OEt the protonated form of a catalytically important enzymic group of pK_a = *ca.* 3.5 is necessary, and the conclusion that the hydrolysis of a cationic substrate is favored by the apparent deprotonation of an enzymic group also having a pK_a value near 3.5. It is implicit in the usual interpretation of the pH dependence of kinetic parameters that, for an enzyme which acts on several substrates, the k_{cat}/K_M *vs.* pH plots should be the same for all substrates (whether they are neutral or charged) provided they all interact with the same groups in the enzyme (Peller and Alberty, 1959). We have therefore undertaken the systematic study of the pH dependence of the kinetic parameters in the peptic hydrolysis of a variety of synthetic substrates. In the present communication, we report data for the hydrolysis of Z-His-Phe-Phe-OEt at 37° in buffered systems containing no added organic solvents. The initial rate of formation of Phe-OEt was determined by means of an automatic ninhydrin method, using units of the Technicon Autoanalyzer assembly.

In connection with these studies, it was of interest to examine the kinetics of the hydrolysis of Z-His-Phe-Phe-OEt by pepsin that had been treated with acetylimidazole at pH 5.8. As shown by Lokshina and Orekhovich (1966) and by Perlmann (1966), such acetylation causes a marked decrease in the proteinase activity toward hemoglobin and a marked enhancement of activity toward acetyl dipeptides at pH 2. In addition to the estimation of the kinetic parameters for the hydrolysis of Z-His-Phe-Phe-OEt by acetylated pepsin, such modified enzyme preparations have also been examined in the present studies with Z-His-Phe(NO_2)-Phe-OMe and Z-His-Phe(NO_2)-Pla-OMe as synthetic substrates. The hydrolysis of the latter two compounds was followed spectrophotometrically at 310 mμ, by means of the method recently developed in this laboratory for the rapid measurement of the peptidase and esterase activity of pepsin preparations (Inouye and Fruton, 1967).

Experimental Section

The synthesis of Z-His-Phe-Phe-OEt, Z-His-Phe(NO_2)-Phe-OMe, and Z-His-Phe(NO_2)-Pla-OMe has been described in previous communications from this laboratory (Inouye *et al.*, 1966; Inouye and Fruton, 1967). Twice-crystallized pepsin (Worthington Biochemical Corp., lot PM 708) was used in all experiments, and fresh enzyme solutions were prepared before each set of kinetic runs. Except where otherwise stated, the enzyme concentration was determined spectrophotometrically at 278 mμ, with the assumption that pepsin has a molar absorptivity of 50,900 (Perlmann, 1966) and a molecular weight of 34,163 (Rajagopalan *et al.*, 1966a).

The proteinase activity of pepsin preparations was determined spectrophotometrically with hemoglobin as the substrate, in the manner described previously (Anson, 1938; Delpierre and Fruton, 1965); one unit of pepsin activity is defined as the amount of enzyme that produces an increase of 0.01 in absorbance at 280 mμ under the conditions of the assay. The rate of the peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe (peptidase activity) and of Z-His-Phe(NO_2)-Pla-OMe (esterase activity) was measured spectrophotometrically at 310 mμ, as described by Inouye and Fruton (1967); unless otherwise stated, the substrate concentration was 0.25 mM and the reaction was conducted at pH 4.0 (0.04 M citrate buffer) and 37°. In the peptidase assay, the enzyme concentration was 0.02 mg of pepsin/ml; in the esterase assay, it was 0.005 mg/ml. For the comparison of various pepsin preparations, the measured initial rates of peptidase and esterase activity are expressed as μmoles/min per mg of pepsin.

The kinetics of the hydrolysis of Z-His-Phe-Phe-OEt was followed by means of a continuous automatic method for the measurement of the rate of formation of Phe-OEt. This method is based on the procedures described by Lenard *et al.* (1965) and Cornish-Bowden and Knowles (1965). A solution of the substrate in the appropriate buffer was kept at 37.0 ± 0.1° for 10 min before the addition of enzyme to initiate the reaction. The reaction mixture was pumped into an assembly consisting of a proportionating pump, heating bath, flow-through colorimeter, and single-pen recorder (all ob-

tained from the Technicon Corp.), and the enzymic action was stopped within about 30 sec when contact was made with a ninhydrin solution (Stein and Moore, 1948). The development of the color with ninhydrin was performed in a glass coil immersed in an oil bath at 97°. The reaction mixture was pumped at about 0.42 ml/min and the rate of N_2 flow was 0.60 ml/min, using Tygon tubing of i.d. 0.035 and 0.040 in., respectively. The ninhydrin solution and the waste solution were pumped at about 1.06 ml/min through Solvaflex tubing of i.d. 0.056 in. The over-all time for the passage of the reaction mixture through the assembly and for the recording of the absorbance at 570 mμ was about 25 min. The initial linear rate of increase in absorbance was determined for the first 20 min of the enzymic reaction (corresponding to about 10–15% hydrolysis of the substrate present) and was converted into initial velocity (v) in millimicromoles of substrate per milliliter of reaction mixture per minute, based on a standard curve obtained by passing solutions of Phe-OEt through the assembly. Each determination of the rate of hydrolysis of Z-His-Phe-Phe-OEt under a given set of conditions was run at least in duplicate, and usually in triplicate.

In the studies on the pH dependence of the hydrolysis of Z-His-Phe-Phe-OEt, the initial substrate concentration (S) ranged from 0.016 to 1.0 mM, except at pH values at which the solubility of the substrate limited the top concentration to values less than 1 mM. Buffer systems between pH 2.5 and 5.5 were prepared by mixing 0.1 mM solutions of critic acid and trisodium citrate, and the total concentration of all forms of citrate in the incubation mixtures was 0.04 M, to give the following ionic strengths: pH 2.5, $I = 0.008$; pH 3.0, $I = 0.017$; pH 3.5, $I = 0.029$; pH 4.0, $I = 0.035$; pH 4.5, $I = 0.039$; pH 5.0, $I = 0.039$; pH 5.5, $I = 0.043$. Buffer systems for the pH range 1.0–2.5 were prepared from 0.1 N solutions of HCl and NaCl to give an ionic strength of 0.036–0.40 in the incubation mixtures. The pH determinations were performed with a Beckman Expandomatic pH meter standardized against the Beckman pH 4.0 standard buffer.

At each pH value, 10–20 determinations of v were made for each value of S, and satisfactory linear plots of v *vs.* v/S were obtained, from which values of V_{max} and K_M were estimated. The data were subjected to computer analysis, using a FORTRAN IV program kindly provided by Dr. K. R. Hanson of the Connecticut Agricultural Experimental Station. This program (Hanson *et al.*, 1967) is based on an iteration procedure for fitting v *vs.* S on a rectangular hyperbola (Bliss and James, 1966), and the analysis permitted the calculation of 95% confidence limits given in Tables I and II for the values of k_{cat} and K_M. The IBM 7090/7094 coupled system of the Yale Computer Center was employed.

The acetylation of pepsin was performed with acetylimidazole, prepared in the following manner. A suspension of imidazole (10 g, 147 mmoles) in dry pyridine (16 ml) was chilled, acetic anhydride (13.8 ml, 147 mmoles) was added, and the mixture was stirred for 40 min. After being left at room temperature for 1 hr, the solution was concentrated *in vacuo*, and the residue was recrystallized from benzene to yield 7.7 g (48%) of the product, mp 102–103.5°. Boyer (1955) has reported mp 101.5–102.5°.

Before being acetylated, pepsin (598 mg) was dissolved in 74 ml of 0.1 M acetate buffer (pH 5.8) and the solution was dialyzed against 2 l. of the buffer at 4° for 18 hr; the solution was then diluted with the buffer to give a protein concentration of 6.25 mg/ml. To each 8-ml sample of the resulting pepsin solution, a freshly prepared solution (2 ml) of acetylimidazole in the acetate buffer was added, and the reaction mixture was kept at 30° for 1 hr. For the preparation of pepsin-A1 and pepsin-A2, the concentration of acetylimidazole in the reaction mixture was 0.16 (1.4 mM, 10-fold M excess) and 0.64 mg/ml (5.6 mM, 40-fold M excess), respectively. A third sample of the dialyzed pepsin solution was subjected to the same treatment as that used to prepare pepsin-A1 and pepsin-A2, except that acetylimidazole was omitted; the resulting preparation (pepsin-AC) served as the control for the other two preparations. The pepsin solutions were then passed through a column of Sephadex G-25, and the protein fraction was eluted with 0.01 M acetate buffer (pH 5.0). After extensive dialysis against distilled water, the protein solutions were lyophilized. The proteinase activity (in units per milligrams) of the lyophilized preparations of pepsin-A1, pepsin-A2, and pepsin-AC was 2015, 740, and 3060, respectively; the peptidase activity (in micromoles per minute per milligram; substrate Z-His-Phe(NO_2)-Phe-OMe) was 0.22, 0.27, and 0.14, respectively. Absorbance measurements at 278 mμ of the reaction mixture (before passage through Sephadex G-25) for the preparation of pepsin-A1 showed a decrease of 9%, as compared with the control sample; in the case of pepsin-A2, the decrease in absorbance was 11% of the control value. If the value of $\Delta\epsilon$ of 1160 (Simpson *et al.*, 1963) is used to calculate the number of tyrosine residues acetylated per molecule of pepsin, the above changes indicate that *ca.* four tyrosines were blocked in pepsin-A1 and *ca.* five tyrosines were blocked in pepsin-A2. These changes in absorbance were used as correction factors in the spectrophotometric determination of the concentration of solutions of the acetylated preparations.

In separate experiments, the procedure outlined above for the preparation of pepsin-A1 and pepsin-A2 was used for the acetylation of pepsin with acetylimidazole at reagent concentrations of 8.4 mM (60-fold M excess) and of 25.2 mM (180-fold M excess) to give pepsin-A3 and pepsin-A4, respectively. During the course of the procedure, samples were taken for assay of the proteinase activity (substrate, hemoglobin) and the peptidase activity (substrate, Z-His-Phe(NO_2)-Phe-OMe) after the 1-hr incubation with acetylimidazole (step I), after passage through Sephadex G-25 (step II), after dialysis against distilled water (step III), and after lyophilization (step IV).

For the determination of the values of K_I for Phe-OMe and Phe-OEt as competitive inhibitors of the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe by acetylated pepsin, initial rates were determined in the manner described by Inouye and Fruton (1968) at substrate concentrations of 0.10, 0.175, and 0.25 mM; at each level of S the inhibitor concentrations I were 10, 20, and 36 mM.

TABLE I: Kinetic Parameters for the Hydrolysis of Z-His-Phe-Phe-OEt by Pepsin.[a]

pH	S (mM)[b]	K_M (mM)	k_{cat} (10^2 sec^{-1})[c]	k_{cat}/K_M (10^2 mM^{-1} sec^{-1})
1.0[d]	0.05–0.80 (12)	0.26 ± 0.07	10 ± 1	39
1.5[d]	0.10–0.80 (13)	0.37 ± 0.15	13 ± 2	35
2.0[d]	0.10–1.00 (12)	0.33 ± 0.04	11 ± 1	31
2.5[d]	0.05–1.00 (11)	0.30 ± 0.06	11 ± 1	35
2.5[e]	0.05–0.70 (22)	0.37 ± 0.05	14 ± 1	37
3.0[d]	0.16–1.00 (20)	0.18 ± 0.05	11 ± 1	63
3.0[e]	0.05–0.60 (16)	0.28 ± 0.04	15 ± 1	53
3.0[f]	0.016–0.50 (10)	0.17 ± 0.03	16 ± 1	93
3.5[e]	0.05–0.60 (19)	0.21 ± 0.03	21 ± 1	99
4.0[e]	0.05–0.50 (10)	0.17 ± 0.05	33 ± 7	190
4.0[f]	0.016–0.50 (22)	0.14 ± 0.03	33 ± 3	233
4.5[e]	0.05–0.60 (18)	0.18 ± 0.02	47 ± 2	265
4.5[g]	0.05–0.40 (12)	0.37 ± 0.05	47 ± 4	127
5.0[e]	0.05–0.40 (14)	0.09 ± 0.04	28 ± 4	295
5.5[e]	0.02–0.40 (22)	0.04 ± 0.01	8 ± 1	213

[a] Enzyme concentration, 2.8×10^{-4} mM; temperature, 37° (for details, see Experimental Section). [b] The numbers in parentheses denote the total number of runs at this pH value. [c] Calculated from V_{max} values on the assumption that the molecular weight of pepsin is 34,163. [d] HCl–NaCl buffer. [e] 0.04 M citrate buffer. [f] 0.04 M citrate buffer and 0.2 M NaCl. [g] 0.04 M citrate buffer and 5% methanol.

The resulting data gave satisfactory linear plots ($1/v$ *vs.* I) for competitive inhibiton by the method of Dixon (1953).

To determine the number of acetyl groups present in the acetylated pepsin, a sample of [1-^{14}C]acetylimidazole (Calbiochem, lot 901565) was mixed with unlabeled reagent and recrystallized to constant radioactivity (4420 cpm/μmole). One sample of pepsin was treated with reagent in the manner described for the preparation of pepsin-A2; another sample of pepsin was treated with 14 mM reagent (100-fold M excess) in a similar manner. A control experiment was performed in which labeled acetylimidazole was hydrolyzed with 0.1 N HCl, diluted with acetate buffer (pH 5.8), and mixed with pepsin; the concentration of [1-C^{14}]acetate was 28 mM. The resulting solution was passed through a Sephadex G-25 column as before. The radioactivity of all the pepsin samples was determined by means of a Packard scintillation counter (Model 3375), with 2,5-diphenyloxazole, 2,2-*p*-phenylenebis(5-phenyloxazole), and naphthalene in dioxane as the scintillator solution. There was no measurable radioactivity in the control pepsin, whereas the acetylated pepsin corresponding to pepsin-A2 contained 3.9 ± 0.3 acetyl groups per molecule of protein (average of duplicate determinations). The other acetyl-pepsin contained 5.5 ± 0.1 acetyl groups per molecule of pepsin. Measurement of the change in absorbance at 278 mμ of the two acetylated pepsin samples, as compared to the control sample, gave values corresponding to the acetylation of 3.7 ± 0.5 and 5.0 ± 0.4 tyrosine residues per molecule, respectively.

The iodination of pepsin was performed at 0° by mixing 4 ml of the enzyme solution (1.25 mg/ml) in 0.15 M phosphate buffer (pH 5.6) with 1 ml of an aqueous solution of iodine monochloride. For the preparation of pepsin-I1 and pepsin-I2, the concentration of iodine monochloride in the reaction mixture was 0.116 (5-fold M excess) and 0.58 mg/ml (25-fold M excess), respectively. Before the reaction, the pepsin solution had been dialyzed overnight at 2° against the phosphate buffer. A control solution, from which ICl had been omitted, was run in parallel (pepsin-IC). The reaction mixtures were kept at 0° for 30 min and then were treated with 0.1 M $Na_2S_2O_3$ (1 ml). Each of the resulting solutions (pH 5.5) was passed through Sephadex G-25, the elution being performed with 0.01 M citrate buffer (pH 4.0); the protein fraction was collected and assayed for proteinase (hemoglobin), peptidase (Z-His-Phe(NO_2)-Phe-OMe), and esterase (Z-His-Phe(NO_2)-Pla-OMe) activity.

Results

The data in Table I show that, in the peptic hydrolysis of Z-His-Phe-Phe-OEt, both K_M and k_{cat} are invariant over the pH range 1–3, within the precision of the measurements. In the pH range 3–5.5, the value of K_M drops somewhat and that of k_{cat} increases about fourfold to an apparent maximum at pH 4.5, and drops thereafter. These changes with pH are reflected in a k_{cat}/K_M *vs.* pH plot with an apparent maximum at pH 4.5. It may be noted that the values of K_M and k_{cat} given in Table I for the rate of hydrolysis at pH 4.0 (citrate buffer) are 0.17 mM and 0.33 sec^{-1}, respectively; they are in good agreement with those reported previously (K_M = 0.18 mM and k_{cat} = 0.31 sec^{-1}) by Inouye *et al.* (1966).

Examination of the kinetic parameters at pH 2.5 and

TABLE II: Kinetic Parameters for the Hydrolysis of Z-His-Phe-Phe-OEt by Acetyl-Pepsin.[a]

Preparation	pH	S (mM)[b]	K_M (mM)	k_{cat} (10^2 sec^{-1})
Pepsin-AC[c]	2.5	0.05–0.80 (12)	0.34 ± 0.08	13 ± 2
	4.0	0.05–0.70 (12)	0.11 ± 0.04	32 ± 3
	5.0	0.05–0.48 (11)	0.07 ± 0.02	24 ± 2
Pepsin-A1[c]	3.0	0.05–0.80 (10)	0.43 ± 0.06	44 ± 3
	4.0	0.05–0.48 (10)	0.32 ± 0.08	91 ± 11
	4.5	0.05–0.40 (9)	0.32 ± 0.05	113 ± 10
	5.0	0.05–0.40 (11)	0.12 ± 0.05	62 ± 9
Pepsin-A2[d]	2.5	0.016–0.40 (15)	0.43 ± 0.12	70 ± 11
	4.0	0.016–0.40 (11)	0.23 ± 0.03	142 ± 10
	4.5	0.016–0.40 (9)	0.22 ± 0.05	115 ± 13

[a] Citrate buffers, temperature, 37° (for details, see Experimental Section). [b] The numbers in parentheses denote the total number of runs at this pH value. [c] Enzyme concentration, 2.9×10^{-4} mM. [d] Enzyme concentration, 1.45×10^{-4} mM.

TABLE III: Acetylation of Pepsin with Acetylimidazole.[a]

	Pepsin-AC		Pepsin-A3		Pepsin-A4	
Prepn Tested[a]	Assay I[b]	Assay III[c]	Assay I[b]	Assay II[c]	Assay I[b]	Assay II[c]
Step I	0.14	3350	0.28	440	0.18	140
Step II	0.155	3400	0.30	470	0.20	150
Step III	0.17	3400	0.35	425	0.22	140
Step IV	0.15	3050	0.29	320	0.19	70

[a] The definition of pepsin preparations obtained at each step of the procedure is given in the Experimental Section. [b] Peptidase assay with Z-His-Phe(NO_2)-Phe-OMe as the substrate at pH 4 and 37°; the enzymic activity is given in micromoles per minute per milligram of pepsin. [c] Proteinase assay with hemoglobin as the substrate; the enzymic activity is given in units, as defined in the Experimental Section.

3.0 with either HCl–NaCl or citrate buffer showed little evidence of a specific buffer effect at the buffer concentrations used. Furthermore, the addition of 0.2 M NaCl to the pH 4 citrate buffer did not alter the kinetic parameters significantly. The apparent absence of a general ionic strength effect in the case of the hydrolysis of Z-His-Phe-Phe-OEt is similar to the results reported by Jackson *et al.* (1965) and Zeffren and Kaiser (1967) for the hydrolysis of Ac-Phe-TyrI_2 and of Ac-Phe-TyrBr_2. It will be noted from Table I that the presence of 5% methanol at pH 4.5 causes a doubling of the value of K_M without changing k_{cat}.

In Table II are given the kinetic parameters for the hydrolysis of Z-His-Phe-Phe-OEt by pepsin that had been treated with acetylimidazole at pH 5.8. It will be seen that the kinetic properties of the control preparation (pepsin-AC) were similar to those for the untreated enzyme used for the experiments reported in Table I. The data in Table II show that, in the action of two acetylated pepsin preparations (pepsin-A1 and pepsin-A2) on Z-His-Phe-Phe-OEt, the K_M values are similar to those for the hydrolysis of this substrate by untreated pepsin, and that the effect of pH also is the same for the acetylated and control enzyme preparations. On the other hand, the acetylated pepsins exhibit a striking increase in the value of k_{cat}, as compared to the control preparation, the ratio of the k_{cat} values at a given pH value being nearly the same over the pH range 2.5–4.5.

In exploratory experiments on the acetylation of pepsin by acetylimidazole, the effect of the individual steps in the procedure used to isolate the modified protein on the proteinase and peptidase activity of pepsin were examined. In these experiments, pepsin was treated with higher concentrations of acetylimidazole than those used to prepare pepsin-A1 and pepsin-A2. As shown in Table III, the peptidase activity (substrate, Z-His-Phe(NO_2)-Phe-OMe) was unchanged, but a slight loss in proteinase activity was noted at the step involving lyophilization of both the control preparation and the acetylated pepsins (pepsin-A3 and pepsin-A4). As with Z-His-Phe-Phe-OEt, a marked increase in the rate of cleavage of Z-His-Phe(NO_2)-Phe-OEt was observed.

Qualitatively, therefore, the action of acetylated pepsins of these two synthetic substrates at pH 4 is similar to that previously reported for the action of such modified enzyme preparations on acetyl dipeptides at pH 2 (Lokshina and Orekhovich, 1966; Perlmann, 1966). It may be added that the esterase activity of acetylated pepsin toward Z-His-Phe(NO_2)-Pla-OMe at pH 4 is increased roughly to the same degree as is the peptidase activity. For example, whereas the peptidase activity (in micromoles per minute per milligram) of pepsin-A2 and pepsin-AC was 0.27 and 0.14, respectively, the esterase activity of the two preparations was 0.78 and 0.42, respectively.

In considering the increased k_{cat} exhibited by acetylated pepsin toward synthetic substrates, the possibility presented itself that the greater rate of decomposition of the ES complex might be related to more rapid release of cleavage products. If this were so, a difference might be expected in the K_I values for the cleavage products, when they act as competitive inhibitors in the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe (Inouye and Fruton, 1968). The experiments with Phe-OMe as the inhibitor of pepsin-A2 gave a value of K_I = 29 mM at pH 4 and 37°; this may be compared with the value of 22 mM found with untreated pepsin (Inouye and Fruton, 1968). With Phe-OEt as the inhibitor, K_I values of 8 and 9 mM were obtained for pepsin-A1 and pepsin-A2, respectively, with a value of 7 mM for the control preparation pepsin-AC. Earlier studies had given a value of 10 mM for Phe-OEt as an inhibitor of the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe by untreated pepsin (Inouye and Fruton, 1968). It may be concluded, therefore, that within the precision of the method employed there is no marked change in the K_I for Phe-OMe or for Phe-OEt upon acetylation of pepsin. The K_I for Z-His-Phe(NO_2) could not be determined because of its limited solubility at pH 4.

Previous work has indicated that treatment of pepsin with acetylimidazole at pH 5.8 leads to the acetylation of the phenolic groups of tyrosyl residues of the enzyme. Perlmann (1966) reported that an acetylated preparation having 40% of the proteinase activity of the untreated enzyme contained nine to ten acetyl groups per pepsin molecule, as judged either by the change in absorbance at 278 mμ (Simpson *et al.*, 1963) or by the reaction with NH_2OH to form acethydroxamic acid. Lokshina and Orekhovich (1966) reported the presence of nine to ten *O*-acetyl groups (and less than one *N*-acetyl group) in acetylated pepsin having 10–15% of the original proteinase activity. The fact that the proteinase activity can be restored by mild deacylation is consistent with the conclusion that substitution of phenolic groups of tyrosine residues inhibits the peptic cleavage of protein substrates. Under the conditions used in the present work, it was found that pepsin-A2, which had 25% of the proteinase activity of the control pepsin-AC, exhibited a decrease in absorbance at 278 mμ corresponding to the acetylation of *ca.* five tyrosyl residues per molecule of pepsin. As an additional check, pepsin was treated with [1-^{14}C]acetylimidazole, and it was found that, under the conditions for the preparation of pepsin-A2, the incorporated radioactivity corresponded to the introduction of *ca.* four acetyl groups per molecule of pepsin. This result gives further support to the conclusion that acetylimidazole had reacted almost exclusively with the phenolic groups of tyrosyl residues.

In contrast to the effect of acetylation, iodination of pepsin under the conditions described in the Experimental Section caused a concomitant inactivation of the enzyme as a proteinase, peptidase, and esterase (Table IV).

TABLE IV: Inactivation of Pepsin by Iodine Monochloride.[a]

	Specific Activity[b]		
	Pepsin-IC	Pepsin-I1	Pepsin-I2
Proteinase activity[c]	3190	2560 (83)	750 (23)
Peptidase activity[d]	0.14	0.11 (78)	0.04 (28)
Esterase activity[e]	0.41	0.34 (83)	0.13 (32)

[a] For details, see Experimental Section. [b] The numbers in parentheses denote the percentages of the appropriate control values. [c] Substrate, hemoglobin; specific activity in units per milligram of pepsin. [d] Substrate, Z-His-Phe(NO_2)-Phe-OMe; specific activity in micromoles per minute per milligram of pepsin. [e] Substrate, Z-His-Phe(NO_2)-Pla-OMe; specific activity in micromoles per minute per milligram of pepsin.

Discussion

The data presented above show that the kinetics of the peptic hydrolysis of a cationic substrate such as Z-His-Phe-Phe-OEt exhibits a different pH dependence from that previously reported for acetyl dipeptides (*e.g.*, Ac-Phe-Tyr) or acetyl dipeptide esters or amides (*e.g.*, Ac-Phe-Tyr-OEt or Ac-Phe-Tyr-NH_2). In considering this difference it may be useful first to note the relative magnitudes of the kinetic parameters for the various substrates studied recently. The data in Table V, taken from the cited publications, are given in the same units as those used in Table I. A comparison shows that, of the compounds listed in Table V, only Ac-Phe-TyrBr$_2$ and Ac-Phe-TyrI$_2$ exhibit k_{cat}/K_M values of the same magnitude as those found for Z-His-Phe-Phe-OEt. The relatively slow hydrolysis of Ac-Phe-Tyr and its esters or its amide is especially noteworthy; even at pH 2, near the optimum for their hydrolysis, the value of k_{cat}/K_M is less than 10% of that for Z-His-Phe-Phe-OEt.

Since the ionic state of Z-His-Phe-Phe-OEt may be considered to be unchanged over the pH range 1–5, the increase in k_{cat}/K_M with increasing pH (from 2.5 to 4.5) may be considered to reflect the presence, in the enzyme, of a catalytically important prototropic group that is effective in its dissociated form. A calculated curve based

TABLE V: Kinetic Parameters for the Hydrolysis of Acetyl Dipeptides and Their Derivatives by Pepsin.

Substrate	pH	Temp	k_{cat} (10^2 sec^{-1})	K_M (mM)	k_{cat}/K_M (10^2 sec^{-1} mM^{-1})
Ac-Phe-Tyr	2.0	37[a]	6.8	2.4	2.8
	2.0	37[b]	8.5	2.2	3.9
	2.0	35[c]	3.5	1.2	2.9
	3.9	35[c]	1.5	5.9	0.25
	4.0	37[a]	1.6	4.4	0.4
Ac-Phe-Tyr-OEt	2.0	37[a]	2.1	0.94	2.2
	4.0	37[a]	1.1	1.8	0.6
Ac-Phe-Tyr-OMe	2.1	25[d]	1.9	2.35	0.8
	4.1	25[d]	1.3	1.5	0.9
Ac-Phe-Tyr-NH_2	2.0	35[c]	7.8	2.4	3.3
	3.9	35[c]	7.3	8.1	0.9
Ac-Phe-TyrI_2	2.0	37[e]	20	0.075	270
	4.5	37[e]	70	0.84	84
Ac-Phe-TyrBr_2	2.0	25[f]	7.3	0.14	51
	4.0	25[f]	2.7	0.67	4
Ac-Phe-Trp	2.0	35[c]	5.2	7.0	0.7
	3.9	35[c]	2.8	33.4	0.08

[a] Lutsenko *et al.* (1967); Ac-Phe-Tyr-OEt in 10% ethanol. [b] Jackson *et al.* (1966). [c] In 3% methanol; Denburg *et al.* (1968). [d] In 3.16% dioxane; Clement and Synder (1966). [e] Jackson *et al.* (1965). [f] In 5–5.8% methanol; Zeffren and Kaiser (1967).

on the equation $k_{cat}/K_M = (k_{cat}/K_M)(\text{lim})/(1 + (H^+/K_a))$, with $pK_a = 3.8$, appears to accord with the data presented in Table I. We do not believe that the precision of our data, or of those cited in Table V, is sufficient to allow more than a rough estimate of such pK_a values. A pK_a value of about 3.8 is consistent, however, with those of the side-chain carboxyl groups of aspartyl and glutamyl residues (Nozaki and Tanford, 1967), and we may therefore infer that the presence of a carboxylate group in the enzyme favors the cleavage of Z-His-Phe-Phe-OEt. One possible function of such a carboxylate group would be to form an ion pair with the cationic group of the substrate, in a manner analogous to that postulated for the interaction of acetylcholinesterase with its specific substrates (Wilson, 1960). In the case of pepsin, such an ion pair may not, in itself, contribute extensively to the binding of Z-His-Phe-Phe-OEt to the enzyme, but may cooperate with the strong binding at the catalytic site of the side chains of adjacent apolar amino acids (Inouye and Fruton, 1968).

As noted earlier in this paper, the comparison of k_{cat}/K_M *vs.* pH plots for different substrates of an enzyme, and the interpretation of such plots in terms of assumed pK_a values of catalytically important prototropic groups in the protein, are only justified if the various substrates all combine with the same groups in the enzyme. In view of the difference in the k_{cat}/K_M *vs.* pH plots for Z-His-Phe-Phe-OEt and Ac-Phe-Tyr-OEt, the possibility must be considered that, during the course of their hydrolysis by pepsin, these two substrates do not combine with the enzyme in the same manner. In particular, it would seem that the interaction of a suitably located cationic group in a substrate with a carboxylate group at the catalytic site of pepsin may favor hydrolysis at the sensitive peptide bond. Conversely, the presence in a pepsin substrate of a carboxylate group adjacent to a sensitive bond (as in acetyl dipeptides) may inhibit enzymic action, possibly through repulsion by a carboxylate group of the enzyme. These considerations suggest that the usual interpretation of the pH-dependence curves of kinetic parameters should be applied with caution, especially in the case of pepsin. It may well be that the catalytic region of the enzyme contains several carboxyl groups that may or may not be catalytically important groups, depending on the structure of the peptide substrate. This view is not inconsistent with the complete inactivation of pepsin by the stoichiometric reaction of one molecule of suitable diazo compound with one molecule of pepsin (Delpierre and Fruton, 1966; Rajagopalan *et al.*, 1966b; Hamilton *et al.*, 1967; Ong and Perlmann, 1967; Kozlov *et al.*, 1967), as it may be sufficient and sterically possible to block only one of the catalytically important carboxyl groups of the enzyme to abolish activity. The possibility that pepsin contains more than one catalytically important carboxyl group is consistent with the finding that deactivation of the enzyme by *p*-bromophenacyl bromide is accompanied by the substitution of the β-carboxyl group of an aspartyl residue (Erlanger *et al.*, 1966; Gross and Morell, 1966) and that inactivation of pepsin by α-diazo-*p*-bromacetophenone involves the substitution of an enzymic group different from the one that reacts with *p*-bromophenacyl bromide (Erlanger *et al.*, 1967). Various mechanisms have been proposed (Bender and Kézdy,

1965; Delpierre and Fruton, 1965; Zeffren and Kaiser, 1967) for the participation of carboxyl groups in the catalytic process; when based on the pH dependence of the kinetic parameters of selected synthetic substrates, such proposals must be viewed cautiously until a more extensive body of kinetic data is available and knowledge is gained of the amino acid sequence and the three-dimensional structure in the catalytic region of pepsin.

It was of interest to find that, upon acetylation of tyrosyl residues in pepsin with acetylimidazole, the enhanced rate of hydrolysis of Z-His-Phe-Phe-OEt is reflected almost entirely in an increase in k_{cat}, the value of K_M being essentially unaffected. It has been inferred that in the peptic hydrolysis of synthetic substrates $K_M = K_S$ (Clement and Snyder, 1966; Inouye and Fruton, 1967); if this is true, the enhancement of the hydrolysis of synthetic substrates by acetylated pepsin would be a consequence of a more efficient catalytic mechanism rather than of tighter binding of the substrates.

It has been shown that the interaction of the substrate analog Z-His-Phe(NO_2)-Pol with pepsin involves strong binding at a primary site, identified as the substrate-binding site for Z-His-Phe(NO_2)-Phe-OEt, and weaker binding at multiple secondary sites; in acetylated pepsin, some of the secondary binding sites are blocked (Humphreys and Fruton, 1968). It may be surmised that the substitution of tyrosyl residues near such secondary binding sites has altered the structure of the catalytic region of pepsin so as to prevent the effective cleavage of long-chain peptides (presumably because of their inability to interact with the enzyme at the secondary sites) and to promote the more effective hydrolysis of small synthetic substrates. In view of the finding, mentioned above, that the enhanced rate of cleavage of Z-His-Phe-Phe-OEt by acetylated pepsin is reflected in an increased k_{cat}, it would seem that acetylation near the secondary binding sites has altered the structure of the active site of pepsin so as to increase the efficiency of catalysis. Further studies are needed to elucidate this phenomenon; the possibility that the split product Phe-OEt is bound less firmly by acetylated pepsin, thus favoring more rapid turnover, has not been supported by the data presented above.

The importance of the secondary binding sites for the cleavage of long-chain peptides by pepsin may be related to the failure of some pepsin-like proteinases (rennin, cathepsin D) to effect the rapid hydrolysis of small synthetic substrates for pepsin. Through the kind generosity of Dr. B. Foltmann and of Dr. J. Garnier, samples of crystalline rennin (for a review, see Foltmann, 1966) were made available to us for a preliminary estimate of the rate of its action on Z-His-Phe(NO_2)-Phe-OMe. At pH 4 (0.04 M citrate buffer) and 37°, this substrate (0.25 mM) was cleaved by rennin at a rate of 0.8 mμmole/min per mg of rennin, a value about 0.5% of that for pepsin (*cf.* data for for pepsin-AC in Table III). Studies are in progress to synthesize long-chain peptide substrates for rennin and cathepsin D, so that the kinetics of the action of these enzymes may be studied quantitatively and compared to the kinetics of pepsin action.

As noted above, acetylation of pepsin causes a parallel rise in the specific activity of the enzyme as a peptidase and as an esterase. This finding is in contrast to the results of Simpson *et al.* (1963) with carboxypeptidase, whose peptidase activity is suppressed by acetylation, whereas the esterase activity is markedly enhanced. Simpson and Vallee (1966) further showed that iodination of carboxypeptidase causes a similar change in the specificity of this enzyme. With pepsin, however, iodination with iodine monochloride at pH 5.6 effects parallel decrease of proteinase, peptidase, and esterase activity. The preferential attack of iodine at the tyrosyl side chains of pepsin has long been known (Herriott, 1937, 1947), and it will be of interest to examine more closely the difference between acetylated and iodinated pepsin.

Added in proof

Since the submission of this paper, Keilova and Keil (1968) have reported that Gly-Phe-Phe-Tyr-Thr-Pro-Lys is cleaved by cathepsin D predominantly at the Phe-Phe linkage.

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