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The Observation of Acyl–Enzyme Intermediates in the α-Chymotrypsin-Catalyzed Reactions of N-Acetyl-L-tryptophan Derivatives at Low pH[1]

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Investigations of the mechanism of catalysis by α-chymotrypsin were carried out in the region of pH 2 to 4. Three criteria indicate that these investigations are pertinent to the α-chymotrypsin mechanism: (1) quantitative titration of the enzyme active sites at low pH is identical with that at high pH; (2) the catalytic rate constants of the hydrolysis of the ethyl and *p*-nitrophenyl esters of N-acetyl-L-tryptophan are identical from pH 7 to 2; (3) the rate constants of several reactions form a continuous set from pH 7 to 2 dependent solely on a basic group of intrinsic pK_a 7.1. The specific acyl–enzyme, N-acetyl-L-tryptophanyl–α-chymotrypsin, at pH 2 to 4 has been observed as: (1) an intermediate in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-tryptophan *p*-nitrophenyl ester, as observed in the initial "burst" of *p*-nitrophenol under conditions when $S_0 > E_0$; (2) an intermediate in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester, as observed spectrophotometrically when $E_0 > S_0$; and (3) the product of the reaction of N-acetyl-L-tryptophan and α-chymotrypsin which consists of an equilibrium mixture of this acyl–enzyme and the parent acid, as measured spectrophotometrically and titrimetrically. Calculations of the previously measured α-chymotrypsin-catalyzed isotopic oxygen exchange of N-acetyl-L-tryptophan at pH 7.9 in terms of a mechanism involving an acyl–enzyme intermediate are consistent with the direct kinetic measurements at low pH. Thus, the previous kinetic arguments for the formation of specific acyl–enzymes are corroborated by direct observation.

Introduction

In the hierarchy of evidence for intermediate formation in a chemical reaction, indirect kinetic evidence is surpassed by both isolation of the intermediate and by direct observation of the intermediate by some chemical or physical method.[2–4] Although isolation of the acyl–enzyme intermediate in the α-chymotrypsin-catalyzed hydrolysis of a specific substrate appears difficult, the possibility of observation of the intermediate by some chemical or physical method, such as has been demonstrated with nonspecific substrates,[2,3] depends only on the development of techniques of observation which are sensitive and fast enough for such reactions. The α-chymotrypsin-catalyzed hydrolyses of the ethyl, methyl, and *p*-nitrophenyl esters of N-acetyl-L-tryptophan were shown by means of a kinetic argument to proceed through the formation of a common N-acetyl-L-tryptophanyl–α-chymotrypsin intermediate.[5,6] For the methyl ester, the half-lives at pH 7 for the formation, k_2, and the decomposition, k_3, of this intermediate (eq. 1) were calculated to be approximately 1 and 30 msec., respectively. These times are of course too fast for ordinary, or even most stopped-flow, instrumentation to measure directly. However, it was found in the previous paper[7] that both k_2 and k_3 are dependent on a basic group with a pK_a of *ca.* 7 (among other things). Therefore, at pH 3, the half-lives of the above individual steps would be expected to be of the order of tens of seconds rather than milliseconds, and thus the individual steps of the α-chymotrypsin-catalyzed hydrolysis of this specific substrate should be amenable to direct measurement at low pH.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_1} E + P_2 \qquad (1)$$
$$\qquad\qquad +P_1$$

The low pH region (from pH 2 to 7) has received surprisingly little attention in kinetic studies, although it has been known for a long time that the enzyme is much more stable in this region[8]; for example, the maximum stability of the enzyme is at pH 2 to 4, and even at pH 1.5, the enzyme is more stable than at pH 7, the pH of many kinetic investigations. Furthermore, pH 2 is often used to crystallize α-chymotrypsin.[9] The present paper gives spectrophotometric and kinetic evidence for the applicability of eq. 1 to the α-chymotrypsin-catalyzed reactions of N-acetyl-L-tryptophan, methyl ester, ethyl ester, and *p*-nitrophenyl ester in the low pH region.

Experimental

Materials.—The enzyme and the determination of the normality of its solution have been described previously.[6,7] Special attention was given here to the centrifugation of the enzyme solution at 20,000 r.p.m. for at least 45 min. in order to produce optically clear solutions of high enzyme concentration, which solutions were used within 20 min. of centrifugation. Most substrates and buffers have been described previously.[6,7] N-Acetyl-L tryptophan (Mann Research Laboratories) was used without further purification; m.p. 180°, $[\alpha]^{25}D$ +31.7° (*c* 1.23, as the anion in H_2O); lit.[10] m.p. 180–181°, $[\alpha]^{25}D$ +30 ± 1°.

Kinetic Measurements.—All kinetic measurements were carried out using a Cary 14PM recording spectrophotometer equipped with a thermostated cell compartment. The pH's of all solutions were measured at the end of each reaction, using a Radiometer 4C pH meter. Below pH 5, the following difference spectra were obtained (25.0°): (1) N-acetyl-DL-

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

(2) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(3) M. L. Bender and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2550 (1962).

(4) M. L. Bender in "Technique of Organic Chemistry," A. Weissberger, Ed., 2nd Ed., Vol. VIII, Part 2, John Wiley and Sons, Inc., New York, N. Y., 1963, Chapter 25.

(5) B. Zerner and M. L. Bender, *ibid.*, **86**, 3669 (1964).

(6) B. Zerner, R. P. M. Bond, and M. L. Bender, *ibid.*, **86**, 3674 (1964).

(7) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *ibid.*, **86**, 3680 (1964).

(8) F. L. Aldrich, Jr., and A. K. Balls, *J. Biol. Chem.*, **233**, 1355 (1958).

(9) M. Laskowski, "Methods in Enzymology," Vol. 2, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, Inc., New York, N. Y., 1955, p. 12.

(10) H. T. Huang and C. Niemann, *J. Am. Chem. Soc.*, **73**, 1541 (1951).

tryptophan + *p*-nitrophenol *vs.* N-acetyl-DL-tryptophan *p*-nitrophenyl ester: $\Delta\epsilon_{340} = 6360$; (2) N-benzyloxycarbonyl-L-tyrosine + *p*-nitrophenol *vs.* N-Benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester: $\Delta\epsilon_{340} = 6014$. N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester gave 100 ± 1% of *p*-nitrophenol, and N-acetyl-DL-tryptophan *p*-nitrophenyl ester gave 50 ± 1% of *p*-nitrophenol on enzymatic hydrolysis, as determined by these extinction coefficients. This operational criterion is the best method for determining optical purity. At 311 mμ, the following extinction coefficients were obtained: (1) N-acetyl-L-tryptophan methyl ester, $\epsilon = 40.7$; (2) N-acetyl-L-tryptophan (acid form), $\epsilon = 41.1$; (3) N-acetyl-L-tryptophan (anionic form), $\epsilon = 71.7$. The pH-dependent difference spectrum of N-acetyl-L-tryptophan ethyl ester *vs.* hydrolytic products has been described previously.[5] The ionization constant of N-acetyl-L-tryptophan was measured spectrophotometrically at 300 mμ; $pK_a = 3.63$ at 25.0° and $\mu \sim 0.1$.

Results

Titration of Active Sites at Low pH.—The spectrophotometric titration of the active sites of α-chymotrypsin by N-*trans*-cinnamoylimidazole at pH 5.05 has been described previously.[11] We have now carried out similar titrations at lower pH's in order to determine if the number of active sites is dependent on pH. It is seen in Table I that the normality of active sites of α-chymotrypsin is independent of pH from at least pH 5.05 to 3.33.

TABLE I

TITRATION OF α-CHYMOTRYPSIN BY N-*trans*-CINNAMOYLIMIDAZOLE[a]

pH	$E_0 \times 10^5$,[b] *M*	$E_0 \times 10^5$, *M*, measured	ϵ_{335} of titrant[c]
5.05	5.21	5.21	9370
3.63	5.21	5.34	13270
3.33	5.21	5.33	15140

[a] 25°; initial concentration of titrant = 9.72×10^{-5} *M*; 335 mμ; 3.17% (v./v.) acetonitrile–water. [b] As titrated at pH 5.05. [c] The extinction coefficients of the titrant were determined at each pH, because of protonation of the titrant.

Since the rate of reaction of specific substrates is diminished considerably at low pH's, it should be possible to use a *p*-nitrophenyl ester of a specific substrate as a titrant of the enzyme by observation of the stoichiometric amount of *p*-nitrophenol in the presteady-state portion of the reaction. An apparent instantaneous release of *p*-nitrophenol approximately equivalent to the enzyme concentration, followed by a slower turnover rate, has indeed been observed in the reaction of N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester with α-chymotrypsin at pH 7.2,[12] but the high rate of the reaction prohibited a quantitative estimate of the titration. Table II shows the titrations of α-chymotrypsin at low pH by two *p*-nitrophenyl ester specific substrates, N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester and N-acetyl-DL-tryptophan *p*-nitrophenyl ester. A typical spectrophotometric titration consists of: (1) the spontaneous hydrolysis of the substrate which is essentially negligible at these low pH's; (2) the rapid liberation (burst) of the stoichiometric amount of *p*-nitrophenol after the addition of the enzyme; and (3) the zero-order turnover reaction. Since the reaction is absolutely zero order over a wide substrate concentration range, $S_0 >> K_m(\text{app})$ and therefore $V = k_{cat}E_0$. The condition $S_0 >> K_m(\text{app})$ is one of two conditions that must be met so that the initial amount of liberated *p*-nitrophenol exactly equals the enzyme concentration.[13] The other condition, that $k_2 >> k_3$, is certainly satisfied with a *p*-nitrophenyl ester.

TABLE II

TITRATION OF α-CHYMOTRYPSIN BY *p*-NITROPHENYL ESTERS OF N-BENZYLOXYCARBONYL-L-TYROSINE AND N-ACETYL-DL-TRYPTOPHAN[a,d]

pH	$S_0 \times 10^5$, *M*	$E_0 \times 10^6$,[b] *M*	Measured % E_0
N-Benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester			
2.18	2.10	4.78	96.5
2.18	2.12	2.41	101.3
2.18	1.48	2.41	100
N-Acetyl-DL-tryptophan *p*-nitrophenyl ester[c]			
1.74	20.2	5.57	96.5
2.3	2.7	4.81	92
2.27	2.7	2.43	92.9
2.47	2.7	2.43	91.7
2.56	2.7	2.43	92.3
2.74	2.7	2.43	94.8
2.96	2.7	2.43	98.8
3.18	2.7	2.43	95.5
3.19	2.7	2.43	98.8
3.41	2.7	2.43	91.0
3.61	2.7	2.43	92.3
3.49	20.2	5.57	97

[a] 1.62% (v./v.) acetonitrile–water; 25.0°; 0.05 *M* citrate buffer except pH 1.74 which is HCl–KCl. [b] E_0 was determined by N-*trans*-cinnamoylimidazole titration at pH 5.05. [c] Concentrations of this substrate correspond to the L-ester only. [d] Using enzyme stock solutions made up at pH's lower than 6 gave titrations 10–30% less than theoretical at pH 2.6 with N-acetyl-DL-tryptophan *p*-nitrophenyl ester. This phenomenon is associated with the presence of an inactive enzyme in these solutions. However, the catalytic rate constant of these solutions is identical with those of pure α-chymotrypsin. This problem will be the subject of a future communication.

Since one of the *p*-nitrophenyl esters used in the titration is a DL-compound, it is pertinent to consider the complications to the titration (and the kinetics) which the racemate introduces. One possible complication is that the D-compound may act as a competitive inhibitor of the reaction of the L-compound. It may be shown mathematically that this complication can be disregarded. Another complication is the reaction of the D-compound with the enzyme to form a stable acyl-enzyme,[6] which of course prevents the enzyme from reacting with the L-ester and thus prevents a correct titration value. However, the rate constant of acylation by the D-compound is independent of the substrate concentration, a result which is found theoretically and experimentally. Under the usual conditions of our experiments, when $S_0^L >> K_m(\text{app})^L$, the turnover reaction of the L-ester will be zero order ($V = k_3E_0$) and thus the time of total hydrolysis will be inversely proportional to the initial enzyme concentration. On the other hand, the acylation by the D-ester is a reaction first order in E_0, the half-life being independent of E_0. Thus, by using high enough E_0, it is always possible to make the secondary reaction of the enzyme with the D-compound negligible with respect to the turnover reaction of the L-ester. Furthermore, by using high enough enzyme concentration, it is possible to titrate the L-ester before the much slower reaction of the D-ester occurs.

(11) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(12) H. Gutfreund and B. R. Hammond, *Biochem. J.*, **73**, 526 (1959).

(13) L. Ouellet and J. A. Stewart, *Can. J. Chem.*, **37**, 737 (1959).

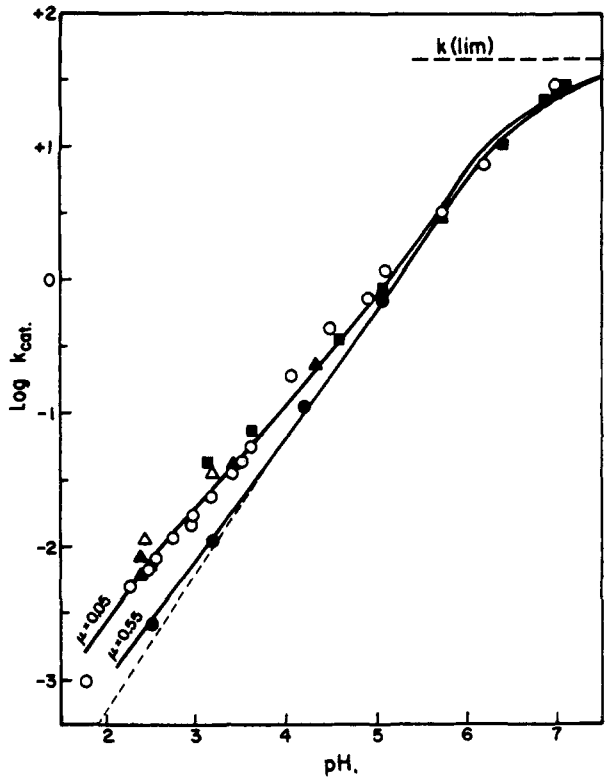

Fig. 1.—The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan derivatives at 25.0°, 1.6% acetonitrile–water. μ = 0.05: O, catalytic rate constant of *p*-nitrophenyl ester; ■, catalytic rate constant of ethyl ester; Δ, acylation constant of acid with E > S; ▲, deacylation constant of methyl ester with E > S. All rate constants in 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 = 0.051 for μ = 0.05, and 0.025 for μ = 0.55.

Table II indicates that it is indeed possible to titrate the active sites of α-chymotrypsin quantitatively with both N-benzyloxycarbonyl-L-tyrosine and N-acetyl-DL-tryptophan *p*-nitrophenyl esters at pH's from 1.74 to 3.61, and furthermore that the titrations with these specific substrates are within the experimental error of those determined by titration with N-*trans*-cinnamoylimidazole.

Kinetic Studies with N-Acetyl-DL-tryptophan *p*-Nitrophenyl Ester.—The catalytic rate constants of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl ester were determined from pH 6.97 to 1.79 using conditions in which $S_0 >> E_0$ and $S_0 >> K_m$(app). Table III and Fig. 1 summarize these results. The most important result of this study is the finding of a continuum of catalytic rate constants from pH 7 to 2, which decrease almost 10^5-fold over this range. This pH dependence may be attributed to an enzymatic basic group of $pK_a \sim 7$ over this entire range.

A large effect of ionic strength on the rate constants of this hydrolysis at low pH is seen in the data of Table III. Such an effect of ionic strength is not seen at higher pH.[7] The plot of log k_{cat} *vs.* pH (Fig. 1) is a straight line of slope ~1 from pH 7 to 2.5 at μ = 0.55; on the other hand, at μ = 0.05 this plot shows a positive deviation from that line at low pH's. These effects may be rationalized in terms of the effect of ionic strength on the high positive charge on the enzyme

TABLE III

THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-DL-TRYPTOPHAN *p*-NITROPHENYL ESTER[a]

μ, *M*	Buffer	pH	$E_0 \times 10^6$, *M*	$k_{cat} \times 10^3$, sec.$^{-1}$
0.05	Citrate	1.79	55.5	0.965
.05	Citrate	2.27	2.43	5.05
.05	Citrate	2.47	2.43	6.67
.05	Citrate	2.56	2.43	7.83
.05	Citrate	2.74	2.43	11.65
.05	Citrate	2.94	11.1	14.1
.05	Citrate	2.96	2.43	17.4
.05	Citrate	3.18	2.43	24.2
.05	Citrate	3.41	2.43	35.3
.05	Citrate	3.49	5.57	43.8
.05	Citrate	3.61	2.43	54.8
.05	Acetate	4.04	5.56	199
.05	Acetate	4.48	3.56	424
.05	Acetate	4.90	2.24	742
.05	Acetate	5.10	5.56	1200
.05	Acetate	5.72	1.13	3130
.05	Phosphate	6.19	1.14	7350
.05	Phosphate	6.97[c]		30500
.025	Citrate	2.62	1.27	8.53
.025	Citrate	3.39	1.27	33.0
.05	Acetate	4.32	0.511	239
.05	Acetate	5.14	0.103	831
.25	Citrate[b]	2.54	1.27	4.20
.25	Citrate	3.24	1.27	15.3
.25	Acetate	4.25	0.511	128
.25	Acetate	5.10	0.256	766
.35	Citrate	2.56	1.27	3.58
.35	Citrate	3.23	1.27	12.85
.35	Acetate	4.28	0.511	125
.35	Acetate	5.08	0.256	746
.45	Citrate	2.54	1.27	3.46
.45	Citrate	3.20	1.27	11.4
.45	Acetate	4.24	0.511	121
.45	Acetate	5.10	0.256	747
.55	Citrate	2.51	1.27	2.82
.55	Citrate	3.18	1.27	10.8
.55	Acetate	4.21	0.511	112
.55	Acetate	5.08	0.256	700

[a] At 25° in 0.82% (v./v.) acetonitrile–water; S_0^L = 1.788 to 20.1 × 10^{-6} *M*. [b] Potassium chloride added to adjust the ionic strength. [c] From ref. 5.

at low pH; this charge will perturb the ionization of the basic group of the active site electrostatically, tending to convert it to the free base, relative to that expected from the ionization constant in aqueous solution. When the ionic strength is low, this effect will be the most important, whereas when the ionic strength is high, this effect will tend to disappear. Such electrostatic effects on protein ionizations have been encountered many times before,[14] and can be treated by the use of an electrostatic model which assumes that the protein is a symmetrically charged sphere whose charge varies with pH.[14] The equation of this model is

$$pK_{int} = pH - \log(\alpha/(1-\alpha)) + 0.868\omega Z \quad (2)$$

where pK_{int} is the intrinsic pK_a of the catalytically active basic group of the enzyme, α is the fraction of the prototropic group in its catalytically active (basic) form (in this case, equal to k_{cat}/k_{cat}(lim), while $(1-\alpha)$ equals $(k_{cat}(\text{lim}) - k_{cat})/(k_{cat}(\text{lim}))$, Z is the total charge on the enzyme, and ω is an empirical parameter which is dependent on the ionic strength among other things.

(14) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, p. 516.

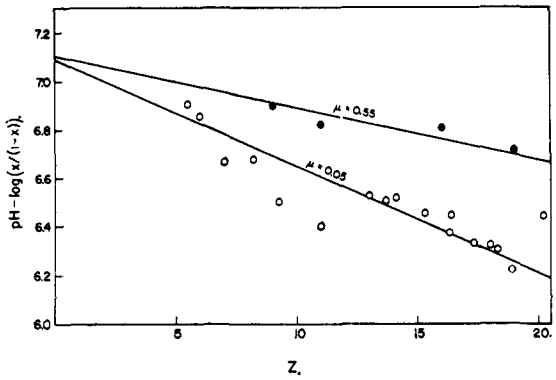

Fig. 2.—Electrostatic effects in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl ester; μ = 0.05: ω = 0.0507, pK_{int} = 7.09; μ = 0.55: ω = 0.025, pK_{int} = 7.16.

Figure 2 shows plots of this equation for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl ester at μ = 0.05 and 0.55. Satisfactory linear plots are observed, leading to an intrinsic pK_a of the catalytically important basic group of the enzyme of 7.1.

The plot of Fig. 2 requires a knowledge of Z at all pH's at the appropriate ionic strength. The prototropic titration of α-chymotrypsin at μ = 0.15[15] allows the calculation of ω at μ = 0.05 and 0.55 M, and thus the calculation of Z at any pH at these two ionic strengths. Using the intrinsic pK_a of the catalytically important basic group of 7.1 and the values of ω at the two ionic strengths, it is possible to calculate a theoretical log k_{cat} vs. pH curve according to eq. 2. The comparison of the theoretical curve and the experimental points at both ionic strengths is quite satisfying (Fig. 1).[16]

Spectrophotometric Studies with N-Acetyl-L-tryptophan Methyl Ester.—At this juncture, the catalytic rate constant of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl ester at various pH's is known. This constant may be reasonably equated to the deacylation constant, k_3, of eq. 1, on the basis of the observations at pH 7,[6] and of the stoichiometric and instantaneous burst of *p*-nitrophenol from this compound. This is indeed a slow reaction at pH's 2 to 4, with half-lives of the order of 500 to 5 sec., respectively. The k_3 of the methyl ester must be equivalent to that of the *p*-nitrophenyl ester and, furthermore, k_2 of the methyl ester is only of the order of 20 times larger than k_3.[6] Thus, in the pH range of 2 to 4, both k_2 and k_3 should have half-lives of the order of seconds and be directly observable.

The technique of the methyl ester experiments consisted in spectrophotometrically observing the time course of the reaction under conditions of $E_0 > S_0$ and $E_0 > K_s$ so that two consecutive first-order processes are observed, as demonstrated with methyl cinnamate.[3] When the substrate is saturated with enzyme (*sic*), when the enzyme is in greater concentration than the substrate, and when k_2 is greater than k_3, as is expected for the methyl ester by analogy with the results at pH 7, the initial first-order process should measure k_2 and the final first-order reaction should measure k_3. Unfortunately, a large background absorbance caused by the enzyme (which leads to stray light problems) and a small change in absorbance resulting from the small difference in extinction coefficients between the methyl ester, the acyl–enzyme, and the acid prevents the attainment of clean first-order conditions; E_0 was always greater than S_0 but the ratios E_0/S_0 were only ~2.5 to 5. Furthermore, for reasons mentioned above, the attainment of the condition $E_0 > K_s$ is also problematical. Therefore, the rate constants observed in these experiments may not be true maximal rate constants at saturation conditions, and thus must be considered only as semiquantitative measurements.

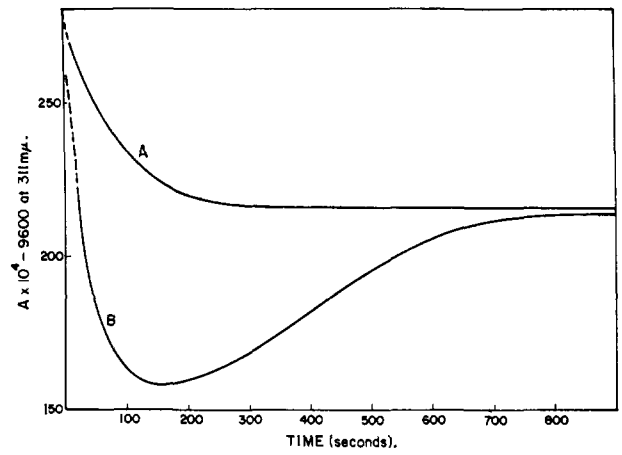

Fig. 3.—The reactions of N-acetyl-L-tryptophan (A) and N-acetyl-L-tryptophan methyl ester (B) with α-chymotrypsin at pH 2.42 and 25.0°; 0.05 M citrate buffer; 1.64% acetonitrile–water $E_0 = 1.83 \times 10^{-3}$ M; $(acid)_0 = (ester)_0 = 6.8 \times 10^{-4}$ M. K_{obsd} (acid) ≦ 1.1×10^{-2} sec.$^{-1}$; k_{tail} (ester) ≦ 0.7×10^{-2} sec.$^{-1}$; k_3 (NP ester) = 0.66×10^{-2} sec.$^{-1}$. The curves are Cary 14 PM spectrophotometer traces in which the noise level was $\pm 3 \times 10^{-4}$ absorbance unit.

Nonetheless, it is possible to observe the stepwise process in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester. A typical experiment is shown in Fig. 3. In each experiment, the initial absorbance of the ester decreased rapidly, reached a minimum, and then slowly rose, reaching an infinity absorbance which is characteristic either of the anion of the acid and/or a mixture of the acid and acyl–enzyme. The infinity absorbance of the ester hydrolysis was identical with the infinity absorbance of the reaction of the acid, N-acetyl-L-tryptophan, with α-chymotrypsin under comparable conditions, indicating a true equilibrium between acyl–enzyme and acid under these conditions. The maximal amount of acyl–enzyme (as determined by the position of the minimum of the absorbance *vs.* time curve) formed in this reaction is consistent with a crude calculation carried out using the kinetics of two consecutive first-order reactions, the relative rate constants determined here, and the extinction coefficient of the acyl–enzyme. 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 leads to reasonable first-order rate constants. The first-order rate constants from the "tail" of each reaction are listed in Table IV and are plotted in Fig. 1.

(15) M. A. Marini and C. Wunsch, *Biochemistry*, **2**, 1457 (1963).

(16) These results do not substantiate the recent kinetic evidence for a functional carboxy group; J. A. Stewart, H. S. Lee, and J. E. Dobson, *J. Am. Chem. Soc.*, **85**, 1537 (1963).

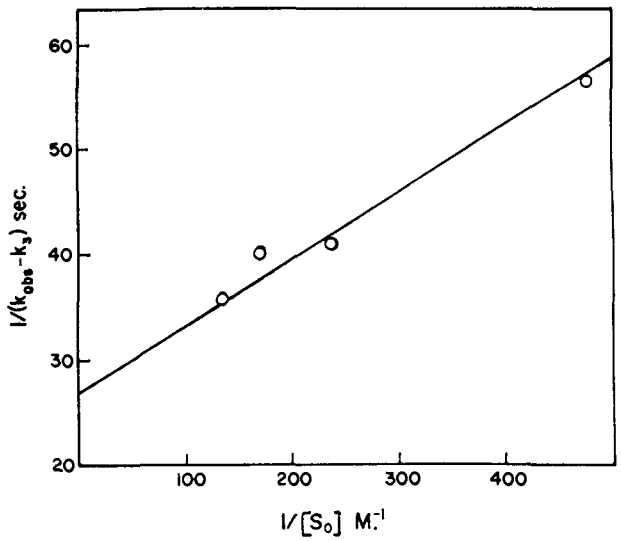

Fig. 4.—The kinetics of acylation of α-chymotrypsin by N-acetyl-L-tryptophan at 25° and pH 2.3.

The Acylation of α-Chymotrypsin by N-Aceyl-L-tryptophan.—α-Chymotrypsin can catalyze not only the hydrolysis of carboxylic acid derivatives but also the conversion of carboxylic acids to carboxylic acid derivatives.[16] The α-chymotrypsin-catalyzed isotopic oxygen exchange of carboxylic acids and water[17-19] has been measured kinetically and found to follow Michaelis–Menten kinetics.[20] Using the hypothesis that the α-chymotrypsin-catalyzed oxygen exchange of carboxylic acids occurs through the intermediacy of an acyl–enzyme, and that only the undissociated form of the carboxylic acid would be the reactive species, even at pH 8, since the carboxylate ion should be unreactive in this nucleophilic[21,22] reaction, one may write eq. 3, where E is enzyme; EAH and EA, the adsorption complexes of the protonated acid and anion, respectively; and EA' the acyl–enzyme.

TABLE IV

THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-L-TRYPTOPHAN METHYL ESTER AT LOW pH[a]

pH	$S_0 \times 10^4$, M	$E_0 \times 10^4$, M	$k_{(tail)}$, sec.$^{-1}$
2.41	7.0	3.86	$\sim 6.8 \times 10^{-3}$
3.12	7.8	2.12	1.4×10^{-2}
3.18	7.0	3.02	3.14×10^{-2}
3.35	7.8	2.12	3.56×10^{-2}
3.47	7.0	3.19	4.7×10^{-2}
4.32[b]	7.0	3.16	2.39×10^{-1}

[a] At 25.0° in 1.6% (v./v.) acetonitrile; 0.05 M citrate buffer; $\mu = 0.05$. [b] Acetate buffer; $\mu = 0.05$.

$$\begin{array}{ccccc} E + AH & \underset{}{\overset{K_s^{AH}}{\rightleftharpoons}} & EAH & \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} & EA' + H_2O \\ K_s^{AH}\uparrow\downarrow & & \uparrow\downarrow K_s^{EAH} & & \\ E + H^+ + A^- & \underset{}{\overset{K_i^A}{\rightleftharpoons}} & EA + H^+ & & \end{array} \quad (3)$$

The isotopic oxygen exchange experiments were carried out at pH 7.9, where essentially all of the acylamino acid is in the anionic form, A^-, a form not conducive to reaction. However, at low pH, essentially all of the acylamino acid is in the reactive protonated acid form, AH. Under these conditions, the top line of eq. 3 is solely operative and an equilibrium acylation of the enzyme by an acylamino acid such as N-acetyl-L-tryptophan should be observable. From eq. 3 when $H > K_s^{AH}$ and $S >> E$

$$1/(k_{obsd} - k_{-3}) = 1/k_3 + K_s^{AH}/k_{-3}S \quad (4)$$

and

$$(E_0/EA') - 1 = (k_3/k_{-3}) + K_s^{AH}k_3/k_{-3}S_0 \quad (5)$$

where k_{obsd} is the first-order rate constant for the equilibrium disappearance of enzyme.

Equations 4 and 5 were tested by determining the amount of N-acetyl-L-tryptophanyl-α-chymotrypsin formed from the acid. The titration of the active sites of the enzyme with N-acetyl-L-tryptophan *p*-nitrophenyl ester described previously was used as an analytical tool since this reaction occurs much faster than the acylation by the acid and since both the carboxylic acid, N-acetyl-L-tryptophan, and the titrating agent, N-acetyl-L-tryptophan *p*-nitrophenyl ester, should give a common acyl–enzyme intermediate. Under these conditions, the following phenomena should occur. (1) If the enzyme, the acid, and (a high concentration of) the *p*-nitrophenyl ester are mixed simultaneously, the presence of the acid should only have a small inhibitory effect on the initial burst of *p*-nitrophenol and on the steady-state rate constant, as shown in eq. 6 and 7, respectively. In eq. 6, it is assumed that $k_3 << k_2$ for the *p*-nitrophenyl ester and π is the observed burst of *p*-nitrophenol.[13]

$$\pi/E_0 = 1/(1 + (K_m(app)/S_0)(1 + I/K_i))^2 \quad (6)$$

$$V = k_{cat}E_0/(1 + (K_m(app)/S_0)(1 + I/K_i) \quad (7)$$

(2) On the other hand, if the acid and the enzyme are premixed so that reaction may take place to form the acyl–enzyme, the addition of the *p*-nitrophenyl ester should result in a smaller burst as a consequence of the smaller number of unoccupied active sites. However, the steady-state rate should remain identical with that found in (1).

The results of such experiments at pH 2.3 as exemplified by Table V show that the amount of titratable enzyme active sites, in the presence of N-acetyl-L-tryptophan, decreases slightly at zero time as predicted by eq. 6, but decreases markedly at finite times, approaching finally a limiting value. On the other hand, the zero-order velocity of the reaction is constant irrespective of the time of contact between the enzyme and N-acetyl-L-tryptophan, although all measured velocities are slightly diminished in the presence of the acid owing to the inhibition predicted by eq. 8. From the data of Table V, a first-order rate constant for the disappearance of enzyme (k_{obsd} of eq. 4) may be calculated. Using different values of the substrate concentration, values of k_{-3} and K_s^{AH} were obtained according to eq. 4 as shown in Fig. 4. Furthermore, the equilibrium concentration of acyl–enzyme (EA') was measured as a function of the substrate concentra-

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TABLE V

THE ACYLATION OF α-CHYMOTRYPSIN BY N-ACETYL-L-TRYPTOPHAN AS DETERMINED BY TITRATION WITH N-ACETYL-L-TRYPTOPHAN *p*-NITROPHENYL ESTER[a]

N-Acetyl-L-tryptophan × 10³, *M*	Time,[b] sec.	Burst × 10⁴, A. U.	Free enzyme, %	V/E_0 of ester × 10², sec.⁻¹
0	0	281	100[d]	1.013[c]
5.94	0	237	100	0.886
5.94	10	217	91.6	.889
5.94	20	177	74.7	.912
5.94	30	142	59.9	.902
5.94	40	105	44.3	.882
5.94	70	98	41.3	.915
5.94	400	74	31.2	.873

[a] 25.3; pH 2.3; citrate buffer, $\mu = 0.01$; burst of *p*-nitrophenol measured at 340 mμ; N-acetyl-DL-tryptophan *p*-nitrophenyl ester = 5.46×10^{-5} *M*; $E_0 = 4.82 \times 10^{-6}$ *M*, 0.8% (v./v.) acetonitrile–water. [b] Time elapsed between the addition of the enzyme and the *p*-nitrophenyl ester to the solution. [c] This value corresponds to k_{cat}; all others in this column are slightly (and equally) depressed owing to inhibition. [d] After 500 sec., the enzyme gave exactly the same titration value.

tion, leading to the calculation of k_{-3}/k_3 according to eq. 5 as shown in Fig. 5. The results of these calculations are shown in Table VII.

It is also possible to carry out a direct spectrophotometric observation of the formation of the acyl-enzyme (Fig. 3). The experiment was carried out in a manner similar to the experiment described previously for the corresponding methyl ester, that is, with $E_0 > S_0$ and $E_0 > K_s$. As with the methyl ester experiments, the twin difficulties of a high background absorbance caused by the enzyme and a small difference in extinction coefficients between the acid and the acyl-enzyme prevented full attainment of pure maximal first-order constants. The infinity absorbance (percentage acyl-enzyme at equilibrium) and the rate of approach to infinity ($k_3 + k_{-3}/(1 + K_s/E_0)$) are essentially identical for reactions starting with both the acid and the methyl ester as seen in Table VII and Fig. 3.

Discussion

This paper describes a number of investigations of the mechanism of catalysis of α-chymotrypsin in the region of pH 2 to 4. In order that these investigations have validity in the general problem of α-chymotrypsin mechanism, it is necessary to prove that no extraneous factors intrude in the low pH region. Titration of the concentration of enzymatic active sites by N-*trans*-cinnamoylimidazole at pH 5.05 has been shown to be equivalent to titration of the enzymatic sites by the same compound as low as pH 3.33, indicating no change in the intervening region. Furthermore, titration by N-*trans*-cinnamoylimidazole at pH 5.05 has been shown to be equivalent to titration by the specific *p*-nitrophenyl esters, N-benzyloxycarbonyl-L-tyrosine and N-acetyl-DL-tryptophan *p*-nitrophenyl esters, from pH 1.74 to 3.61. Finally it is known that titration by N-*trans*-cinnamoylimidazole at pH 5.05 is equivalent to titration by *p*-nitrophenyl acetate at pH 7.[23] At pH 7, the catalytic rate constants (turnover) 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.[6] The catalytic rate constants of these two reactions are essentially identical from pH 7 down to 2 (see Fig. 2). Thus the

(23) F. J. Kézdy, unpublished observations in this laboratory.

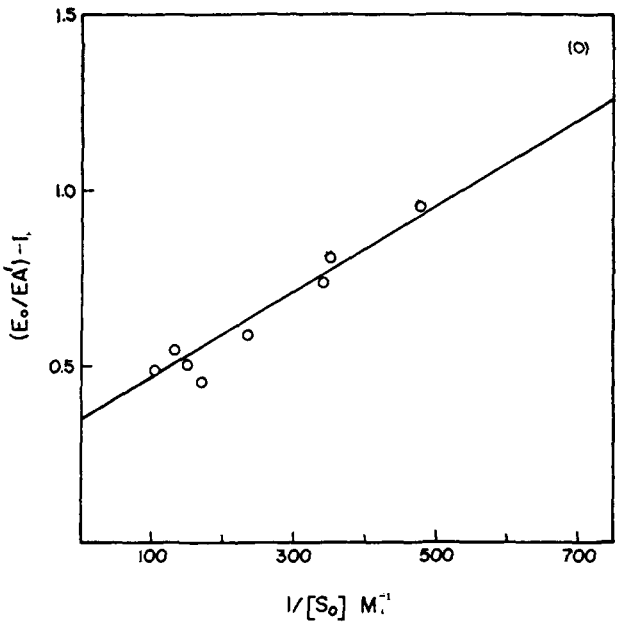

Fig. 5.—Equilibrium measurements of the acylation of α-chymotrypsin by N-acetyl-L-tryptophan at 25° and pH 2.3.

rate-determining decomposition of a common N-acetyl-L-tryptophanyl-α-chymotrypsin intermediate for these two reactions is independent of pH from pH 7 to 2. Finally, the catalytic rate constant of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl ester is dependent on a group with an intrinsic pK_a of 7.1 over the entire pH range from pH 7 to 2. As discussed above, sizable ionic strengths become important at low pH because of the high positive charge of the enzyme in that region, but these ionic strength effects have been quantitatively treated in terms of the "charged sphere" model of the enzyme. Thus the titration of active sites, the relative rates of reaction, and the pH dependence of the enzyme appear to be normal as low as pH 2, and it appears feasible to carry out mechanistic investigations at low pH.

It is of interest to compare the catalytic rate constants of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl and ethyl esters ($S_0 > E_0$) with the rate constants of the tail of the reaction of α-chymotrypsin with N-acetyl-L-tryptophan methyl ester ($E_0 > S_0$) and with the rate constants of the reaction of α-chymotrypsin with N-acetyl-L-tryptophan ($E_0 > S_0$). Such a comparison is shown in Table VI. The data of Table VI indicate that the rate constant from the "tail" of the methyl ester reaction and the rate constant from the acid reaction are as large as twice the catalytic rate constant of the turnover reactions of the ethyl and *p*-nitrophenyl ester reactions. This result is to be expected since the former rate constants are equal to $(k_3 + k_{-3}/(1 + K_s/E_0))$ while the latter catalytic rate constants are simply equal to k_3. Since $k_{-3}/(1 + K_s/E_0)$ ($\cong k_{-3}$ under these conditions) has approximately the same value as k_3 at this pH (*vide infra*), the value of the combined constant observed in reaction when $E_0 > S_0$ should be approximately twice that of k_3 alone determined when $S_0 > E_0$. The numbers quoted in Table VI for the methyl ester and acid have associated with them rather large errors (*vide supra*); in spite of these difficulties, however, a reasonable correspondence is seen between

TABLE VI

REACTIONS OF α-CHYMOTRYPSIN WITH N-ACETYL-L-TRYPTOPHAN DERIVATIVES AT pH 2.42[a]

Derivative	Condition	$k \times 10^4$, sec.$^{-1}$
p-Nitrophenyl ester[b]	$S_0 > E_0$	0.66
Methyl ester	$E_0 > S_0$	~ .7
Acid	$E_0 > S_0$	.9–1.1

[a] 25°; μ = 0.05. [b] Interpolated from Table III.

the methyl ester and acid, and also a semiquantitative comparison between these two reactions and the *p*-nitrophenyl ester reaction is found.

The data for the acylation of α-chymotrypsin by N-acetyl-L-tryptophan at pH 2.3 presented in Fig. 4 and 5 have been used to calculate the values of the acylation rate constant, k_5, and the Michaelis constant, K_s^{AH} (see eq. 4 and 5), in conjunction with the value of k_3 determined from the turnover of the *p*-nitrophenyl ester. These values are listed in Table VII.

TABLE VII

THE KINETICS OF ACYLATION OF α-CHYMOTRYPSIN BY N-ACETYL-L-TRYPTOPHAN[a]

pH	k_{-3}, sec.$^{-1}$	k_3, sec.$^{-1}$	k_{-3}/k_3	$K_s^{AH} \times 10^3$, *M*
7.9[b]	323	46.5	6.96	1.18
2.3[f]	3.77×10^{-2}[c]	1.0×10^{-2}[d]	3.77	2.4
2.3[f]	2.86×10^{-2}	1.0×10^{-2}[d]	2.86[e]	3.46

[a] 25.0°. [b] From isotopic oxygen exchange experiments using eq. 2. [c] From eq. 3. [d] k_3 determined from the steady-state portion of the *p*-nitrophenyl ester hydrolysis. [e] From eq. 4. [f] Citrate buffer, μ = 0.01; these rate constants cannot be directly compared with those of the *p*-nitrophenyl ester because of the difference in ionic strength.

Also listed in Table VII is a value for the acylation constant k_{-3} at pH 7.9, determined from the kinetics of the α-chymotrypsin-catalyzed isotopic oxygen exchange of N-acetyl-L-tryptophan at pH 7.9.[19] On the basis of eq. 3, the relationship between the observed rate constant of isotopic oxygen exchange and k_{-3} at $H << K_i$ is

$$k_{obsd} = k_{-3}K_I^A(H)/2K_s^{AH}K_i \qquad (8)$$

Equation 8 takes into account the ionization of the carboxylic acid, AH, the inhibitory properties of the carboxylate ion, A^-, and a difference of two necessitated by the equivalence of the two oxygen atoms in the carboxylic acid group. At pH 7.9 and 25.0°, the oxygen exchange of N-acetyl-L-tryptophan exhibited: $k_{obsd} = 2.98 \times 10^{-2}$ sec.$^{-1}$ and $K_m(app) = 4.0 \times 10^{-3}$ *M* (essentially equivalent to K_i^A).[19] Using these constants, an experimental value of $K_i = 2.34 \times 10^{-4}$ *M*, and a value of K_s^{AH} equal to the (real) K_s for the corresponding methyl ester, N-acetyl-L-tryptophan methyl ester, a compound which is structurally and electronically similar, 1.18×10^{-3} *M*,[24] it is then calculated that k_{-3} = 323 sec.$^{-1}$. Thus at pH 7.9, acylation of α-chymotrypsin by N-acetyl-L-tryptophan (the undissociated acid), k_{-3} = 323 sec.$^{-1}$, is faster than deacylation of N-acetyl-L-tryptophanyl-α-chymotrypsin, k_3 = 46.5 sec.$^{-1}$.[7]

(24) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3697 (1964).

The agreement in k_{-3}/k_3 ratios shown in Table VII between the two methods at pH 2.3 and between these and the calculations at pH 7.9 are reasonably good, confirming the various experimental approaches to the elucidation of eq. 3. Furthermore, the absolute rate constants, k_{-3}, at the two pH's may be used to calculate the pK_a of the enzymatic group on which this reaction depends; two independent sets of calculations which combine lines one and two or lines one and three of Table VII yield pK_a's of 6.56 and 6.86, respectively, quite similar to that found for the acylation with N-acetyl-L-tryptophan ethyl ester, 6.77.[7]

A considerable body of evidence indicates that the spectrophotometric observations carried out at pH 2.4 of excess α-chymotrypsin with both N-acetyl-L-tryptophan and N-acetyl-L-tryptophan methyl ester do actually demonstrate the formation of the specific acyl–enzyme, N-acetyl-L-tryptophanyl-α-chymotrypsin. This evidence includes: (1) the rate of formation of the minimum in the ester reaction and the rate of formation of the equilibrium in the acid reaction are too slow to reflect the formation of Michaelis complexes since the latter usually form with rate constants approaching diffusion-controlled reactions[25]; (2) the rate constant of the (equilibrium) decomposition of the minimum in the ester reaction is equal to the rate constant of the (equilibrium) acylation of the enzyme by the acid; (3) the equilibrium (infinity) position of both the ester and acid reactions are identical with one another under identical conditions; (4) the spectrophotometric experiments are consistent with the titration of the active sites of the enzyme using the specific titrant, N-acetyl-L-tryptophan *p*-nitrophenyl ester; (5) the pH dependence of the rate constants of both the ester and acid reactions are consistent with those of normal enzymatic (turnover) reactions; (6) the rate constants of the ester and acid reactions are closely related to the catalytic rate constants of the ethyl and *p*-nitrophenyl ester hydrolyses; and (7) the relative rate constants of the formation and decomposition of the intermediate in the methyl ester reaction are consistent with those calculated at pH 7.0.

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. This conclusion is substantiated by the demonstration of the equilibrium formation of this stable acyl–enzyme from the parent acid, N-acetyl-L-tryptophan. The experimental evidence presented here indicates that these α-chymotrypsin-catalyzed reactions proceed exclusively through acyl–enzyme intermediates. The experimental evidence presented here is completely consistent with the kinetic arguments concerning reactions at neutrality and above, described previously.[6] Thus, it is possible to generalize the acyl–enzyme mechanism represented by eq. 1.

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