The Effect of pH on the Rates of Hydrolysis of Three Acylated Dipeptides by Pepsin[1]

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Abstract: The pH dependence of k_0/K_0, k_0, and K_0 for the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine, N-acetyl-L-phenylalanyl-L-tyrosine amide, and N-acetyl-L-phenylalanyl-L-tryptophan have been determined. The main conclusions derived from analysis of the data are that a base of $pK_a \simeq 1.4$ and an acid of $pK_a \simeq 4.35$ on the enzyme are important in these reactions and that both groups are still free to undergo ionization in the enzyme–substrate complex. A brief discussion of what the ionizable groups may be is presented. The kinetic data rule out a mechanism for pepsin action postulating equilibrium formation of an anhydride of the enzyme but, taken in conjunction with other observations on pepsin, do not permit formulation of a satisfactory mechanism at this time. A discussion of the probability that K_0 is an equilibrium constant concludes the paper.

Little detailed information on the pH dependence of the rate of pepsin-catalyzed hydrolyses is available. Development of a spectrophotometric technique for measuring the rate of hydrolysis of synthetic substrates by pepsin has much simplified obtaining such information[3,4] and has enabled us to determine pH–rate profiles for the compounds Ac-Phe-Tyr,[5] Ac-Phe-TyrNH$_2$, and Ac-Phe-Trp.

Each peptide offers one particular advantage. The general properties of Ac-Phe-Tyr are familiar from previous investigations.[3,6,7] The enzymatic hydrolysis of Ac-Phe-TyrNH$_2$ is not complicated by the presence of a free carboxyl group in the substrate. Finally, for reasons discussed below it is much easier experimentally to observe the hydrolysis of Ac-Phe-Trp than of the tyrosine-containing substrates.

Experimental Section

Chemicals. Inorganic compounds were reagent grade. Pepsin was lots PM 699 and 709 of the Worthington Biochemical Corp., Freehold, N. J. Ac-Phe-Tyr was lots S-1020-B[8] and S-1020-C (mp 221–225°) of the Cyclo Chemical Corp., Los Angeles, Calif. Cyclo lot M-1616 of Ac-Phe-Trp was recrystallized once from ethanol–water to yield material, mp 221–225.5°, $[\alpha]^{22}D$ +56° (*c* 0.6, acetic acid). Its pK_a was determined spectrophotometrically.

Anal.[8] Calcd for $C_{22}H_{23}N_3O_4$: C, 67.16; H, 5.89; N, 10.68. Found: C, 67.16; H, 6.14; N, 10.85.

Cyclo lot M-2474 of Ac-Phe-TyrNH$_2$ was used as received, mp 249–252°, $[\alpha]^{22}D$ −20° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{20}H_{23}N_3O_4$: C, 65.02; H, 6.27. Found: C, 64.95; H, 6.47.

Some controls on Ac-Phe-TyrNH$_2$ were performed on Cyclo lot M-2868, mp 243–246°, which was slightly yellow but had the same kinetic and spectrophotometric properties as M-2474. TyrNH$_2$·HCl was Cyclo lot K-4501, mp 247–250°. To a solution of the salt in water was added exactly enough sodium hydroxide to neutralize the hydrochloric acid, and a rotation measured and calculated for the free base, TyrNH$_2$; $[\alpha]^{30}D$ +18.3° (*c* 4, water); Koenigs and Mylo[9] report $[\alpha]^{20}D$ +19.5° (*c* 6, water) for TyrNH$_2$. The following compounds were purchased from Cyclo and were used as received: Tyr, Trp, Ac-Phe, Trp-Trp, Tyr-TyrNH$_2$·HCl, Phe-Tyr, and Phe-TyrNH$_2$.

Kinetic Methods. The technique for making the kinetic measurements with a Cary 14 recording spectrophotometer has been described.[3] Reactions were carried out at 35.0 ± 0.3° in a solvent mixture containing 3.0 ml of aqueous buffer, μ = 0.5,[10] 0.100 ml of aqueous enzyme solution, and 0.100 ml of methanolic substrate solution. The pH of typical reaction mixtures was measured with a Radiometer PHM 4c or 22 meter standardized against the solutions recommended by Bates.[12] These readings were used in all plots and calculations, except that the pH of reactions in 0.5, 0.3, and 0.2 *M* hydrochloric acid was taken as 0.30, 0.50, and 0.70, respectively. These three solutions actually gave meter readings of 0.40, 0.60, and 0.75, respectively, when the meter was set at pH 1.09 while the electrodes were immersed in 0.1 *M* hydrochloric acid.[12] The ionic strength was maintained with either sodium or potassium chloride.

The solubility of the three substrates in the methanolic stock solutions ultimately defined the maximum substrate concentrations attainable in the kinetic runs. The aqueous solutions of Ac-Phe-Trp and Ac-Phe-TyrNH$_2$ for kinetics also approached saturation, but for Ac-Phe-Tyr much higher substrate concentrations can be achieved by other techniques.[6]

The wavelengths used to monitor the hydrolyses were 237 and 240 mμ for Ac-Phe-Tyr, 237 mμ for Ac-Phe-TyrNH$_2$, and 294 mμ for Ac-Phe-Trp. Measurements at 294 mμ were extremely easy to perform, but some technical problems associated with the kinetics at 237 mμ should be mentioned. The slit width on the Cary spectrophotometer was 0.4–0.8 mm. For widths within this range, the difference in molar extinction coefficient (ϵ) between reactants and products ($\Delta\epsilon$) was independent but the ϵ's of substrate and products were highly dependent on the exact slit width. In any series of runs at a given pH, the slit width was held constant. The optical densities (OD) of solutions of different initial substrate concentrations ($[S]_0$) at t = 0 could then be employed as a means of verifying that the OD of the solutions adhered to the Beer–Lambert law. Rather dilute acetate, tartrate, and citrate buffers had to be used because of the appreciable absorbance of these buffers at 237 mμ. Several runs with Ac-Phe-Tyr in acetate and tartrate buffers were monitored at 240 mμ, where the ϵ's of buffer, enzyme, substrate,

(1) Supported by Grant AM 08005 of the U. S. Public Health Service.
(2) Taken in part from the B.A. thesis of J. L. Denburg.
(3) M. S. Silver, J. L. Denburg, and J. J. Steffens, *J. Am. Chem. Soc.*, **87**, 886 (1965).
(4) The automated ninhydrin procedure of A. J. Cornish-Bowden and J. R. Knowles, *Biochem. J.*, **95**, 71P (1965), should nicely supplement the spectrophotometric method.
(5) All amino acid residues are of the L configuration unless otherwise indicated. The following abbreviations are used: Ac = acetyl, Phe = phenylalanine, Tyr = tyrosine, Trp = tryptophan, TyrNH$_2$ = tyrosine amide, I$_2$Tyr = diiodotyrosine, TyrOMe = tyrosine methyl ester, TyrOEt = tyrosine ethyl ester, TrpOEt = tryptophan ethyl ester, Z = benzyloxycarbonyl, Glu = glutamic acid, His = histidine, PheOEt = phenylalanine ethyl ester, and dehydroPhe = α-acetaminocinnamoyl.
(6) L. E. Baker, *J. Biol. Chem.*, **193**, 809 (1951); **211**, 701 (1954).
(7) W. T. Jackson, M. Schlamowitz, and A. Shaw, *Biochemistry*, **5**, 4105 (1966).
(8) Analyses were performed by Micro-Tech Laboratory, Skokie, Ill.
(9) E. Koenigs and B. Mylo, *Ber.*, **41**, 4427 (1908).
(10) Kinetics on Ac-Phe-Tyr in the pH range 1.5–3.0 employed an aqueous buffer of μ = 0.2. The points for μ = 0.2 and μ = 0.5 formed a smooth continuous curve over the entire pH range. The pH–activity curve for the hydrolysis of Ac-Phe-I$_2$Tyr by pepsin is reported[11] to be insensitive to μ.
(11) W. T. Jackson, M. Schlamowitz, and A. Shaw, *Biochemistry*, **4**, 1537 (1965).
(12) R. G. Bates, "Determination of pH," John Wiley and Sons, Inc., New York, N. Y., 1964, Chapters 4 and 5.

Table I. Hydrolysis of N-Acetyl-L-phenylalanyl-L-tyrosine by Pepsin at 35° in 3% Methanol[a]

No.	pH and buffer[b]	$[S]_0 \times 10^4$, M	$[E]_0 \times 10^5$, M	Pts[c]	$K_0 \times 10^3$, M	$k_0 \times 10^2$, sec^{-1}
1	0.50	5.16–11.6	1.43	10	2.05 ± 0.76	1.09 ± 0.28
2	0.70	4.03–12.1	1.60	11	6.05 ± 1.94	4.18 ± 1.52
3	1.11P	1.93–11.6	1.42	13	2.01 ± 0.35	2.70 ± 0.33
4	1.40P	2.66–12.9	1.40	26	2.23 ± 0.28	5.10 ± 0.47
5	1.50P	1.93–15.5	1.36	12	1.69 ± 0.14	3.85 ± 0.20
6	1.75P	2.66–12.9	1.34	9	2.56 ± 0.60	5.63 ± 1.00
7	1.95P[d]	1.16–13.6	1.46	24	1.43 ± 0.13	3.70 ± 0.38
8	2.05P[d]	1.17–12.9	1.36	18	1.15 ± 0.15	3.47 ± 0.28
9	2.25P	2.66–12.9	1.45	18	1.51 ± 0.18	3.77 ± 0.29
10	2.60P	2.74–12.9	1.40	18	1.87 ± 0.24	4.12 ± 0.38
11	2.80P	2.66–12.9	1.44	20	1.64 ± 0.22	3.11 ± 0.29
12	3.15P	4.09–12.9	1.40	15	2.00 ± 0.31	3.09 ± 0.33
13	3.56T	1.93–15.5	1.40	15	9.23 ± 3.27	7.50 ± 2.44
14	3.60T	2.42–15.1	1.96	12	9.13 ± 1.62	9.38 ± 1.58
15	3.90A	3.09–15.5	1.98	17	5.87 ± 3.91	1.45 ± 0.74
16	4.06T	4.03–15.1	2.39	10	−19.2 ± 44.5	−6.14 ± 10.17

[a] See text for details. [b] No letter = hydrochloric acid, P = phosphate, T = tartrate, A = acetate, and C = citrate buffer. [c] Number of points in the Lineweaver–Burk plots. [d] These values differ from those in ref 3 because a weighted least-squares treatment was not used previously.

and products are all much lower than they are at 237 mμ. Runs routinely were observed for 27–60 min.

Buffers Used. A brief series of experiments on the hydrolysis of Ac-Phe-Trp demonstrated that a consistent set of data throughout the pH range studied could be obtained with the following buffers: pH <1, hydrochloric acid; 1 < pH < 3.15, phosphate; 3.5 < pH < 4.7, tartrate; pH 5.0, citrate; pH 5.4, phosphate. Molecular chloroacetic and acetic acids appeared to inhibit the reaction. Some data for hydrolyses in acetic acid buffers are included in the appropriate tables and figures but were ignored in all calculations for Table IV. The tendency of the data for runs in acetic acid to give values for k_0/K_0 lower than those obtained with the preferred buffers is noticeable in Figures 1 and 4.

Lineweaver–Burk Plots. The initial velocity, v_0, of each kinetic run was determined by two procedures. First, v_0 was estimated either visually or by the polynomial method of Booman and Niemann,[13] depending upon whether the OD *vs.* time curve was straight or noticeably curved in the first 15–20 min. In addition, a first-order plot of the points of the run was made and from that portion of the run which adhered to the first-order law the first-order rate constant k and thus $v_0 = k[S]_0$ was extracted.[3,14] Weighted least-squares analysis[15] of the Lineweaver–Burk plots of $1/v_0$ *vs.* $1/[S]_0$ provided a visual-polynomial and a first-order determination of the k_0 and K_0 of eq 1 and 2. Agreement between the results of the two methods was generally excellent. For each substrate only one set of data has been reported. However, the analyses described in the Appendix were carried through on both sets

$$E + S \underset{k_y}{\overset{k_x}{\rightleftharpoons}} ES \xrightarrow{k_0} E + \text{products} \qquad (1)$$

$$v = k_0[E]_0[S]/(K_0 + [S]) \qquad K_0 = (k_y + k_0)/k_x \qquad (2)$$

of data for each compound, and in all three cases the results obtained from the two calculations agreed within experimental error.

All computations were performed on the IBM 1620 or CDC 3600 computers of the University of Massachusetts.

Hydrolysis of Ac-Phe-Tyr. Table I. For nearly every pH $\Delta\epsilon$ was both calculated ($\Delta\epsilon_c$) from the ϵ's of Ac-Phe-Tyr, Ac-Phe, and Tyr and measured directly ($\Delta\epsilon_0$) by allowing reaction mixtures to come to equilibrium ("infinity" mixtures, characterized by no further change in OD for 0.5–2 hr). The two methods always agreed within 5%, thus establishing both the purity of the substrate and the (predominant) nature of the reaction products.

Trial kinetic runs obeyed the first-order law up to 60% reaction in some instances. The satisfactory nature of the first-order plots and the knowledge that the polynomial method heavily emphasizes the less reliable early points of a run led us to prefer the first-order results of Table I as the basis for subsequent calculations.[16] The sole difficulty encountered was in runs of pH <1, where curves obtained from the spectrophotometer were irregular for the first 10 or 15 min of hydrolysis. Addition of methanol to an enzyme solution of pH <1 in a blank reaction caused an irreproducible slow decrease in OD, which stopped after 10–15 min. Since hydrolyses at these pH's were very slow and should be zero order for at least 60 min, we took the slope of the experimental OD–time curve after 15 min as a measure of v_0.

Hydrolysis of Ac-Phe-Trp. Table II. The $\Delta\epsilon_0$ and $\Delta\epsilon_c$ (from the ϵ's of Ac-Phe-Trp, Ac-Phe, and Trp) always agreed within 5%. Runs adhered to first-order kinetics for up to 90% reaction in some instances. The first-order results were consequently again preferred as the basis for further analysis. The spectrophotometric behavior at 294 mμ of runs at pH <1 was perfectly normal.

Hydrolysis of Ac-Phe-TyrNH$_2$. Table III. The calculated $\Delta\epsilon$ for reaction of this substrate (based on the ϵ's of Ac-Phe-TyrNH$_2$, Ac-Phe, and TyrNH$_2$) was 360–420. It is slightly pH dependent because of the decrease in ϵ of AcPhe as ionization of the terminal carboxyl group occurs. The $\Delta\epsilon_0$ was not in good agreement with $\Delta\epsilon_c$, but was consistently too small. For example, $\Delta\epsilon_0/\Delta\epsilon_c$ was 0.75, 0.83, and 0.73 in 0.2 M hydrochloric acid, pH 3 phosphate, and pH 4.25 tartrate, respectively. The *observed* $\Delta\epsilon$ has been used in all calculations for Table III except entries 55 and 56, for which it was not available.

Much effort was expended in futile attempts to account for the discrepancy between $\Delta\epsilon_c$ and $\Delta\epsilon_0$. At pH <1.2, $\Delta\epsilon_c$ was in close agreement with $\Delta\epsilon$ calculated and observed for Ac-Phe-Tyr, as might be expected. This suggests that $\Delta\epsilon_c$ for Ac-Phe-TyrNH$_2$ is correct and that something has gone awry in the determination of $\Delta\epsilon_0$. But what? And why only with Ac-Phe-TyrNH$_2$? When a series of reaction mixtures in the same buffer but of differing $[S]_0$ were allowed to come to equilibrium, there appeared to be a trend toward greater $\Delta\epsilon_0$ with lower $[S]_0$, but the effect was barely beyond the experimental error of the determinations. Incubation of Ac-Phe and TyrNH$_2$ with pepsin for several hours produced no detectable change in OD of the solutions. Chromatographic experiments described below revealed that infinity reaction mixtures contained only the expected[17] products and no unreacted substrate. For example, cleavage of the terminal CO–NH$_2$ bond of either

(13) K. A. Booman and C. Niemann, *J. Am. Chem. Soc.*, 78, 3642 (1956).

(14) An analysis of how exactly first order the kinetics of the hydrolysis of Ac-Phe-Tyr are has recently appeared.[7] We attach no theoretical significance to first-order kinetics but merely use them, where appropriate, as a tool for obtaining v_0.

(15) G. N. Wilkinson, *Biochem. J.*, 80, 324 (1961).

(16) According to A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed, John Wiley and Sons, Inc., New York, N. Y., 1961, p 49, the most accurate points for a first-order reaction are found at ≃two-thirds of complete reaction.

(17) Ac-Phe-TyrOMe[18] and several histidyl-containing tripeptide derivatives with blocked terminal carboxyl group[19] are cleaved as expected by pepsin, between the two aromatic amino acid residues.

(18) G. E. Clement and S. L. Snyder, *J. Am. Chem. Soc.*, 88, 5338 (1966).

(19) K. Inouye, I. M. Voynick, G. R. Delpierre, and J. S. Fruton, *Biochemistry*, 5, 2473 (1966).

Table II. Hydrolysis of N-Acetyl-L-phenylalanyl-L-tryptophan by Pepsin at 35° in 3% Methanol[a]

No.	pH and buffer	$[S]_0 \times 10^5$, M	$[E]_0 \times 10^5$, M	Pts	$K_0 \times 10^4$, M	$k_0 \times 10^2$, sec^{-1}
17	0.30	7.32–58.6	1.38	12	18.1 ± 2.6	1.18 ± 0.15
18	0.50	7.41–51.9	2.04	11	10.9 ± 1.8	1.35 ± 0.18
19	0.70	12.2–61.1	2.02	10	17.1 ± 2.1	2.64 ± 0.26
20	1.11P	4.72–51.9	1.56	15	9.3 ± 0.7	2.11 ± 0.12
21	1.50P	4.72–51.9	1.49	13	10.1 ± 0.8	5.24 ± 0.31
22	1.89P	5.71–50.1	1.43	15	9.6 ± 1.5	6.38 ± 0.75
23	1.95P	5.97–59.7	1.42	10	8.2 ± 1.0	5.70 ± 0.47
24	1.96P	7.41–51.9	1.47	11	7.0 ± 0.4	5.24 ± 0.20
25	2.50P	4.72–51.9	1.46	15	6.6 ± 0.4	5.05 ± 0.20
26	2.94P	5.71–50.1	1.43	16	7.6 ± 1.0	4.51 ± 0.54
27	3.01P	4.72–51.9	1.87	14	12.3 ± 1.0	6.14 ± 0.40
28	3.54A	4.72–63.1	1.75	14	29.1 ± 5.5	4.18 ± 0.69
29	3.55T	5.33–58.6	1.48	15	13.1 ± 1.2	3.64 ± 0.25
30	3.56T	4.72–63.1	1.76	13	10.3 ± 1.1	3.06 ± 0.23
31	3.73T	5.33–58.6	1.40	16	17.1 ± 1.5	4.03 ± 0.55
32	3.86A	9.76–58.6	1.48	11	45.2 ± 15.7	2.96 ± 1.05
33	3.90A	7.41–63.1	2.34	14	33.4 ± 11.6	2.77 ± 0.85
34	4.06T	7.41–63.1	2.25	13	24.1 ± 2.5	2.24 ± 0.20
35	4.20T	12.2–61.1	1.96	10	53.9 ± 21.9	3.82 ± 1.54
36	4.26A	10.4–63.1	2.31	11	27.6 ± 5.3	0.94 ± 0.17

[a] Footnotes *a–c* of Table I apply where appropriate.

Table III. Hydrolysis of N-Acetyl-L-phenylalanyl-L-tyrosine Amide by Pepsin at 35° in 3% Methanol[a]

No.	pH and buffer	$[S]_0 \times 10^4$, M	$[E]_0 \times 10^6$, M	Pts	$K_0 \times 10^3$, M	$k_0 \times 10^2$, sec^{-1}
37	0.50	1.43–7.14	2.20	11	1.67 ± 0.50	0.99 ± 0.29
38	0.70	1.96–7.28	2.16	9	2.28 ± 0.66	1.64 ± 0.44
39	1.11P	1.43–7.14	1.82	23	2.34 ± 0.56	3.48 ± 0.92
40	1.50P	1.46–7.14	1.42	13	2.36 ± 0.50	5.05 ± 0.94
41	1.93P	1.17–7.28	1.45	10	31.6 ± 54.4	93 ± 220
42	1.95P	0.85–8.45	1.41	20	2.41 ± 0.38	7.80 ± 1.07
43	2.50P	1.46–7.14	1.41	10	1.88 ± 0.33	5.59 ± 0.81
44	3.15P	0.87–8.49	1.40	19	2.33 ± 0.39	7.02 ± 1.04
45	3.54A	1.46–7.14	1.46	12	4.79 ± 1.17	4.73 ± 1.12
46	3.67T	1.80–8.49	1.41	13	2.73 ± 0.81	5.46 ± 1.42
47	3.91A	1.43–7.14	1.49	11	8.05 ± 3.68	7.32 ± 3.95
48	4.06T	1.54–7.71	1.41	14	28 ± 32	50 ± 80
49	4.20T	1.17–7.28	2.15	12	2.54 ± 0.69	4.70 ± 1.11
50	4.26A	1.43–7.14	1.39	16	2.55 ± 0.85	2.47 ± 0.70
51	4.68T	1.46–7.14	1.81	14	1.87 ± 0.47	1.82 ± 0.39
52	4.70T	1.71–8.49	1.42	14	1.68 ± 0.21	2.07 ± 0.20
53	4.87A	1.85–8.49	1.45	12	−6.5 ± 2.8	−2.3 ± 1.4
54	4.97C	1.96–7.28	2.17	8	4.47 ± 2.63	3.59 ± 1.95
55	5.43P	3.01–8.49	1.42	9	1.94 ± 0.89	0.76 ± 0.29
56	5.44P	3.87–7.14	2.21	6	0.72 ± 0.39	0.24 ± 0.08

[a] Footnotes *a–c* of Table I apply where appropriate.

Ac-Phe-TyrNH$_2$ or TyrNH$_2$ is ruled out by the failure to detect either Ac-Phe-Tyr or Tyr and also by the knowledge that formation of free Tyr would have caused $\Delta\epsilon_0$ to exceed $\Delta\epsilon_c$. Finally, the ninhydrin color produced by several infinity reaction mixtures and by appropriate solutions of the expected reaction products were in excellent agreement. The experimental artifact (?) apparently arises only in the spectrophotometric method.

Since trial kinetic runs did not give as good first-order plots as were obtained with the other substrates, the data derived by the polynomial method were selected for Table III and subsequent analysis. The difficulty in runs at pH <1 mentioned for Ac-Phe-Tyr also appeared with Ac-Phe-TyrNH$_2$.

Chromatographic Analyses. The purity of the substrates and the nature of the products of the enzymatic hydrolyses were examined with the aid of thin layer chromatography. Techniques described by Stahl[20] were used. The plates were coated with silica gel G and were activated for 30 min at 110°. Either butanol–acetic acid–water (4:1:1) or methyl acetate–isopropyl alcohol–water–ammonia (45:35:15:5) was used as the solvent system. The following sprays were employed: (a) for tryptophan residues, Stahl No. 64 (formaldehyde–hydrochloric acid) followed by 50% aqueous morpholine to enhance the fluorescence; (b) for tyrosine residues, either Stahl No. 38 (diazotized *p*-nitroaniline) or diazotized sulfanilic acid;[19] (c) ninhydrin.

The three substrates were chromatographically homogeneous. No unreacted substrate could ever be detected in an infinity reaction mixture. The predominant reaction products in all cases were as expected for cleavage between the two aromatic amino acid fragments. Other products were as follows.

(a) **From Ac-Phe-Tyr.** This was not examined in detail since it is known[21] to give the transpeptidation product Tyr-Tyr. Considerable amounts of Tyr-Tyr were detected in a reaction run in pH 4.25 acetate buffer.

(b) **From Ac-Phe-Trp.** Small amount of Trp-Trp formed in pH 2 phosphate and somewhat more in pH 4.25 acetate. For the latter reaction it was estimated that less than 7% of the amount of Trp-Trp theoretically possible had actually been produced.

(c) **From Ac-Phe-TyrNH$_2$.** We were unable to detect Ac-Phe-Tyr, Tyr-TyrNH$_2$, Phe-Tyr or Phe-TyrNH$_2$.

(20) E. Stahl, "Thin-Layer Chromatography," Academic Press Inc., New York, N. Y., 1965.

(21) F. A. Bovey and S. S. Yanari in "The Enzymes," Vol. 4, 2nd ed, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press Inc. New York, N. Y., 1960, Chapter 4.

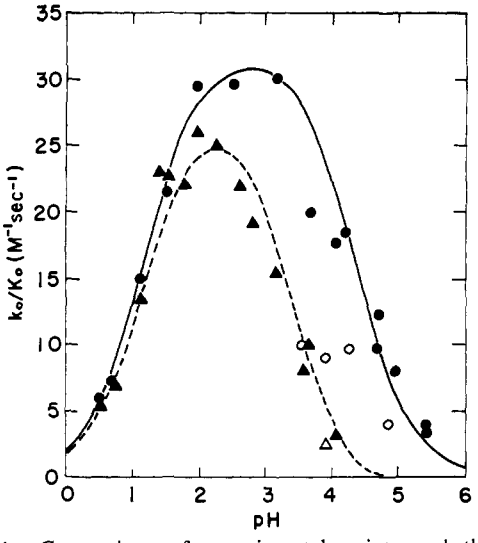


Figure 1. Comparison of experimental points and theoretical curves for the pH dependence of k_0/K_0 in the hydrolysis of Ac-Phe-Tyr (▲ and △) and Ac-Phe-TyrNH$_2$ (● and ○) by pepsin. As in all subsequent figures, unfilled points refer to data obtained in acetic acid buffers. The theoretical curve for Ac-Phe-Tyr (----) was calculated from eq 8 and the data of Table IV; that for Ac-Phe-TyrNH$_2$ (——) from eq 11 and the same table.

High voltage electrophoretic examination of Ac-Phe-Tyr and Ac-Phe-TyrNH$_2$ and their infinity reaction mixtures supported all the above conclusions.

Test for Enzyme Denaturation. Two kinds of tests were performed. First, pepsin was preincubated in buffers of extreme pH (0.3 and 0.5 *M* hydrochloric acid and pH 5.4 phosphate) for 60 min and the rate of hydrolysis of added substrate compared to that in a duplicate experiment in which the preincubation was omitted. In all cases the two rates were identical within experimental error. This rules out significant denaturation of pepsin in the time interval between the initiation and termination of a kinetic run. Both instantaneous and slow denaturation of the enzyme was shown to be negligible in 0.5 *M* hydrochloric acid and pH 5.4 phosphate in a second set of controls. Pepsin was incubated in the buffer for 30 min, the pH was adjusted to 2–3 (exact value unimportant), and the rate of hydrolysis of added Ac-Phe-Trp determined and found to be identical with that in a solution where the pH adjustment was made prior to enzyme addition.[22]

Kinetic Schemes. General Treatment for Ac-Phe-Tyr and Ac-Phe-Trp. A complete discussion of the influence of pH on the rate of hydrolysis of these substrates by pepsin must consider the ionization of both substrate and enzyme, since each of these amides has a terminal carboxyl group of p$K_a \simeq 3.4$. Frieden[23] has described a kinetic scheme which meets this condition and which satisfactorily correlates all our experimental findings. Alternative methods of analysis of the data have not been examined.

The treatment for an ionizable substrate assumes that both forms of the substrate can undergo reaction (eq 3 and 4) and defines acid

$$HE + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HES \xrightarrow{k_3} HE + \text{products} \qquad (3)$$

$$K_{ES} = (k_2 + k_3)/k_1$$

$$HE + SH \underset{k_5}{\overset{k_4}{\rightleftharpoons}} HESH \xrightarrow{k_6} HE + \text{products} \qquad (4)$$

$$K_{ESH} = (k_5 + k_6)/k_4$$

dissociation constants for substrate, enzyme, and two enzyme–substrate complexes according to (5). Solution of the appropriate

$$SH \overset{K_S}{\rightleftharpoons} S \qquad H_2E \overset{K_{1E}}{\rightleftharpoons} HE \overset{K_{2E}}{\rightleftharpoons} E$$

$$H_2ESH \overset{K_{1ESH}}{\rightleftharpoons} HESH \overset{K_{2ESH}}{\rightleftharpoons} ESH \qquad H_2ES \overset{K_{1ES}}{\rightleftharpoons} HES \overset{K_{2ES}}{\rightleftharpoons} ES \qquad (5)$$

(22) The remarkable stability of pepsin in strongly acidic solutions was established by J. H. Northrup, *J. Gen. Physiol.*, **16**, 33 (1932).

(23) C. Frieden, *J. Am. Chem. Soc.*, **80**, 6519 (1958).

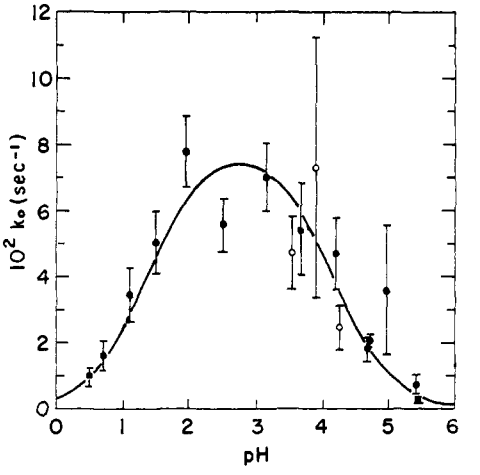


Figure 2. Comparison of experimental points and the theoretical curve (based on eq 10 and Table IV) for the pH dependence of k_0 in the hydrolysis of Ac-Phe-TyrNH$_2$ by pepsin.

steady-state equations yields eq 13–15 of the Appendix, where $f_A = (1 + [H^+]/K_{1A} + K_{2A}/[H^+])$ for the acid H_2A. When preliminary evaluation of the data of Tables I–III suggested that the anionic forms (S) of Ac-Phe-Tyr and Ac-Phe-Trp do not undergo hydrolysis readily, data analysis proceeded with a simplified set of equations, 6–8, derived from 13–15 by introducing the condition

$$K_0 = K_{ESH}(f_E/f_{ESH})(1 + K_S/[H^+]) \qquad (6)$$

$$k_0 = k_6/f_{ESH} \qquad (7)$$

$$k_0/K_0 = (k_6/K_{ESH})(1/f_E)(1/(1 + K_S/[H]^+)) \qquad (8)$$

$1/K_{ES} = 0$. This is equivalent to assuming that S does not bind to pepsin. Further discussion of this point may be found in the Appendix.

Kinetic Scheme for Ac-Phe-TyrNH$_2$. The treatment is unexceptional since substrate ionization is not a factor. Equations 9–11

$$K_0 = K_{ESH}/(f_E/f_{ESH}) \qquad (9)$$

$$k_0 = k_6/f_{ESH} \qquad (10)$$

$$k_0/K_0 = (k_6/K_{ESH})(1/f_E) \qquad (11)$$

define the experimental parameters in terms of the rate constants of eq 4. Equations 7 and 10 for k_0 are identical and independent of substrate ionization while 6 and 8 are 9 and 11, respectively, modified for substrate ionization.

Source of Data of Table IV. The appropriate equation from the group 6–11 was used to obtain a "best" calculated curve to fit the experimental values for each parameter by a process described in the Appendix. The calculated curves and experimental points are shown in Figures 1–8.[24] The k_0/K_0 plot for Ac-Phe-TyrNH$_2$ provided the only determination of pK_{2E} since the effect of changing pK_{2E} from 4 to 5 on the theoretical k_0/K_0 (or K_0) curves for Ac-Phe-Tyr and Ac-Phe-Trp was undetectable. The term reflecting substrate ionization of these last two substrates completely overwhelms the influence of pK_{2E} in the pH region investigated.

Discussion

Five points, for which experimental evidence has been cited above, should be kept in mind when evaluating the present results.

(a) The predominant net reaction for all three substrates is hydrolytic cleavage of the amide bond joining the two aromatic amino acid residues. The kinetic data must reflect primarily the rate of this cleavage.

(b) The maximum $[S]_0$ attainable was always somewhat less than K_0. Since k_0/K_0 depends on the slope,

(24) Entries 41, 48, and 53 of Table III are omitted from Figures 2 and 3 and entry 16 of Table I is omitted from Figures 7 and 8.

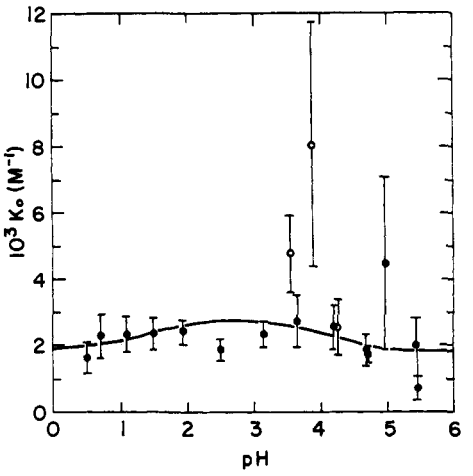


Figure 3. Comparison of experimental points and the theoretical curve (based on eq 9 and Table IV) for the pH dependence of K_0 in the hydrolysis of Ac-Phe-TyrNH$_2$ by pepsin.

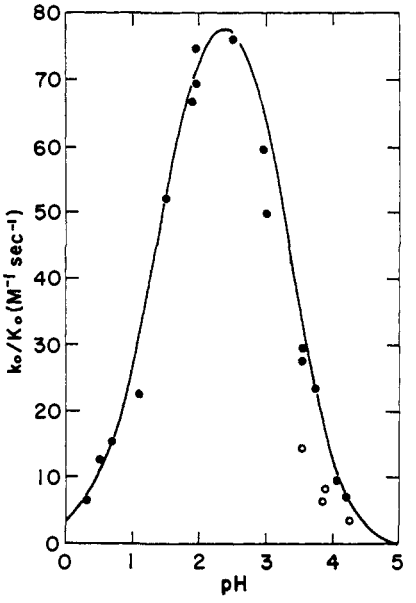


Figure 4. Comparison of experimental points and the theoretical curve (based on eq 8 and Table IV) for the pH dependence of k_0/K_0 in the hydrolysis of Ac-Phe-Trp by pepsin.

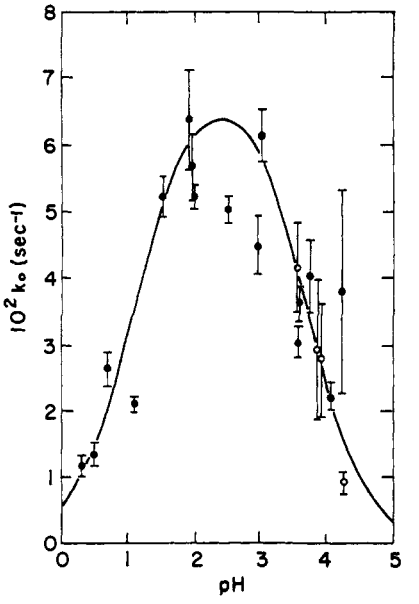


Figure 5. Comparison of experimental points and the theoretical curve (based on eq 7 and Table IV) for the pH dependence of k_0 in the hydrolysis of Ac-Phe-Trp by pepsin.

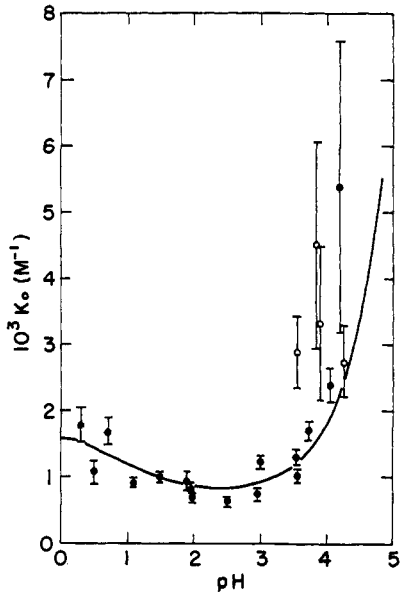


Figure 6. Comparison of experimental points and the theoretical curve (based on eq 6 and Table IV) for the pH dependence of k_0 in the hydrolysis of Ac-Phe-Trp by pepsin.

but k_0 and K_0 on the intercepts of the Lineweaver–Burk plots, quantities derived from k_0/K_0 are more reliable than those derived from k_0 or K_0. The plot of k_0 *vs.* pH for Ac-Phe-Tyr was especially poor.

(c) The hydrolysis of Ac-Phe-Trp provided the most accurate data for pH <1.

(d) The transition from hydrochloric acid to phosphate to tartrate buffer in passing from low to high pH introduced no perturbing buffer effects.

(e) Pepsin is stable throughout the pH range investigated for the duration of time required for measuring v_0.

The following comments summarize the major conclusions that can be drawn from Figures 1–8 and Table IV.

(a) The k_0/K_0–pH curve for Ac-Phe-TyrNH$_2$ establishes that two kinetically important ionizations occur on pepsin.

(b) Two kinetically important ionizations of the enzyme–substrate complexes are revealed by all three k_0–pH curves.

(c) The preferred value for pK_{1E} is 1.40, from the Ac-Phe-Trp data.

(d) The single value for pK_{2E} should be reliable.

(e) No importance should be attached to the differences between pK_{1E} and pK_{1ESH}, but it may be that pK_{2ESH} for Ac-Phe-Trp is significantly less than pK_{2E}. Since none of the determinations of the pK_a's for the complexes can be regarded as highly accurate, average values of pK_{1ESH} = 1.17 and pK_{2ESH} = 3.85 are probably preferable.

(f) Although the kinetic parameters for Ac-Phe-Tyr and Ac-Phe-TryNH$_2$ are similar, the rates of hydrolysis of the two substrates at pH >2 are very different (see

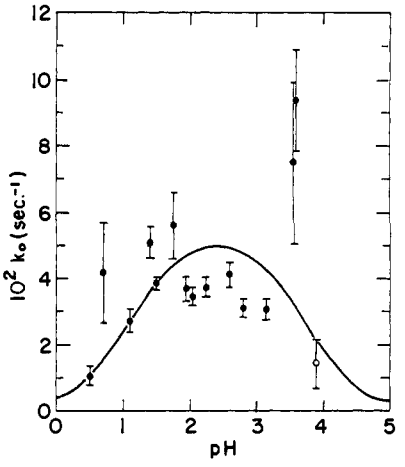


Figure 7. Comparison of experimental points and the theoretical curve (based on eq 7 and Table IV) for the pH dependence of k_0 in the hydrolysis of Ac-Phe-Tyr by pepsin.

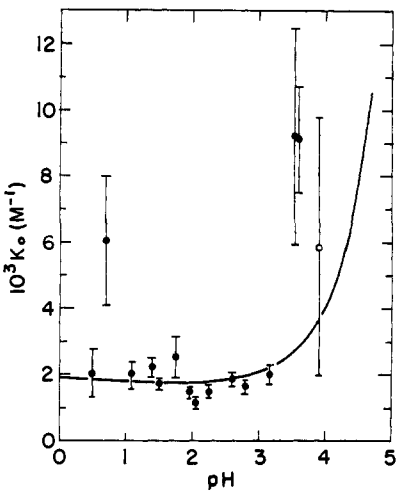


Figure 8. Comparison of experimental points and the theoretical curve (based on eq 6 and Table IV) for the pH dependence of K_0 in the hydrolysis of Ac-Phe-Tyr by pepsin.

Figure 1). The difference stems primarily from the ability of Ac-Phe-Tyr to form a carboxylate anion which is hydrolyzed slowly if at all by the enzyme (*cf.* ref 19).

Table IV. Summary of Kinetic and Ionization Constants Characterizing the Hydrolysis of Three Synthetic Substrates by Pepsin at 35° in 3% Methanol[a]

Quantity[b]	Compound		
	Ac-Phe-TyrNH$_2$	Ac-Phe-Tyr	Ac-Phe-Trp
$10^2 \times k_6$, sec^{-1}	8.0 ± 0.5	5.5 ± 0.5	7.0 ± 0.5
$10^3 \times K_{ESH}$, M	2.8 ± 0.3	1.7 ± 0.3	0.75 ± 0.15
k_6/K_{ESH}, M^{-1} sec^{-1}	32.5 ± 2.5[c] 29[d]	29 ± 1[c] 32[d]	95 ± 5[c] 93[d]
pK_{1E}	1.17 ± 0.05	1.17 ± 0.05	1.40 ± 0.10
pK_{2E}	4.35 ± 0.10	(4.35)[e]	(4.35)[e]
pK_{1ESH}	1.35 ± 0.10	1.12 ± 0.10	1.05 ± 0.05
pK_{2ESH}	4.15 ± 0.15	3.70 ± 0.15	3.70 ± 0.10
pK_S		3.40 ± 0.05	3.40 ± 0.05

[a] The methods of deriving the data of this table are given in detail in the text. [b] Defined by eq 4 and 5. [c] Preferred value, from fit of a k_0/K_0 *vs.* pH curve. [d] The quotient obtained by dividing k_6 of line 1 by K_{ESH} of line 2 of this table. [e] Assumed.

Our experiments clearly reveal a serious shortcoming of the spectrophotometric method as applied to pepsin,[3] at least for the substrates thus far employed by ourselves or by Clement and Snyder.[18] The inability to attain sufficiently great initial substrate concentrations combines with the relatively poor binding of the substrates to the enzyme to render impossible accurate determinations of k_0 and K_0. Where the spectrophotometric method can provide the desired information, Ac-Phe-Trp is the compound of choice both by virtue of its higher reactivity[25] and of the ease of following its hydrolysis.

The results in this paper strictly refer only to the properties of commerical pepsin. Several reports[7,19,26] substantiate the belief that a highly homogeneous preparation of enzyme[26] would not exhibit markedly different kinetic behavior.

(25) Inouye, *et al.*,[19] found that replacement of a carboxyl-terminal TyrOEt with TrpOEt led to increased susceptibility to hydrolysis by pepsin. The effect was exclusively upon k_0 in their case.

(26) T. G. Rajagopalan, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **241**, 4940 (1966).

Comparison to Other Investigations. There are only three previous reports on pepsin kinetics to which our data can be compared. Clement and Snyder[18] spectrophotometrically determined a k_0–pH profile for the hydrolysis of Ac-Phe-TyrOMe at 25° in 3.2% dioxane and obtained pK_{1ESH} = 1.62 and pK_{2ESH} = 3.48.[27] Jackson, Schlamowitz, and Shaw[11] utilized the ninhydrin method to obtain k_0 for Ac-Phe-I$_2$Tyr at pH 2 and 4.5 at 37° in pure water. From their values for k_0/K_0 and k_0 we estimate[28] pK_{2E} = 4.5 and pK_{2ESH} = 4.15. From their measured $K_0 = 7.5 \times 10^{-5}$ M at pH 2 and our pK's we calculate[28] $K_0 = 61 \times 10^{-5}$ M at pH 4.5, *vs.* the observed 88×10^{-5} M. The data of these two groups and of ourselves is in reasonably good agreement.

Very recently Lutsenko, Ginodman, and Orekhovich[30] have described pH–rate profiles for the hydrolysis of Ac-Phe-Tyr (in water) and Ac-Phe-TyrOEt (in 10% ethanol). They obtained pK_{2ESH} = 3.8 for the former and 4.2 for the latter, in excellent accord with our work. However, the identical k_0/K_0–pH curves for the two substrates, and the value pK_{2E} = 3.0 derived therefrom, must be in error. The curve for Ac-Phe-Tyr can only yield pK_S while competitive inhibition by ethanol[31] may have shifted the k_0/K_0–pH curve for Ac-Phe-TyrOEt in some undetermined manner.[32]

(27) Since only a preliminary report on Ac-Phe-TyrOMe has yet appeared, detailed comparisons with Ac-Phe-TyrNH$_2$ cannot be made and it is impossible to tell if the data for Ac-Phe-TyrOMe were significantly influenced by the acetic acid buffers employed.

(28) The calculations were based on eq 6–8 and pK_S = 3.20,[29] pK_{1E} = 1.40, pK_{1ESH} = 1.17. The values for k_0 and K_0 at pH 2, which are not influenced by pK_{2E} or pK_{2ESH}, served to establish k_0/K_{ESH} k_6, and K_{ESH}. From k_0/K_0 and k_0 at pH 4.5, pK_{2E} and pK_{2ESH} were then estimated. K_0 at pH 4.5 was calculated from K_{ESH} and the required pK's (pK_{2E} = 4.35, pK_{2ESH} = 3.85).

(29) (a) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p 487, give the pK_a of I$_2$Tyr as 2.12 and that of Tyr as 2.20 in water at 25°; (b) E. Zeffren and E. T. Kaiser, *J. Am. Chem. Soc.*, **88**, 2139 (1966), report that the pK_S of Ac-Phe-I$_2$Tyr is 0.3 unit lower than that of Ac-Phe-Tyr in 50% methanol at room temperature.

(30) N. G. Lutsenko, L. M. Ginodman, and V. N. Orekhovich, *Biokhimiya*, **32**, 223 (1967).

(31) J. Tang, *J. Biol. Chem.*, **240**, 3810 (1965).

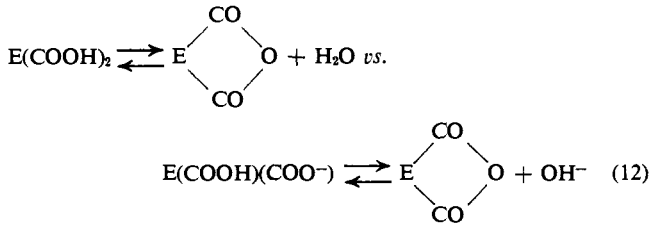
(32) In contrast to our results, E. Zeffren and E. T. Kaiser, *J. Am. Chem. Soc.*, **89**, 4204 (1967), have obtained pK_{2E} = 2.67 from the k_0/K_0–pH curve for the hydrolysis of N-acetyl-L-phenylalanyl-L-dibromotyrosine. Their contention that this curve reflects pK_{2E} and not pK_S relies on a measured pK_S = 4.06, a value which appears in-

All other investigations on the effect of pH upon the hydrolysis of synthetic or protein substrates[33] by pepsin have involved the determination of pH–activity curves, the meaning of which is highly ambiguous. Observation that the curves for the hydrolysis of Ac-Phe-I_2Tyr[11] and Ac-Phe-Tyr[19] exhibit a decrease in rate dependent on a group of $pK_a \simeq 3.2$ and 3.4, respectively, probably reflects the intrusion of the appropriate pK_S's. The pH–activity curves for histidyl-[19] or glutamyl-containing[6,34,35] peptides almost certainly are perturbed by the presence of the charged groups of the substrates. Detailed analysis of the kinetics of the hydrolysis of such substrates should be quite challenging.

Identification of Kinetically Important Groups. An acid and a base essential to pepsin action are detected kinetically on both the free enzyme and the enzyme–substrate complex. The acid of $pK_a = 4.35$ is most probably an undissociated carboxyl group. The only obvious candidate for the base of $pK_a = 1.4$, the phosphate of pepsin,[36] is eliminated by Perlmann's demonstration[37] that removal of this phosphate leaves intact pepsin's activity toward both hemoglobin and Ac-Phe-I_2Tyr. Perhaps the base is the anion of a highly acidic carboxylic acid. Bender and Kézdy[38] have suggested a mechanism for pepsin action, discussed below, which postulates the presence of two catalytically active carboxyl groups on the enzyme. Either the carboxyl of $pK_a = 4.35$ or that of $pK_a = 1.4$ (if it is a carboxyl) may be the β-carboxyl of aspartic acid which is inactivated by *p*-bromophenacyl bromide.[39,40]

The speculation that a carboxylic acid of $pK_a = 1.4$ is involved in the catalytic reaction has further implications if one assumes that the cause of its high acidity is a positive charged environment. Pepsin possesses only five formal positive charges, those on N-terminal isoleucine, on a single histidine, and on the two arginines and sole lysine which are found among the 20 amino acids of the C-terminal peptide.[26,41,42] At least one of these three parts of the protein molecule would then have to lie near this carboxyl group.

Mechanistic Considerations. Bender and Kézdy[40] have suggested the only semidetailed mechanism for pepsin action of which are are aware, and Clement and Snyder[18] have supported their proposal. The principal feature of the mechanism is reversible formation of an anhydride on the enzyme and subsequent slow reaction of the anhydride with substrate. The proposal is not compatible with the present data, for the anhydride is kinetically equivalent to H_2ESH and not to HESH (eq 12). We briefly explored the possibility that our data



for Ac-Phe-TyrNH_2 could be explained by a scheme where three ionizations of the enzyme were allowed (H_3E and H_3ESH), H_2ESH^- or the corresponding anhydride was assumed to be the reactive species, $pK_{2E} = pK_{3E}$, and $pK_{2ESH} = pK_{3ESH}$. The scheme predicts curves for k_0/K_0 and k_0 which have the same general form as those derived from eq 10 and 11 but which decrease much more rapidly on the alkaline side of the optimum, especially for $pH > pK_{2E}, pK_{2ESH}$. The fit to the experimental data was not as good as for the preferred treatment. However, we cannot state unequivocally that a satisfactory fit could not be achieved with such a scheme, given a suitable set of pK_a's. Our data are of course also compatible with rate-limiting formation of an anhydride by the enzyme–substrate complex, the speed of which depends upon the nature of the substrate.

We are unable to offer a mechanism for pepsin action which satisfactorily explains the many observations on the mode of action of this enzyme. Particularly vexing is the problem of rationalizing both acyl and amino activation, and we plan to reexamine the evidence in this area in the near future.

Conclusions on the mode of action of hog pepsin based on studies with synthetic substrates are hopefully applicable to proteolytic reactions of the enzyme. In contrast, pepsin C[43] and chicken pepsin[44,45] contain no phosphorus and show essentially no activity toward Ac-Phe-I_2Tyr while exhibiting "normal" activity with hemoglobin.

Meaning of K_0. Several authors have suggested that K_0 represents an equilibrium binding constant for a Michaelis–Menten complex in the hydrolysis of synthetic substrates by pepsin.[7,11,18,46,47] Three kinds of evidence support but do not prove this proposal. First, the inhibitors Ac-D-Phe-I_2Tyr,[11] Ac-dehydroPhe-I_2Tyr,[4] Ac-D-Phe-D-TyrOMe,[18] Z-His-Phe-D-PheOEt,[47] and Z-His-D-Phe-PheOEt[47] have inhibitor dissociation constants, K_I, which are the same as the K_0's of their corresponding all L-amino acid substrates. However, K_I for Ac-D-Phe-Phe or for Ac-Phe-D-Phe differs significantly from K_0 for Ac-Phe-Phe.[4] Second, kinetic data for the hydrolysis of Ac-Phe-Phe, Ac-Phe-Tyr, Ac-Tyr-Phe, and Ac-Tyr-Tyr accord with this view of the meaning of K_0 and do not permit rate-determining breakdown of a covalent complex which contains solely either the acetyl amino acid or C-terminal amino acid fragments of the substrates.[14] The argument is valid only if the substrates have a common rate-determining step. Also,

consistent with both our data and with their own report that the pK_a of acetylphenylalanine is 3.60.

(33) *E.g.*, M. Schlamowitz and L. U. Peterson, *J. Biol. Chem.*, **234**, 3137 (1959).

(34) J. S. Fruton and M. Bergmann, *ibid.*, **127**, 627 (1939).

(35) The position of the optimum in the pH–activity profile for the hydrolysis of Z-Glu-Tyr is apparently[6] a function of $[S]_0$.

(36) W. D. Kamler and J. J. Eiler, *J. Am. Chem. Soc.*, **65**, 2355 (1943), tabulated and discussed the pK_a's of many phosphoric acid derivatives.

(37) G. E. Perlmann, *J. Gen. Physiol.*, **41**, 441 (1958).

(38) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).

(39) B. F. Erlanger, S. M. Vratsanos, N. Wassermann, and A. G. Cooper, *J. Biol. Chem.*, **240**, PC 3447 (1965); *Biochem. Biophys. Res. Commun.*, **23**, 243 (1966).

(40) E. Gross and J. L. Morell, *J. Biol. Chem.*, **241**, 3638 (1966).

(41) T. A. A. Dolpheide, S. Moore, and W. H. Stein, *ibid.*, **242**, 1833 (1967).

(42) The observation of E. B. Ong, Y. Tsang, and G. E. Perlmann, *ibid.*, **241**, 5661 (1966), that tryptic digestion of pepsinogen leads to a loss of potential peptic activity is in accord with this or innumerable other interpretations.

(43) A. P. Ryle and M. P. Hamilton, *Biochem. J.*, **101**, 176 (1966).

(44) Commercial pepsin is derived from hogs.

(45) T. P. Levchuk and V. N. Orekhovich, *Biokhimiya*, **28**, 1004 (1963).

(46) M. S. Silver, *J. Am. Chem. Soc.*, **87**, 1627 (1965).

(47) (a) K. Inouye and J. S. Fruton, *ibid.*, **89**, 187 (1967); (b) *Biochemistry*, **6**, 1765 (1967).

slow breakdown of a covalent complex containing the entire substrate molecules cannot be excluded.[14] Third, we have detected kinetically two functional groups whose ionization affects pepsin activity and which are present in both the enzyme and the enzyme–substrate complex. Any covalent bond between substrate and enzyme in this complex must consequently involve still a third enzymatic functional group, whose ionization on the enzyme occurs outside the pH range investigated. Such groups obviously exist, but a carboxylic acid is not likely to belong in this category.

Acknowledgment. This manuscript was written while M. S. S. enjoyed the hospitality of the Biophysics Department of the Weizmann Institute as a National Science Foundation Senior Postdoctoral Fellow.

Appendix

Kinetic Analysis. Solution of Eq 3–5. The general solution of the steady-state equations yields eq 13–15.

$$K_0 = \frac{K_{ESH}(1 + K_S/[H^+])f_E}{f_{ESH} + f_{ES}(K_S K_{ESH})/([H^+]K_{ES})} \quad (13)$$

$$k_0 = \frac{k_6 + (K_{ESH}K_S k_3)/(K_{ES}[H^+])}{f_{ESH} + (f_{ES}K_S K_{ESH})/([H^+]K_{ES})} \quad (14)$$

$$k_0/K_0 = \frac{k_6 + (K_{ESH}K_S k_3)/(K_{ES}[H^+])}{K_{ESH}(1 + K_S/[H^+])f_E} \quad (15)$$

The condition that the anionic form, S, of the ionizable substrates is unreactive may be introduced in two ways. Approximation I does not allow S to bind to the enzyme and has been discussed previously (eq 6–8). In approximation II, S may bind to the enzyme but HES is not converted to product, $k_3 = 0$, K_0 is given by (13)

$$k_0 = k_6/(f_{ESH} + f_{ES}K_{ESH}K_S/K_{ES}[H^+]) \quad (16)$$

and k_0/K_0 is given by (8).

Determining Best Fit to Experimental Data. Computer programs were written, the outputs of which were a tabulation of values for the desired experimental parameter at intervals of 0.2 pH unit from pH 0 to 6 as calculated from the appropriate equation. The input consisted of trial values for each quantity needed to define the given parameter. The input was varied until the best fit was achieved, as determined by visual comparison of the theoretical curves and the experimental points.[48] Efforts were made to vary the input over a wide range, but obviously all combinations of input information could not be tried for an equation such as (16). The error assigned to each quantity of Table IV in most cases indicates that value of the quantity which gave a visibly poorer calculated curve when all other relevant parameters were held at their best values.

The general sequence of events in analyzing the data for each substrate was as follows.

(a) Fit the k_0/K_0–pH curve; determine pK_{1E}, pK_{2E}, and the preferred value for k_6/K_{ESH} (eq 8 or 11).

(b) Fit the k_0–pH curve; determine pK_{1ESH}, pK_{2ESH}, and k_6 (eq 7 or 10).

(c) Use pK_{1E} and pK_{2E} from and pK_{1ESH} and pK_{2ESH} from b and vary K_{ESH} to fit the K_0–pH curve (eq 6 or 9). The only exception to this general procedure was that for Figure 7 the curve through the amorphous pattern of points was generated by assuming $pK_{1ESH} = 1.12$ and taking pK_{2ESH} equal to pK_{2ESH} found for Ac-Phe-Trp. The agreement between the values for k_6/K_{ESH} determined directly from a and from the quotient of k_6 divided by K_{ESH} (Table IV) indicates the satisfactory nature of the visual comparison procedure.

Consequences of Approximation II. We briefly explored the implications of this scheme, utilizing the experimental data for Ac-Phe-Trp. The calculations required estimating three new parameters, pK_{1ES}, pK_{2ES}, and K_{ES}, and examining the pH dependence of K_0 and k_0. since approximations II and I give an identical expression for k_0/K_0. Values for k_6, K_{ESH}, pK_{1E}, and pK_{2E} were fixed according to Table IV, but pK_{1ESH} and pK_{2ESH} were treated as variables.

When the calculated curves were found to be insensitive to a change of pK_{1ES} from 0.5 to 1.5, pK_{1ES} was fixed at 1.0 and pK_{1ESH} at 1.05. Only pK_{2ES}, pK_{2ESH}, and K_{ES} remained as significant variables. The condition $K_{ES} < K_{ESH}$ led to K_0–pH curves which were qualitatively incorrect, since they did not reproduce the rapid rise in K_0 in the region of substrate ionization (Figure 6). On the other hand, with $K_{ES} > 5 \times 10^{-3}$ M, $pK_{2ESH} = 3.7$, and $pK_{2ES} = 4$, curves could be generated which were indistinguishable from those obtained under approximation I. Binding by the anionic form of the substrate cannot be ruled out from our data, but the neutral species is bound at least five times more strongly than the anionic form.

(48) All these calculations were performed on the shared time computer of Dartmouth College, Hanover, N. H., *via* a teletype console at Amherst College. With this equipment, the trial and error method was convenient and rapid.