to be homogeneous in paper chromatography: R_f(BAW) 0.05, R_f(SBA) 0.61; R_f(BPAW) 0.86, $[\alpha]^{27}D$ −40.0° (*c* 0.5, acetic acid). The yield of tosyl-S-benzylcysteinyllysylasparaginylglycylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester was 21% (0.45 g).

Cysteinyllysylasparaginylglycylglutaminylthreonylasparaginylcysteine (XIII). Tosyl-S-benzylcysteinyllysylasparaginylglycylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester (0.42 g, 0.32 mmol) was dried in a vacuum desiccator for 24 hr over P_2O_5 and NaOH. The material was dissolved in 50 ml of liquid ammonia in a 250-ml, round-bottomed flask with rigid exclusion of moisture. Stirring was accomplished by means of a glass-coated magnetic bar. Small pieces of sodium were added to the solution until a blue color persisted. Ammonium chloride (20 mg) was then added, and the liquid ammonia was allowed to evaporate. The residue was dissolved at room temperature in 2 l. of 0.001 *M* acetic acid which had been boiled and flushed with nitrogen. The pH was carefully adjusted to 6.5 with 0.1 *M* NaOH, and CO_2-free oxygen was bubbled through the solution for 3 hr. At the end of this time, the solution was acidified with 1 ml of glacial acetic acid, and the solvent was removed in a rotary evaporator. The residue was dissolved in 2 ml of 50% acetic acid and chromatographed on a 0.9 × 150 cm column of Sephadex G-25 with 50% acetic acid as eluent. The pattern obtained is shown in Figure 5. The cyclic octapeptide was found to be homogeneous in paper chromatography in three solvent systems: R_f(BAW) 0.00, R_f(SBA) 0.80, R_f(BPAW) 0.47. Quantitative amino acid analysis of an acid hydrolysate of the peptide is given in Table I. The yield of the cyclic octapeptide after chromatography was 36% (0.1 g), $[\alpha]^{27}D$ −82.0° (*c* 0.5, water). Molecular weight by titration was found to be 871 (theoretical value, 866). Electrophoresis on Whatman No. 1 filter paper for 3 hr at 20 V/cm gave a single ninhydrin-positive spot at pH 5.6 with pyridine–acetate buffer and at pH 7.9 with *N*-ethylmorpholine–acetate. Digestion with leucine aminopeptidase gave quantitative recovery of free amino acids, indicating that no racemized amino acid residues were in the peptide (Table II).

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The pH Dependence of the Pepsin-Catalyzed Hydrolysis of Neutral Dipeptides[1a]

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Abstract: The pH dependences of the kinetic constants for the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester were determined in the pH range 1.05–5.05 at 25° using twice-crystallized pepsin. The pH *vs.* k_{cat} and K_m figures were bell-shaped curves. The pH *vs.* k_{cat} curves depend on a pair of catalytic groups with pK_a's in water and deuterium oxide of 1.62, 3.48, and 1.92, 4.01, respectively. For this reaction, there is no deuterium oxide solvent isotope effect with $k_{H_2O\ lim}/k_{D_2O\ lim} = 1.05 \pm 0.30$. The competitive inhibition constant, K_I, for N-acetyl-D-phenylalanyl-D-tyrosine methyl ester was found to be equal to the Michaelis constant, K_m, for the L–L substrate at various pH's between 1.10 and 4.05. This indicates that the K_m measured under turnover conditions is a true equilibrium constant not modified by additional rate constants. The pH dependences of the kinetic constants for the pepsin- (prepared according to Rajagopalan, Moore, and Stein) catalyzed hydrolyses of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester, N-acetyl-L-phenylalanyl-L-phenylalanine methyl ester, and N-acetyl-L-tyrosyl-L-phenylalanine methyl ester were also determined in this pH range. The pH *vs.* k_{cat} and K_m profiles were all bell-shaped curves. Evidence is presented that $k_{cat} = k_2$, the first catalytic step of the reaction. Based on these results a mechanism is proposed for these reactions which accounts for the prototropic equilibria and a rate-determining step which does not involve a proton transfer. Finally the inhibition constants for some of the products and dioxane were also determined in this pH range.

Previous work has established that pepsin catalyzes the hydrolysis of N-acyl-L-dipeptides[1c,2–6] and N-acyl-L-tripeptide esters.[7,8] All but one of the N-acyl-L-dipeptides, which exhibit maximum hydrolysis near pH 2, contained a free C-terminal carboxyl group which ionizes in this low pH region of pepsin activity. The N-acyl-L-tripeptide esters all contained a positively charged histidine moiety which was included to help increase the substrate solubility. These positively charged compounds (*e.g.*, Z-His-Phe-Trp-OET)[9] are the most catalytically active peptide substrates which have been prepared for pepsin. Unfortunately the electric charge of these substrates makes them less

(1) (a) This research was supported in part by Grant GM12022 from the National Institutes of Health. (b) Author to whom all inquiries should be addressed at the Department of Chemistry, Kenyon College, Gambier, Ohio 43022. (c) A preliminary report of some of this work has appeared: G. E. Clement and S. Snyder, *J. Am. Chem. Soc.*, **88**, 5338 (1966).

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(9) Abbreviations used: His, L-histidyl; Phe, L-phenylalanyl; Tyr, L-tyrosine; I_2Tyr, L-diiodotyrosine; Br_2Tyr, L-dibromotyrosine; Trp, L-tryptophyl; Ac, acetyl; Z, benzyloxycarbonyl; OEt, ethyl ester; OMe, methyl ester; Glu, L-glutamyl.

suitable for mechanistic studies owing to the possible complication of charge interaction between enzyme and substrate. In some very valuable studies of the kinetics of pepsin-catalyzed hydrolyses, Baker[10,11] showed that Ac-Phe-Tyr which is electrically neutral at low pH was hydrolyzed by pepsin to give Ac-Phe and Tyr. Therefore, we have extended this work to a study of the pepsin-catalyzed hydrolyses of neutral dipeptides containing Phe and Tyr.

The aim of the present work is to determine in detail the kinetic parameters and prototropic equilibria relating to the rate-determining step for the pepsin-catalyzed hydrolysis of three neutral substrates, Ac-Phe-Tyr-OMe, Ac-Phe-Phe-OMe, and Ac-Tyr-Phe-OMe. The observed pK_a's of the catalytically important groups as obtained from these pH–rate profiles should be a direct measure of those functioning on the enzyme. An additional reason for protecting the carboxyl group is to eliminate the possibility of transpeptidation.[12,13] There is ample evidence that pepsin-catalyzed hydrolyses proceed with more than one catalytically important step. Therefore, by studying the hydrolyses of three similar but different substrates, information can also be obtained which indicates which of the proposed catalytic steps is rate limiting for these reactions. While this work was in progress, Lutsenko and coworkers[14] described the pH–rate profile of Ac-Phe-Tyr-OEt in 10% ethanol.

Experimental Section

Materials. Pepsin, Worthington Lot PM703 twice crystallized, and pepsinogen, Worthington Lot PG6LB crystalline, were both used without additional purification. The activation of pepsinogen to pepsin was carried out exactly as described by Rajagopalan and coworkers[15] and is referred to as activated pepsin. Both pepsin preparations were assayed with hemoglobin using a modified procedure.[15] The hemoglobin activities of the commercial and activated pepsin are 1.76×10^6 and 1.97×10^6, respectively, in units of change in absorbance (ΔA) at 278 mμ per hour per mole of pepsin. The activities as expressed in these units are reproducible to ±5%. The absorbance at 278 mμ was preferred over 280 mμ because the pepsin concentration was also determined at 278 mμ.[16]

Ac-Phe-Tyr-OMe was obtained from Cyclo Chemical Corp. in Lots M-2145, M-2010, M-2755, and M-2546.[17] After three recrystallizations from ethyl acetate–petroleum ether (bp 38–52°) this material gave a reproducible sharp melting point, 125–126°. These lots were used with the twice-crystallized pepsin, $[\alpha]^{25}_{300}$ +119.6° (*c* 0.05, methanol); $[\theta]^{25}_{277}$ +500° (*c* 0.05, methanol).

Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.48; H, 6.29; N, 7.29. Found: C, 65.48; H, 6.40; N, 6.98.

A fifth lot, M-3396-R, was used without additional purification, mp 136–137°, with pepsin obtained from the activation of pepsinogen,[17] $[\alpha]^{25}_{300}$ +125.9° (*c* 0.05, methanol); $[\theta]^{25}_{277}$ +521° (*c* 0.05, methanol).

Anal. Found: C, 65.31; H, 6.29; N, 7.05.

Ac-Phe-Phe-OMe was obtained from Cyclo Chemical Corp. Lot G-1033/R, mp 174–175°, $[\alpha]^{25}_{300}$ +59.7° (*c* 0.05, methanol).

Anal. Calcd for $C_{21}H_{24}N_2O_4$: C, 68.45; H, 6.57; N, 7.60. Found: C, 68.72; H, 6.77; N, 7.54.

Ac-Tyr-Phe-OMe was obtained from Cyclo Chemical Corp. Lot G-1055, mp 106–109°, $[\alpha]^{25}_{300}$ +67.8° (*c* 0.05, methanol).

Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.48; H, 6.29; N, 7.29. Found: C, 65.67; H, 6.47; N, 7.32.

All substrates were homogeneous in three solvent systems in either paper or thin layer chromatography.

Ac-D-Phe-D-Tyr-OMe was Cyclo Lot M-2907.[17] After recrystallization from a benzene–petroleum ether (bp 38–52°) mixture followed by fractional recrystallization from ethyl acetate–petroleum ether (bp 38–52°), two main fractions were isolated. One gave mp 114–115° and the other melted at 155–160°. The material used herein was the lower melting fraction which had an ultraviolet spectrum identical with the L-L isomer (λ_{max} 275 mμ), exhibited no reaction with the enzyme, and had a molar absorptivity at 237 mμ of 1450; $[\theta]^{25}_{277}$ −503° (*c* 0.05, methanol).

Ac-Phe was Cyclo Lot M-1113, mp 172–173°. Tyr-OMe·HCl was obtained from Mann Research Laboratories, Lot 2220. The hydrochloride was decomposed by treatment with 5% sodium bicarbonate solution. The free methyl ester was recrystallized from methanol–water and gave mp 134–135°; Almond and Niemann[18] report mp 134–135°. Tyrhydroxamic acid, Mann Lot 1646, was recrystallized from water, mp 179–180°; Foster and coworkers[19] report mp 161–162°.

Anal. Calcd for $C_9H_{12}N_2O_3$: C, 55.09; H, 6.17; N, 14.27. Found: C, 55.01; H, 6.14; N, 14.24.

Z-Tyr-NH_2 was Cyclo Lot R-3772, mp 120–121°. Phe-OMe·HCl was Cyclo Lot K-4332, mp 160–162°.

Buffers. Aqueous buffer solutions were prepared by mixing appropriate volumes of acetic acid–sodium acetate, phosphoric acid–potassium dihydrogen phosphate, dipotassium citrate–hydrochloric acid, and hydrochloric acid–water. The solutions of ionic strength 0.05–0.10 were prepared from Fisher reagent grade chemicals and distilled demineralized water. When dioxane was employed, it was freshly distilled from sodium. All glassware was cleaned with chromic acid, thoroughly rinsed, and steamed out. When deuterium oxide was employed as a solvent, 0.1 *M* stock solutions of DCl, sodium acetate, potassium phosphate, and deuterioacetic acid–sodium chloride were prepared. The deuterium oxide buffers were prepared by mixing appropriate volumes of these stock solutions. pH measurements were made at room temperature on a Radiometer Model 4C pH meter which had been standardized against a potassium phthalate buffer solution, pH 4.00 ± 0.02. The pH of the reaction mixture was measured at the end of the kinetic run. For solutions in D_2O, the pD was calculated by adding 0.40 pD unit to the glass electrode reading.[20]

Kinetic Measurements. The kinetics of the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe in water or deuterium oxide, using twice-crystallized pepsin, normally were followed spectrophotometrically at 237 mμ using a Cary 14 PM recording spectrophotometer equipped with a thermostated cell compartment.[3] The stock enzyme solutions were dissolved in a pH 4.60 acetate buffer, or, for reactions in deuterium oxide, a pD 4.60 deuterium oxide buffer. The concentrations of all enzyme solutions were determined spectrophotometrically at 278 mμ assuming a molar absorptivity of 50,900.[16] Since the measured changes in optical density were small (about 0.04–0.015), while the total absorption of the cuvette containing the reaction mixture was large (up to 2.5 optical density units), it was necessary to employ a blank in the reference compartment to keep the pen on the recording chart. This blank was prepared by adding the appropriate amount of substrate or Tyr or pepsin to a cuvette containing buffer solution. For the hydrolysis of Ac-Phe-Tyr-OMe, a convenient standard slit opening of 1.8 mm was always employed. When inhibitors were used or the reaction was run in dioxane concentrations greater than 3.17%, these substances were dissolved directly in the buffer solutions. When the D-D isomer was used as an inhibitor it was prepared as a stock solution in dioxane and 50 μl of this solution was added to the cuvette.

Absorption Measurements. The molar absorptivities for Ac-Phe-Tyr-OMe, Tyr-OMe, and Ac-Phe were determined at 237

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mμ, at 25°, in 0.1 *M* acetate or phosphate buffer solutions, which were 3.17% in dioxane. The molar absorptivities of Ac-Phe-Tyr-OMe and Tyr-OMe were found to be 1460 ± 12 and 1107 ± 10, respectively, for 24 and 16 determinations. Both were found to be independent of pH changes. The molar absorptivity for Ac-Phe was found to change with varying acidity and gave the values of 109 ± 4 at pH 1.05 and 68 ± 4 at pH 5.05. The $\Delta\epsilon$'s calculated from these data ranged from 248 at pH 1.05 to 289 at pH 5.05. The $\Delta\epsilon$'s calculated from infinity determinations at a slit opening of 1.8 mm for the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe ranged from 251 ± 20 at pH 1.10 to 275 ± 19 at pH 4.70. Therefore, the apparent $\Delta\epsilon$'s determined from the kinetic runs were in good agreement with those determined by subtracting the ϵ of the starting material from the ϵ of the products. The reactions in 1.59% dioxane had the same $\Delta\epsilon$'s as the reactions in 3.07% dioxane. In 6.17% dioxane a $\Delta\epsilon$ of 196 ± 5 at pH 2.10 was taken directly from five infinity determinations and for pH 4.70 in 6.17% dioxane a $\Delta\epsilon$ of 237 ± 9 was determined.

Automatic Ninhydrin Method. The kinetic measurements on the Cary spectrophotometer required the measurement of small changes in optical density in a solution of high optical density. The points at pH's 1.15, 2.57, and 4.65 in Table I were repeated on a Technicon automatic amino acid analyzer. This method is based on a continuous automatic system by following the development of color with ninhydrin reagent. The Technicon automatic amino acid analyzer system and procedure is exactly the same as described by Lenard and coworkers.[21] At the beginning and end of each day's experiments, standard Tyr-OMe solutions at various concentrations were prepared in the buffer being used for the kinetic study. These standards were run through the instrument and the color values were used to plot a standard curve in order to convert the rates from OD/min to moles of Tyr-OMe produced/sec. The molar absorptivities determined for the ninhydrin color development for Tyr-OMe were in the range 5300–5700.

All experiments using pepsin obtained from pepsinogen[15] were carried out on the Technicon automatic analyzer. A 1-ml aliquot of the freshly prepared pepsin in the pH 4.40 acetate buffer was added to a thermostated cell containing 15 ml of the appropriate buffer. The reaction was initiated by adding 500 μl of a stock substrate solution in dioxane. The molar absorptivities determined for the ninhydrin color development for Phe-OMe were in the range 4800–5000. All three substrates exhibited an enzymatic reaction of 100 ± 3% as measured on the automatic analyzer. The initial rates were obtained from a 4–6% enzymatic hydrolysis of the substrate. Pepsin was completely stable in this pH range during the time required for a kinetic determination. Both the amide and ester bonds of the substrate are stable to nonenzymatic hydrolysis in the pH range during the time required for the measurement of an initial rate. This time varied from 30 min to 4 hr depending on the particular substrate. The background absorbance due to the reaction of pepsin with ninhydrin was 0.3 and 0.1 absorbance unit for commercial and activated pepsin, respectively.

Table I. The Kinetics of the Pepsin-Catalyzed Hydrolysis of Ac-Phe-Tyr-OMe in Aqueous Media

pH	$k_{cat} \times 10^3$ sec^{-1}	$K_m \times 10^3$ *M*	Buffer
5.05[a]	3.8 ± 0.4	1.6 ± 0.1	Acetate
4.70	4.7 ± 0.7	1.7 ± 0.3	Acetate
4.65[b]	5.0 ± 0.5	1.4 ± 0.4	Acetate
4.05	6.2 ± 0.6	1.5 ± 0.2	Acetate
3.50	11.1 ± 2.9	2.7 ± 0.9	Acetate
3.10	13.5 ± 1.0	3.2 ± 0.3	Phosphate
2.57[b]	19.2 ± 2.4	2.9 ± 0.3	Phosphate
2.57	17.1 ± 3.0	3.2 ± 0.7	Phosphate
2.10	18.7 ± 2.6	2.4 ± 0.4	Phosphate
1.75	11.5 ± 2.2	1.7 ± 0.4	Phosphate
1.15[b]	7.9 ± 1.1	1.4 ± 0.7	HCl
1.05	6.8 ± 0.4	1.3 ± 0.1	HCl
1.16[c]	14.0 ± 1.9	2.3 ± 0.3	HCl
2.00	29.1 ± 6.0	3.9 ± 0.9	Phosphate
1.97	30.9 ± 2.3	3.1 ± 0.3	HCl
2.68	42.7 ± 9.0	5.1 ± 1.5	Phosphate
2.65	41.0 ± 3.0	5.2 ± 0.5	Citrate
4.07	27.3 ± 6.0	4.6 ± 1.3	Acetate
4.42	15.0 ± 2.0	2.7 ± 0.4	Citrate
4.61	11.0 ± 0.7	2.6 ± 0.2	Acetate
4.70	10.1 ± 0.8	1.9 ± 0.2	Acetate
4.86	12.9 ± 2.0	3.3 ± 0.5	Acetate

[a] The data for the following nine points were obtained using twice-crystallized pepsin; 25.0° in 3.17% (v/v) dioxane–water; $[E_0] = 1.5 \times 10^{-5}$ *M*, $[S_0] = 0.26$–1.10×10^{-3} *M*. [b] Determined on the Technicon analyzer system for comparison with the spectrophotometric method; $[E_0] = 3$–6×10^{-6} *M*. [c] The data for the following ten points were obtained using pepsin obtained by activation of pepsinogen; 25.0° in 3.03% (v/v) dioxane–water; $[E_0] = 3$–6×10^{-6} *M*, $[S_0] = 0.30$–1.26×10^{-3} *M*.

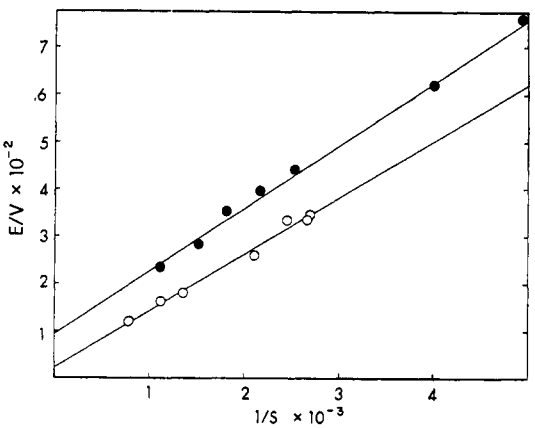

Figure 1. Typical Lineweaver–Burk plots for the pepsin-catalyzed hydrolysis of Ac-Phe-Phe-OMe, pH 2.30 (●), and Ac-Phe-Tyr-OMe, pH 2.68 (○). Pepsin obtained from activation of pepsinogen was used for both determinations.

Results

The kinetic parameters for the pepsin-catalyzed hydrolyses of Ac-Phe-Tyr-OMe, Ac-Phe-Phe-OMe, and Ac-Tyr-Phe-OMe were obtained from initial rate data by applying a least-squares computer program to the Lineweaver–Burk[22] form of the Michaelis–Menten equation. The error in k_{cat} reflects the error in the intercept and the error in K_m is a summation of the errors in the slope and intercept. Figure 1 shows typical Lineweaver–Burk plots for the pepsin-catalyzed hydrolysis of Ac-Phe-Phe-OMe at pH 2.30 and Ac-Phe-Tyr-OMe at pH 2.68.

The kinetic parameters k_{cat} and K_m are listed in Tables I and II for the reaction of Ac-Phe-Tyr-OMe in water and deuterium oxide, respectively. Table I

Table II. The Kinetics of the Pepsin-Catalyzed Hydrolysis of Ac-Phe-Tyr-OMe in Deuterium Oxide[a]

pD	$k_{cat} \times 10^3$ sec^{-1}	$K_m \times 10^3$ *M*	Buffer
5.10	5.1 ± 1.5	1.4 ± 0.5	Acetate
4.51	6.2 ± 0.4	1.6 ± 0.1	Acetate
4.05	13.6 ± 3.9	2.0 ± 0.9	Acetate
3.54[b]	15.9 ± 1.8	2.8 ± 0.4	Acetate
3.10[b]	18.1 ± 2.3	2.9 ± 0.3	Phosphate
2.65[b]	15.2 ± 2.0	2.5 ± 0.8	Phosphate
2.03	13.7 ± 2.3	2.7 ± 0.5	Phosphate
1.65	8.1 ± 1.5	1.6 ± 0.4	DCl

[a] 3.17% (v/v) dioxane–deuterium oxide; 25.0°. The pepsin was twice crystallized; $[E_0] = 1.5 \times 10^{-5}$ *M*, $[S_0] = 0.26$–1.10×10^{-3} *M*. [b] Determined on the Technicon analyzer system; $[E_0] = 3$–6×10^{-6} *M*.

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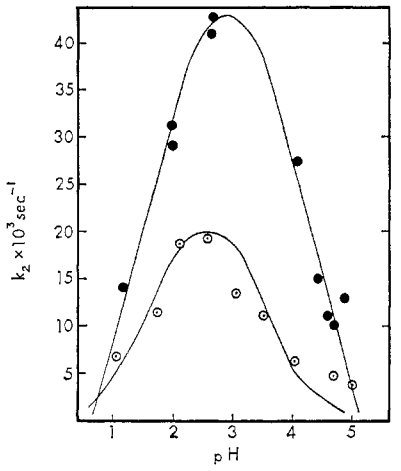


Figure 2. The pH–k_{cat} profiles for the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe in dixoane–water using twice-crystallized pepsin (⊙) and pepsin obtained from activation of pepsinogen (●).

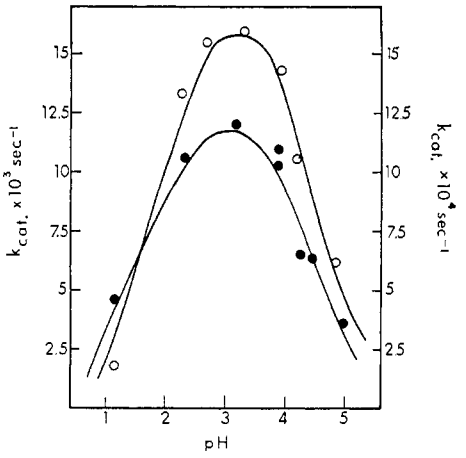


Figure 3. The pH–k_{cat} profiles for the pepsin-catalyzed hydrolysis of Ac-Phe-Phe-OMe (●), right-hand scale, and Ac-Tyr-Phe-OMe (○), left-hand scale. The pepsin was obtained from activation of pepsinogen.

also contains a comparison of the hydrolysis of Ac-Phe-Tyr-OMe by two pepsin samples: commercial twice-crystallized pepsin *vs.* pepsin obtained by activation of pepsinogen. This comparison of the k_{cat} *vs.* pH profiles with pepsin preparation is graphically represented in Figure 2. The kinetic parameters k_{cat} and K_m are listed in Tables III and IV for the pepsin-

Table III. The Kinetics of the Pepsin-Catalyzed Hydrolysis of Ac-Phe-Phe-OMe in Aqueous Media[a]

pH	$k_{cat} \times 10^3$ sec^{-1}	$K_m \times 10^3$ M	Buffer
1.16	4.5 ± 0.4	0.60 ± 0.1	HCl
2.30	10.7 ± 0.8	1.4 ± 0.1	Phosphate
3.24	12.0 ± 0.6	2.1 ± 0.3	Citrate
3.90	11.0 ± 1.1	2.0 ± 0.3	Citrate
3.90	10.4 ± 0.7	1.9 ± 0.2	Acetate
4.24	6.5 ± 0.4	1.1 ± 0.1	Citrate
4.42	6.3 ± 0.5	1.1 ± 0.1	Citrate
4.59	3.6 ± 0.3	0.55 ± 0.1	Acetate

[a] The pepsin was obtained from the activation of pepsinogen; 25.0°, 3.03% (v/v) dioxane–water; $[E_0] = 3–6 \times 10^{-6}$ M, $[S_0] = 0.20–1.04 \times 10^{-3}$ M.

Table IV. The Kinetics of the Pepsin-Catalyzed Hydrolysis of Ac-Tyr-Phe-OMe in Aqueous Media[a]

pH	$k_{cat} \times 10^4$ sec^{-1}	$K_m \times 10^3$ M	Buffer
1.17	1.7 ± 0.8	0.4 ± 0.1	HCl
2.24	13.4 ± 0.9	2.8 ± 0.3	Phosphate
2.70	15.4 ± 3.0	5.7 ± 1.3	Phosphate
3.32	16.1 ± 3.7	4.7 ± 1.5	Citrate
3.94	14.3 ± 2.5	5.4 ± 1.6	Acetate
4.24	10.7 ± 2.0	4.8 ± 1.3	Acetate
4.84	6.1 ± 0.8	2.7 ± 0.4	Acetate

[a] The pepsin was obtained from the activation of pepsinogen; 25.0°, 3.03% (v/v) dioxane–water; $[E_0] = 3–6 \times 10^{-6}$ M, $[S_0] = 0.46–1.64 \times 10^{-3}$ M.

catalyzed hydrolysis of Ac-Phe-Phe-OMe and Ac-Tyr-Phe-OMe. The pH *vs.* k_{cat} profiles for these two compounds are compared in Figure 3. The calculated pH *vs.* k_{cat} bell-shaped curves of Figures 2 and 3 were determined according to the procedures described in Dixon and Webb[23] and eq 1.

(23) M. Dixon and E. C. Webb, "Enzymes," Academic Press Inc., New York, N. Y., 1964, p 116.

$$k_{cat} = \frac{k_{cat(lim)}}{1 + (H/K_1') + (K_2'/H)} \quad (1)$$

The pH and pD *vs.* k_{cat} profiles and the absence of a deuterium oxide solvent isotope effect for the pepsin-cataylzed hydrolysis of Ac-Phe-Tyr-OMe were previously reported.[1b] However, the data from which these curves were obtained were not reported and are included in Tables I and II. In order to determine the effect of the pepsin preparation on the deuterium oxide solvent isotope effect, pepsin obtained from the activation of pepsinogen was desalted and lyophilized.[15] This pepsin yielded rate constants in H_2O at pH 3.00 and D_2O at pD 3.15 of 10.3 ± 1.1 and 10.5 ± 1 × 10^{-3} sec^{-1}, respectively, for the hydrolysis of Ac-Phe-Tyr-OMe. The absence of a deuterium oxide solvent isotope effect is still observed, but the activity of the pepsin has been greatly diminished. Similar results were obtained with the lyophilized pepsin-catalyzed hydrolysis of Ac-Phe-Phe-OMe. The rate constants for this hydrolysis in H_2O at pH 0.82 and D_2O at pD 1.30 were 1.80 ± 0.40 and 1.90 ± 0.50 × 10^{-3} sec^{-1}, respectively. Again, these rate constants indicate a less active enzyme. The deuterium oxide solvent isotope effect on the pepsin-catalyzed hydrolysis of Ac-Phe-Phe-OMe at other pH's and Ac-Tyr-Phe-OMe at all pH's could not be determined due to precipitation of the substrate during the time required for a kinetic measurement.

The competitive inhibition constants reported in Table V were calculated from eq 2. A plot of E/V *vs.* $1/S$ at constant inhibitor concentration [I] yields a

$$V = \frac{k_{cat}E_0S}{K_m + S + [I]K_m/K_I} \quad (2)$$

straight line whose slope can be used to calculate K_I (eq 3). The K_m is the Michaelis constant for the reac-

$$K_I = \frac{K_m[I]}{(\text{slope})k_{cat} - K_m} \quad (3)$$

tion in the absence of inhibitor. Since it was experimentally convenient to use solutions containing 3.17% dioxane for the kinetic runs, it was of interest to investigate the effect of dioxane on the pepsin-catalyzed hy-

Table V. Competitive Inhibition Constants[a-c]

Inhibitor	$K_I \times 10^3$ M at pH 1.05	2.10	4.05	[I] $\times 10^3$ M
N-Ac-L-Phe[e]	15.1 ± 4.1	10.4 ± 3.8	100 ± 24	10.0
L-Tyr-OMe	23.0 ± 6.0	...	17.0 ± 10.0	2.30
L-Phe-OMe	...	8.0 ± 1.1	...	4.70
L-Tyr-hydroxamic acid	8.2 ± 1.9	...	6.8 ± 2.2	2.60
CBZ-L-TyrNH$_2$	2.0 ± 0.4	1.7 ± 0.6	2.6 ± 0.7	1.20
N-Ac-D-Phe-D-Tyr-OMe	2.3 ± 0.4	1.9 ± 0.7	2.5 ± 0.8	0.80
Dioxane		451 ± 173	215 ± 86[d]	187
		622 ± 295	152 ± 75[d]	622

[a] Determined from the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe. An average of five different substrate concentrations was used to determine the inhibitor constant, $[S_0] = 0.26\text{–}1.1 \times 10^{-3}$ M. [b] 25.0°, 3.17% (v/v) dioxane–water. [c] Twice-crystallized pepsin was used for these determinations. [d] These determinations were obtained at pH 4.70. [e] Jackson and coworkers[6] reported K_I's for Ac-Phe and Ac-Tyr of 23 and 41 mM, respectively, and Zeffren and Kaizer[5] report a K_I of 2 mM for Ac-Phe all in aqueous solution at pH 2.0.

drolysis of Ac-Phe-Tyr-OMe. The competitive inhibition constants obtained for dioxane are listed in Table V for pH's 2.10 and 4.70. In an attempt to evaluate directly the effect of increasing dioxane concentration on K_m, we have made the plot shown in Figure 4. The intercept, k_{cat}^{-1}, for the hydrolysis reaction for all concentrations of dioxane employed was assumed to be that found for the hydrolysis in 3.17% dioxane. That is, within experimental error, dioxane is a competitive inhibitor. K_m was then calculated from the slopes of the respective Lineweaver–Burk plots in 1.59, 3.17, and 6.17% dioxane and the following expression: K_m = slope × k_{cat}. Plots of K_m at pH 2.10 and 4.70 (Figure 4) *vs.* dioxane concentration resulted in straight lines which allowed the evaluation of the Michaelis constant in the absence of dioxane. From Figure 4, it can be seen that the slopes of both lines for such a plot are quite large and that the K_m's determined in 3.17% dioxane are considerably larger than the Michaelis constants estimated in aqueous buffers. At pH 2.10, for example, the Michaelis constant (K_m) in 3.17% dioxane was found to be 2.35 mM, while the corresponding value in 0% dioxane ($K_m°$) taken from Figure 4 is 0.75 mM. It is not surprising that dioxane is a competitive inhibitor since Tang[24] found that aliphatic alcohols were competitive inhibitors of the pepsin-catalyzed hydrolysis of Z-Glu-Tyr.

It has been reported that the pepsin-catalyzed hydrolysis for peptides containing a free carboxyl group follow first-order kinetics.[3,7,11] This phenomenon has generally been ascribed to competitive inhibition by products.[25] In the present study, the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe does not follow first-order kinetics at pH's 1.05, 2.10, 3.00, and 4.05 with either pepsin preparation used herein. A similar behavior was observed for the pepsin-catalyzed hydrolysis of Ac-Phe-Phe-OMe at pH 3.0. This is the expected result since the inhibition constants of the products (Table V) are larger than the Michaelis constants for the two neutral substrates.[26]

When it is noted that esterified substrates do not follow these apparent first-order kinetics, it seems reasonable to ascribe the apparent inhibition to the presence of the free carboxyl group. Possibly an electrostatic or charger interaction between this free carboxyl group and the enzyme could cause the substrate to bind to the enzyme in such an orientation as to cause apparent inhibition to be observed. The possibility that the α-carboxyl group might have an inhibitory effect on pepsin action was first noted by Inouye and coworkers.[7]

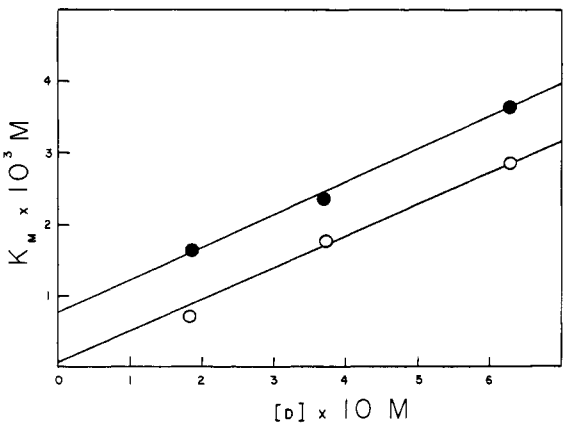


Figure 4. The effect of dioxane on the apparent Michaelis constant for the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe: (○) pH 4.70; (●) pH 2.10. The pepsin preparation was twice crystallized.

Discussion

Until very recently the pH–rate studies on the pepsin-catalyzed hydrolysis of synthetic substrates have given little indication as to the relative importance of binding or catalysis on pepsin activity. These studies have shown that the pepsin-catalyzed hydrolysis of synthetic substrates exhibits bell-shaped pH–activity profiles.[7,10,27,28] These previous studies were all conducted with substrates which are themselves capable of ionizing in the region of pepsin activity. This could mean that their pH–activity profiles do not only reflect the apparent pK_a's of the catalytically important groups on pepsin. For example, the maximum activity occurs at pH 4 and 4.5 for the pepsin-catalyzed hydrolysis of Z-Glu-Tyr and Z-Glu-Phe, respectively.[27] Inouye and coworkers[7] found bell-shaped pH–activity curves for their positively charged tripeptides to exhibit maximum activity around pH 4. Moreover, it is still not clear whether charged substrates can significantly

(24) J. Tang, *J. Biol. Chem.*, **240**, 3810 (1965).
(25) N. M. Green, *Nature*, **178**, 145 (1956).
(26) We have observed that the pepsin-catalyzed hydrolysis of Ac-Phe-I$_2$Tyr follows apparent first-order kinetics at pH 2.00, at 25° in 3.17% dioxane. The competitive inhibition constant for Ac-Phe (K_I = 17.7 mM) determined from the hydrolysis of Ac-Phe-I$_2$Tyr at pH 2.00 is an excellent agreement with the value listed in Table V. The competitive inhibition constants for Ac-Phe and Tyr at pH 2.00 are 20.2 and 8.0 mM, respectively, as determined from the hydrolysis of Ac-Phe-Tyr at 25.0° in 3.17% methanol. A K_m of 1.77 mM was observed for this substrate at the pH and temperature.
(27) J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **127**, 627 (1939).
(28) W. T. Jackson, M. Schamowitz, and A. Shaw, *Biochemistry*, **4**, 1537 (1965).

affect the enzyme specificity. The effect of pH on the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr in water and Ac-Phe-Tyr-OEt in 10% ethanol has been reported.[14] In the reaction of Ac-Phe-Tyr-OEt, the ethanol could affect the stability of pepsin at the high pH's, thereby causing the K_m to increase.

Zeffren and Kaiser[5] recently did report a bell-shaped pH–k_{cat} profile for the pepsin hydrolysis of Ac-Phe-Br_2Tyr with a maximum around pH 2 and pK_a's of 0.89 and 3.44. More recently, Silver and coworkers[3] have shown that the pepsin-catalyzed hydrolysis of three dipeptides, including one which cannot ionize, also exhibits bell-shaped pH–k_{cat} profiles with pK_a's in the range of 1.4 and 4.35. In this respect these workers' results are very similar to those reported herein for that portion of this work using commercial pepsin.

The pH *vs.* k_{cat} profiles for the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe, Ac-Phe-Phe-OMe, and Ac-Tyr-Phe-OMe were all bell-shaped curves with maximum activity at pH's 2.8, 3.0, and 3.2, respectively. The pK'_a's of the groups controlling k_{cat} in water are in the range of 1.4–1.8 and 4.1–4.6 for these substrates using pepsin obtained from the activation of pepsinogen (Table VI). A comparison of the k_{cat} at

Table VI. Limiting Rate Constants and pK_a's Associated with the Catalytic Groups Controlling the Pepsin-Catalyzed Hydrolysis of Three Neutral Substrates

Quality	Ac-Phe-Tyr-OMe	Ac-Phe-Phe-OMe	Ac-Tyr-Phe-OMe
k_{cat}(lim) × 10^3 sec^{-1}	51.9 ± 7.0[a]	11.8 ± 0.9[a]	1.6 ± 0.2[a]
	24.6 ± 3.6[b]		
	23.4 ± 3.7[c]		
pK_1'	1.6[a]	1.4[a]	1.8[a]
	1.6[b]		
	1.9[c]		
pK_2'	4.1[a]	4.6[a]	4.6[a]
	3.5[b]		
	4.0[c]		

[a] The pepsin was obtained from activation of pepsinogen; 3.03% (v/v) dioxane–water. [b] The pepsin was commercial twice crystallized; 3.17% (v/v) dioxane–water. [c] The pepsin was commercial pepsin twice crystallized; 3.17% (v/v) dioxane–deuterium oxide.

the maximum pH for these three neutral substrates shows that they react with different rate constants: the k_{cat} for Ac-Phe-Tyr-OMe is *ca.* four times larger than k_{cat} for Ac-Phe-Phe-OMe which in turn is *ca.* seven times larger than that for Ac-Tyr-Phe-OMe. This must mean that the rate-determining step for the hydrolysis of these substrates does not involve the breakdown of a common intermediate. A similar observation has been reported[6] for the corresponding free acids at pH 2.0. For the hydrolysis of Ac-Phe-Tyr-OMe two different pepsin preparations were used: commercial twice-crystallized and "pure" pepsin obtained from the activation of pepsinogen.[15] Both pepsin preparations gave bell-shaped pH–k_{cat} profiles and the "pure" pepsin had twice the activity (Tables I and VI and Figure 2).

The absence of a deuterium oxide solvent isotope effect, $k_{H_2O\,lim}/k_{D_2O\,lim}$ = 1.05 ± 0.30, for the commercial pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe (Tables I and II) has already been reported.[1b] The pK_a's for the groups controlling k_{cat} in water were 1.62 and 3.48 and for the reaction in deuterium oxide were 1.92 and 4.01. The positive shift of 0.30–0.50 pK_a unit in going from water to deuterium oxide is an expected result since acids are weaker in deuterium oxide than in water.[29] This was the first proteolytic enzyme hydrolysis reaction which had been found to proceed with no deuterium oxide solvent isotope effect. The deuterium oxide solvent isotope effect observed for other proteolytic enzymes such as α-chymotrypsin is in the range of 2–3.[30] More recently Reid and Fahrney[31] also reported the absence of a deuterium oxide solvent isotope effect for the pepsin-catalyzed hydrolysis of methyl phenyl sulfite.

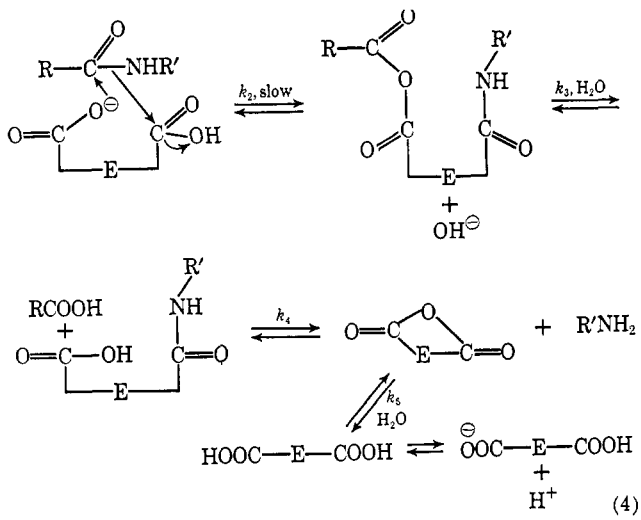
Since this was the first proteolytic enzyme which did not exhibt a deuterium oxide solvent isotope effect, it was of interest to extend this study to other substrates and other enzyme preparations. Unfortunately, Ac-Phe-Phe-OMe and Ac-Tyr-Phe-OMe were insoluble in D_2O solutions at the concentrations required for kinetic measurements. However, Ac-Phe-Phe-OMe does have a favorable K_m at low pH so that one preliminary comparison could be made for this reaction: k_{H_2O}(pH 0.8)/k_{D_2O}(pD 1.3) = 0.9 ± 0.3. The desalted lyophilized "pure" pepsin used for these additional studies unfortunately exhibited denaturation during the desalting and/or lyophilization steps. Although the desalted lyophilized enzyme exhibited no D_2O effect for the hydrolysis of Ac-Phe-Tyr-OMe at pH 3.0, the k_{cat} was *ca.* four times less than the value observed with the freshly prepared pepsin (*cf.* Results section).

A comparison of the K_m's for the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe with K_I's for Ac-D-Phe-D-Tyr-OMe which shows that K_I/K_m was unity in the pH range 1–4 has already been reported.[1c] This result provides evidence that K_m is a true binding constant, not modified by rate constants, and further suggests that k_{cat} = k_2, the first catalytic step of the reaction. Finally, the replacement of a Tyr by a Phe leads to an increase in binding. Other workers[6,8] have also observed that replacement of Tyr by Phe increases the binding.

There is a growing body of evidence which implicates carboxyl groups as being the catalytically important groups at the active site of pepsin. First the pK_a's determined from bell-shaped pH–k_{cat} profiles are in the correct range for carboxyl groups.[1c,3] Secondly, an aspartic acid residue has been implicated in pepsin action by the inactivation of pepsin with *p*-bromophenacyl bromide.[32] Thirdly, many different diazo compounds have been employed to specifically inactivate pepsin in a mole for mole ratio.[33] This reaction would normally be expected to involve the esterification of a carboxyl group. Finally, the phosphate group can be rejected as being catalytically important because Perlmann[34] found dephosphorylated pepsin to be catalytically active toward hemoglobin and Ac-Phe-I_2Tyr.

(29) R. P. Bell, "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959.
(30) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. A. Heck, *J. Am. Chem. Soc.*, **86**, 3680 (1964).
(31) T. W. Reid and D. Fahrney, *ibid.*, **89**, 3941 (1967).
(32) E. Gross and J. L. Morell, *J. Biol. Chem.*, **241**, 3638 (1966); B. F. Erlanger, S. K. Vratsanos, N. Wassermann, and A. G. Cooper, *Biochem. Biophys. Res. Commun.*, **23**, 243 (1966).
(33) G. A. Hamilton, J. Spona, and L. D. Crowell, *ibid.*, **26**, 193 (1967); T. G. Rajagopalan, W. H. Stein, and T. G. Moore, *J. Biol. Chem.*, **241**, 4295 (1966); G. R. DelPierre and J. S. Fruton, *Proc. Natl. Acad. Sci. U. S.*, **56**, 1817 (1966); E. B. Org and G. E. Perlmann, *Nature*, **215**, 1492 (1967).
(34) G. Perlmann, *J. Gen. Physiol.*, **46**, 441 (1958).

A mechanism for the pepsin-catalyzed hydrolysis of neutral dipeptides has to be consistent with the following facts: (1) two carboxyl groups are kinetically involved in the rate-limiting step; (2) both the acyl and amine half of the dipeptide are covalently bonded to the enzyme during hydrolysis;[12,13,35] and (3) the rate-determining step does not involve a proton transfer, as reflected by the absence of a deuterium oxide isotope effect. A simplified mechanism which is consistent with these observations is described in eq 4.

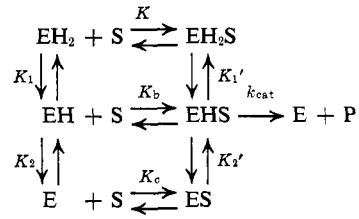


(4)

Although the total model system for the first rate-determining step (k_2) has not been observed, each half has been independently studied. In one-half of this step, the carboxylate on the enzyme acts as a nucleophile toward the carbonyl group of the amide bond and the second half of the reaction involves the concerted nucleophilic attack by the liberated amine anion on the carboxyl group of the enzyme. The first half is similar to the phthalamic acid hydrolysis[36] and the second half is similar to the formation of N-methylphthalimide during the hydrolysis of N-methylphthalamic acid.[37] The k_3 step is the rapid hydrolysis of an anhydride, the k_4 step is identical with the phthalamic acid hydrolysis, and step k_5 is again the rapid hydrolysis of an anhydride to re-form the active enzyme. Since the mechanism of action of pepsin has not been as intensively studied as other proteolytic enzymes, eq 4 is not proposed as the final mechanism but only as one which is consistent with the known data.

The simplest assignment of the two catalytically important groups at the active site of pepsin was to one carboxyl and one carboxylate acting in a concerted manner. However, two other alternatives cannot absolutely be discarded at the present time. The first is that the pK_a's around 1.5 involve amide or peptide bonds which can be protonated around a pH of 1.[38] This alternative can generally be rejected by considering that a protonated amide or peptide bond would be expected to be a very good leaving group resulting in some hydrolysis of the enzyme. It has been shown that pepsin exhibits unusual stability in acid solutions.[39] The second alternative could involve the participation by a tetrahedral intermediate as was observed for the hydrolysis of O-carboxyphthalimide.[40] Although the participation by a tetrahedral intermediate cannot be absolutely rejected, it has been detected only in very selected ester hydrolyses.[41] Finally, the anhydride mechanism originally proposed by Bender and Kézdy[42] can be rejected because the pH dependence of the enzyme–anhydride active species involves a double sigmoid curve rather than a bell-shaped curve.

Meaning of k_{cat}/K_m. The k_{cat}/K_m–pH profile for the commercial pepsin catalyzed hydrolysis of Ac-Phe-Try-OMe (Table I) could be flat, but we feel that the data generally describe a curve with a maximum around pH 2.5. Bell-shaped pH–k_{cat}/K_m profiles have been reported by Zeffren and Kaiser[5] for Ac-Phe-Br_2Tyr and by Silver and coworkers[3] for Ac-Phe-Tyr, Ac-Phe-Trp, and Ac-Phe-Tyr-NH_2, a neutral substrate. The pK_a's for Ac-Phe-Br_2Tyr were 0.75 and 2.67 and for the last three substrates 1.17–1.40 and 4.35. All these studies were carried out with commercial pepsin. The ionization described by these bell-shaped curves should be a measure of the ionizations occurring on the free enzyme and are consistent with eq 5.



(5)

The equations for the theoretical pH dependence of these catalytic parameters that result from eq 5 are

$$\frac{k_{cat}}{K_m} = \frac{\frac{k_{obsd}}{K_b}}{1 + \frac{H}{K_1} + \frac{K_2}{H}} \quad (6)$$

$$K_m = K_b \frac{1 + \frac{H}{K_1} + \frac{K_2}{H}}{1 + \frac{H}{K_1'} + \frac{K_2'}{H}} \quad (7)$$

However, the pH–k_{cat}/K_m dependence of the pepsin-(obtained from pepsinogen) catalyzed hydrolysis of our three neutral dipeptides is linear, within experimental error, in the pH range studied (*cf.* Tables I, III, and IV). An examination of eq 6 and 7 shows that this invariance of k_{cat}/K_m with pH could still be consistent with eq 5 if K_1 and K_2 for "pure" pepsin were outside the pH range of 1–5; *i.e.*, p$K_1 < 1$ and p$K_2 > 5$. Although this explanation is consistent with our results, we cannot offer, at this time, any reasons why the apparent pK_a's should change with pepsin preparation.

An examination of Figure 2 and the pH–k_{cat} profile for Ac-Phe-Br_2Tyr as reported by Zeffren and Kaiser[5] indicates that the k_{cat}'s at pH's greater than pK_2' are

(35) N. Sharon, V. Grisaro, and H. Neumann, *Arch. Biochem. Biophys.*, **97**, 219 (1962).
(36) M. L. Bender, Y. Chow, and F. Chloupek, *J. Am. Chem. Soc.*, **80**, 5380 (1958).
(37) J. Brown, S. C. K. Su, and J. A. Shafer, *ibid.*, **88**, 4468 (1966).
(38) A. R. Goldfarb, A. Mele, and N. Gutstein, *J. Am. Chem. Soc.*, 77, 6194 (1955).
(39) J. H. Northrup, *J. Gen. Physiol.*, **16**, 33 (1932).
(40) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **83**, 2267 (1961).
(41) T. C. Bruice and L. Fedor, *ibid.*, **86**, 738 (1964).
(42) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).

larger than predicted by eq 1. The mechanistic implications of these observations are not clear at this time. However, we feel that these observations are also directly related to the k_{cat}/K_m–pH dependency problem. We have initiated an extensive examination of pepsin hydrolyses at pH's above 5 which should give some meaningful insite into this ambiguity in pepsin action.

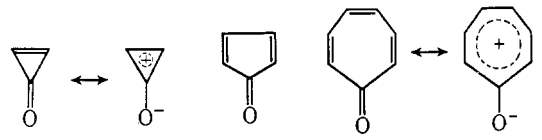
Acknowledgment. The authors thank Mr. Steven Frolow for technical assistance and Professor F. J. Kézdy for many useful discussions.

Communications to the Editor

The Synthesis of 4,5:10,11-Bis(tetramethylene)-4,10-cyclotridecadiene-2,6,8,12-tetrayn-1-one, a Derivative of [13]Annulenone[1]

Sir:

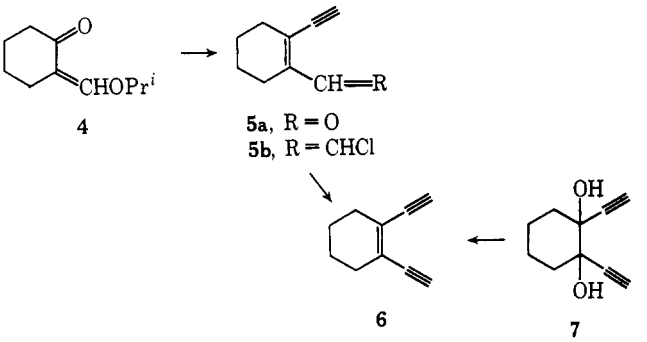
Monocyclic conjugated ketones are an interesting class of compounds, for which we propose the generic name "annulenones." Such compounds are expected to be aromatic if they contain a $(4n + 3)$-membered ring and nonaromatic if they contain a $(4n + 1)$-membered ring, due to the electron-withdrawing properties of the oxygen of the carbonyl group. In agreement with this are the properties of the $[4n + 3]$annulenones cyclopropenone (**1**) and cycloheptatrienone (tropone, **3**) on



1 **2** **3**

the one hand and the $[4n + 1]$annulenone cyclopentadienone (**2**) on the other.[2] No annulenone, or derivative of an annulenone, containing a ring larger than the seven-membered one has been prepared previously. We now describe the synthesis of 4,5:10,11-bis(tetramethylene)-4,10-cyclotridecadiene-2,6,8,12-tetrayn-1-one [4,5:10,11-bis(tetramethylene)-2,6,8,12-tetradehydro[13]annulenone, **11**], a derivative of [13]annulenone (*e.g.*, **13**).

1,2-Diethynylcyclohexene (**6**) was prepared by two routes. The preferred one proceeded from 2-isopropoxymethylenecyclohexanone (**4**),[3] which was treated with lithium acetylide–ethylenediamine[4] in tetrahydrofuran at room temperature and then with dilute sulfuric acid; Wittig reaction of the resulting aldehyde **5a** (38% yield, mp 49.5–50°)[5] with chloromethylenetriphenylphosphorane[6] in tetrahydrofuran at room temperature



4 **5a**, R = O; **5b**, R = CHCl **6** **7**

led to 58% of the chloromethylene compound **5b** as a mixture of *cis* and *trans* isomers (*ca* 1:2), which was dehydrochlorinated to **6** with sodamide in liquid ammonia (60% yield) or with potassium *t*-butoxide in boiling ether (46% yield). Alternatively, **6** was obtained in 19% yield by the reduction of 1,2-diethynylcyclohexane-1,2-diol (**7**, *trans* isomer or mixture of *cis* and *trans* isomers)[7] with diphosphorus tetraiodide in pyridine and carbon disulfide at room temperature.[8] The enediyne **6** obtained by either method proved to be an unstable liquid, bp 52–54° (18 mm); λ_{max}^{EtOH} 249 sh mμ (ε 10,200), 256 (12,400), 260 sh (11,100), and 269 (9700); nmr spectrum (all in CCl_4, 100 Mcps), two-proton singlet at τ 7.02 (acetylenic protons), four-proton multiplet at 7.65–7.95 (allylic protons), and four-proton multiplet at 8.20–8.50 (methylene protons).

Treatment of the mono-Grignard derivative of **6** with 0.5 molar equiv of ethyl formate in tetrahydrofuran at room temperature gave 27% of the liquid alcohol **8** which was coupled with oxygen, cuprous chloride, and ammonium chloride in aqueous ethanol and benzene. The resulting 13-membered ring alcohol **9a**,[11] isolated in *ca.* 40% yield by chromatography on silicic acid, was a very unstable yellow oil; λ_{max}^{ether} 258 mμ (ε 24,800), 278 sh (8020), 348 sh (3860), 365 (5200), and 391 (4310). Attempts were made to convert **9a** to the fully conjugated cyclic bromoallene **10** by reaction with phosphorus tribromide (see ref 12). Although a nonpolar

(1) Unsaturated Macrocyclic Compounds. LXII. For part LXI, see R. H. Mitchell and F. Sondheimer, *Tetrahedron Letters*, 2873 (1968).

(2) For relevant references, see A. Streitwieser, "Molecular Orbital Theory for Organic Chemists," John Wiley & Sons, Inc., New York, N. Y., 1961, Chapter 10; R. Breslow and L. J. Altman, *J. Am. Chem. Soc.*, **88**, 504 (1966); R. Breslow and G. Ryan, *ibid.*, **89**, 3073 (1967).

(3) W. S. Johnson and H. Posvic, *ibid.*, **69**, 1361 (1947); R. B. Woodward and W. M. McLamore, *ibid.*, **71**, 379 (1949); A. S. Dreiding and S. N. Nickel, *ibid.*, **76**, 3965 (1954).

(4) Supplied by Foote Mineral Co., Exton, Pa.; see O. F. Beumel and R. F. Harris, *J. Org. Chem.*, **28**, 2775 (1963); **29**, 1872 (1964).

(5) All new compounds were characterized by uv, ir, and nmr spectra, as well as by determination of the elemental composition (microanalysis or high-resolution mass spectroscopy).

(6) This ylide was generated by treatment of (chloromethyl)triphenylphosphonium chloride with butyllithium at −70°, according to G. Köbrich, H. Trapp, K. Flory, and W. Drischel, *Chem. Ber.*, **99**, 689 (1966).

(7) W. Ried and H. J. Schmidt, *ibid.*, **90**, 2499 (1957).

(8) The Inhoffen modification[9] of the Kuhn–Winterstein reduction[10] was used.

(9) H. H. Inhoffen, K. Radscheit, U. Stache, and V. Koppe, *Ann.*, **684**, 24 (1965).

(10) R. Kuhn and A. Winterstein, *Helv. Chim. Acta*, **11**, 87 (1928).

(11) The synthesis of **9a** from **6** is based on the analogous synthesis of 2,6,8,12-cyclotridecatetrayn-1-ol from 1,5-hexadiyne,[12] but in the present case it was found necessary to use double the amount of cuprous chloride and prolong the reaction to 2 hr in order to effect complete conversion.

(12) C. C. Leznoff and F. Sondheimer, *J. Am. Chem. Soc.*, **90**, 731 (1968).