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# The Correlation of the pH (pD) Dependence and the Stepwise Mechanism of α-Chymotrypsin-Catalyzed Reactions[1]

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The pH dependence of the acylation of α-chymotrypsin by *p*-nitrophenyl acetate, and of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide, N-acetyl-L-phenylalanine amide, and N-acetyl-L-tryptophan ethyl ester have been determined. The acylation of α-chymotrypsin by *p*-nitrophenyl acetate exhibits a bell-shaped pH–$k_2$ profile, indicating that the reaction is dependent on two ionizable groups, an acid of p$K_a$ 9 and a base of p$K_a$ 7. The deacylation of several acyl-α-chymotrypsins exhibits a sigmoid pH–$k_3$ profile, indicating that the reaction is dependent on one ionizable group, a base of p$K_a \sim 7$. The pH–$k_{cat}$ profiles of the α-chymotrypsin-catalyzed hydrolysis of the two specific amide substrates named above whose catalytic rate constants have been previously postulated to be determined solely by acylation, $k_2$, are predicted on this basis to exhibit bell-shaped curves; this is found experimentally. The pH–$k_{cat}$ profiles of the α-chymotrypsin-catalyzed hydrolysis of the specific ester substrate named above, whose catalytic rate constant has been previously postulated to be determined by deacylation, $k_3$, at pH 7, is predicted on this basis to exhibit a sigmoid curve up to some high pH at which point a change in rate-determining step is predicted which will result in the sigmoid curve changing to a bell-shaped curve; this behavior is found experimentally and can be quantitatively fitted by such a scheme. Thus all pH dependencies of specific substrates are consistent with the stepwise mechanism of the reaction involving an acyl–enzyme intermediate. The effects of deuterium oxide on the α-chymotrypsin-catalyzed hydrolyses of N-acetyl-L-tryptophan amide and of N-acetyl-L-tryptophan ethyl ester were determined. As found in the hydrolyses of nonspecific substrates, a deuterium oxide kinetic isotope effect ($k^{H_2O}/k^{D_2O}$) of 2–3 is found, the isotope effect being slightly higher when $k_{cat}$ reflects deacylation (ethyl ester) than when $k_{cat}$ reflects acylation (amide). An equilibrium isotope effect is also found, the p$K_a$'s of the groups on which the enzymatic reactions are dependent being 0.5 ± 0.2 p$K$ unit higher in deuterium oxide than in water. These isotope effects are attributed to the involvement of a proton transfer in the rate-determining steps of the enzymatic catalysis.

## Introduction

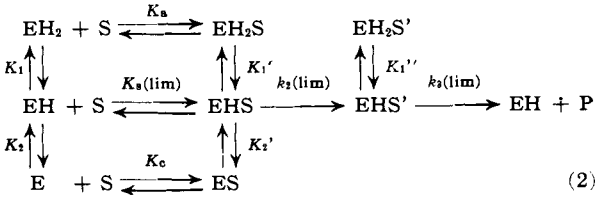
The stepwise mechanism of α-chymotrypsin reactions shown in eq. 1 leads to the possibility that more than one pH dependence may be seen, depending on the rate-

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

determining step of the reaction[5] and on the pH dependence of the individual steps. An analysis of fragmentary literature data on the α-chymotrypsin-catalyzed hydrolyses of specific ester and amide substrates indicated that more than one pH dependency may in fact be observable.[6] The pH dependency of the deacylation, $k_3$, of acyl-α-chymotrypsins has been determined in detail previously.[7] We therefore have investigated the pH dependency of a discrete acylation step, the acylation of α-chymotrypsin by *p*-nitrophenyl acetate, and the pH dependency of the α-chymotrypsin-catalyzed hydrolysis of two specific amide substrates, N-acetyl-L-tryptophan amide and N-acetyl-L-phenylalanine amide, whose rate-determining step has previously been postulated to be acylation,[5] and one specific ester substrate, N-acetyl-L-tryptophan ethyl ester, whose rate-determining step has previously been postulated to be deacylation.[5]

The pH dependence of a discrete acylation step, $k_2$, will give a direct measure of the kinetically important prototropic equilibria of ES; $k_2$ is found here to be dependent on two ionizable groups, an acid and a base. The pH dependence of $k_2/K_s$ will give a direct measure of the kinetically important prototropic equilibria of E; $k_2/K_s$ is found here to be dependent on two ionizable groups, an acid and a base, in a similar fashion to $k_2$. The pH dependence of the discrete deacylation step, $k_3$, will give a direct measure of the kinetically important prototropic equilibria of ES'; $k_3$ has previously been found to be dependent on one ionizable group, a base.

On the basis of the above facts, a minimal elaboration of eq. 1 may be proposed to take into account the effect of pH on each of the constituent parts of the reaction.




(2)

Equation 2 leads directly to the pH dependencies of $K_s$, $k_2$, and $k_3$ given in eq. 3–5.

$$K_s = K_s(\text{lim})\frac{1 + (H/K_1) + (K_2/H)}{1 + (H/K_1') + (K_2'/H)} \qquad (3)$$

$$k_2 = \frac{k_2(\text{lim})}{1 + (H/K_1') + (K_2'/H)} \qquad (4)$$

$$k_3 = k_3(\text{lim})/(1 + (H/K_1'')) \qquad (5)$$

These equations are quite similar to those given previously[8-11] for similar processes, with the exception that here only one prototropic ionization is given for ES,

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(2) (a) N. I. H. Postdoctoral Fellow; (b) N. S. F. Predoctoral Fellow.

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whereas two such ionizations are postulated for ES and E.

The pH dependence of the reaction of a specific substrate whose rate-determining step is solely acylation should be that of a discrete acylation step, namely a bell-shaped pH–$k_{cat}$ profile. In a like manner, the pH dependence of the reaction of a specific substrate whose rate-determining step is solely deacylation should be that of a discrete deacylation step, namely a sigmoid pH–$k_{cat}$ profile. However, in the general case, provision must be made for a change in rate-determining step with pH. This can be done with the use of eq. 6 and 7, which have been shown to pertain to eq. 1,[6] in conjunction with eq. 3, 4, and 5.

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (6)$$

$$K_m(app) = (k_3/(k_2 + k_3))K_s \quad (7)$$

A plot of $V_{max}/K_m(app)$ *vs.* pH (or $k_{cat}/K_m(app)$ *vs.* pH) yields the dissociation constants of the free enzyme irrespective of the number of intermediates in the system, and hence for an enzyme which catalyzes the reaction of several substrates, the pH dependence of $k_{cat}/K_m(app)$ must be the same provided that the substrates all interact with the same groups in the enzyme.[11] This conclusion may be seen in eq. 3 and 4. The quotient of eq. 4/3 (and the quotient of eq. 6/7) give

$$k_2/K_s = k_{cat}/K_m(app) = \frac{k_2/K_s(lim)}{1 + (H/K_1) + (K_2/H)} \quad (8)$$

Thus eq. 8, 4, and 5 give the pH dependencies of the free enzyme, enzyme–substrate complex, and acyl–enzyme, respectively. The pH dependencies are seen from these equations to be a bell-shaped curve, a bell-shaped curve, and a sigmoid curve, respectively. It is within this context that the pH dependence of α-chymotrypsin-catalyzed reactions will be discussed.

As an adjunct to the studies of pH–rate constant profiles of α-chymotrypsin-catalyzed hydrolyses of specific substrates, we have also investigated the pD–rate constant profiles of the hydrolyses of two specific substrates— N-acetyl-L-tryptophan amide and N-acetyl-L-tryptophan ethyl ester. The effects of deuterium oxide on the profiles of these specific substrates supplement earlier investigations with nonspecific substrates[12a] concerning the involvement of a rate-determining proton transfer in α-chymotrypsin reactions.

## Experimental

**Materials.**—α-Chymotrypsin was a Worthington three-times crystallized product. Enzyme solutions were made up in acetate or phosphate buffers; they were centrifuged for 30 min. at 15,000 r.p.m. and their normality was determined by spectrophotometric titration with N-*trans*-cinnamoylimidazole at 335 mμ.[12b] N-*trans*-Cinnamoylimidazole was recrystallized from *n*-hexane immediately before use; m.p. 134.0–134.5°. The Worthington three-times crystallized enzyme gives a titration value of about 70–85% of that calculated on a weight basis, assuming a mol. wt. of 24,800. *p*-Nitrophenyl acetate was described previously.[13] N-Acetyl-L-tryptophan ethyl ester has been described elsewhere.[5] N-Acetyl-L-tryptophan amide was a Mann Biochemicals Co. product: it was recrystallized from methanol–ether–hexane; m.p. 194–194.5°, lit.[14] m.p. 192–193°, $[\alpha]^{23.8}D$ +18.5° (*c* 1.5, methanol), lit.[14] $[\alpha]^{23}D$ +20° (*c* 2, methanol). N-Acetyl-L-phenylalanine amide was a Cyclo Chemical Corp. product, recrystallized once from water and once from reagent grade acetone; m.p. 182–183°, $[\alpha]^{23}D$ +26.5° (*c* 1, methanol); lit.[15] m.p. 176–177°, $[\alpha]^{25}D$ 27° (*c* 1, methanol). Acetate, phosphate, Tris, and carbonate buffers were prepared from doubly distilled water and analytical reagent grade products.[16,17] Acetonitrile (Eastman Kodak spectral grade) was distilled over phosphorus pentoxide.

The deuterium oxide was General Dynamics Corp. Batch XX (> 99.5%). A solution of deuterium chloride in deuterium oxide was kindly prepared by Dr. E. Euranto according to Holmberg.[18] The buffers in deuterium oxide were prepared by dissolving dried solid disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium carbonate, and sodium hydrogen carbonate in deuterium oxide, keeping the amount of hydrogen introduced into solutions below 1%. For deuterium oxide experiments, the stock enzyme solution was also made up in deuterium oxide. The pH (pD) of all reactions was determined immediately after reaction using a Radiometer 4c pH meter; pD = meter reading + 0.40.[19]

**Kinetic Measurements.**—The kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide and N-acetyl-L-tryptophan ethyl ester in water or deuterium oxide were determined spectrophotometrically using a Cary 14 PM recording spectrophotometer equipped with a thermostated cell compartment. The observance of Beer's law was checked, and the initial and infinite absorbancies were used to check the stoichiometry of the reaction.

For N-acetyl-L-tryptophan amide, a 200- to 500-μl. aliquot of a concentrated enzyme solution was added to 3.0 ml. of the substrate in the appropriate buffer, previously equilibrated in the cell compartment of the spectrophotometer. The reference compartment of the spectrophotometer held two cells in tandem, one containing 3.0 ml. of substrate plus a 200- to 500-μl. aliquot of buffer and the other containing 3.0 ml. of buffer plus the appropriate aliquot of enzyme. In this manner it was possible to blank out *ca.* 3 absorbance units of background, and to measure initial rates of reaction using the 0 to 0.1 slide wire of the Cary spectrophotometer by following the increase in absorbance at 306 mμ for the transformation of the amide to the acid, $\Delta\epsilon = 73.8$, an average of six determinations.

The kinetics of the hydrolysis of N-acetyl-L-tryptophan ethyl ester have been described.[5] The extinction coefficient for the transformation of ester to carboxylic acid is pH dependent below pH 5, diminishing as the carboxylic acid becomes protonated so that at pH 3.12 and 2.52 the extinction coefficient difference is 65 and 28, respectively, small values that increase the experimental error of the rate constants at these pH's considerably.

The kinetics of the hydrolysis of *p*-nitrophenyl acetate was followed spectrophotometrically on both the Cary 14 PM recording spectrophotometer and the recording spectrophotometer equipped with a stopped-flow mixing device, as described previously.[13]

The kinetics of the hydrolysis of N-acetyl-L-phenylalanine amide was followed spectrophotometrically using Nessler reagent for determination of the liberated ammonia.[20]

Since in the determination of the pH dependence of various α-chymotrypsin-catalyzed hydrolyses, we were interested in investigating as wide a pH range as possible, it was mandatory to determine the stability of the enzyme over the entire pH region. In acidic solutions (pH 2.5 to 5.0) stock solutions of α-chymotrypsin of the order of $10^{-3}$ *M* are stable, as determined by titration with N-*trans*-cinnamoylimidazole,[12] for several weeks and thus the problem of denaturation at room temperature in this pH region is negligible.[21] Above pH 6 the rate of irreversible denaturation of α-chymotrypsin becomes finite and studies were made of this rate in order to determine over what pH region we

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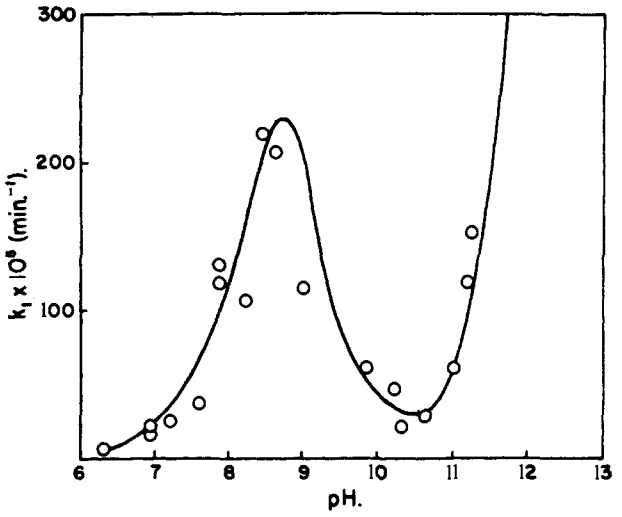


Fig. 1.—The kinetics of irreversible denaturation of α-chymotrypsin at 25.0°; buffers, 0.05 to 0.10 *M* phosphate, barbital–HCl, Tris–HCl, carbonate and sodium hydroxide.

could observe kinetics of hydrolysis without complications from irreversible denaturation. In the previous investigations, using an N-acetyl-L-tyrosine ethyl ester rate assay,[22] it was found that the rate of denaturation increased roughly with the pH with a small indication of a minimum at pH 9.15, and further that the rate of denaturation was buffer dependent. In the present investigation the rate of irreversible denaturation was determined by the spectrophotometric titration of the active sites of the enzyme with N-*trans*-cinnamoylimidazole[8] after removing aliquots from the reaction mixture and diluting them in pH 5.05 acetate buffer where any reversibly denatured enzyme would be reactivated. Most denaturations were studied kinetically to *ca.* 50% reaction where precipitation started to occur. The data shown in Fig. 1 are not comprehensive, but rather serve to illustrate the denaturation kinetics under the conditions of the present hydrolysis reactions.

Several interesting conclusions may be seen from this work. At pH 6.9, the rate of irreversible denaturation is first-order in enzyme concentration since the half-life of denaturation is independent of enzyme concentration from 8.9 to 106 9 × $10^{-5}$ *M*.[23] Although irreversible denaturation is usually described as an autolysis involving the cleavage of the peptide bonds of the enzyme,[24] the first-order kinetics, the position of the maximum in Fig. 1, and the absolute value of the minimum indicate that a hydrolytic reaction is not being observed but rather a rate-determining reversible denaturation followed by a fast hydrolytic reaction. Although it is not possible to characterize the irreversible denaturation process with these experiments, especially since salt or buffer effects were not investigated in detail, the results indicate that denaturation at pH 11.5 is not faster than it is at pH 8.5, making it possible to determine the kinetics of α-chymotrypsin catalyzed reactions up to about pH 12. In all kinetic experiments at high pH, initial rates were determined, and the time of observation of these initial rates was always regulated so that less than 2% denaturation, and in most instances less than 1% denaturation, occurred.

## Results

**The pH Dependence of the Acylation of α-Chymotrypsin by *p*-Nitrophenyl Acetate.**—The pH dependence of this reaction has been investigated at least four times. In all the previous investigations, evidence for a sigmoid pH–rate constant profile was obtained which indicated the kinetic importance of a single basic group of p$K_a$ 6.22,[25] 6.7,[26] 7.0,[27] and 7.14.[13] Whereas in previous investigations kinetics were not determined above pH 7.8, in the current investigation the kinetics of acylation were determined from pH 4.18 to 9.98 in order to determine whether the pH–$k_2$ profile is a sigmoid curve or a bell-shaped curve.

Two kinetic approaches were used. The first approach, using the stopped-flow technique, permits one to determine $k_2$ and $K_s$ separately, as defined in eq. 9

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \qquad (9)$$

These experiments were carried out with $[E]_0 << [S]_0$, as described previously.[13] At a given pH, the apparent first-order rate constants of acylation, $k_{expt1}$, were calculated using at least two of the three methods described previously and good agreement was always obtained. From $k_{expt1}$, $k_2$ and $K_s$ were calculated at a given pH using Lineweaver–Burk plots and the method of least squares. Table I summarizes the data.

TABLE I

THE KINETICS OF THE ACYLATION OF α-CHYMOTRYPSIN BY *p*-NITROPHENYL ACETATE[a]

| pH | Buffer[b,c] | $k_2$, sec.$^{-1}$ | $K_s \times 10^3$, *M* | $k_2/K_s$, $M^{-1}$ sec.$^{-1}$ |
|---|---|---|---|---|
| 4.18[d] | Acetate | ... | ... | 14.6 ± 1 |
| 5.08[d] | Acetate | ... | ... | 78.5 ± 4 |
| 5.38[d] | Acetate | ... | ... | 186 ± 15 |
| 5.91[d] | Phosphate | 0.18 ± 0.02 | 0.37 ± 0.03 | 487 ± 15 |
| 6.47[d] | Phosphate | 0.81 ± .13 | 0.72 ± .22 | 1120 ± 220 |
| 6.58 | Phosphate | 2.31 ± .31 | 1.71 ± .32 | 1350 ± 70 |
| 6.74 | Phosphate | 2.43 ± .30 | 1.24 ± .24 | 1970 ± 130 |
| 6.83 | Phosphate | 3.23 ± .24 | 1.74 ± .19 | 1850 ± 50 |
| 6.97[d] | Phosphate | ... | ... | 2350 ± 130 |
| 7.09[a] | Phosphate | 3.66 ± 0.21 | 1.47 ± 0.08 | 2490 ± 52 |
| 7.21[d] | Phosphate | ... | ... | 2850 ± 150 |
| 7.46 | Phosphate | 3.99 ± 0.44 | 1.25 ± 0.22 | 3190 ± 200 |
| 7.66 | Tris–HCl | ... | ... | 3000 ± 300 |
| 7.80[b,d] | Phosphate | 3.96 ± 0.08 | 1.12 ± 0.16 | 3600 ± 450 |
| 7.83 | Tris–HCl | ... | ... | 3410 ± 400 |
| 8.13 | Tris–HCl | ... | ... | 3330 ± 400 |
| 8.22 | Barbital | 2.82 ± 0.28 | 1.17 ± 0.19 | 2400 ± 140 |
| 8.71 | Tris–HCl | ... | ... | 2660 ± 300 |
| 8.73 | Barbital | 1.30 ± 0.10 | 0.71 ± 0.12 | 1850 ± 540 |
| 9.21 | Carbonate | 1.045 ± 0.09 | 0.58 ± 0.11 | 1810 ± 190 |
| 9.38 | Carbonate | ... | ... | 1420 ± 150 |
| 9.98 | Carbonate | ... | ... | 670 ± 70 |

[a] 1.6% (v./v.) acetonitrile–water at 25.0°. [b] The rate constants appear to be independent of the nature of the buffer. For example, $k_2/K_s$ at 7.83 = 3410 $M^{-1}$ sec.$^{-1}$ in Tris–HCl buffer while previously at pH 7.8 in phosphate buffer values from 3600–3900 $M^{-1}$ sec.$^{-1}$ were obtained.[13] [c] The buffer concentration appears to have little if any effect; buffer concentrations were 0.05–0.10 *M* total concentration. [d] From ref. 13.

The second kinetic approach involves the use of second-order kinetics with $E_0 \cong S_0 << K_s$, according to the method described previously.[13] The second-order constant, $k_2/K_s$, was used to check the results obtained from the first set of conditions, since the slope of the Lineweaver–Burk plots mentioned above directly yields $k_2/K_s$. At higher pH's, the hydroxide ion-catalyzed hydrolysis may be taken into account by the use of eq. 10.

$$(dP_1/dt)_{t=0} = S_0[(k_2/K_s)E_0 + k_{OH^-}(OH^-)] \qquad (10)$$

The second-order rate constants, $k_2/K_s$, are also listed in Table I.

Figures 2 and 3 show the pH–rate constant profiles for the acylation of α-chymotrypsin by *p*-nitrophenyl acetate using the pure (first-order) rate constant $k_2$ and the

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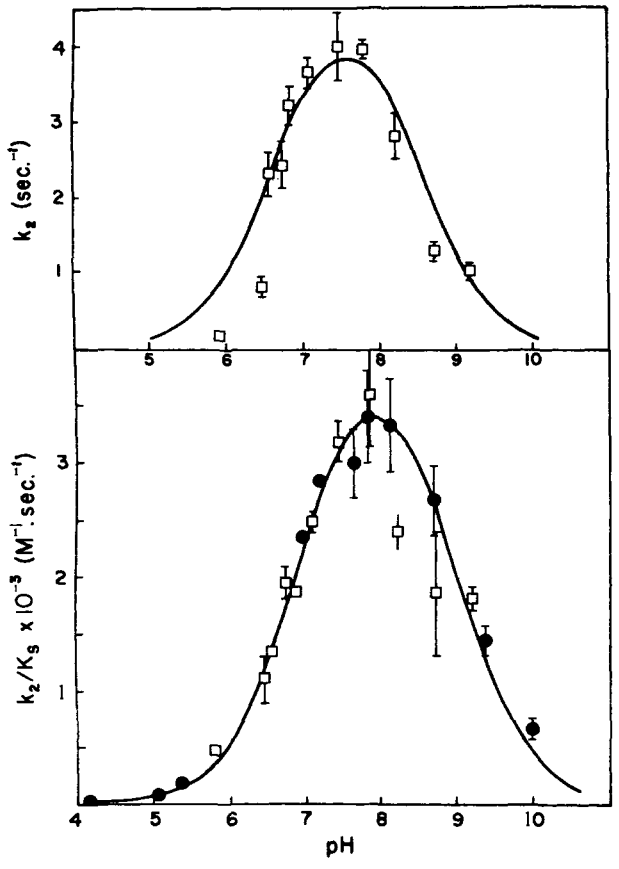


Fig. 2 and 3.—The acylation of α-chymotrypsin by *p*-nitrophenyl acetate at 25.0°: ●, from second-order kinetics; □, from first-order kinetics.

complex (second-order) constant $k_2/K_s$, respectively. Neither pH–rate profile is a sigmoid curve but rather each approximates a bell-shaped curve, which may be interpreted in terms of a double prototropic ionization. The pH–$k_2/K_s$ profile fits such a scheme, in this instance eq. 11.

Calculations of $K_1$ and $K_2$ of eq. 11 were made using Alberty's method.[8,10] In the calculation, only those

$$\begin{array}{ccc} & EH_2 & \\ & K_1 \uparrow\downarrow & \\ E \underset{K_1}{\rightleftharpoons} EH + S & \xrightarrow{(k_2/K_s)(\text{lim.})} & ES' + P_1 \end{array} \qquad (11)$$

$k_2/K_s$ values obtained experimentally from second-order kinetics (which are more reliable than the quotient derived from separate determination of $k_2$ and $K_s$) were used. This calculation, utilizing the pH's of the two half-maximal values as well as the pH of the maximum, leads to values of $pK_1 = 6.85$, $pK_2 = 9.04$, and $(k_2/K_s)(\text{lim}) = 3940\ M^{-1}$ sec.$^{-1}$. Using this set of data, the smooth curve was drawn through the experimental points of Fig. 3, which experimental points include all points from both first- and second-order kinetic experiments.

The pH–$k_2$ profile of *p*-nitrophenyl acetate also fits a scheme similar to eq. 11, except that the prototropic equilibria involve the enzyme–substrate complex, and not the enzyme. A more satisfactory method for the calculation of $K_1'$ and $K_2'$ was developed for the analysis of this curve; this graphical procedure involves the linear equation $1/k_2 = 1/k_2(\text{lim}) + (H + (H_{max}^2/H))/k_2(\text{lim})K_1'$ which is derived by an algebraic transformation of eq. 4, together with the relationship $H_{max}^2 = K_1'K_2'$. This method necessitates only the prejudging of $H_{max}$. The results of these calculations give $pK_1' = 6.59$, $pK_2' = 8.61$, and $k_2(\text{lim}) = 4.55$ sec.$^{-1}$. Both $pK_1'$ and $pK_2'$ are smaller than $pK_1$ and $pK_2$, indicating a slight dependence of $K_s$ on pH. Using the above set of data, the calculated curve of Fig. 2 was drawn.

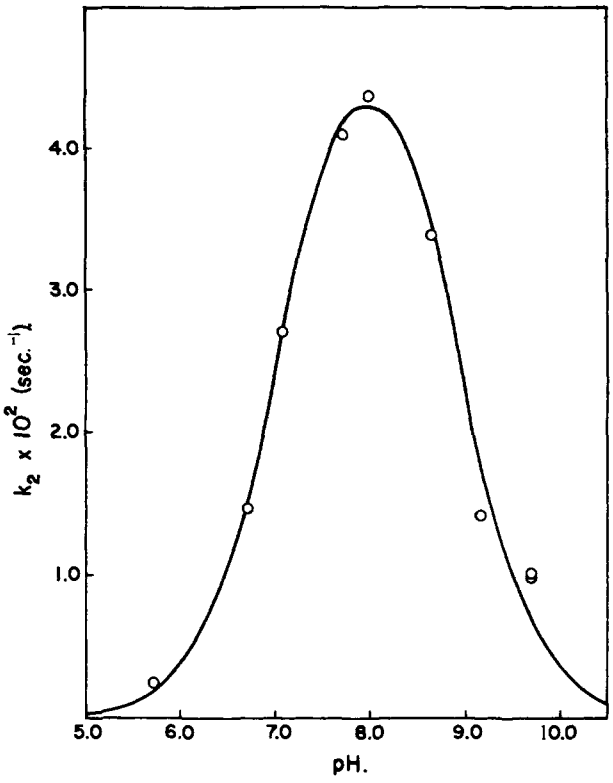


Fig. 4.—The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide in water at 25.0°.

The pH dependence of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide is presented in Table II and Fig. 4; it is seen that the pH–$k_{cat}$ profile for the amide defines a precise bell-shaped curve. The calculated curve of Fig. 4, determined according to the method of Alberty,[28] is derived from $k_{cat} = k_{cat}$

TABLE II

THE KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-L-TRYPTOPHAN AMIDE[a,b]

| pH | Buffer, 0.1 *M* | $k_{cat} \times 10^2$, sec.$^{-1}$ | $K_m$(app) × 10$^3$, *M* | $k_{cat}/K_m$(app) × 10$^1$, $M^{-1}$ sec.$^{-1}$ |
|---|---|---|---|---|
| 5.73 | Phosphate | 0.249 ± 0.013 | 3.30 ± 0.33 | 7.55 |
| 6.72 | Phosphate | 1.44 ± .11 | 3.91 ± .51 | 36.8 |
| 7.09 | Phosphate | 2.70 ± .43 | 3.26 ± .75 | 82.8 |
| 7.73 | Phosphate | 4.10 ± .76 | 3.24 ± .96 | 126.5 |
| 8.00 | Tris-HCl | 4.37 ± .90 | 4.81 ± 1.39 | 90.1 |
| 8.66 | Tris-HCl | 3.40 ± .70 | 4.55 ± 1.23 | 74.7 |
| 9.17 | Carbonate | 1.42 ± .36 | 3.21 ± 1.08 | 44.2 |
| 9.70 | Carbonate | 1.02 ± .05 | 5.61 ± 0.35 | 18.2 |
| 9.70 | Carbonate | 0.974 ± .09 | 5.38 ± .69 | 18.1 |
| 10.60 | Carbonate | .106 ± .008 | 2.47 ± .42 | 4.3 |
| 10.84 | Carbonate | .103 ± .003 | 1.23 ± .17 | 8.3 |

[a] Aqueous solution at 25.0°; $[S_0] = 0.708$ to $12.73 \times 10^{-3}$ *M*, $[E_0] = 1.0$ to $2.5 \times 10^{-4}$. The higher $[E_0]$ were used at the extreme pH's where the rate constants are smaller. [b] Computations carried out with an IBM 709 computer program designed by A. M. Myers based on a weighted regression analysis of $1/v$ *vs.* $1/S$.[28]

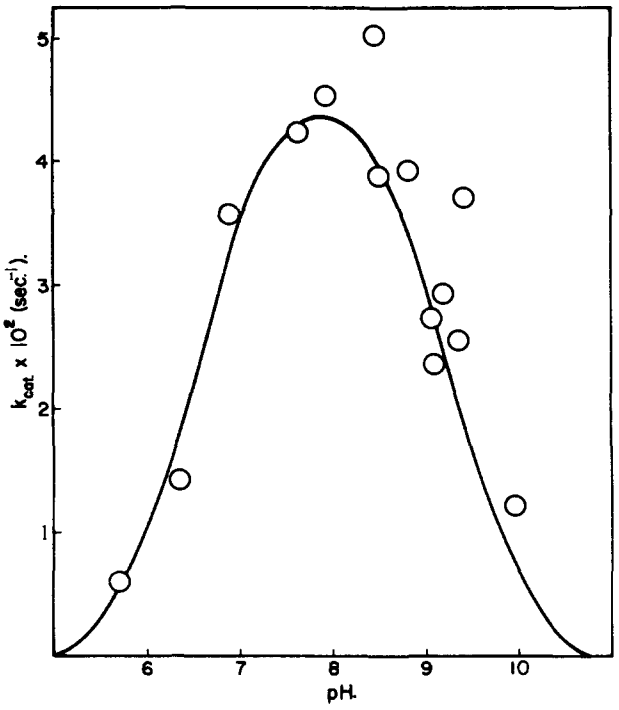


Fig. 5.—The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-phenylalanine amide in water at 25.0°.

(theoretical max)/(1 + (H/$K_1'$) + ($K_2'$/H)) using the values $k_{cat}$(theoretical max) = 5.08 × $10^{-2}$ sec.$^{-1}$, p$K_1'$ = 7.16, and p$K_2'$ = 8.9. At pH 9.05 with $E_0$ = 1.84 × $10^{-4}$ $M$, $S_0$ = 0.918 × $10^{-2}$ $M$, and ammonia = 0 to 1.5 × $10^{-2}$ $M$, no effect of ammonia on the initial rate was observed.[14]

The pH dependence of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-phenylalanine amide was determined. The values in Table III are those for the L-compound at pH 7.92, 8.44, and 9.95, while the values at other pH's are values for the DL-compound which have been corrected to the L-compound using the ratio $K_s/K_i$ found graphically to equal 2.52 (this value compares favorably with that found by Niemann, 2.4 ± 0.4).[15] The calculated bell-shaped curve of Fig. 5 was determined according to the method of Alberty,[28] using the values of $k_{cat}$ (theoretical max) = 4.76 × $10^{-2}$ sec.$^{-1}$, p$K_1'$ = 6.55, and p$K_2'$ = 9.23.

TABLE III

THE KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-L-PHENYLALANINE AMIDE[a]

| pH | Buffer | $k_{cat}$ × $10^2$, sec.$^{-1}$ | $K_m$(app) × $10^2$, $M$ | $k_{cat}/K_m$(app), $M^{-1}$ sec.$^{-1}$ |
|---|---|---|---|---|
| 5.71 | Phosphate[b] | 0.61 | 2.0 | 0.32 |
| 6.34 | Phosphate | 1.4 | (0.97) | 1.5 |
| 6.87 | Phosphate | 3.6 | 2.0 | 1.8 |
| 7.62 | Phosphate | 4.3 | 1.6 | 2.6 |
| 7.92 | Tris-HCl | 4.6 | 3.0 | 1.5 |
| 8.44 | Tris-HCl | (5.0) | 4.1 | 1.2 |
| 8.49 | Tris-HCl | 3.9 | 3.5 | 1.1 |
| 8.81 | Tris-HCl[c] | 3.9 | 4.2 | 0.93 |
| 9.05 | Carbonate[d] | 2.8 | 3.0 | .93 |
| 9.08 | Carbonate | 2.4 | 3.3 | .73 |
| 9.18 | Carbonate | 3.0 | 3.3 | .88 |
| 9.35 | Carbonate | 2.6 | 5.2 | .50 |
| 9.41 | Carbonate | (3.7) | (8.3) | .45 |
| 9.95 | Carbonate | 1.2 | 5.4 | .23 |

[a] Aqueous solution at 25.0°; ($S_0$) = 0.35 to 3.5 × $10^{-3}$ $M$ and ($E_0$) = 2 to 5 × $10^{-5}$ $M$. Calculated from a least squares analysis of $1/v$ vs. $1/S$. [b] 0.1 $M$ phosphate buffer, ionic strength 0.6. [c] 0.1 $M$ tris(hydroxymethyl)aminomethane–hydrochloric acid buffer, ionic strength 0.1. [d] 0.1 $M$ carbonate buffer, ionic strength 0.15.

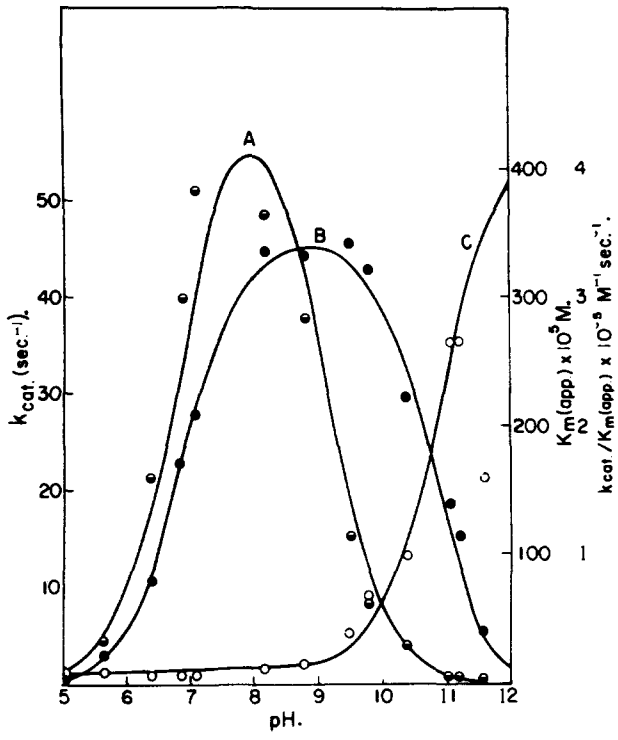


Fig. 6.—The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester at 25.0° in acetonitrile–water: A, $k_{cat}/K$(app); B, $k_{cat}$; C, $K_m$(app).

The pH dependence of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester is presented in Table IV and in Fig. 6: the pH–$k_{cat}$ curve (B) is a hybrid between a sigmoid curve and a bell-shaped

TABLE IV

THE KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-L-TRYPTOPHAN ETHYL ESTERS[a,b,c]

| pH | Buffer | $k_{cat}$, sec.$^{-1}$ | $K_m$(app) × $10^5$, $M$ | $k_{cat}/K_m$(app) × $10^{-5}$, $M^{-1}$ sec.$^{-1}$ |
|---|---|---|---|---|
| 2.52 | HCl | 0.0073 ± 0.0001 | 24.3 ± 10.5 | 0.000303 |
| 3.12 | HCl | .043 ± .002 | 16.9 ± 2.1 | .00398 |
| 3.60 | 0.1 $M$ acetate | .076 ± .002 | 7.4 ± 1.0 | .0104 |
| 4.57 | .1 $M$ acetate | .35 ± .01 | 6.6 ± 1.3 | .0527 |
| 5.04 | .1 $M$ acetate | .84 ± .02 | 8.3 ± 1.0 | .101 |
| 5.71 | .1 $M$ acetate | 3.03 ± .12 | 8.7 ± 2.0 | .348 |
| 6.40 | .1 $M$ phosphate | 10.7 ± .35 | 6.7 ± 1.6 | 1.61 |
| 6.87 | .1 $M$ phosphate | 23.0 ± 1.2 | 7.7 ± 2.5 | 3.00 |
| 7.09 | .1 $M$ phosphate | 27.8 ± 1.4 | 7.3 ± 2.5 | 3.82 |
| 8.18 | .1 $M$ Tris | 44.9 ± 1.8 | 12.3 ± 2.3 | 3.65 |
| 8.79 | .1 $M$ Tris | 44.3 ± 1.8 | 15.6 ± 2.6 | 2.85 |
| 9.49 | .1 $M$ carbonate | 45.6 ± 4.0 | 39.4 ± 8.6 | 1.16 |
| 9.81 | .1 $M$ carbonate | 43.0 ± 2.8 | 68.1 ± 8.8 | 0.631 |
| 10.40 | .1 $M$ carbonate | 29.8 ± 1.2 | 100.3 ± 7.2 | .297 |
| 11.08 | NaOH–$Na_2HPO_4$ | 18.7 ± 2.3 | 267.8 ± 44.2 | .0698 |
| 11.23 | NaOH–$Na_2HPO_4$ | 15.3 ± 1.7 | 267.9 ± 40.7 | .570 |
| 11.59 | NaOH | 5.53 ± 0.55 | 159.4 ± 23.6 | .0347 |

[a] 0.81% (v./v.) acetonitrile–water at 25.0°. [b] Computations carried out with an IBM 709 computer program designed by A. M. Myers based on a weighted regression analysis of $1/v$ vs. $1/S$.[28] Four to seven substrate concentrations were used. [c] [$E_0$] = $10^{-5}$ to $10^{-7}$ $M$. [$S_0$] = 0.23 to 1.55 × $10^{-3}$ $M$. The higher [$E_0$] were used at the extreme pH's where the rate constants are smaller.

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

curve; the pH–$k_{cat}/K_m$(app) curve (A) is a bell-shaped curve; and the pH–$K_m$(app) curve (C) is independent of pH at low pH but rises sharply at high pH. The pH–$k_{cat}$ profiles of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine ethyl ester,[29] acetyl-L-tryptophan ethyl ester, and acetyl-L-tyrosine ethyl ester[30] were previously found to have sigmoid shapes from pH 6.5–9.0, 5.5–9.0, and 5.5–8.3, respectively. Furthermore, the pH–$K_m$(app) curve of the hydrolysis of acetyl-L-phenylalanine ethyl ester was found to increase sharply from pH 8 to 9.[29] Thus the same phenomena have been observed before as are seen here in a more extended range.

The experimental data in Fig. 6 can be explained by the use of eq. 1–8. Using these equations together with the conclusions of the previous paper,[5] namely that the rate-determining step of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester at pH 7 is deacylation ($k_3$), the following qualitative predictions can be made: (1) $k_{cat}$ of the hydrolysis will follow a sigmoid curve to some high pH (while $k_3$ is rate determining), but above some pH, $k_{cat}$ will decrease (as $k_2$ becomes rate determining); (2) $K_m$(app) will increase (as the rate-determining step changes from $k_3$ to $k_2$) leading eventually to the limiting value which is the true $K_s$. The data in Fig. 6 bear out these predictions.

In Fig. 6, the change in rate-determining step from $k_3$ to $k_2$ may be seen both in curve B, $k_{cat}$, which changes from a sigmoid curve to a bell-shaped curve at about pH 10, and in curve C, $K_m$(app), which changes at about the same pH from its low pH-independent value to its high value. Furthermore, Fig. 6 shows that $k_{cat}/K_m$(app) (curve A) is a true bell-shaped curve as it must be from eq. 6 and 7.

The circles of Fig. 6 are experimental points. The solid curves of Fig. 6 are theoretical curves calculated from eq. 1–8 in the following way. Equations 6 and 7 can be combined and rearranged to

$$1/K_m(\text{app}) = 1/K_s + (k_{cat}/(K_m(\text{app}))(1/k_3) \quad (12)$$

At pH values greater than 8, $k_3 = k_3$(lim), a constant; therefore, above pH 8 a plot of $1/K_m$(app) *vs.* $k_{cat}/K_m$(app) should be linear with a slope of $1/k_3$(lim) and an intercept of $1/K_s$; excellent linearity was observed. Knowing $K_s$ and $k_3$(lim), it is possible to calculate values of $k_2$ at pH's over 8 from eq. 13 which is eq. 7 in rearranged form with the proviso that $k_3 = k_3$(lim).

$$k_2 = k_3(\text{lim})((K_s/K_m(\text{app})) - 1) \quad (13)$$

Then using eq. 4 at pH's above 8 where the term ($H/K_1'$) is negligible, it is possible to calculate directly $k_2$(lim) and $pK_2'$. $pK_1'$ may be calculated in a similar manner by first determining $k_2'$ values below pH 8 by the use of a modified eq. 13 in which $k_3$(lim) is replaced by $k_{cat} = k_3$. The values of $k_2$(lim) determined from the data below and above pH 8 agree well with one another. $pK_1$ and $pK_2$ may be calculated from the bell-shaped curve of pH–$k_{cat}/K_m$(app). $pK_1''$ may be calculated from the pH–$k_{cat}$ profile below pH 8. The six kinetic parameters so calculated from the $k_{cat}$ and $K_m$(app) data include: (1) $k_2$(lim) = 1818 sec.$^{-1}$; (2) $k_3$(lim) = 46.5 sec.$^{-1}$; (3) $pK_1' = 6.77$; (4) $pK_2' = 9.21$; (5) $pK_1'' = 6.86$; and (6) $K_s = 4.1 \times 10^{-3}$ *M*.[31] Using these data together with the proper combination of eq. 4–7 the theoretical curves of Fig. 6 were calculated. The agreement between experiment and theory is quite good. The pH dependence of $K_s$, the true equilibrium binding constant, for two uncharged substrates, *p*-nitrophenyl acetate and N-acetyl-L-tryptophan amide, can be seen in Tables II and III. In neither reaction is there a significant pH dependence of $K_s$. This result is in accord with some fragmentary data in the literature of the pH dependence of $K_i$ for uncharged inhibitors.[32] This result validates the calculations of the kinetic parameters of the hydrolysis of N-acetyl-L-tryptophan ethyl ester, in which this assumption was made.

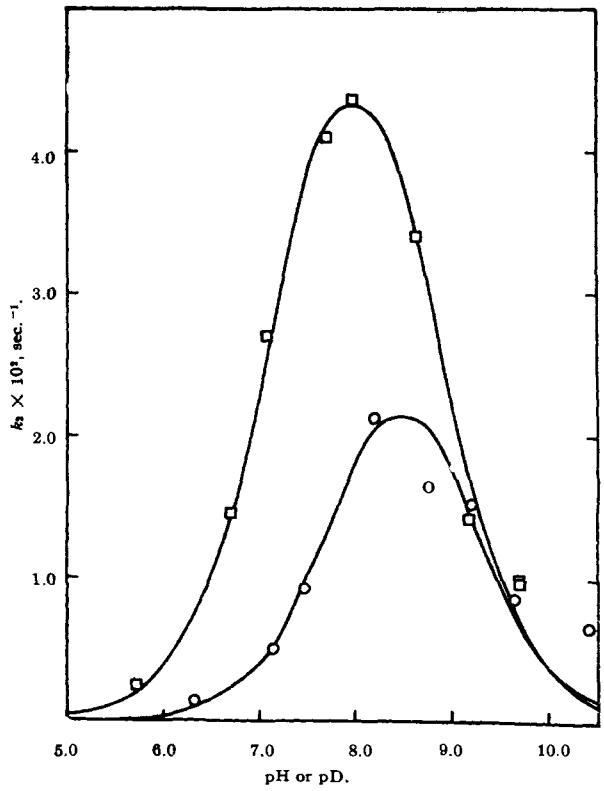


Fig. 7.—The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide in water, □, and in deuterium oxide, O, at 25.0°

**Kinetics in Deuterium Oxide Solvent.**—The kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide in deuterium oxide are presented in Table V and Fig. 7. The kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester in deuterium oxide are presented in Table VI and Fig. 8.

It is seen from these data that the shapes of the pH–rate profiles of both the amide and the ethyl ester do not change from water to deuterium oxide. Equation 5 applies to the pD–$k_{cat}$ profile for the hydrolysis of N-acetyl-L-tryptophan ethyl ester in the pD range under investigation, since in this region $k_3$ is completely rate controlling; further, eq. 4 applies to the pD–$k_{cat}$

(29) B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955).

(30) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).

(31) This may be compared with $2.5 \times 10^{-3}$ *M* calculated by an independent route.[5]

(32) R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 3365 (1955); F. Vaslow and D. G. Doherty, *ibid.*, **75**, 928 (1953).

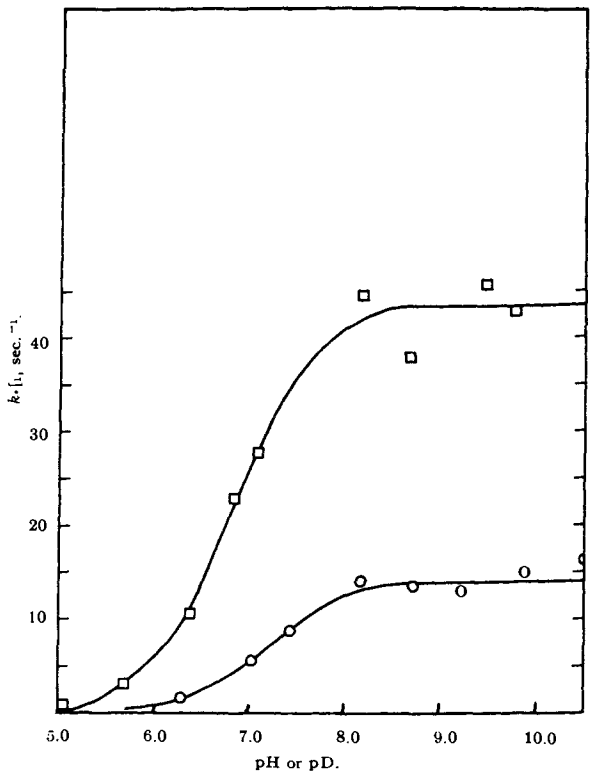


Fig. 8.—The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester at 25.0° in 0.81% (v./v.) acetonitrile–water □, and in 0.81% (v./v.) acetonitrile–deuterium oxide, O.

profile for the hydrolysis of N-acetyl-L-tryptophan amide. Using these equations and the method of Alberty[28] for the calculation of the bell-shaped curves,

TABLE V

KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-L-TRYPTOPHAN AMIDE IN DEUTERIUM OXIDE[a,b]

| pD | Buffers, 0.1 $M$ | $k_{cat} \times 10^2$, sec.$^{-1}$ | $K_m$(app) $\times 10^3$, $M$ |
|---|---|---|---|
| 6.33 | Phosphate | 0.134 ± 0.003 | 3.47 ± 0.15 |
| 7.16 | Phosphate | .507 ± .063 | 7.13 ± 1.10 |
| 7.47 | Phosphate | .922 ± .068 | 2.69 ± 0.44 |
| 8.21 | Phosphate | 2.12 ± .17 | 2.74 ± .40 |
| 8.75 | Tris–HCl | 1.64 ± .19 | 4.21 ± .79 |
| 9.21 | Tris–HCl | 1.53 ± .13 | 4.45 ± .61 |
| 9.64 | Carbonate | 0.867 ± .089 | 4.09 ± .67 |
| 10.41 | Carbonate | 0.670 ± .128 | 9.49 ± 2.20 |

[a] Aqueous solution at 25.0°; $[S_0] = 0.708$ to $12.73 \times 10^{-3}$ $M$, $[E_0] = 1.0$ to $2.5 \times 10^{-4}$ $M$. [b] Weighted regression analysis carried out with an IBM 709 computer program designed by A. M. Myers.[28]

the calculated curves of Fig. 7 and 8 and the p$K_a$'s for the pD–rate profiles were determined; the latter are listed in Table X, together with comparable values in aqueous solution.

## Discussion

**pH Dependencies and the Stepwise Mechanism.**—A summary of the pH–rate constant profiles for discrete acylation and deacylation steps determined here and in previous investigations is given in Table VII.[33-35] A considerable number of investigations consistently point to a sigmoid pH–$k_3$ profile which can be analyzed in terms of dependence of the reaction on a basic group of p$K_a \sim 7$. Only one investigation, the present one, has determined the complete pH–$k_2$ profile, which can be analyzed in terms of dependence of the reaction on two groups, a basic group of p$K_a \sim 7$ and an acidic group of p$K_a \sim 9$.

TABLE VI

KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-TRYPTOPHAN ETHYL ESTER IN DEUTERIUM OXIDE[a,b,c]

| pD | Buffer, 0.1 $M$ | $k_{cat}$, sec.$^{-1}$ | $K_m$(app) $\times 10^4$, $M$ | $k_{cat}/K_m$(app) $\times 10^{-5}$, $M^{-1}$ sec.$^{-1}$ |
|---|---|---|---|---|
| 6.29 | Phosphate | 1.59 ± 0.01 | 2.50 ± 0.01 | 0.64 |
| 7.05 | Phosphate | 5.50 ± .08 | 4.81 ± .68 | 1.14 |
| 7.45 | Phosphate | 8.76 ± .12 | 3.86 ± .46 | 2.27 |
| 8.19 | Phosphate | 14.14 ± .23 | 3.98 ± .69 | 3.56 |
| 8.74 | Tris | 13.45 ± .25 | 6.63 ± .73 | 2.03 |
| 9.25 | Tris | 13.55 ± .32 | 12.97 ± 1.28 | 1.04 |
| 9.90 | Carbonate | 14.94 ± .79 | 16.15 ± 2.69 | 0.93 |
| 10.57 | Carbonate | 16.38 ± .15 | 50.17 ± 1.11 | 0.33 |

[a] 0.81% (v./v.) acetonitrile–deuterium oxide; 25.0°. [b] Computations carried out with an IBM 709 computer program designed by A. M. Myers.[28] [c] $[E_0] = 10^{-5}$ to $10^{-7}$ $M$, $[S_0] = 0.23$ to $1.55 \times 10^{-4}$ $M$.

TABLE VII

THE PH DEPENDENCE OF THE ACYLATION AND DEACYLATION RATE CONSTANTS IN α-CHYMOTRYPSIN-CATALYZED REACTIONS

| Substrate | p$K_1$ | p$K_2$ | pH optimum | pH range | Reference |
|---|---|---|---|---|---|
| **Acylation ($k_2$)** | | | | | |
| *p*-Nitrophenyl acetate | 6.59 | 8.61 | 7.8 | 5.91–9.21 | [a] |
| **Deacylation ($k_3$)** | | | | | |
| Acetyl-α-chymotrypsin | 6.96–7.3 | .. | ... | 5.6–8.8 | 25, 26, 33 |
| *trans*-Cinnamoyl-α-chymotrypsin | 7.15 | .. | ... | 6–13 | 34, 35 |
| Trimethylacetyl-α-chymotrypsin | 6.8 | .. | ... | 5.58–9.04 | 35 |

[a] This research.

The On the basis of these observations of different pH dependencies of the discrete acylation and deacylation steps, it is of interest to consider the pH dependence of the α-chymotrypsin-catalyzed hydrolysis of specific substrates. Table VIII gives a summary of the current results pertaining to $k_{cat}/K_m$(app) *vs.* pH, as well as a reasonably complete presentation of the pertinent literature results.[36-42] It is seen that all $k_{cat}/K_m$(app) *vs.* pH profiles are bell-shaped curves, no matter whether the substrate is a specific or a nonspecific substrate and no matter whether the substrate is a peptide, amide, ethyl ester, or *p*-nitrophenyl ester. Although there is a small spread in the values of p$K_1$ and p$K_2$, the near equivalence of these profiles is excellent evidence that the same enzymatic groups are involved in all the reactions listed in Table VIII, providing strong

(33) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).
(34) M. L. Bender, G. R. Schonbaum, G. A. Hamilton, and B. Zerner, *J. Am. Chem. Soc.*, **83**, 1255 (1961).
(35) M. L. Bender, G. R. Schonbaum, and B. Zerner, *ibid.*, **84**, 2562 (1962).
(36) D. W. Thomas, R. V. MacAllister, and C. Niemann, *ibid.*, **73**, 1548 (1951).
(37) D. S. Hogness and C. Niemann, *ibid.*, **75**, 884 (1953).
(38) T. Yamashita, *J. Biochem.*, **48**, 651 (1960).
(39) J. H. Northrop, M. Kunitz, and R. M. Herriott, "The Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948, p. 118.
(40) C. J. Martin, J. Golubow, and A. E. Axelrod, *J. Biol. Chem.*, **234**, 294 (1958).
(41) B. F Erlanger, H Castleman, and A. G. Cooper, *J. Am. Chem. Soc.*, **85**, 1872 (1963).
(42) H. T. Huang and C. Niemann, *ibid.*, **74**, 4634 (1952).

TABLE VIII

pH–$k_{cat}/K_m$(APP) PROFILES OF SOME α-CHYMOTRYPSIN-CATALYZED HYDROLYSES

| Substrate | $pK_1$ | $pK_2$ | pH optimum | pH range | Reference | Calculation of $k_{cat}/K_m$(app) |
|---|---|---|---|---|---|---|
| N-Acetyl-L-tryptophan amide | 7.07 | 8.64 | 7.85 | 5.73–9.70 | [h] | [a] |
| N-Acetyl-L-tyrosine amide | 6.7(6.85) | 9 ? | 7.8[g] | 6.7–8.5 | 30, 36 | [c,d] |
| N-Acetyl-L-phenylalanine amide | ~6.5 | ~8.5 | ~7.8 | 5.71–9.95 | [h] | [a] |
| N-Acetyl-L-tyrosine hydroxamide | 6.5 | 8.5 | 7.6 | 6.5–8.8 | 37 | [c,e] |
| N-Acetylglycyl-L-tyrosine amide | ~6.7 | ~8.8 | 7.8 | 6.4–8.5 | 38 | [a] |
| Casein | 6.3 | 9.5 | ~8 | 6–11 | 39 | [a] |
| N-Benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester | 7 | 9 | 8 | 6.5–9 | 40 | [b] |
| N-Acetylphenylalanine ethyl ester | 6.8 | 8.7 | 7.8 | 6.5–9 | 29 | [a] |
| N-Acetyl-L-tryptophan ethyl ester | 6.77 | 9.22 | 7.8 | 5–11.6 | [h] | [a,f] |
| *p*-Nitrophenyl acetate | 6.85 | 9.04 | 7.9 | 5.91–9.21 | [h] | [b] |
| Diphenylcarbamyl chloride | 6.6 | 8.4 | 7.6 | 6–9 | 41 | [b] |

[a] $k_{cat}/K_m$(app) calculated from the slope of the Lineweaver–Burk plot which is equal to $k_{cat}/K_m$(app). [b] $k_{cat}/K_m$(app) calculated from second-order kinetics, where the rate constant = $k_2/K_s$ = $k_{cat}/K_m$(app). [c] $k_{cat}/K$(app) calculated from the velocity of the reaction at $S_0 < K_m$(app), whose rate constant = $k_{cat}/K_m$(app). [d] $S_0/K_s$ = 0.67 at pH 7.9. [e] $S_0/K_s$ = 0.39 at pH 7.9. [f] See text. [g] This pH optimum is a broad maximum extending over 1.5 pH units. [h] This research.

TABLE IX

pH–$k_{cat}$ PROFILES OF SOME α-CHYMOTRYPSIN-CATALYZED HYDROLYSES

| Substrate | $pK_1$ | $pK_2$ | pH optimum | pH range | Reference |
|---|---|---|---|---|---|
| Bell-shaped pH–rate profiles | | | | | |
| N-Acetyl-L-tryptophan amide | 7.16 | 8.9 | 7.91 | 5.73–9.70 | [a] |
| N-Acetyl-L-tryptophan amide[c] | 6.5 ? | 9 ? | 7.8[b] | 6.6–8.8 | 14 |
| N-Acetyl-L-phenylalanine amide | 6.55 | 9.23 | 7.89 | 5.71–9.95 | [a] |
| Methyl hydrocinnamate | 7.0 | 8.4 | 7.8 | 5.0–9.5 | 43 |
| N-Acetyl-L-tyrosine hydroxamic acid | ~6.8 | ~8.8 | 7.9 | 6.3–9.1 | 44 |
| N-Nicotinyl-L-tryptophan amide[d] | 6.5 | 9 ? | 7.8[b] | 6.6–8.8 | 14 |
| Sigmoid pH–rate profiles | | | | | |
| N-Acetyl-L-tryptophan ethyl ester | 6.86 | ... | ... | 5.04–9.81 | [a] |
| N-Acetyl-L-phenylalanine ethyl ester | 6.8 | .. | .. | 6.05–9.0 | 29 |
| N-Acetyl-L-tryptophan ethyl ester | 6.7 | .. | .. | 5.5–9.0 | 30 |
| N-Acetyl-L-tyrosine ethyl ester | 6.74 | .. | .. | 5.5–8.3 | 30 |

[a] This research. [b] A broad pH optimum. [c] $k_{cat}$ and $K_m$(app) not separated but $k \sim k_{cat}$ since $S_0/K_m$(app) = 2.0 at pH 7.9. [d] $k_{cat}$ and $K_m$(app) not separated but $k \sim k_{cat}$ since $S_0/K_m$(app) = 3.7 at pH 7.9.

evidence of a common mechanism for all reactions of α-chymotrypsin which have been investigated.[45]

Table IX summarizes the current results pertaining to pH–$k_{cat}$ profiles of specific amide substrates (up to pH 10), as well as pertinent literature results. It is seen that the pH–$k_{cat}$ profiles of the two specific amide substrates investigated here as well as others in the literature are bell-shaped curves. These profiles indicate two kinetically important ionizations of the enzyme–substrate complex, ES. Furthermore, the parameters of the pH–$k_{cat}$ profiles of the specific amide substrates are similar to the parameters of those pH–$k_{cat}/K_m$(app) bell-shaped curves listed in Table VIII. This equivalence of pH dependencies may be explained on the basis of two earlier conclusions: (1) the pH dependence of the enzyme–substrate complex (ES) and of the enzyme (E) are identical with one another which implies that $K_m$(app) is pH independent; and (2) the rate-determining step of the specific amide substrates is acylation $k_2$.[5] Thus a completely consistent picture arises in the identification of the pH–$k_{cat}$ profiles of specific amide substrates as pH–$k_2$ profiles.

The pH–$k_{cat}$ profile of one ester, methyl hydrocinnamate, is a bell-shaped curve; the implication here is that acylation is the rate-determining step in the hydrolysis of this *nonspecific* ester substrate. Other nonspecific ester substrates which presumably fit into this same category include methyl N-acetylglycinate[6] and methyl hippurate[41] (*vide infra*).

Table IX summarizes the current results pertaining to pH–$k_{cat}$ profiles of specific ester substrates (up to pH 10), as well as a complete list of the pertinent literature results. The pH–$k_{cat}$ profiles of specific ester substrates are sigmoid curves, up to pH 9 in three instances and up to pH 9.81 in a fourth investigation. Thus the identification of the pH–$k_{cat}$ profiles of specific ester substrates as pH–$k_3$ profiles[5] appears to be correct.

In Table IX, two investigations of the pH–$k_{cat}$ profile of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester are given, each result being characterized by a sigmoid curve, with $pK_a$'s of 6.86 and 6.7, respectively. However, the current investigation has shown that the pH–$k_{cat}$ profile of this reaction is a sigmoid curve only up to pH 9.81, degenerating at higher pH's into a curve which resembles a bell-shaped curve (Fig. 6). However, whereas the true bell-shaped curves have rather sharp maxima and inflection points which are usually within 2.5 pH units of one another, the pH–$k_{cat}$ profile of N-acetyl-L-tryptophan ethyl ester has a maximum which is completely flat for 2 pH units and has inflection points which are 4 pH units apart. Furthermore the right-hand leg of this profile does not conform to a segment of a bell but can be analyzed in terms of eq. 1–8 to give a coherent explanation of the entire profile.

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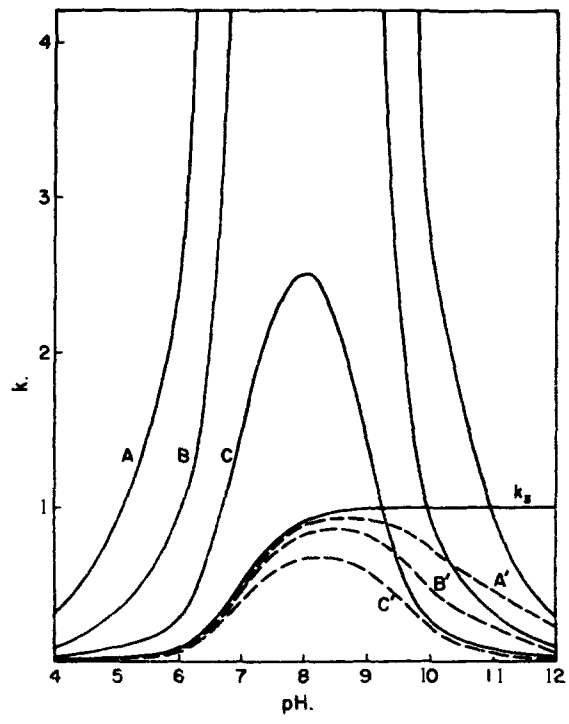


Fig. 9.—A hypothetical family of pH–rate constant profiles for a series of α-chymotrypsin-catalyzed hydrolyses in which $pK_1$ = 7.0 and $pK_2$ = 9.0. Curves A, B, and C are pH–$k_2$ curves. The common pH–$k_3$ curve is so labeled. The curves A', B', and C' are pH–$k_{cat}$ curves based on A, B, and C, respectively. In curves A and A', $k_2 = 30k_3$; in curves B and B', $k_2 = 10k_3$; in curves C and C' $k_2 = 3k_3$.

From this analysis, given in the section of Results, the following conclusions can be drawn: (1) the rate-determining step of $k_{cat}$ changes with pH, being $k_3$ at pH's lower than 10.7 and $k_2$ at pH's higher than 10.7 (from eq. 6, when $k_2 = k_3$, $k_{cat} = k_3/2$ which is ~pH 10.7); (2) because of this change in rate-determining step, $K_m$(app) changes with pH, the large change occurring when the ratio $k_3/k_2$ changes radically at higher pH (eq. 7 indicates that when $k_2 = k_3$, $K_m$(app) = $K_s/2$ which is ~pH 10.7); (3) the pH–$k_{cat}/K_m$(app) profile is essentially identical with that of all other α-chymotrypsin-catalyzed reactions. Since this analysis is in agreement with the kinetic arguments of the preceding paper[5] and since the experimental data fit this analysis quite well (Fig. 6), it must be concluded that the explanation for the pH–rate profiles of the ethyl ester can be made in terms of eq. 1–8 and that this profile is indeed a combination of sigmoid and bell-shaped pH–rate profiles.

This analysis immediately leads to the conclusion that the pH–$k_{cat}$ profiles of α-chymotrypsin-catalyzed hydrolyses should encompass a spectrum of curves. At one extreme of this spectrum lies the pure bell-shaped curve which reflects a reaction in which $k_{cat}$ is controlled solely by acylation, $k_2$. There are many examples of such reactions, encompassing all hydrolyses of specific amides and peptides. At the other extreme of this spectrum lies the pure sigmoid curve which reflects a reaction in which $k_{cat}$ is controlled by deacylation, $k_3$, across the whole pH spectrum. There are essentially no known reactions of this kind, for this limiting case requires that the ratio $k_2/k_3$ be infinite and thus may be reached only asymptotically or achieved when $k_3$ is examined in isolation.

In addition to the two extreme pH–$k_{cat}$ profiles described above, a whole spectrum of intermediate profiles should be found. The profile of the hydrolysis of N-acetyl-L-tryptophan ethyl ester is an example of such an intermediate profile. In general any reaction in which $k_3$ is (fully or partially) rate determining at low pH will fall into this category, for since $k_3$ exhibits a sigmoid pH–rate profile while $k_2$ exhibits a bell-shaped pH–rate profile, there must necessarily be a crossover of the profiles resulting in a change in rate-determining step at some pH according to the diagram in Fig. 9.

**Deuterium Oxide Isotope Effects.**—α-Chymotrypsin-catalyzed hydrolyses of a specific ester substrate and a specific amide substrate are seen to be as well behaved in deuterium oxide as were the nonspecific substrates investigated earlier,[12a] in the sense that the shapes of the respective pH–rate profiles remain intact in going from water to deuterium oxide (Fig. 7 and 8). A summary of the deuterium oxide kinetic and equilibrium isotope effects are given in Table X for the present and previous results. Two effects are seen. One is that the rate constant in deuterium oxide is one-half to one-third of that in water. The other effect is that the pH–rate profiles are displaced about 0.3 to 0.7 pH unit to higher values in deuterium oxide than in water. This result is reflected in the kinetically-determined $pK_a$'s of Table X which are correspondingly 0.3 to 0.7 unit higher in deuterium oxide than in water. It is known that the $pK_a$ of imidazole changes from 7.09 in water to 7.65 in deuterium oxide[46,47] and therefore the changes in $pK_1$ ($pK_1'$, $pK_1''$) are entirely consistent with specifying the responsible enzymatic group as an imidazole group of a histidine moiety of the enzyme. However, the observation of $\Delta pK$ of 0.5 ± 0.2 for $pK_1$ is certainly not definitive evidence for an imidazole group, for practically all acids have $\Delta pK'$s of this order of magnitude. Högfeldt and Bigeleisen[48] have analyzed the effect of deuterium oxide on the $pK_a$'s of acids with respect to type of compound and inherent $pK_a$. Without exception, all acids with $pK_a$'s from −5 to +12.5 possess $\Delta pK$'s of 0.5 ± 0.2. Therefore, no identification can be made of specific enzymatic groups on the basis of the deuterium oxide isotope effects alone. However, all the $\Delta pK$'s found in this investigation with both specific and nonspecific substrates are within the range of normal $\Delta pK$'s noted in simple systems This fact indicates that the effect of deuterium oxide on α-chymotrypsin-catalyzed reactions is a simple one of perturbation of the ionization constant of the groups on the enzyme and not one of perturbation of the mechanism or of the configuration of the enzyme which would be expected to have a further, profound effect on the $pK$'s of ionizable groups.

The most straightforward comparison of rates in deuterium oxide and water solutions involves a comparison of comparable pH and pD independent regions. When this comparison cannot be made, the next best approach is to compare a solution containing a given percentage of the pertinent enzymatic ionization in deuterium oxide with that same percentage of

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TABLE X

THE EFFECTS OF DEUTERIUM OXIDE ON SOME α-CHYMOTRYPSIN-CATALYZED REACTIONS[a]

| Reaction | $k^{H_2O}/k^{D_2O}$ | $pK_1$ | | $pK_2$ | | Reference |
|---|---|---|---|---|---|---|
| | | $H_2O$ | $D_2O$ | $H_2O$ | $D_2O$ | |
| $k_3$ of *trans*-cinnamoyl-α-chymotrypsin | 2.5 | 7.15 | 7.75[d] | | | 12a |
| $k_3$ of benzoyl-α-chymotrypsin[c] | 2.4 | | | | | 46 |
| $k_3$ of trimethylacetyl-α-chymotrypsin | 3.0 | | | | | 12a |
| $k_2$ of *p*-nitrophenyl trimethylacetate | 2.2 | | | | | 12a |
| $k_{cat}$ of N-acetyl-L-tryptophan amide | 2.0 | 7.16 | 7.84[e] | 8.66 | 9.06[e] | b |
| $k_{cat}$ of N-acetyl-L-tryptophan ethyl ester | 2.67 | 6.86 | 7.19[d] | | | b |
| $k_{cat}/K_m$(app) of N-acetyl-L-tryptophan ethyl ester | | 6.77 | 7.49[f] | | | b |
| $k_{cat}$ of N-acetyl-L-tryptophan methyl ester | 2.83 | | | | | 12a |

[a] 25.0° in either aqueous solution or 0.81% (v./v.) acetonitrile–water. [b] This research. [c] Recalculated from the data of ref. 28, using a pD 0.6 higher than pH (see text). [d] $pK_1''$. [e] $pK_1'$ and $pK_2'$. [f] $pK_1$.

enzymatic ionization in water. Since the $\Delta pK$ of enzymatic groups between water and deuterium oxide appears to be 0.5 ± 0.2, the comparison of rates must utilize a pD in deuterium oxide 0.5 unit higher than the pH in water. This can be accomplished reasonably well by using a solution of deuterium oxide which has the same buffer ratio as the solution in water. This method has been used to determine the rate ratios $k^{H_2O}/k^{D_2O}$ in Table X. Table X indicates that for both specific and nonspecific substrates of α-chymotrypsin, $k^{H_2O}/k^{D_2O}$ = 2 to 3 and further that this ratio holds for both acylation and deacylation rate constants. The ratio appears to be somewhat smaller for acylation (2.0 for $k_{cat}$ of N-acetyl-L-tryptophan amide and 2.2 for $k_2$ of *p*-nitrophenyl trimethylacetate) than for deacylation (2.4 for $k_3$ of benzoyl-α-chymotrypsin, 2.5 for $k_3$ of *trans*-cinnamoyl-α-chymotrypsin, 2.67 for $k_{cat}$ of N-acetyl-L-tryptophan ethyl ester, 2.83 for $k_{cat}$ of N-acetyl-L-tryptophan methyl ester, and 3.0 for $k_3$ of trimethylacetyl-α-chymotrypsin). However, it is not certain that these differences are beyond unknown constant experimental errors.

The interpretation of kinetics of enzyme reactions in deuterium oxide is beset with complications. Some possible extraneous influences of deuterium oxide include: (1) changes in the conformation of the protein and/or the active site resulting from changes in the helix-coil transition temperature in deuterium oxide, differences in helices of marginal stability, or differences in the tertiary structure due to differences in solvation by deuterium oxide; (2) differences in the solvation of the transition state; and (3) a change in the activity of the nucleophile.[49]

Arguments have been given previously that α-chymotrypsin is not irreversibly inactivated in deuterium oxide.[12a] Further, the similarity of both the kinetic and the equilibrium isotope effects found with α-chymotrypsin to those found in simple organic systems[50] is a strong argument that no profound conformational changes are being observed in the α-chymotrypsin reactions. Completely deuterated α-chymotrypsin has been made by heating the enzyme to 100° at pD 3 in deuterium oxide and cooling. In this process *all* exchangeable hydrogens were replaced by deuterium. When dissolved in water, an inner core of deuterium atoms remains, but the rate with such an enzyme is the same as with native α-chymotrypsin.[51] Thus even the introduction of deuterium into the innermost parts of the enzyme apparently has no effect on the conformation of the enzyme.

Finally the effect of deuterium oxide on the kinetics of other enzyme-catalyzed reactions are identical with the effect found in α-chymotrypsin reactions. With trypsin $k_{cat}$ of the hydrolysis of benzoyl-L-arginine ethyl ester is lowered by about 2.6-fold in deuterium oxide at approximately pH 8.[52] We have investigated the eel acetylcholinesterase-catalyzed hydrolysis of phenyl acetate.[53] The ratio $k^{H_2O}/k^{D_2O}$ is 2.4 and the $pK_a$ of the basic group on which the reaction is dependent changes from 6.3 in water to 6.8 in deuterium oxide. If conformational changes were responsible for these kinetic and equilibrium isotope effects, the effect on different enzyme systems should be different. The fact that the effect of deuterium oxide on several different enzyme systems appears to be identical is further evidence that true kinetic and equilibrium isotope effects of deuterium oxide are being observed.

If, in fact, deuterium oxide does not cause conformational changes in the enzyme, the kinetic isotope effects noted above may be due to differences in solvation, differences in nucleophilicity, or differences in the rate of proton transfer. The kinetic isotope effects found in α-chymotrypsin reactions do not appear to be compatible with solvation effects or nucleophilicity effects. It is known that solvation effects give rise to $k^{H_2O}/k^{D_2O}$ ratios of 1.0 to 1.5[50]; since the group of $pK_a \sim 7$ is most probably imidazole,[45] the deuterium oxide solvation effects in nucleophilic reactions of imidazole are of interest: they are essentially nil.[50] Therefore one may rule out solvation effects of deuterium oxide. Deuterium oxide does have a nucleophilicity which is somewhat different from that of water. However, nucleophilicity differences cannot explain the present kinetic isotope effects, for such effects are seen in both acylation, $k_2$, in which water (or $D_2O$) does not serve as a nucleophile and in deacylation, $k_3$, in which water (or $D_2O$) does serve as a nucleophile. The fact that the kinetic isotope effects are larger in deacylation than in acylation may be explained in terms of the difference in nucleophilicity of water and $D_2O$. However, the major portion of the kinetic isotope effect is common to both reactions and thus cannot be caused by differences in nucleophilicity. Therefore, one must conclude that the kinetic isotope effects reflect a proton (deuteron) transfer in the transition state of both the acylation and deacylation reactions.

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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.

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

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-

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