ists, in enzymatic digests of thymus nucleic acid, a substance possessing the properties and constitution of a desoxynucleotide with 5-methylcytosine as its base component. Unless it is assumed that desoxycytidylic acid is partially methylated (or thymidylic acid partially aminated) during the enzymatic digestion period, the desoxynucleotide of 5-methylcytosine must be regarded as a constituent of thymus nucleic acid analogous to those of adenine, guanine, thymine and cytosine.

Two factors set this nucleotide apart from the others, however. One is its low molar concentration, which here approximates 3% of the amount of desoxycytidylic acid found (Wyatt[15] has reported, in preliminary form, analyses of DNA samples derived from a multitude of biological sources which indicate that 5-methylcytosine may range from zero to as high as 30% of the cytosine found). The other is that 5-methylcytosine is structurally related to both of the two pyrimidine bases of DNA, thymine and cytosine.

(15) G. R. Wyatt, *Biochem. J. (Proceedings)*, **47**, vii (1950); *Nature*, **166**, 237 (1950).

OAK RIDGE, TENNESSEE RECEIVED OCTOBER 5, 1950

[CONTRIBUTION NO. 1439 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

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

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

The kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tryptophanamide have been determined at 25° and *p*H 7.9 and it has been shown that the hydrolytic reaction is competitively inhibited by the corresponding acylated L-amino acids and by the D-antipodes of the above substrates.

The α-chymotrypsin catalyzed hydrolysis of certain acylated α-amino acid amides to the corresponding acylated α-amino acids and ammonia[3] is one of the simplest reactions that can be used for the determination of the mode of action of this enzyme. Therefore, with this latter goal in mind we have examined the kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tryptophanamide at 25° and *p*H 7.9 in an aqueous solution 0.02 molar with respect to a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. In this study consideration was given to (a) the possibility of interaction of the enzyme with the buffer components; (b) the dependence of the activity of the enzyme upon the *p*H of the reaction system; (c) the possibility of inhibition of the hydrolytic reaction by one or both of the hydrolysis products; (d) the development of suitable rate expressions and the determination of rate constants; and (e) the possibility of inhibition of the hydrolysis of the above substrates by their D-antipodes. Since it is known[3] that α-chymotrypsin requires neither a coenzyme nor an activator it shall be assumed that the catalytically active species is identical with crystalline α-chymotrypsin.

In a previous study[4] it was found that the activity of α-chymotrypsin, when determined by its effect upon the extent of hydrolysis of nicotinyl-L-tryptophanamide, in systems buffered at *p*H 7.8 with either a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, an ethylenediamine–hydrochloric acid buffer, or a potassium phosphate buffer, was independent of the nature of the buffer. Furthermore, with the first two buffers the activity of the enzyme was found to be independent of the concentration of the buffer when it was varied from 0.005 molar to 0.05 molar with respect to the amine component.[4] Therefore, in the subsequent discussion consideration will be limited to a reaction system containing only enzyme, *i.e.*, α-chymotrypsin, substrate and reaction

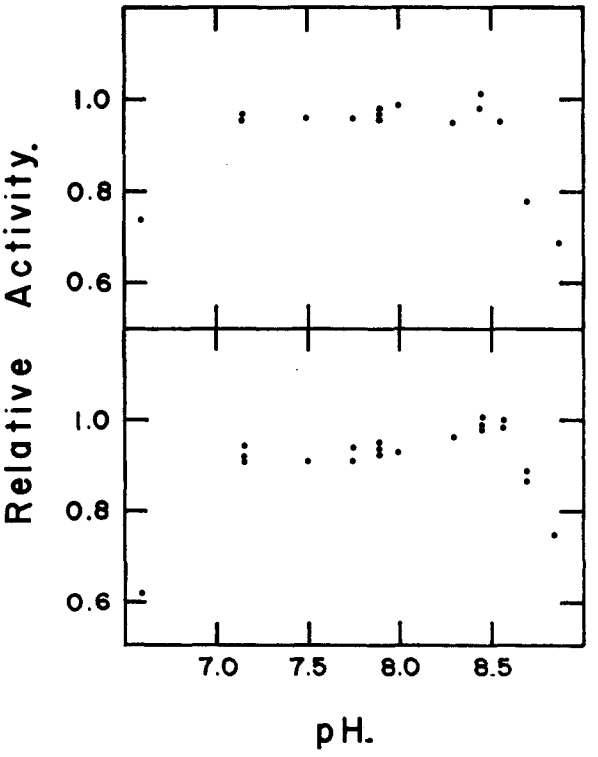

Fig. 1.—Lower half; acetyl-L-tryptophanamide, $[S]_0 = 10 \times 10^{-3}$ *M*, [E] = 0.208 mg. protein-nitrogen per ml.: upper half; nicotinyl-L-tryptophanamide, $[S]_0 = 10 \times 10^{-3}$ *M*, [E] = 0.069 mg. protein-nitrogen per ml.

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

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

(3) See H. Neurath and G. Schwert, *Chem. Revs.*, **46**, 69 (1950), for a resumé of previous studies on the enzymatic properties of α-chymotrypsin.

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

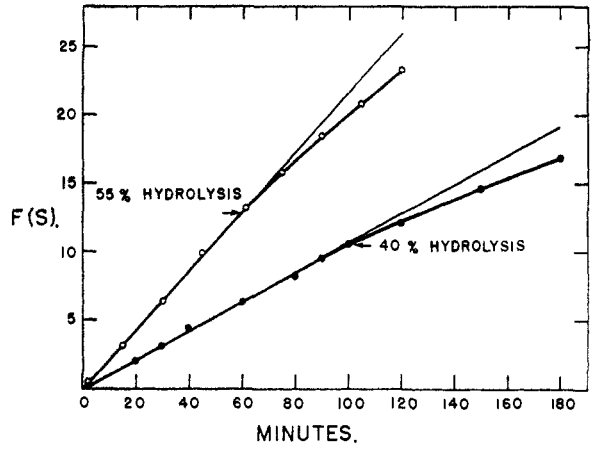

Fig. 2.—$F(S)$ in units of 10^{-3} M; ●, acetyl-L-tryptophanamide, $[S]_0 = 20 \times 10^{-3}$ M, $[E] = 0.208$ mg. protein-nitrogen per ml.; ○, nicotinyl-L-tryptophanamide, $[S]_0 = 20 \times 10^{-3}$ M, $[E] = 0.146$ mg. protein-nitrogen per ml.

products, except in those cases where an inhibitor was knowingly added.[5]

A plot of the relative activity of α-chymotrypsin *versus* the *p*H of the reaction system, as determined by the extent of hydrolysis of acetyl- and nicotinyl-L-tryptophanamide, is given in Fig. 1. It is beyond the scope of this communication to analyze the effect of *p*H upon the various factors that are operative in the α-chymotrypsin catalyzed hydrolysis of specific acylated α-amino acid amides, and Fig. 1 is given only to illustrate the character of the *p*H-activity relationship and to emphasize the point that the present study is limited to a description of the hydrolysis of two specific substrates at a particular *p*H, *i.e.*, 7.9.

As a first approximation, the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tryptophanamide was formulated in terms of the classical intermediate enzyme-substrate complex theory[6-13] in its simplest form, *i.e.*

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

With

$[E]$ = molar concentration of total enzyme
$[E_f]$ = molar concentration of free enzyme
$[S]$ = molar concentration of total substrate
$[S_f]$ = molar concentration of free substrate
$[ES]$ = molar concentration of enzyme-substrate complex
$[P_{1f}], [P_{2f}]$ = molar concentration of the two free hydrolysis products

(5) It will be seen from data given later that the rate of hydrolysis is directly proportional to the enzyme concentration when the latter is expressed in terms of the amount of protein nitrogen added to the reaction system. Therefore it is reasonable to assume that the principal non-protein constituent of the enzyme preparation, *i.e.*, magnesium sulfate, is without effect, in the concentrations added (0.001 to 0.003 molar), upon the reaction system.

(6) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

(7) D. D. Van Slyke and G. E. Cullen, *J. Biol. Chem.*, **19**, 141, 211 (1914).

(8) G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

(9) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930.

(10) B. Chance, *J. Biol. Chem.*, **151**, 553 (1943).

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

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

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

it has been shown[8-13] that for the case where all reactants possess unit activity

$$d[ES]/dt = k_1[E_f][S_f] - (k_2 + k_3)[ES] \qquad (2)$$

$$-d[S_f]/dt = k_1[E_f][S_f] - k_2[ES] \qquad (3)$$

and

$$d([ES] + [S_f])/dt = -k_3[ES] \qquad (4)$$

For the condition that

$$d[ES]/dt = k_1([E] - [ES])[S] - (k_2 + k_3)[ES] \doteq 0 \qquad (5)$$

i.e., where $d[S]/dt \gg d[ES]/dt$ and $[S] \doteq [S_f] \gg [ES]$ it follows[8-13] that

$$\frac{k_2 + k_3}{k_1} = \frac{([E] - [ES])[S]}{[ES]} = K_S \qquad (6)$$

$$-\frac{d[S]}{dt} = \frac{k_3[E][S]}{K_S + [S]} \qquad (7)$$

and

$$k_3[E]t = 2.3\, K_S \log [S]_0/[S] + ([S]_0 - [S]) \qquad (8)$$

where $[S] = [S]_0$ when $t = 0$. The constant K_S appearing in equation (8), *i.e.*, the so-called Michaelis constant, was evaluated from the relation

$$\frac{1}{v} = \frac{K_S}{V}\left(\frac{1}{[S]}\right) + \frac{1}{V} \qquad (9)$$

where $V = k_3[E]$ and $v = -d[S]/dt$, by the customary plot of $1/v_0$ *versus* $1/[S]_0$.[14]

The experimental data obtained with the use of acetyl-L-tryptophanamide as a substrate was tested for congruity with equation (8) by a plot of $F(S) = 2.3\, K_S \log ([S]_0/[S]) + ([S]_0 - [S])$ *versus* time in minutes (*cf.* Fig. 2) and it was observed that after 40% hydrolysis of the substrate the experimental curve deviated markedly from the calculated linear one. A similar behavior was noted when nicotinyl-L-tryptophanamide was used as a substrate (*cf.* Fig. 2). The observation that the experimental and calculated plots were congruous up to approximately 40% hydrolysis of the substrate and that negative deviations were obtained beyond this point suggested the possibility of interaction of the enzyme with one or both of the reaction products. The fact that the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tryptophanamide is inhibited by acetyl-L-tryptophan and the corresponding hydrolysis of nicotinyl-L-tryptophanamide is inhibited by nicotinyl-L-tryptophan and that neither of the above reactions is inhibited by reasonable concentrations of ammonia or ammonium ion was established when it was observed that the initial rates of hydrolysis of the acylated α-amino acid amides were decreased by the addition of the corresponding acylated-L-amino acid and remained unchanged upon the addition of ammonia in concentrations up to 0.01 molar.

For the reaction system

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

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \qquad (10)$$

where

$[P_{1f}]$ = molar concentration of free inhibitory hydrolysis product
$[EP_1]$ = molar concentration of enzyme-inhibitor complex

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

with the condition as before that $d[ES]/dt \doteq 0$ and that $[S] \doteq [S_f]$ and $[P_1] \doteq [P_{1f}]$ it follows[8-13,15,16] that

$$\frac{k_2 + k_3}{k_1} = \frac{([E] - [ES] - [EP_1])[S]}{[ES]} = K_S \quad (11)$$

$$\frac{k_5}{k_4} = \frac{([E] - [ES] - [EP_1])([S]_0 - [S])}{[EP_1]} = K_{P_1} \quad (12)$$

$$-\frac{d[S]}{dt} = \frac{k_3[E][S]}{K_S\left(1 + \frac{([S]_0 - [S])}{K_{P_1}}\right) + [S]} \quad (13)$$

and

$$k_3[E]t = 2.3K_S\left(1 + \frac{[S]_0}{K_{P_1}}\right)\log\frac{[S]_0}{[S]} + \left(1 - \frac{K_S}{K_{P_1}}\right)([S]_0 - [S]) \quad (14)$$

The constant K_{P_1} appearing in equation (14) was evaluated[14] by plots of $1/v_0$ *versus* $1/[S]_0$ taking advantage of equation (15)

$$\frac{1}{v} = \frac{K_S}{V}\left((1 + \frac{[P_1]}{K_{P_1}}\right)\frac{1}{[S]} + \frac{1}{V} \quad (15)$$

The plots given in Fig. 3 are representative of the data obtained when acetyl-L-tryptophanamide was used as a substrate and from these and similar

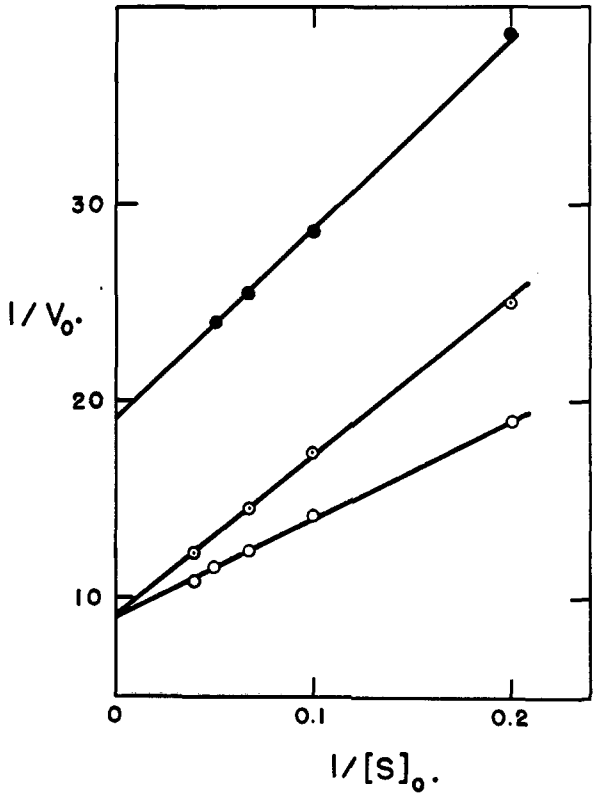

Fig. 3.—$[S]_0$ in mole × 10^{-3} per liter of acetyl-L-tryptophanamide, v_0 (initial velocities) in mole × 10^{-3} per liter per min.: ●, [E] = 0.104 mg. protein-nitrogen per ml.; O, [E] = 0.208 mg. protein-nitrogen per ml., ⊙, [E] = 0.208 mg. protein-nitrogen per ml. and [I] = 10 × 10^{-3} *M* added acetyl-L-tryptophan.

(15) I. D. Franz, Jr., and M. Stephensen, *J. Biol. Chem.*, **169**, 359 (1947).

(16) K. Harmon and C. Niemann, *ibid.*, **178**, 743 (1949).

data it was found that the inhibition by acetyl-L-tryptophan was competitive in nature and that for the above system $K_S = 5.3 \pm 0.2 \times 10^{-3}$ molar and $K_{P_1} = 17.5 \pm 1.5 \times 10^{-3}$ molar. With the preceding values for K_S and K_{P_1} a plot of $F(S) = 2.3\ K_S\ (1 + [S]_0/K_{P_1})\ \log\ ([S]_0/[S]) + (1 - K_S/K_{P_1})\ ([S]_0 - [S])$ *versus* time in minutes was made using the same data as were used for the plot given in Fig. 2 and it will be seen from Fig. 4 that the experimental values are in excellent agreement with those expected on the basis of equation (14). A similar treatment of the data obtained

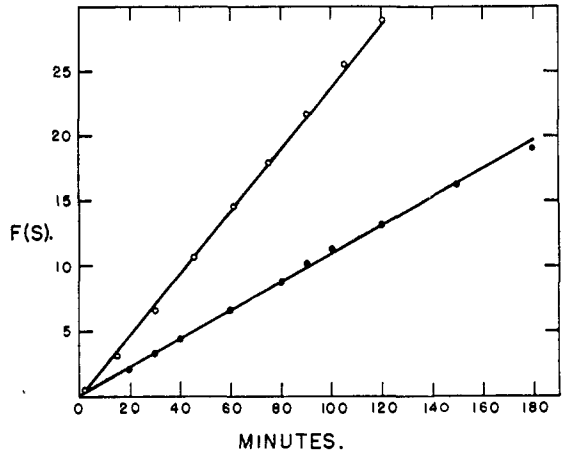

Fig. 4.—$F(S)$ in units of 10^{-3} *M*: ●, acetyl-L-tryptophanamide, $[S]_0 = 20 \times 10^{-3}$ *M*, [E] = 0.208 mg. protein-nitrogen per ml.; O, nicotinyl-L-tryptophanamide, $[S]_0 = 20 \times 10^{-3}$ *M*, [E] = 0.146 mg. protein nitrogen per ml.

when nicotinyl-L-tryptophanamide was used as a substrate (*cf.* Figs 5 and 7) established the fact that nicotinyl-L-tryptophan is a competitive inhibitor in the α-chymotrypsin catalyzed hydrolysis of nicotinyl-L-tryptophanamide under the conditions specified, that for this system $K_S = 2.7 \pm 0.2 \times 10^{-3}$ molar and $K_{P_1} = 8.8 \pm 1.0 \times 10^{-3}$ molar and that with these latter values equation (14) describes the course of the reaction within the limits of experimental error (*cf.* Fig. 4). From the data given in Fig. 4, with the enzyme concentration expressed in terms of mg. of protein-nitrogen per ml. of reaction mixture, k_3 for acetyl-L-tryptophanamide was estimated to be 0.50×10^{-3} mole/liter/min./mg. protein-nitrogen/ml. and for nicotinyl-L-tryptophanamide 1.60×10^{-3} mole/liter/min./mg. protein-nitrogen/ml. The fact that consistent values of k_3 were obtained with two different enzyme concentrations in the case of acetyl-L-tryptophanamide and three different enzyme concentrations in the case of nicotinyl-L-tryptophanamide appears to establish the point that the rates of hydrolysis of these two substrates are directly proportional to the enzyme concentration as would be expected from equation (14).

While it is true that within the limits of experimental error equation (14) is a satisfactory rate equation for the α-chymotrypsin catalyzed hydrolysis of both acetyl- and nicotinyl-L-tryptophanamide at 25° and *p*H 7.9 it must be remembered that in the derivation of equation (14) in addition to the assumption that $d[ES]/dt \doteq 0$ it was also

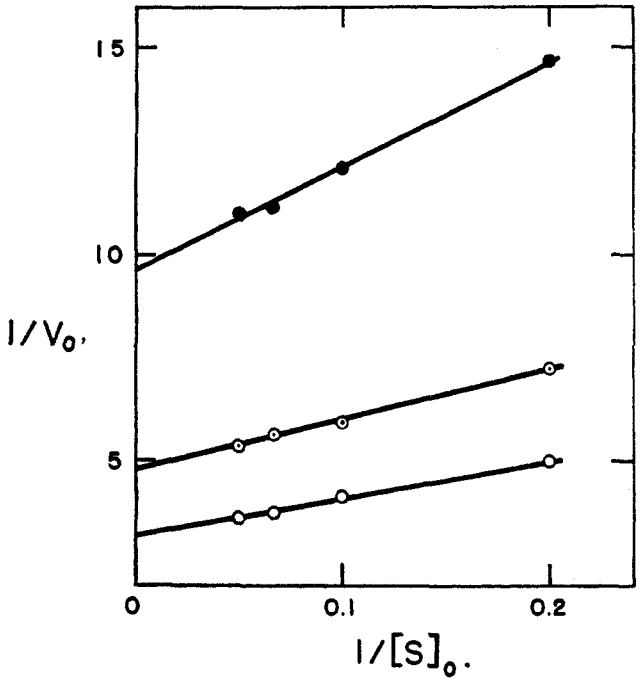

Fig. 5.—$[S]_0$ in mole $\times 10^{-3}$ per liter of nicotinyl-L-tryptophanamide, v_0 (initial velocities) in mole $\times 10^{-3}$ per liter per min.: ●, [E] = 0.069 mg. protein-nitrogen per ml.; ⊙, [E] = 0.145 mg. protein-nitrogen per ml.; O, [E] = 0.208 mg. protein-nitrogen per ml.

assumed that $[S] \doteq [S_f] \gg [ES]$ and $[P_1] \doteq [P_{1f}] \gg [EP_1]$, *i.e.*, that the systems were, in the terminology of Straus and Goldstein,[11,12] in zone A. If it be assumed, with some justification, that α-chymotrypsin possesses an equivalent weight of 27,000[17,18] and that the upper boundary of zone A for our experiments corresponds to a specific enzyme concentration, $E'_S = [E]/K_S$, or $E'_{P_1} = [E]/K_{P_1}$, of 0.1 it can be shown that for a reaction system containing 0.208 mg. of enzyme protein-nitrogen per ml., the maximum enzyme concentration employed in the present study, the enzyme concentration is approximately 4.8×10^{-5} normal with respect to the reaction under investigation. With the aid of this latter value the following maximum values for E'_S and E'_{P_1} were obtained; acetyl-L-tryptophanamide, 0.91×10^{-2}; nicotinyl-L-tryptophanamide, 1.78×10^{-2}; acetyl-L-tryptophan, 0.27×10^{-2}; and nicotinyl-L-tryptophan, 0.53×10^{-2}. In experiments containing initially enzyme, substrate, and added inhibitor the net effect of the substrate and inhibitor would be to transfer the system as a whole further into zone A than would be expected on the basis of separate consideration of the above values for E'_S and E'_{P_1}. Therefore it can be concluded that the experimental conditions used in this investigation were such as to ensure the validity of the assumption previously made that $[S] \doteq [S_f] \gg [ES]$ and $[P_1] \doteq [P_{1f}] \gg [EP_1]$.

The observation that the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosylglycinamide is inhibited by acetyl-D-tyrosylglycinamide[19] led us to investigate the systems: α-chymotrypsin; acetyl-L-tryptophanamide; acetyl-D-tryptophanamide and α-chymotrypsin; nicotinyl-L-tryptophanamide; nicotinyl-D-tryptophanamide. On the basis of present knowledge it is reasonable to postulate that for these systems

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

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftarrows}} EP_1 \qquad (10)$$

$$E_f + I_f \underset{k_7}{\overset{k_6}{\rightleftarrows}} EI \qquad (16)$$

With [I] = molar concentration of added inhibitor, in this case the D-antipode of the substrate S, [EI] = molar concentration of the enzyme-added inhibitor complex, $d[ES]/dt \doteq 0$, $[S_f] \doteq [S]$, $[P_{1f}] \doteq [P_1]$ and $[I_f] \doteq [I]$ it follows that

$$\frac{k_2 + k_3}{k_1} = \frac{([E] - [ES] - [EP_1] - [EI])[S]}{[ES]} = K_S \qquad (17)$$

$$\frac{k_5}{k_4} = \frac{([E] - [ES] - [EP_1] - [EI])([S]_0 - [S])}{[EP_1]} = K_{P_1} \qquad (18)$$

$$\frac{k_6}{k_7} = \frac{([E] - [ES] - [EP_1] - [EI])[I]}{[EI]} = K_I \qquad (19)$$

$$-\frac{d[S]}{dt} = \frac{k_3[E][S]}{K_S\left(1 + \frac{[I]}{K_I} + \frac{([S]_0 - [S])}{K_{P_1}}\right) + [S]} \qquad (20)$$

and

$$k_3[E]t = 2.3K_S\left(1 + \frac{[S]_0}{K_{P_1}} + \frac{[I]}{K_I}\right)\log\frac{[S]_0}{[S]} + \left(1 - \frac{K_S}{K_{P_1}}\right)([S]_0 - [S]) \qquad (21)$$

In the absence of inhibition by one of the hydrolysis products, *i.e.*, in this case for initial rates, equations (20) and (21) are reduced to

$$-\frac{d[S]}{dt} = \frac{k_3[E][S]}{K_S\left(1 + \frac{[I]}{K_I}\right) + [S]} \qquad (22)$$

and

$$k_3[E]t = 2.3K_S\left(1 + \frac{[I]}{K_I}\right)\log\frac{[S]_0}{[S]} + ([S]_0 - [S]) \qquad (23)$$

Values of K_I for both acetyl- and nicotinyl-D-tryptophanamide were obtained in the usual manner[14] and it is clear from Figs. 6 and 7 that in both cases the inhibition is competitive in nature. Thus at 25° and *p*H 7.9 for acetyl-D-tryptophanamide $K_I = 2.7 \pm 0.2 \times 10^{-3}$ molar and for nicotinyl-D-tryptophanamide $K_I = 1.4 \pm 0.1 \times 10^{-3}$ molar. The validity of the assumption that for the experiments described in this communication $[I_f] \doteq [I]$ was established when it was shown that the maximum value of E_f used in the evaluation of K_I for acetyl-D-tryptophanamide was 1.78×10^{-2}, and in the evaluation of K_I for nicotinyl-D- tryptophanamide, 3.43×10^{-2}. The suitability of equation (21) as a rate expression

(17) E. Jansen, M. D. Fellows-Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949).

(18) E. Jansen, M. D. Fellow-Nutting and A. K. Balls, *ibid.*, **179**, 201 (1949).

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

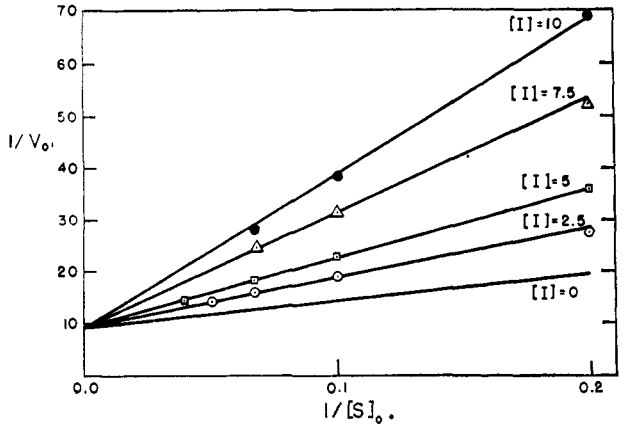

Fig. 6.—$[S]_0$ in mole × 10^{-3} per liter of acetyl-L-tryptophanamide, v_0 (initial velocities) in mole × 10^{-3} per liter per min., inhibition by acetyl-D-tryptophanamide, [I] in mole × 10^{-3} per liter, [E] = 0.208 mg. protein-nitrogen per ml.

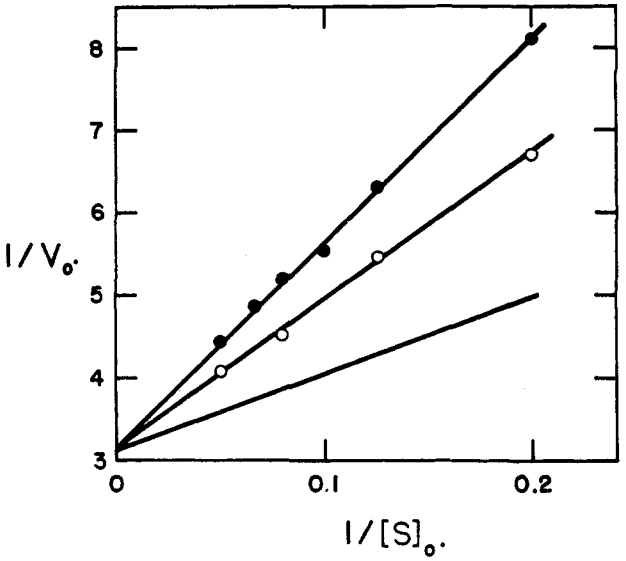

Fig. 7.—$[S]_0$ in mole × 10^{-3} per liter of nicotinyl-L-tryptophanamide, v_0 (initial velocities) in mole × 10^{-3} per liter per min., [E] = 0.208 mg. protein-nitrogen per ml., base line no added inhibitor: O, nicotinyl-L-tryptophan, [I] = 9.7 × 10^{-3} *M*; ●, nicotinyl-D-tryptophanamide, [I] = 2.5 × 10^{-3} *M*.

for the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tryptophanamide in the presence of acetyl-D-tryptophanamide, and of nicotinyl-L-tryptophanamide in the presence of nicotinyl-D-tryptophanamide is apparent from the data given in Fig. 8. When the same data were evaluated on the basis of equation (23) a negative deviation was observed after 40% hydrolysis of either of the above substrates.

On the basis of information now available it is apparent that the α-chymotrypsin catalyzed hydrolysis of both acetyl- and nicotinyl-L-tryptophanamide can be described in terms of classical Michaelis–Menten kinetics. It is evident from equations (8), (14) and (21) that one effect associated with the generation of a competitive inhibitor during the course of the reaction is the enhancement of the importance of the first order term of the rate expression at the expense of the zero order term. The importance of the first order term is still further increased by the initial addition of the same or a second competitive inhibitor to the system. Thus with competitive inhibition by a hydrolysis product, or by the D-antipode of the substrate, one may observe apparent congruity to a first order rate expression throughout the course of the reaction[3,20–22] though it is probable that more extended observations would reveal the biphasic character of the reaction.

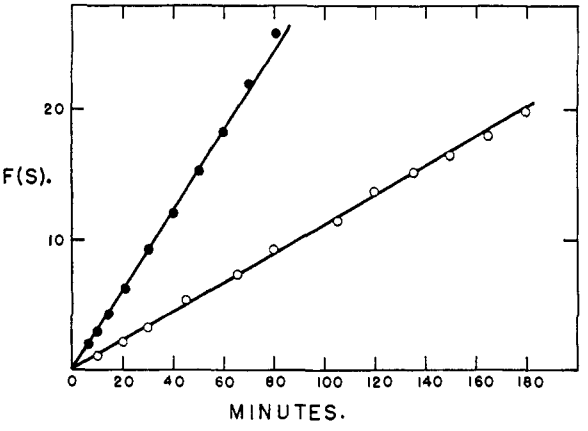

Fig. 8.—$F(S)$ in units of 10^{-3} *M*; O, acetyl-L-tryptophanamide, $[S]_0 = 20 \times 10^{-3}$ *M*, acetyl-D-tryptophanamide, [I] = 2.5 × 10^{-3} *M*, [E] = 0.208 mg. protein-nitrogen per ml.; ●, nicotinyl-L-tryptophanamide, $[S]_0$ = 20 × 10^{-3} *M*, nicotinyl-D-tryptophanamide, [I] = 2.5 × 10^{-3} *M*, [E] = 0.208 mg. protein-nitrogen per ml.

The phenomenon of competitive inhibition of the hydrolysis of typical L-substrates by their D-antipodes raises the question of the significance of apparent K_S values (K_S') evaluated from data on the hydrolysis of DL-mixtures on the tacit assumption that the D-antipode is without effect upon the reacting system.[3,22] K_S' is obviously a function of K_S for the L-substrate and K_I for the D-inhibitor. For a typical plot of $1/v_0$ *versus* $1/[S]_0$ for a DL-mixture

$$\frac{1}{v} = \frac{K_S}{V}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V} \qquad (24)$$

and since [I] = [S]

$$\frac{1}{v} = \frac{K_S}{V} \times \frac{1}{[S]} + \left(1 + \frac{K_S}{K_I}\right)\frac{1}{V} \qquad (25)$$

Comparing equations (9), (15) and (25) it is seen that the over-all effect of using a DL-mixture is to increase the true value of $1/V$, *i.e.*, the intercept, by an amount equal to $K_S/K_I \times 1/V$ without affecting the slope of the plot. Thus

$$K_S' = K_S/V \times \frac{V}{(1 + K_S/K_I)} = \frac{K_S \times K_I}{K_S + K_I} \qquad (26)$$

If the respective K_S values for two substrates S_1 and S_2 are in inverse order of their respective k_3 values it follows[3,23,24] that the corresponding

(20) S. Kaufman, H. Neurath and G. S. Schwert, *J. Biol. Chem.*, **177**, 793 (1949).
(21) G. S. Schwert and S. Kaufman, *ibid.*, **180**, 517 (1949).
(22) S. Kaufman and H. Neurath, *Arch. Biochem.*, **21**, 437 (1949).
(23) J. E. Snoke and H. Neurath, *ibid.*, **21**, 351 (1949).
(24) J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **181**, 789 (1949).

values for k_2/k_1 will be greater for S_1 than for S_2. Therefore, on the basis of the summarized kinetic constants given in Table I it can be concluded that

TABLE I

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

Compound	$K \times 10^3$ molar[a] Value	Constant	$k_3 \times 10^{3a,b}$
Acetyl-L-tryptophanamide	5.3 ± 0.2	K_S	0.50
Acetyl-L-tryptophan	17.5 ± 1.5	K_{P_1}	
Acetyl-D-tryptophanamide	2.7 ± 0.2	K_I	
Nicotinyl-L-tryptophanamide	2.7 ± 0.2	K_S	1.60
Nicotinyl-L-tryptophan	8.8 ± 1.0	K_{P_1}	
Nicotinyl-D-tryptophanamide	1.4 ± 0.1	K_I	

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

the affinity of α-chymotrypsin for nicotinyl-L-tryptophanamide is greater than that for acetyl-L-tryptophanamide. In contrast to K_S, K_{P_1} and K_I, when properly determined[11,12] are true equilibrium constants and quantitative comparisons can be made. Thus for acetyl-L-tryptophan and nicotinyl-L-tryptophan where the ratio of the respective K_{P_1} values = 17.5/8.8 = 2.0, and for acetyl-D-tryptophanamide and nicotinyl-D-tryptophanamide where the ratio of the respective K_I values = 2.7/1.4 = 1.9 it appears that replacement of an acetyl group by a nicotinyl group results in an increase in the affinity constant, *i.e.*, $1/K_{P_1}$ or $1/K_I$, of enzyme and inhibitor by a factor of approximately 2.0. The fact that for acetyl-L-tryptophanamide and nicotinyl-L-tryptophanamide the ratio of the respective K_S values = 5.3/2.7 = 2.0, *i.e.*, the same value noted above for the two sets of inhibitors, suggests, but most certainly does not prove, that in this instance the values of k_3 may be very much smaller than those of k_2 and that $K_S \doteq k_2/k_1$. It should be noted that the above interpretation of K_S is at variance with the interpretation given by Kaufman and Neurath[3,25] for the case of acetyl-L-tyrosinamide. From the observation that $1/K_S$ decreases linearly with increasing methanol concentration while k_3 remains invariant these authors conclude that k_3 cannot be of the same order of magnitude as k_2 and that $K_S \doteq k_3/k_1$. An examination of their data reveals, however, that their findings can be explained equally well by the alternative interpretation that $K_S \doteq k_2/k_1$, *i. e.*, $k_3 \ll k_2$ since shifts in the equilibrium position of a reversible reaction with changes in the nature of the solvent are relatively common.[26] The suggestion that the values of k_3 are small in the case of the α-chymotrypsin catalyzed hydrolysis of both acetyl and nicotinyl-L-tryptophanamide derives some support from a consideration of the so-called turnover numbers of these reactions. If it be assumed that the enzyme is saturated with respect to substrate, has but one reactive site per molecule,[17,18] possesses a molecular weight of 27,000[17,18] and contains 16% of nitrogen, it can be shown that for acetyl-L-tryptophanamide the turnover number is approximately 2 molecules/enzyme molecule/min. and for nicotinyl-L-tryptophanamide 7 molecules/enzyme molecule/min. These latter values are considerably smaller than those for the turnover numbers of fifteen representative enzyme–substrate systems listed by McIlvain[27] where the values varied from approximately 8×10^2 to 26×10^5 molecules/enzyme molecule/min.

It will be seen from the data given in Table I that the affinity of α-chymotrypsin is greater for both acetyl- and nicotinyl-D-tryptophanamide than for the corresponding acylated L-acids. Although there is an indication that the affinity of α-chymotrypsin for the L-substrates is less than for the D-antipodes of these substrates it is clear that additional evidence will have to be provided before this point can be regarded as being established. Experiments directed toward this end are now in progress.

TABLE II

SUMMARY OF EXPERIMENTS USED FOR THE EVALUATION OF KINETIC CONSTANTS

S; I	[E][a]	No. of experiments at $[S]_0 = 10^{-3}$ molar × 5	8	10	12.5	15	20	25
II	0.208	3		3		3	2	1
II	.104	1		1		1	1	1
II; VII[b]	.208	2		2		2	1	1
II; VI[c]	.208	2		2		2	2	
II; VI[d]	.208	1		1		1		1
II; VI[e]	.208	1		1		1		
II; VI[f]	.208	1		1		1		
VIII	.208	3		3		3	3	
VIII	.145	2		2		2	2	
VIII	.069	2		2		2	2	
VIII; XI[g]	.208	1		1		1	1	
VIII; X[h]	.208	1	1	1	1	2	2	

[a] Mg. protein-nitrogen per ml. reaction mixture. [b] $[I]_0 = [P_1]_0 = 10 \times 10^{-3}$ molar. [c] $[I]_0 = 2.5 \times 10^{-3}$ molar. [d] $[I]_0 = 5 \times 10^{-3}$ molar. [e] $[I]_0 = 7.5 \times 10^{-3}$ molar. [f] $[I]_0 = 10 \times 10^{-3}$ molar. [g] $[I]_0 = [P_1]_0 = 9.7 \times 10^{-3}$ molar. [h] $[I]_0 = 2.5 \times 10^{-3}$ molar.

Experimental[28,29]

Acetyl-L-tryptophan Methyl Ester (I).—Acetylation of 10 g. of L-tryptophan methyl ester hydrochloride with 5 ml. of acetic anhydride in the presence of aqueous potassium carbonate gave 9.6 g. of I, small dense prisms, m.p. 152.5°, after recrystallization from ethyl acetate; $[\alpha]^{23}D$ +11.5 ± 0.5° (*c*, 2% in methanol).

Anal. Calcd. for $C_{14}H_{16}O_3N_2$ (260): C, 64.6; H, 6.2; N, 10.8. Found: C, 64.6; H, 6.1; N, 10.8.

Acetyl-L-tryptophanamide (II).—Ammonolysis of 4 g. of I gave 2.7 g. of II, clusters of fine thin needles, m.p. 192–193°, after recrystallization from a mixture of methanol and ether; $[\alpha]^{23}D$ +20 ± 1° (*c*, 2% in methanol).

Anal. Calcd. for $C_{13}H_{15}O_2N_3$ (245): C, 63.7; H, 6.2; N, 17.2. Found: C, 64.0; H, 6.0; N, 17.1.

Acetyl-DL-tryptophan Methyl Ester (III).—Acetylation of 15 g. of DL-tryptophan methyl ester hydrochloride as described for I gave 15 g. of III, small dense prisms, m.p. 153.5°, after recrystallization from ethyl acetate.

Anal. Calcd. for $C_{14}H_{16}O_3N_2$ (260): C, 64.6; H, 6.2; N, 10.8. Found: C, 64.5; H, 6.2; N, 10.7.

Acetyl-DL-tryptophanamide (IV).—Ammonolysis of 3 g. of III gave 2 g. of IV, fluffy needles, m.p. 219°, after recrystallization from water.

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

(26) G. W. Wheland, "Advanced Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1949.

(27) H. McIlvain, *Nature*, **158**, 898 (1946).

(28) All melting point values are corrected.

(29) The authors are indebted to Dr. A. Elek for all the microanalyses reported in this communication.

Anal. Calcd. for $C_{13}H_{15}O_2N_3$ (245): C, 63.7; H, 6.2; N, 17.2. Found: C, 63.7; H, 6.2; N, 17.1.

Acetyl-D-tryptophan Methyl Ester (V).[30]—To the sirupy ester obtained from 15 g. of DL-tryptophan methyl ester hydrochloride was added 100 mg. of α-chymotrypsin in 1.5 ml. of water, the clear sirup incubated at 28° for 40 hours, the solid mass triturated with ethyl acetate, the solid phase collected to give 5.6 g. of crude L-tryptophan, the filtrate evaporated to a thick sirup and acetylated in the usual manner to give 5.4 g. of V, small dense prisms, m.p. 152.5° after two recrystallizations from ethyl acetate; $[\alpha]^{23}D$ −12.0 ± 0.5° (*c*, 2% in methanol).

Anal. Calcd. for $C_{14}H_{16}O_3N_2$ (260): C, 64.6; H, 6.2; N, 10.8. Found: C, 64.5; H, 6.2; N, 10.7.

Acetyl-D-tryptophanamide (VI).—Ammonolysis of 2 g. of V gave 1.5 g. of VI, clusters of fine needles, m.p. 192–193°, after recrystallization from aqueous methanol; $[\alpha]^{25}D$ −19 ± 1° (*c*, 2% in methanol).

Anal. Calcd. for $C_{13}H_{15}O_2N_3$ (245): C, 63.7; H, 6.2; N, 17.2. Found: C, 63.6; H, 6.2; N, 17.1.

Acetyl-L-tryptophan (VII).—To a suspension of 10 g. of III in 500 ml. of 40% aqueous methanol, contained in a beaker thermostated at 30° and fitted with a stirrer and electrodes, was added 50 mg. of α-chymotrypsin and the *p*H of the reaction system maintained at *p*H 7.8 by the addition of 1 *N* aqueous sodium hydroxide. Although the reaction appeared to be complete after 3 hours the mixture was stirred for an additional 3 hours and then left at 25° overnight. The precipitate was collected to give 5 g. of crude V, the filtrate evaporated *in vacuo* to a volume of 200 ml., the solution filtered and the filtrate acidified to give an oily precipitate. The latter product was induced to crystallize by trituration with water and the crude VII recrystallized once from ethyl acetate and then three times from aqueous methanol to give 3.0 g. of VII, fine needles, m.p. 180–181°; $[\alpha]^{25}D$ +30 ± 1° (*c*, 1.23% in water containing an equivalent amount of sodium hydroxide).

Anal. Calcd. for $C_{13}H_{14}O_3N_2$ (246): C, 63.4; H, 5.7; N, 11.4. Found: C, 63.5; H, 5.6; N, 11.3.

Sealock and du Vigneaud[31] gave the following constants for VII, m.p. 189–190°: $[\alpha]^{31}D$ +29° (*c*, 1% in aqueous sodium hydroxide).

Nicotinyl-L-tryptophanamide (VIII).—VIII, soft long needles, m.p. 180–181°, was prepared as previously described.[32] When a concentrated aqueous solution of VIII was quickly cooled, VIII crystallized in clusters of short thick needles, m.p. 194–195°. This latter product when recrystallized from dilute aqueous solutions gave VIII, soft long needles, m.p. 180–181°; $[\alpha]^{24}D$ −34.5 ± 1° (*c*, 2% in methanol).

Nicotinyl-DL-tryptophanamide (IX).—Ammonolysis of 3.8 g. of sirupy nicotinyl-DL-tryptophan methyl ester, prepared exactly as described for the L-compound,[32] gave 2.0 g. of IX, fine short needles, m.p. 209–210°, after two recrystallizations from methanol.

Anal. Calcd. for $C_{17}H_{16}O_2N_4$ (308): C, 66.2; H, 5.2; N, 18.2. Found: C, 66.2; H, 5.3; N, 18.1.

Nicotinyl-D-tryptophanamide (X).—To a solution of 620 mg. of IX in 300 ml. of water and 5 ml. of 0.5 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer of *p*H 8.0 was added 50 mg. of α-chymotrypsin and the reaction mixture incubated at 25° for 2 days. The solution was then evaporated at 25°, to a volume of 50 ml., the oil which had separated crystallized by vigorous rubbing, and the crude X recrystallized from methanol to give 240 mg. of X, fine needles, m.p. 194–195°; $[\alpha]^{25}D$ +34.5 ± 1° (*c*, 2% in methanol).

Anal. Calcd. for $C_{17}H_{16}O_2N_4$ (308): C, 66.2; H, 5.2; N, 18.2. Found: C, 66.3; H, 5.3; N, 18.1.

Nicotinyl-L-tryptophan (XI).—To a solution of 0.5 g. of VIII in 150 ml. of water and 5 ml. of 0.5 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer of *p*H 8.0 was added 20 mg. of α-chymotrypsin and the reaction mixture allowed to stand at 25° for 24 hours. The clear solution was evaporated, at 25°, to a volume of 125 ml., the solution acidified with dilute hydrochloric acid, the gelatinous precipitate recovered and reprecipitated after solution in aqueous sodium hydroxide. The gelatinous precipitate so obtained was dissolved in ethyl acetate, the solution dried over anhydrous calcium sulfate, and the solvent evaporated *in vacuo* to give an apparently amorphous powder, m.p. 167–169°. Attempts to recrystallize this latter product from methanol, ethyl acetate or water gave only gelatinous precipitates. The preparation of XI, m.p. 167–169°, used in the enzyme studies was twice precipitated from a hot ether solution. Thus 200 mg. of a fine powder was obtained; $[\alpha]^{25}D$ −7.5 ± 0.5° (*c*, 0.6% in water containing an equivalent amount of sodium hydroxide).

Anal. Calcd. for $C_{17}H_{15}O_3N_3$ (309): C, 66.0; H, 4.9; N, 13.6. Found: C, 65.9; H, 4.8; N, 13.4.

Buffer Solutions.—Technical tris-(hydroxymethyl)-aminomethane (Commercial Solvents) was recrystallized twice from aqueous methanol to give a product, colorless thick rods, m.p. 168–169°. A stock solution 0.2 *M* with respect to the amine component was prepared by the addition of sufficient 1.0 *N* hydrochloric acid to an aqueous solution of the amine to give a solution of *p*H 8.05 at 25°, after the stock solution was made up to volume. This stock solution was used in all of the studies conducted at *p*H 7.9 since it was found that in the presence of enzyme, substrate and inhibitor, a 1:10 dilution of the above stock solution gave a reaction mixture of *p*H 7.90 ± 0.02 at 25°. Other stock solutions 0.2 *M* with respect to the amine component were prepared for studies at *p*H values other than 7.9, *e.g.*, in the determination of the *p*H-activity curves, and in these cases an allowance was made for a decrease in *p*H upon a 1:10 dilution of 0.15 *p*H units for final *p*H values between 7.5 and 8.4, and a decrease of 0.2 to 0.3 *p*H unit for final *p*H values > 8.4 but < 9.2 and < 7.5 but > 6.8.

Aqueous Formaldehyde.—Merck and Co., Inc., reagent grade formaldehyde, 36–38%, was kept in contact with magnesium carbonate and the solution, *p*H 8.0, filtered for use each day.

Enzyme Solutions.—Crystalline α-chymotrypsin (Armour, Lot No. 90402), containing magnesium sulfate, was used in all experiments reported in this communication. Enzyme stock solutions were prepared daily and were kept at 4° except for 4 periods during the 24-hour interval when they were brought to 25° to permit the withdrawal of a 1.0-ml. aliquot. Independent experiments based upon the use of nicotinyl-L-tryptophanamide as a substrate established the fact that the activity of the enzyme solution maintained at 25° remained substantially constant during a 24-hour period. The protein nitrogen content of the enzyme solutions was determined by the Kjeldahl method after precipitation of the protein with trichloroacetic acid.

Procedure.—The substrate, or the substrate and inhibitor, was dissolved, with the aid of heat if necessary, in 5 to 7 ml. of water contained in a 10-ml. G. S. volumetric flask; 1.0 ml. of the appropriate 0.2 *M* buffer solution was then added and the clear solution placed in a 25.0 ± 0.1° bath for 20–25 minutes.[33] At minus 20 seconds from zero time the volumetric flask was withdrawn from the bath, 1.0 ml. of enzyme solution added, and the solution made up to volume. At zero time the flask was stoppered, gently inverted 10–12 times to ensure adequate mixing, returned to the bath and 1.0-ml. aliquots withdrawn at convenient intervals and delivered into a 10-ml. shell vial containing 1.0 ml. of formaldehyde solution. The solutions were titrated as described previously.[4] In general the time consumed in the withdrawal of an aliquot and the attendant titration did not permit the withdrawal of subsequent aliquots at intervals of less than 5 minutes. For each titration a titration curve was constructed from 3 to 8 points in order to determine accurately the extent of hydrolysis.[4] It should be noted that in none of the experiments herein reported did the total reaction time exceed 3 hours and in no case was a significant change in the *p*H of the reaction system observed. Blanks for the autolysis of the enzyme at *p*H 7.9 and 25° were performed and at enzyme concentrations of < 0.208 mg. of protein-nitrogen per ml. of reaction mixture were found to be negligible. At enzyme concentrations of 0.208 mg. of protein-nitrogen per ml. of reaction mixture

(30) M. Brenner, E. Sailer and V. Kocher, *Helv. Chim. Acta*, **31**, 1909 (1948).

(31) R. Sealock and V. du Vigneaud, *J. Biol. Chem.*, **96**, 511 (1932).

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

(33) With nicotinyl-L-tryptophanamide at an initial substrate concentration of 20×10^{-3} *M* the solution occasionally was cloudy prior to the addition of the enzyme solution and final dilution but was invariably clear after the latter operation.

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 $[S]_0 = 5 \times 10^{-3}$ molar and to within ±3% at $[S]_0 = 20 \times 10^{-3}$ 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.

PASADENA, CALIFORNIA RECEIVED JULY 28, 1950

[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

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

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

and that the rate equation for the above reactions is

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

provided conditions are selected so that $d[ES]/dt \doteq 0$, $[S_f] \doteq [S] \gg [ES]$, and $[P_{1f}] \doteq [P_1] \gg [EP_1]$.[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 $K_S = 30.5 \pm 1.0 \times 10^{-3}$ molar.

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

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

and provided $d[ES]/dt \doteq 0$ and $[S_f] \doteq [S] \gg [ES]$, 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).