for the smaller metachromatic effect of the calcium chondroitin sulfate as compared with the potassium salt. Neutral salts at high concentrations produce an increase in values of R either in the presence or absence of chondroitin sulfate. This is the effect Michaelis and Granick referred to as molecular aggregation when the salting out point is approached.

The locations of the minima in the extinctions at 665 and 610 mμ of Figs. 2 and 6 suggest that there is a loose binding of dye cations and chromotrope polyanions. Adsorption of dye ions to colloidal surfaces may cause a change in their color. Fajans and Hassel[17] showed this with anionic fluoresceins adsorbed to silver halides. Scheibe[14] showed that a shift in absorption maxima of some polymethine dyes occurs on adsorption to surfaces such as mica. Since chondroitin sulfate is polyanionic, it may be expected to have some of the properties of the water soluble synthetic linear polycations described by Fuoss and Strauss.[18] In particular its solutions are likely to have micro regions of high and low anionic density. Regions of high anionic density will tend to fix dye cations. If the dye is one whose energy levels are easily influenced by external electrical fields, then energy level differences and absorption band positions will be likely to change. Addition of neutral salts to a concentration of 10^{-3} M would supply competing cations that could displace most of the dye cations (10^{-5} M) and metachromasy would disappear. Increasing chondroitin sulfate concentration would tend to level out the anionic density throughout the solution and at the same time increase the average cation density and again metachromasy would disappear. Why dyes which show Beer's law deviations also show metachromasy cannot be accounted for except to suggest that in these dyes energy levels are more sensitive to external electrical fields. That the metachromatic effect is quite different from the Beer's law deviation effect has been sufficiently emphasized.

There is left for discussion the difference between the amorphous potassium chondroitin sulfate and the corresponding salt made from crystalline calcium chondroitin sulfate. The difference appears at concentrations of chondroitin sulfate above 10^{-4} period per liter. It might indicate the presence of a chromotropic component in the amorphous potassium salt that has been removed in the further purification leading to the crystallization of the calcium salt. Work is in progress dealing with this point.

The results of this study of metachromasy in dilute aqueous solutions of a purified chromotrope are not directly applicable to the interpretation of results of histological metachromatic staining. The latter is carried out on heterogeneous systems and is subject to far more complex influences.

(17) K. Fajans and O. Hassel, *Z. Elektrochem.*, **29**, 495 (1923).
(18) R. M. Fuoss and U. P. Strauss, *J. Polymer Sci.*, **3**, 246 (1948).

NEW YORK 116, N. Y. RECEIVED JUNE 26, 1951

[CONTRIBUTION NO. 1557 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin-Catalyzed Hydrolysis of Chloroacetyl- and Trifluoroacetyl-L-tyrosinamide in Aqueous Solutions at 25°[1]

BY HENRY J. SHINE AND CARL NIEMANN[2]

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of chloroacetyl- and trifluoroacetyl-L-tyrosinamide have been determined in aqueous solutions at 25° and at the optimum *p*H for each substrate; *i.e.*, between 7.7 and 7.8 for chloroacetyl-L-tyrosinamide, and between 7.8 and 7.9 for trifluoroacetyl-L-tyrosinamide. The kinetic constants so obtained appear to support the proposition that the principal forces involved in the combination of the enzyme with specific substrates and competitive inhibitors are van der Waals forces rather than intermolecular hydrogen bonds.

Results of investigations on the nature of the α-chymotrypsin-catalyzed hydrolysis of amino acid derivatives have led to the hypothesis[3,4] that an α-amino acid derivative of the general formula $R_1CHR_2R_3$ which can combine with the enzyme at its catalytically active site does so *via* combination with three centers which are complementary to the three prominent structural features of the attached molecule, *viz.*, R_3, the functional derivative of the carboxyl group; R_2, the α-amino acid side chain; and R_1, the remaining substituent of the α-carbon atom. It is fortunate that the nature of the group R_1 can be varied over rather wide limits without causing a critical loss of those properties which are characteristic of experimentally useful specific substrates. Thus, with an appropriate choice in respect to the nature of R_2 and R_3, an examination of the effect of variation in the character of R_1, where R_2 and R_3 remain invariant within a given series, might be expected to yield information relative to the nature of the forces involved in the combination of the enzyme with the various specific substrates and, possibly, to the mechanism of the subsequent hydrolytic process.

The immediate objective of the present investigation was to determine the effects on the course of the reaction due to the presence of electron-attracting groups in an amide type R_1 substituent. The specific substrates selected for study were chloroacetyl-L-tyrosinamide and trifluoroacetyl-L-tyrosinamide. The *p*H-activity relationship was determined for each of these specific substrates, and the optimum *p*H for the hydrolysis of chloroacetyl-L-tyrosinamide was found to be between 7.7 and 7.8, and for trifluoroacetyl-L-

(1) Supported in part by a grant from Eli Lilly and Company.
(2) To whom inquiries regarding this article should be sent.
(3) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).
(4) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

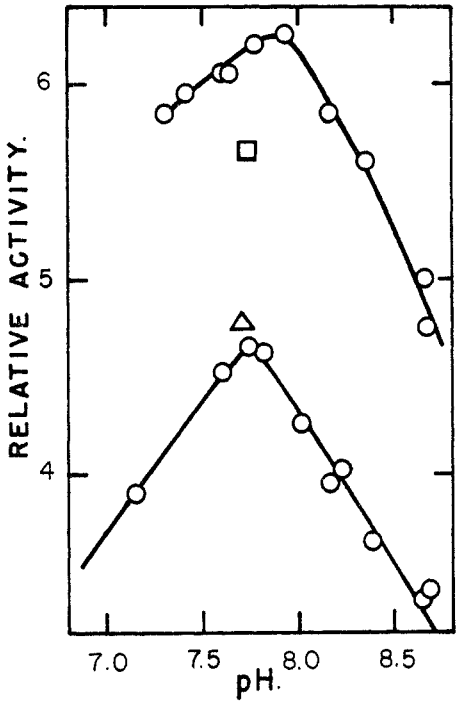

Fig. 1.—*p*H-activity relationships: upper curve, trifluoroacetyl-L-tyrosinamide, $[S]_0 = 15 \times 10^{-3}$ *M*, [E] = 0.208 mg. protein-nitrogen per ml., 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer; lower curve, chloroacetyl-L-tyrosinamide, $[S]_0 = 15 \times 10^{-3}$ *M*, [E] = 0.0677 mg. protein-nitrogen per ml., 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. Relative activity is expressed in [S] $\times 10^{-3}$ *M* hydrolyzed in 40 min.; Δ and □ refer to the lower curve and relate, respectively, to experiments in which the solutions were 0.00947 and 0.0947 *M* in added magnesium sulfate.

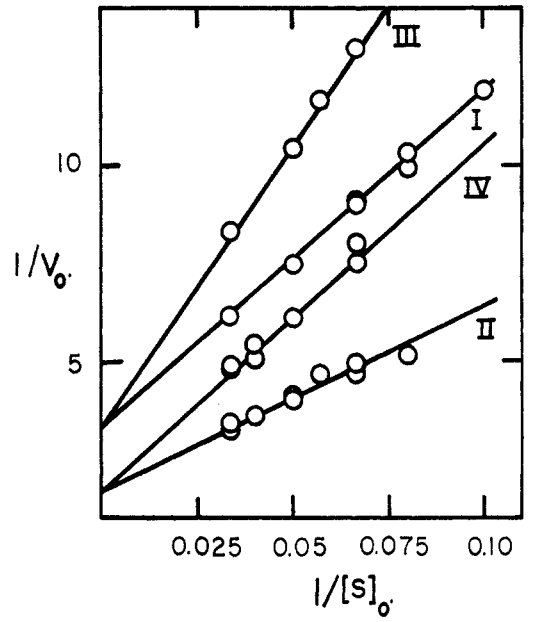

Fig. 2.—Determination of K_s for chloroacetyl-L-tyrosinamide and of K_{P_1} for chloroacetyl-L-tyrosine: v_0 in units of 10^{-3} *M* per min.; $[S]_0$ in units of 10^{-3} *M* of chloroacetyl-L-tyrosinamide; buffer, 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid. I and III, [E] = 0.0677 mg. protein-nitrogen per ml.; II and IV, [E] = 0.1354 mg. protein-nitrogen per ml.; III and IV, $[P_1] = 80 \times 10^{-3}$ *M* chloroacetyl-L-tyrosine.

tyrosinamide between 7.8 and 7.9, *cf.*, Fig. 1. The Michaelis constants and the rate constants for the two specific substrates, and the inhibition constants for the two acidic hydrolysis products were evaluated from plots of $1/[S]_0$ *vs.* $1/v_0$, *cf.* Figs. 2–4, the initial velocities, v_0, being obtained from the relation $v_0 = k[S]_0$, where k is the rate constant for the apparent first-order hydrolysis. As before[5–7] all measurements were made in aqueous solutions at 25° buffered at the appropriate *p*H with a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer 0.02 *M* with respect to the amine component. The kinetic constants obtained are given in Table I.

The enzyme-inhibitor dissociation constants, *i.e.*, the K_{P_1} values, for the three acylated-L-tyrosines, *cf.* Table I and Figs. 2 and 4, must be regarded as approximate values, correct only as to order of magnitude. The reasons for this are at least twofold, and are a consequence of the relatively high concentration of inhibitor needed to cause a measurable decrease in the rate of hydrolysis. First, the experimental errors are increased, particularly at the higher enzyme concentrations, as is indicated by the scatter of the experimental values given in Figs. 2 and 4. Furthermore at the higher enzyme concentrations a small change in the shallow slope of the $1/[S]_0$ *vs.* $1/v_0$ plot causes a large change in the value of K_{P_1}. Second, it appears that there is a positive salt effect upon the rate caused by the added inhibitor. The magnitude of this effect is uncertain. Jandorf[8] has reported that magnesium sulfate and other salts increase the so-called "esterase activity" of α-chymotrypsin. We have observed with chloroacetyl-L-tyrosinamide that there is a 2.4 and a 24% increase in the rate of hydrolysis when the magnesium sulfate concentration is increased

TABLE I

KINETIC CONSTANTS

Compound	$K \times 10^3$ *M*[a] Value	Constant	k_s $\times 10^{3a,b}$
Acetyl-L-tyrosinamide	30.5[c]	K_S	2.4[c]
Trifluoroacetyl-L-tyrosinamide	30.0	K_S	2.8
	30.2	K_S	2.9
Chloroacetyl-L-tyrosinamide	26.0[d]	K_S	4.3
	27.6[e]	K_S	4.2
Acetyl-L-tyrosine	115[c]	K_{P_1}	
Trifluoroacetyl-L-tyrosine	119	K_{P_1}	
Chloroacetyl-L-tyrosine	122	K_{P_1}	

[a] At 25° in aqueous solution at the *p*H optima for each substrate: *viz.*, 7.8–8.0 for acetyl-L-tyrosinamide, 7.8–7.9 for trifluoroacetyl-L-tyrosinamide, and 7.7–7.8 for chloroacetyl-L-tyrosinamide. [b] Moles/liter/min./mg. protein-nitrogen/ml. [c] Values reported by Thomas, MacAllister and Niemann.[5] [d] [E] = 0.0677 mg. protein nitrogen/ml. [e] [E] = 0.1354 mg. protein nitrogen/ml.

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

(6) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, *ibid.*, **73**, 3231 (1951).

(7) H. T. Huang and C. Niemann, *ibid.*, **73**, 1541 (1951).

(8) B. J. Jandorf, *Federation Proc.*, **9**, 186 (1950).

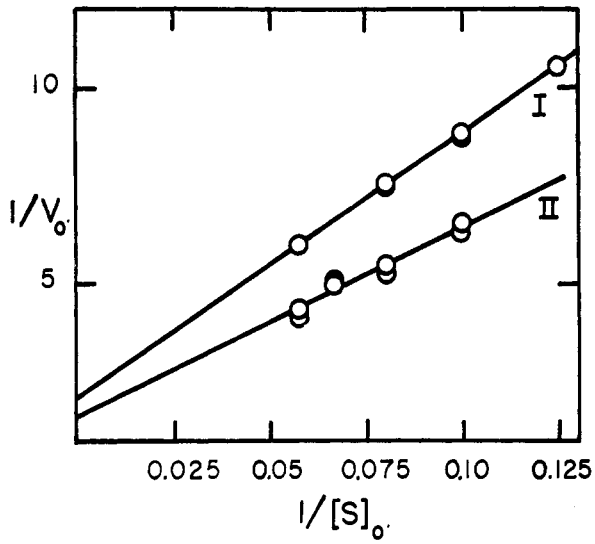

Fig. 3.—Determination of K_s for trifluoroacetyl-L-tyrosinamide: v_0 in units of 10^{-3} M per min.; $[S]_0$ in units of 10^{-3} M of trifluoroacetyl-L-tyrosinamide; buffer, 0.02 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid. I, [E] = 0.1560 mg. protein-nitrogen per ml.; II, [E] = 0.208 mg. protein-nitrogen per ml.

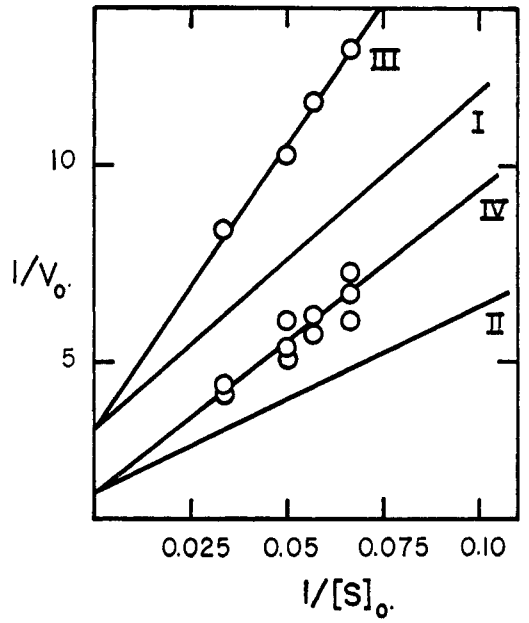

Fig. 4.—Inhibition of hydrolysis of chloroacetyl-L-tyrosinamide by trifluoroacetyl-L-tyrosine: v_0 in units of 10^{-3} M per min.; $[S]_0$ in units of 10^{-3} M of chloroacetyl-L-tyrosinamide; buffer, 0.02 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid. I and III, [E] = 0.0677 mg. protein-nitrogen per ml.; II and IV, [E] = 0.1354 mg. protein-nitrogen per ml.; III and IV, [I] = 80×10^{-3} M trifluoroacetyl-L-tyrosine.

to 10 and 100 times that normally present[7] in the enzyme solution, *cf.*, Fig. 1. The contributions of the added magnesium sulfate to the ionic strengths of the solutions were, respectively, 0.038 and 0.38. In the experiments reported in this communication the inhibitor concentration was 80 μM/ml., and since an equivalent amount of sodium hydroxide was added to the solutions the inhibitors may be regarded as being introduced as the sodium salts which are completely dissociated at the obtaining *p*H. Therefore, the contribution of the added inhibitors to the ionic strengths of the solutions is 0.08. Although the quantitative relationship between the changes in ionic strength and the changes in rate has not yet been determined it is clear that in addition to the anticipated *decrease in rate* associated with normal competitive action one must allow for an *increase in rate* when ionizable competitive inhibitors are used in high concentrations, *i.e.*, of the order of 0.1 M. In view of the fact that the K_{P_1} values reported in this communication were evaluated with inhibitor concentrations of 0.08 M it follows from the above argument that the effectiveness of the inhibitor in the competitive process is underestimated.

Although the value of K_{P_1} for chloroacetyl-L-tyrosine is only an approximate value, it is noteworthy that the use of this value in the integrated rate equation (1) gave an $F(S)$ *versus* t plot that was linear over the entire time interval, corresponding to approximately 80% hydrolysis, whereas a comparable plot based upon equation (2), wherein

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

$$k_3[E]t = 2.3K_S \log [S]_0/[S] + ([S]_0 - [S]) \quad (2)$$

inhibition by the acidic hydrolysis product was ignored, exhibited a negative deviation after approximately 50% hydrolysis. The k_3 values determined from all of the plots agreed within the probable limits of experimental error.

It has been shown previously[4-7] that it is reasonable to assume that K_S can be taken as a measure of the affinity of the enzyme for specific substrates which are acylated α-amino acid amides. If this assumption is admitted for the cases at hand, the data given in Table I lead one to the conclusion that the relation between the affinity of the enzyme for a specific substrate and the susceptibility to hydrolysis of the corresponding enzyme–substrate complex is by no means clear. Indeed, in the extreme case one is confronted by reasonably effective D and L competitive inhibitors whose K_I values are comparable to the K_S values of a number of specific substrates[4] and whose rates of hydrolysis are for all practical purposes zero. Of course there is no *a priori* reason why affinity and susceptibility to hydrolysis should be directly related so that the problem can be divided into two elements, *viz.*, what is responsible for affinity and what determines susceptibility to hydrolysis, *cf.* ref. 4.

In respect to the relation between the structure of the specific substrate and the affinity of the enzyme for the substrate it has been suggested[3] that intermolecular hydrogen bonding may be involved in the formation of the enzyme–substrate complex. If it is postulated that the specific substrate functions as the hydrogen donor, *via* the amide fragment of the R_1 group, it would be expected that the values of K_S, when taken to be approximately equal to k_2/k_1,[4-7] would be in the order acetyl > chloroacetyl > trifluoroacetyl. If the enzyme were the hydrogen donor and the acylamino, *i.e.*, the R_1 group the hydrogen acceptor,

the expected order would be either the same as that given above, or possibly acetyl ≐ chloroacetyl ≫ trifluoroacetyl. Actually the observed order is acetyl ≐ trifluoroacetyl > chloroacetyl with no great differences obtaining between the three members of the series.

If consideration is limited to specific substrates of the acylated α-amino acid amide type the only characteristic of the R_1 groups, *i.e.*, the initial acylamino groups, that appears to be related to the corresponding K_S values is the molecular refractivity of these groups, *i.e.*, acetyl = 10.3, trifluoroacetyl = 10.2, chloroacetyl = 15.1 and nicotinyl = 25.8. It is clear that the relation is not a simple one and will be subject to perturbation by effects associated with the hydration of both substrate and enzyme, the presence of formal charges in the two reacting species and steric factors. However, on the basis of existing evidence it appears that in so far as the R_1 group is concerned the forces responsible for the formation of the enzyme–substrate complex are primarily van der Waals forces.[9] It is hoped that studies now in progress with other acylated α-amino acid amides will be of value in determining the validity of this hypothesis.

There is no apparent correlation between the electrophilic character of the R_1 substituent and the susceptibility to hydrolysis of the corresponding enzyme–substrate complex. On the basis of hydrolysis by a nucleophilic attack by hydroxyl ion, or by a water molecule, it would be expected that the k_3 values of the three specific substrates under consideration would be in the order, acetyl < chloroacetyl < trifluoroacetyl. When it was found that the pK_A' values of acetyl-, chloroacetyl- and trifluoroacetyl-L-tyrosine were, respectively, 3.68, 3.50, and 3.37 at 25°, it was surmised that even though the above order prevailed one might be unable to determine it in respect to k_3 because of the small differences involved. The fact that the k_3 values were found to be in the order acetyl < trifluoroacetyl < chloroacetyl clearly demonstrates that the hydrolysis of these amides at the enzyme surface is governed by a mechanism still to be determined.

Experimental[10,11]

Chloroacetyl-L-tyrosinamide (I).—Six grams (0.0332 mole) of L-tyrosinamide was dissolved in 150 ml. of acetone by boiling under reflux on a steam-bath. To the warm solution was added 450 ml. of ethyl acetate, and the solution was allowed to cool to 25°. A solution of 2 ml. (0.027 mole) of chloroacetyl chloride in 10 ml. of ethyl acetate was then added in small portions with continual swirling. Two minutes after the addition of the chloroacetyl chloride the turbid mixture was shaken with 30 ml. of saturated potassium carbonate solution until turbidity had disappeared. The mixture was allowed to stand until the two liquid phases had separated, and then the addition of chloroacetyl chloride and shaking with carbonate solution was repeated until no further precipitation of L-tyrosinamide hydrochloride occurred on addition of chloroacetyl chloride. The alkaline aqueous layer was then withdrawn, and the ethyl acetate solution evaporated to small volume at room temperature in a current of air. The precipitated chloroacetyl-L-tyrosinamide was recovered and dried to give 5 g. (64.5%) of crude I, m.p. 198–199°. This product was recrystallized twice from ethyl acetate, by continuous extraction, to give 4.4 g. of I, m.p. 203–204°, with decomposition; $[\alpha]^{20}D$ +19.75° (*c* 2% in pyridine).

Anal. Calcd. for $C_{11}H_{13}O_3N_2Cl$ (256.6): C, 51.5; H, 5.1; N, 10.9; Cl, 13.9. Found: C, 51.4; H, 5.1; N, 10.9; Cl, 13.7.

Trifluoroacetyl-L-tyrosinamide (II).—To a solution of 3.6 g. (0.02 mole) of L-tyrosinamide in 150 ml. of acetone and 400 ml. of ethyl acetate was added, in small portions, a solution of 4 ml. of trifluoroacetic anhydride in 10 ml. of ethyl acetate. The solution was then washed with aqueous sodium bicarbonate, dilute hydrochloric acid, water and dried over sodium sulfate. Evaporation of the solvent on a steam-bath gave 1.3 g. (23.5%) of II. Recrystallization from a mixture of benzene and ethyl acetate gave 1.15 g. of II, m.p. 228–228.5°, with decomposition; $[\alpha]^{26.5}D$ +39.8° (*c* 2% in absolute ethanol); $[\alpha]^{20}D$ +20° (*c* 2% in pyridine).

Anal. Calcd. for $C_{11}H_{11}O_3N_2F_3$ (276.2): C, 47.8; H, 4.0; N, 10.1. Found: C, 48.2; H, 4.2; N, 10.2.

The above preparation was varied many times by increasing the amount of anhydride and time of contact, with very little change in the yield. Experiments with pyridine as the solvent gave even poorer yields. Attempts to prepare the amide by ammonolysis of the easily prepared trifluoroacetyl-L-tyrosine methyl ester resulted in the loss of the trifluoroacetyl group.

Chloroacetyl-L-tyrosine (III).—III was prepared by the chloroacetylation of L-tyrosine in sodium hydroxide solution. Recrystallization of the crude product from water gave III, fine needles, m.p. 152.5–153.5°; $[\alpha]^{22.5}D$ +54.25° (*c* 2% in water containing an equivalent amount of sodium hydroxide).

Trifluoroacetyl-L-tyrosine (IV).—A solution of 9.05 g. (0.031 mole) of trifluoroacetyl-L-tyrosine methyl ester in 150 ml. of 50% methanol was adjusted to *p*H 7.9 by the addition of standard sodium hydroxide. A solution of 0.05 g. of α-chymotrypsin in 5 ml. of water was then introduced and standard hydroxide solution slowly added from a buret so as to keep the *p*H of the solution between 7.5 and 8.0. The enzymatic hydrolysis was complete after 45 min. as evidenced by a steady *p*H and the addition of the required amount of sodium hydroxide. The solution was acidified with hydrochloric acid and evaporated to small volume at room temperature in a stream of air. The precipitate obtained (9.0 g., 105%) was recrystallized from water to give IV, needles, m.p. 192.5–193.5°; $[\alpha]^{25}D$ +45.0° (*c* 2% in water containing an equivalent amount of sodium hydroxide).

Anal. Calcd. for $C_{11}H_{10}O_4NF_3$ (277.2): N, 5.1. Found: N, 5.2.

Enzyme Experiments.—The method of following the rate of hydrolysis, and the procedure used in determining the kinetic constants for the systems were the same as those described by Huang and Niemann.[7] Because of the low solubility of trifluoroacetyl-L-tyrosinamide, the determination of K_{P_1} for trifluoroacetyl-L-tyrosine was carried out using chloroacetyl-L-tyrosinamide as the specific substrate. The experiments used for the evaluation of the kinetic constants are summarized in Table II. The enzyme preparation used in these experiments was an Armour product, lot no. 90402.

TABLE II

SUMMARY OF EXPERIMENTS USED FOR THE EVALUATION OF KINETIC CONSTANTS

		No. of experiments at $[S]_0 = 10^{-3}$ molar x							
S:I	[E][a]	8	10	12.5	15	17.5	20	25	30
I	0.0677		1	2	2		2		2
I	.1354			1	2	1	2	1	2
I:III[b]	.0677				2	1	1		2
I:III[b]	.1354				1	1	1	1	1
I:IV[c]	.0677				1	1	2		1
I:IV[c]	.1354				3	3	3		2
II	.1560	1	2	2		1			
II	.2080		3	2	3	2			

[a] Mg. protein-nitrogen per ml. reaction mixture. [b] $[I]_0 = [P_1]_0 = 80 \times 10^{-3}$ *M*. [c] $[I]_0 = 80 \times 10^{-3}$ *M*.

(9) It does not follow that hydrogen bonding can be ignored in speculations relative to the mode of formation of the enzyme–substrate complex since it is probable that a dehydration process involving the *rupture* of hydrogen bonds is a feature of this process.

(10) All melting points are corrected.

(11) Microanalyses by Dr. A. Elek.

Determination of Dissociation Constants.—One hundredth molar solutions of acetyl-, chloroacetyl- and trifluoroacetyl-L-tyrosine in 0.1 *M* sodium chloride were titrated potentiometrically at 25° with 0.01 *M* sodium hydroxide. The half-neutralization points were taken as the respective pK_A' values. Each determination was repeated three times.

PASADENA 4, CALIFORNIA RECEIVED MAY 29, 1951

[CONTRIBUTION NO. 1560 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Enzyme-Inhibitor Dissociation Constants of α-Chymotrypsin and Three Series of Competitive Inhibitors Derived from D-Tryptophan[1]

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

The enzyme-inhibitor dissociation constants of α-chymotrypsin and three series of competitive inhibitors derived from D-tryptophan, *i.e.*, a series of acylated-D-tryptophanamides, a series of derivatives of acetyl-D-tryptophan, and a series of acylated tryptamines, have been evaluated for aqueous solutions of these substances at 25° and *p*H 7.9.

It was pointed out in a previous communication[3] that the elucidation of the mode of action of α-chymotrypsin by studies on the structural requirements of synthetic specific substrates for this enzyme is predicated on the recognition of two distinct series of relationships, *i.e.*, one, that between the structure of the specific substrate and the affinity of the enzyme for that substrate and two, that between the structure of the specific substrate and the susceptibility to hydrolysis of the corresponding intermediate enzyme–substrate complex. While the syntheses of numerous structurally desirable specific substrates can be achieved by known methods, the sparing solubility of many of these compounds in aqueous media, or their slow rates of hydrolysis, has severely limited the number that can be used in detailed kinetic studies with present analytical techniques. Therefore, increasing attention has been paid in recent communications from these laboratories[3,4] to the more modest objective of securing data on relation one above, *viz.*, the effect of structure upon affinity, by determining the enzyme-inhibitor dissociation constants, *i.e.*, the K_I values[5] of selected series of competitive inhibitors. Since there is reason to believe that the mode of combination with the active site of the enzyme is the same for D- and L-enantiomorphs of the general formula $R_1CHR_2R_3$[3] we have, in an attempt to obtain information that would be pertinent to the bonding of both D- and L-compounds, determined the enzyme-inhibitor dissociation constants of two series of competitive inhibitors derived from D-tryptophan, *i.e.*, where R_2 = β-indolylmethyl and remains invariant. The first was a series of acyl-D-tryptophanamides, *i.e.*, R_1 variant with R_3 constant, and the second, a series of derivatives of acetyl-D-tryptophan, *i.e.*, R_3 variant with R_1 constant. The selection of derivatives of tryptophan rather than those of tyrosine or phenylalanine was based upon the knowledge that the affinity of the enzyme for the β-indolylmethyl side chain is considerably greater than that for either the *p*-hydroxybenzyl or benzyl side chains.[4,6] In order to provide information relative to the case where R_3 = H several derivatives of tryptamine were also evaluated.

The specific substrate used in this study was nicotinyl-L-tryptophanamide for which K_S = 2.7×10^{-3} *M*[5]. All experiments were performed at 25° and *p*H 7.9 in aqueous solutions 0.02 *M* in respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer in the presence of an enzyme concentration corresponding to 0.208 mg. of protein nitrogen per ml. reaction mixture. In view of the fact that in every case the reaction was limited to 30% hydrolysis, it is permissible to ignore inhibition by one of the hydrolysis products,[5] and to formulate the systems in terms of equations (1) and (2)

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

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

where $K_S = (k_2 + k_3)/k_1$ and $K_I = k_5/k_4$

The results of these experiments are summarized in Figs. 1–4 and Tables I–III. To facilitate discussion we have included in Tables I and II previous data pertinent to the two series under consideration. The K_I values in Figs. 2–4 were calculated from $1/v_0$ *versus* $1/[S]_0$ plots based upon equation (3)[7]

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

and it will be noted that in every case the inhibition is competitive in nature. While the data for the four inhibitors summarized in Fig. 1, *i.e.*, *p*-methoxybenzoyl-D-tryptophanamide, acetyl-D-tryptophan ethyl ester, *p*-nitrobenzoyl-D-tryptophanamide and benzoyl tryptamine when plotted according to equation (3) also gave straight lines suggesting competitive inhibition, the apparent K_I values so calculated were significantly less than 0.5×10^{-3} *M*. Since it is probable that the enzyme concentration in these systems is of the order of 0.05×10^{-3} *M*,[8] the specific enzyme concentration with respect to the inhibitor in each case is $E'_I = [E]/K_I = 0.1$, *i.e.*, probably beyond the upper limit for zone A.[9,10] Thus these systems

(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**, 3223 (1951).

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