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The Kinetics of the α-Chymotrypsin-catalyzed Hydrolysis of α-N-Carbethoxy-L-tyrosinamide and its Inhibition by α-N-Carbethoxy-D-tyrosinamide[1]

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The constants K_S and k_3 for the α-chymotrypsin-catalyzed hydrolysis of α-N-carbethoxy-L-tyrosinamide, in aqueous solutions at 25° and *p*H 7.9 and 8.2 and 0.02 *M* in the THAM component of a THAM–HCl buffer, have been determined, as has the constant K_I for α-N-carbethoxy-D-tyrosinamide which functions as a competitive inhibitor in the above reaction. A consideration of these values of K_S, K_I and k_3 and those of other α-N-acylated-L- and D-tyrosinamides has shown that when K_S can be interpreted as an apparent enzyme–substrate dissociation constant the ratio of the value of K_S of the L-enantiomorph, functioning as a specific substrate, to the value of K_I of the corresponding D-enantiomorph, functioning as a competitive inhibitor, is determined by the nature of the α-N-acyl moiety and that the value of this ratio may be greater than, equal to or less than unity.

Values of K_S or K_I characteristic of the interaction of α-chymotrypsin, in aqueous solutions at 25° and *p*H 7.9 ± 0.2, with fourteen enantiomorphic pairs of α-N-acylated α-amino acid derivatives, which are either specific substrates or competitive inhibitors of this enzyme,[3-8] are summarized in Table I. For two of the fourteen pairs, *i.e.*, α-N-acetyl-L- and D-tryptophan ethyl ester and α-N-acetyl-L- and D-tyrosine ethyl ester, the ratio K_{S_L}/K_{I_D} is significantly less than unity whereas in all cases this ratio, or K_{I_L}/K_{I_D}, is either close to or greater than unity.

In contrast to the α-N-acylated α-amino acid amides where there is reason to believe that K_S may be interpreted as an apparent equilibrium constant,[9-15] it is possible that this may not be true for the above two L-ethyl esters, particularly since their respective values of k_3 are several orders of magnitude greater than those of the corresponding amides.[5] This situation coupled with what appears to be an anomalous behavior with respect to the corresponding values of K_{S_L}/K_{I_D} suggests the desirability of deferring consideration of these two cases until sufficient information is available with respect to the proper interpretation of K_S for the ethyl esters of α-N-acetyl-L-tryptophan and α-N-acetyl-L-tyrosine.

In the two cases where K_{I_L}/K_{I_D} was determined, *i.e.*, α-N-acetyl-L- and D-tryptophanate and α-N-acetyl-L- and D-tryptophanmethylamide the ratio appears to be greater than unity and it may be concluded that in each of these cases the enzyme has a greater affinity for the D-enantiomorph than for the L. The fact that the same situation appears to prevail with respect to values of K_{S_L}/K_{I_D} for all of the hydroxamides, amides and hydrazides listed in Table I, *i.e.*, a total of twelve pairs, could lead to the impression that the affinity of the enzyme for a specific substrate is invariably less than that for the corresponding enantiomorphic competitive inhibitor. In fact a partial theory of the mechanism of enzyme action has been based upon such a supposition.[16]

TABLE I

SUMMARY OF K_S AND K_I VALUES FOR SYSTEMS INVOLVING α-CHYMOTRYPSIN AND ENANTIOMORPHIC PAIRS OF α-N-ACYLATED α-AMINO ACID DERIVATIVES[a]

Ref.	Substrate or inhibitor	K_S or K_I × 10³ *M*	K_{S_L}/K_{I_D}
3	α-N-Acetyl-L-tryptophanate	10 ± 2	1.5 ± 0.5[b]
4	α-N-Acetyl-D-tryptophanate	7.5 ± 1.5	
5	α-N-Acetyl-L-tryptophan ethyl ester	0.09 ± 0.02	0.4
6	α-N-Acetyl-D-tryptophan ethyl ester	0.25	
7	α-N-Acetyl-L-tryptophanamide	5.0 ± 0.5	2.3 ± 0.6
3	α-N-Acetyl-D-tryptophanamide	2.3 ± 0.4	
4	α-N-Acetyl-L-tryptophanmethylamide	6.5 ± 1.5	
4	α-N-Acetyl-D-tryptophanmethylamide	1.8 ± 0.3	3.9 ± 1.5[b]
7	α-N-Nicotinyl-L-tryptophanamide	2.5 ± 0.2	1.6 ± 0.5
3	α-N-Nicotinyl-D-tryptophanamide	1.6 ± 0.3	
5	α-N-Acetyl-L-tyrosine ethyl ester	0.7 ± 0.05	0.14 ± 0.02
4	α-N-Acetyl-D-tyrosine ethyl ester	5.0 ± 1.0	
7	α-N-Acetyl-L-tyrosinhydroxamide	43 ± 4	6.1 ± 1.7
3	α-N-Acetyl-D-tyrosinhydroxamide	7.5 ± 1.5	
7	α-N-Acetyl-L-tyrosinamide	32 ± 2	2.8 ± 0.6
3	α-N-Acetyl-D-tyrosinamide	12 ± 2	
7	α-N-Trifluoroacetyl-L-tyrosinamide	26 ± 5	1.4 ± 0.6
3	α-N-Trifluoroacetyl-D-tyrosinamide	20 ± 5	
7	α-N-Chloroacetyl-L-tyrosinamide	27 ± 2	4.5 ± 1.3
3	α-N-Chloroacetyl-D-tyrosinamide	6.5 ± 1.5	
7	α-N-Nicotinyl-L-tyrosinamide	12 ± 3	1.4 ± 0.6
3	α-N-Nicotinyl-D-tyrosinamide	9 ± 2	
8	α-N-Acetyl-L-tyrosinhydrazide	22 ± 8	3.3 ± 1.6
4	α-N-Acetyl-D-tyrosinhydrazide	7.5 ± 1.5	
7	α-N-Acetyl-L-phenylalaninamide	31 ± 3	2.8 ± 1.0
3	α-N-Acetyl-D-phenylalaninamide	12 ± 3	
7	α-N-Nicotinyl-L-phenylalaninamide	19 ± 4	2.3 ± 1.0
3	α-N-Nicotinyl-D-phenylalaninamide	9 ± 2	

[a] In aqueous solutions at 25° and *p*H 7.9 ± 0.2. [b] Ratio actually K_{I_L}/K_{I_D}.

In 1948 Bennett and Niemann[17] noted that the papain-catalyzed synthesis of α-N-acylated α-amino acid phenylhydrazides, from α-N-acylated

(1) Supported in part by a grant from the National Institutes of Health, Public Health Service.

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TABLE II

EVALUATION OF K_S AND k_3 FOR α-N-CARBETHOXY-L-TYROSINAMIDE[a]

pH[b]	[E] c	[E] d	$[S]_0$[e]	K_S[e]	k_3[f]	E_S'[g]	S_S'
7.9	0.208[h]	5.91	5.0–20.0[i]	6.4 ± 0.5[i]	0.68 ± 0.04[i]	0.9	0.78–3.13
8.2	0.208[k]	5.91	2.0–20.0[l]	7.3 ± 0.6[m,n]	0.84 ± 0.03[m]	0.8	0.72–2.74

[a] In aqueous solutions at 25.0° and 0.02 *M* in the THAM component of a THAM–HCl buffer. [b] To within ±0.1 of a *p*H unit. [c] In units of mg. protein-nitrogen per ml. [d] In units of 10^{-5} *M* based upon an assumed molecular weight of 22,000 and a nitrogen content of 16.0%. [e] In units of 10^{-3} *M*. [f] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [g] In units of 10^{-2}. [h] Armour preparation no. 90402. [i] 56 values including replicates within this range. [j] From a least squares fit to the relation $([E]/v_0) = (1/k_3) + (K_S/k_3)(1/[S]_0)$. [k] Armour preparation no. 10705. [l] 18 values including replicates within this range. [m] Evaluated by the method of Foster and Niemann.[26,27] [n] $K_p = 15 \pm 3 \times 10^{-3}$ *M*.

α-amino acid and phenylhydrazine, is not necessarily restricted to the L-isomers. This observation was verified and extended in subsequent studies[18–20] where it was found that the degree of antipodal specificity, in favor of the L-isomer, was greatest when the α-N-acyl moiety was an acetyl group and least when it was a carbethoxy group.[20] While Schuller and Niemann[21] observed a complete retention of stereochemical specificity, in favor of the L-isomer, in the comparable α-chymotrypsin-catalyzed syntheses, these experiments do not exclude the possibility that the interaction of α-chymotrypsin with the α-N-carbethoxy derivatives of L- and D-tyrosinamide could lead to a value of K_{S_L}/K_{I_D} substantially different from that of α-N-acetyl-L- and D-tyrosinamide. Therefore, it was decided to evaluate K_S and k_3 and K_I for the former enantiomorphic pair.

The α-chymotrypsin catalyzed hydrolysis of α-N-carbethoxy-L-tyrosinamide was first examined in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.02 *M* in the THAM[22] component of a THAM–HCl buffer in order that a direct comparison could be made with the comparable hydrolysis of α-N-acetyl-L-tyrosinamide.[7,23] The former reaction was followed with the aid of a formol titration,[9] the initial velocities determined by the procedure of Jennings and Niemann[24] and K_S and k_3 evaluated by a least squares fit to the relation $([E]/v_0) = (1/k_3) + (K_S/k_3)(1/[S]_0)$.[25] The results obtained are summarized in Table II.

Since there was no reason to assume that the *p*H dependency of the α-chymotrypsin-catalyzed hydrolysis of α-N-carbethoxy-L-tyrosinamide should be identical with that of α-N-acetyl-L-tyrosinamide,[10] the *p*H–activity relationship was determined for the former system. From the data presented in Fig. 1 it is clear that the *p*H-optimum for the hydrolysis of α-N-carbethoxy-L-tyrosinamide lies in a more alkaline region than that of α-N-acetyl-L-tyrosinamide and that the *p*H optima are 8.2 ± 0.1 and 7.9 ± 0.1, respectively.

In order to provide values of K_S and k_3 for the hydrolysis of α-N-carbethoxy-L-tyrosinamide at the *p*H-optimum, a second series of experiments were performed essentially as before except that K_S and k_3 were evaluated by the method of Foster and Niemann,[26,27] which in addition led to a value of K_P for α-N-carbethoxy-L-tyrosinate. The results of this series of experiments are given in Table II.

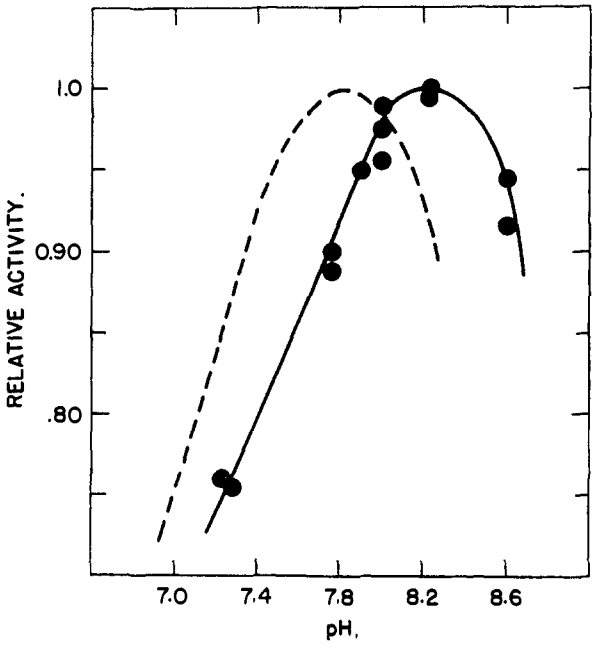

Fig. 1.—Activity *vs.* *p*H for a α-chymotrypsin-catalyzed hydrolysis of α-N-carbethoxy-L-tyrosinamide: dotted line comparable relationship for α-N-acetyl-L-tyrosinamide.

In a third and fourth series of experiments α-N-carbethoxy-D-tyrosinamide was found to be a competitive inhibitor of the α-chymotrypsin catalyzed hydrolysis of α-N-carbethoxy-L-tyrosinamide, in aqueous solutions at 25.0° and *p*H 7.9 ± 0.1 and 0.02 *M* in the THAM component of a THAM–HCl buffer, and of α-N-acetyl-L-tyrosinhydroxamide,[7,28] in aqueous solutions at 25.0° and *p*H 7.6 ± 0.1 and 0.27 *M* in the amine component of the same buffer. The values of K_I were determined by a least squares fit of the initial velocities, determined by the procedure of Jennings and Niemann,[24] to the relation $([E]/v_0) = (1/k_3) + (K_S'/k_3)(1/[S]_0)$ where $K_S' = K_S(1 + [I]/K_I)$. The values so obtained are given in Table III.

A summary of all known values of K_S, K_P, K_I and k_3 for the α-N-acylated L- and D-tyrosinamides, with reference to their interaction with α-chymo-

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TABLE III

EVALUATION OF K_I FOR α-N-CARBETHOXY-D-TYROSINAMIDE[a]

pH[b]	[E][c]	[S]$_0$[d]	[I][d]	K_I[d]	E_S'[e]	E_I'[e]	S_S'	I_I'
7.9[f]	0.208[g]	5.0–14.28[h]	5.0	21.1 ± 2.5[i]	0.9	0.28	0.71–2.04	0.24
7.9[f]	.208[g]	4.0–10.0[j]	10.0	20.3 ± 2.5[i]	.9	.29	.57–1.42	.49
7.6[k]	.0266[l]	5.0–10.0[m]	10.0	17.3 ± 1.2[i]	.02	.04	.12–0.23	.58

[a] In aqueous solutions at 25.0°. [b] To within ±0.1 of a *p*H unit. [c] In units of mg. protein-nitrogen per ml. [d] In units of 10^{-3} *M*. [e] In units of 10^{-2}. [f] Buffer 0.02 *M* in the THAM component of a THAM–HCl buffer. [g] Armour preparation no. 90402. [h] Seven values including replicates within this range with α-N-carbethoxy-L-tyrosinamide as the specific substrate. [i] Initial velocities determined by the method of Jennings and Niemann[24] and K_I evaluated by a least squares fit to the relation $([E]/v_0) = 1/k_3 + (K_S'/k_3)(1/[S]_0)$ where $K_S' = K_S(1 + [I]/K_I)$. [j] Nine values including replicates within this range with α-N-carbethoxy-L-tyrosinamide as the specific substrate. [k] Buffer 0.27 *M* in the THAM component of a THAM–HCl buffer. [l] Armour preparation no. 10705. [m] Six values including replicates within this range with α-N-acetyl-L-tyrosinhydroxamide as the specific substrate.

trypsin in aqueous solutions at 25.0°, and *p*H 7.8 ± 0.2 and 0.02 *M* in the THAM component of a THAM–HCl buffer is given in Table IV.

TABLE IV

SUMMARY OF VALUES OF K_S, K_P, K_I AND k_3 FOR THE α-N-ACYLATED-L- AND D-TYROSINAMIDES[a,b]

α-N-Acyl moiety	K_S[c]	K_P[c]	K_I[c]	k_3[d]
Formyl-L-	12 ± 3	.....	.....	0.45 ± 0.05
Acetyl-L-	32 ± 4	80 ± 20	.....	2.5 ± 0.1
Acetyl-D-	.....	.....	12 ± 2	.....
Trifluoroacetyl-L-	26 ± 5	120 ± 50	.....	2.6 ± 0.5
Trifluoroacetyl-D-	.....	.....	20 ± 5	.....
Chloroacetyl-L-	27 ± 2	150 ± 50	.....	4.0 ± 0.2
Chloroacetyl-D-	.....	.....	6.5 ± 1.5	.....
Carbethoxy-L-	6.4 ± 0.5	15 ± 3	.....	0.68 ± 0.04
Carbethoxy-D-	.....	.....	21 ± 3	.....
Benzoyl-L-	2.5 ± 0.3	.....	.....	4.0 ± 0.5
Nicotinyl-L-	12 ± 3	.....	.....	5.0 ± 1.0
Nicotinyl-D-	.....	.....	9 ± 2	.....
Isonicotinyl-L-	9 ± 2	.....	.....	6.4 ± 0.6

[a] *Cf.*, ref. 3, 7, 23. [b] For aqueous solutions at 25.0° and *p*H 7.8 ± 0.2 and 0.02 *M* in the THAM component of a THAM–HCl buffer. [c] In units of 10^{-3} *M*. [d] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml.

A comparison of the values of K_S and k_3 for α-N-carbethoxy-L-tyrosinamide, *i.e.*, 6.4 ± 0.5[29] and 0.68 ± 0.04[29] with those of α-N-acetyl-L-tyrosinamide, *i.e.*, 32 ± 4 and 2.5 ± 0.1 illustrates the effectiveness of the carbethoxy group, in α-N-carbethoxy-L-tyrosinamide, in diminishing the magnitude of K_S but at the expense of a concomitant decrease in the value of k_3. This effect, which is also seen in the case of replacement of an acetyl group by a formyl group, *cf.*, Table IV, stands in marked contrast to all other cases summarized in Table IV where replacement of an acetyl group by a trifluoroacetyl-, chloroacetyl-, benzoyl-, nicotinyl- or isonicotinyl-group either causes no significant change in the values of K_S or k_3 or leads to a decrease in the value of K_S and an increase in the value of k_3.

The observation that $K_{S_L}/K_{I_D} = 0.30 \pm 0.08$ for the system α-chymotrypsin α-N-carbethoxy-L- and D-tyrosinamide, *cf.*, Table IV, allows us to establish the order, chloroacetyl = 4.5 ± 1.3, > acetyl = 2.8 ± 0.6, > trifluoroacetyl = 1.4 ± 0.6, ≐ nicotinyl = 1.4 ± 0.6 > carbethoxy = 0.3 ± 0.08 with reference to the influence of the nature of the α-N-acyl moiety on values of K_{S_L}/K_{I_D} observed for the α-N-acylated-L- and D-tyrosinamides when such observations are made under apparently equivalent experimental conditions. It is clear that this ordering allows us to conclude that when K_S may be interpreted as an apparent enzyme–substrate dissociation constant the value of K_S of the L-enantiomorph functioning as a specific substrate may be greater than, equal to or less than the value of K_I of the D-enantiomorph functioning as a competitive inhibitor. This generalization and its consequences must be recognized in any reasonably complete theory of the mechanism of action of α-chymotrypsin.

That the nature of an α-N-acyl moiety devoid of the elements of asymmetry may have a profound influence on the ability of L- and D-enantiomorphs to combine with the catalytically active site of the enzyme, even to the point of causing a reversal of the relative combining abilities of L- and D-enantiomorphs, appears to be due to an inherent asymmetry of the catalytically active site of the enzyme that extends beyond those regions directly concerned with the asymmetry arising from the single asymmetric carbon atom present in the enantiomorphic pairs under consideration. A detailed explanation of how a carbethoxy group, present in an α-N-acylated-L- and D-tyrosinamide, can lead to more favorable modes of combination, in the enzyme complex, for the L-enantiomorph, possibly at the expense of those modes of combination capable of eventually leading to reaction products[30–32] than are available to the corresponding D-enantiomorph and how a chloroacetyl group in the same situation can lead to an apparent reversal of the above effect must await further information either with respect to the conformation of the active site of the enzyme or with respect to the behavior of other enantiomorphic α-N-acylated α-amino acid derivatives from which the first might be inferred.

Experimental[33,34]

α-N-Carbethoxy-L-tyrosinamide.—A solution of 3.34 g. of L-tyrosinamide in aqueous dioxane was allowed to react with a 10% excess of ethyl chlorocarbonate and aqueous potassium carbonate. The crude product was recrystallized from water to give α-N-carbethoxy-L-tyrosinamide, colorless needles, m.p. 156.6–158.1°; lit.[35] m.p. 155–157°, $[\alpha]^{25}D$ +19.0° (*c* 5% in methanol).

Anal. Calcd. for $C_{12}H_{16}O_4N_2$ (252): C, 57.1; H, 6.4; N, 11.1. Found: C, 57.0; H, 6.4; N, 11.2.

(29) All values of K_S and K_I are in units of 10^{-3} *M* and those of k_3 in units of 10^{-3} *M*/min./mg. protein-nitrogen per ml.

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(33) All melting points are corrected.

(34) Microanalysis by Dr. A. Elek.

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α-N-Carbethoxy-DL-tyrosine Ethyl Ester.—To a solution of 100 g. of DL-tyrosine ethyl ester in 500 ml. of pyridine, maintained at 5–10°, was added dropwise and with stirring over a period of 1 hr., 58 g. of ethyl chlorocarbonate. The pyridine was removed *in vacuo*, the resulting sirup dissolved in 300 ml. of chloroform, the solution extracted with cold 1 *N* aqueous hydrochloric acid, the chloroform phase dried and the solvent removed *in vacuo* to give 91 g. of colorless prisms, m.p. 87.2–90.3°.

α-N-Carbethoxy-D-tyrosine Ethyl Ester.—α-Chymotrypsin, 75 mg., was added to a stirred solution of 75 g. of α-N-carbethoxy-DL-tyrosine ethyl ester in 500 ml. of methanol and 1400 ml. of water, maintained at 30–35° and containing sufficient phenol red indicator to follow visibly the *p*H of the reaction mixture. As the asymmetric hydrolysis proceeded, 5.46 g. of sodium hydroxide in 336 ml. of water was added at a rate sufficient to maintain the *p*H of the reaction mixture at *p*H 7–8. After 4 hr. the reaction was essentially complete and the volume of the solution was reduced by *ca.* 400 ml. by evaporation *in vacuo*. The residue was adjusted to *p*H 8.5 and extracted with four 200-ml. portions of chloroform. Evaporation of the chloroform phase gave a sirup which could not be crystallized.

α-N-Carbethoxy-D-tyrosinamide.—A sirupy preparation of α-N-carbethoxy-D-tyrosine ethyl ester obtained from the enzymatic resolution of 55 g. of α-N-carbethoxy-DL-tyrosine ethyl ester was dissolved in 150 ml. of absolute methanol and the solution saturated with anhydrous ammonia at 0–5°. The reaction mixture was allowed to stand at room temperature for 2 days, the excess ammonia and solvents removed by evaporation *in vacuo* and the residue allowed to crystallize. Recrystallization of the crude product from water gave 7.6 g. of α-N-carbethoxy-D-tyrosinamide, colorless needles, 156.6–158.1°, $[\alpha]^{25}D$ −19.4° (*c* 5% in methanol).

Anal. Calcd. for $C_{12}H_{16}O_4N_2$ (252): C, 57.1; H, 6.4; N, 11.1. Found: C, 57.4; H, 6.2; N, 11.1.

Enzyme Solutions.—Aqueous stock solutions of crystalline α-chymotrypsin were prepared daily and were kept at 4° between the intervals in which they were brought to 25° prior to introduction into the reaction mixtures.

Buffer Solutions.—THAM was recrystallized three times from distilled water prior to the preparation of stock solutions which were adjusted to the desired *p*H by titration with aqueous hydrochloric acid.

Formaldehyde.—Merck reagent grade formaldehyde (36–38% in water) was adjusted to *p*H 8 prior to its use in the formol titrations.

Ferric Chloride Solution.—The ferric chloride solution used for the spectrophotometric determination of α-N-acetyl-L-tyrosinhydroxamide was prepared as follows: 54.0 g. of reagent grade ferric chloride hexahydrate was dissolved in 500 ml. of water and 16.3 ml. of concd. hydrochloric acid. The solution was then made up to 1000 ml. with absolute methanol. This solution was turbid when first prepared but became clear after filtration through a Whatman No. 1 paper and standing for two days.

Enzyme Experiments.—All enzymatic hydrolyses were conducted at 25.0° in aqueous solutions containing a THAM–HCl buffer and in the manner described by Huang and Niemann.[9] The titrimetric method of analysis used in all experiments with α-N-carbethoxy-L-tyrosinamide was identical with that described by Huang and Niemann[9] and earlier by Iselin and Niemann.[36] The experiments involving the use of α-N-acetyl-L-tyrosinhydroxamide as a specific substrate were followed by a modification of the procedure of Hogness and Niemann.[28] In the present experiments, the absorbance, at any time *t*, of unhydrolyzed α-N-acetyl-L-tyrosinhydroxamide, as its ferric chloride complex, was determined at 515 mμ with a Beckman Model B Spectrophotometer. From the reaction mixtures at *p*H 7.6 and 0.27 *M* in the THAM component of the THAM–HCl buffer 1.0-ml. aliquots were withdrawn at pre-selected time intervals and delivered into 10-ml. G. S. volumetric flasks containing 1.0 ml. of the ferric chloride solution and 7.5 ml. of absolute methanol. The solutions were adjusted to 10.0 ml. with absolute methanol, mixed and the absorbance determined with the spectrophotometer set at 100% transmission for a blank, prepared in the same manner as above, but containing instead of 10 ml. of reaction mixture, 1.0 ml. of a solution consisting of enzyme, buffer and water. In the case of those experiments with $[S]_0 = 15$ to 40×10^{-3} *M* it was necessary to increase the dilution of the colored ferric complex in order to facilitate measurement. This was achieved by employing 25-ml. G. S. volumetric flasks and delivering the 1.0-ml. aliquot of the reaction mixture into 1.0 ml. of the ferric chloride solution and 22.5 ml. of absolute methanol and then making up to volume with absolute methanol.

Determination of *p*H-Optimum.—The experiments, which led to the data summarized in Fig. 1, were conducted in aqueous solutions at 25° and 0.02 *M* in the THAM component of a THAM–HCl buffer with [E] = 0.266 mg. protein-nitrogen per ml. of Armour preparation no. 10705, $[S]_0 = 10 \times 10^{-3}$ *M* and *t*, time of reaction, of 20 min.

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The Effect of Various Salts on the α-Chymotrypsin-catalyzed Hydrolysis of Two Acylated α-Amino Acid Esters[1]

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A detailed analysis has been made of the effect of sodium and calcium chlorides on the rate of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate and of sodium, calcium and magnesium chlorides on the rate of the comparable reaction involving acetyl-L-valine methyl ester. Less extensive studies with seven additional salts also are reported.

It has been shown[3] that in the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate, in aqueous solutions at 25° and *p*H 7.90 ± 0.01 in the presence of sodium chloride, at concentrations of sodium chloride greater than 1 *M* the value of K_S'[4] is essentially constant but as the concentration of sodium chloride is decreased below 1 *M*, the value of K_S' begins to increase, slowly and then rapidly, and as the system approaches zero ionic strength the value of K_S' tends to become large. While the value of k_3' generally decreases with decreasing concention of sodium chloride, at concentrations below 1 *M* the value of k_3' decreases more rapidly than at concentrations above 1 *M* and as the system approaches zero ionic strength, the value of k_3' appears to become small. In this investigation the above studies have been extended to include ex-

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

(3) R. B. Martin and C. Niemann, THIS JOURNAL, **79**, 4814 (1957).

(4) The symbols K_S' and k'_3 are used herein with reference to the dependence of the rate upon the initial specific substrate concentration and refer to apparent constants which may be composite in nature. However, for the purposes of this communication it is unimportant whether the constants are simple or complex.