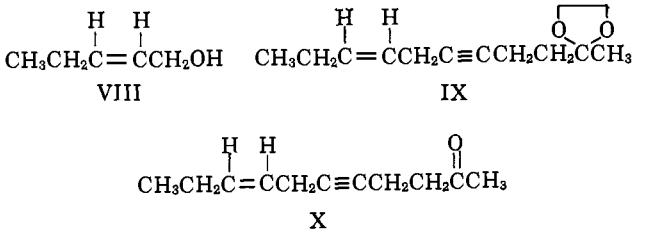

VIII IX

X

temperature gave pure *cis*-8-undecen-5-yn-2-one (X), b.p. 81–84° (0.2 mm.), one peak on v.p.c. (20% Craig, 215°), no ultraviolet absorption, and no *trans* double bond peak at 10.3 μ. The *p*-nitrophenylhydrazone had m.p. 95–96°.

The second problem came from the fact that it was now necessary to hydrate the acetylenic link in X without affecting the *cis* double bond. Here the remarkable acceleration of the rate of hydrolysis by γ-carbonyl participation became crucially important. Whereas the usual hydration conditions (hot aqueous methanolic sulfuric acid and mercuric ion) led to almost complete *cis–trans* isomerization, it was possible to effect the desired reaction without any involvement of the *cis* double bond by keeping X at room temperature for 1.5 hr. with dilute aqueous methanolic sulfuric acid–mercuric sulfate.[6] The resulting 1,4-diketone III was then cyclized with dilute aqueous base, in the usual manner, to jasmone (75% over-all from ketal IX). This showed only one peak on v.p.c. (20% Craig, 220°) and was identified as *cis*-jasmone by its ultraviolet spectrum [λ_{max}^{EtOH} 237 mμ (ϵ 11,000)], the identity of its infrared spectrum with that of authentic material,[7] and by its 2,4-dinitrophenylhydrazone, m.p. 117.5° as reported.[7]

(6) Under these conditions, the δ-ketoacetylenes are completely unchanged.

(7) L. Crombie and S. H. Harper, *J. Chem. Soc.*, 869 (1952).

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The Observation of Acyl–Enzyme Intermediates in the α-Chymotrypsin-Catalyzed Hydrolysis of Specific Ester Substrates at Low pH[1]

Sir:

The α-chymotrypsin-catalyzed hydrolyses of the ethyl, methyl, and *p*-nitrophenyl esters of N-acetyl-L-tryptophan were shown by means of an indirect kinetic argument to proceed through the formation of a common N-acetyl-L-tryptophanyl-α-chymotrypsin intermediate.[2] For the methyl ester, the half-lives at pH 7 for the formation, k_2, and decomposition, k_3, of this intermediate (eq. 1) were calculated to be 1 and 30 msec., respectively, too fast for ordinary or even most stopped-flow instrumentation to measure directly. However, both k_2 and k_3 are dependent on

$$E + S \overset{K_m}{\rightleftarrows} ES \xrightarrow{k_2} \underset{+\ P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

a basic group with a pK_a of *ca.* 7.[3] Therefore, at pH 3, the half-lives of the above individual steps should be of the order of seconds rather than milliseconds, and thus amenable to direct measurement.

(1) This research was supported by grants from the National Institutes of Health, part XXV in the series: The Mechanism of Action of Proteolytic Enzymes.

(2) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **85**, 356 (1963).

(3) M. L. Bender, G. E. Clement, F. J. Kézdy, and B. Zerner, *ibid.*, **85**, 358 (1963).

At pH 7, the catalytic rate constants (of the turnover, $k_2k_3/(k_2 + k_3)$) of the α-chymotrypsin-catalyzed hydrolysis of the ethyl and *p*-nitrophenyl esters of N-acetyl-L-tryptophan were shown to be equivalent to one another.[2] The catalytic rate constants of these two reactions are essentially identical from pH 7 down to pH 2 (see Fig. 1). Since the enzyme contains many carboxylate ions, the protonation of these groups should electrostatically perturb the ionization constant of the basic group of pK_a = 7. Thus, whereas ionic strength effects on the rate are negligible near neutrality, they are important at low pH because of the large positive charge on the enzyme.[4] The intrinsic pK_a of the group, on which both these ester hydrolyses are dependent, is 7.2, using data from pH 2 to 7. Furthermore, titration of the concentration of enzymatic active sites by N-*trans*-cinnamoylimidazole around neutrality is exactly equivalent to titration of that same solution by the burst of *p*-nitrophenol in the reaction of N-acetyl-L-tryptophan *p*-nitrophenyl ester[5] at pH 2.3, using pure α-chymotrypsin. Thus the relative rates of reaction, titration of active sites, and the pH dependence of the enzyme appear to be normal as low as pH 2, and thus it is feasible to carry out mechanistic investigations at low pH.[6]

The object of such investigations is to spectrophotometrically observe the individual steps of eq. 1, which are not discretely observable at pH 7. The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl ester at pH 2.3 shows a typical initial burst followed by a steady-state production of *p*-nitrophenol. This observation can only be interpreted in terms of the rapid formation and slow decomposition of an N-acetyl-L-tryptophanyl-α-chymotrypsin intermediate.[7] The formation of this intermediate is stoichiometric, as mentioned above, by comparison with titration of the enzymatic sites with N-*trans*-cinnamoylimidazole.

The technique of the observation of the acyl–enzyme in the hydrolysis of N-acetyl-L-tryptophan methyl ester consisted in spectrophotometrically observing the time course of the reaction under conditions of $(E)_0 > (S)_0$ and $(E)_0 > K_m$, so that two consecutive first-order processes are observed. When the substrate is saturated with enzyme [*sic*] and when the enzyme is in greater concentration than the substrate, the initial first-order process measures k_2 and the final first-order reaction measures k_3 (if $k_2 > k_3$).[8] These are approximately the conditions[9] under which the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester was carried out at pH values of 2.3, 3.4, and 4.3. A typical spectrophotometric experiment is shown in Fig. 1 of the accompanying communication.[10] In each experiment, the initial absorbance of the ester decreased rapidly, reached a minimum, and then slowly rose, reaching an infinity

(4) Evidence for a functional carboxyl group [J. A. Stewart, H. S. Lee, and J. E. Dobson, *ibid.*, **85**, 1537 (1963)] is probably related to these large ionic strength effects.

(5) The DL-ester was used at an $(E)_0/(S)_0$ ratio high enough to obviate the side reaction of the D-compound noted earlier.[2]

(6) The enzyme is stable at pH values of 2 to 3: in fact pH 2 is often used to crystallize α-chymotrypsin [M. Laskowski, "Methods in Enzymology," Vol. 2, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, New York, N. Y., 1955, p. 12].

(7) H. Gutfreund and B. R. Hammond [*Biochem. J.*, **73**, 526 (1959)] report a similar phenomenon in the α-chymotrypsin-catalyzed hydrolysis of N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester at pH 7.2, but do not indicate that the reaction is stoichiometric.

(8) M. L. Bender and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2550 (1962); F. J. Kézdy and M. L. Bender, *Biochem.*, **1**, 1097 (1962).

(9) Although the requirement of $(E)_0 > (S)_0$ was always obeyed, the requirement of $(E)_0 > K_m$ is problematical because of experimental limitations so that some rate constants may not be true maximal rate constants at saturation conditions.

(10) F. J. Kézdy and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 938 (1964).

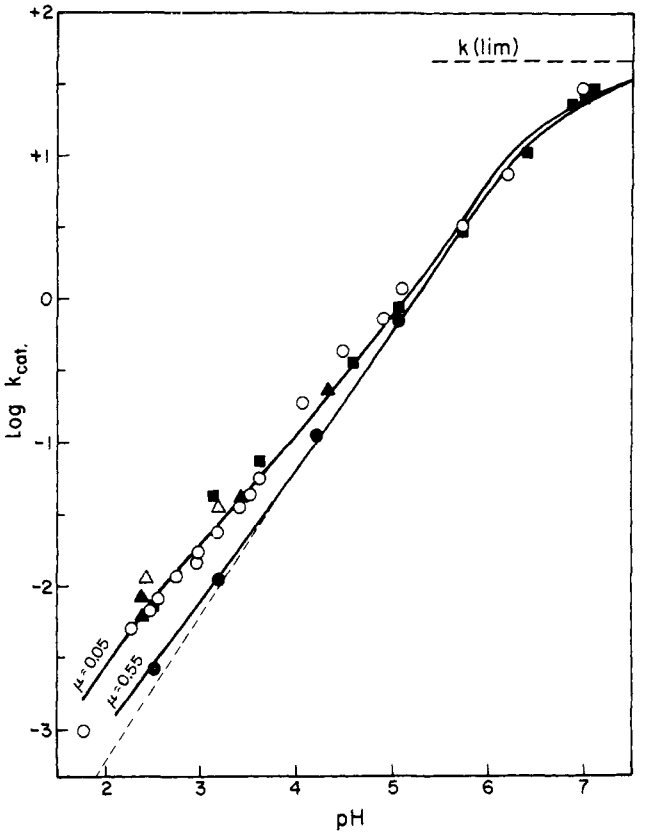

Fig. 1.—The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan derivatives at 25.0°, 1.6% acetonitrile–water. μ = 0.05: ○, catalytic rate constant of *p*-nitrophenyl ester; ■, catalytic rate constant of ethyl ester; △, deacylation constant of acid with (E) > (S); ▲, deacylation constant of methyl ester with (E) > (S). All rate constants are sec.$^{-1}$; μ = 0.55: ●, catalytic rate constant of *p*-nitrophenyl ester. Lines are theoretical lines using pH − log $\alpha/(1-\alpha)$ = $pK'_{int} - 0.868\omega Z$; ω = 0.062 for μ = 0.05 and 0.025 for μ = 0.55. Dashed line = slope of 1.

absorbance which is characteristic either of the anion of the acid and/or a mixture of the acid and acyl-enzyme.[10] The infinity absorbance of the ester hydrolysis is identical with the infinity absorbance of the reaction of the acid, N-acetyl-L-tryptophan, with α-chymotrypsin under comparable conditions,[10] indicating a true equilibrium between acyl-enzyme and acid under these conditions. The maximal amount of acyl-enzyme (position of the minimum) formed in this reaction is consistent with the calculation of two consecutive first-order reactions, the relative rate constants determined here, and the extinction coefficient of the acyl-enzyme determined later.[10] Other arguments indicating that the minimum is the acyl-enzyme include: (1) formation of the minimum is too slow to be a Michaelis complex since the latter form with rate constants approaching diffusion controlled reactions, and (2) the rate constant of the (equilibrium) decomposition of this minimum is equal to the rate constant of the (equilibrium) acylation of the enzyme by the acid.[10]

The rate constants of the reactions of the methyl ester at pH values of 2.3, 3.4, and 4.3 increase in that order, in agreement with the catalytic rate constants of the ethyl and *p*-nitrophenyl esters. It is not possible to determine precise rate constants for the formation of the intermediate, since the experimental data are too sketchy. However, the data for the decomposition of the acyl-enzyme lead to excellent first-order rate constants. The first-order rate constants from the "tail" of each reaction ($(E)_0$ = 1.8 to 3.8 × 10^{-3} *M* and $(S)_0$ = 6.8 to 7.4 × 10^{-4} *M*; $(E)_0/(S)_0$ = 3 to 5.2) are plotted in Fig. 1. These rate constants are similar to the catalytic rate constants (normal turnover constants when $(S)_0 > (E)_0$) of the corresponding ethyl and *p*-nitrophenyl esters, indicating that the "tail" of the present reactions is related to k_3, the rate-determining step of the ethyl and *p*-nitrophenyl ester reactions.[2] However, the rate constants from the "tails" of the reactions when $(E)_0 > (S)_0$ are slightly higher than the catalytic rate constants when $(S)_0 > (E)_0$, for example, k_{obsd}(tail) = 0.7 × 10^{-2} sec.$^{-1}$, whereas k_3 = 0.51 × 10^{-2} sec.$^{-1}$. This observation, however, is readily explained since $k_{obsd}(\text{tail}) = k_3 + k_5/(1 + K_m/(S))$ (a combination of deacylation and acylation of the acid) and thus k_{obsd}(tail) must be larger than k_3.[10]

Thus, the spectrophotometric observations of the hydrolysis of N-acetyl-L-tryptophan methyl ester, the pH dependence of the rate constants of the "tails" of these reactions, and the relationship of their rate constants to the catalytic rate constants of the ethyl and *p*-nitrophenyl esters indicate that direct observation indeed has been made of the formation and decomposition of an acyl–enzyme in the hydrolysis of the specific substrate, N-acetyl-L-tryptophan methyl ester.

Both the burst of *p*-nitrophenol from N-acetyl-L-tryptophan *p*-nitrophenyl ester and the time course of the reaction of N-acetyl-L-tryptophan methyl ester unequivocally point to the formation of an N-acetyl-L-tryptophanyl-α-chymotrypsin intermediate in the hydrolysis of these specific substrates. It is thus possible to generalize the acyl–enzyme mechanism represented by eq. 1.

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The Acylation of α-Chymotrypsin by N-Acetyl-L-tryptophan[1]

Sir:

α-Chymotrypsin can catalyze not only the hydrolysis of carboxylic acid derivatives but also the conversion of carboxylic acids to carboxylic acid derivatives.[2] One of these reactions, the α-chymotrypsin-catalyzed isotopic oxygen exchange of carboxylic acids and water,[3,4] has been measured kinetically and found to follow Michaelis–Menten kinetics.[5] More recently an acyl-enzyme has been postulated as an intermediate in α-chymotrypsin-catalyzed reactions,[6] which may be characterized as nucleophilic reactions.[6] One would expect that the α-chymotrypsin-catalyzed oxygen exchange of carboxylic acids would occur through the intermediacy of an acyl-enzyme, and that only the undissociated carboxylic acid would be the reactive species, even at pH 8 (since the carboxylate ion is unreactive toward nucleophiles). Thus one may write eq. 1, where E is enzyme, EAH and EA are the adsorption complexes of the protonated acid and anion, respectively, and EA′ is the acyl-enzyme. The

(1) This research was supported by grants from the National Institutes of Health, part XXVI in the series: The Mechanism of Action of Proteolytic Enzymes.

(2) M. L. Bender, *Chem. Rev.*, **60**, 95 (1960).

(3) D. B. Spriuson and D. Rittenberg, *Nature*, **167**, 484 (1951); F. Vaslow, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **30**, 45 (1956).

(4) M. L. Bender and K. C. Kemp, *J. Am. Chem. Soc.*, **79**, 116 (1957).

(5) The kinetics of the α-chymotrypsin-catalyzed synthesis of an amide from a carboxylic acid has also been investigated [O. Gawron, *et al.*, *Arch. Biochem. Biophys.*, **95**, 203 (1961)].

(6) See M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962), for a summary of current information.