the correction due to "self-hydrolysis" was never greater than 0.02 ml. of 0.01 N sodium hydroxide. No substrate or inhibitor used in this study was found to be hydrolyzed at 25° and pH 7.9 in the absence of enzyme.

The extent of hydrolysis during a given time interval in terms of ml. of standard alkali was plotted against time and the initial velocity at zero time was estimated by an extrapolation procedure based upon the construction of tangents to the slopes near this point. When the above data were plotted as log ([S]$_0$ − [S]) *versus* t a curve was obtained which clearly showed that the apparent first order rate constant increased with increasing extent of hydrolysis. Exclusive of the experiments concerned with the determination of the pH-activity relationships and other supplementary determinations 83 separate experiments were judged to be necessary to evaluate the kinetic constants given in this communication. A summary of these experiments is given in Table II. The values for K_S, K_{P_1} and K_I given previously (*cf.* Table I) are believed to be reliable within the indicated limits of error. It is difficult to evaluate the reliability of the two k_3 values since these values are based upon the assumption that all of the protein nitrogen is derived from catalytically active material. If this assumption is valid it should be noted that initial velocities can be estimated to within ±5% at [S]$_0$ = 5 × 10^{-3} molar and to within ±3% at [S]$_0$ = 20 × 10^{-3} molar. Therefore in a relative sense the k_3 values are probably accurate to within ±5% in view of the fact that they were obtained from a large number of experiments.

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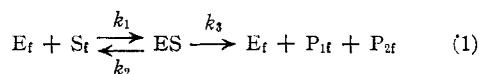
[CONTRIBUTION NO. 1467 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-tyrosinamide in Aqueous Solutions at 25° and pH 7.8–8.0[1]

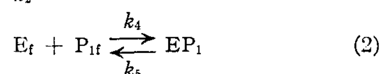
BY DUDLEY W. THOMAS,[2] ROBERT V. MACALLISTER AND CARL NIEMANN[3]

The kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide at 25° and pH 7.8–8.0 have been found to be similar to those noted previously for other synthetic substrates, and from the kinetic constants of these reactions conclusions have been drawn relative to the effect of replacement of a β-indolylmethyl group by a p-hydroxybenzyl group in both substrates and competitive inhibitors. It also has been established that for three different buffers the reaction kinetics are independent of the nature of the buffer.

It has been shown previously[4] that the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tryptophanamide at 25° and pH 7.9 can be formulated as



$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad (1)$$



$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \quad (2)$$

and that the rate equation for the above reactions is

$$k_3[E]t = 2.3K_S\,(1 + [S]_0/K_{P_1})\log [S]_0/[S] + (1 - K_S/K_{P_1})([S]_0 - [S]) \quad (3)$$

provided conditions are selected so that d[ES]/dt ≐ 0, [S$_f$] ≐ [S]≫[ES], and [P$_{1f}$] ≐ [P$_1$]≫[EP$_1$].[5] In other words, in the absence of added inhibitors, the reactions can be described in terms of a steady state process which includes competitive inhibition of the hydrolytic reaction by one of the hydrolysis products, in this case the acylated α-amino acids. It is the purpose of this communication to show that the kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide at 25° and pH 7.8–8.0 are similar to those noted above, and that from the kinetic constants so obtained conclusions can be drawn which are of importance in the further definition of the mode of action of α-chymotrypsin.

It will be seen from Fig. 1 that the so-called pH-activity curve of the system α-chymotrypsin–acetyl-L-tyrosinamide in water at 25° possesses a maximum in the region between pH 7.8 and 8.0 and in this respect is similar to the pH-activity curves given earlier by Kaufman, Neurath and Schwert[6] for α-chymotrypsin–benzoyl-L-tyrosinamide and α-chymotrypsin–benzoyl-L-tyrosine ethyl ester in 30 volume % aqueous methanol at 25°. The fact that the pH-activity curves observed with substrates derived from L-tyrosine are different from those observed with substrates derived from L-tryptophan[4] suggests that the nature of the characteristic amino acid side chain, even though it bears no formal charge, is of considerable importance in determining the nature of the pH-activity curve.

It has been observed that the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide at 25° and pH 7.8–8.0 is biphasic in character as would be expected from the nature of equation (3). The initial velocities were estimated as before,[4,7] and K_S, the so-called Michaelis constant, was evaluated by the usual plot of $1/v_0$ *versus* $1/[S]_0$.[8] A typical plot is given in Fig. 2. Five independent determinations with three different enzyme concentrations and two different buffer systems gave a mean value of K_S = 30.5 ± 1.0 × 10^{-3} molar.

In the absence of competitive inhibition of the hydrolytic reaction by the hydrolysis products, the rate equation is simply

$$k_3[E]t = 2.3\,K_S \log [S]_0/[S] + ([S]_0 - [S]) \quad (4)$$

and provided d[ES]/dt ≐ 0 and [S$_f$] ≐ [S]≫[ES], this equation can be taken as the exact rate

(1) Supported in part by a grant from Eli Lilly and Company.

(2) Allied Chemical and Dye Corp. Fellow 1949–1950.

(3) To whom inquiries regarding this article should be sent.

(4) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(5) *Cf.* ref. 4 for definitions of terms used in equations.

(6) S. Kaufman, H. Neurath and G. W. Schwert, *J. Biol. Chem.*, **177**, 793 (1949).

(7) Where the reaction was apparently zero order with respect to the substrate concentration the initial velocities were estimated from a plot of ([S]$_0$–[S]) *versus* time; where the reaction was approximately first order with respect to the substrate concentration the initial velocities were estimated from a plot of log ([S]$_0$–[S]) *versus* time.

(8) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

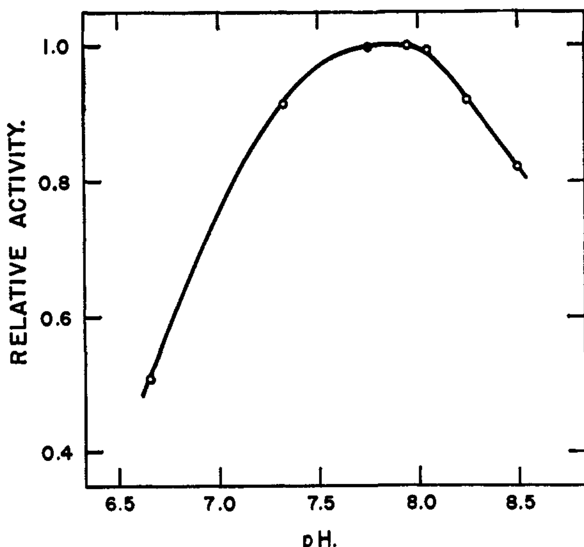


Fig. 1.—Acetyl-L-tyrosinamide, $[S]_0 = 20 \times 10^{-3}$ *M*; [E] = 0.125 mg. protein-nitrogen per ml.; O, 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer; ●, 0.02 *M* ethylenediamine–hydrochloric acid buffer.

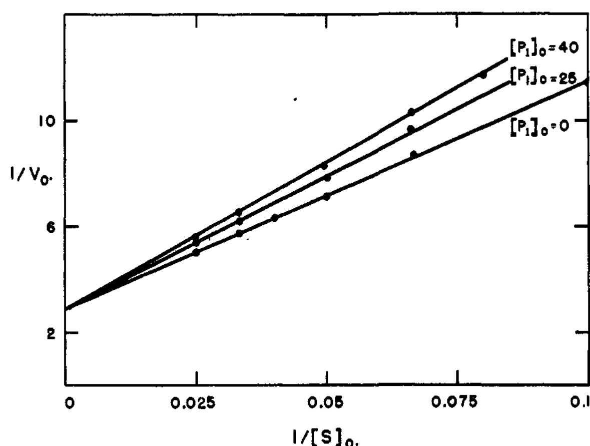


Fig. 2.—$[S]_0$ in units of 10^{-3} *M* of acetyl-L-tyrosinamide; v_0 (initial velocities) in mole $\times$ 10^{-3} per liter per min.; inhibition by acetyl-L-tyrosine, $[P_1]_0$ in units of 10^{-3} *M* [E] = 0.139 mg. protein-nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

expression for the reaction given in equation (1)[9] and the approximate rate expression for the initial stages of the two simultaneous reactions described by equations (1) and (2). The data obtained from experiments in which the reaction was allowed to proceed to near completion were tested for congruity with equation (4), and it will be seen from Fig. 3, where $F(S) = 2.3\ K_S \log [S]_0/[S] + ([S]_0 - [S])$, that after approximately 50% hydrolysis the reaction velocity is less than that expected on the basis of equation (4). The fact that the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide at 25° and *p*H 7.8–8.0 is competitively inhibited by acetyl-L-tyrosine is obvious from the data given in Fig. 2, and from these data a mean value of $K_{P_1} = 115 \pm 15 \times 10^{-3}$ molar was obtained. With the above values of

(9) E. Elkins-Kaufman and H. Neurath, *J. Biol. Chem.*, **175**, 893 (1948).

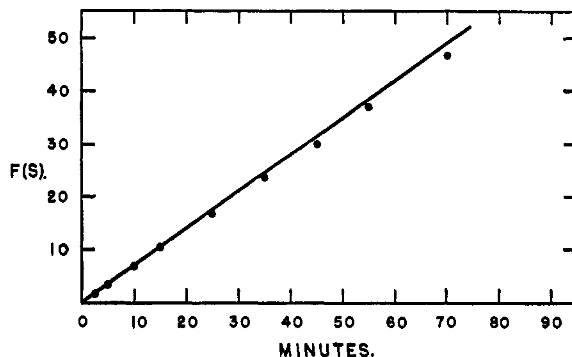


Fig. 3.—$F(S)$ in units of 10^{-3} *M*; acetyl-L-tyrosinamide, $[S]_0 = 20 \times 10^{-3}$ *M*; [E] = 0.312 mg. protein-nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

K_S and K_{P_1} and with the same experimental data as were used for the plot given in Fig. 3, a plot of $F(S) = 2.3K_S(1 + [S]_0/K_{P_1}) \log [S]_0/[S] + (1 - K_S/K_{P_1}) ([S]_0 - [S])$ *versus* time was made, and this plot is given in Fig. 4. It is clear that the experimental data are in excellent agreement with those predicted on the basis of equations (1), (2) and (3), and that equation (3) is a satisfactory rate expression for the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide at 25° and *p*H 7.8–8.0. The constant k_3 was evaluated either from equation (3), or for initial rates from equation (4),[10] and a mean value of $k_3 = 2.4 \pm 0.1 \times 10^{-3}$ mole/liter/min./mg. protein-nitrogen/ml. was obtained. With the same assumptions as were made previously,[4] it can be shown that the above value of k_3 corresponds to a turnover number for the system α-chymotrypsin–acetyl-L-tyrosinamide at 25° and *p*H 7.8–8.0 of approximately 10 molecules/enzyme molecule/min.

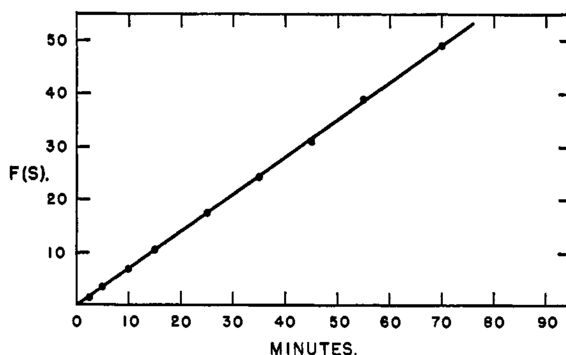


Fig. 4.—$F(S)$ in units of 10^{-3} *M*; acetyl-L-tyrosinamide, $[S]_0 = 20 \times 10^{-3}$ *M*; [E] = 0.312 mg. protein-nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

On the basis of previous observations[4] it was expected that the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide would be competitively inhibited by acetyl-D-tyrosinamide. It is obvious from the data given in Fig. 5 that this is indeed the case, and from these data the dissociation constant K_I of the system α-chymotrypsin–acetyl-D-tyrosinamide was found to be 12.0 $\pm$

(10) This practice is permissible in this particular case because of the relatively large value of K_{P_1}.

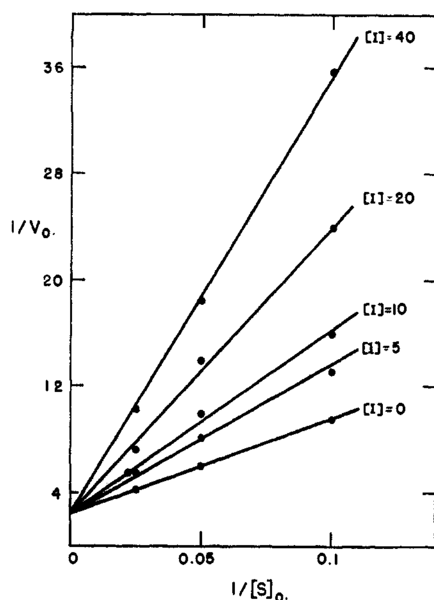


Fig. 5.—$[S]_0$ in units of 10^{-3} *M* of acetyl-L-tyrosinamide; v_0 (initial velocities) in mole $\times$ 10^{-3} per liter per min.; inhibition by acetyl-D-tyrosinamide, [I] in units of 10^{-3} *M;* [E] = 0.150 mg. protein-nitrogen per ml.; 0.02 *M* ethylenediamine–hydrochloric acid buffer.

1.0×10^{-3} molar at 25° and *p*H 7.8–8.0. From the data given in Fig. 6, it will be seen that acetyl-D-tyrosine ethyl ester is also a competitive inhibitor of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide, and in this case the dissociation constant K_I for the system α-chymotrypsin–acetyl-D-tyrosine ethyl ester was found to be $3.5 \pm 0.5 \times 10^{-3}$ molar at 25° and *p*H 7.8–8.0.

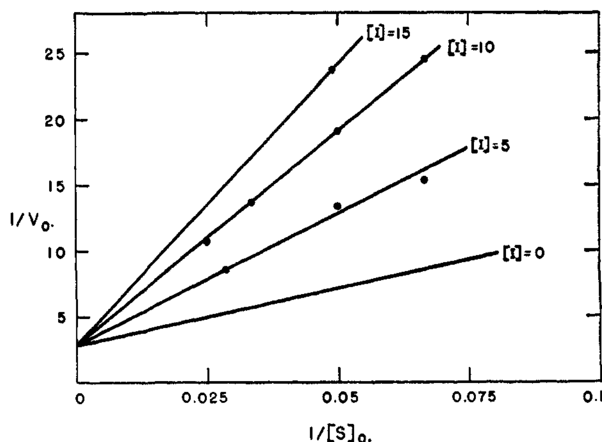


Fig. 6.—$[S]_0$ in units of 10^{-3} *M* of acetyl-L-tyrosinamide; v_0 (initial velocities) in mole $\times$ 10^{-3} per liter per min.; inhibition by acetyl-D-tyrosine ethyl ester, [I] in units of 10^{-3} *M;* [E] = 0.139 mg. protein-nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

It has been shown[4,11,12] that $K_S = ([E] - [ES] - [EP_1])\,[S]/[ES]$ and $K_{P_1} = ([E] - [ES] - [EP_1])\,[P_1]/[EP_1]$ only if $[S] \doteq [S_f] \gg [ES]$ and $[P_1] \doteq [P_{1f}] \gg [EP_1]$ or, in the terminology of Straus and Goldstein,[11,12] if the systems under consideration are in zone A. As has been indicated previously[4] it appears to be reasonable to accept for $E_S' = [E]/K_S$ and $E_{P_1}' = [E]/K_{P_1}$ a value of 0.1 for the upper boundary of zone A. In view of the fact that the enzyme concentrations used in this study for the evaluation of K_S did not exceed 4.8×10^{-5} normal,[13] or for the evaluation of K_{P_1}, 3.2×10^{-5} normal,[13] it follows that the maximum values of $E_S' = 0.16 \times 10^{-2}$ and $E_{P_1}' = 0.03 \times 10^{-2}$ are such as to place the systems used for the evaluation of K_S and K_{P_1} well within zone A. A similar treatment leading to the same conclusions in respect to zone behavior can be given for the experiments used for the evaluation of K_I for both acetyl-D-tyrosinamide and acetyl-D-tyrosine ethyl ester. In the former case, with an enzyme concentration of 3.5×10^{-5} normal[13] $E_I' = 0.3 \times 10^{-2}$, and in the latter, with an enzyme concentration of 3.2×10^{-5} normal[13] $E_I' = 0.9 \times 10^{-2}$.

The individual values of K_S and k_3 evaluated in this study are given in Table I, and from these data it may be concluded that (a) the buffer components have no function in the reaction systems other than to control the *p*H of these systems; (b) the rate of hydrolysis is directly proportional to the enzyme concentration; and (c) because of the nature of the *p*H-activity curve (*cf.* Fig. 1), the mean values of $K_S = 30.5 \pm 1.0 \times 10^{-3}$ molar and $k_3 = 2.4 \pm 0.1$ moles/liter/min./mg. protein-nitrogen/ml. can be taken as the values of these constants at 25° and *p*H 7.9 ± 0.1.

TABLE I

VALUES OF K_S AND k_3 FOR THE SYSTEM α-CHYMOTRYPSIN–ACETYL-L-TYROSINAMIDE AT 25°

Expt.	[E]^a	*p*H	K_S^{b}	k_3^{c}	Buffer system
1	0.139	7.8	31.5	2.3	0.02 *M* EDA-HCl^d
2	.204	7.8	31.5	2.3	.02 *M* EDA-HCl^d
3	.150	8.0	29.5	2.5	.02 *M* EDA-HCl^d
4	.139	8.0	29.5	2.4	.02 *M* THM-HCl^e
5	.139	7.8	29.5	2.1^f	.02 *M* EDA-HCl^d
Mean			30.5^g	2.4^h	

^{a} Mg. protein nitrogen per ml. ^{b} $\times$ 10^3 molar. ^{c} $\times$ 10^3 mole/liter/min./mg. protein-nitrogen/ml. ^{d} Ethylenediamine-hydrochloric acid buffer of designated *p*H, 0.02 molar with respect to the amine component. ^{e} Tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer of designated *p*H, 0.02 molar with respect to the amine component. ^{f} Not included in average because of poor agreement of $1/v_0$ value with those of other experiments. ^{g} ±1.0. ^{h} ±0.1.

During the period that the present study was in progress, four apparently independent determinations of K_S and k_3 for the system α-chymotrypsin–acetyl-L-tyrosinamide at 25° and *p*H 7.8 have been reported,[14–17] *viz.*, K_S = 23,[14] 32.6,[15] 27[16] and 29[17] $\times 10^{-3}$ molar, and k_3 = 2.7,[14] 2.7,[15] 3.0[16] and 3.1[17] moles/liter/min./mg. protein-nitrogen/ml. Of the preceding values of K_S, it appears that only the latter two are regarded as being reasonably accu-

(11) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).

(12) A. Goldstein, *ibid.*, **27**, 529 (1944).

(13) Based upon the reasonable assumption that α-chymotrypsin has a molecular weight of 27,000 and contains but one reactive site per molecule. *cf.* ref. 4.

(14) S. Kaufman and H. Neurath, *Arch. Biochem.*, **21**, 245 (1949).

(15) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **180**, 181 (1949).

(16) G. W. Schwert and S. Kaufman, *ibid.*, **180**, 517 (1949).

(17) S. Kaufman and H. Neurath, *ibid.*, **181**, 623 (1949).

rate.[16,18] Therefore the mean value of $K_S = 28 \pm 1 \times 10^{-3}$ molar should be compared with the mean value of $K_S = 30.5 \pm 1.0 \times 10^{-3}$ molar obtained in this investigation. In view of the fact that these two values were obtained from independent investigations in which different enzyme preparations, buffer systems, and analytical methods were used, it is clear that with α-chymotrypsin it is possible to obtain accurate values of K_S which are dependent only upon substrate, temperature, *p*H, and the dielectric properties of the solvent system. The preferred value of K_S is that determined in this investigation principally because it is based upon a greater number of experiments conducted in solutions of lower ionic strength than those reported previously. In regard to the k_3 values, where the agreement does not appear to be as satisfactory as with the K_S values, it may be concluded by some that the higher value is to be favored, since a k_3 value is necessarily dependent upon the assumption that all of the protein-nitrogen present in the reaction system is catalytically active material. However, before this conclusion is made, it should be realized that the evaluation of k_3 is subject to errors which are considerably greater than the limits indicated above. Therefore, in the absence of data relative to the precision of the k_3 values reported previously, we prefer the value obtained in this study.

In several of the experiments described in this communication, use was made of mixtures of acetyl-D- and L-tyrosinamide. Although the contention that a racemic DL-compound can exist in aqueous solutions[19] has been contested previously,[20] it is of importance to point out that with crystalline acetyl-DL-tyrosinamide the space group can accommodate both D- and L-isomers only if the asymmetric unit contains both a D- and an L-molecule,[21] and it was predicted[21] that the above DL-mixture must be capable of spontaneous resolution on crystallization. This prediction has been verified by the observation that preparations of crystalline acetyl-DL-tyrosinamide can be resolved by the classical method of Pasteur, *i.e.*, by mechanical separation. Thus, with no evidence for interaction leading to racemate formation in the solid state, it is obvious that such interaction in aqueous solutions is most unlikely.

From the kinetic constants given in Table II, it will be seen from the ratio of the K_{P_1} values for acetyl-L-tryptophan and acetyl-L-tyrosine, *i.e.*, 115/17.5 = 6.6, and from the ratio of the K_I values for acetyl-D-tryptophanamide and acetyl-D-tyrosinamide, *i.e.*, 12.0/2.7 = 4.4, that the inhibitors derived from D- or L-tryptophan are bonded more firmly to the enzyme than are the corresponding inhibitors derived from D- or L-tyrosine. While it appears likely that the value of k_2/k_1 for the system α-chymotrypsin–acetyl-L-tryptophanamide will be found to be smaller than the value of k_2/k_1 for the system α-chymotrypsin–acetyl-L-tyrosinamide, it is not possible at present to provide rigorous proof that this is the case.[18] From the ratio of the values of K_I for acetyl-D-tyrosinamide and acetyl-D-tyrosine ethyl ester, *i.e.*, 12.0/3.5 = 3.4, it follows that in this case, the replacement of an $-NH_2$ group by an $-OC_2H_5$ group results in an increase in the bonding forces between enzyme and inhibitor.

TABLE II

KINETIC CONSTANTS OF SEVERAL SUBSTRATES AND COMPETITIVE INHIBITORS OF α-CHYMOTRYPSIN

Compound	$K \times 10^3$ *M*[a] Value	Constant	$k_3 \times 10^3$[a,b]
Acetyl-L-tryptophanamide[c]	5.3 ± 0.2	K_S	0.50
Acetyl-L-tyrosinamide	30.5 ± 1.0	K_S	2.4
Acetyl-L-tryptophan[c]	17.5 ± 1.5	K_{P_1}	
Acetyl-L-tyrosine	115 ± 15	K_{P_1}	
Acetyl-D-tryptophanamide[c]	2.7 ± 0.2	K_I	
Acetyl-D-tyrosinamide	12.0 ± 1.0	K_I	
Acetyl-D-tyrosine ethyl ester	3.5 ± 0.5	K_I	

[a] At 25° and *p*H 7.9 in an aqueous solution. [b] Moles/liter/min./mg. protein-nitrogen/ml. [c] *Cf.* ref. 4.

In a previous communication, data were presented relative to the affinity of α-chymotrypsin for a substrate of the L-configuration and the corresponding competitive inhibitor of the D-configuration.[4] At this time, we wish to point out that for α-chymotrypsin it appears that a competitive inhibitor, which is the D-enantiomorph of a substrate of the L-configuration, is bonded more firmly to the enzyme than is the enantiomorphic L-substrate and it will be shown in a subsequent communication, that for two enantiomorphic competitive inhibitors, the one possessing the D-configuration is bonded more strongly to the enzyme than is the one possessing the L-configuration.[22] It is possible that an explanation of the apparent preference of α-chymotrypsin for a molecule of the unnatural or D-configuration may also provide at least a partial answer to the question as to the mode of action of this enzyme.[23]

Experimental[24,25]

Acetyl-L-tyrosine (I).—I, colorless rods, m.p. 151–153°, $[\alpha]^{25}D$ +47.4° (*c* 2% in water) was prepared as directed by du Vigneaud and Meyer[26]; lit.,[26] m.p. 152–154°, $[\alpha]^{26}D$ +47.5°.

Anal. Calcd. for $C_{11}H_{13}O_4N$ (223): C, 59.2; H, 5.9; N, 6.3. Found: C, 59.2; H, 5.8; N, 6.2.

Acetyl-L-tyrosinamide (II).—I was esterified in the usual manner with ethanolic hydrogen chloride to give acetyl-L-tyrosine ethyl ester (III), m.p. 96–97°, $[\alpha]^{25}D$ +24.7° (*c* 7% in ethanol), in an 80% yield. III was dissolved in methanol, the solution saturated with ammonia at 0°, the reaction mixture allowed to stand at 25° for 2 days, the solution evaporated to dryness *in vacuo*, and the residue recrystallized twice from aqueous ethanol to give 60% of II, m.p. 226–228°, $[\alpha]^{25}D$ +49.7° (*c* 0.8% in water); lit.,[15] m.p. 222–224°.

Anal. Calcd. for $C_{11}H_{14}O_3N_2$ (222): C, 59.5; H, 6.4; N, 12.6. Found: C, 59.5; H, 6.4; N, 12.6.

Acetyl-D-tyrosine Ethyl Ester (IV).—A solution of 16 g. of acetyl-DL-tyrosine ethyl ester (V)[27] in 160 ml. of meth-

(18) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(19) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **124**, 321 (1938).

(20) R. V. MacAllister, K. M. Harmon and C. Niemann, *ibid.*, **177**, 767 (1949).

(21) G. B. Carpenter, *Acta Crystall.*, **2**, 260 (1949).

(22) Unpublished data obtained in these laboratories by Dr. H. T. Huang.

(23) *Cf.* L. Pauling, *Am. Scientist*, **36**, 51 (1948).

(24) All microanalyses by Dr. A. Elek.

(25) All melting points are corrected.

(26) V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **98**, 295 (1932).

(27) C. Niemann and G. E. McCasland, THIS JOURNAL, **66**, 1870 (1944).

anol and 720 ml. of water was adjusted to *p*H 8.0 with 1.0 *N* aqueous sodium hydroxide, 100 mg. of α-chymotrypsin added and the *p*H of the reaction mixture maintained at *p*H 8.0 by the addition of 1.0 *N* aqueous sodium hydroxide. After 30 minutes at 25° the reaction mixture was evaporated *in vacuo* to a volume of 150 ml. whereupon crude IV crystallized from the concentrate. Crude IV was recrystallized twice from aqueous ethanol to give 5.5 g. of IV, m.p. 95–97°, $[\alpha]^{25}D$ −24.8° (*c* 7% in ethanol).

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): C, 62.1; H, 6.8; N, 5.6. Found: C, 62.2; H, 6.8; N, 5.6.

Acetyl-D-tyrosinamide (VI).—VI, m.p. 225–226°, $[\alpha]^{25}D$ −49.4° (*c* 0.9% in water) was prepared from IV in the same manner and in approximately the same yield as was II from III.

Anal. Calcd. for $C_{11}H_{14}O_3N_2$ (222): C, 59.5; H, 6.4; N, 12.6. Found: C, 59.7; H, 6.6; N, 12.5.

Acetyl-DL-tyrosinamide (VII).—VII, optically inactive, m.p. 197–198°, was prepared from V in exactly the same way as was II from III and VI from IV.

Anal. Calcd. for $C_{11}H_{14}O_3N_2$ (222): C, 59.5; H, 6.4; N, 12.6. Found: C, 59.4; H, 6.4; N, 12.5.

Mechanical Separation of Acetyl-D- and L-Tyrosinamides from Acetyl-DL-Tyrosinamide.—A preparation of VII, m.p. 197–198°, was recrystallized slowly from aqueous ethanol so as to give large well-defined single crystals. The m.p. of any single crystal was found to be 226–228°, which is the m.p. of either II or IV. The mixed m.p. of a single crystal isolated from the DL-mixture with II was found to be either 197–198° or 226–228°, depending upon whether the single crystal selected from the DL-mixture was VI or II.

Enzyme Experiments.—The only departure from the methods described earlier[4,28] was the use of 36–38% Merck and Co., Inc., reagent grade aqueous formaldehyde, adjusted to *p*H 8.0 with aqueous sodium hydroxide instead of with solid magnesium carbonate.

The precision of the measurements reported in this study was identical with that obtained previously.[4] Two enzyme preparations, both obtained from Armour and Company, were used in the present study. Of the constants summarized in Table I, those obtained in experiment no. 3 were with lot no. 70902, and all of the others with lot no. 90402. The initial conditions used for the evaluation of the kinetic constants described in this communication are summarized in Table III. It will be noted that a total of 53 experiments were performed exclusive of those required for the determination of the *p*H-activity curve and those required for the reactions which were allowed to proceed to near completion.

(28) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).

TABLE III

SUMMARY OF EXPERIMENTS USED FOR THE EVALUATION OF KINETIC CONSTANTS

S:I	$[E]^a$	No. of experiments at $[S]_0 = 10^{-3}$ molar × 5	10	15	20	30	40	y
II	0.204^b	1	1	1	1	1	1	1^c
II	.150^d	1	1		1		1	
II	.139^b		3	2	3	2	3	1,^{e} 1^f
II:I^g	.139^b			1	1	1	1	
II:I^h	.139^b			1	1	1	1	
II:IV^i	.139^b			1	1			1^j
II:IV^k	.139^b			1	1	1	1	
II:IV^l	.139^b							1^m
II:VI^i	.150^d		1		1		1	
II:VI^k	.150^d		1		1			1^n
II:VI°	.150^d		1		1		1	
II:VI^p	.150^d		1		1		1	

[a] Mg. protein-nitrogen per ml. [b] Lot no. 90402. [c] $y = 25$. [d] Lot no. 70902. [e] $y = 8$. [f] $y = 13.3$. [g] $[I]_0 = [P_1]_0 = 25 \times 10^{-3}$ *M*. [h] $[I]_0 = [P_1]_0 = 40 \times 10^{-3}$ *M*. [i] $[I]_0 = 5 \times 10^{-3}$ *M*. [j] $y = 35$. [k] $[I]_0 = 10 \times 10^{-3}$ *M*. [l] $[I]_0 = 15 \times 10^{-3}$ *M*. [m] $y = 20.5$. [n] $y = 45$. [o] $[I]_0 = 20 \times 10^{-3}$ *M*. [p] $[I]_0 = 40 \times 10^{-3}$ *M*.

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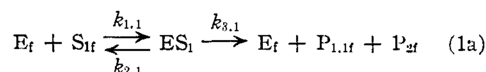
The Kinetics of the α-Chymotrypsin-Catalyzed Competitive Hydrolysis of Acetyl-L-tryptophanamide and Acetyl-L-tyrosinamide in Aqueous Solutions at 25° and *p*H 7.9[1]

BY ROBERT J. FOSTER AND CARL NIEMANN[2]

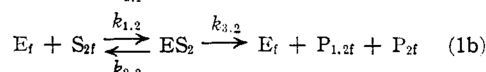
An investigation of the kinetics of the α-chymotrypsin-catalyzed competitive hydrolysis of acetyl-L-tryptophanamide and acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 has provided independent evidence that these two substrates are hydrolyzed to the corresponding acylated α-amino acids and ammonia *via* combination at the same catalytically active site of the enzyme molecule.

The question as to whether two or more substrates do or do not react at the same catalytically active site of an enzyme molecule can be answered if data are available relative to the reaction kinetics of systems containing the enzyme and substrates, the latter both singly and in competition. In view of the fact that data are now available for the kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tryptophanamide and of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9[3,4] the kinetics of the α-chymotrypsin-catalyzed competitive hydrolysis of these two substrates, at 25° and *p*H 7.9 in aqueous solutions 0.02 *M* with respect to a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, was investigated in order to determine whether the above substrates were hydrolyzed *via* combination at the same reactive site of the enzyme molecule.

For reasons given previously[3,4] the reaction system can be limited to enzyme, substrates and reaction products and the reactions can be formulated in terms of the classical intermediate enzyme–substrate complex theory,[5] *i.e.*



$$E_f + S_{1f} \underset{k_{2.1}}{\overset{k_{1.1}}{\rightleftarrows}} ES_1 \xrightarrow{k_{3.1}} E_f + P_{1.1f} + P_{2f} \quad (1a)$$



$$E_f + S_{2f} \underset{k_{2.2}}{\overset{k_{1.2}}{\rightleftarrows}} ES_2 \xrightarrow{k_{3.2}} E_f + P_{1.2f} + P_{2f} \quad (1b)$$

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(2) To whom inquiries regarding this article should be sent.

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(4) D. W. Thomas, R. V. MacAllister and C. Niemann, *ibid.*, **73**, 1548 (1951).

(5) J. B. S. Haldane, "Enzymes," Longmans-Green, London, 1930.