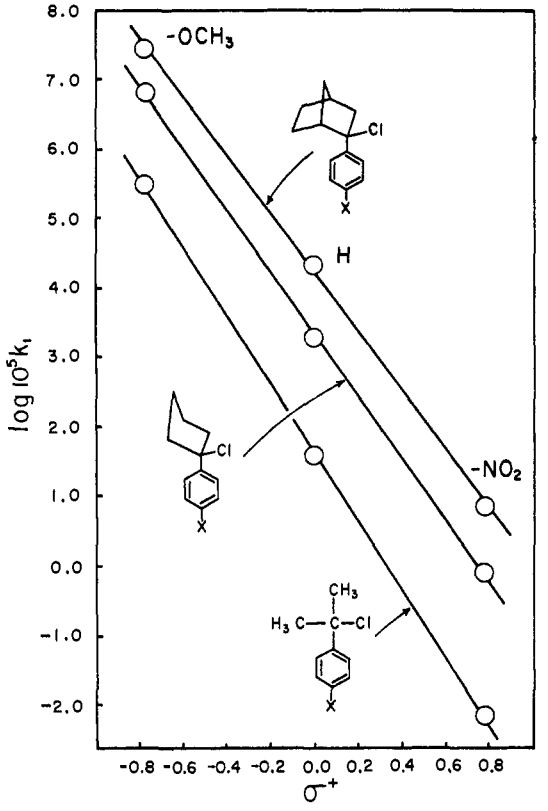

Figure 1. Plot of log k *vs.* σ^+ for the ethanolysis of substituted aryldimethylcarbinyl, 1-arylcyclopentyl, and 2-aryl-*exo*-norbornyl chlorides.

enormous range of electron demand—from the highly stabilized 2-anisylnorbornyl derivative to the much more electron-demanding 2-*p*-nitrophenylnorbornyl derivative.

The existence of π participation in the norbornyl system is clearly indicated by Wiley's results with 3′,6′-dimethoxy-*exo*-benznorbornyl tosylate[13] and by Tanida's elegant studies with substituted 7-benznorbornyl tosylates.[14] However, all of our attempts to confirm the frequently postulated σ participation in saturated norbornyl derivatives not undergoing rearrangement to more stable structures have uniformly yielded negative answers. At the present time we know of no experiment which provides independent confirmation for the proposal that the high *exo*/*endo* rate ratios in saturated norbornyl derivatives are due to σ participation. Accordingly, we are abandoning further efforts along this line[15] and are turning our attention to exploration of steric hindrance to ionization as a possible explanation for the behavior of norbornyl derivatives.

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(15) Schleyer has informed us that he is examining norbornyl derivatives containing methyl and methoxy substituents in the 6 position in a further effort to obtain evidence for the postulated 2,6 bridging in the transition state for solvolysis of such derivatives.

(16) Research assistant on a grant (G 19878) supported by the National Science Foundation.

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The Kinetics of the Pepsin-Catalyzed Hydrolysis of N-Acetyl-L-phenylalanyl-L-tyrosine Methyl Ester[1]

Sir:

Previous work has established that pepsin catalyses the hydrolysis of N-acyl L-dipeptides[2-4] and N-acyl L-tripeptide esters.[5] The N-acyl dipeptides contain a free C-terminal carboxyl group which ionizes in the low pH region of pepsin activity and the N-acyl tripeptide esters all contained a positively charged histidine moiety. Baker[6] showed that N-acetyl-L-phenylalanyl-L-tyrosine was hydrolyzed by pepsin to give N-acetyl-L-phenylalanine and tyrosine. Therefore, we have studied the pH dependence of the pepsin-catalyzed hydrolysis of a neutral substrate, N-acetyl-L-phenylalanyl-L-tyrosine methyl ester. This eliminates correcting the pH–rate profile (Figure 1) for the ionization of the substrate. The observed pK_a values of the catalytically important groups as obtained from such a pH–rate profile are a direct measure of those functioning on the enzyme.

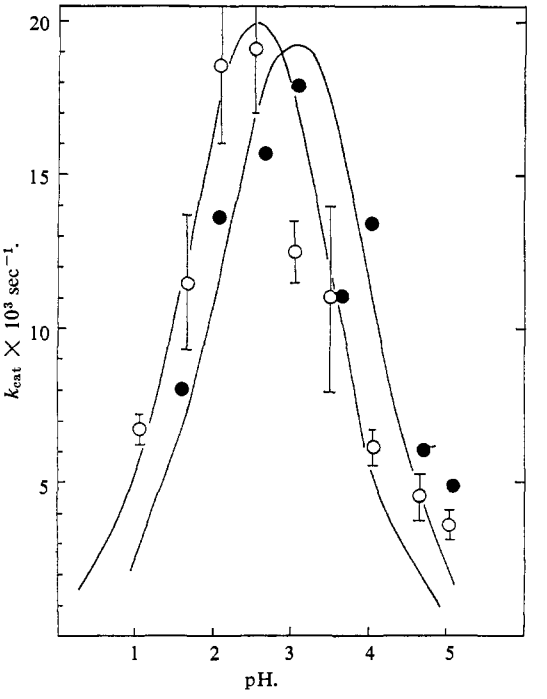

Figure 1. The pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester in water (○) and in deuterium oxide (●) at 25.0° in 3.16% dioxane.

Figure 1 shows the bell-shaped pH–rate profiles for the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester in water and deuterium oxide containing 3.16% dioxane.[7,8] The pK_a's

(1) This research was supported by Grant GM12022 from the National Institutes of Health.

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(3) W. T. Jackson, M. Schlamowitz, and A. Shaw, *Biochemistry*, **4**, 1537 (1965).

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

(6) L. E. Baker, *J. Biol. Chem.*, **193**, 809 (1951); L. E. Baker, *ibid.*, **211**, 701 (1954).

(7) Kinetic runs were obtained by using the spectrophotometric method of Silver[2] by measuring initial rates at 237 mμ and plotting the data according to the procedure of H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934). ($E_0 = 1.5 \times 10^{-5}$ *M*; $S_0 = 0.26 - 1.10$ m*M*.) Pepsin, Worthington Lot PM703 two times crystallized, was

for the catalytically important groups for the hydrolysis in water are 1.62 and 3.48; for the same reaction in deuterium oxide, 1.92 and 4.01.[9] The pK_a's determined in deuterium oxide are increased 0.3–0.5 pK_a unit as compared to those in water.[10] The observation of bell-shaped pH–rate profiles for pepsin-catalyzed hydrolyses has been observed by other investigators. Fruton and Bergmann[11] reported bell-shaped curves for the per cent hydrolysis in 24 hr *vs.* pH for the pepsin-catalyzed hydrolysis of CBZ-L-glutamyl-L-tyrosine and CBZ-L-glutamyl-L-phenylalanine. The pH maximum for these negatively charged substrates are 4 and 4.5, respectively. More recently, Inouye, *et al.*,[5] found bell-shaped pH–activity curves for positively charged tripeptides to exhibit maximum activity around pH 4. The maximum rate constant for the pepsin-catalyzed hydrolysis of our neutral substrate occurs at a lower pH, 2.55. This shows that charged substrates can significantly affect the pK_a's of the groups on the enzyme responsible for catalytic activity. However, it is still not clear whether charged substrates can significantly affect the enzyme specificity.

The pepsin-catalyzed hydrolysis of our substrate, within experimental error, exhibits no deuterium oxide solvent isotope effect; $k_{H_2O}(\text{lim})/k_{D_2O}(\text{lim}) = 1.05 \pm 0.30$. This implies that there is no proton transfer in the rate-determining step of the reaction. This is the first proteolytic enzyme hydrolysis reaction which proceeds with no deuterium oxide solvent effect. The deuterium oxide solvent isotope effect observed for other proteolytic enzymes such as α-chymotrypsin is in the range of 2–3.[12]

There is compelling evidence reported by Neumann and co-workers[13,14] that both the acyl and amine half of the dipeptide are covalently bound to the enzyme during the pepsin-catalyzed hydrolysis of a dipeptide. Since covalently bound pepsin–amino acid intermediates have been implicated, we wanted to determine the meaning of our K_m as obtained under turnover conditions. Zerner and Bender[15] found for α-chymotrypsin-catalyzed hydrolyses that K_m for an L-amide was only twice K_I for the corresponding D-amide, a competitive inhibitor. These authors use this comparison as part of their evidence that acylation is the rate-limiting step for the hydrolysis of amides.

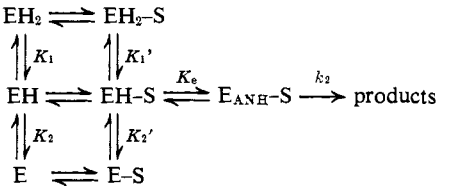
Table I. Comparison of the Inhibition Constant, K_I, for N-Acetyl-D-phenylalanyl-D-tyrosine Methyl Ester[a,b] with the Michaelis Constant, K_m, for N-Acetyl-L-phenylalanyl-L-tyrosine Methyl Ester

	pH			
	4.05	3.10	2.10	1.10
K_I D–D compound, mM	2.49	3.12	1.92	2.33
K_m L–L compound, mM	1.52	3.16	2.35	1.25

[a] This material was obtained from Cyclo Chemical Corp. and recrystallized from ethyl acetate–petroleum ether, mp 114–115°. This material exhibited no reaction with the enzyme. [b] Inhibition constants were obtained from measurements of initial rates for substrate hydrolyses in solutions containing a fixed concentration of inhibitor: [I] = 0.80 mM.

Table I shows a similar comparison for pepsin where K_m for the L–L substrate is equal, within the experimental error, to the K_I for the corresponding D–D inhibitor.

On the basis of a bell-shaped pH–rate profile, no deuterium oxide solvent isotope effect, and the equality of K_I and K_m, we propose the following scheme where the active center of pepsin contains two important carboxyl groups which react to form a catalytically important anhydride.



The absence of a deuterium oxide solvent isotope effect supports and is consistent with the original proposal of Bender and Kézdy[16] that an anhydride (E_{ANH}–S) is the catalytically active species of pepsin.[17] The anhydride reacts with the dipeptide in a rate-limiting four-center type reaction to give the acylated and aminated pepsin which rapidly reacts with water to give products and regenerate the free enzyme.[18]

Acknowledgment. The authors gratefully acknowledge many useful discussions with Professor Ferenc Kézdy.

used throughout this study. The buffers employed for the reported pH range were 0.10 N hydrochloric acid, 0.10 N phosphoric acid–potassium dihydrogen phosphate, and 0.10 N acetic acid–sodium acetate, all at an ionic strength of 0.10 M. The $\Delta\epsilon$ for the reaction was found to be slightly pH dependent varying from 248 at pH 1.10 to 289 at pH 5.0. The solid curves are theoretical curves calculated from the equation $k_{cat} = k_{cat}(\text{lim})/(1 + H^+/K_1 + K_2/H^+)$. The enzyme concentration was based on a molar absorptivity of 50,900 A l. mole^{-1} cm^{-1} at 278 mμ: G. Perlmann, *J. Biol. Chem.*, **241**, 153 (1966). The substrate was obtained from Cyclo Chemical Corp. and recrystallized from ethyl acetate–petroleum ether (bp 38–52°); mp 125–126°. *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.

(8) Both Baker[6] and Silver, *et al.*,[2] found that the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine follows first-order kinetics at pH 2.00. However, we find that the hydrolysis of the corresponding methyl ester follows first-order kinetics only to *ca.* 70% reaction at pH 2.10. This result is in accord with the suggestion of Inouye, *et al.*,[5] that the presence of an α-carboxylate group adjacent to the point of peptide cleavage inhibits the hydrolysis, whereas no similar inhibition would be expected for our neutral substrate.

(9) The pK_a's were calculated according to the procedure reported in M. Dixon and E. C. Webb, "Enzymes," Academic Press Inc., New York, N. Y., 1964, p 116.

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(17) B. F. Erlanger, S. M. Vratsanos, N. Wassermann, and A. G. Cooper, *Biochem. Biophys. Res. Commun.*, **23**, 243 (1966). These authors suggest that commercial pepsin contains two ester-like linkages involving α-carboxylates of glutamic acid.

(18) Many workers in this field, including F. A. Bovey and S. S. Yanari, *Enzymes*, **4**, 63 (1960), and more recently Jackson, *et al.*,[3] have suggested that a protonated carbonyl group on the enzyme is important for cleavage of the peptide linkage of the substrate. However, one would expect that the participation by one or more protonated carboxyl groups in the rate-determining step would involve a proton transfer. Therefore, a deuterium oxide solvent isotope effect would be expected. It would not be unexpected to be able to find a substrate for pepsin which has a different rate-limiting step from that proposed herein, for which a deuterium oxide solvent effect would be observed.

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