[CONTRIBUTION NO. 1516 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Evaluation of the Enzyme-Inhibitor Dissociation Constant of α-Chymotrypsin and Acetyl-D-tryptophan Methyl Ester[1]

BY H. T. HUANG AND CARL NIEMANN[2]

The value of the apparent dissociation constant of α-chyomotrypsin and acetyl-D-tryptophan methyl ester, at 25° and *p*H 7.9, determined by the competitive inhibition of the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide by acetyl-D-tryptophan methyl ester at an enzyme concentration of 0.208 mg. protein nitrogen per ml. and the customary $1/v_0$ *versus* $1/[S]_0$ plot, suggests that the affinity of the enzyme for this competitive inhibitor is of such a magnitude as to invalidate the usual assumption that the molar concentration of the inhibitor is very much greater than the molar concentration of the enzyme-inhibitor complex. An attempt has been made to determine the true value of the above enzyme-inhibitor dissociation constant, and to evaluate the equivalent weight of the enzyme, by a more detailed analysis of the kinetic data.

The simplifying zone A assumptions employed in the derivation of rate equations to describe the course of the α-chymotrypsin-catalyzed hydrolysis of specific substrates in the presence of competitive inhibitors were briefly discussed in a previous communication.[3] Specifically, these assumptions refer to the experimental conditions under which the approximation that $[S] \doteq [S_f] \gg [ES]$ and $[I] \doteq [I_f] \gg [EI]$ may be considered as being valid in the quantitative formulation of the reactions

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

$$E_f + I_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI \qquad (2)$$

It has been observed[3] that in general the upper limits for zone A behavior recommended by Straus and Goldstein,[4,5] *i.e.*, $E'_S = [E]/K_S$ and $E'_I = [E]/K_I > 0.1$, can serve as a convenient guide to determine if a given system is in zone A. Thus in those cases where the highest enzyme concentration is of the order of 5×10^{-5} *M*[3,6] deviations from zone A conditions would not be expected unless the system contained a specific substrate or competititive inhibitor with a K_S or K_I value of less than 0.5×10^{-3} *M*. It will be noted that to date no specific substrate of α-chymotrypsin has been found to have a K_S value of less than 1×10^{-3} *M*[3,7,8] and in view of the enzyme concentrations that have been employed for the evaluation of this constant it is not surprising that no deviations from zone A relations have been reported for specific substrates. However, in a systematic study of the affinity of α-chymotrypsin for a series of competitive inhibitors derived from D-tryptophan several compounds were found to have an apparent K_I value (K'_I) of less than 0.5×10^{-3} *M* when nicotinyl-L-tryptophanamide was used as the specific substrate.[9] It is evident from the low K'_I values and the enzyme concentrations employed in these experiments that for these systems the assumption that $[I] \doteq [I_f] \gg [EI]$ may not be valid. In other words, while these systems are in zone A with respect to the specific substrate they appear to be in zone B with respect to the competitive inhibitor. The fact that, in all cases, the customary $1/v_0$ *versus* $1/[S]_0$ plot of the data[10] showed a satisfactory linear relationship within the limits of experimental error clearly suggested the need of a more rigorous analysis of the data to disclose deviations from zone A behavior and to provide reliable information as to the extent to which the K'_I values evaluated by the Lineweaver and Burk zone A equation for competitive inhibition,[10] *i.e.*, equation (3), may differ from the corresponding values of K_I. For this

$$\frac{1}{v} = \frac{K_S}{V}\left(1 + \frac{[I]}{K'_I}\right)\frac{1}{[S]} + \frac{1}{V} \qquad (3)$$

study acetyl-D-tryptophan methyl ester was chosen since it is an effective competitive inhibitor in systems containing α-chymotrypsin and a hydrolyzable specific substrate and is sufficiently soluble in aqueous media to permit its use at concentrations from 0.05 to 1.00×10^{-3} *M*.

All experiments were conducted at 25° and *p*H 7.9 in the presence of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer 0.02 *M* with respect to the amine component. The main series of experiments were performed with nicotinyl-L-tryptophanamide as the specific substrate and at an enzyme concentration corresponding to 0.208 mg. protein nitrogen per ml. The supplementary experiments were conducted with nicotinyl-L-tyrosinamide as the specific substrate at an enzyme concentration of 0.047 mg. protein nitrogen per ml. Since the extent of hydrolysis of the above specific substrates in all experiments was less than 30% the inhibition of the hydrolytic reaction by the corresponding liberated acylamino acids[3,8] may be ignored, and equations (1) and (2) can be taken as an accurate representation of the system under discussion. By definition

$$K_S = \frac{([E] - [ES] - [EI])([S] - [ES])}{[ES]} \qquad (4)$$

and

$$K_I = \frac{([E] - [ES] - [EI])([I] - [EI])}{[EI]} \qquad (5)$$

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

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

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

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

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

(6) Computed for an enzyme concentration of 0.208 mg. protein nitrogen per ml. on the basis of one reactive site per enzyme molecule and a molecular weight of 27,000. *Cf.* E. F. Jansen, M. D. Fellows-Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **185**, 209 (1950).

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

(8) D. Thomas, R. V. MacAllister and C. Niemann, THIS JOURNAL, **73**, 1548 (1951).

(9) H. T. Huang, unpublished observations.

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

It follows that

$$[EI] = \frac{K_S[ES][I]}{K_I([S] - [ES]) + K_S[ES]} \quad (6)$$

and

$$K_S[ES] = \left([E] - [ES] - \frac{K_S[ES][I]}{K_I([S] - [ES]) + K_S[ES]}\right)([S] - [ES]) \quad (7)$$

Since the system is in zone A with respect to substrate, *i.e.*, $[S] \gg [ES]$, equation (7) can be reduced to

$$K_S[ES] = \left([E] - [ES] - \frac{K_S[ES][I]}{K_I[S] + K_S[ES]}\right)[S] \quad (8)$$

which on rearrangement gives a quadratic equation in [ES], *i.e.*

$$K_S(K_S[S])[ES]^2 + [S](K_SK_I + K_I[S] + K_S[I] - [E]K_S)[ES] - [E]K_I[S]^2 = 0 \quad (9)$$

It will be noted that equation (9) can be obtained by the expansion and suitable rearrangement of equation $6B_IA_S$ of Goldstein,[5] which refers specifically to a system in zone A with respect to substrate but in zone B with respect to inhibitor. The most important difference between equation (9) and pure zone A type expressions is that it is not possible to find a solution for [ES] in which [E] can be conveniently eliminated such as in equation (3). Therefore, with equation (9) K_I cannot be evaluated without a previous or simultaneous evaluation of [E]. While a knowledge of [E] can be derived from existing data on the molecular weight of α-chymotrypsin and the number of reactive sites per molecule,[6] it is of interest to see if an independent evaluation of [E], based entirely upon kinetic data, is possible with our present analytical methods.

In order to effect a solution of equation (9) it has been found desirable to resort to the practice of Straus and Goldstein[4,5] and to use the term "fractional activity" which is defined as $\alpha = v/V = [ES]/[E]$. Substituting the quantity $\alpha[E]$ for [ES] in equation (9) and rearranging one obtains the relation

$$\frac{[I]}{(\alpha^{-1} - 1)\frac{[S]}{K_S} - 1} = K_I + \alpha[E]\frac{K_S}{[S]} \quad (10)$$

Equation (10), while identical in content with Goldstein's equation $6B_IA_S$, is in a more convenient form for the evaluation of K_I and [E]. Thus a plot of $Q = [I]/[(\alpha^{-1} - 1)[S]/K_S - 1]$ *versus* $\alpha K_S/[S]$ should give K_I as the intercept and [E] as the slope. The results of competition experiments at different inhibitor concentrations with nicotinyl-L-tryptophanamide as the specific substrate, and with [E] invariant, are summarized in the plot given in Fig. 1. An attempt has been made to indicate the range in which Q must lie for each individual experiment, based on the possible variations in α, as calculated from the probable limits of error in the determination of the experimental values of v. It should be pointed out that if the system is entirely in zone A, *i.e.*, $[E] \rightarrow 0$, then $Q = K_I$ and a line parallel to the abscissa is obtained. Q, however, is extremely sensitive to errors in α, since a small deviation in α is grossly magnified in the function $(\alpha^{-1} - 1)$. Furthermore, considering the large error in Q for each measurement, the range in $\alpha K_S/[S]$ is too small to allow a good estimation of a difference in Q to be made. It is evident that while the data do indicate a significant slope, *i.e.*, [E] is finite and the system is in zone B with respect to the inhibitor, the plot given in Fig. 1 cannot be expected to yield a value of [E] with a precision of better than 50%. Thus forced to an alternative procedure the best lines have been drawn to fit [E], on the basis of 27,000 as the molecular weight of α-chymotrypsin[6] for different values of n, where n = the number of reactive sites per molecule. It will be noted that only when $n = 1$ does the corresponding line touch all of the points on the plot within the indicated limits of experimental error. Therefore, it may be concluded that $n = 1$, and $K_I = 8.9 \pm 0.4 \times 10^{-5}$ M although $n = 2$ is not clearly excluded.

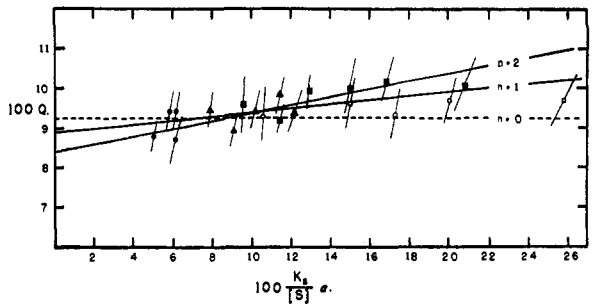

Fig. 1.—Inhibition of the hydrolysis of nicotinyl-L-tryptophanamide by acetyl-D-tryptophan methyl ester; plot of Q *versus* $\alpha K_S/[S]$; [E] = 0.208 mg. protein nitrogen per ml., [S] and [I] in units of 10^{-3} M, $K_S = 2.7 \times 10^{-3}$ M. ●, $[I] = 1.0 \times 10^{-3}$ M; ▲, $[I] = 0.40 \times 10^{-3}$ M; ■, $[I] = 0.20 \times 10^{-3}$ M; ○, $[I] = 0.10 \times 10^{-3}$ M. The points for experiments at $[I] = 0.05 \times 10^{-3}$ M are not included because the error is too large to be of value in the plot. Each point represents the mean of the values obtained in two separate experiments.

In spite of the extreme sensitivity of the function Q to errors in α, the present results indicate that this approach has definite possibilities as a method for the estimation of [E] and hence to provide an independent check on the equivalent weight or the number of reactive sites per molecule of enzyme. It is clear that the precision of the evaluation of [E] can be improved by a substantial increase in the precision of present experimental methods, and by the use of higher enzyme concentrations. An increase in the enzyme concentration will have the effect of increasing both the value of the slope of the Q *versus* $\alpha K_S/[S]$ plot and the range of the $\alpha K_S/[S]$ values. A further advantage of working at higher enzyme concentrations is that it should be possible to obtain more reliable values of the initial velocities v_0 at lower values of [S].

The value of $K_I = 8.9 \pm 0.4 \times 10^{-5}$ M for the system α-chymotrypsinacetyl-D-tryptophan methyl ester, at 25° and *p*H 7.9, is significantly smaller than the corresponding K'_I values calculated from the same experimental data with the aid of equation (3) by the customary $1/v_0$ *versus* $1/[S]_0$ plot, *cf.*, Fig. 2 and Table I. The fact that the values for K'_I decrease as [I] is increased is con-

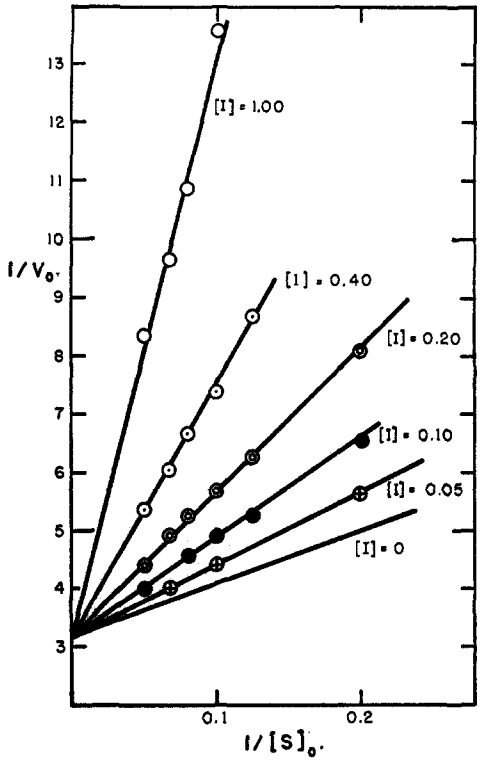

Fig. 2.—Inhibition of the hydrolysis of nicotinyl-L-tryptophanamide by acetyl-D-tryptophan methyl ester; plot of $1/v_0$ *versus* $1/[S]_0$; [E] = 0.208 mg. protein nitrogen per ml., [S] and [I] in units of 10^{-3} *M*, $K_S = 2.7 \times 10^{-3}$ *M*. Each point represents the mean of the values obtained in two separate experiments.

sistent with the expectation that as [I] is increased the approximation that $[I] \gg [EI]$ assumes a greater degree of validity. If it is assumed, with some justification, that the value of [E] which was used for the evaluation of K_I is correct then $E'_I = 0.54$ and the system appears to be close to the lower boundary of zone B, *i.e.*, $E'_I = 0.1$. When it is recalled that the effect of the presence of substantial amounts of the specific substrate is to shift the system as a whole toward zone A,[3] the actual deviations from zone A behavior will be even smaller than those expected from a consideration of the value of E'_I alone, *i.e.*, the systems are closer to zone A than the value of E'_I would indicate. Therefore, with a lower enzyme concentration it should be possible to evaluate K_I under essentially zone A conditions. For this reason

TABLE I

APPARENT ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF α-CHYMOTRYPSIN AND ACETYL-D-TRYPTOPHAN METHYL ESTER[a]

Substrate	[I][b]	[E][c]	K'_I[d]
Nicotinyl-L-tryptophanamide[e]	0.05	0.208	0.11
Nicotinyl-L-tryptophanamide[e]	.10	.208	.10
Nicotinyl-L-tryptophanamide[e]	.20	.208	.10
Nicotinyl-L-tryptophanamide[e]	.40	.208	.097
Nicotinyl-L-tryptophanamide[e]	1.00	.208	.091
Nicotinyl-L-tyrosinamide[f]	0.10	.047	.088

[a] At 25° and *p*H 7.9. [b] In units of 10^{-3} *M*. [c] [E] in mg. protein nitrogen per ml. [d] In units of 10^{-3} *M*. [e] $K_S = 2.7 \times 10^{-3}$ *M*. [f] $K_S = 15.0 \times 10^{-3}$ *M*.

K_I has been evaluated from a series of experiments with nicotinyl-L-tyrosinamide as the specific substrate and where $[E] = 1.08 \times 10^{-5}$ *M*, *i.e.*, 0.047 mg. protein nitrogen per ml. The value obtained for K'_I in this instance was 8.8×10^{-5} *M*, *cf.* Fig. 3, which is in excellent agreement with the previously estimated value of K_I of 8.9×10^{-3} *M*. It should be noted that in the experiments where $[E] = 1.08 \times 10^{-5}$ *M* the value of E'_I was 0.13.

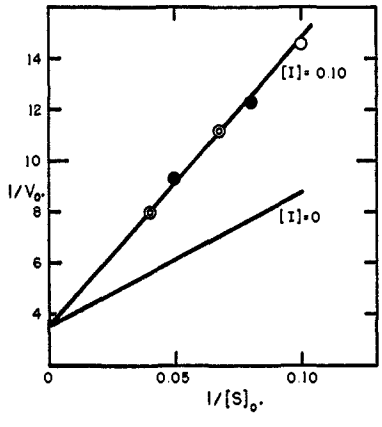

Fig. 3.—Inhibition of the hydrolysis of nicotinyl-L-tyrosinamide by acetyl-D-tryptophan methyl ester; plot of $1/v_0$ *versus* $1/[S]_0$; [E] = 0.047 mg. protein nitrogen per ml., [S] and [I] in units of 10^{-3} *M*, $K_S = 15.0 \times 10^{-3}$ *M*: ⊙, mean of three experiments; ●, mean of two experiments; O, single experiment.

From *a priori* considerations it may be expected that for larger concentrations of the enzyme a plot of $1/v_0$ *versus* $1/[S]_0$ may give rise to a curve with decreasing slope as the value of $1/[S]_0$ is increased. However, in the present series of experiments, no such deviation was observed even for measurements with [I] as low as 0.05×10^{-3} *M*. No unambiguous relation could be obtained from approximate solutions of equation (9) for [ES] which would justify a linear relationship between $1/v_0$ and $1/[S]_0$. For the condition that $K_S(K_S + [S])[ES]/[S](K_S K_I + K_I[S] + K_S[I] - K_S[E])$ is less than 0.1, a perturbation solution of equation (9) gives the relation

$$\frac{1}{v} = \frac{1}{V} + \frac{K_S}{V}\left(1 + \frac{[I]}{K_I} - \frac{[E]}{K_I} + \frac{[E]}{K_I} \cdot \frac{1}{1+\delta}\right)\frac{1}{[S]} \quad (11)$$

where $\delta = K_S([I] - [E])/K_I(K_S + [S])$. Thus for each series of experiments at a given value of [I] and [E], δ decreases as [S] is increased. However, it can be seen that the variation in δ is small within the range of [S] ordinarily encountered and considering the change a variation in δ causes in the terms $[I]/K_I - [E]/K_I + [E]/K_I(1 + \delta)$ as a whole it would not be expected, in our experiments, that the plot of $1/v_0$ *versus* $1/[S]_0$ would be other than linear. It is obvious that the observed linear relationship between $1/v_0$ and $1/[S]_0$ is more apparent than real. It will be appreciated that under favorable conditions equation (11) can be used to give a corrected value of K'_I by making use of the value of K'_1 derived from equation (3). In general, once the correct slope is determined, an evaluation of K_I from a plot based on equation

(10) is less ambiguous. Applications of equation (10) will be illustrated by the evaluation of the K_I values of various competitive inhibitors in a subsequent communication.

We wish to express our thanks to Dr. Verner Schomaker for valuable suggestions and discussions in relation to this work.

Experimental

The preparation of acetyl-D-tryptophan methyl ester,[3] nicotinyl-L-tryptophanamide[3] and nicotinyl-L-tyrosinamide[11] has been described previously. All enzymatic experiments are conducted at 25° and *p*H 7.9 ± 0.02 in aqueous solution 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. The methods used are identical with those described previously.[3] The α-chymotrypsin was an Armour preparation lot no. 90402 of bovine origin.

(11) B. M. Iselin, H. T. Huang, R. V. MacAllister and C. Niemann, THIS JOURNAL, **72**, 1729 (1950).

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

BY H. T. HUANG, R. V. MACALLISTER, D. W. THOMAS AND CARL NIEMANN[2]

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 have been found to be similar to those reported previously for this enzyme and other specific substrates of the acylated α-amino acid amide type. For the system α-chymotrypsin–nicotinyl-L-tyrosinamide at 25° and *p*H 7.9 K_S was found to have a value of $15.0 \pm 1.0 \times 10^{-3}$ *M* and k_3 a value of 6.2×10^{-3} *M*/mg. protein nitrogen/ml./min.

It has been noted previously that whereas the *p*H–activity curves for α-chymotrypsin and acetyl- and nicotinyl-L-tryptophanamide are practically identical[3] there is a significant difference between the above curves and that observed for α-chymotrypsin and acetyl-L-tyrosinamide.[4] From the data given in Fig. 1 it will be seen that the *p*H–activity curve for α-chymotrypsin and nicotinyl-L-tyrosinamide is identical, within the limits of experimental error, with that observed for this enzyme and acetyl-L-tyrosinamide. The fact that substantially different *p*H–activity relationships have now been observed for α-chymotrypsin and acetyl- and nicotinyl-L-tryptophanamide on one hand, and α-chymotrypsin and acetyl- and nicotinyl-L-tyrosinamide on the other, clearly indicates that for this enzyme and specific substrates of the general formula $RCONHCHR_1CONH_2$, the nature of the amino acid side chain, *i.e.*, R_1, may have a profound influence upon the *p*H–activity relationship. In contrast, it appears, from the two cases at hand, that the role of the acyl group, *i.e.*, of R, in the above process is relatively unimportant.[5] Experiments designed to provide data relative to effect of *p*H upon the K_S and k_3 values of the above specific substrates are now in progress.

The kinetics of the α-chymotrypsin catalyzed hydrolysis of nicotinyl-L-tyrosinamide, at 25° and *p*H 7.9, are similar to those noted previously for this enzyme and other specific substrates[3,4] in that the course of the hydrolytic reaction is described by equations (1) and (2)

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

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

and, when $E'_S < 0.1$, by the rate equation (3)

$$k_3[E]t = 2.3K_S\left(1 + \frac{[S]_0}{K_{P_1}}\right)\log\frac{[S]_0}{[S]} + \left(1 - \frac{K_S}{K_{P_1}}\right)([S]_0 - [S]) \qquad (3)$$

However, with the system α-chymotrypsin–nicotinyl-L-tyrosinamide at 25° and *p*H 7.9, the relation between the values of K_S, K_{P_1} and k_3 is such that the experimental data can also be described, within the limits of experimental error, by rate equation (4)

$$k_3[E]t = 2.3K_S\log\frac{[S]_0}{[S]} + ([S]_0 - [S]) \qquad (4)$$

wherein inhibition of the hydrolytic reaction by the liberated nicotinyl-L-tyrosine is ignored. Therefore, in this instance, and when $E'_S < 0.1$, equation (4) may be used with confidence for the evaluation of k_3, even though the extent of hydrolysis may be of the order of 80% (*cf.* Fig. 2). K_S was determined in the usual manner[6] from a plot of $1/v_0$ *versus* $1/[S]_0$, and three independent determinations at two different enzyme concentrations and in two different buffer systems, *i.e.*, tris-(hydroxymethyl)-aminomethane–hydrochloric acid and ethylenediamine–hydrochloric acid, gave a mean value of $K_S = 15.0 \pm 1.0 \times 10^{-3}$ *M*. A typical plot is given in Fig. 3. The value of k_3 was found to be 6.2×10^{-3} *M*/mg. protein nitrogen/ml./min.

The fact that nicotinyl-L-tyrosine must compete, at least to some degree, with nicotinyl-L-tyrosinamide for the catalytically active site on the enzyme molecule was established indirectly when it was shown that the α-chymotrypsin catalyzed hydroly-

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

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

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

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

(5) It should be understood that this generalization relates only to specific substrates derived from acylated α-amino acids. There is evidence to show that replacement of an acylamino group by an amino or ammonium group is accompanied by a change in the position of the maximum of the *p*H-activity curve; *cf.* E. F. Jansen, M. D. Fellows-Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **185**, 209 (1950); H. Goldenberg and V. Goldenberg, *Arch. Biochem.*, **29**, 154 (1950).

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