[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY, CHICAGO 16, ILL., AND NORTHWESTERN UNIVERSITY, EVANSTON, ILL.]

# The *p*H Dependence of Some α-Chymotrypsin-catalyzed Hydrolyses[1-3]

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Investigations have been made of the *p*H dependency of three α-chymotrypsin-catalyzed hydrolyses in which both the acylation and deacylation steps can be observed separately: the hydrolyses of N-*trans*-cinnamoylimidazole, *o*-nitrophenyl cinnamate and *p*-nitrophenyl trimethylacetate. The reactions were followed spectrophotometrically. With the exception of the acylation of N-*trans*-cinnamoylimidazole, each acylation and deacylation reaction exhibits a sigmoid *p*H–rate profile with an inflection point around *p*H 7. This *p*H dependence indicates the involvement of a basic group on the enzyme with an apparent $pK_a$ of 7. The *p*H dependence of the acylation with N-*trans*-cinnamoylimidazole has been analyzed in terms of the ionization of both the substrate and of a group on the enzyme with an apparent $pK_a$ of 6.8. The finding that the acyl-enzyme, *trans*-cinnamoyl-α-chymotrypsin, is considerably more resistant to base (and urea) denaturation than the enzyme itself and that this resistance is enhanced by the addition of sodium chloride enabled the investigation of the effect of *p*H on its deacylation up to 0.1 *N* sodium hydroxide. It was found that the deacylation reaction is independent of *p*H from *p*H 9 to 0.1 *N* sodium hydroxide (and possibly to 1.0 *N* sodium hydroxide). Thus it appears that if an acid group of the enzyme is operative (in conjunction with the basic group of $pK_a \sim 7$), the acid group must possess a $pK_a$ greater than 13.9. Furthermore, the deacylation reaction must involve a water molecule as the nucleophile, rather than a hydroxide ion.

## Introduction

One of the most distinctive features of enzymatic reactions is their facile occurrence at or near neutrality. This behavior has led to many investigations of the effect of *p*H on enzymatic reactions. The effects of *p*H on enzymatic processes may take the form of reversible or irreversible denaturation of the enzyme protein, changes in the three-dimensional configuration of the enzyme protein or in the degree of aggregation of the protein, changes in the prototropic equilibria of the enzyme (with possible associated changes in binding of counterions, etc.) and changes in the prototropic equilibria of the substrate. Despite these manifold possibilities there appear to be two prevalent effects of *p*H on many enzymatic processes. These two *p*H dependencies are characterized by a bell-shaped or a sigmoid *p*H–rate profile.

In earlier studies an ill-defined activity was determined as a function of *p*H. But in recent years enzyme kinetics has been subjected to careful scrutiny and with it *p*H effects on enzyme kinetics have been put on a rigorous basis.[5-7] Specifically the separation of *p*H effects into effects on $K_m$, the Michaelis constant, which in many cases of interest is an equilibrium constant, and on $k_{cat}$, the catalytic rate constant, is established practice. Analysis of the *p*H dependence of $k_{cat}$, the catalytic rate constant, enables one to study the ionizable groups which are closely connected with catalytic activity.

Chymotrypsin catalyses have in general shown a sigmoid *p*H dependence of the catalytic rate constant, $k_{cat}$, although there is one report of a bell-shaped rate profile for $k_{cat}$.[8] The *p*H dependencies of chymotrypsin reactions have been determined at two levels of detail: (1) *p*H dependence of the classical $K_m$ and $k_{cat}$; and (2) *p*H dependence of $K_m^{acylation}$ and of each of the individual catalytic steps $k_2$ (acylation) and $k_3$ (deacylation) as defined by eq. 1.[9]

$$E + S \underset{k_{-1}}{\overset{K_m;\ k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} R\overset{O}{\overset{\|}{C}}E \xrightarrow{k_3} RCOOH) + E \quad (1)$$
$$\qquad\qquad\qquad\qquad\quad + \qquad\qquad\quad (P$$
$$\qquad\qquad\qquad\qquad\quad P_1$$

The *p*H dependencies of $K_m$ (based on the classical breakdown into $K_m$ and $k_3$) indicate that $K_m$ is essentially independent of *p*H from *p*H 6 to 8[8,10,11] but may become larger above *p*H 8.[10,12] The *p*H dependence of the $k_{cat}$ step of a number of specific substrates of chymotrypsin, including acetyl-L-phenylalanine ethyl ester, acetyl-L-tyrosine ethyl ester, acetyl-L-tryptophan ethyl ester and acetyl-L-tryptophan amide, indicate that a group with an apparent $pK_a$ of 6.7 to 6.85 is responsible (in its basic form) for the catalysis.[10,11,13] In the most careful investigations, determinations were carried out over the region from *p*H 5.5 to 9, and the data fit the theoretical curve for a single ionizing species.[11]

The *p*H dependencies of the individual acylation and deacylation steps of eq. 1 have been carried out for the chymotrypsin-catalyzed hydrolysis of several labile esters including *p*-nitrophenyl acetate and 2,4-dinitrophenyl acetate. It has been found that both the acylation and deacylation rate constants, like the classical catalytic rate constant, show a sigmoid dependency on *p*H, with approximately the same inflection point. The apparent $pK_a$ of the group on which the acylation steps are dependent varied from 6.2 to 6.7 and the apparent

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

(2) Paper XIII in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, **84**, 2556 (1962).

(3) Some of the results of this paper appeared in preliminary form; M. L. Bender, G. R. Schonbaum, G. A. Hamilton and B. Zerner, *ibid.*, **83**, 1255 (1961).

(4) Alfred P. Sloan Foundation Research Fellow; present address; Northwestern University, Evanston, Ill.

(5) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, N. Y., 1958, pp. 120-150.

(6) K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford University Press, London, 1958, Chap. V.

(7) R. A. Alberty, *J. Cell. Comp. Physiol.*, **47**, 245 (1956)

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

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

(12) *Cf.*, however, R. A. Dickie and J. A. Stewart, Abstracts of 140th Meeting of the Am. Chem. Soc., Chicago, Ill., Sept., 1961, p. 10-C.

(13) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **42**, 719 (1956).

$pK_a$ of the group on which the deacylation steps are dependent varied from 6.96 to 7.3.[14,15] These results of a number of different investigators are reasonably consistent, considering the different conditions under which they were carried out.

The purpose of the present paper is to describe a careful study of the $p$H dependency of some $\alpha$-chymotrypsin-catalyzed hydrolyses in which both the acylation and deacylation steps can be observed separately: the hydrolyses of N-*trans*-cinnamoylimidazole, *o*-nitrophenyl cinnamate and *p*-nitrophenyl trimethylacetate. These investigations seek to determine the presence of a sigmoid or of a bell-shaped $p$H–rate profile, and of course to determine the apparent $pK_a$'s on which the $p$H dependence rests. It was of special interest to investigate the behavior of the acyl-enzyme, *trans*-cinnamoyl-$\alpha$-chymotrypsin, over a large range of $p$H, especially in the high $p$H region which is normally not accessible. Since this compound can be readily prepared, since it is reasonably stable at high $p$H's and since its hydrolysis (a deacylation reaction) can be quantitatively and easily followed, an ideal situation presented itself for a detailed investigation of an enzymatic process up to $p$H 14.

## Experimental

**Materials.**—N-*trans*-Cinnamoylimidazole was prepared by the reaction of cinnamoyl chloride with imidazole in benzene solution; m.p. 133.5–134°.[16] *o*-Nitrophenyl cinnamate and *p*-nitrophenyl trimethylacetate were prepared from the appropriate acid chlorides and phenols in pyridine solution and were purified by recrystallization from alcohol.[17]

The solvents, buffers, enzyme and added salts have been described previously.[9] Carbonate-free sodium hydroxide solutions were prepared from saturated solutions of sodium hydroxide and were standardized against N.B.S. primary standard potassium hydrogen phthalate.

A Radiometer $p$H meter, model 4b or 4c, was used to determine the $p$H. The meter was standardized as recommended by Bates, *et al.*,[18,19] for use with solutions of high $p$H, against 0.01 $M$ trisodium phosphate or against an accurately standardized solution of 0.1 $N$ NaOH. The $p$H values in salt solutions have been corrected for sodium ion error from a nomograph supplied by Radiometer for the G200B glass electrode. These values, however, should be viewed with reserve.

**Kinetics.**—A description of the spectrophotometric apparatus and procedure has been given previously.[9]

The second-order acylation rate constants were calculated using either eq. 2 or 3.

$$\text{(enzyme)} \neq \text{(substrate)}: \quad kt/\delta - c = \log [1 + r/(A_\infty - A_t)] \quad (2)$$

$$\text{(enzyme)} = \text{(substrate)}: \quad kat = (A_t - A_0)/(A_\infty - A_t) \quad (3)$$

where $\delta = 2.303/b(n-1)$, $a$ is the initial concentration of the species in excess, $b$ is the initial concentration of the other species, $a = nb$, $r = (n-1)(A_\infty - A_0)$, $c$ is a constant, and $A_0$, $A_t$, $A_\infty$ are the measured absorbances at the time indicated. Under the conditions of these experiments, the deacylation of the acyl-enzyme (and the spontaneous hydrolysis of the substrate) can be reasonably assumed to be negligibly slow with respect to the acylation reaction. This assumption is borne out by the fact that the second-order plots were invariably linear to greater than two half-lives and also by the fact that the second-order rate constants were constant over a range of enzyme and substrate concentrations (see Results section). It should be pointed out that knowledge of the absolute enzyme concentration is essential in these experiments.

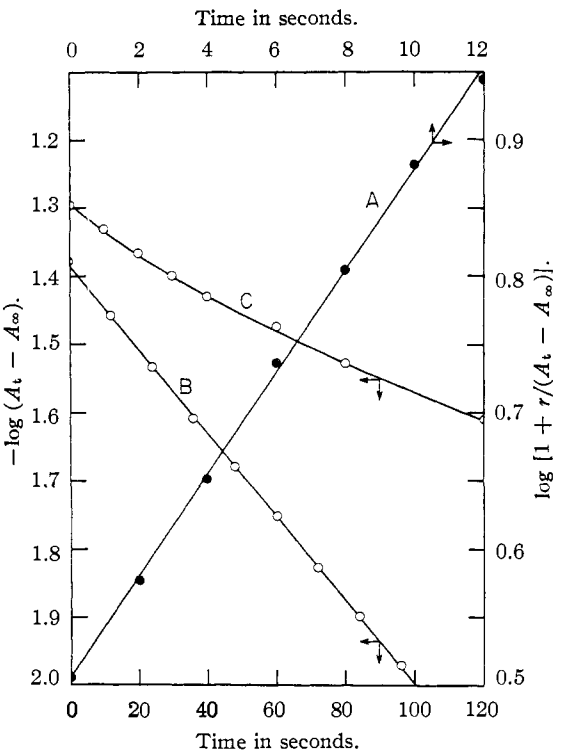


Fig. 1.—The $\alpha$-chymotrypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole: A, second-order acylation, $p$H 6.20, $[E]_0 = 1.27 \times 10^{-5}$ $M$, $[S]_0 = 8.43 \times 10^{-6}$ $M$; B, deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin in $0.107_5$ $N$ NaOH, 0.200 $M$ in NaCl; C, deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin in $0.107_5$ $N$ NaOH alone.

The first-order constants for the deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin were calculated by either the method of Guggenheim[20] or by using conventional first-order kinetics employing an infinity reading of the absorbance. In these experiments a slight excess of enzyme over substrate was generally used in order to eliminate turnover of the enzyme which produces initially a zero-order reaction. However, experiments in which there was an initial deficit of enzyme gave good first-order deacylation rates after the initial zero-order portion of the rate curve. The rate constants so obtained were in excellent agreement with those obtained under the usual conditions in which there was excess enzyme. Typical first-order deacylation plots and second-order acylation plots are given in Fig. 1. These plots were in general linear to 75–90%.

The first-order constants for the turnover of *p*-nitrophenyl trimethylacetate using an excess of substrate is a direct measure of the deacylation of trimethylacetyl-$\alpha$-chymotrypsin.[13,14] The ratio of substrate to enzyme used in these experiments was varied from 2 to 4, both values leading to the same zero-order velocity. The first-order rate constants for this process were calculated by dividing the zero-order velocity by the enzyme concentration.

## Results

**Kinetics.**—In the experiments reported in this paper, the kinetics of the deacylation process, $k_3$, have been determined according to two procedures: (1) by the determination of the turnover of the enzymatic process under conditions of excess sub-

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

(15) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1957).

(16) G. R. Schonbaum, B. Zerner and M. L. Bender, *ibid.*, **236**, 2930 (1961).

(17) M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962).

(18) R. G. Bates and V. E. Bower, *Anal. Chem.*, **28**, 1322 (1956).

(19) R. G. Bates, G. D. Pinching and E. R. Smith, *J. Research Natl. Bur. Standards*, **45**, 418 (1950).

(20) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed., John Wiley and Sons, Inc., New York, N. Y., 1961, p. 49.

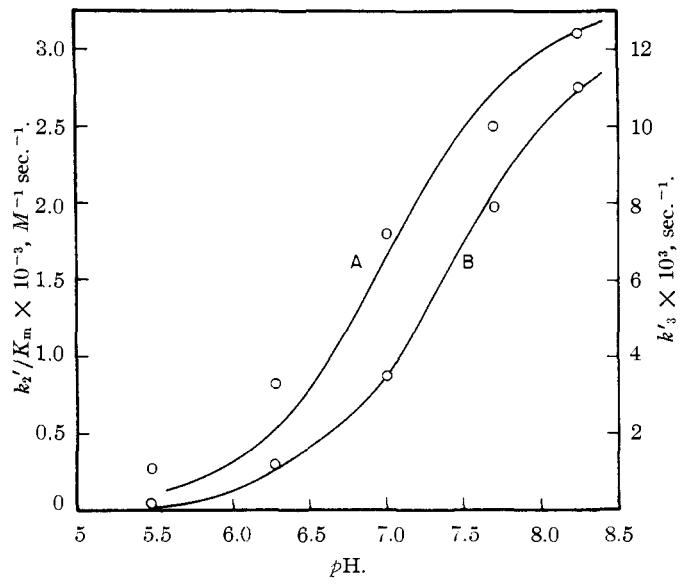


Fig. 2.—Acylation (A) and deacylation (B) of α-chymotrypsin with *o*-nitrophenyl cinnamate in 10% acetonitrile–water; phosphate buffer, 0.061 *M* total phosphate; 25°; acylation, left-hand scale; deacylation, right-hand scale.

strate; or (2) by the direct observation of the decomposition of the acyl-enzyme intermediate (or the production of the carboxylate ion from an acyl-enzyme intermediate) which has been prepared *in situ* in a stoichiometric manner. The latter procedure, although unconventional, possesses the great advantage that observations are made on a first-order process, the rate constant being independent of the concentration of the reactant, the acyl-enzyme.

A somewhat unconventional approach to the kinetics of the acylation process has been taken and therefore it is desirable to discuss the kinetics of this process in some detail. The experiments reported in this paper were carried out before a stopped-flow reactor was available in this Laboratory for measurement of fast reactions. Therefore it was necessary to determine second-order acylation constants for the three reactions of α-chymotrypsin reported here ($k_{obs} = k_2'/K_m$). These rate studies were carried out under conditions in which the ratio [substrate]/[enzyme] was usually about 2:1 but did vary from 0.5 to about 4. Under these conditions we may write (neglecting the deacylation reaction)

$$\begin{array}{ccccc} & & k_1 & k_2 & \\ E + S & \rightleftharpoons & ES & \longrightarrow & P_1 + ES' \\ K_1 \downarrow\uparrow & & k_{-1} & K_2 \downarrow\uparrow & \\ EH^+ & & & ESH^+ & \end{array} \qquad (4)$$

The rate equations are

$$dP_1/dt = k_2(ES) = k_{obs}(a - x)(b - x) \qquad (5)$$

From the conservation equations, one may obtain

$$(a - x) = (ES)[K_m/(S)(1 + [H^+]/K_1) + (1 + [H^+]/K_2)] \qquad (6)$$

$$(b - x) = (S)[1 + (E)/K_m(1 + [H^+]/K_2)] \qquad (7)$$

Substitution of eq. 6 and 7 into 5 gives

$$k_{obs} = k_2/[K_m(1 + [H^+]/K_1) + (S)(1 + [H^+]/K_2)][1 + (E)/K_m(1 + [H^+]/K_2)] \qquad (8)$$

where (E) and (S) represent the concentrations of unbound enzyme and substrate, respectively. We may reasonably assume in these experiments that $K_m$ is much greater than either (S) or (E), both of which are of the order of $10^{-5}$ *M*. For the type of substrate utilized here, $K_m$ is probably greater than $10^{-3}$ *M*. For example, the $K_m$ (acylation) for *p*-nitrophenyl acetate has been found to be $5 \times 10^{-3}$ *M*[14]; furthermore the $K_m$ (acylation) for *p*-nitrophenyl trimethylacetate was found to be similar, $1.6 \times 10^{-3}$ *M*.[21,22] $K_m(1 + [H^+]/K_1)$ is thus much greater than (S)(1 + $[H^+]/K_2$) and therefore eq. 8 becomes

$$k_{obs} = \frac{k_2}{[K_m(1 + [H^+]/K_1) + (E)(1 + [H^+]/K_1)(1 + [H^+]/K_2)]} \qquad (9)$$

If we assume that $K_1 \simeq K_2 \simeq 10^{-7}$, $K_m \simeq 10^{-3}$ and $(E)_0 \simeq 10^{-5}$, from eq. 9 it follows that the [enzyme] dependent term in the denominator will always be small compared with $K_m$ (1 + $[H^+]/K_1$) and may therefore be neglected, giving

$$k_{obs} = k_2/K_m(1 + [H^+]/K_1) = k_2'/K_m \qquad (10)[23]$$

**α-Chymotrypsin-catalyzed Hydrolysis of *o*-Nitrophenyl Cinnamate.**—The reaction of *o*-nitrophenyl cinnamate with α-chymotrypsin has previously been shown to follow eq. 1.[9] The rate of the acylation reaction [d(*o*-nitrophenol)/d*t*] = $[k_2'(E)(S)/K_m]$ is considerably faster than the deacylation reaction [d(cinnamate)/d*t*] = $[k_3'(ES')]$. Furthermore, the appearance of *o*-nitrophenol may be followed at wave lengths where no other species absorbs. Therefore the kinetics of the acylation reaction may be observed without interference by any deacylation process and *vice versa*. The kinetics of the acylation and deacylation from *p*H 5.48 to 8.24 are presented in Table I and graphically in Fig. 2.

TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *o*-NITROPHENYL CINNAMATE[a]

| | Acylation[b] | | Deacylation[c] | |
|---|---|---|---|---|
| *p*H | $k_2'/K_m \times 10^{-3}$,[d] $M^{-1}$ sec.$^{-1}$ | No. of runs | $k_3' \times 10^3$,[d] sec.$^{-1}$ | No. of runs |
| 5.48 | 0.27 ± 0.03 | 4 | 0.18 | 1 |
| 6.27 | 0.82 | 1 | 1.2 ± 0.1 | 3 |
| 7.00 | 1.8 | 1 | 3.5 ± .2 | 3 |
| 7.70 | 2.5 ± 0.3 | 2 | 7.9 ± .1 | 2 |
| 8.24 | 3.1 | 1 | 11.0 ± .0 | 2 |

[a] In 10% acetonitrile–water, phosphate buffer, 0.061 *M* total phosphate, 25°. [b] Followed at 410 or 350 mμ. Absolute values should be taken with some reserve since enzyme concentrations were determined by weight rather than by titration. [c] Followed at 250 mμ. [d] Deviations are average deviations from the mean.

(21) M. L. Bender and G. A. Hamilton, *J. Am. Chem. Soc.*, **84**, 0000 (1962).

(22) Many specific substrates possess $K_m$'s which are no smaller than these values.

(23) Second-order acylation constants have been observed previously in the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate, but no theoretical interpretation was given at that time.[15]

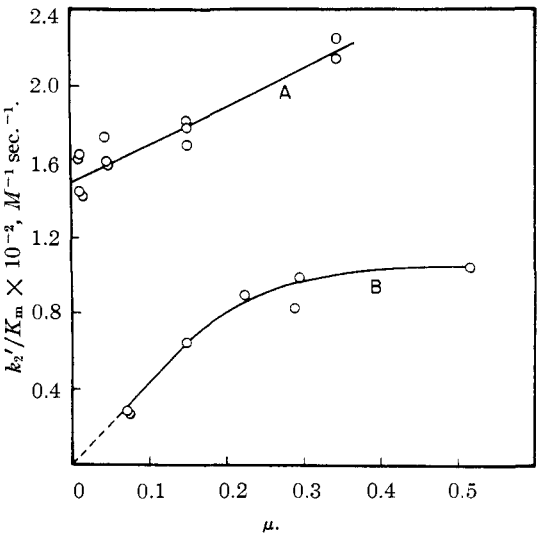


Fig. 3.—The effect of ionic strength on the second-order acylation rate constants of α-chymotrypsin with *p*-nitrophenyl trimethylacetate: A, *p*H 7.0; B, *p*H 5.0.

The *o*-nitrophenyl cinnamate results are the least extensive and least accurate results of those presented here. However, they clearly indicate a sigmoid relationship between the acylation or deacylation rate constant and *p*H. The apparent $pK_a$ of the acylation curve is 7.0 and that for the deacylation curve is 7.3. The smooth curves are the theoretical curves drawn using these respective $pK_a$ values and assuming maximum rate constants of $3.3 \times 10^3$ $M^{-1}$ sec.$^{-1}$ and $12 \times 10^{-3}$ sec.$^{-1}$ for acylation and deacylation, respectively. The fit of the deacylation data to the theoretical line is quite satisfactory, but the fit of the acylation data to the theoretical line is somewhat unsatisfactory at lower *p*H's.[24]

**α-Chymotrypsin-catalyzed Hydrolysis of *p*-Nitrophenyl Trimethylacetate.**—The reaction of *p*-nitrophenyl trimethylacetate with α-chymotrypsin has previously been shown to follow eq. 1.[25] The difference between the rates of acylation and deacylation in this instance is so great that it has been possible to isolate and crystallize the acyl-enzyme intermediate, trimethylacetyl - α - chymotrypsin.[26] Determination of the kinetics of acylation in this system is particularly attractive because acylation with the bulky trimethylacetyl compounds is considerably slower, and therefore more experimentally accessible, than with the corresponding acetyl derivative which has been used extensively before.[13-15]

The kinetics of the acylation of α-chymotrypsin with *p*-nitrophenyl trimethylacetate were determined in great detail. It was found that the second-order acylation rate constants were a function of the ionic strength of the medium. Therefore careful studies were carried out at several *p*H's

(24) The apparent deviations at low *p*H are arbitrary since they could be transformed into deviations at high *p*H by a lateral shift of the curve.

(25) C. E. McDonald and A. K. Balls, *J. Biol. Chem.*, **227**, 727 (1957).

(26) A. K. Balls, C. E. McDonald and A. S. Brecher, Proc. International Symposium on Enzyme Chemistry, Maruzen Co., Tokyo, 1957, p. 392.

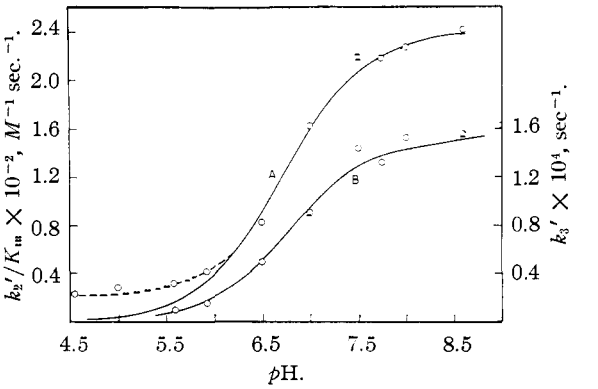


Fig. 4.—Acylation (A) and deacylation (B) of α-chymotrypsin with *p*-nitrophenyl trimethylacetate in aqueous solution at 25°: acylation, $\mu = 0.075$, left-hand scale; deacylation, right-hand scale.

to determine the effect of ionic strength on the rate constant. At *p*H 7.0 and above, a linear relationship was found between the rate constant and $\mu$; at *p*H 5.0, a non-linear relationship was found (Fig. 3). The rate constants for acylation presented in Table II and Fig. 4 are values at 0.075 ionic strength, where most data were available. The rate constants in Table II are either values found at that ionic strength or are interpolated values from curves such as Fig. 3. In two cases, *p*H 4.56 and 6.49, small extrapolations were used.

TABLE II

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *p*-NITROPHENYL TRIMETHYLACETATE[a]

| | Acylation[b] | | Deacylation | |
|---|---|---|---|---|
| *p*H | $k_2'/K_m \times 10^{-2}$,[h] $M^{-1}$ sec.$^{-1}$ | No. of runs[c] | $k_3' \times 10^4$,[g,h] sec.$^{-1}$ | No. of runs |
| 4.56[d] | 0.22 ± 0.04 | 2 | | |
| 5.0[d] | .28 ± .02 | 8 | | |
| 5.58[d,e] | .32 ± .02 | 2 | 0.10 | 1 |
| 5.92[e] | .42 ± .02 | 4 | .15 | 1 |
| 6.49[e] | .83 | 1 | .50 ± 0.01 | 2 |
| 7.00[e,f] | 1.63 ± 0.02 | 12 | .91 ± .03 | 8 |
| 7.51[f] | 2.18 ± .03 | 2 | 1.44 ± .02 | 2 |
| 7.74[e,f,i] | 2.18 ± .04 | 3 | 1.32 ± .04 | 5 |
| 8.0[e,f] | 2.27 ± .04 | 12 | 1.53 ± .10 | 7 |
| 8.59[f] | 2.42 ± .04 | 2 | 1.56 ± .02 | 3 |
| 9.04[f] | 2.2 ± .1 | 3 | 1.63 ± .02 | 2 |
| 9.44[f] | 1.0 ± .05 | 4 | | |

[a] In 0.8–1.6% acetonitrile–water at 25.2 ± 0.2°. [b] Ester concentration = 2 to 6 × $10^{-5}$ *M*; [ester]/[enzyme] varied from 0.5 to 4.0. [c] This is the number of runs that was used to interpolate or extrapolate the rate constant at ionic strength 0.075. [d] Acetate buffer. [e] Phosphate buffer. [f] Tris buffer. [g] Calculated from $V_{max} = k_3'[E]_0$. [h] Deviations are average deviations from the mean. [i] Borate appears to exert a specific buffer effect in the region of *p*H 7.74–8.75, depressing the rate considerably.

It is seen from Fig. 4 that the second-order acylation constants for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl trimethylacetate (up to *p*H 8.59) show a sigmoid dependence on a group with an apparent ionization constant of 6.7. However, in the low *p*H region of the curve there is considerable deviation, the actual rate constants being much higher than those predicted by the sigmoid relationship. A preliminary account of this work[27]

(27) M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).

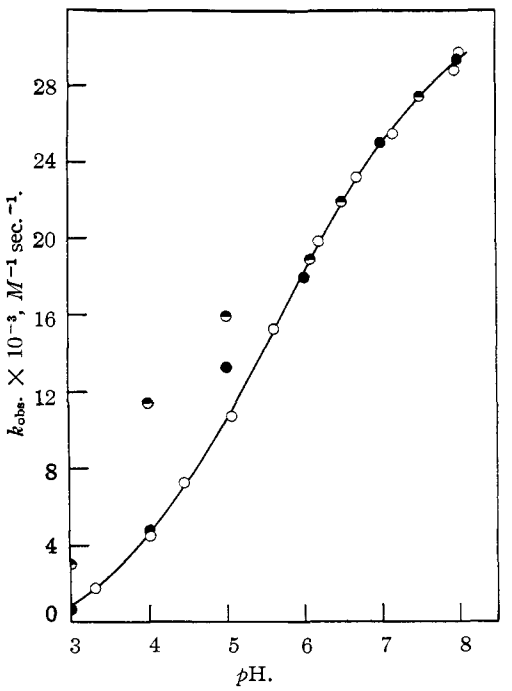


Fig. 5.—The acylation of α-chymotrypsin with N-*trans*-cinnamoylimidazole at 25.0 ± 0.1°: open circles, experimental data; full circles, fit of eq. 13 to experimental data, using $pK_1 = 6.78$ and $pK_a' = 4.40$; half-filled circles, fit of eq. 13, using $pK_1 = 6.78$ and $pK_a' = 3.65$.

indicated that the deviations at low *p*H could be accounted for on the basis of the variation of total charge on the protein with variation in the *p*H. That is, an electrostatic effect of the charge of the protein on the ionization of the group involved in the catalytic action could produce this deviation. However, inspection of the effects of ionic strength on the acylation rate constants (Fig. 3) indicates that at low *p*H, the region of interest here, the acylation rate constants are extremely sensitive to ionic strength and therefore the choice of a particular ionic strength for comparison of the data is arbitrary. It appears from the extrapolation shown in Fig. 3 that if zero ionic strength had been chosen, the positive deviations from a sigmoid curve at low *p*H, evident in Fig. 4, would disappear. Zero ionic strength was not chosen simply because insufficient data were available to make suitable extrapolations at all *p*H's. However, it is now apparent that the deviations from the sigmoid curve at low *p*H's are not due to the electrostatic effect of the protein on the ionization of the catalytic group but rather are due to powerful ionic strength effects at low *p*H. The effect of ionic strength on the dimerization of α-chymotrypsin does not appear to offer an explanation for this phenomenon.[28]

Configurational changes brought about by specific binding of ions might offer some explanation. It has been shown that the addition of sodium chloride increases the classical $k_{cat}$ and decreases $K_m$ in the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate, both of these effects leading to a faster over-all reaction.[29] The effect was particularly large at low ionic strength (in agreement with the present results). The solution to this problem will ultimately depend on an analysis of the effects of ionic strength on both $K_m$ and $k_2$, data which are not available at the moment.

Table II also contains data on the *p*H dependence of deacylation of trimethylacetyl-α-chymotrypsin calculated from the zero-order velocity at [substrate]/[enzyme] ratios greater than one. The deacylation of trimethylacetyl-α-chymotrypsin, as evident from Table II and Fig. 4, is dependent on a group with an apparent $pK_a$ of 6.8. The deacylation reaction in contrast to the acylation reaction is insensitive to the ionic strength of the medium.

**α-Chymotrypsin-catalyzed Hydrolysis of N-*trans*-Cinnamoylimidazole.**—The reaction of N-*trans*-cinnamoylimidazole with α-chymotrypsin has previously been shown to follow eq. 1.[9] As with the other substrates utilized in this study, the acylation reaction is considerably faster than the deacylation reaction and it is therefore possible to determine the kinetics of each process independently of the other. Second-order kinetics were followed precisely for the acylation reaction. The value of the absorbance of N-*trans*-cinnamoylimidazole at zero time is required for computation of the second-order constants. The absorbance of the substrate was found to be *p*H dependent owing to its protonation. Therefore initial absorbances were obtained by a spectrophotometric titration procedure, independently of the kinetic experiments. The spectral change observed was of sufficient magnitude to allow the $pK_a'$ of N-*trans*-cinnamoylimidazole to be determined with good precision. The value obtained for the $pK_a'$ was 3.65 ± 0.01.[30] The results of the acylation experiments from *p*H 3.3 to 9 are shown in Table III and Fig. 5.

The *p*H dependence of the acylation of α-chymotrypsin with N-*trans*-cinnamoylimidazole appears to be exceptional, when compared to other chymotrypsin-catalyzed reactions. Instead of the usual sigmoid relationship, a considerably flattened *p*H–rate profile is found. The explanation for the unusual *p*H dependence may lie in the fact that N-*trans*-cinnamoylimidazole possesses a $pK_a'$ of 3.65, while substrates investigated previously do not possess an ionizable group near neutrality. If this is the case, this acylation reaction cannot conform to eq. 4 and 10. Taking into account both the ionization of the enzyme and the ionization of the substrate, a possible scheme for the acylation of α-chymotrypsin with N-*trans*-cinnamoylimidazole is

$$\begin{array}{ccccccc} E & + & S & \overset{K}{\rightleftharpoons} & ES & \xrightarrow{k_2} & ES' + P_1 \\ K_1 \downarrow\uparrow & & K_S \downarrow\uparrow & & K_2 \downarrow\uparrow & & \\ EH^+ & & SH^+ & & ESH^+ & \xrightarrow{k_2^*} & ES' + P_1 + H^+ \end{array} \qquad (11)$$

Using a derivation similar to that used in the derivation of eq. 8 it may be shown that

(28) R. F. Steiner, *Arch. Biochem. Biophys.*, **53**, 457 (1954).

(29) C. Niemann and R. B. Martin, *J. Am. Chem. Soc.*, **79**, 4814 (1957).

(30) W. P. Jencks and J. Carriuolo, *J. Biol. Chem.*, **234**, 1272 (1959), found a $pK_a$ of 3.6 for N-acetylimidazole from kinetic measurements.

$$k_{obs} = \frac{k_2/K_m + k_2^*[H^+]/K_mK_2}{(1 + [H^+]/K_1)[(1 + [H^+]/K_s) + (E)/K_M(1 + [H^+]/K_2)]} \quad (12)$$

By arguments similar to those elaborated before in simplification of eq. 8, the last term in the denominator of eq. 12 may be dropped, leading to

$$k_{obs} = \frac{k_2/K_m + k_2^*[H^+]/K_mK_2}{(1 + [H^+]/K_1)(1 + [H^+]/K_s)} \quad (13)^{31}$$

Since the value of $K_s$ ($pK_s' = 3.65$) is known, at $p$H's higher than 5.5 the second term in the denominator of eq. 13 may be neglected; it is then possible to calculate the value of $K_1$, $k_2/K_m$ and $k_2^*/K_mK_2$. From the solution of simultaneous equations using the data from $p$H's 6, 7 and 8, it is calculated that $pK_1 = 6.8$, in reasonable agreement with the values of the apparent $pK_a$'s of the other acylation reactions.[32] Using the values of $K_1$, $k_2/K_m$ and $k_2^*/K_mK_2$ calculated above and eq. 13, it is then possible to determine kinetically the value of $K_s$, using data at $p$H's 3, 4 and 5. The value of

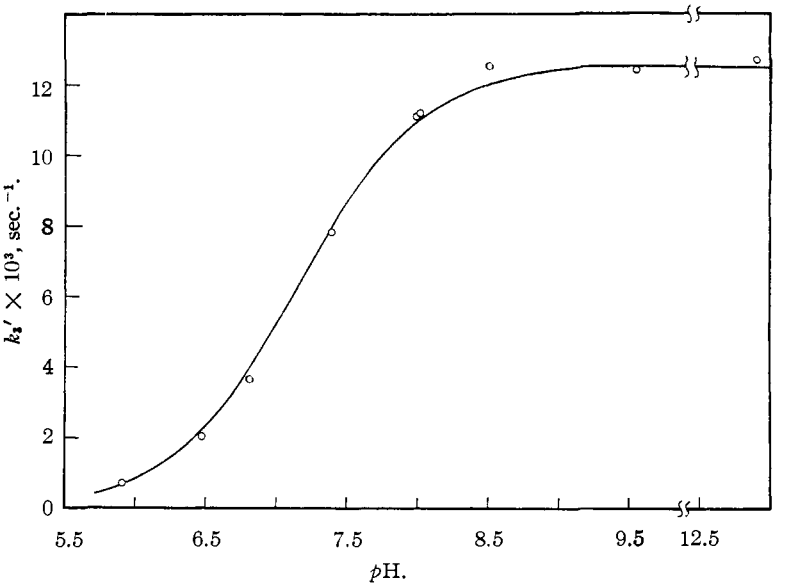


Fig. 6.—Deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin in 1% acetonitrile–water at 25°.

TABLE III

ACYLATION OF $\alpha$-CHYMOTRYPSIN WITH N-*trans*-CINNAMOYLIMIDAZOLE[a]

| $p$H | Buffer | [E]$_0$/[S]$_0$ | $k_{obs} \times 10^{-3}$,[g] $M^{-1}$ sec.$^{-1}$ |
|---|---|---|---|
| 3.31 | Acetate[b] | 3.02, 1.51 | 1.77 ± 0.06 |
| 4.02 | Acetate | 3.02, 1.51 | 4.46 ± .06 |
| 4.46 | Acetate | 1.51, 1.51 | 7.22 ± .06 |
| 5.06 | Acetate | 1.51, 1.51 | 10.75 ± .04 |
| 5.60 | Phosphate[b] | 1.51, 1.51 | 15.32 ± .12 |
| 6.20 | Phosphate | 1.51, 2.42 | 19.9 ± .1 |
| 6.70 | Phosphate | 1.21, 1.21 | 23.2 ± .7 |
| 7.16 | Phosphate | 1.22, 2.24 | 25.5[d,f] |
| | | 1.12, 1.55 | |
| 7.97 | Phosphate | 1.22, 1.22 | 28.8 ± 1.0 |
| 8.02 | Barbital[c] | 1.12, 1.55 | 29.8[e,f] |
| | | 1.18 | |
| 7.22 | Tris[b] | 1.18, 1.18 | 38.8 ± 2.0 |
| 7.87 | Tris[b] | 1.16, 1.16 | 39.5 ± 0.2 |
| 8.44 | Tris | 1.15, 1.15 | 24.4 ± .2 |
| 9.88 | Tris | 1.16, 1.16 | 24.2 ± .2 |
| 8.95 | Barbital[c] | 1.18, 1.18 | 11.04 ± .05 |

[a] In 1.6% acetonitrile–water at 25.0°; [enzyme] varied from $9 \times 10^{-6}$ to $2.5 \times 10^{-5}$ $M$; [substrate] was ~$8 \times 10^{-6}$ $M$. [b] 0.1 $M$ total. [c] 0.1 $M$ sodium barbital and 0.1 $M$ hydrochloric acid. [d] $\sigma_M$ (standard error of mean) = $1.29 \times 10^3$—7 determinations. [e] $\sigma_M = 1.04 \times 10^3$ — 6 determinations. [f] Data obtained with three independently standardized enzyme solutions. [g] Deviations are average deviations from the mean.

$pK_s$ found kinetically is 4.4, differing significantly from that found by spectrophotometric titration (3.65). The reason for this large discrepancy is not immediately apparent; it may be connected with the reason for the discrepancy between the theoretical and observed acylation curves of the other compounds investigated here. At any rate, a reasonable extrapolation of eq. 4 is able to account qualitatively if not quantitatively for the $p$H dependence of the acylation reaction of N-*trans*-cinnamoylimidazole with $\alpha$-chymotrypsin.

The acylation rate constant with N-*trans*-cinnamoylimidazole falls sharply above $p$H 8, as does that with $p$-nitrophenyl trimethylacetate above 8.6. This effect has been explained by Laidler in some cases as being due to a second protonic equilibrium of the enzyme. The deacylation results for *trans*-cinnamoyl-$\alpha$-chymotrypsin and for trimethylacetyl-$\alpha$-chymotrypsin, particularly the former, cast serious doubt on this interpretation. The rate constant of the deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin is independent of $p$H from $p$H 8.5 to $p$H 13. In deacylation, a single step, uncomplicated by binding equilibria, is being observed and denaturation of the enzyme has been shown not to produce a significant effect on the kinetics. The acylation data, on the other hand, are complicated by the $p$H dependence of $K_m$ and possible denaturation of the enzyme above $p$H 8.[33] Therefore the acylation results of interest with N-*trans*-cinnamoylimidazole and $p$-nitrophenyl trimethylacetate are only those obtained up to $p$H 8 or 8.6, respectively.

It is clear from Table III that Tris is exerting a specific buffer effect on the acylation rate at the concentration of buffer used. Both barbital and phosphate buffers yield consistent results at $p$H~8, and it is clear from Fig. 5 that the cross-over from acetate to phosphate buffers is quite smooth.

The effect of $p$H on the deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin is shown in Table IV and Fig. 6. These data are the most extensive and most carefully collected data reported in this paper. This reaction provides kinetics which are

(31) It has been shown that a mechanism omitting $k_2^*$ does not satisfy the data, and several more complex schemes give no better fit than that obtained here.

(32) The results of this calculation indicate that $k_2$ and $k_2^*$ are of the same order of magnitude.

(33) The slow deterioration of active $\alpha$-chymotrypsin in mildly alkaline solution is well-known.[28]

convenient to measure and which are capable of high accuracy. As seen from Table IV, the average deviations are rarely over 5% and in most cases considerably better than that. For an enzymatic process, this reproducibility is indeed welcome. Taking advantage of this situation, it was decided to extend the *p*H range of inquiry from the acid region (*p*H 5.91) to as far on the basic side as was practical. Valid results were obtained up to 0.1 *N* sodium hydroxide and an approximate result was obtained in 1 *N* sodium hydroxide. The *p*H's quoted sound as if one is dealing with a base-catalyzed reaction, but in fact the reaction in 0.1 *N* sodium hydroxide (and perhaps even in 1.0 *N* sodium hydroxide) is an enzymatic one.

TABLE IV

DEACYLATION OF *trans*-CINNAMOYL-α-CHYMOTRYPSIN[a]

| *p*H | Buffer | [NaOH] | [NaCl] | No. of detn. | $10^3\ k_3'$, sec.$^{-1}$[b] |
|---|---|---|---|---|---|
| 5.91[e] | Phosphate[c] | | | 1 | 0.73 |
| 6.48 | Phosphate | | | 2 | 2.02 ± 0.03 |
| 6.82 | Phosphate | | | 2 | 3.65 ± .07 |
| 7.39 | Phosphate | | | 2 | 7.82 ± .14 |
| 7.94 | Borax[d] | | | 1 | 11.1 |
| 8.06 | Phosphate | | | 5 | 11.2 ± 0.3 |
| 8.49 | Tris[h] | | | 15[i] | 12.5 ± .5 |
| 9.54 | Tris[g] | | | 2 | 12.4 ± .1 |
| 10.95 | Carbonate | | | 2 | 13.4 ± .4[i] |
| 11.8[f] | | $0.0103_5$ | 0.100 | 2 | 12.4 ± .2 |
| 12.9[f] | | $.107_5$ | | 2 | 12.7 ± .7 |
| 12.9[f] | | $.107_5$ | 0.100 | 2 | 12.9 ± .1 |
| 12.8[f] | | $.107_5$ | .200 | 2 | 14.4 ± .3 |
| 12.8[f] | | $.107_5$ | .400 | 2 | 15.6 ± .2 |
| 12.8[f] | | $.107_5$ | $1.00_0$ | 2 | 24.5 ± .0 |
| 12.7[f] | | $.107_5$ | $1.80_0$ | 2 | 37.6 ± 1.0 |
| 13.7[f] | | $1.07_5$ | | 1 | 35.0 |

[a] In 1% acetonitrile–water at 25.0°. [b] Deviations are average deviation for two runs, or convey the range for more than two runs. [c] $^1/_{15}$ *M*. [d] 0.05 *M* borax + 0.1 *N* HCl. [e] In experiments from *p*H 5.91 to 10.95 the acyl-enzyme was generated directly in the spectrophotometer cuvette. [f] In these experiments an aliquot of acyl-enzyme prepared at *p*H 4.2 was used. [g] 0.1 *M*. [h] 0.05 *M*. [i] This value is not believed to be significantly different from $12.5 \times 10^{-3}$. In general, values obtained with the DK-2 for these fast reactions are higher than those obtained with Cary 14 PM. [j] These runs were carried out with various enzyme preparations over a considerable period of time. The reproducibility in general was excellent.

Up to *p*H 10.95 it was possible to produce the acyl-enzyme from the enzyme plus substrate directly in the reaction medium. Above this *p*H, very little acyl-enzyme was formed in this manner, presumably due to the rapid denaturation of the enzyme, coupled with the rapid base-catalyzed hydrolysis of the substrate. Even at *p*H 11, the amount of acyl-enzyme formed was considerably reduced below the stoichiometric amount. Therefore, for work at the high *p*H values the acyl-enzyme was prepared at *p*H 4.2, where the rate of deacylation is very slow. An aliquot of this solution was then added to the appropriate base solution, and the deacylation reaction followed. These observations indicate considerably greater stability of the acyl-enzyme toward hydroxide ion than of the enzyme itself. This phenomenon has been noted many times in these laboratories. Not only is stability toward denaturation by hydroxide ion enchanced when the enzyme is converted to the acyl-enzyme, but also stability toward denaturation by urea is enhanced by this transformation. The enhanced stability of the acyl-enzyme with respect to the enzyme implies that the important part of denaturation occurs in the area immediately surrounding the active site; if one protects this area of the enzyme by derivatization, resistance toward various forms of denaturation is thus enhanced.

It may be further noted that sodium chloride stabilizes both the enzyme and the acyl-enzyme. This effect can be seen from the data in 0.1075 *N* sodium hydroxide. In the absence of sodium chloride, normal first-order kinetics were not obtained, the rate constant drifting rapidly to lower values as the reaction progressed (see Fig. 1).[34] On the other hand, in the presence of 0.100 *M* or higher sodium chloride, precise first-order plots were obtained, indicating that the acyl-enzyme was sufficiently stable to deacylate in a normal fashion. The presence of large concentrations of salt produces another effect besides stabilization of the acyl-enzyme—an ionic strength effect. Since determinations of the deacylation rate constant at lower *p*H's were carried out at 0.1 *M* ionic strength or lower, it was desired to have a value for *p*H 12.9 which would be comparable. This was obtained by determining the effect of ionic strength on the rate constant including determination of the initial rate for the deacylation in the absence of sodium chloride (*vide supra*). The data shown in Table IV lead to a continuous although non-linear function of $k_3$ *vs.* $\mu$, indicating that the value of $k_3$ in the absence of sodium chloride is an excellent value and may be used for comparison with other values of $k_3$ in the Table.

The ionic strength data in 0.1 *N* sodium hydroxide also provide information applicable to the reaction carried out in 1.0 *N* sodium hydroxide. The rate constant expected in 1.0 *N* sodium hydroxide on the basis of the effect of ionic strength on the reaction in 0.1 *N* sodium hydroxide is approximately $24.5 \times 10^{-3}$ sec.$^{-1}$. The value obtained was somewhat higher, $35 \times 10^{-3}$ sec.$^{-1}$. An explanation of this discrepancy lies in the fact that the enzyme produced upon deacylation undergoes denaturation at high *p*H, accompanied by an increase in absorbance. The rate of change of absorbance with time in base solutions containing enzyme alone has been measured independently to ascertain whether denaturation was producing a significant effect on the deacylation rate constants. It was found in 0.01 *N* and 0.1 *N* sodium hydroxide (with added sodium chloride) that the denaturation of the enzyme was not affecting the deacylation rate constants. However, there is considerable denaturation in 1.0 *N* sodium hydroxide and it is for this reason that the rate constant for 1 *N* sodium hydroxide must not be considered an accurate value. It is almost certainly less than $35 \times 10^{-3}$ sec.$^{-1}$, for the effect of denaturation is to raise the apparent rate constant, indicating that the independence of the deacylation rate constant of *p*H may extend even up to 1.0 *N* sodium hydroxide.

(34) Initial rates were obtained using a prism as a tangimeter; V. L. Frampton, *Science*, **107**, 323 (1948).

## Discussion

Table V summarizes the $p$H effects found in the second-order acylation rate constants and in the deacylation rate constants for the $\alpha$-chymotrypsin-catalyzed hydrolyses of *o*-nitrophenyl cinnamate, *p*-nitrophenyl trimethylacetate and N-*trans*-cinnamoylimidazole.

TABLE V

$p$H DEPENDENCE OF SOME $\alpha$-CHYMOTRYPSIN-CATALYZED REACTIONS[d]

| Substrate | Apparent $pK_a$ of enzymatic group on which step is dependent | |
|---|---|---|
| | Acylation[d] | Deacylation |
| *o*-Nitrophenyl cinnamate[a] | 7.0 | 7.3 |
| *p*-Nitrophenyl triethylacetate[b] | 6.7 | 6.8 |
| N-*trans*-Cinnamoylimidazole[c] | 6.8 | 7.15 |

[a] 10% acetonitrile–water. [b] 0.8–1.6% acetonitrile–water. [c] 1% acetonitrile–water; the acylation reaction also depends on the prototropic equilibrium of the substrate. [d] 25°. [e] Derived from second-order kinetic constants which means that any dependency of $K_m$ on $p$H is included.

The results shown in Table V are in reasonable agreement with each other, and with comparable results in the literature. The $pK_a$ of the deacylation steps of the reactions of *o*-nitrophenyl cinnamate and of N-*trans*-cinnamoylimidazole should be identical with one another for these reactions involve the reaction of the same acyl-enzyme intermediate, *trans* - cinnamoyl - $\alpha$ - chymotrypsin. The discrepancy between the $pK$'s of these reactions reflects the difference in solvent and the experimental error in these determinations which is of the order of 0.1 $pK$ unit. It should be pointed out that the value of the $pK_a$ of 7.15 determined from N-*trans*-cinnamoylimidazole is a more precise value for a number of reasons: the data are more extensive; the determinations were carried out after considerable preliminary experience with other systems; the enzyme concentrations were always determined by titration of the active sites; and finally the scatter is less extensive. Therefore the $pK_a$ of 7.15 is to be preferred for the deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin. The $pK_a$ of 6.8 for the deacylation of trimethylacetyl-$\alpha$-chymotrypsin is somewhat lower than the $pK_a$'s of the other deacylation reactions but is still reasonably close. The values in the literature for the deacylation of acetyl-chymotrypsin (either $\alpha$ or $\delta$) include $pK_a$'s of 6.96,[15] 7.2,[25] 7.28,[14] 7.32[32] and 7.44.[35] Certainly there is excellent agreement, considering the differences in solvents and buffers used, between the results obtained in this investigation and those in the literature.

The most significant new results of these determinations of the $p$H dependence concerns the effect of $p$H's greater than 9 on the deacylation reaction. The rate constant of the deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin is essentially invariant from $p$H 9 to 0.1 $N$ sodium hydroxide and may perhaps be invariant to 1.0 $N$ sodium hydroxide. No one has ventured into these non-physiological regions with an enzyme before because of the obvious obstacle of enzyme denaturation. Our finding that the acyl-enzyme is considerably more resistant to base (and urea) denaturation than the enzyme itself and that this resistance is enhanced by the addition of sodium chloride allowed us to investigate the effect of $p$H in this region. The result is that we can extend the region of applicability of the sigmoid relationship now to $p$H 12.9. Thus it appears that if an acid group is operative (in conjunction with the basic group of $pK_a \sim 7$), the acid group must possess a $pK_a$ greater than 13.9, for we should be able to pick up a diminution in rate constant one unit lower than the $pK_a$.

Apparently the deacylation reaction, even in this high $p$H region, involves a water molecule as the nucleophile rather than hydroxide ion. At $p$H $\sim$ 13 the rate constant of the alkaline hydrolysis of *trans*-cinnamoyl-$\alpha$-chymotrypsin in 7.74 $M$ urea is equal to that of the deacylation of *trans*-cinnamoyl-$\alpha$-chymotrypsin in water.[9] If hydroxide ion were the nucleophile in the deacylation reaction, one would expect a dependency on the hydroxide ion concentration at $p$H $\sim$ 13 such as that found with *trans*-cinnamoyl-$\alpha$-chymotrypsin in 7.74 $M$ urea, assuming that the acyl-enzyme is a simple serine ester. Since such a dependency is not found it must be concluded that the enzyme must require the reaction of a water molecule with the acyl-enzyme to the exclusion of hydroxide ion.

The $pK_a$'s of the acylation reactions cannot be analyzed in as straightforward a manner as those of the deacylation reactions for in the former we have measured a complex rate constant consisting of a pre-equilibrium ($K_m$) followed by a rate-determining step ($k_2$). This of course means that the $pK_a$ may reflect the $p$H dependence of the pre-equilibrium or of the rate-determining step or of both steps. The $pK_a$'s of the acylation steps of *o*-nitrophenyl cinnamate and of *p*-nitrophenyl trimethylacetate are reasonably consistent with one another and with results in the literature. The $pK_a$'s of 6.7 and 7.0 found in these investigations may be compared with values of 6.22, 6.7 and 7.0 reported for the acylations of *p*-nitrophenyl acetate,[15] 2,4-dinitrophenyl acetate[13] and *p*-nitrophenyl acetate,[35] respectively. The first and third values are measurements taken from second-order acylation constants while the second value is one determined from first-order acylation constants ($k_2$ itself). There does not seem to be any difference between results obtained from first-order or second-order acylation constants; the results obtained here may therefore reflect $k_2$. The reason that this approximation may be valid is that in the acid region, $K_m$ appears to be independent of $p$H.[8,10,11] The only result showing a dependence of $K_m$ on $p$H indicates that the change of $K_m$ with $p$H does not start until above $p$H 8.[10,12] It should be pointed out that the second-order acylation rate constants are complicated by ionic strength effects, in contrast to the deacylation rate constants which are essentially independent of ionic strength. This result required the use of an arbitrary ionic strength close to zero ionic strength for a comparison of the acylation rate constants at various $p$H's. Therefore the $pK_a$'s determined from the acylation measurements may be open to criticism.

The acylation of $\alpha$-chymotrypsin with N-*trans*-

(35) E. Awad and H. Neurath, quoted by H. Neurath and B. S. Hartley, *J. Cell. Comp. Physiol.*, **54**, Supp. 1, 185 (1959).

cinnamoylimidazole is complicated by the protonation of the substrate as well as the protonation of the enzyme, and by the apparent reaction of both protonated and unprotonated substrate species. Although the correspondence of this scheme (eq. 13) to the experimental data is not completely satisfying quantitatively, it is the simplest one which at least qualitatively fits the data.

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# Kinetic Isotope Effects of Deuterium Oxide on Several α-Chymotrypsin-catalyzed Reactions[1,2]

BY MYRON L. BENDER[3] AND GORDON A. HAMILTON

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The kinetics of several reactions catalyzed by α-chymotrypsin have been determined in $D_2O$ as solvent, and the results have been compared with the kinetics of the same reactions in $H_2O$ as solvent. By a suitable choice of substrates it has been possible to estimate the effect of $D_2O$ on the binding of the substrate to the enzyme, on the acylation of the enzyme and on the deacylation of the acyl-enzyme intermediate. The substrate *p*-nitrophenyl trimethylacetate was used for studying the acylation reaction, and *trans*-cinnamoyl-α-chymotrypsin and trimethylacetyl-α-chymotrypsin were used for studying the deacylation reaction. A sizable isotope effect was observed for both the acylation and deacylation reactions ($k^H/k^D$ = 2 to 3). The kinetics of the hydrolysis of N-acetyl-L-tryptophan methyl ester were also compared in $D_2O$ and $H_2O$ and it was observed that at high substrate concentrations the limiting rate constant is greater in $H_2O$ than in $D_2O$ by a factor of 2.83. The results indicate a rate-determining proton transfer in the catalytic steps of the enzymatic reaction.

## Introduction

Several mechanisms for the chymotrypsin-catalyzed hydrolysis of carboxylic acid derivatives have been proposed; in general these mechanisms involve a catalytic group on the enzyme acting as a general base or as a nucleophile or, in some cases, as both.[4] Usually it has been proposed that this group is an imidazole group of a histidine residue. Evidence in the literature suggests that the rate of a general base-catalyzed reaction is decreased in deuterium oxide relative to that in water by approximately 2- to 3-fold, whereas the rate of a reaction subject to nucleophilic catalysis may be affected to only a small extent by deuterium oxide.[5] Therefore, from the effect of deuterium oxide on the rate of the chymotrypsin-catalyzed reactions, one should be able to distinguish between general base and nucleophilic catalysis by the enzyme. The mechanisms proposed for the enzyme-catalyzed reactions are probably oversimplified; however, from the effect of deuterium oxide on each step of the reaction it should at least be possible to determine whether a proton is transferred in the rate-determining part of each step. Consequently, the effects of deuterium oxide on the rate of each of the various steps of the enzyme reaction were studied and are reported in this part.

From previous work[4] on the mechanism of chymotrypsin catalysis it appears that the reaction involves initially a reversible adsorption of the substrate (S) on the enzyme (E) to give an enzyme-substrate complex (ES). For many substrates the complex then reacts to give an acyl-enzyme (ES′) with concomitant release of the alcohol or amine portion ($P_1$) of the substrate, and the final step of the sequence involves deacylation of the acyl-enzyme to give the free enzyme (E) and carboxylic acid ($P_2$). By a suitable choice of substrates it is

$$\begin{aligned} &E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES && \text{adsorption} \\ &ES \xrightarrow{k_2} ES' + P_1 && \text{acylation} \qquad (1) \\ &ES' \xrightarrow{k_3} E + P_2 && \text{deacylation} \end{aligned}$$

possible to study the effect of deuterium oxide on each of these steps.

Various workers[1,4,6] using several different acyl-enzymes have studied the kinetics of the deacylation step in water. In the present research the kinetics of the deacylation of *trans*-cinnamoyl-α-chymotrypsin was studied thoroughly using deuterium oxide as solvent; this acyl-enzyme was used because the kinetics of its deacylation in water had been studied extensively and the reaction can be followed conveniently.[1]

Gutfreund and Sturtevant,[7] by using fast reaction techniques, were able to study the kinetics of the acylation of chymotrypsin by *p*-nitrophenyl acetate and 2,4-dinitrophenyl acetate. Since the ester *p*-nitrophenyl trimethylacetate reacts more slowly than the acetate esters with the enzyme,[1] its kinetics are more easily studied and thus this ester was used in the present research. The kinetics of the acylation reaction in both water and deuterium oxide were determined. Gutfreund

(1) This research was supported by grants from the National Institutes of Health and the U. S. Atomic Energy Commission. Paper XIV in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962).

(2) Some of the present results have been presented in preliminary form: M. L. Bender, G. R. Schonbaum, G. A. Hamilton and B. Zerner, *ibid.*, **83**, 1255 (1961).

(3) Alfred P. Sloan Foundation Research Fellow; present address: Northwestern Univ., Evanston, Ill.

(4) For recent reviews see (a) M. L. Bender, *Chem. Revs.*, **60**, 53 (1960); (b) M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).

(5) M. L. Bender, E. J. Pollock and M. C. Neveu, *J. Am. Chem. Soc.*, **84**, 595 (1962).

(6) M. L. Bender, G. R. Schonbaum and B. Zerner, *ibid.*, **84**, 2540 (1962).

(7) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956); *Proc. Natl. Acad. Sci., U. S.*, **42**, 719 (1956).