[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY, EVANSTON, ILL.]

The Kinetics of α-Chymotrypsin Reactions in the Presence of Added Nucleophiles[1]

BY MYRON L. BENDER, GERALD E. CLEMENT,[2] CLAUDE R. GUNTER, AND FERENC J. KÉZDY

RECEIVED FEBRUARY 12, 1964

The effect of added nucleophiles such as methanol, ethanol, and hydroxylamine on the kinetics of α-chymotrypsin-catalyzed reactions is interpreted in terms of the competitive partitioning of the acyl–enzyme intermediate by water and the added nucleophile according to eq. 2. In agreement with this hypothesis, the rate of deacylation of *trans*-cinnamoyl-α-chymotrypsin in methanol–water solutions is dependent on the methanol concentration, and the products of the reaction are both methyl cinnamate and cinnamate ion. Data at high methanol concentrations do not indicate a saturation phenomenon, thus giving no evidence for a binding of methanol (or water) to the enzyme. The α-chymotrypsin-catalyzed hydrolysis and alcoholysis of both specific and nonspecific substrates of α-chymotrypsin conform to the kinetics predicted by eq. 2. The kinetics of hydrolysis and methanolysis of N-acetyl-L-tyrosineamide and of N-acetyl-L-phenylalanineamide must be explained on the basis of eq. 2. A complete set of rate constants can be calculated from the alcoholysis experiments for the specific substrates, N-acetyl-L-tryptophan ethyl and methyl esters and N-acetyl-L-phenylalanine methyl ester. The α-chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis of N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosine hydroxamic acid may be interpreted in terms of an expansion of eq. 2, eq. 16, which takes into account both the enzymatic and nonenzymatic fates of the labile initial product O-(N-acetyl-L-tyrosyl)hydroxylamine.

Introduction

The reactions catalyzed by α-chymotrypsin have in the preceding papers been shown to follow eq. 1. This

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

equation omits the fact that every hydrolytic reaction catalyzed by α-chymotrypsin involves a molecule of water. Although it is possible to vary the concentration of the principal substrate, the carboxylic acid derivative, it is unfortunately not possible to vary the concentration of water in water. However, it is possible to use nucleophiles other than water in α-chymotrypsin-catalyzed reactions, including alcohols, and amines such as amino acids, hydroxylamine, and phenylhydrazine.[3] Presumably the role of these nucleophiles in α-chymotrypsin reactions is the same as that of water, namely to react with the acyl–enzyme intermediate to form the product of the reaction. Two particularly pertinent indications that added nucleophiles such as alcohols do in fact participate directly in α-chymotrypsin reactions are seen in the observation of an increase in the rate constant of deacylation of acetyl-α-chymotrypsin in the presence of ethanol and the isolation of ethyl acetate from the reaction mixture,[4] and the observation of an exchange reaction between N-acetyl-L-phenylalanine methyl-C^{14} ester and unlabeled methanol of the solution catalyzed by α-chymotrypsin.[5]

Several years ago Koshland and Herr[6] suggested that the role of water in enzymatic reactions could be elucidated by the use of water analogs such as alcohol, using the water analog to elucidate a possible water site on the surface of the enzyme. It is also possible to use a nucleophilic "water analog" to describe the transition state of deacylation which must include a nucleophilic species, and also to probe for the presence of the acyl–enzyme intermediate itself.

Hydroxylamine has been used as a water analog for the study of α-chymotrypsin-catalyzed reactions, with reference to the identification of the stepwise nature of the process.[7-13] The initial use of hydroxylamine for this purpose gave results which apparently contradict the hypothesis of an acyl–enzyme intermediate. For example, Bernhard[7-9] stated that the kinetics of simultaneous hydrolysis and hydroxylaminolysis are incompatible with an acyl–enzyme intermediate. More recently, Caplow and Jencks[10] have reported that trapping experiments involving hydroxylamine are at variance with the acyl–enzyme hypothesis. Thus, it is necessary to probe this controversial area.

The approach taken in the present paper presupposes the correctness of eq. 1. By introducing a nucleophile in addition to water, eq. 1 is expanded to eq. 2 where P_1 represents an alcoholic portion of an ester

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \begin{cases} \xrightarrow{k_3[H_2O]} E + P_2 \\ \xrightarrow{k_4[N]} E + P_3 \end{cases} \qquad (2)$$
$$\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\quad P_1$$

substrate, S, and P_2 represents the carboxylic acid; if the nucleophile N is the same as P_1 then P_3 must be equivalent to S.

From eq. 2, eq. 3, 4, and 5 can be derived using the usual steady-state assumption ($k_3' = k_3[H_2O]$). In this treatment it is assumed that the enzyme does not contain a specific site for a water molecule or other nucleophile; no evidence for binding of methanol to the enzyme is found here.

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

(2) N. I. H. Postdoctoral Research Fellow.

(3) M. L. Bender, *Chem. Rev.*, **60**, 95 (1960), and references cited therein.

(4) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

(5) M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960); *cf.* M. L. Bender and K. C. Kemp, *ibid.*, **79**, 111 (1957).

(6) D. E. Koshland, Jr., and E. B. Herr, Jr., *J. Biol. Chem.*, **228**, 1021 (1957).

(7) S. A. Bernhard and H. Gutfreund, "Proceedings of the International Symposium on Enzyme Chemistry, Tokyo," 1957, p. 124.

(8) S. A. Bernhard in "The Enzymes," Vol. I, P. D. Boyer, H. Lardy, and K. Myrback, Ed., 2nd Ed., Academic Press, Inc., New York, N. Y., 1959, p. 126.

(9) S. A. Bernhard, W. C. Coles, and J. F. Nowell, *J. Am. Chem. Soc.*, **82**, 3043 (1960).

(10) M. Caplow and W. P. Jencks, *J. Biol. Chem.*, **238**, PC 1907 (1963).

(11) M. Caplow and W. P. Jencks, *ibid.*, **238**, PC 3139 (1963).

(12) F. J. Kézdy, G. E. Clement, and M. L. Bender, *ibid.*, **238**, PC 3141 (1963).

(13) R. M. Epand and I. B. Wilson, *ibid.*, **238**, PC 3137 (1963).

$$\frac{dP_1}{dt} = \frac{\frac{k_2(k_3' + k_4N)}{k_2 + k_3' + k_4N} E_0S_0}{S_0 + K_s\frac{k_3' + k_4N}{k_2 + k_3' + k_4N}} \quad (3)$$

$$\frac{dP_2}{dt} = \frac{\frac{k_2k_3'}{k_2 + k_3' + k_4N} E_0S_0}{S_0 + K_s\frac{k_3' + k_4N}{k_2 + k_3' + k_4N}} \quad (4)$$

$$\frac{dP_3}{dt} = \frac{\frac{k_2k_4N}{k_2 + k_3' + k_4N} E_0S_0}{S_0 + K_s\frac{k_3' + k_4N}{k_2 + k_3' + k_4N}} \quad (5)$$

The catalytic rate constant (k_{cat}) is equal to the complex constant in the numerator of each equation and the K_m(app) is equal to K_s times the complex constant in the denominator of each equation. With eq. 3 through 5, which have the form of Michaelis–Menten equations, it is possible to account for the kinetics of α-chymotrypsin-catalyzed reactions involving added nucleophiles.

Experimental

Materials.—The enzyme, the determination of the normality of its solution, most substrates, and the buffers have been described previously.[14] The methanol was Baker analyzed reagent grade methanol or Eastman Kodak Co. methanol redistilled from calcium hydride. Hydroxylamine hydrochloride (Baker analyzed reagent) was used without further purification. The ammonia was a Baker analyzed reagent. N-Acetyl-L-tyrosine ethyl ester (Cyclo Chemical Co.) was recrystallized from ethanol–water; m.p. 73–77°; an infinity reading of an enzymatic hydrolysis on the pH stat showed 95.8% purity. However, the alkaline hydroxylamine test[10] gave *ca.* 85% of the theoretical N-acetyl-L-tyrosinehydroxamic acid. A gift of N-acetyl-L-tyrosinehydroxamic acid from Professor W. P. Jencks is gratefully acknowledged.

Kinetic Measurements.—The kinetics of the deacylation of *trans*-cinnamoyl-α-chymotrypsin in water and in methanol–water solutions have been described.[15,16] A spectrophotometric determination of the products of the methanolysis–hydrolysis reaction was made using a method for the simultaneous determination of two components of a mixture, knowing the extinction coefficients of methyl cinnamate and cinnamate ion at two wave lengths. The decomposition of methyl cinnamate by α-chymotrypsin under these conditions is negligible. The error in the product determination varies from 5% at low methanol concentration to about 15% at 6.17 *M* methanol. At concentrations of methanol approaching 35% by volume, enzyme precipitation causes any kinetic studies to be entirely qualitative.

The kinetics of the α-chymotrypsin-catalyzed hydrolysis and alcoholysis of N-acetyl-L-tryptophan methyl and ethyl esters was determined spectrophotometrically.[14]

The kinetics of the α-chymotrypsin-catalyzed hydrolysis and methanolysis of N-acetyl-L-phenylalanine methyl ester were determined using a Radiometer TTT1C pH stat.[17] The K_i of N-acetyl-L-phenylalanine was determined by using it as an inhibitor for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate using second-order conditions ($E_0 \cong S_0 << K_s$). The inhibition constant determined from the equation $(k_2/K_s)'/(k_2/K_s)^{H_2O} = (1 + (I/K_i))$ was found to be 4.44 × 10^{-3} *M* in 0.82% (v./v.) acetonitrile–water at pH 7.0.

The calculation of the kinetics of hydrolysis of both the tryptophan compounds, observed spectrophotometrically, and of the phenylalanine compound, determined with the pH stat, utilized one kinetic run to determine the complete Lineweaver–Burk plot. This convenient method necessitates an initial substrate concentration 2 to 5 times that of K_m(app) and utilizes data for the reaction up to 100% completion. Ordinarily data for the entire reaction is treated by means of an integrated equation. However, it is more profitable to utilize a complete reaction curve by means of the usual differential form of the Lineweaver–Burk equation because correction for spontaneous hydrolysis may easily be made. One can thus determine many S_i and the corresponding V_i from one kinetic experiment. The V_i's may be corrected for spontaneous hydrolysis of the substrate, knowing S_i and the rate constant for spontaneous hydrolysis. The amount of substrate remaining at each time, S_i, can be readily calculated knowing the experimental observation at 100% reaction and the increment due to a change in substrate concentration. Thus one can plot 15 to 30 points in a usual Lineweaver–Burk plot over a 10–20-fold change in substrate concentration from one experiment.

In some instances inhibition by product will occur, which can be corrected by means of the equation

$$k = \frac{k_{cat}S_i}{S_i + K_s(1 + (S_0 - S_i)/K_i)} \quad (6)$$

where the concentration of the inhibitor equals ($S_0 - S_i$) and E_0 is incorporated into k_{cat}. The reciprocal of this equation with suitable transformations is

$$1/k = 1/k_{cat}(1 - K_s/K_i) + 1/S_i[(K_s/k_{cat}) + (K_sS_0/k_{cat}K_i)] \quad (7)$$

Thus a Lineweaver–Burk plot of eq. 7 (plotting $1/S$ *vs.* $1/V$) gives a straight line with an intercept and slope having different meanings from the usual Lineweaver–Burk plot. In order to complete the analysis, it is obviously necessary to determine the inhibition constant, K_i, of the product of the reaction. If K_s is not close to K_i, one may completely determine the kinetics.

In all kinetic experiments infinity readings were used to determine the purity of the starting material and to check that the correct stoichiometry was followed.

Results

Hydrolysis and Methanolysis of *trans*-Cinnamoyl-α-chymotrypsin.—One may determine directly whether an added nucleophile such as methanol can participate in the enzymatic reaction as a nucleophilic competitor of water for the acyl-enzyme intermediate, using the discrete deacylation step, the deacylation of *trans*-cinnamoyl-α-chymotrypsin. In methanol–water solutions at pH 8.5, the deacylation of *trans*-cinnamoyl-α-chymotrypsin is found spectrophotometrically to produce cinnamate ion and methyl cinnamate. It is possible to determine both the rate of disappearance of the reactant and also the rate of appearance of the two products, cinnamate ion and methyl cinnamate, since they absorb differently from one another.[18] The partitioning of the acyl–enzyme can be envisioned kinetically in two ways: (1) competitive reactions of water and methanol from solution (eq. 8) or (2) com-

$$ES' \xrightarrow{k_3[H_2O]} E + P_2 \qquad ES' \xrightarrow{k_4[MeOH]} E + P_3 \quad (8)$$

$$ES' \underset{H_2O}{\overset{K_w}{\rightleftharpoons}} ES'W \xrightarrow{k_1^*} E + P_2 \qquad ES' \underset{MeOH}{\overset{K_s}{\rightleftharpoons}} ES'M \xrightarrow{k_4^*} E + P_3 \quad (9)$$

(14) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *J. Am. Chem. Soc.*, **86**, 3680 (1964).

(15) M. L. Bender, G. R. Schonbaum, and B. Zerner, *ibid.*, **84**, 2542 (1962).

(16) M. L. Bender, G. R. Schonbaum, and B. Zerner, *ibid.*, **84**, 2562 (1962).

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

(18) M. L. Bender, G. R. Schonbaum, and B. Zerner, *ibid.*, **84**, 2542 (1962).

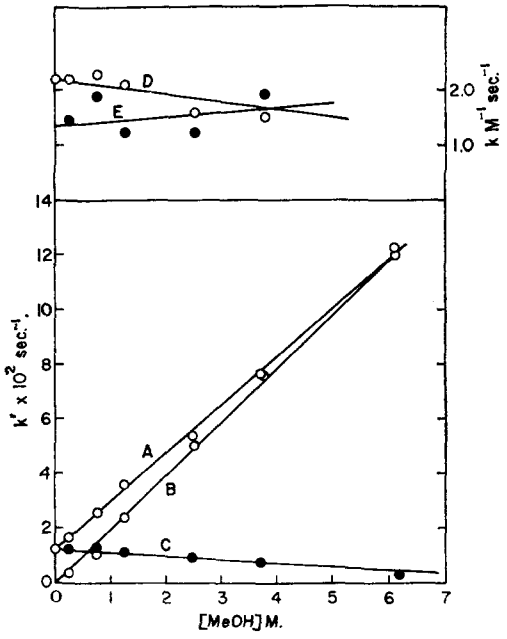


Fig. 1.—The kinetics of the simultaneous hydrolysis and methanolysis of *trans*-cinnamoyl-α-chymotrypsin, pH 8.5, Tris-HCl buffer (0.1 *M*), 0.63% acetonitrile, 25.0°: A, $k_{obsd} \times 10^2$ sec.$^{-1}$; B, $k_4' \times 10^2$ sec.$^{-1}$; C, $k_3' \times 10^2$ sec.$^{-1}$; D, $k_3 \times 10^4$ M^{-1} sec.$^{-1}$; E, $k_4 \times 10^2$ M^{-1} sec.$^{-1}$.

petitive formation of complexes of acyl-enzyme and water or methanol followed by reaction (eq. 9). ES′ is the acyl-enzyme, ES′W is the water complex, ES′M is the methanol complex, P_2 is the carboxylate product, and P_3 is the methyl ester. This formulation assumes that P_3 does not react with the enzyme. Equations 10 and 11 are the kinetic equations of the disappearance of ES′ corresponding to eq. 8 and 9, respectively.

$$k_{obsd} = k_3[H_2O] + k_4[MeOH] \quad (10)$$

$$k_{obsd} = \frac{k_3^*[H_2O]/K_w + k_4^*[MeOH]/K_s}{1 + [H_2O]/K_w + [MeOH]/K_s} \quad (11)$$

Equation 10 predicts that k_{obsd} will be a linear function of the methanol concentration, while eq. 11 predicts that k_{obsd} will in general not be a linear function of the methanol concentration, but rather a saturation by methanol will be observed at some point. Experimentally the predictions of eq. 8 and 10 are found (see Table I and Fig. 1). Spectrophotometric analysis of the products, together with

TABLE I

THE EFFECT OF METHANOL CONCENTRATION ON THE KINETICS OF METHANOLYSIS AND HYDROLYSIS OF *trans*-CINNAMOYL-α-CHYMOTRYPSIN[a]

Methanol, *M*	$k_{obsd} \times 10^2$, sec.$^{-1}$	$k_3' \times 10^1$, sec.$^{-1}$	$k_4' \times 10^1$, sec.$^{-1}$	$k_3 \times 10^1$, l. mole^{-1} sec.$^{-1}$	$k_4 \times 10$, l. mole^{-1} sec.$^{-1}$
0.00	12.5[b]	12.5	...	0.22	..
.25	17.0	12.5	3.6	.22	14.5
.75	26.0	12.6	10.5	.23	14.1
1.25	35.6	11.4	24.2	.21	19.3
2.47	59.2	9.0	50.2	.17	20.3
3.71	83.7	7.6	76.1	.15	20.5
6.18	123	3.0	120	.06	19.4

[a] pH 8.5 at 25.0°, 0.63% (v./v.) acetonitrile, 0.1 *M* Tris-HCl buffer. [b] Reference 16 reports $k_{obsd} = 12.5 \times 10^{-3}$ sec.$^{-1}$ for this reaction in 1% (v./v.) acetonitrile–water solution.

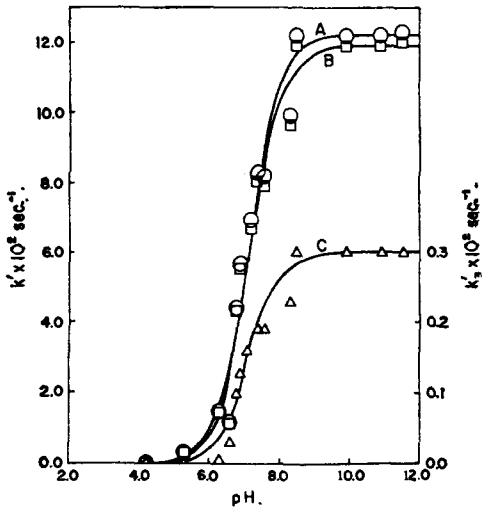


Fig. 2.—Deacylation of *trans*-cinnamoyl-α-chymotrypsin in 25% (6.18 *M*) methanol–water containing 0.63% acetonitrile, 0.1 *M* buffers, 25.0°: A, $k_{obsd}' \times 10^2$ sec.$^{-1}$; B, $k_4' \times 10^2$ sec.$^{-1}$; C, $k_3' \times 10^2$ sec.$^{-1}$; $pK_A = 7.15$, $pK_B = 7.25$, $pK_C = 7.3$.

eq. 10, allows the calculation of $k_4' = k_4[MeOH]$ and $k_3' = k_3[H_2O]$. Both k_4' and k_3' are linear functions of methanol concentration, whereas k_4 and k_3 are *independent* of methanol concentration as shown in Fig. 1.

The pH selected for the above determination of the effect of methanol concentration on the deacylation of *trans*-cinnamoyl-α-chymotrypsin was greater than 8.5. By carrying out these determinations in the "flat" of the sigmoid curve (Fig. 2), it is possible to eliminate the possibility that varying concentrations of methanol cause a change in the activity of the hydrogen ion not reflected by the pH meter reading. However, recent measurements indicate the essential identity of the hydrogen ion activities and pH meter readings in methanol–water solutions up to 68% methanol.[19]

The pH-k_{obsd} profile for the deacylation of *trans*-cinnamoyl-α-chymotrypsin in 25% methanol–water (Fig. 2 and Table II) is a sigmoid curve, quite similar to that found in water alone.[16] Both profiles have inflection points implying the dependence of the reaction on groups with pK_a's of 7.15, and thus indicating that the

TABLE II

THE EFFECT OF pH ON THE KINETICS OF METHANOLYSIS AND HYDROLYSIS OF *trans*-CINNAMOYL-α-CHYMOTRYPSIN[a]

pH	$k_{obsd} \times 10^2$, sec.$^{-1}$	$k_4' \times 10^2$, sec.$^{-1}$	$k_3' \times 10^2$, sec.$^{-1}$
4.2	0.0452	0.0442	0.001
5.3	0.268	0.262	.006
6.3	1.52	1.48	.04
6.6	1.20	1.17	.03
6.8	4.43	4.33	.10
6.9	5.67	5.54	.13
7.1	6.85	6.69	.16
7.4	8.28	8.09	.19
7.6	8.18	7.95	.19
8.3	9.90	9.67	.23
8.5	12.2	11.9	.3
9.9	12.2	11.9	.3
10.9	12.2	11.9	.3
11.5	12.3	12.0	.3

[a] 25% methanol–water at 25.0°, 0.63% acetonitrile, 0.1 *M* buffers.

(19) R. G. Bates, M. Paabo, and R. A. Robinson, *J. Phys. Chem.*, **67**, 1833 (1963).

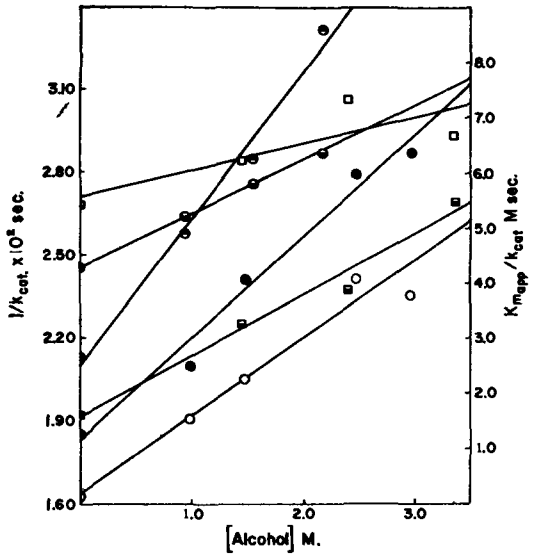


Fig. 3.—The kinetics of the simultaneous hydrolysis and alcoholysis of some specific substrates of α-chymotrypsin: ○, $1/k_{cat}$ for N-acetyl-L-phenylalanine methyl ester at pH 7.00; ●, $K_m(app)/k_{cat} \times 10^5$ for N-acetyl-L-phenylalanine methyl ester at pH 7.00; ◒, $1/k_{cat}$ for N-acetyl-L-tryptophan ethyl ester at pH 7.65; ⊖, $K_m(app)/k_{cat} \times 10^6$ for N-acetyl-L-tryptophan ethyl ester at pH 7.65; □, $1/k_{cat}$ for N-acetyl-L-tryptophan methyl ester at pH 7.65; ■, $K_m(app)/k_{cat} \times 10^6$ for N-acetyl-L-tryptophan methyl ester at pH 7.65.

same mechanism is operative and that methanol and water must be true competitors of the acyl-enzyme. The function k_{obsd} measures both methanolysis and hydrolysis. The ratios of methyl cinnamate/cinnamate in 25% MeOH was spectrophotometrically found to be 40.0 ± 0.2 at pH's 8.5, 7.4, and 6.8. This constancy allowed us to separate k_{obsd} with the aid of eq. 10 into its constituent parts. On this basis the pH–k_4' and pH–k_3' profiles may be calculated. As shown in Fig. 2, all these profiles are also sigmoid curves with approximately the same pK_a.

The Effect of Alcohols on the Hydrolysis of Specific Substrates.—In the hydrolysis of N-acetyl-L-tryptophan methyl ester in methanol–water, N-acetyl-L-tryptophan ethyl ester in ethanol–water, and N-acetyl-L-phenylalanine methyl ester in methanol–water, eq. 4 is applicable. Equation 4 can be transformed into eq. 12 and 13, which are useful for the above systems, since previous kinetic results indicate that k_2 is greater than k_3' for these systems.[14,17]

$$K_m(app)/k_{cat} = K_s/k_2 + K_s k_4 N/k_2 k_3' \quad (12)$$

$$1/k_{cat} = (k_2 + k_3')/k_2 k_3' + k_4 N/k_2 k_3' \quad (13)$$

Table III gives the experimental data for the hydrolyses of these specific ester substrates in alcohol–water solutions of varying concentration and Fig. 3 indicates that the data do in fact fit eq. 12 and 13 up to alcohol concentrations of 5 *M*.

The Hydrolysis and Hydroxylaminolysis of N-Acetyl-L-tyrosine Ethyl Ester.—The effect of enzyme concentration on the yield of N-acetyl-L-tyrosinehydroxamic acid from the α-chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis of N-acetyltyrosine ethyl ester is shown in Table IV.

TABLE III

THE EFFECT OF ALCOHOLS ON THE HYDROLYSIS OF SPECIFIC ESTER SUBSTRATES[a]

Alcohol, *M*	k_{cat}, sec.$^{-1}$	$K_m(app) \times 10^5$, *M*
N-Acetyl-L-tryptophan methyl ester in methanol–water solution[b]		
0	37.3 ± 1.0[c]	6.0 ± 0.7
1.44	35.2 ± 1.4	13.0 ± 1.3
2.41	32.6 ± 1.3	16.8 ± 1.4
3.37	34.0 ± 2.0	30.1 ± 3.0
N-Acetyl-L-tryptophan ethyl ester in ethanol–water solution[b]		
0	40.7 ± 1.3	10.8 ± 0.8
0.93	37.9 ± 1.3	19.9 ± 1.3
1.55	36.3 ± 2.0	26.5 ± 2.5
2.18	34.8 ± 1.3	38.1 ± 2.2
N-Acetyl-L-phenylalanine methyl ester in methanol–water solution[d]		
0	61.6 ± 0.3	75.9 ± 3
0.99	52.5 ± .4	141 ± 5
1.48	48.7 ± .7	227 ± 10
2.47	41.4 ± .4	332 ± 8
2.97	42.5 ± 1.7	398 ± 50

[a] 25.0°; all solutions contained 0.80% (v./v.) acetonitrile. [b] pH 7.65. [c] Computations carried out with an IBM 709 computer program designed by A. M. Myers.[14] [d] pH 7.0. [e] K_m(app)'s have been corrected for inhibition by methanol.[20]

TABLE IV

HYDROLYSIS AND HYDROXYLAMINOLYSIS OF ACETYL-L-TYROSINE ETHYL ESTER CATALYZED BY α-CHYMOTRYPSIN[a]

$[NH_2OH]_{free}$, *M*	pH	$E_0 \times 10^5$, *M*	$S_0 \times 10^3$, *M*	μ, *M*	Yield of ATHA, %
0.413	6.16	96.5	5.00	0.8	18.5
.423	6.16	1.49	6.01	0.8	39.1
.8	8.09	94.4	4.93	2.6	30.0
.8	8.09	9.65	5.03	2.6	36.7
.8	8.09	0.97	5.04	2.6	44.6
.8	7.22	1.95 ÷ 2.48	5.05×10^{-3}	2.7	53[b]
.8	7.68			2.7	50[b]
.8	8.27			2.7	49[b]

[a] 25°, 0.2 *M* Tris–HCl buffer. [b] Reference 10.

Discussion

The Methanolysis of *trans*-Cinnamoyl-α-chymotrypsin.—The identity of the pH dependence of the methanolysis and the hydrolysis of *trans*-cinnamoyl-α-chymotrypsin indicates that these two reactions follow the same mechanism, confirming the postulate of a competition between the nucleophiles methanol and water for the acyl–enzyme.[21] This competition is supported by the effect of methanol concentration on the observed rate constant of deacylation. These data require that methanol (and water, by analogy) must participate in the rate-determining process of deacylation.

The fact that the rate of the methanolysis reaction is linear with methanol concentration even up to very high methanol concentration indicates that no saturation phenomenon and therefore no binding of methanol occurs on the enzyme. This indication, however, is not a proof that at some higher methanol concentration binding does not occur. The site for water, if it does exist, must be quite different in chemical characteristics from the site for the principal substrate.

(20) M. Caplow and W. P. Jencks, *J. Biol. Chem.*, **239**, 164 (1964).

(21) D. Findlay, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **85**, 134 (1962), report a similar competition between methanol and water in the ribonuclease-catalyzed reaction.

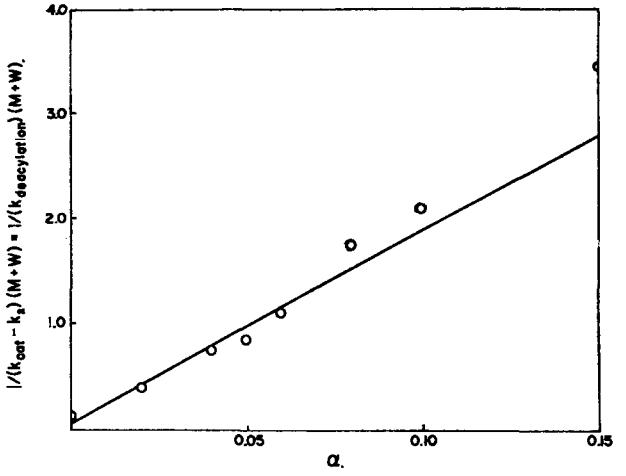


Fig. 4.—The α-chymotrypsin-catalyzed hydrolysis and methanolysis of *p*-nitrophenyl acetate at 12° and pH 7.85.[25]

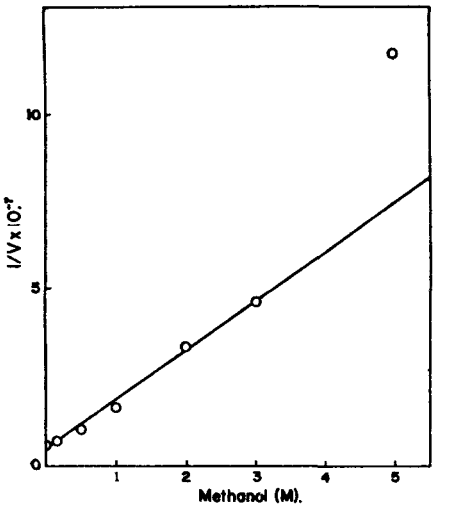


Fig. 5.—The hydrolysis of methyl hydrocinnamate in methanol–water solutions at pH 7.8.[26]

Whereas the former must be mainly hydrophobic in character, the hypothetical site for water may be most easily discussed in terms of a hydrogen bonding atom or group. Whether or not water and/or an added nucleophile is bound to the active site, the general postulate that water and nucleophiles compete for the acyl–enzyme is confirmed by the observations with *trans*-cinnamoyl-α-chymotrypsin.

The Effect of Alcohols on the Hydrolysis of Specific Substrates.—Equations 3 through 5 which were introduced to describe the effect of added nucleophiles, including alcohols, on various aspects of the hydrolysis of specific substrates, are not tractable as they stand. However, there are five simplifying conditions which lead to predictable and testable equations of all known data.

I. If one measures dP_1/dt and if k_3' is much greater than k_2, then $k_{cat} = k_2$ and $K_m(app) = K_s$, and k_{cat} should be independent of N. The chymotrypsin-catalyzed hydrolyses of N-acetyl-L-tyrosineamide,[22] and N-acetyl-L-phenylalanineamide[23] are predicted to fall in this category, by analogy with the kinetic constants and the pH–rate profile in the hydrolysis of N-acetyl-L-tryptophan amide and N-acetyl-L-phenylalanineamide. In these reactions, k_{cat} is *completely* independent of methanol concentration from 0 to 20.8% and from 0 to 25% methanol, respectively. The data on the effect of methanol on the rate constants of these amide hydrolyses are in agreement with the prediction made above using eq. 3. If the amide were to bypass the acyl–enzyme and react directly with water or methanol, k_{cat} should be dependent on either the water or the methanol concentration assuming that the reaction is either an hydrolysis or a methanolysis. Since high concentrations of methanol do not affect the k_{cat} of N-acetyl-L-tyrosineamide or of N-acetyl-L-phenylalanineamide at all, the amide reaction must not contain a water or methanol molecule in the rate-determining step and must therefore follow eq. 3.

II. If one measures dP_1/dt and if k_2 is much greater than k_3', $k_{cat} = k_2(k_3' + k_4N)/(k_2 + k_4N)$ from eq. 3; at low N, $k_{cat} = k_3' + k_4N$ but as N increases, $k_2 = k_{cat}$. In the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate, k_2 is much greater than k_3',[24] and one measures dP_1/dt. The data of Awad[25] permit one to test the above predictions; in agreement with prediction, the observed k_{cat} is *ca.* one-twenty-fourth the observed k_2 at 0% methanol while $k_{cat} = k_2$ at 0.5 mole fraction of methanol. The data of Awad may be treated using eq. 14 derived from eq. 3 ($\alpha = (M)/(W + M)$ = mole fraction of methanol). In Fig. 4 Awad's data are plotted according to eq. 14

$$1/(1/k_{cat} - 1/k_2)(W + M) = k_3 + (k_4 - k_3)\alpha \quad (14)$$

confirming the predicted relationship.

III. If one measures dP_2/dt and if k_3' is much greater than k_2, from eq. 4 k_{cat} can be expressed as $1/k_{cat} = 1/k_2 + k_4N/k_2k_3'$. The α-chymotrypsin-catalyzed hydrolysis of methyl hydrocinnamate in methanol–water should conform to this condition since the pH–rate profile of this reaction is a bell-shaped curve.[26] In agreement with prediction, a plot of $1/V$ *vs.* N for this reaction is indeed linear (Fig. 5).

IV. If one measures dP_2/dt and if k_2 is much greater than k_3', $k_{cat} = k_3'$ and thus k_{cat} would be independent of N. This condition has not as yet been observed and is included only for completeness.

V. If one measures dP_2/dt and if $k_2 \geqq k_3'$, the most common and interesting conditions are found. These are the conditions which apply to the hydrolysis of N-acetyl-L-tryptophan methyl ester and ethyl ester and of N-acetyl-L-phenylalanine methyl ester, fitted by eq. 12 and 13. The two slopes and two intercepts of plots of the left-hand sides of eq. 12 and 13 *vs.* N, as shown in Fig. 3, enable one to determine *all* rate and association constants, namely k_2, k_3, k_4, and K_s. The rate constants and association constants calculated in this way are summarized in Table V.

It is of interest to examine the details of the partitioning of the acyl–enzyme intermediate by water and alcohol. Table VI gives a summary of the partitioning of the acyl–enzymes by water and alcohol, and also a summary of corresponding nonenzymatic reactions. Toward carboxylic acid derivatives, alcohol is a

(22) F. Kaufman and H. Neurath, *J. Biol. Chem.*, **180**, 181 (1949).
(23) Unpublished experiments of Mr. H. d'A. Heck in this laboratory.
(24) F. J. Kézdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962).
(25) E. S. Awad, Doctoral Dissertation, University of Washington, 1959, p. 7.
(26) D. R. Stein and K. J. Laidler, *Can. J. Chem.*, **37**, 1272 (1959).

TABLE V

KINETIC CONSTANTS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS AND ALCOHOLYSIS OF SOME SPECIFIC SUBSTRATES[a]

Substrates, N-acetyl- ester	k_2, sec.$^{-1}$	k_1', sec.$^{-1}$	k_4,[b] M^{-1} sec.$^{-1}$	$K_s \times 10^3$, M
L-Tryptophan methyl[c]	755 ± 110	39 ± 7	28 ± 8	1.18 ± 0.1
L-Tryptophan ethyl[c]	553 ± 85	44 ± 8	47 ± 14	1.38 ± 0.13
L-Phenylalanine methyl[d]	581	68.3	108	6.70

[a] 25.0°. [b] k_4 pertains to methanolysis for the two methyl esters and ethanolysis for the ethyl ester. [c] pH 7.65. [d] pH 7.0.

better nucleophile than water and alkoxide ion is a better nucleophile than hydroxide ion, both in enzymatic and nonenzymatic reactions. The relative nucleophilicity of water and alcohol in the enzymatic reactions (A) appear to parallel the nonenzymatic intramolecular general basic catalysis mechanism (B5) more closely than the nonenzymatic intermolecular specific lyoxide reactions (B1–4). However, relative nucleophilicity in the latter reactions is not far different from that in the former reactions.

TABLE VI

RELATIVE REACTIVITIES OF ALCOHOL AND WATER IN VARIOUS REACTIONS OF CARBOXYLIC ACID DERIVATIVES

	Compound	$k_{methanol}/k_{H_2O}$	Reference
	A. α-Chymotrypsin reactions		
1	*trans*-Cinnamoyl-α-chymotrypsin	76.2	a
2	Furoyl-α-chymotrypsin	584	20
3	N-Acetyl-L-tryptophan methyl ester	39	a
4	N-Acetyl-L-tryptophan ethyl ester	51.7[b]	a
5	N-Acetyl-L-phenylalanine methyl ester	87.2	a
6	N-Acetyl-L-phenylalanine methyl ester	123–160[c]	5
7	*p*-Nitrophenyl acetate	123, 160	25, 31
	B. Nonenzymatic reactions		
1	*p*-Nitrophenyl acetate	46[d,g]	27
2	*p*-Nitrophenyl acetate	17[e,g]	27
3	N-Acetylphenylalanine methyl ester	10.3[d,h]	28[h]
4	N-Acetylphenylalanine ethyl ester	3.6[e,h]	28[h]
5	*p*-Nitrophenyl 5-nitrosalicylate	123[f]	29

[a] This research. [b] $k_{ethanol}/k_{H_2O}$. [c] Corresponding to 0.25 M methanol to 0.50 M methanol. [d] $k_{methoxide}/k_{OH^-}$. [e] $k_{ethoxide}/k_{OH^-}$. [f] $k_{methanol}/k_{H_2O}$ in intramolecular general basic catalysis. [g] Based on product analysis. [h] Recalculated from the data of ref. 28 on the basis of the ionization constants of Ballinger and Long[30] (pK(H_2O) = 15.75, pK(methanol) = 15.5, and pK(ethanol) = 16.0) which were used by Jencks and Gilchrist.[27] In this way, it is possible to make a direct comparison of reactions B1 and B3, and of reactions B2 and B4.

The principle of selectivity–reactivity would predict that a larger $k_{alcohol}/k_{H_2O}$ ratio would be observed in the reactions of an alkyl ester than in the reactions of a *p*-nitrophenyl ester, but just the opposite is found. The two investigations were carried out by completely different experimental techniques; nevertheless, no simple explanation can be found for this obvious problem.

The similarity of the $k_{alcohol}/k_{H_2O}$ ratios in the enzymatic reactions and in the model reaction, the solvolysis of *p*-nitrophenyl 5-nitrosalicylate, is a confirmation of the general basic catalysis in deacylation, for the latter reaction has been shown to involve an intramolecular general basic catalysis.[29] Of course, this is not a mandatory argument, since the reactions of specific lyoxide ions show ratios not much different from that of general basic catalysis.

Hydroxylaminolysis Reactions.—Caplow and Jencks[10] made two observations using hydroxylamine: (1) the rate of hydrolysis of N-acetyl-L-tyrosine ethyl ester by α-chymotrypsin is essentially independent of the hydroxylamine concentration while the rate of hydrolysis of N-acetyl-L-tyrosinehydroxamic acid by chymotrypsin decreases with increasing hydroxylamine concentration; and (2) these two substrates are partitioned differently between hydroxylamine and water. The first of these observations is compatible with the acyl–enzyme hypothesis embodied in eq. 2 while the second is apparently not.

If one assumes a single mechanism for these two substrates, the first observation is incompatible with a one-step mechanism, in which one should observe either independence or dependence of the hydrolysis on the hydroxylamine concentration with *both* substrates. This behavior cannot be explained in terms of differing solvent effects on the ethyl ester and hydroxamic acid reactions, for solvent effects of small molecules on chymotrypsin rate constants are small and independent of substrate.[32] However, this behavior can be readily explained, assuming the formation of an acyl–enzyme intermediate. From eq. 4 the maximal rate of hydrolysis is

$$dP_2/dt = k_W E_0 = E_0 k_2 k_3'/(k_2 + k_3' + k_4N) \quad (15)$$

In the hydrolysis of N-acetyl-L-tyrosine ethyl ester, since $k_2 > k_3'$[33] and since k_3' is of the same order of magnitude as k_4N (from product analysis),[10] $k_W = k_3'$; that is, the rate of hydrolysis is independent of the hydroxylamine concentration, as found experimentally. On the other hand, in the hydrolysis of N-acetyl-L-tyrosinehydroxamic acid, k_3' and hence also $k_4N > k_2$[34]; under these circumstances $k_W = k_2k_3'/(k_3' + k_4N)$; that is, the rate of hydrolysis is inversely related to the hydroxylamine concentration as found experimentally.[35]

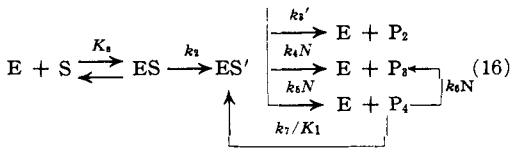
Why, then, are different partitionings between hydroxylamine and water found in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester and hydroxamic acid? To answer this question, consider the chemistry of hydroxylamine. Hydroxylamine acts at neutrality as an ambident anion toward activated acyl groups giving two products: hydroxamic acid and O-acylhydroxylamine.[36] Hydroxylamine may act as an ambident anion in an enzymatic reaction: the reaction of furoylchymotrypsin with hydroxylamine yields largely O-furoylhydroxylamine as well as furoylhydroxamic acid.[10] Therefore the hydrolysis and hydroxylaminolysis of N-acetyl-L-tyrosine ethyl ester should produce a significant amount of the O-(N-acetyl-L-tyrosyl)hydroxylamine, which can react either nonenzymatically[36] with the excess hydroxyl-

(27) W. P. Jencks and M. Gilchrist, *J. Am. Chem. Soc.*, **84**, 2912 (1962).
(28) M. L. Bender and W. A. Glasson, *ibid.*, **81**, 1590 (1959).
(29) M. L. Bender, F. J. Kézdy, and B. Zerner, *ibid.*, **85**, 3017 (1963).
(30) P. Ballinger and F. A. Long, *ibid.*, **82**, 795 (1960).
(31) R. J. Foster, *J. Biol. Chem.*, **236**, 2461 (1961).
(32) G. E. Clement and M. L. Bender, *Biochemistry*, **2**, 836 (1963).
(33) This conclusion is reached by analogy with the kinetic constants in the hydrolysis of acetyl-L-tryptophan ethyl ester (Table V and ref. 17).
(34) This conclusion is reached by analogy with the kinetic constants in the hydrolysis of N-acetyl-L-tryptophan amide.[17]
(35) This kinetic argument ignores the formation of O-(acetyl-L-tyrosyl)-hydroxylamine, but this omission (for the sake of clarity) can be mathematically shown not to change the argument.
(36) W. P. Jencks, *J. Am. Chem. Soc.*, **80**, 4581 (1958).

amine present or can react enzymatically (since it has the backbone of a specific substrate and since O-acylhydroxylamine compounds are labile[36]) returning first to the acyl-enzyme. If the O-acylhydroxylamine compound follows the latter pathway, this compound has an additional opportunity to produce N-acetyl-L-tyrosine.

The reaction paths listed above can be employed to explain the main features of the anomalous partitionings in the acetyl-L-tyrosine ethyl ester and hydroxamic acid reactions. In 0.8 M hydroxylamine, reaction of the former compound yielded 50% N-acetyl-L-tyrosine hydroxamic acid and 50% N-acetyl-L-tyrosine while hydrolysis of the hydroxamic acid was slowed down by only 20% rather than 50% which would be predicted on the basis of equipartitioning of a common N-acetyl-L-tyrosyl–chymotrypsin intermediate.[10] However, the two experiments employed different concentrations of enzyme (for practical reasons).[11] Since one pathway for the decomposition of the O-acylhydroxylamine mentioned above involves a non-enzymatic reaction (with hydroxylamine) while the other pathway involves an enzymatic reaction, the eventual fate of the O-acylhydroxylamine would be expected to be dependent on enzyme concentration. Since a higher enzyme concentration will return the O-acylhydroxylamine to the acyl-enzyme which then has a second opportunity to produce acetyl-L-tyrosine, a higher enzyme concentration would be expected to lead to a lower ratio of hydroxamic acid product/carboxylate product, as found experimentally.

The above argument may be expressed in a quantitative manner, using eq. 16 which is an expansion of eq. 2



where P_4 is the O-acylhydroxylamine. Assuming that P_4 has a reactivity of the same order of magnitude as N-acetyl-L-tyrosine ethyl ester ($k_7 >> k_3W$), that the enzymatic and nonenzymatic decomposition of P_4 is of the same order of magnitude, and P_4 exists in low, steady-state concentration, the following equation can be obtained (using the steady-state assumption for all enzyme compounds)

$$\frac{dP_3}{dP_2} = \frac{V_N}{V_W} \cong \frac{[P_3]_\infty}{[P_2]_\infty} = \frac{k_4N}{k_3'} + \frac{k_6N}{k_3'} \cdot \frac{k_5N}{k_6N + k_7E/K_1} \quad (17)$$

In this equation E is the free enzyme concentration.

From eq. 16 and 17 the following predictions can be made: (1) at constant hydroxylamine concentration, V_N/V_W should decrease as the free enzyme concentration increases; (2) changing the pH from 8.2 to 7.2 should increase k_6[36] and hence V_N/V_W should increase; (3) V_N/V_W will not change significantly with the substrate concentration since a change in the latter can only affect the amount of free enzyme by two- or three-fold, which on the basis of Table IV should produce only a small change in V_N/V_W.

These three predictions are borne out in experiment. The differences in partitioning in the reactions of the ethyl ester and the hydroxamic acid found previously[10] bear out prediction 1. The free enzyme in these experiments may be calculated from the equation K_m-(app)/S = free enzyme/bound enzyme. Using stated values of K_m(app) and S,[10] this ratio is 0.14 for N-acetyl-L-tyrosine ethyl ester and 0.31 for N-acetyl-L-tyrosinehydroxamic acid. Thus, for a given E_0, more free enzyme will exist in the reaction of the hydroxamic acid than in the reaction of the ethyl ester. Furthermore, the absolute concentration of the E_0 was 500-fold greater in the former experiment.[11] Therefore, the difference in free enzyme in the two experiments was about 1500-fold. Prediction 1 is further borne out by the experiments listed in Table IV, describing the partitioning in the simultaneous hydrolysis and hydroxylaminolysis of N-acetyl-L-tyrosine ethyl ester at 100-fold different enzyme concentrations. Prediction 2 is borne out by the pH dependence of the partitioning in this reaction found previously[10] (see Table IV). Prediction 3 is borne out by the small dependence of the partitioning of this reaction on substrate concentration.[37]

Both Caplow and Jencks[11] and Epand and Wilson[13] as well as ourselves[12] independently found that a higher enzyme concentration leads to a lower ratio of hydroxamic acid. Epand and Wilson also propose the theory embodied in eq. 16 to explain this result. One shred of doubt exists that eq. 16 completely explains the hydroxylaminolysis data: at high enzyme concentrations, the ratio of hydroxamic acid product/carboxylate product from the ethyl ester does not quite reach the value predicted from the slowdown of the N-acetyl-tyrosinehydroxamic acid under the same conditions. This result is found in both our hands (Table IV) and that of Caplow and Jencks.[11] However, the main features of these reactions are certainly amenable to interpretation in terms of a mechanism related to eq. 2, and the present authors believe that the iconoclasts of the acyl-enzyme theory must use a reaction more straightforward than the terrifically complicated hydroxylamine reactions to destroy the evidence of this series of papers as well as elsewhere. In contrast to reactions involving hydroxylamine as an added nucleophile, reactions involving methanol and ethanol as added nucleophiles are straightforward kinetically and chemically, and give results fully compatible with the acyl–enzyme hypothesis.

(37) W. P. Jencks, personal communication.