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Use of the *p*H-Stat in Kinetic Studies of Reactions Whose Products Are Capable of Functioning as Buffers*

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The *p*H-stat has been used to follow the α-chymotrypsin–catalyzed hydrolysis of α-*N*-acylated L-tyrosinamides, hydrazides, and hydroxamides under conditions where one of the reaction products was capable of functioning as a buffer. The limits of usefulness of this application have been defined and α-*N*-acetyl-L-tyrosinhydroxamide has been found to be a particularly useful substrate. Attention has been directed to the nature of the dependence of the kinetic constants of a variety of substrates upon the concentration of added salts.

The kinetics of many enzyme-catalyzed reactions are determinable through use of the *p*H-stat (Jacobsen *et al.*, 1957; Applewhite *et al.*, 1958). These reactions include a number whose reaction products are capable of functioning as buffers (Waley and Watson, 1953; Richards, 1955; Haugaard and Haugaard, 1955; Ottesen, 1956). However, with the latter substrates no attempt has been made to determine the limits of applicability of the *p*H-stat, or to compare reaction kinetics so determined with those evaluated by other procedures. Since kinetic data of the latter kind are available for many systems involving α-chymotrypsin and substrates whose reaction products are capable of buffering the reaction system, we have examined the usefulness of the *p*H-stat for evaluation of their kinetic constants.

We may represent the general case by reactions (1) to (4) inclusive,

$$RCOB + H_2O \longrightarrow RCO_2H + BH \qquad (1)$$

$$RCO_2H \overset{K_1}{\rightleftharpoons} RCO_2^{\ominus} + H^{\oplus} \qquad (2)$$

$$BH + H^{\oplus} \overset{K_2}{\rightleftharpoons} BH_2^{\oplus} \qquad (3)$$

$$BH \overset{K_3}{\rightleftharpoons} B^{\ominus} + H^{\oplus} \qquad (4)$$

where B may be OR′, NHOH, NH_2, $NHNH_2$, or NHR″, when RCOB is an α-*N*-acylated α-amino acid ester, hydroxamide, amide, hydrazide, or a peptide, representing the range of substrates normally encountered in α-chymotrypsin–catalyzed hydrolyses. In all preceding examples, except for phenolic esters, $B^{\ominus}$ is a strong base, hence as a product is present in aqueous reaction systems of $pH < 10$ as the species BH. Since α-chymotrypsin–catalyzed reactions are ordinarily studied in the region from *p*H 6 to 9, reaction (4) may be ignored, except for phenolic esters.

The dissociation constant $K_1 = [RCO_2^{\ominus}][H^{\oplus}]/[RCO_2H]$ of reaction (2) must satisfy the condition, $pK_1 \leq (pH_R - 2)$, where pH_R is the *p*H of the reaction system if more than 99% of the acid is to be titrated. This latter condition is usually satisfied, since for α-*N*-acylated α-amino acids and peptides $pK_1 \leq 3.4$ (Greenstein and Winitz, 1961). Therefore, for most cases we shall be concerned with the consequences of participation of reaction (3).

Kinetic studies require knowledge of the degree to which the stoichiometry, in terms of addition of standard base to the reaction system, is altered by the presence of buffer and in what way, if any, the

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TABLE I

DISSOCIATION CONSTANTS OF CONJUGATE ACIDS OF BASIC REACTION PRODUCTS[a,b]

NaCl (M)	$pK_A' = pK_2'$	NaCl (M)	$pK_A' = pK_2'$	NaCl (M)	$pK_A' = pK_2'$
$NH_3 + H^\oplus \rightleftharpoons NH_4^\oplus$		$NH_2OH + H^\oplus \rightleftharpoons NH_3OH^\oplus$		$NH_2OCH_3 + H^\oplus \rightleftharpoons NH_3OCH_3^\oplus$	
0.002	9.20	0.002	5.99	0.002	4.59
0.460	9.26	0.032	6.04	0.032	4.55
0.920	9.27	0.064	6.03	0.064	4.58
1.84	9.24	0.128	5.98	0.128	4.59
		0.256	6.01	0.256	4.62
$NH_2NH_2 + H^\oplus \rightleftharpoons NH_2NH_3^\oplus$		0.460	6.04	0.460	4.70
		0.920	6.00	1.38	4.62
0.02	8.10	1.38	6.01	1.84	4.62
0.10	8.10	1.84	5.93	2.30	4.64
1.00	8.00	2.30	5.97		

[a] In aqueous solutions at 25.0°. [b] The precision of a pair of measurements was ±0.01 *pK* unit.

kinetic constants are related to the dissociation constant of the buffer system.

The question of stoichiometry is readily answered. For the condition, $pK_1 \ll pH_R$ and $pK_1 \ll pK_2$, the species BH may be regarded as simply removing $H^\oplus$ from the reaction system, thereby diminishing the requirement for added $OH^\ominus$ to neutralize $H^\oplus$ resulting from dissociation of RCO_2H. The product balance is such that $[RCO_2^\ominus] = [BH] + [BH_2^\oplus]$, hence the apparent concentration of product at time t, $[RCO_2^\ominus]_t'$, must be corrected to the true concentration, $[RCO_2^\ominus]_t$, by relation (5).

$$[RCO_2^\ominus]_t = [RCO_2^\ominus]_t'[1 + ([BH_2^\oplus]/[BH])] \quad (5)$$

For reactions conducted at a constant *p*H, and described by equation (6),

$$-d[S]/dt = d[P]/dt = k_0[E][S]/(K_0 + [S]) \quad (6)$$

it has been shown (Kurtz, 1960) that when one of the products, P, is a buffer only the value of the constant k_0 is affected and that the true value of k_0 is related to the apparent value, k_0', through equation (7).

$$k_0 = k_0'[1 + ([BH_2^\oplus]/[BH])] \quad (7)$$

The preceding considerations led us to determine the kinetics of the α-chymotrypsin–catalyzed hydrolysis of a number of α-*N*-acylated-L-tyrosine derivatives, including hydroxamides, amides, and hydrazides, for which the value of pK_2 of the buffering reaction product varied from 5.99 to 9.24, a range sufficiently large to permit evaluation of the usefulness of the *p*H-stat in kinetic studies involving not only α-*N*-acylated α-amino acid derivatives but also peptides. As it was necessary to have precise values of pK_2, the dissociation constants of the conjugate acids of hydroxylamine, *O*-methylhydroxylamine, ammonia, and hydrazine were evaluated in aqueous solutions containing varying amounts of sodium chloride, a component of the reaction systems. The dependence of the constants K_0 and k_0 upon the concentration of sodium chloride was also determined.

EXPERIMENTAL

Determination of Dissociation Constants.—The dissociation constants of the conjugate acids of hydroxylamine, *O*-methylhydroxylamine, ammonia, and hydrazine were determined by titration of 0.002 M aqueous solutions of the hydrochlorides, containing varying amounts of sodium chloride, with 0.0536 N aqueous sodium hydroxide, using a Di-Functional Recording Titrator manufactured by the International Instrument Co., Canyon, Calif. It can be used for constant or variable *p*H titrations. The results obtained are summarized in Table I.

Substrates.—Four of the five substrates were prepared as described previously: α-*N*-acetyl-L-tyrosinamide (Thomas *et al.*, 1951); α-*N*-nicotinyl-L-tyrosinamide (Iselin *et al.*, 1950), and α-*N*-acetyl- and α-*N*-trimethylacetyl-L-tyrosinhydrazide (Lutwack *et al.*, 1957a). The preparation of the fifth, α-*N*-acetyl-L-tyrosinhydroxamide, has been described by Hogness and Niemann (1953). However, the monosodium salt, obtained as an intermediate by the preceding investigators, is more accessible than the acid and is equally useful as a substrate. Sodium α-*N*-acetyl-L-tyrosinhydroxamate was conveniently prepared by the following procedure.

Sixty ml of 3.5 M methanolic sodium methoxide was added to 12.2 g (0.175 mole) of hydroxylamine hydrochloride in 70 ml of methanol at 40°. The reaction mixture was stirred for 30 minutes and filtered, and to the filtrate was added 10.4 g (0.044 mole) of methyl α-*N*-acetyl-L-tyrosinate and 14 ml of 3.5 M methanolic sodium methoxide. The solution was stored at 4° for 3 days and the crystalline product was collected, washed with 30 ml of cold methanol, and dried *in vacuo* to give 7.1 g (62%) of product, m.p. 189.0–189.5°, with decomposition. This product was recrystallized from aqueous-methanol to give sodium α-*N*-acetyl-L-tyrosinhydroxamate, m.p. 190.5–191.0°, with decomposition, $[\alpha]_D^{25}$ 35.0°(*c*, 5% in 0.21 N hydrochloric acid).

Anal. Calcd. for $C_{11}H_{13}O_4N_2Na$(260): C, 50.8; H, 5.0; N, 10.8. Found: C, 50.7; H, 4.8; N, 10.8. Potentiometric titration of the sodium salt gave an equivalent weight of 262; theory, 260.

Kinetic Studies.—The procedure, based upon use of a Di-Functional Recording Titrator, was essentially that developed by Applewhite *et al.* (1958) for ester-type substrates. The *p*H-stat was cali-

brated, as described by these investigators, Beckman standard buffer solutions being used. The *p*H of the reaction systems was constant to within 0.02 of a *p*H unit as determined by the absence of observable fluctuations of the monitoring *p*H meter and significant stepping in the recorder traces. The attainment of these conditions is dependent upon proper choice of drive speed of the syringe buret introducing base into the reaction system and upon the concentration of base so introduced. Other controllable factors are the relative position of buret tip and glass electrode and speed of stirring of the reaction mixture. The enzyme preparation was Armour bovine crystalline salt-free α-chymotrypsin, lot no. 238. The enzyme and substrate solutions were prepared and adjusted to the reaction *p*H as described earlier (Applewhite *et al.*, 1958). The only departure from previous practice occurred when sodium α-*N*-acetyl-L-tyrosinhydroxamate was used as a substrate in lieu of the acid. In this instance adjustment of the *p*H of the substrate stock solution with aqueous hydrochloric acid was accompanied by formation of sufficient sodium chloride to require sodium chloride stock solutions of lesser concentration than ordinarily used in order to achieve the final concentrations specified in Table III.

The initial velocities were determined from the recorder traces by the orthogonal polynomial procedure of Booman and Niemann (1956), with the aid of a program written for the Datatron 205 computer (Abrash *et al.*, 1960) except for linear traces of extent of reaction *vs.* time. Initial velocities for the latter were evaluated by a linear least-squares procedure. The kinetic constants K_0 and k_0 were obtained by a least-squares procedure with use of a program for the Datatron 205 computer (Abrash *et al.*, 1960), in which the enzyme blank reaction was treated as described by Martin and Niemann (1957). Non-enzyme-catalyzed hydrolysis of the substrates was negligible.

Results

The values obtained for the dissociation constants of the basic reaction products, *cf.* Table I, are in good agreement with those obtained previously. Harned and Owen (1930) report a value of $pK_0' = 9.25$ for ammonium ion, which may be compared with the values of $pK_0' = 9.20$ and $\overline{pK_A}' = \overline{pK_2} = 9.24 \pm 0.02$ obtained in the present investigation. For monoprotonated hydrazine, at 20°, Schwarzenbach (1933) gives a value of $pK_0' = 8.12$. At 25°, we find $pK_0 = 8.10$ and $\overline{pK_A}' = \overline{pK_2} = 8.10 \pm 0.01$. Values of pK_0' for the conjugate acid of hydroxylamine have been reported as 5.96 (Briegleb 1949) and 5.97 (Bissot *et al.*, 1957). Our values are $pK_0' = 5.99$ and $\overline{pK_A}' = \overline{pK_2}' = 5.99 \pm 0.02$. Anticipating future use of *O*-methylhydroxamides as substrates of α-chymotrypsin, we determined the dissociation constant of the conjugate acid of *O*-methylhydroxylamine. The values of $pK_0' = 4.59$ and $\overline{pK_A}' = \overline{pK_2}' = 4.61 \pm 0.03$ obtained are in good agreement with the value of $pK_0' = 4.60$ reported by Bissot *et al.* (1957).

The apparent initial velocity, v_0', is directly proportional to k_0', which in turn is lower than k_0 by the factor $1/[1 + ([BH_2^{\oplus}]/[BH])]$. Accordingly, values of v_0' are lower than in the absence of buffer by the same factor. The extent to which v_0' exceeds that of the enzyme blank reaction, v_E, will, within limits, determine the precision of the over-all procedure which in general will increase as the value of pK_2' decreases. Thus, for amides, hydrazides, and hydroxamides, where $\overline{pK_2}' = 9.24$, 8.10, and 5.99, respectively, the expected precision will be lowest for amides and greatest for hydroxamides.

Amides.—The α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinamide and α-*N*-nicotinyl-L-tyrosinamide was examined in aqueous solutions at 25.0°, *p*H 7.90, and 0.116 or 1.93 M in sodium chloride with $[E] = 0.293$ mg protein-nitrogen per ml and $[S] = 6.42$ to 28.9×10^{-3} M for the former substrate and 3.0 to 18.0×10^{-3} M for the latter. The reactions were followed over the time interval from $t = 1$ to 9 minutes. Values of v_0' were obtained from nonlinear recorder traces of amount of base added *vs.* time through use of a 17-point orthogonal polynomial at a $t_{0.10}$ significance level. The enzyme blank reaction gave values of $v_E = 0.14 \pm 0.02 \times 10^{-4}$ M/minute at 0.116 M sodium chloride and $0.067 \pm 0.001 \times 10^{-4}$ M/minute at 1.93 M sodium chloride.

For systems 0.116 M in sodium chloride values of v_0' for α-*N*-acetyl-L-tyrosinamide were of the same order of magnitude as those of v_E, the enzyme blank reaction. Therefore, in this instance no attempt was made to evaluate K_0 or k_0. The other results are summarized in Table II.

The experiments conducted with α-*N*-acetyl- and α-*N*-nicotinyl-L-tyrosinamide gave values of K_0 and k_0 of relatively low precision but of comparable magnitude to those obtained previously, *cf.* Table II. The lack of precision was caused by the relatively limited demand of the reaction system for added base to maintain the *p*H at a constant value of 7.90. As a consequence of the substantial buffering capacity of the liberated ammonia at this *p*H the apparent rate of reaction, as indicated by the demand for added base, was of similar magnitude as that of the enzyme blank reaction. Since this situation would prevail for all amides it is apparent that use of a *p*H-stat for evaluation of the reaction kinetics of systems in which the substrates are α-*N*-acylated α-amino acid amides is limited to exploratory studies of relatively low precision.

Hydrazides.—The α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl- and α-*N*-trimethylacetyl-L-tyrosinhydrazide was studied under the conditions specified in Table II, with $[E'] = 0.1464$ mg protein-nitrogen per ml and $[S] = 3.0$ to 12.0×10^{-3} M for the former substrate and 10.0 to 100.0×10^{-3} M for the latter. Relative to the amides, a substantial increase in precision was obtained and in general the values of K_0 and k_0 were in reasonable

TABLE II

KINETIC CONSTANTS FOR THE α-CHYMOTRYPSIN–CATALYZED HYDROLYSIS OF SEVERAL α-*N*-ACYLATED L-TYROSINAMIDES AND L-TYROSINHYDRAZIDES[a]

Substrate	NaCl (M)	K_0[b]	k_0'[c]	$k_0 = xk_0'$ x	k_0[c]
α-*N*-Ac-L-Tyr-NH_2[d]	1.93	38.5 ± 9	0.287 ± 0.059	23.0	6.6 ± 1.3
	[e]	30 ± 2			3.85 ± 0.3
	[f]	31 ± 3			5.0 ± 0.3
α-*N*-Ni-L-Tyr-NH_2[g]	0.116	16 ± 6	0.202 ± 0.055	23.0	4.6 ± 1.3
	1.93	2.3 ± 1.0	0.262 ± 0.026	23.0	6.0 ± 0.6
	[h]	12 ± 3			5.0 ± 1.0
α-*N*-Ac-L-Tyr-$NHNH_2$[i]	0.10	23.3 ± 4.2	0.390 ± 0.066	2.58	1.0 ± 0.2
	1.93	18.6 ± 0.9	0.936 ± 0.042	2.58	2.4 ± 0.1
	[j]	29.5 ± 6.0			1.1 ± 0.2
α-*N*-Tr-L-Tyr-$NHNH_2$[k]	0.92	56 ± 3	0.51 ± 0.02	2.58	1.3 ± 0.05
	[l]	40 ± 20			0.4 ± 0.2

[a] In aqueous solutions at 25.0° and *p*H 7.90. [b] In units of 10^{-3} M. [c] In units of 10^{-3} M/min./mg protein-nitrogen per ml. [d] α-*N*-Acetyl-L-tyrosinamide. [e] Buffer contained 0.2 M phosphate component (Bernhard and Niemann, 1957). [f] Buffer contained 0.4 M phosphate component (Bernhard and Niemann, 1957). [g] α-*N*-Nicotinyl-L-tyrosinamide. [h] Buffer contained 0.02 M Tris component (Foster and Niemann, 1955). [i] α-*N*-Acetyl-L-tyrosinhydrazide. [j] Buffer contained 0.02 M Tris component (Braunholtz *et al.*, 1959). [k] α-*N*-Trimethylacetyl-L-tyrosinhydrazide. [l] Buffer contained 0.02 M Tris component (Lutwack *et al.*, 1957).

agreement with those obtained previously. With the hydrazides the values of v_0' were an order of magnitude greater than those of v_E, which were 0.025×10^{-4} M/minute and 0.019×10^{-4} M/minute at 0.10 M and 1.93 M sodium chloride respectively.

Our experiments with the two α-*N*-acylated α-amino acid hydrazides and the similarity of the pK_A' value of the monoconjugate acid of hydrazine and those of the conjugate acids of many peptides demonstrates that the kinetics of the α-chymotrypsin–catalyzed hydrolysis of peptides are determinable with a *p*H-stat. Reasonable precision can be obtained provided values of pK_A' are available for the conjugate acids of the basic reaction products.

Hydroxamides.—It was anticipated from the preceding experiments and the relatively low value of $\overline{pK_2}'$ for the conjugate acid of hydroxylamine that kinetic constants for the α-chymotrypsin–catalyzed hydrolysis of α-*N*-acylated α-amino acid hydroxamides could be evaluated with considerable precision with a *p*H-stat. Since this technique offered promise of a convenient procedure for evaluation of the inhibition constants of reversible inhibitors of this enzyme, the hydrolysis of α-*N*-acetyl-L-tyrosinhydroxamide, a readily available substrate, was examined in considerable detail.

With α-acylaminohydroxamic acids it is necessary to consider the consequences of the equilibrium shown in equation (8).

$$\text{RCONHOH} \rightleftharpoons \text{RCONHO}^{\ominus} + \text{H}^{\oplus} \qquad (8)$$

For α-*N*-acetyl-L-tyrosinhydroxamide, pK_{A1} = 9.0 and pK_{A2} = 10.2 (Hogness and Niemann, 1953). The former value may be assigned to the hydroxamic acid function and the latter to the phenolic hydroxyl group (Hogness and Niemann, 1953; Almond *et al.*, 1959). Thus, in the region between *p*H 6 and 9 the substrate may be partially ionized, the ionization diminishing the effective concentration of the substrate (Hogness and Niemann, 1953) and, because of the presence of anionic species, further decreasing the demand of the reaction system for added base. However, below *p*H 8 less than 10% and below *p*H 7.6 less than 5% of the substrate will be ionized. Thus, under these conditions buffering of the reaction system by substrate will be relatively unimportant. To establish this point experimentally, the *p*H optimum for the α-chymotrypsin–catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinhydroxamide in aqueous solutions at 25.0° and 0.04 M in sodium chloride, was evaluated with the *p*H-stat. It was found to be 7.60, a value in excellent agreement with that obtained previously by a colorimetric procedure in which the rate of disappearance of substrate was determined (Hogness and Niemann, 1953). All subsequent experiments were conducted at *p*H 7.60 ± 0.01 unless otherwise noted.

The kinetic constants were evaluated from five sets of experiments, performed in aqueous solutions at 25.0° and 0.040, 0.20, 0.50, 1.0, and 2.0 M in sodium chloride, respectively, with $[E]$ = 0.0209 mg protein-nitrogen per ml. For each set of experiments five substrate concentrations varying from $[S] = 5.0$ to 40.0×10^{-3} M were used. Duplicate or triplicate experiments were performed at each substrate concentration. The recorder traces showed slight curvature and accordingly were evaluated by a nine-point orthogonal polynomial at a $t_{0.10}$ significance level for reactions times of t = 1 to 9 minutes. It was noted later that values of v_0' so obtained were identical within the limits of error with those computed from a least-squares fit to an assumed linear trace. Since values of v_0' were several orders of magnitude greater than v_E, correction for the enzyme blank reaction was insignificant and could have been ignored. The kinetic constants obtained are given in Table III.

It is apparent from the data of Table III that the precision obtained in evaluation of the constants K_0 and k_0 of equations (6) is as good as or better than that obtained by other procedures and is at the same level as that observed when the *p*H-stat is used for evaluation of kinetic constants of ester-type substrates where there is no significant buffering of the system by reaction products. From the viewpoint of convenience, precision, and

TABLE III

KINETIC CONSTANTS FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-*N*-ACETYL-L-TYROSINHYDROXAMIDE[a]

NaCl (M)	K_0[b]	k_0'[c]	k_0[c,d]
0.040	51.4 ± 3.6	28.4 ± 1.8	29.2 ± 1.8
0.10	47.0 ± 4.0	30.2 ± 1.9	31.0 ± 2.0
0.20	46.2 ± 3.8	30.6 ± 2.3	31.4 ± 2.4
0.50	38.3 ± 3.9	35.7 ± 3.1	38.3 ± 3.9
1.00	26.7 ± 2.7	31.8 ± 2.4	32.6 ± 2.5
2.00	15.1 ± 0.8	29.9 ± 0.9	30.7 ± 1.0
[e]	43 ± 4		33 ± 3

[a] In aqueous solutions at 25.0° and *p*H 7.60. [b] In units of 10^{-3} M. [c] In units of 10^{-3} M/min./mg protein-nitrogen per ml. [d] $k_0 = 1.025\ k_0'$. [e] Buffer contained 0.3 or 0.5 M Tris component (Foster and Niemann, 1955).

automation, use of the *p*H-stat with an α-*N*-acylated α-amino acid hydroxamide type substrate provides one of the more attractive methods of examining the kinetic behavior of systems involving α-chymotrypsin. It has found extensive application in inhibition studies (Kurtz and Niemann, 1961; Abrash, 1961; Almond *et al.*, 1962).

The variation of values of k_0 with sodium chloride concentration, with an apparent maximum at 0.50 M sodium chloride, is within the limits of experimental error, and it may be concluded that the value of k_0 is essentially independent of the concentration of sodium chloride. From 0.040 to 2.0 M sodium chloride the mean value of $\bar{k}_0 = 32.4 \pm 2.8 \times 10^{-3}$ M/minute/mg protein-nitrogen per ml. This value is in excellent agreement with $\bar{k}_0 = 33 \pm 3 \times 10^{-3}$ M/minute/mg protein-nitrogen per ml obtained previously for systems that are 0.3 or 0.5 M with respect to the tris(hydroxymethyl)aminomethane component of a Tris-HCl buffer of *p*H 7.60. The reaction was followed previously by a colorimetric procedure which determined the rate of disappearance of substrate (Foster and Niemann, 1956) instead of the rate of appearance of the anionic reaction product.

In contrast to values of k_0, those of K_0 are markedly dependent upon the concentration of sodium chloride and are given by relation (9).

$$\log K_0 = -(1.283 \pm 0.0008) - (0.273 \pm 0.007)[\mathrm{NaCl}] \quad (9)$$

The independence of values of k_0 and dependence of values of K_0 of α-*N*-acetyl-L-tyrosinhydroxamide upon the concentration of sodium chloride is exactly the opposite of that observed by Shine and Niemann (1955) for α-*N*-chloroacetyl-L-tyrosinamide and sodium chloride, by Bernhard and Niemann (1957) for α-*N*-acetyl-L-tyrosinamide and sodium or potassium phosphate, and by Kerr and Niemann (1958) for α-*N*-nicotinyl-L-tyrosinhydrazide and tris(hydroxymethyl)aminomethane hydrochloride, where in all cases K_0 remained invariant and k_0 increased with increasing concentration of salt. With the observation of Martin and Niemann (1958) that both K_0 and k_0 for methyl hippurate and acetyl-L-valine methyl ester varied with the concentration of sodium or potassium chloride, it is evident that examples of all possible dependencies of K_0 and k_0 upon salt concentration have now been observed except that of simultaneous independence of both constants. It is clear that any explanation of the variation of K_0 and k_0 with salt concentration must provide for the above three possibilities and recognize that a determining factor is the structure of the substrate molecule. It is likely that α-*N*-acetyl-L-tyrosinhydroxamide is not a unique example of the independence of k_0 and dependence of K_0 upon salt concentration, since the data of Table II indicate that the same situation may prevail for α-*N*-nicotinyl-L-tyrosinamide. In contrast, the data for α-*N*-acetyl-L-tyrosinhydrazide suggest essential independence of K_0 but dependence of k_0 upon concentration of sodium chloride.

In the experiments with α-*N*-acetyl-L-tyrosinhydroxamide at *p*H 7.60 the correction factor for conversion of values of k_0' to those of k_0 is 1.025. In order to explore further the limits of usefulness α-*N*-acetyl-L-tyrosinhydroxamide as a substrate when used with a *p*H-stat several experiments were conducted at *p*H 6.70, where the correction factor is 1.195. The data given in Table IV provide evidence for the usefulness of the above system over a considerable range of *p*H and in addition indicate an increase in K_0 and a decrease in k_0 with a decrease in *p*H from 7.60 to 6.70.

TABLE IV

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-*N*-ACETYL-L-TYROSINHYDROXAMIDE AT *p*H 6.70 AND ITS INHIBITION BY 1-ACETYL-2-(L-TYROSYL)HYDRAZINE[a]

$[I] = 0$			$[I] = 0.237 \times 10^{-3}$ M		
$[S]$[d]	v_0'[b]	P_m[c]	$[S]$[d]	v_0'[b]	P_m[c]
40	0.988	2	35	0.533	2
25	0.699	2	25	0.391	2
20	0.616	2	20	0.329	2
15	0.476	2	15	0.260	2
10	0.342	2	10	0.177	2
7.5	0.270	3	7.5	0.140	2
$K_0 = 65.2 \pm 3.9$[d]			$K_0' = 117 \pm 14$[d]		
$k_0' = 17.6 \pm 1.0$[e]			$k_0' = 15.5 \pm 1.9$[e]		
$k_0 = 21.0 \pm 1.2$[e]			$k_0 = 18.6 \pm 2.2$[e]		

$K_1 = 0.22 \pm 0.02$[d]

[a] In aqueous solutions at 25.0° and 0.1 M with respect to sodium chloride, with $[E] = 0.1464$ mg protein-nitrogen per ml. [b] In units of 10^{-3} M/min. [c] Order of orthogonal polynominal. [d] In units of 10^{-3} M. [e] Units of 10^{-3} M/min./mg protein-nitrogen per ml.

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Interaction of α-Chymotrypsin with Several α-Methyl-α-Acylamino Acid Methyl Esters*

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Methyl (+)- and (−)-*N*-acetyl-α-methylphenylalaninate, methyl (+)- and (−)-α-*N*-acetyl-α-methyltyrosinate, and methyl (±)- and (−)-*N*-acetyl-α-methyl-β-(2-naphthyl)-alaninate have been evaluated as competitive inhibitors of the α-chymotrypsin–catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinhydroxamide and as substrates of this enzyme. Replacement of the α-hydrogen atom of representative enantiomorphic α-*N*-acyl aromatic α-amino acid esters by a methyl group changes the substration or inhibition constants by less than an order of magnitude but for substrates decreases the rate of formation of products by a factor of about 10^5. These results have been explained on the basis of steric interference by the α-alkyl group with attack of the potentially hydrolyzable carboalkoxy group by electro- or nucleophilic groups present at the active site of the enzyme.

Hein and Niemann (1961) proposed that α-*N*-acylated aromatic α-amino acid esters such as methyl α-*N*-acetyl-L-phenylalaninate and L-tyrosinate are substrates approximating the $S^{3E}_{R_2R_3}$ limit type, a designation for trifunctional ester-type substrates where orientation at the active site of the enzyme is achieved through interaction of R_1, the α-acylamino moiety, R_2, the α-amino acid side-chain, and COR_3, the reactive carboalkoxy group, with their respective complementary loci, ρ_1, ρ_2, and ρ_3, but where the enzyme-substrate dissociation constants are determined by the degree to which R_2 interacts with ρ_2 and COR_3 with ρ_3.

The orientation of an $S^{3E}_{R_2R_3}$ limit type substrate with R_1 of adequate size and structure to achieve its stereochemical role at the active site is represented schematically in Figure 1, as is that of its inhibitory D-antipode. This representation implies that the rate of formation of products from the D-antipode is suppressed by several orders of magnitude because R_1, in the position occupied by the α-hydrogen atom of the L-antipode, not only is incapable of assuming its critical orienting role, which for the L-antipode is associated with a high rate of formation of products, but in addition sterically limits attack on the carbonyl group of COR_3 of the D-antipode by an electro- or nucleophilic group of the site that has ready access to the same group in the L-antipode. These two effects, augmenting each other, result in a decrease in the rate of formation of products from the D-antipode of several orders of magnitude and a substantial relative stereospecificity in favor of the L-antipode. Absolute specificity for the L-antipode would demand the absence of a COR_3-ρ_3 interaction for the D-antipode, an unreasonable situation for an en-

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