STUDIES ON THE MODE AND MECHANISM OF ACTION OF alpha-CHYMOTRYPSIN

Thesis by

Richard Allan Bernhard

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1955

ACKNOWLEDGMENTS

To Professor Carl Niemann I wish to express my appreciation for his aid and counsel during my years at the Institute, and for his valuable guidance throughout the course of my research.

I should like to thank Professor James Bonner for permitting me to put "hand to plant", and for his patient efforts to teach me a smattering of plant physiology.

The list is long of those to whom I am indebted. May it suffice to say a simple thank you to you all.

I am profoundly grateful to the California Institute of Technology for making me the recipient of the Laws, Dobbins, and Institute Scholarships during my first three years of residence, and to the National Institutes of Health, United States Public Health Service for a grant during these past two years.

ABSTRACT

The enzyme-inhibitor dissociation constants, i.e., K_I values, for alpha-chymotrypsin and two competitive inhibitors, indole-2-carboxylate and cinchoninamide, have been determined at pH 7.9 and 25°.

A new colorimetric procedure for the determination of proteolytic activity employing the reaction of ninhydrin with ammonia has been developed.

A study has been made of the effect of buffer species and ions upon the course of alpha-chymotrypsin catalyzed hydrolyses. It has been demonstrated for the case at hand that K_S is essentially independent of buffer species and ionic strength. The discrepancies between values of k_3, evaluated in the presence of THAM-HCl buffers and in the presence of phosphate buffers, appear to be due principally to the effects of the increased ionic strength of the phosphate buffers. A number of enzyme-inhibitor dissociation constants have been evaluated in different buffer systems. During the evaluation of K_I values for anionic, bifunctional, competitive inhibitors of alpha-chymotrypsin, it was determined that the presence of phosphate buffers apparently increased the affinity of the enzyme for the inhibitor. A possible mechanism for this phenomenon has been proposed and has been supported by experimental observations.

It has been demonstrated that in a typical alpha-chymotrypsin

catalyzed hydrolysis, the reaction proceeds in solution insofar as can be experimentally determined, and that wall effects are unimportant within the limits of experimental error.

An investigation has been made of the possible use of dilatometry as a means of following the course of enzyme catalyzed hydrolyses. A number of instruments have been developed and discussed. Some typical data have been experimentally determined and analyzed in the usual manner.

TABLE OF CONTENTS

PART	TITLE	PAGE
I	THE ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF alpha-CHYMOTRYPSIN AND SOME COMPETITIVE INHIBITORS	1
	Formulation of the Kinetics of the Enzymatic Reaction	2
	Inhibition by a Trifunctional Compound	18
	Inhibition by Bifunctional Compounds	25
	Experimental	34
	Tables and Figures	42
	References	51
II	A NEW COLORIMETRIC PROCEDURE FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITY	54
	Apparatus	61
	Reagents	62
	Analytical Procedure	65
	Calibration Curves	66
	Color Development	71
	References	79
III	THE EFFECT OF BUFFER SPECIES AND IONS UPON THE KINETICS OF alpha-CHYMOTRYPSIN CATALYZED REACTIONS	80
	The Kinetics of the alpha-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-tyrosinamide In the Presence of THAM-HCl Buffer	87

PART	TITLE	PAGE
	In the Presence of Phosphate Buffers	91
	Effect of Phosphate Buffer Concentration and Ionic Strength	98
	The Kinetics of the alpha-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-tryptophanamide	104
	Enzyme-Inhibitor Dissociation Constants for Some Competitive Inhibitors of alpha-Chymotrypsin	
	In the Presence of THAM-HCl Buffer	107
	In the Presence of Phosphate Buffers	110
	Effect of Ionic Environment	116
	Experimental	140
	Tables and Figures	146
	References	197
IV	THE ABSENCE OF WALL EFFECTS IN A TYPICAL alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS	199
	Experimental	204
	Tables	206
	References	210
V	DILATOMETRIC INVESTIGATIONS	211
	Experimental	224
	Tables and Figures	229
	References	233
VI	PROPOSITIONS	235

PART I

THE ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF alpha-CHYMOTRYPSIN AND SOME COMPETITIVE INHIBITORS

FORMULATION OF THE KINETICS OF THE ENZYMATIC REACTION

Introduction

The necessity of a theoretical foundation for integration of the mass of confusing experimental findings in enzyme chemistry has encouraged efforts to apply physical-chemical theories to such studies. The formulation of suitable mathematical expressions for the interpretation of enzymatic reactions has been a logical consequence of this need.

The formulation of the kinetics of enzyme action and the derivation of the resulting rate equations have been extensively covered in several review articles (1-4), and therefore the following brief treatment is presented as an aid in the interpretation of the experimental results given in the next sections.

If the effect of buffer ions and molecules, the hydrogen ion activity, and the temperature are excluded from consideration by carrying out all experiments with these quantities held constant, the variables determining the reaction rate can be limited to the concentrations of the following substances: the enzyme, the substrates, the products of the reaction, and any inhibitor that may be added. In the case of alpha-chymotrypsin catalyzed hydrolysis, the concentration of one of the components, water, may be

eliminated from this list of variables, since in aqueous solutions it remains essentially constant. If all inhibitors are considered to react in a competitive manner, and if the reaction is considered irreversible such that the only influence of the products of the reaction is that of inhibition, the reaction can be formulated in terms of hypothetical enzyme-substrate and enzyme-inhibitor complexes as follows:

Simple Hydrolysis

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

Where

$[E]$ = 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

and k_1, k_2, and k_3 are the rate constants for the reactions denoted by the respective arrows. Consider the case in which all reactants possess unit activity coefficients, $[S] \doteq [S_f]$ and $[S] \gg [E]$. The

concentration of free enzyme, $\left[E_f\right]$, equals $\left[\left[E\right] - \left[ES\right]\right]$.

The rate of formation of $\left[ES\right]$ is given by

$$d\left[ES\right]/dt = k_1\left\langle\left[\left[E\right] - \left[ES\right]\right]\left[S\right]\right\rangle - \left\langle\left(k_2 + k_3\right)\left[ES\right]\right\rangle \quad (2)$$

The rate of disappearance of the substrate is given by

$$-d\left[S\right]/dt = k_1\left\langle\left[\left[E\right] - \left[ES\right]\right]\left[S\right]\right\rangle - k_2\left\langle\left[ES\right]\right\rangle \quad (3)$$

The rate of the overall reaction is given by summation of equations (2) and (3):

$$d\left\langle\left[ES\right] + \left[S\right]\right\rangle/dt = -k_3\left[ES\right]. \quad (4)$$

A complete solution of equation (4) was obtained by Chance (5) with the aid of the differential analyzer and has been given in graphical form. It suffices for the present discussion to consider the restricted conditions usually met with in hydrolytic enzymatic reactions. This condition is that of the steady state, i.e., the concentration of ES is constant, or else the rate of change of $\left[ES\right]$ is negligibly small as compared to the rate of change of $\left[S\right]$.

Accordingly, for $d\left[ES\right]/dt \doteq 0$, equations (2) and (3) reduce to

$$-d\left[S\right]/dt = k_3\left[ES\right]. \quad (5)$$

Hence, the overall reaction velocity is proportional only to the

concentration of the enzyme-substrate complex, ES. It also follows from equation (2) that since $d[ES]/dt \doteq 0$,

$$k_1 \left[[E] - [ES]\right] [S] = (k_2 + k_3)[ES] \tag{6}$$

and

$$\frac{k_2 + k_3}{k_1} = \frac{\left[[E] - [ES]\right][S]}{[ES]} = \frac{[E_f][S]}{[ES]} = K_S \tag{7}$$

Solving equation (7) for $[ES]$, and substituting this value into equation (5), one obtains the overall reaction velocity as a function of enzyme and substrate concentrations:

$$v = -d[S]/dt = \frac{k_3 [E][S]}{K_S + [S]}. \tag{8}$$

Equation (8) is formally identical with that of Michaelis and Menten (6), but differs in the interpretation of K_S. It should be pointed out that K_S is not a dissociation constant as the right side of equation (7) would seem to indicate. Only in the case where $k_2 \gg k_3$ does K_S approach a true dissociation constant of the enzyme-substrate complex.

Since, according to the mass law, the concentration of ES increases as $[S]$ is increased, the velocity, v, increases hyperbolically with increasing substrate concentration to a maximum value, V, which is reached when all the enzyme is bound in ES,

i.e., when $[E] \doteq [ES]$. Under these conditions equation (8) becomes

$$v = \frac{V\ [S]}{K_S + [S]} \tag{9}$$

where

$$V = k_3\ [E] \tag{10}$$

Estimation of K_S from a graph of v *versus* $-\log[S]$ is somewhat cumbersome and inaccurate. Lineweaver and Burk (4) have pointed out that if the reciprocal of both sides of equation (8) is taken, a linear form is obtained which is superior for determination of the values of V and K_S.

$$\frac{1}{v} = \frac{K_S + [S]}{V\ [S]} = \frac{K_S}{V}\left[\frac{1}{[S]}\right] + \frac{1}{V} \tag{11}$$

It can be seen from equation (11) that if $1/v$ is plotted against $1/[S]$ a straight line results with slope equal to K_S/V and an intercept of $1/V$. A convenient method of estimating both K_S and V from the experimental data is thus provided. There are several variations of this procedure (7-9), a very effective one being a plot of v *versus* $v/[S]$. This gives a straight line of slope $-K_S$, an ordinate intercept of $k_3\ [E]$, and an abscissa intercept of $k_3\ [E]/K_S$.

Integration of equation (8) gives

$$k_3 [E] t = K_S \ln \left[[S]_o / [S]_t \right] + \left[[S]_o - [S]_t \right]^1 \qquad (12)$$

This integrated rate equation contains both a zero and first order term. The zero order will predominate when $[S]$ is large and K_S is small. The first order will predominate when $[S]$ is small and K_S is large.

Inhibition

Inhibitors of enzymatic reactions are traditionally divided into two broad classifications: competitive and non-competitive. In competitive inhibition, the inhibitor is regarded as competing with the substrate for specific sites on the enzyme so that the apparent decrease in activity of the enzyme depends on the relative concentration of both substrate and inhibitor. In non-competitive inhibition, the inhibitor inactivates the enzyme by combination at sites not concerned with substrate; hence inactivation depends only on the concentration of inhibitor.

It has been demonstrated by Huang and Niemann (10) that there may be inhibition by the products of the hydrolytic reaction.

For the reaction system

---

[1] $[S]_o$, initial substrate concentration; $[S]_t$, substrate concentration at time t.

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \tag{12}$$

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \tag{13}$$

where

$[P_{1f}]$ = molar concentration of free inhibitory hydrolysis product

$[EP_1]$ = molar concentration of enzyme-inhibitor complex

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

$$\frac{k_2 + k_3}{k_1} = \frac{\left([E] - [ES] - [EP_1]\right)[S]}{[ES]} = K_S \tag{14}$$

$$\frac{k_5}{k_4} = \frac{\left([E] - [ES] - [EP_1]\right)\left([S]_o - [S]_t\right)}{[EP_1]} = K_{P_1} \tag{15}$$

$$\frac{-d[S]}{dt} \quad \frac{k_3[E][S]}{K_S\left[1 + \frac{[S]_o - [S]_t}{K_{P_1}}\right] + [S]} \tag{16}$$

and

$$k_3[E]t = K_S\left\langle 1 + \frac{[S]_o}{K_{P_1}}\right\rangle \ln\frac{[S]_o}{[S]_t} + \left\langle 1 - \frac{K_S}{K_{P_1}}\right\rangle [S]_o - [S]_t \tag{17}$$

A generalized form of equation (17) is:

$$k_3[E]t = K_S\left[1 + [S]_o\sum_{j=1}^{n}\frac{1}{K_{p_j}}\right]\ln\frac{[S]_o}{[S]_t} + \left[1 - K_S\sum_{j=1}^{n}\frac{1}{K_{p_j}}\right]\left[[S]_o - [S]_t\right] \qquad (18)$$

The evaluation of the kinetic constants K_S and K_{p_j} is a problem which is complicated not only by the possible competitive interaction of the free enzyme with one or more of the reaction products, but also by the fact that the integrated rate expression contains both a zero and a first order term.

The traditional solution to this problem has been to study the reaction in its initial stages so as to minimize the difficulties arising from the possible interaction of the free enzyme with one or more of the reaction products, and to estimate the initial velocities, at the various initial specific substrate concentrations, from assumed zero and first order plots. The initial velocities so obtained are then used to evaluate K_S and k_3 by a variety of graphical procedures based upon the differential form of equation (18) in which all interactions between the free enzyme and the reaction products are ignored, i.e., equation (19).

$$-d[S]/dt = k_3[E][S]/(K_S + [S]) \qquad (19)$$

The above procedure is at best a compromise and since

it can be criticized on both practical and theoretical grounds, Foster and Niemann (11) instituted a search for more rational methods. They have developed a procedure which eliminates much of the subjective error inherent in the earlier methods. The method of Foster and Niemann (11) is described in detail in Part III of this thesis, and where applicable, it has been employed to evaluate all experimental data.

For systems in which there is an added inhibitor on the basis of present knowledge it is reasonable to postulate

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

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \qquad (13)$$

$$E_f + I_f \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EI \qquad (20)$$

With $[I]$ = molar concentration of added inhibitor,

$[EI]$ = molar concentration of the enzyme-added inhibitor complex,

$d[ES]/dt \doteq 0$, $[S] \doteq [S_f]$, $[P_1] \doteq [P_{1f}]$, and $[I] = [I_f]$, it follows that

$$\frac{k_2 + k_3}{k_1} = \frac{\left[[E] - [ES] - [EP_1] - [EI]\right][S]}{[ES]} = K_S \qquad (21)$$

$$\frac{k_5}{k_4} = \frac{\left[[E] - [ES] - [EP_1] - [EI]\right][S]_o - [S]_t}{[EP_1]} = K_{P_1} \tag{22}$$

$$\frac{k_7}{k_6} = \frac{\left[[E] - [ES] - [EP_1] - [EI]\right][I]}{[EI]} = K_I \tag{23}$$

$$-\frac{d[S]}{dt} = \frac{k_3[E][S]}{K_S\left(1 + \frac{[I]}{K_I} + \frac{[S]_o - [S]_t}{K_{P_1}}\right) + [S]} \tag{24}$$

and

$$k_3[E]\,t = K_S\left[1 + \frac{[S]_o}{K_{P_1}} + \frac{[I]}{K_I}\right]\ln\frac{[S]_o}{[S]_t} + \left[1 - \frac{K_S}{K_{P_1}}\right]\left[[S]_o - [S]_t\right] \tag{25}$$

In the absence of inhibition by one of the hydrolysis products, i.e., in the case for initial rates, equations (24) and (25) are reduced to

$$-\frac{d[S]}{dt} = \frac{k_3[E][S]}{K_S\left[1 + \frac{[I]}{K_I}\right] + [S]} \tag{26}$$

and

$$k_3[E]\,t = K_S\left[1 + \frac{[I]}{K_I}\right]\ln\frac{[S]_o}{[S]_t} + \left[[S]_o - [S]_t\right] \tag{27}$$

Equation (26) may be transformed into

$$\frac{1}{v_o} = \frac{K_S}{V}\left[1 + \frac{[I]}{K_I}\right]\frac{1}{[S]_o} + \frac{1}{V} \tag{28}$$

When $1/v_o$ is plotted against $1/[S]_o$ a straight line is obtained with slope of $K_S/V\{1 + [I]/K_I\}$. Comparison of equation (11) with equation (28) shows that the effect of a competitive inhibitor is to increase by the quantity $(1 + [I]/K_I)$ the slope of the line obtained when $1/v_o$ is plotted versus $1/[S]_o$. Hence competitive inhibition is indicated by an increase in the slope of the line of a $1/v_o$ versus $1/[S]_o$ plot accompanied by no significant change in the intercept.

While it is true that within the limits of experimental error equation (17) is a satisfactory rate equation for the alpha-chymotrypsin catalyzed hydrolysis of both acetyl-L-tyrosinhydroxamide, and acetyl-L-tyrosinamide at 25° and pH 7.6 and 7.9 respectively, it must be remembered that in the derivation of equation (17) in addition to the assumption that $d[ES]/dt \doteq 0$, it was also assumed that $[S] \doteq [S_f] \gg [ES]$ and $[P_1] \doteq [P_{1f}] \gg [EP_1]$. A much more rigorous derivation of the above kinetic expressions has been made by Straus and Goldstein (12, 13). In their terminology the above conditions specify that the systems described herein are in zone A, and indeed such has been demonstrated (14, 15), Thus the assumptions as used herein appear to be valid.

The Generalized Theory of Straus and Goldstein

The following is taken essentially from Wilson (3) and from discussions with H. T. Huang. A chief source of the criticisms

of the Michaelis-Menten treatment is its assumption of certain properties for the system that may not always obtain. For example, it is assumed that the reaction is pseudo-monomolecular instead of bimolecular as is indicated by the formulation. Straus and Goldstein (12) and later Goldstein (13) derived the velocity equations rigorously so that any simplifying assumptions could be introduced only after they were shown to be valid for a particular system. It is instructive to compare their generalized derivations with the more specific ones already discussed.

Substrate Alone

If $[E]$ is total enzyme, $[E_f]$ is free enzyme, $[S]$, total substrate and $[S_f]$, free substrate, then the correct velocity equation can be derived:

$$E + S \rightleftharpoons ES$$

$$K_S = \frac{[E_f][S_f]}{[ES]}$$

$$[E] = [E_f] + [ES]$$

$$[S] = [S_f] + [ES]$$

Let a, fractional activity be defined:

$$= \frac{[E](1 - a)([S] - a[E])}{a[E]}$$

$$a = \frac{[ES]}{[E]} = \frac{v}{V}$$

$$a[E] = [ES]$$

$$[E_f] = [E](1 - a)$$

$$[S_f] = [S] - a[E]$$

$$[S] = K_S \frac{a}{1 - a} + a[E] \qquad (29)$$

A noteworthy contribution of Straus and Goldstein was to emphasize that it cannot always be assumed that $[E]$ is negligible since the important consideration is not the absolute concentration of E but its concentration with reference to K_S. Accordingly, they expressed $[E]$ and like quantities in specific concentrations, $E' = [E]/K_S$, which, analogous to specific gravities, are dimensionless. Equation (29) is put in the specific concentration form if divided by K_S.

$$S' = a/(1 - a) + aE' \qquad (30)$$

This is the complete equation, but, if E' is very small, as is usually true, the last term is negligible, so it reduces to the simple Michaelis-Menten form. The question of when this can be done is best answered after consideration of inhibition.

Non-competitive Inhibition

Non-competitive inhibition is entirely analogous to the substrate-enzyme equilibrium since the significant reaction is $E + I \rightleftharpoons EI$. It is assumed that concentration of substrate is high enough to saturate the free enzyme; consequently a, the fractional activity, is equal to $[E_f]/[E]$. From the equilibrium

$$K = \frac{[E_f][I_f]}{[EI]} = \frac{[E_f]\ ([I] - [EI])}{[EI]}$$

the complete equation (30 B) is derived as before:

$$I' = (1 - a)/a + (1 - a)\, E'_i \qquad (30\ B)$$

Since $(1 - a)E'_i$ is equal to $[EI]'_i$, the specific concentration of combined inhibitor, the first term in (30 B) must represent the free inhibitor. Non-competitive inhibition therefore is divided into three zones according to how many terms of equation (30 B) it is necessary to use: in zone A essentially all inhibitor is free so that

$$I' = (1 - a)/a; \qquad (30\ A)$$

in zone C the inhibitor is all combined so that

$$I' = (1 - a)\, E'_i \qquad (30\ C)$$

In zone B the complete equation must be employed to describe the behavior of the enzyme-inhibitor system. The boundaries of E' are established by a graphical method based on the choice of a permissible error in a. For a = 0.01, the boundaries are approximately:

Zone A, $E' < 0.10$; Zone C, $E' > 100$

Competitive Inhibition

When the inhibition is competitive, two equilibria must be satisfied

(I) $\frac{[E_f][I_f]}{[EI]} = K_I$ (II) $\frac{[E_f][S_f]}{[ES]} = K_S$

The following relationships are easily verifiable by consideration of these two equilibria:

$$[E] = [ES] + [EI] + [E_f] \qquad [EI] + [E_f]/[E] = (1 - a)$$

$$\text{or} \quad [EI] = (1 - a)[E] - [E_f]$$

in which $\underline{a} = [ES]/[E]$

Solving (II) for $[E_f]$ and substituting in (I), the following complicated expression results:

$$I' = \underbrace{\left\{(S' - aE'_s)\left[\frac{1 - a}{a}\right] - 1\right\}}_{\text{FREE}} + \underbrace{\left\{(1 - a)\left[1 + \frac{1}{S' - aE'_s}\right] E'_i\right\}}_{\text{COMBINED}} \quad (31\ B_iB_s)$$

TOTAL

The letters after the equation number signify that both inhibitor and substrate are in zone B; i.e., this is the most generalized and rigid form of the equation. Simplification is introduced by neglect of $[E_f]$ which is rather small when both $[EI]$ and $[ES]$ are present:

$$I' = \underbrace{[S' - aE'_s]}_{\text{FREE}}\underbrace{[(1 - a)/a]}_{\text{TOTAL}} + \underbrace{(1 - a)\,E'_I}_{\text{COMBINED}} \quad (32\ B_iS_s)$$

The fact that in general enzyme systems will operate in zone A with respect to $\underline{S}$ but some other zone with respect to $\underline{I}$ allows the writing of the following simplified useful variants of $(32\ B_iB_s)$:

$$I' = (S' - aE'_s)\left[(1 - a)/a\right] \quad (32\ A_iB_s)$$

$$I' = S'\left[(1 - a)/a\right] + (1 - a)E'_I \qquad (32\ B_iA_s)$$

$$I' = S'\left[(1 - a)/a\right] \qquad (32\ A_iA_s)$$

Comparison of equation (32 B_iA_s) with (30 B) (the corresponding one for non-competitive inhibition since the system is in zone A with respect to substrate) reveals that they are identical in form except for the multiplier, S'. The corresponding zones for competitive inhibition are accordingly:

Zone A $I' = S'(1 - a)/a$ when $E'_I/S' < 0.1$

Zone C $I' = (1 - a)\,E'_I$ when $E'_I/S' > 100$

INHIBITION BY A TRIFUNCTIONAL COMPOUND

Introduction

While the concept of the enzyme-substrate complex is buttressed by an imposing assemblage of experimental evidence, the extent of the knowledge of the forces operative between the enzyme and substrate and the specific mode of combination of enzyme and substrate remains somewhat limited. It has been assumed and there is evidence which indicates that the enzyme, alpha-chymotrypsin, contains but one active catalytic center, or site, per molecule, in respect to its esterase and proteinase activities (16, 17), and that the combination with substrate occurs at this center.

Numerous investigations on the nature of the alpha-chymotrypsin-catalyzed hydrolysis of amino acid derivatives have led to the hypothesis (18) that an alpha-amino acid derivative of the general formula $R_1CHR_2R_3$ which can combine with the enzyme at its catalytically active site does so by means of combination with three centers, ρ_1, ρ_2, ρ_3, which are complementary to the three prominent structural features of the attached molecule, viz., R_1, the amino or acylamino group of the alpha-carbon atom, R_2, the alpha-amino acid side chain, and R_3, the carboxyl group or a functional derivative of the carboxyl group. The extent to which any given compound of the general formula $R_1CHR_2R_3$ will be bonded

to the active site of the enzyme depends primarily upon the degree to which the molecule and the asymmetric catalytic surface are intrinsically complementary, and secondarily upon the ability of both the combining molecule and the active site to alter their respective conformations in order to improve the closeness of fit during the course of the combining process.

Niemann and co-workers in a series of publications (14, 15, 18-26) have shown that changes in the nature of the R groups may strongly influence the nature of the enzyme-substrate and enzyme-inhibitor dissociation constants. At the time of writing no alpha-amino acid derivatives of the D-configuration have been shown to be substrates of alpha-chymotrypsin. On the contrary, the D-enantiomorphs are excellent competitive inhibitors of the enzyme (20). It may well be that the D-compounds are hydrolyzed, but at such a slow rate that detection of hydrolysis during the course of short term experiments has been impossible with our present analytical methods. It is not impossible that at some future date a specific substrate possessing the D-configuration and derived from an alpha-amino acid will be found for alpha-chymotrypsin.

For acylated alpha-amino acid amides possessing the D-configuration, the affinity of alpha-chymotrypsin for the respective alpha-amino acid side chains is in the order beta-indolylmethyl $\gg$ p-hydroxybenzyl $\gg$ benzyl (20).

This information led to the investigation of the enzyme-inhibitor dissociation constant, K_I, for acetyl-D-tryptophan methyl ester (27) in which R_3 was changed from an amide function to an ester function. It was found that this substance was an extremely effective competitive inhibitor of alpha-chymotrypsin. Subsequently acetyl-D-tryptophan ethyl ester, and acetyl-D-tryptophan isopropyl ester were evaluated as inhibitors (22). It was found that when R_3 equals carbomethoxy, maximum inhibition obtained, see Table II. A logical extension of these investigations was that R_2 and R_3 should be held constant and R_1 should be varied from acetyl to propionyl to butanoyl and so forth. The author undertook the preparation of various acyl derivatives of D-tryptophan isopropyl ester, and a number of these compounds were prepared, (see Table I). Unfortunately, the extremely low solubility of these materials, with the exception of the propionyl derivative, in aqueous solutions precluded their use in further inhibition studies.

In an attempt to increase the solubility of these compounds, the esters were converted to their respective amides, (see Table I). The amides also exhibited extremely low solubility in aqueous media, a fact which precluded their use in further inhibition studies.

Results and Discussion

The inhibition constant for propionyl-D-tryptophan

Table I

INHIBITORS DERIVED FROM D-TRYPTOPHAN

Inhibitor	$[\alpha]_D$	m.p.[a]
Acetyl-L-tryptophan Isopropyl Ester	- 2.9°[b]	132-133.5°
Propionyl-D-tryptophan Isopropyl Ester	- 1.4°[b]	123-124°
Butanoyl-D-tryptophanamide	-	177-178.5°
Hexahydrobenzoyl-D-tryptophanamide	-10.5°[c]	226-227°
Phenylacetyl-D-tryptophanamide	-10.0°[d]	202.5-203°
Dodecanoyl-D-tryptophanamide	-	179-180.5

a., All melting points corrected.

b., At 25°, c 5 % in methanol.

c., At 28°, c 1 % in pyridine.

d., At 26°, c 2 % in methanol.

isopropyl ester was determined at 25° and pH 7.6 in aqueous solutions 0.3 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer in the presence of an enzyme concentration corresponding to 0.0208 mg. protein-nitrogen/ml. of reaction mixture. The specific substrate used in this study was acetyl-L-tyrosinhydroxamide for which $K_S = 43 \pm 4 \times 10^{-3}$ M, and $k_3 = 33 \pm 3 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. (28).

The results of these experiments are summarized in Fig. 1 and Tables II and IV. Data from previous experiments (22) have been included for the purpose of comparison. The results suggest competitive inhibition, and the apparent K_I value calculated from a plot of $1/v_o$ versus $1/[S]_o$ (4), is equal to $0.39 \pm 0.04 \times 10^{-3}$ M. Since all experiments were limited to 30 per cent hydrolysis, the initial velocities, i.e., v_o, were corrected by the method described by Jennings and Niemann (24). The K_I value presented in Table II was calculated from a $1/v_o$ versus $1/[S]_o$ plot (4) and from a plot of v_o versus $v_o/[S]_o$ (7-9) employing corrected initial velocities. These two types of plots gave substantially the same value for K_I.

Care has been taken to assure, as completely as possible, that the enzyme reactions were carried out in such a manner as to maintain zone A conditions (12, 13).

Table II

KINETIC CONSTANTS OF A SERIES OF COMPETITIVE INHIBITORS DERIVED FROM D-TRYPTOPHAN

Inhibitor	K_I [a]
Acetyl-D-tryptophan Methyl Ester	0.089
Acetyl-D-tryptophan Ethyl Ester	0.25
Acetyl-D-tryptophan Isopropyl Ester	0.8 ± 0.2
Propionyl-D-tryptophan Isopropyl Ester	0.37 ± 0.04

a., In units of 10^{-3} M.

There is reason to believe (29-31), that at concentrations of the order of 10^{-5} M or less, alpha-chymotrypsin is present in aqueous solutions essentially in the form of the monomer. The assumption has been made that the molecular weight of monomeric alpha-chymotrypsin is 22,000 and that its nitrogen content is 16 per cent (28). On this basis a solution which contains 0.1 mg. protein-nitrogen/ml. is equal to 2.84×10^{-5} M in this enzyme. Thus reevaluation of the primary data gave the following values.

Propionyl-D-tryptophan isopropyl ester versus acetyl-L-tyrosinhydroxamide.

$K_I = 0.37 \pm 0.04 \times 10^{-3}$ M $\qquad E'_I = 1.6 \times 10^{-2}$

$[E] = 0.59 \times 10^{-5}$ M $\qquad S'_S = 1.16 - 9.3$

$E'_S = 0.014 \times 10^{-2}$ $\qquad I'_I = 1.35$

It has been suggested (22) that the interaction between R_1 and ρ_1 may depend largely upon van der Waals forces. Thus in changes of R_1, keeping R_2 and R_3 constant, in going from acetyl to propionyl one might expect an increase in the affinity of the enzyme for the inhibitor and such appears to be the case.

INHIBITION BY BIFUNCTIONAL COMPOUNDS

Introduction

A necessary condition of the three point attachment theory is that compounds containing two or only one of the three structural elements, R_1, R_2, and R_3, should be capable of functioning as competitive inhibitors of alpha-chymotrypsin. Numerous examples of inhibition by all three possible types of bifunctional inhibitors, i.e., $R_1CH_2R_2$, $R_2CH_2R_3$, and $R_1CH_2R_3$, have been reported (22, 32-36). There is, however, considerably less information on inhibition by monofunctional inhibitors (32, 33, 37). Huang and Niemann (32) have studied five monofunctional inhibitors, and they have reported that indole, itself, is an extremely effective inhibitor of the enzyme, see Table III (32, 33, 37, 38).

In an effort to augment the data in this field, it was felt that inhibition studies should be carried out employing various structural analogs of indole and indole-like compounds, and quantitative determinations should be made to ascertain the effect of the introduction of either an R_1 or R_2 group into the heterocyclic nucleus. A number of these compounds were prepared, and inhibition studies were conducted employing several of these. The enzyme-inhibitor dissociation constants of two of these compounds, indole-2-carboxylate, and cinchoninamide, are discussed in this section because they were evaluated against a common substrate,

Table III

KINETIC CONSTANTS OF A SERIES OF COMPETITIVE INHIBITORS OF alpha-CHYMOTRYPSIN

Inhibitor	K_I [a]
Cyclohexanol	80
Pyridine	50
Nicotinamide	50
Chloramphenicol	25 ± 5
Benzamide	10 ± 2
Phenol	7.0
Skatole	1.0
Indole	0.80 ± 0.2
Cinchoninamide	8.4 ± 1.0
Indole-2-carboxylate	1.5 ± 0.3

a., In units of 10^{-3} M.

acetyl-L-tyrosinhydroxamide, and the kinetics analyzed by a common procedure. Other bifunctional inhibitors of this type are treated in Part III of this thesis.

Results

The inhibition constants for indole-2-carboxylate and cinchoninamide were determined at 25° and pH 7.6 in aqueous solutions 0.3 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer in the presence of an enzyme concentration corresponding to 0.0208 mg. protein-nitrogen/ml. of reaction mixture. The specific substrate used in this study was acetyl-L-tyrosinhydroxamide for which $K_S = 43 \pm 4 \times 10^{-3}$ M, and $k_3 = 33 \pm 3 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. (28).

The results of these experiments are summarized in Fig. 2 and Tables III, V, and VI. Data from previous experiments (32, 33, 37) have been included for the purpose of comparison. The results suggest competitive inhibition, and the apparent K_I values for the two inhibitors calculated from plots of $1/v_o$ versus $1/[S]_o$ (4). The uncorrected K_I values were:

Indole-2-carboxylate $K_I = 1.6 \pm 0.4 \times 10^{-3}$ M

Cinchoninamide $K_I = 8.9 \pm 1.0 \times 10^{-3}$ M

Since all experiments were limited to 30 per cent hydrolysis, the initial velocities, i.e., v_o, were corrected by the method of

Jennings and Niemann (24). The K_I values presented in Tables III, V, and VI were calculated from plots of $1/v_o$ versus $1/[S]_o$ (4) and v_o versus $v_o/[S]_o$ (7-9) employing corrected initial velocities.

Care has been taken to assure, as completely as possible, that the enzyme reactions were performed in such a manner as to maintain zone A conditions (12, 13). Reevaluation of the primary data gave the following values.

Indole-2-carboxylate versus acetyl-L-tyrosinhydroxamide.

$K_I = 1.5 \pm 0.3 \times 10^{-3}$ M $E'_I = 0.39 \times 10^{-2}$

$[E] = 0.59 \times 10^{-5}$ M $S'_S = 1.16 - 9.3$

$E'_S = 0.014 \times 10^{-2}$ $I'_I = 0.67$

Cinchoninamide versus acetyl-L-tyrosinhydroxamide.

$K_I = 8.4 \pm 1.0 \times 10^{-3}$ M $E'_I = 0.07 \times 10^{-2}$

$[E] = 0.59 \times 10^{-5}$ M $S'_S = 1.16 - 9.3$

$E'_S = 0.014 \times 10^{-2}$ $I'_I = 0.595$

It may be noted, from the preceding data, that in every case $[E]$ was of the order of 10^{-5} M, $E'_S = [E]/K_S$ was less than 10^{-2}, $E'_I = [E]/K_I$ less than 1.7×10^{-2}, and $S'_S = [S]/K_S$ between the limits of 1.16 to 9.3. Thus in all of the experiments with the inhibitors discussed above, the conditions were such as to insure the presence of substantially monomeric alpha-chymotrypsin, the maintenance of zone A conditions with respect to both the specific substrate and the competitive inhibitor (12, 13, 28), and the

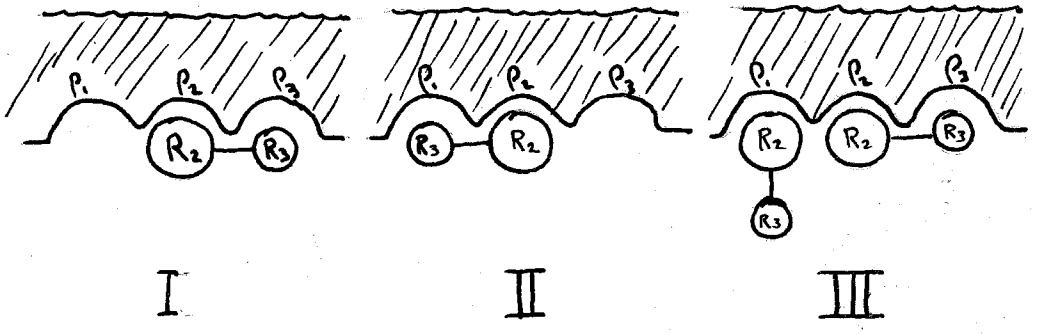
attainment of values of S'_S between the limits necessary for the application of equation (26), in the form of v_o versus $v_o/[S]_o$ plots (7-9) and $1/v_o$ versus $1/[S]_o$ plots (4) within the limits of experimental error. For an experimental error of ± 5 % the permissible limits of S'_S are 0.05 to 20 (28). It will be seen from equation (26) that for a value of I'_I of 0.1 and an experimental error of ± 10 %, the term $K_S (1 + [I]/K_I)$ is

$$-d[S]/dt = k_3[E][S]/K_S (1 + [I]/K_I) + [S] \qquad (26)$$

equal to K_S within the limits of experimental error. Since in the evaluation of K_I, the probable experimental error is likely to be at least 20 %, it is obvious that values of I'_I must exceed 0.2 for K_I values to be of significance, and if they are to be estimated with any reasonable accuracy the value of I'_I should be of the order of 1 or greater. In these experiments described above I'_I varied between the limits of 0.6 to 1.35.

Discussion

It may be expected that indole-2-carboxylic acid, because of its structural relationship to indole, will interact principally with the ρ_2 center of the enzyme, but it cannot be assumed that it will do so exclusively. It is quite possible that there is interaction not only at ρ_2, but also with ρ_3 and possibly ρ_1 to form the binary complexes (I) and (II).



Then either I or II can possibly react with a second molecule of the inhibitor to form the ternary complex III. For the case in which R_2 is indole, Huang and Niemann (32) in a detailed analysis of this problem have shown that the formation of one or more of these complexes prevents the active site from combining with a trifunctional specific substrate, thus with indole inhibition is quite effective.

The introduction of a carboxyl group in the two position of the indole nucleus apparently reduces the affinity of the enzyme for the indole molecule by a factor of about two. It may well be that this reduction in affinity is a reflection of coulombic repulsion between the known negative charge that resides at or near the catalytically active site of alpha-chymotrypsin (1) and the negative charge of the carboxyl group, present at this pH, of the inhibitor.

The marked increase in the K_I value, i.e., the decrease in affinity for the enzyme, exhibited by cinchoninamide as compared to indole may be interpreted in several ways. The problem arises, does cinchoninamide interact with alpha-chymotrypsin as a

monofunctional or bifunctional inhibitor? Cinchoninamide may combine with the enzyme via R_2-ρ_2 interaction in which case it could be thought of as a monofunctional inhibitor such as indole. Two alternate possibilities are that cinchoninamide may interact with the enzyme via a R_1 - ρ_1 and R_2 - ρ_2 interaction, or there may be R_2- ρ_2 and R_3- ρ_3 interaction. For these cases, cinchoninamide would assume the characteristics of a bifunctional inhibitor such as phenylacetamide.

From a comparison of K_I values for several bifunctional competitive inhibitors of the amide type, e.g., benzamide, $K_I = 10 \pm 2 \times 10^{-3}$ M, phenylacetamide, $K_I = 15 \pm 3 \times 10^{-3}$ M, beta-phenylpropionamide, $K_I = 7.0 \pm 2.0 \times 10^{-3}$ M, and gamma-phenyl-butyramide, $K_I = 12 \pm 3 \times 10^{-3}$ M (38), one might well conclude that cinchoninamide falls within this group. Nicotinamide, however, a bifunctional competitive inhibitor, has an unusually large K_I, i.e., approximately 50×10^{-3} M. The large difference between the K_I values of benzamide and nicotinamide has been ascribed to the effects of hydration (22). Nicotinamide is more hydrophilic and hence is presumed to be more heavily hydrated than benzamide. It is reasonable to assume that in the process of formation of the intermediate complex the two reacting molecules, i.e., enzyme and competitive inhibitor, must necessarily approach to within a sufficiently close range to permit the operation of

whatever forces are responsible for the formation of the complex. Thus water molecules on the catalytically active site and on the competitive inhibitor will have to be eliminated, at least in part, during the process of combination. It is very likely that the work involved in the elimination of water molecules at the active site of the enzyme is the same for the formation of the respective intermediate complexes for a series of structurally similar inhibitors, and therefore may be considered as a constant factor. Thus for two inhibitors of approximately the same size and shape, such as benzamide and nicotinamide, the one containing the more hydrophilic group will have more water molecules to be eliminated and hence will require the expenditure of more work in the combination process. This appears to be reflected in a lowering of the affinity of the enzyme for nicotinamide. Possibly a similar effect, although not to such a great extent, is operative in the case of cinchoninamide when compared with monofunctional inhibitors of the indole type.

An additional point to be considered is the fact that there is an available electron pair on the nitrogen of the quinoline ring producing a small negative charge there; this effect is much less pronounced in the case of indole. This negative charge could account for a coulombic repulsion at the bonding site lowering the cinchoninamide molecule's affinity to a marked degree.

Unfortunately, the choice between the various alternatives is by no means clear, and the enzyme-inhibitor relationship might well be a composite of more than one of these effects.

EXPERIMENTAL (39, 40)

Acetyl-L-tyrosinhydroxamide (I). - This material was prepared as directed by Hogness and Niemann (14), colorless needles,[1] m.p. 140-141° with decomposition; $[\alpha]_D^{25}$ -37.0° (c 5 % in water). Lit. (14), m.p. 143-144°, $[\alpha]_D^{25}$ -38.3° (c 5 % in water).

Indole-2-carboxylic Acid (II). - This material was prepared by Dr. H. Rinderknecht, m.p. 198-200°. Recrystallization from methanol and treatment with decolorizing carbon gave long needles of II, m.p. 203-204°. Lit. (41), m.p. 203°.

Anal. Calcd. for $C_9H_7O_2N$ (161): C, 67.1; H, 4.4; N, 8.7. Found: C, 67.2; H, 4.5; N, 8.6.

Cinchoninamide (III). - Ammonolysis of 5 g. of sirupy ethyl cinchoninate, prepared by Dr. J. B. Koepfli, gave 3.8 g. of III, long, colorless needles, m.p. 176.5-178°, after three recrystallizations from water. Lit. (42, 43), m.p. 178°.

DL-Tryptophan Isopropyl Ester (IV). - A suspension of 40 g. of DL-tryptophan in 500 ml. of anhydrous isopropanol was

[1] Some difficulty was encountered in recrystallization. An excellent discussion of the problem and its solution is presented in the Ph.D. thesis of Robert R. Jennings, California Institute of Technology, 1954.

chilled to 0° and saturated with dry hydrogen chloride. The solvent was removed by distillation in vacuo, and the above procedure repeated to give 53.2 g. of the ester hydrochloride. The ester hydrochloride was suspended in 200 ml. of ethyl acetate and treated with a saturated aqueous solution of potassium carbonate until the reaction mixture was alkaline to litmus paper. The ethyl acetate layer was separated from the aqueous layer, and the aqueous layer extracted with two 100 ml. portions of ethyl acetate. All of the ethyl acetate solutions were combined, dried over anhydrous calcium sulfate, and evaporated to dryness in vacuo to give 38 g. of IV, a thick sirup.

D-Tryptophan Isopropyl Ester (V). - A mixture of 15.0 g. of IV and 4 ml. of distilled water was treated with 1 g. of "Viobin" pancreatic extract, and the mixture incubated four days at 25°. The reaction mixture had become solid at the end of this time, and was extracted with four 50 ml. portions of ethyl acetate. The extracts were combined, dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo to dryness to give 6.7 g. of crude V, an amber sirup.

Acetyl-D-tryptophan Isopropyl Ester (VI). - Acetylation of 5.1 g. of crude V with 3 g. of acetic anhydride in ethyl acetate solution, in the presence of aqueous sodium bicarbonate, gave 3.8 g.

of VI, m.p. 132-133.5°, small, stunted needles, after three recrystallizations from ethyl acetate; $[\alpha]_D^{25}$ -2.9° (c 5 % in methanol). No hydrolysis of the ester occurred when tested with alpha-chymotrypsin at pH 7.9.

Anal. Calcd. for $C_{16}H_{20}O_3N_2$ (288): C, 66.7; H, 7.0; N, 9.7. Found: C, 66.7; H, 7.0; N, 9.7.

Propionyl-D-tryptophan Isopropyl Ester (VII). - Acylation of 6.7 g. of crude V in ethyl acetate solution with 8 g. of propionic anhydride, in the presence of aqueous sodium bicarbonate, gave 2.6 g. of crude VII, m.p. 118-120°. Recrystallization from ethyl acetate gave 1.0 g. of VII, m.p. 123-124°, $[\alpha]_D^{25}$ + 1.4° (c 5 % in methanol).

Anal. Calcd. for $C_{17}H_{22}O_3N_2$ (302): C, 67.6; H, 7.3; N, 9.3. Found: C, 67.6; H, 7.3; N, 9.2.

Butanoyl-D-tryptophanamide (VIII). - Ammonolysis of 7.4 g. of crude butanoyl-D-tryptophan methyl ester, obtained by the acylation of crude D-tryptophan methyl ester (44) with butyric anhydride, gave 3.8 g. of VIII, m.p. 178-179.5°, fine needles, after two recrystallizations from methanol. Recrystallization of VIII from a mixture of ethyl acetate and methanol gave fine needles, m.p. 179-181°.

Anal. Calcd. for $C_{15}H_{19}O_2N_3$ (273): C, 65.9; H, 7.0;

N, 15.4. Found: C, 66.3; H, 7.1; N, 15.4.

Hexahydrobenzoyl-D-tryptophanamide (IX). - Ammonolysis of 6.7 g. of crude hexahydrobenzoyl-D-tryptophan methyl ester, obtained by the acylation of crude D-tryptophan methyl ester (44) with hexahydrobenzoyl chloride, gave 2.2 g. of IX, m.p. 226-227°, fine needles, after two recrystallizations from a mixture of methanol and ethyl acetate; $[\alpha]_D^{28}$ -10.5° (c 1 % in pyridine).

Anal. Calcd. for $C_{18}H_{23}O_2N_3$ (313): C, 69.0; H, 7.4; N, 13.4. Found: C, 69.1; H, 7.5; N, 13.4.

Phenylacetyl-D-tryptophanamide (X). - Ammonolysis of 6.7 g. of crude phenylacetyl-D-tryptophan methyl ester, obtained by the acylation of crude D-tryptophan methyl ester (44) with phenylacetyl chloride, gave 1.7 g. of X, m.p. 202.5-203°, fine needles, after three recrystallizations from a methanol-ethyl acetate mixture; $[\alpha]_D^{26}$ -10.0° (c 2 % in methanol).

Anal. Calcd. for $C_{19}H_{19}O_2N_3$ (321): C, 71.0; H, 6.0; N, 13.1. Found: C, 71.3; H, 6.0; N, 13.1.

Dodecanoyl-D-tryptophanamide (XI). - Ammonolysis of 5.1 g. of crude dodecanoyl-D-tryptophan methyl ester, obtained by the acylation of crude D-tryptophan methyl ester (44) with dodecanoyl chloride, gave 2.2 g. of XI, m.p. 173-175.5°, short, stunted

needles, after three recrystallizations of XI from ethyl acetate gave short needles, m.p. 179-180.5°.

Anal. Calcd. for $C_{23}H_{35}O_2N_3$ (385): C, 71.7; H, 9.2; N, 10.9. Found: C, 74.3; H, 9.3; N, 9.8.

Buffer Solutions. - Technical tris-(hydroxymethyl)-aminomethane (Commercial Solvents Corporation) was recrystallized twice from ethanol to give large, colorless, rod-like crystals, m.p. 169-169.5°. A stock solution, 1.5 formal with respect to the amine component, was prepared by the addition of sufficient 6 N hydrochloric acid to an aqueous solution of the amine to give a solution of pH 7.62 at 25° after the stock solution was made up to volume. This stock solution was used in studies conducted at pH 7.6, since it was found that, in the presence of enzyme, substrate, and inhibitor, a 2:10 dilution of this stock solution yielded a reaction mixture, 0.30 formal in the amine component and of pH 7.60 ± 0.02 at 25°.

Enzyme Solutions. - Crystalline alpha-chymotrypsin containing magnesium sulfate (Armour, Lot Nos. 90402 and 10705) was used in these studies. The chymotrypsin preparations contained 10.4 % N and 13.3 % N respectively. Stock solutions of enzyme were prepared daily, brought to 25°, and used immediately. The protein-nitrogen content of the enzyme was determined as follows:

an accurately weighed sample of the alpha-chymotrypsin preparation, dissolved in water, was precipitated with an equal volume of a 5 % aqueous solution of trichloroacetic acid. The precipitate was isolated from the solution by centrifugation in a previously weighed centrifuge tube, followed by careful removal of the solvent. The precipitate was dried in an Abderhalden drying pistol for three hours. The centrifuge tube and its contents were reweighed, and the nitrogen content of the precipitate determined by the Kjeldahl method of analysis.

$$\%\text{protein nitrogen} = \frac{\text{weight of ppt.}}{\text{weight of sample}} \times \%\ \text{N in ppt.}$$

Analytical Procedure. - The substrate plus inhibitor, was placed in a 10-ml. G.S. volumetric flask, and ca. 6.5 ml. of distilled water added. Heat was applied, if necessary, to effect solution; 2.0 ml. of tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer, 1.5 formal with respect to the amine component, was then added, and the stoppered flask placed in a constant temperature bath at $25.0 \pm 0.1^{\circ}$ for at least thirty minutes. After the contents of the flask had reached 25°, the volumetric flask was withdrawn from the bath, 1.0 ml. of enzyme solution added at time zero, and the solution immediately made up to volume with distilled water. The flask was stoppered and inverted gently 12 to 14 times to insure adequate mixing. The flask was then returned to the

constant temperature bath.

Immediately prior to a given experiment 1.0-ml. aliquots of a stock solution 0.2 M in ferric chloride, 0.2 M in hydrochloric acid, and 50 % (by volume) in methanol were introduced into a number of 10-ml. G.S. volumetric flasks followed by the subsequent addition of ca. 7.5 ml. of methanol to each flask. At selected time intervals a 1.0-ml. aliquot of the reaction mixture was added to the contents of one of the above flasks, the solution made up to volume with methanol, and the stoppered flask gently inverted 10 to 12 times to insure thorough mixing.

The optical density of the resulting solution, for a path of 1 cm. and at 505 mμ, was determined in a Beckman model B spectrophotometer. A solution of all the components except the acetyl-L-tyrosinhydroxamide, i.e., the specific substrate, was used to zero the instrument. The dependence of the optical density upon the concentration of acetyl-L-tyrosinhydroxamide was linear over the range of concentrations ordinarily used. When concentrations of acetyl-L-tyrosinhydroxamide were used which were higher than 15×10^{-3} M, 2.5 ml. or 5.0-ml. aliquots of the stock solution were introduced into 25- or 50-ml. flasks and diluted to the appropriate volume after the addition of 1.0 ml. of the reaction mixture.

All reactions were limited to 20 - 30 per cent hydrolysis,

and in each case plots of both ($[S]_o - [S]_t$) *versus* t and $\ln [S]_o/[S]_t$ *versus* t were made and then corrected as described by Jennings and Niemann (24) using a value of $K_S = 43 \times 10^{-3}$ M.

The following data have been determined according to the procedure described in this section. The legend of symbols is as follows:

t = time in minutes

$[E]$ = alpha-chymotrypsin concentration in mg. protein-nitrogen/ml.

$[S]_o$ = initial substrate concentration in units of 10^{-3} molar

$[I]$ = added inhibitor concentration in units of 10^{-3} molar

O.D. = optical density

v_o = initial velocity of reaction in units of 10^{-3} M/min.

Table IV

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINHYDROXAMIDE BY PROPIONYL-D-TRYPTOPHAN ISOPROPYL ESTER AT pH 7.6 AND 25°

$[E]$ = 0.0208 mg. protein-nitrogen/ml.
$[I]$ = 0.5×10^{-3} M
0.30 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer
K_I = $0.37 \pm 0.04 \times 10^{-3}$ M

$[S]_o$	t	O.D.	ln O.D.	v_o
5	2	.658	.419	.033
	6	.636	.453	
	12	.618	.481	
	18	.587	.533	
	24	.557	.585	
	30	.542	.612	
	36	.526	.642	
10	2	1.340	.293	.062
	6	1.310	.270	
	12	1.255	.227	
	18	1.225	.203	
	24	1.165	.152	
	31	1.120	.113	
	37	1.076	.072	
20	2	1.063	.061	1.09
	6	1.032	.031	
	12	1.010	.010	
	18	.988	-.0121	
	24	.951	-.0502	
	30	.914	-.0899	
	36	.893	-.113	

Table IV (cont.)

$[S]_o$	t	O.D.	ln O.D.	v_o
20	2	1.085	.0816	1.13
	6	1.045	.0438	
	12	1.014	.0140	
	18	.985	-.0151	
	24	.945	-.0566	
	30	.915	-.0888	
	36	.892	-.1143	
30	2	1.567	.448	1.47
	6	1.550	.438	
	12	1.514	.415	
	18	1.468	.383	
	24	1.425	.354	
	30	1.382	.323	
	36	1.354	.302	
30	2	1.548	.438	1.47
	6	1.538	.432	
	12	1.476	.392	
	18	1.460	.378	
	24	1.420	.351	
	30	1.378	.322	
	36	1.330	.285	
40	2	2.05	.718	1.73
	7	1.97	.678	
	13	1.92	.652	
	19	1.88	.631	
	25	1.82	.599	
	31	1.77	.571	
	37	1.73	.548	

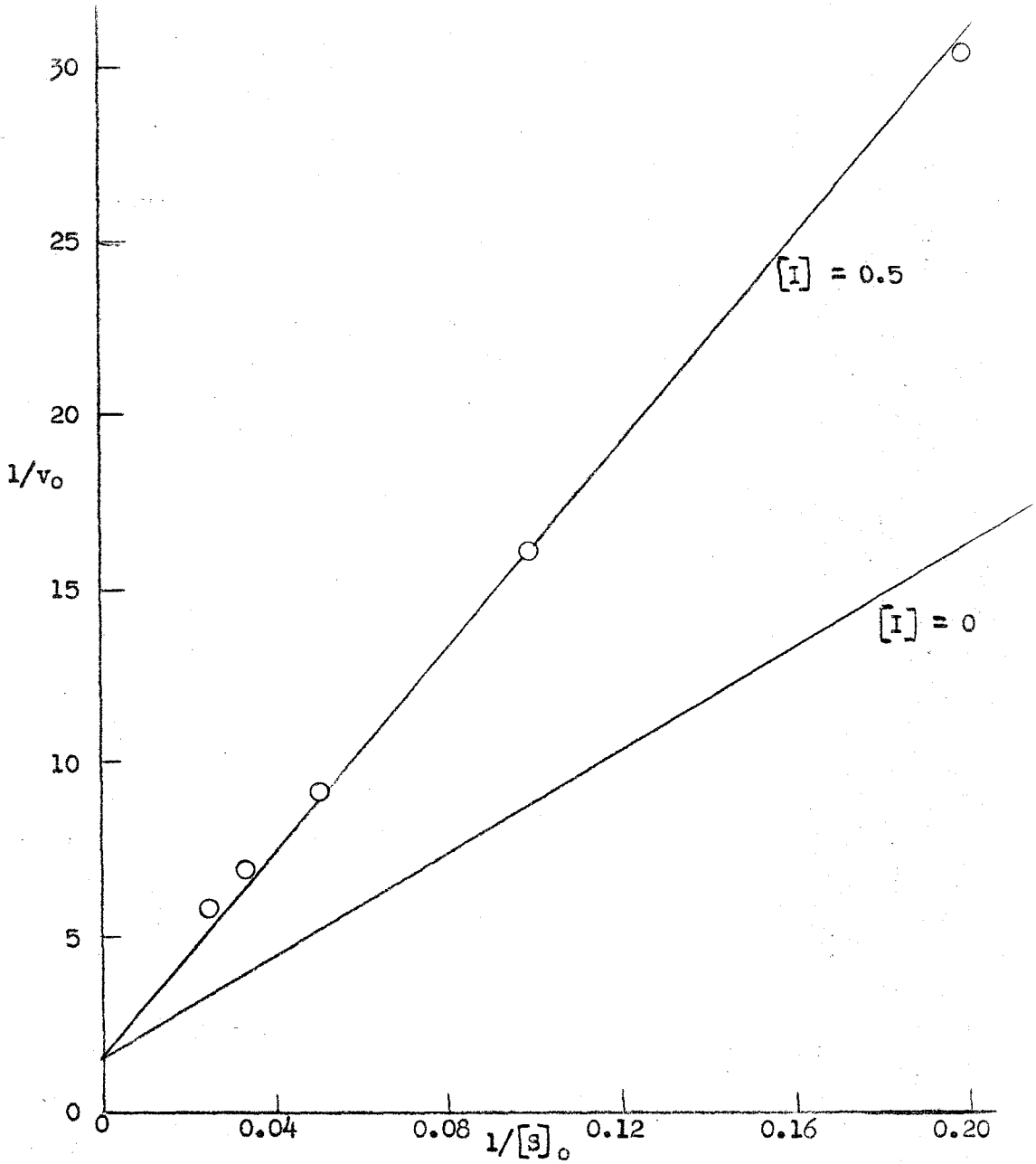


Fig. 1 Inhibition of hydrolysis of acetyl-L-tyrosinhydroxamide by propionyl-D-tryptophan isopropyl ester; v_o in units of 10^{-3} M/min.; $[S]_o$ and $[I]$ in units of 10^{-3} M; $[E]$ = 0.0208 mg. protein-nitrogen/ml.; 0.3 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

Table V

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINHYDROXAMIDE BY INDOLE-2-CARBOXYLATE AT pH 7.6 AND 25°

$[E]$ = 0.0208 mg. protein-nitrogen/ml.
$[I]$ = 1.0×10^{-3} M
0.30 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer
K_I = $1.5 \pm 0.3 \times 10^{-3}$ M

$[S]_o$	t	O.D.	ln O.D.	v_o
5	2	.667	- .405	.444
	6	.642	- .443	
	12	.606	- .501	
	18	.573	- .557	
	24	.552	- .594	
	30	.518	- .658	
	36	.491	- .711	
10	2	1.310	.270	.837
	6	1.261	.231	
	12	1.210	.191	
	18.5	1.130	.122	
	24	1.083	.077	
	30	1.033	.029	
	36	.988	- .013	
20	2	1.066	.063	1.460
	6	1.046	.043	
	12	1.010	.0099	
	18	.958	- .043	
	24	.905	- .099	
	30	.871	- .138	
	36	.832	- .184	

Table V (cont.)

$[S]_o$	t	O.D.	ln O.D.	v_o
30	2	1.553	.439	1.720
	6	1.516	.416	
	12	1.460	.378	
	18	1.420	.351	
	24	1.370	.315	
	30	1.320	.278	
	36	1.263	.233	
30	2	1.555	.441	1.720
	6	1.515	.415	
	12	1.470	.385	
	18	1.410	.344	
	24	1.360	.307	
	30	1.305	.266	
	36	1.250	.223	
40	2	1.970	.678	1.860
	6	1.910	.647	
	12	1.860	.621	
	18	1.830	.604	
	24	1.790	.582	
	30	1.690	.525	
40	2	1.980	.683	1.870
	6	1.945	.666	
	12	1.895	.639	
	18	1.840	.610	
	24	1.780	.577	
	30	1.730	.548	
	36	1.660	.507	

Table VI

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINHYDROXAMIDE BY CINCHONINAMIDE AT pH 7.6 AND 25°

$[E]$ = 0.0208 mg. protein-nitrogen/ml.
$[I]$ = 5.0×10^{-3} M
0.3 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer
K_I = $8.4 \pm 1.0 \times 10^{-3}$ M

$[S]_o$	t	O.D.	ln O.D.	v_o
5	2	.676	- .392	.449
	6	.660	- .416	
	12	.624	- .472	
	18	.586	- .534	
	24	.560	- .580	
	30	.530	- .635	
	36	.500	- .693	
10	2	1.380	.322	.875
	6	1.335	.288	
	12	1.265	.235	
	18	1.186	.170	
	24	1.135	.128	
	30	1.082	.078	
	36	1.023	.021	
20	2	1.094	.0897	1.590
	6	1.054	.0526	
	12	1.008	.0090	
	18	.960	- .0408	
	24	.910	- .0943	
	30	.868	- .1420	
	36	.830	- .1860	

Table VI (cont.)

$[S]_o$	t	O.D.	ln O.D.	v_o
30	2	1.645	.498	2.030
	6	1.605	.473	
	12	1.535	.428	
	18	1.464	.381	
	24	1.413	.346	
	30	1.355	.303	
	36	1.294	.258	
40	2	2.20	.788	2.510
	6	2.18	.779	
	12	2.06	.723	
	18	2.00	.693	
	24	1.92	.652	
	30	1.83	.607	
	36	1.77	.571	

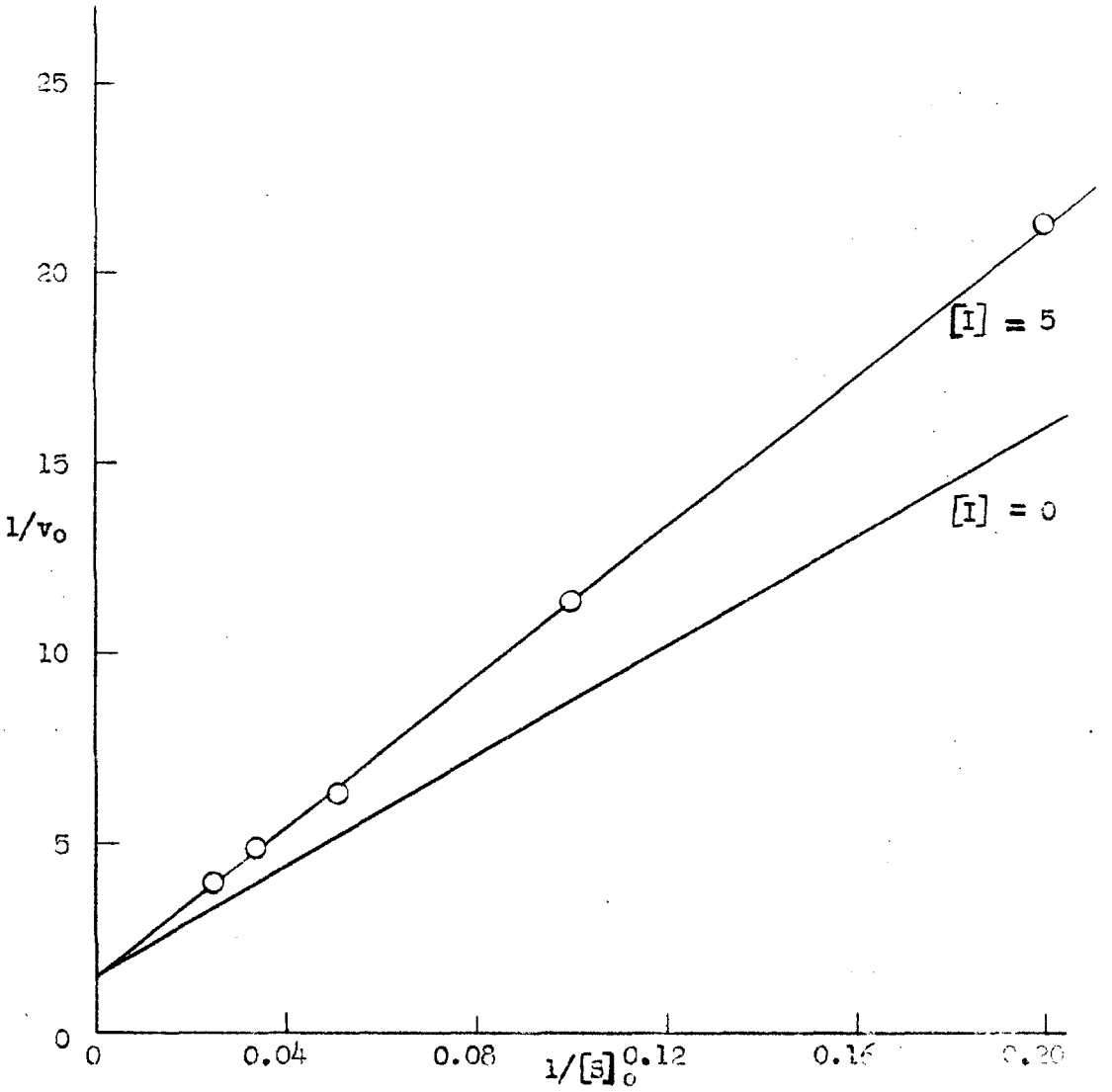


Fig. 2 Inhibition of hydrolysis of acetyl-L-tyrosinhydroxamide by cinchoninamide; v_o in units of 10^{-3} M/min.; $[S]_o$ and [I] in units of 10^{-3} M; [E] = 0.0208 mg. protein-nitrogen per ml.; 0.3 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

REFERENCES

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

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

(3) Wilson, P.H., in "Respiratory Enzymes", by H.A. Lardy, Burgess Publishing Co., Minneapolis, 1949, p. 16ff.

(4) Lineweaver, H., and Burk, D., J.A.C.S., 56, 658 (1934).

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

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

(7) Eadie, G.S., J. Biol. Chem., 146, 85 (1942).

(8) Hofstee, H.J., Science, 116, 329 (952).

(9) Eadie, G.S., ibid., 116, 688 (1952).

(10) Huang, H.T., and Niemann, C., J.A.C.S., 73, 1541 (1951).

(11) Foster, R.J., and Niemann, C., Proc. Natl. Acad. Sci., 39, 999 (1953).

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

(13) Goldstein, A., ibid., 27, 529 (1944).

(14) Hogness, D.S., and Niemann, C., J.A.C.S., 75, 884 (1953).

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

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

(17) Jansen, E., Fellows-Nutting, M.D., and Balls, A.K., ibid., 179, 201 (1949).

(18) Huang, H. T., and Niemann, C., J.A.C.S., 73, 3223 (1951).

(19) Shine, H. J., and Niemann, C., ibid., 74, 97 (1952).

(20) Huang, H. T., and Niemann, C., ibid., 73, 1555 (1951).

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

(22) Huang, H. T., and Niemann, C., ibid., 74, 101 (1952).

(23) Huang, H. T., Foster, R. J., and Niemann, C., ibid., 74, 105 (1952).

(24) Jennings, R. R., and Niemann, C., ibid., 75, 4687 (1953).

(25) Foster, R. J., Jennings, R. R., and Niemann, C., ibid., 76, 3142 (1954).

(26) Niemann, C., Record Chem. Prog., 12, 107 (1951).

(27) Huang, H. T., and Niemann, C., J.A.C.S., 73, 3228 (1951).

(28) Foster, R. J., and Niemann, C., ibid., 77, 0000 (1955).

(29) Schwert, G. W., J. Biol. Chem., 179, 655 (1949).

(30) Schwert, G. W., and Kaufman, S., ibid., 190, 807 (1951).

(31) Smith, E. L., Brown, D. M., and Laskowski, M., ibid., 191, 639 (1951).

(32) Huang, H. T., and Niemann, C., J.A.C.S., 75, 1395 (1953).

(33) Huang, H. T., and Niemann, C., ibid., 74, 5963 (1952).

(34) Kaufman, S., and Neurath, H., J. Biol. Chem., 181, 623 (1949).

(35) Neurath, H., and Gladner, J.A., ibid., 188, 407 (1951).

(36) Huang, H. T., and Niemann, C., J.A.C.S., 74, 4634 (1952).

(37) Huang, H. T., and Niemann, C., ibid., 73, 4039 (1951).

(38) Foster, R. J., and Niemann, C., In Press.

(39) All melting points are corrected.

(40) Microanalyses by Dr. A. Elek.

(41) Elks, J., Elliott, D.F., and Hems, B.A., J. Chem. Soc., 1944, 629.

(42) Wenzel, F., Monatsh., 15, 456 (1894).

(43) Meyer, H., Rec. trav. chim., 44, 323 (1925).

(44) Brenner, M., Sailer, E., and Kochen, V., Helv. Chim. Acta, 31, 1909 (1948).

PART II

A NEW COLORIMETRIC PROCEDURE FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITY

INTRODUCTION

A major problem in the investigation of the reaction kinetics of an enzyme-substrate system is finding a satisfactory means of measuring changes in the system which can be related to suitable kinetic functions. Titration of aliquots from a system containing an acidic or basic substrate or product at periodic intervals allows the investigator to follow the course of a reaction with a satisfactory degree of accuracy, and this technique has been employed extensively in the field of enzyme kinetics. Ideally, a physical method of analysis which does not disturb the system is far more desirable. Unfortunately, a suitable analytical procedure of this type for the accurate investigation of alpha-chymotrypsin catalyzed reactions has yet to be developed.

In order to circumvent the limitations of titration procedures, a varied approach to colorimetric and spectrophotometric methods was investigated. In this connection one may recall the words of Martin and Synge (1):

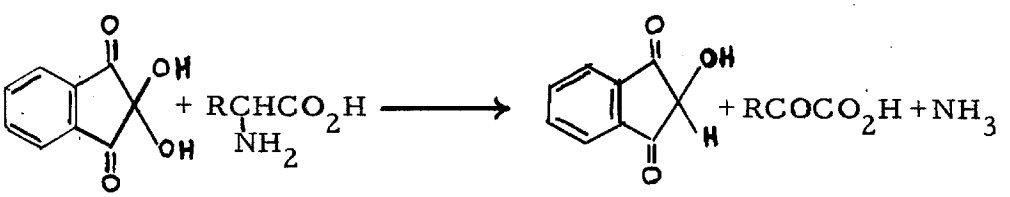
> "The special advantages of these methods are mainly (i) that they require less time and manipulation than most other analytical methods; (ii) they usually consume only minute quantities of material. The combined advantages of speed and ultra-micro scale make them very useful.
>
> On the other hand it is more difficult to convince oneself of the accuracy and specificity of colorimetric methods. Moreover, the extremely close control of conditions which is normally

> recommended as essential, and the generally polemical character of the literature suggest that considerable experience is necessary before even repeatable results can be obtained."

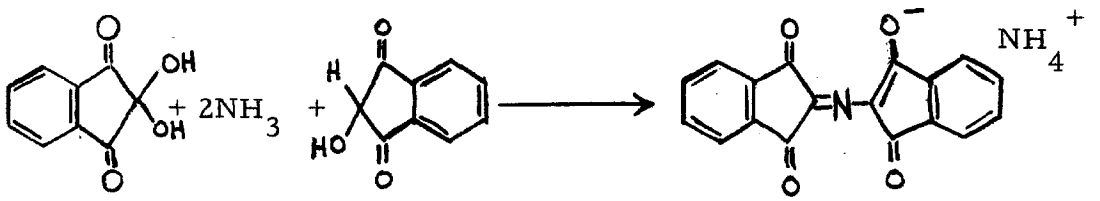
A remarkably sensitive and accurate colorimetric procedure making use of the ferric-hydroxamate color reaction has been developed by Niemann and coworkers (2) which has the advantage of great ease of operation. The procedure is limited in that it requires that the substrate contain the hydroxamic acid group, and that substances which complex with ferric ions cannot be used in the reaction media or the subsequent analytical procedure because they interfere with the color development. In an effort to circumvent these limitations, a search for a new or different colorimetric procedure was begun. Fortunately the outstanding contributions of Moore and Stein (3) were available and an analytical procedure based largely upon their original investigations was elaborated and adapted to the investigation of the enzymatic hydrolysis of acylated alpha-amino acid amides.

Virtually all known alpha-amino acids produce a blue to violet-red color when warmed with a dilute aqueous solution of triketohydrindene hydrate (ninhydrin) (I) (4). Indeed, in a qualitative sense, the reagent has become one of the classic means of detecting alpha-amino acids, peptides, and proteins. Presumably, the reaction proceeds in two steps. First, there is an oxidative deamination of the alpha-amino acid by ninhydrin producing

ammonia, carbon dioxide, the corresponding aldehyde containing one less carbon atom, and a reduced form of ninhydrin, diketohydrindol (II). Second, the ammonia condenses with a molecule of ninhydrin and one of diketohydrindol to form the compound, diketohydrindylidene-diketohydrindamine (5) (6). The blue-violet color associated with this reaction is attributed to the anion (III). This scheme is consistent with the fact that ammonia forms (III) with ninhydrin only in the presence of a reducing agent capable of producing some diketohydrindol (II) (7).



(I) (II)



(III)

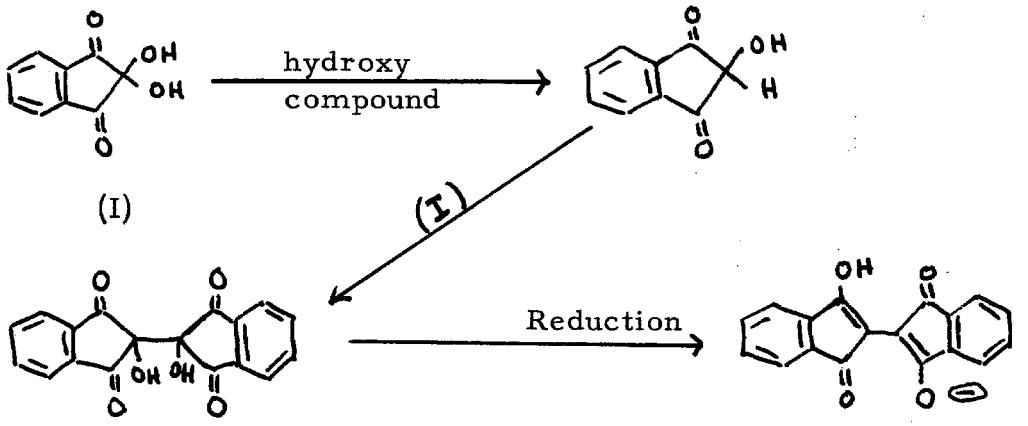
Moore and Stein (3) have developed the reaction into a rapid and convenient photometric method for the quantitative estimation of alpha-amino acids. They demonstrated that the low and variable yields of color usually obtained were due to the autoxidation

of some of the diketohydrindol (II). The relationship between color yield and amino acid concentration was made more nearly linear by the addition of a strong reducing agent directly to the reaction medium. It has been reported that the addition of organic reagents such as ethanol, dioxane, pyridine, phenol (7), and methyl cellosolve (3) will accelerate the development of color to varying degrees.

Numerous researchers (3) (7-9) have now applied this method and its modifications to the determination of alpha-amino acids and have achieved remarkable results. It can be seen that under the proper conditions this method may be employed to determine ammonia or ammonium ions in a reaction system, and developmental work was begun by the author in this direction. Early attempts to react ammonium salts with ninhydrin to produce the blue-violet color met with varied degrees of success. Herzfeld (10) noticed that ammonium carbonate and ammonium oxalate gave colors when evaporated to dryness with ninhydrin. Neuberg (11) found that ammonium salts in general react to produce the characteristic color with ninhydrin, thus contradicting earlier experiments of Abderhalden and Schmidt (12) in which they obtained no such reaction. Later, Harding and coworkers (13) found ammonium chloride gave no color with ninhydrin except when the salt was present at saturation concentrations. This apparent confusion was clarified and

explained by Moore and Stein (3) who demonstrated that some diketohydrindol must be present and that accurate control of pH is vitally important for the production of color especially for the case of ammonia.

Regrettably, ninhydrin exhibits a rather low specificity and reacts to produce colored products not only with numerous nitrogen compounds, e.g., primary amines, but also with certain hydroxy compounds, e.g., glycerol, ethylene glycol, and some sugars. The color associated with the latter compounds has been attributed to bis-1, 3-diketoindanyl (enol form) (V) (14). This compound is presumed to be formed in the following manner (14):



hydrindantin (IV) (V)

The author found that it was possible to make accurate quantitative estimates of small quantities of ammonia in reaction systems if careful attention was given to the control of pH, reagents, and reaction conditions. Thus it has been possible to

develop a procedure which accurately determines the amount of ammonia liberated during the course of an enzyme catalyzed hydrolysis of an alpha-acylamino acid amide.

Briefly, the procedure consisted of placing aliquots of the reaction mixture in a citrate buffer[1] of pH near five.[2] A sample of this solution was then added to ninhydrin reagent and the two heated to develop the characteristic blue-violet color. The highly colored product was diluted with an inert solvent to obtain a convenient volume of solution for the determination of the optical density. The optical density of the resulting solution was then related to the concentration of ammonia in the original reaction mixture.

---

[1] For a discussion pertaining to the choice of buffer and pH see section entitled Color Development.

[2] The enzyme is inactivated immediately by this procedure.

APPARATUS

A considerable advantage to this procedure was that little or no special apparatus was required for its implementation.

Test Tubes

A number of 5 inch, pyrex, rimless test tubes were employed in the color development stage of the procedure. The contents of these tubes were kept free of dust and other foreign matter and excessive evaporation of the contents was reduced by covering the tubes with small cylindrical aluminum caps approximately 7/8 inch in diameter and one inch deep.

Water Bath

A vigorously boiling water bath was required. The author found that a covered eight quart enameled pot heated by a large gas burner, e.g., a Fisher burner, was quite satisfactory for this purpose. The tubes were conveniently held in a metal rack which could be placed directly into the boiling water bath. This allowed all of the tubes to be heated at the same time and for the same length of time. The tubes were immersed in the boiling water to a depth of about three inches. This provided ample heating for a 2 ml. reaction sample. The rate of heat supply was sufficient to bring the bath back up to 99-100^{o} within two minutes after the insertion of a full rack of test tubes.

REAGENTS

Ninhydrin

To insure a low blank reading in the photometric procedure, the ninhydrin was recrystallized from distilled water within thirty days of the time of use.

To 250 ml. of distilled water 100 g. of ninhydrin were added; the hot solution treated with approximately 5 g. of Norit A, filtered, and the filtrate stored at 4° overnight. The ninhydrin crystals were filtered, washed with four 20 ml. portions of ice cold distilled water, and air dried in a dark place. The crystals were pulverized in an agate mortar, and stored in a tightly stoppered brown glass bottle away from the light. The recovery was 85-90 per cent.

Citrate Buffer

The buffer, pH 4.8 ± 0.1 (0.2 M), was prepared by dissolving 42 g. of recrystallized reagent grade citric acid monohydrate in 600 ml. of distilled water. This solution was added with stirring to a solution of 16 g. of C. P. sodium hydroxide pellets dissolved in 400 ml. of distilled water. A few crystals of thymol were added, and the solution stored at 4° until used. This buffer was prepared fresh each week.

Anhydrous Stannous Chloride (15)

In a 600 ml. beaker 100 g. (95 ml., 1 mole) of acetic

anhydride (99-100 %) were placed, and while the liquid was being gently stirred, 84 g. (0.37 mole) of C.P. crystalline stannous chloride dihydrate were added. This operation was performed in a fume hood, for the heat of the reaction was sufficient to cause the acetic anhydride to boil vigorously. After about 90 minutes, the anhydrous stannous chloride was filtered on a large Büchner funnel, rinsed with two 30 ml. portions of dry ether, and dried overnight in a vacuum desiccator. The yield was nearly quantitative (70 g.). The product is stable if kept in a tightly stoppered bottle.

Peroxide-Free Methyl Cellosolve

One liter of methyl cellosolve was heated under reflux with 10-15 g. of solid anhydrous stannous chloride for thirty minutes. The cooled solution was tested for peroxides. If peroxides were detected, additional 5 g. portions of anhydrous stannous chloride were added followed by heating under reflux for thirty minute periods until a negative test for peroxides was obtained. The methyl cellosolve was then distilled through a 60 cm. vigreux column, and the fraction distilling at 123-25^{o} retained. This was stored in a brown glass bottle away from the light.

Ninhydrin Reagent

This reagent was prepared by dissolving 0.0240 g.[1] of

[1] Weighed-out accurately by means of an analytical balance.

C.P. stannous chloride dihydrate in 15.0 ml.[1] of the pH 4.8 citrate buffer and passing dry nitrogen through the solution for three minutes. Dry nitrogen was passed through a solution of 0.600 g.[2] of ninhydrin in 15.0 ml.[1] of peroxide-free methyl cellosolve for three minutes. The stannous chloride solution was then added to the ninhydrin solution, the two solutions thoroughly mixed, and stored in an opaque polyethylene bottle under a nitrogen atmosphere. This reagent was prepared daily just prior to its use as it deteriorates quite rapidly.

Diluent Solution

Equal volumes of distilled water and reagent grade isopropanol were mixed and used as the diluent.

Standard Ammonium Chloride Solutions

For the determination of standard calibration curves, 0.05350 g. of dry C.P. ammonium chloride was weighed into a 10-ml. G.S. volumetric flask and the volume made up to the mark with distilled water. This gives a 0.10 M solution. Suitable dilutions of this solution were made to obtain various concentrations of ammonium chloride.

1 The volumes were measured accurately by means of volumetric pipettes.

2 Weighed-out accurately by means of an analytical balance.

ANALYTICAL PROCEDURE

Prior to a given experiment each of a series of 10-ml. G.S. volumetric flasks was filled with approximately 8.5 ml. of 0.2 M citrate buffer (pH 4.8 ± 0.1). At selected time intervals a 1.0-ml. aliquot of the reaction mixture was added to the contents of one of the above flasks, the solution immediately made up to volume with additional 0.2 M citrate buffer, the flask stoppered and gently inverted 15-20 times.

After completion of the kinetic experiment, a 1.0-ml. aliquot of the above solution was added to 1.0 ml. of the ninhydrin reagent contained in a 5 inch, pyrex, rimless test tube. The tube was shaken by hand for ten seconds to insure adequate mixing of the two solutions, and covered by an aluminum cap. This procedure was repeated for all the aliquots of reaction mixture to be analyzed. The series of test tubes containing the diluted reaction mixture and the ninhydrin reagent were placed in a metal rack, and the whole immersed in a water bath maintained at 99-100° for exactly 20 minutes. Upon completion of the heating period, the rack and tubes were withdrawn from the bath; the tubes were allowed to cool for five minutes, and then wiped dry. A 5.0-ml. aliquot of the diluent solution was added to the contents of each tube, and the contents thoroughly mixed. The optical density of each of these solutions, for a path of 1 cm. and at 565 mμ, was determined in a model B

Beckman spectrophotometer. A sample of diluent solution was used to zero the instrument.

When concentrations of ammonia were encountered which were higher than those indicated on the abscissas of the plots given in Figs. 1 and 2, the volumetric flasks containing the 0.2 M citrate buffer were changed to permit a suitable dilution. The author found the following relationships were conveniently employed.

Concentration of Ammonia to be Analyzed (in 10^{-3} M)	Size of G.S. Volumetric Flask Containing 0.2 M Citrate Buffer (in ml.)
0.0 - 3.0	5
3.0 - 7.0	10
7.0 - 17.5	25
17.5 - 35.0	50

CALIBRATION CURVES

A series of experiments were performed to determine the relationship between the optical density of the colored product and the concentration of ammonia in the reaction solution. It was

found that there was a linear correspondence between the concentration of ammonia and the optical density over the range of concentrations ordinarily employed.

Since it was desired to employ the ninhydrin procedure in the presence of either tris-(hydroxymethyl)-aminomethane-hydrochloric acid or phosphate buffers, experiments were conducted to determine the relationship between optical density and the concentration of ammonia in the presence of each of these buffers. Once again it was found that for both buffers the dependence of the optical density upon the concentration of ammonia was linear over the range of concentrations ordinarily used.

Standard calibration curves were prepared from solutions so adjusted as to duplicate actual experimental conditions and concentrations at selected time intervals of a reaction misture containing varying amounts of acetyl-L-tyrosinamide, acetyl-L-tyrosine, and ammonium chloride, and fixed amounts of enzyme and buffer. Typical calibration curves are presented in Figs. 1 and 2 in which the actual concentration of ammonia in the reaction mixture is a multiple of the abscissa value dependent upon the dilution employed. It was found that for concentrations of ammonia up to 0.04 M in the original reaction solution the optical density was directly proportional, within the limits of experimental error, to the concentration of ammonia.

The proportionality constant was found to be sensitive to the ninhydrin reagent and the amount of tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer present, but essentially independent of the other components of the reaction medium. For this reason, a check was made of the calibration curve each time a fresh sample of ninhydrin reagent was used in a determination. This was conveniently accomplished by preparing a solution containing all the components of the reaction system with the exception of the specific substrate, and another similar solution containing in addition to the above components a known amount of a standard ammonium chloride solution. These two samples were analyzed in conjunction with the reaction mixture in precisely the same manner, and variations in the optical density between the check samples and the standard calibration curve were taken into account in employing the standard calibration curve. This procedure merely represents a refinement of the method, for in no case were any serious discrepancies discovered between the standard calibration curve and the daily checks. For still additional accuracy, duplicate or sometimes triplicate samples were run on all analyses.

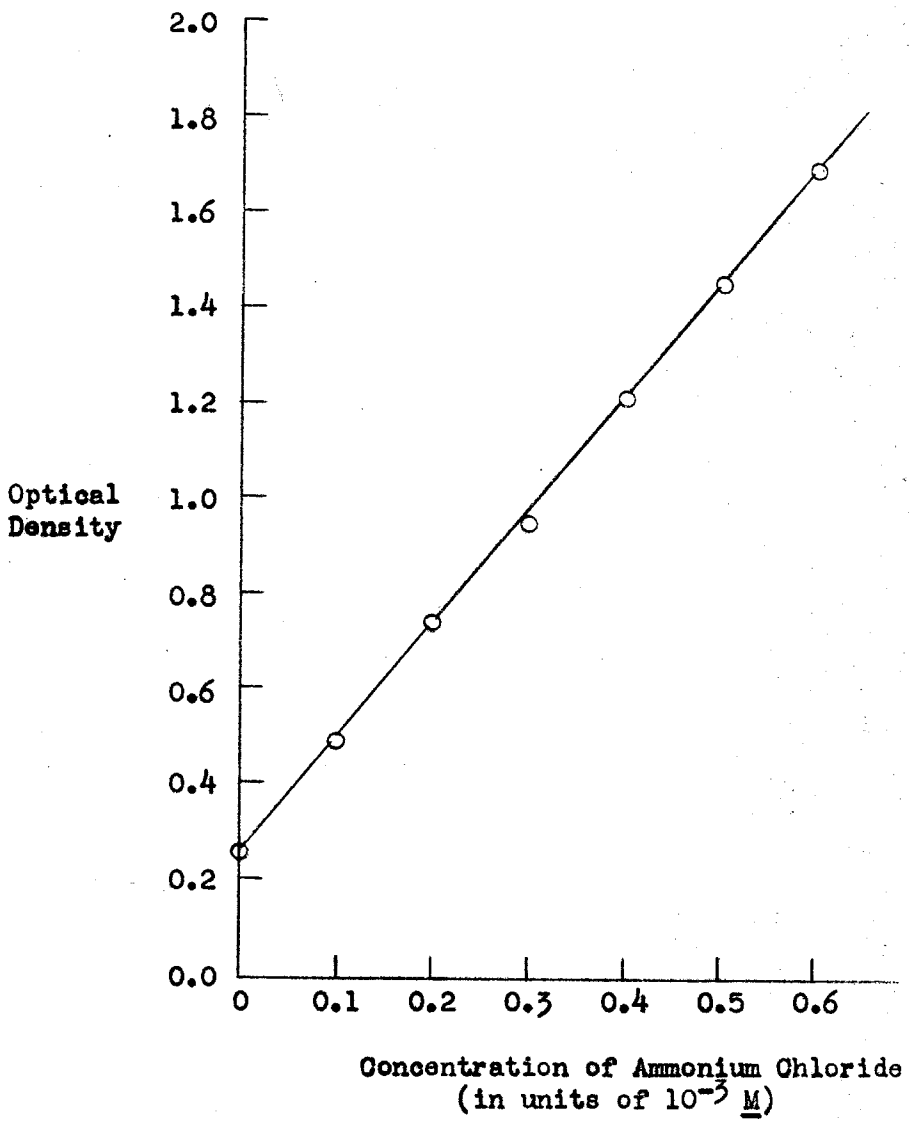


Fig. 1 Standard Calibration Curve for the System Acetyl-L-tyrosinamide, Acetyl-L-tyrosine, alpha-Chymotrypsin, Ammonium Chloride, and tris-(Hydroxymethyl)-aminomethane-hydrochloric Acid Buffer.

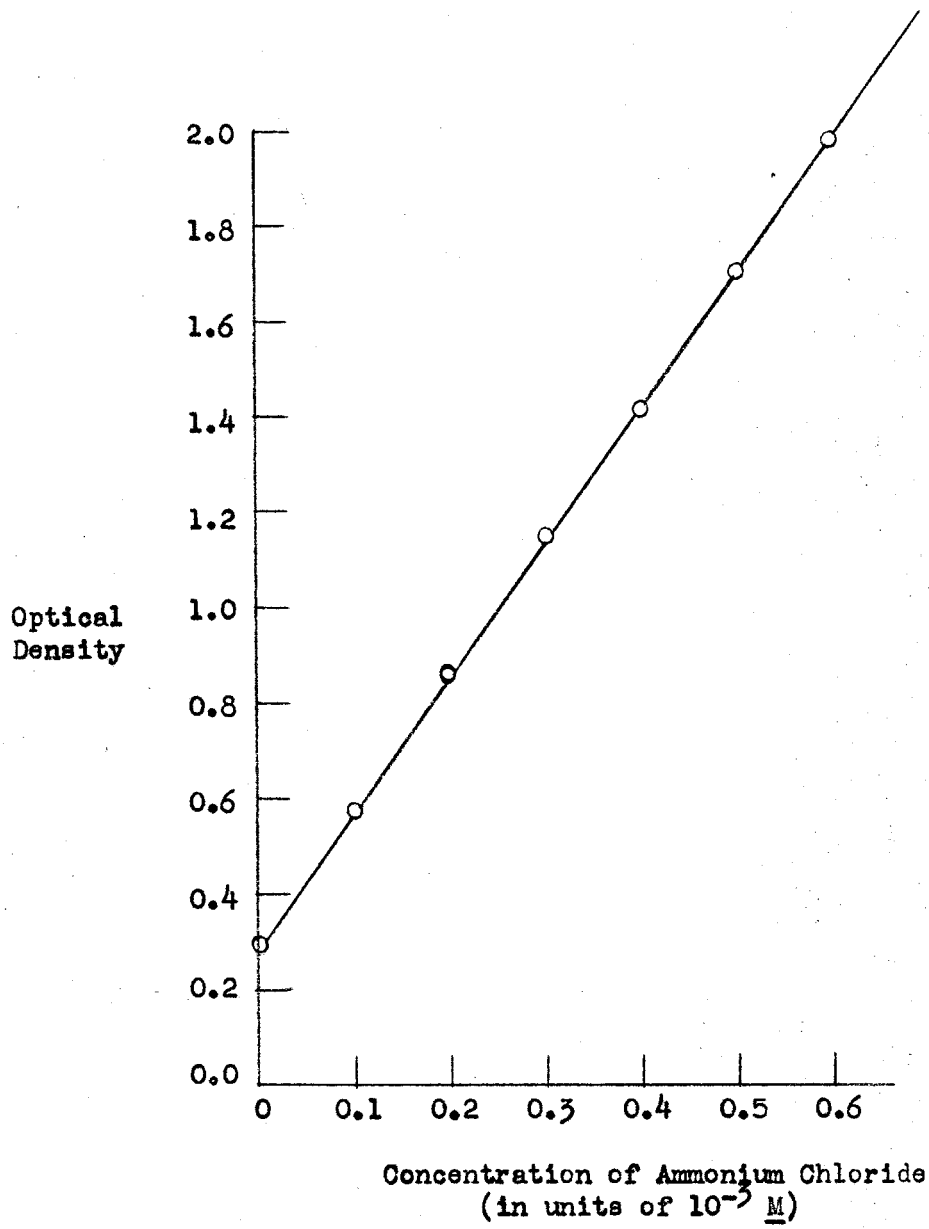


Fig. 2 Standard Calibration Curve for the System Acetyl-L-tyrosinamide, Acetyl-L-tyrosine, alpha-Chymotrypsin, Ammonium Chloride, and Phosphate Buffer.

COLOR DEVELOPMENT

Absorption Spectra

The ultraviolet absorption spectrum of the colored product formed by the reaction of 1.0 ml. of ninhydrin reagent and 4×10^{-4} M ammonium chloride was obtained by means of a Cary automatic recording spectrophotometer employing 5 cm. absorption cells. A plot of optical density as a function of wavelength is presented in Fig. 3. It may be seen (solid curve) that there are two absorption maxima, one being at 406 mμ and the other at 565 mμ.

To determine the effect of an amine buffer upon the colored product and its absorption spectrum, a solution of 1.0 ml. of ninhydrin reagent, 4×10^{-4} M ammonium chloride, and 1.2×10^{-3} M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer was heated, and the spectrum of the resulting product obtained as described above. This revealed (broken line) the appearance of two new maxima, one at 370 mμ and the other at 404 mμ, and a decrease in the optical density of the maximum at 565 mμ. See Fig. 3.

Finally a solution of only ninhydrin reagent and 1.2×10^{-3} M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer was examined. The spectrum of the colored product, obtained as described above, is presented in Fig. 3 (x-marked line).

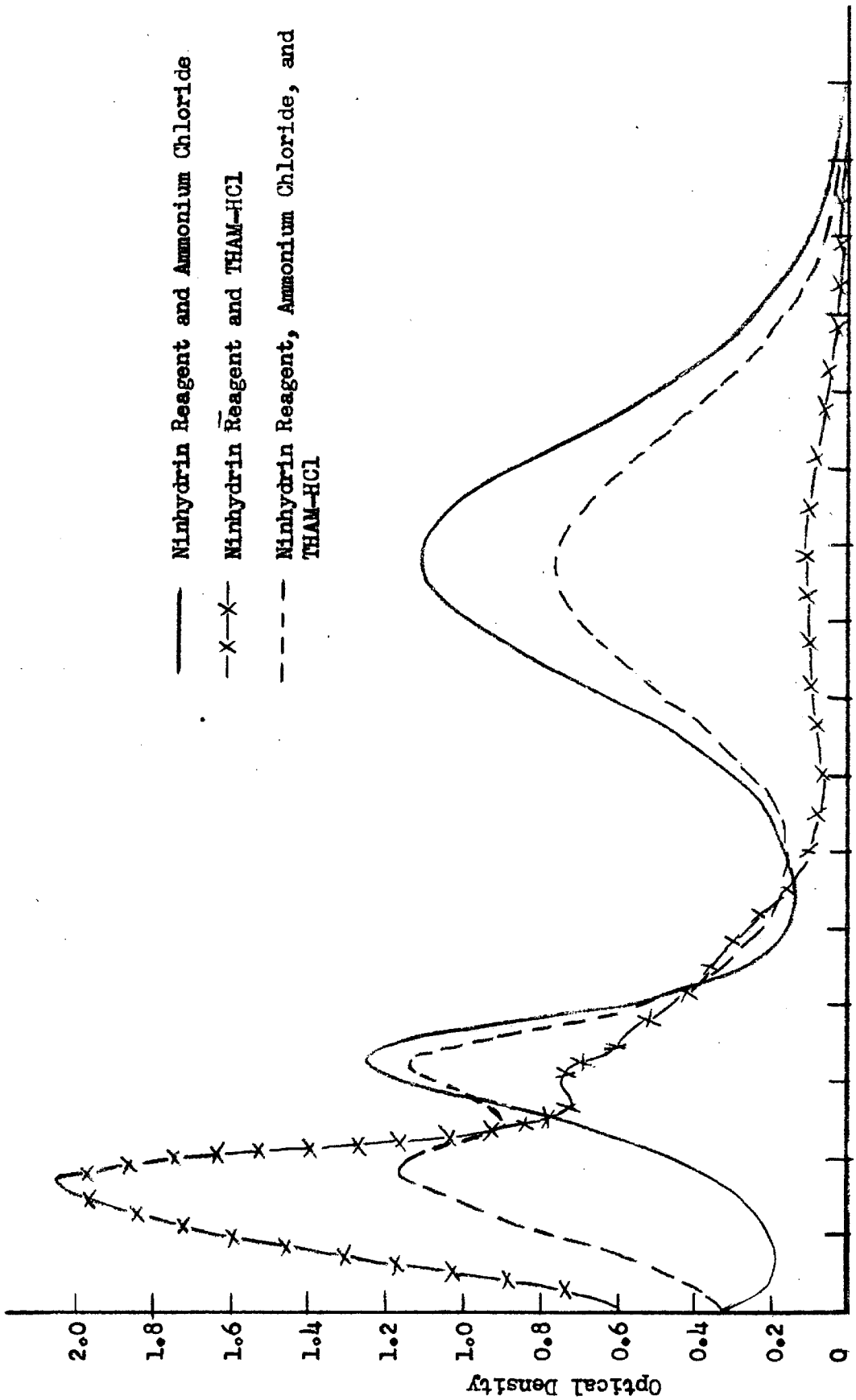


Fig. 3 The U.V. Absorption Spectra of the Colored Products Formed From Ninhydrin Reagent

The maximum at 370 mμ has increased greatly. The maximum at 406 mμ has become a small node, and the maximum at 565 mμ has virtually disappeared.

From the above observations, it was decided that there would be no interference from the amine buffer if the absorption maximum at 565 mμ was used in the analysis procedure.

Rate of Color Formation

The rates of color development have been determined for representative alpha-amino acids over a pH range of 4 - 7 (3). In all cases, at 100° the reaction was complete in less than twenty minutes. The author found, however, that continued heating for any length of time increased the optical density of the colored products, i.e., increased the color yield. Undoubtedly, part of this increased yield was due to the destruction of the reagent. It was therefore decided to heat the reaction for a specific length of time, i.e., twenty minutes. This assured the completion of the desired reaction, and minimized destruction of the reagent. To insure reproducible results, this twenty minute period was accurately timed with a stop watch for each experiment.

Stability of Color

Upon standing the colored product was found to fade gradually to a pale blue color. The optical density of an aliquot of a typical enzyme catalyzed reaction mixture was measured at

various time intervals, and it was found (see Table I) that the optical density decreased about three per cent per hour for the first two hours after the development of the color. Thus it was imperative to determine the optical density of the colored product as rapidly as feasible after the heating period.

Table I

DECREASE IN COLOR INTENSITY WITH TIME

Time (in min.)	Optical Density
0	1.265
8	1.256
26	1.240
62	1.214
73	1.208
108	1.185
120	1.180

Effect of pH on Color Development

Fig. 4 shows a plot of the intensity of color produced, expressed as optical density, for the ninhydrin reaction with various alpha-amino acids and ammonia as a function of the pH of the color development medium. From these data it may be observed that for the case of ammonia, at pH values greater than 5, the color intensity drops off quite rapidly as compared to alpha-amino acids of comparable concentrations. Apparently the reaction of ninhydrin with ammonia proceeds at a rate and in a manner different from that of

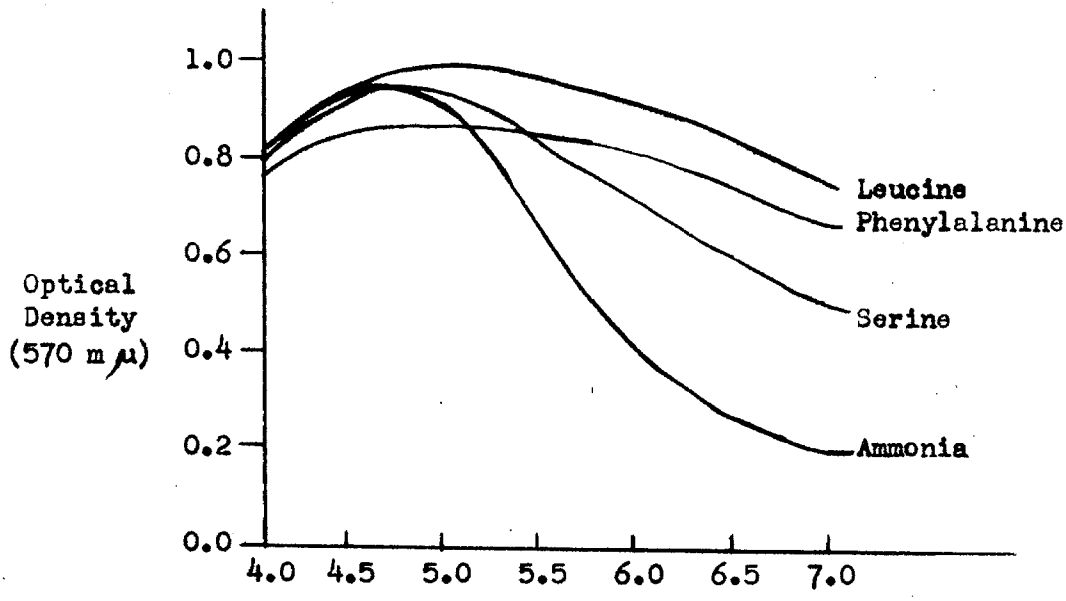


Fig. 4 The Effect of pH on the Intensity of Color Obtained After the Reaction of Ninhydrin With Amino Acids and Ammonia. After Moore and Stein (3).

the reaction of the reagent and alpha-amino acids.

From the above data it was evident that maximum color yields were achieved at pH values at or near 4.8, and the 0.2 M citrate buffer employed in this analytical procedure was adjusted to this value.

Effect of Temperature on Color Development

The color yield was decreased considerably when the color development reaction was carried out at temperatures below 95-100°.

Effect of Stannous Chloride on the Color Development

The presence of a reducing agent improved the color yield to a marked degree. However, the quantity of stannous chloride dihydrate employed was critical for two main reasons. First, for the analytical procedure described herein, the ratio of the molar concentration of ninhydrin to stannous chloride dihydrate was 32:1. When this ratio was markedly changed, e.g., 15.8:1, the color yield decreased about twenty-two per cent; when the ratio was further decreased to 10.5:1, the color yield decreased about twenty-nine per cent. Second, at the lower ratios, due to the relatively high concentration of stannous chloride to citrate buffer, the tin salt tended to precipitate from solution as the hydroxide. This decreased the effective concentration of the reducing agent and produced a turbid solution unsuited for colorimetric determinations.

Effect of Ninhydrin Reagent on Color Development

Several experiments were conducted in which the amount of ninhydrin reagent was varied. It is appropriate to recall that the customary quantity of reagent used in the analytical determinations is 1.0 ml. to 1.0 ml. of the diluted enzyme reaction mixture. When 0.5 ml. of the reagent was used, the color yield was diminished by a factor of approximately 1/4. When 2.0 ml. of the reagent were used, the color yield was increased by a factor of approximately 1/3. It must be emphasized that the ratio of ninhydrin to stannous chloride dihydrate was maintained at a constant value, i.e., 32:1, in the reagents employed for these experiments.

Choice of Diluent Solution

In early experiments water was used as the diluent, and marked fading of the color was noted as a result of the precipitation of the sodium salt of diketohydrindylidene-diketohydrindamine. The use of a 1:1 water-isopropanol solution as the diluent served to keep the relatively insoluble reaction product in solution. It has been reported that n-propanol (3) and ethanol (16) may be used in place of isopropanol with equal success.

Choice of Solvent for Ninhydrin

Among the solvents tested, methyl cellosolve had the highest solvent power for hydrindantin (3). The solvent mixture chosen for the reagent, i.e., a ratio of 1:1 for water (citrate

buffer)-methyl cellosolve, did not readily evaporate in the water bath at 100°, and did not precipitate sodium citrate from the buffer.

Choice of Buffer for Color Development

The primary consideration for the choice of a buffer was that it would be capable of maintaining the pH of the color development medium effectively at values at or near 5. In addition the buffer must not cause interference with the reducing agent. Citrate met these requirements admirably. It prevented the tin from precipitating, and had good buffering capacity at pH 5. Acetate buffer was tested, and it was found that the tin precipitated from this medium, presumably as the hydroxide.

REFERENCES

(1) Martin, A. J. P., and Synge, R. L. M., "Advances in Protein Chemistry", II, Academic Press Inc., New York, 1945.

(2) Iselin, B. M., Huang, H. T., and Niemann, C., J. Biol. Chem., 183, 403 (1950).

(3) Moore, S., and Stein, W. H., ibid., 176, 367 (1948).

(4) Noller, C. R., "Chemistry of Organic Compounds", W. B. Saunders Co., Philadelphia, 1951.

(5) Ruhemann, S., J. Chem. Soc., 97, 1438 (1910); 99, 792; 1491 (1911).

(6) MacFadyen, D. S., J. Biol. Chem., 153, 507 (1944).

(7) Troll, W., and Cannan, R. K., ibid., 200, 803 (1951).

(8) Schwert, G. W., ibid., 174, 411 (1948).

(9) Schwartz, T. B., and Engel, F. L., ibid., 184, 197 (1950).

(10) Herzfeld, E., Biochem. Z., 59, 249 (1914).

(11) Neuberg, C., ibid., 56, 500 (1913).

(12) Abderhalden, R., and Schmidt, H., Z. physiol. Chem., 1911, 37.

(13) Harding, V. J., and Warneford, F. H. S., J. Biol. Chem., 25, 319 (1916).

(14) Halle, W., Loewenstein, E., and Pribram, E., Biochem. Z., 55, 357 (1913).

(15) Stephen, H., J. Chem. Soc., 1930, 2786.

(16) Moore, S., and Stein, W. H., J. Biol. Chem., 211, 907 (1954).

PART III

THE EFFECT OF BUFFER SPECIES AND IONS UPON THE KINETICS OF alpha-CHYMOTRYPSIN CATALYZED REACTIONS

INTRODUCTION

In an effort to clarify and explain the differences existing between the enzyme-inhibitor dissociation constants, i.e., K_I values, reported by Neurath and Gladner (1) and those reported by Huang and Niemann (2) for several anionic, bifunctional, competitive inhibitors of the enzyme alpha-chymotrypsin, it was thought desirable to reexamine several of the experiments performed by both groups of investigators.

Reference to Table I indicates that in each case the K_I values reported by Huang and Niemann (2) are very nearly five times those reported by Neurath and Gladner (1). It should be noted that Neurath and Gladner (1) employed 0.1 M phosphate buffers in their studies, and that Huang and Niemann (2) employed 0.02 M THAM-HCl[1] buffers. Thus it became apparent that there was a fundamental difference in the behavior of the two buffer media toward certain anionic, bifunctional, competitive inhibitors of the enzyme.

With the development of a suitable analytical method for following the kinetics of amide hydrolysis in both phosphate and THAM-HCl buffers (see Part II of this thesis), it was decided to examine the kinetics of the alpha-chymotrypsin catalyzed hydrolysis

[1] A solution 0.02 M in the THAM component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer, henceforth referred to as a THAM-HCl buffer.

Table I

ENZYME-INHIBITOR DISSOCIATION CONSTANTS

Inhibitor	H and N[a] K_I[c]	N and G[b] K_I[d]
beta-(beta-Indole)-propionate	15 ± 3	2.5
gamma-(beta-Indole)-butyrate	23 ± 5	3.6
beta-Phenylpropionate	25 ± 5	5.5
gamma-Phenylbutyrate	60 ± 10[e]	14
Phenylacetate	200 ± 50[e]	42
Benzoate	150 ± 50[e]	42

a., Cf. refs. (7) and (18).

b., Cf. refs. (7) and (14).

c., In units of 10^{-3} M at 25° and pH 7.9 in aqueous systems 0.02 M in respect to the THAM component of a THAM-HCl buffer.

d., In units of 10^{-3} M at 25° and pH 7.8 in systems 0.1 M in phosphate buffer.

e., Value subject to correction for ionic strength effects.

of a specific substrate, i.e., acetyl-L-tyrosinamide, in both buffer systems, and to evaluate the enzyme-inhibitor dissociation constants of selected competitive inhibitors of the enzyme against this substrate in the presence of each of the buffer systems.

Some preliminary kinetic experiments were performed in which the hydrolytic reaction was limited to thirty per cent. For these cases plots of optical density versus time were used to estimate initial velocities, and the corrected initial velocities at zero time, v_o, estimated by the procedure described by Jennings and Niemann (3). The quantity of ammonia in each of the samples at various times was determined by means of a previously prepared plot of optical density versus ammonia concentration, and from this information the various values of $[S]_t$ determined. Then $\ln[S]_t$ was plotted against time, and the corrected initial velocities estimated as described by Jennings and Niemann (3). The corrected initial velocities obtained from both "zero" and "first" order plots differed in no case by more than $\pm$ 5 %. A plot of v_o versus $v_o/[S]_o$ (4-6) was then used to determine K_S and k_3.

The above procedure has been modified by Foster and Niemann (7) in an effort to achieve greater precision, and the vast majority of the data described in this part of the thesis has been evaluated by their method. The following is a brief description of their procedure.

For zone A conditions (8, 9) and where $1/K_P = \sum_{j=1}^{n} 1/K_{P_j}$, equation (18)[1] may be simplified and rearranged to give equation (33),

$$k_3 [E] t = (1 - K_S/K_P)([S]_o - [S]_t) + K_S (1 + [S]_o/K_P) \ln [S]_o/[S]_t \qquad (33)$$

from which it may be seen that for an experiment conducted at a particular initial specific substrate concentration, a plot of $([S]_o - [S]_t)/t$ versus $\ln([S]_o/[S]_t)/t$ will be linear and will have a slope equal to $-K_S(K_P + [S]_o)/(K_P - K_S)$. A valuable feature of a plot with the above parameters is that straight lines drawn through the origin will have slopes equal to the respective $[S]_o$ values, and therefore, the intersection of a line drawn through the origin with a slope corresponding to the initial specific substrate concentration used in a particular experiment with a line defined by the experimental data obtained from that experiment, i.e., the line whose slope is equal to $-K_S(K_P + [S]_o)/(K_P - K_S)$, will define in terms of the ordinate the corresponding initial velocity, i.e., v_o. Furthermore, a straight line drawn through a series of these intersections, derived from a series of experiments with the same enzyme concentration but with different initial specific substrate concentrations, will have a slope of $-K_S$, an ordinate intercept of $k_3 [E]$ and an abscissa

---

[1] See Part I, Page 9, of this thesis.

intercept of $k_3[E]/K_S$. While it is true that a line possessing the same characteristics may also be obtained by a plot of v_o versus $v_o/[S]_o$ (4-6), it should be noted that in this instance the initial velocities, i.e., the v_o values, must be evaluated by separate procedures whose disadvantages and limitations have been discussed above and by Foster and Niemann (7).

In previous studies conducted by Niemann and co-workers (7, 10-13) it has been shown that with a number of acylated alpha-amino acid amides only one of the hydrolysis products, i.e., the carboxylate ion derived from the acylated alpha-amino acid, may competitively inhibit the hydrolytic reaction, and that the other hydrolysis product, i.e., ammonia, or the corresponding monoprotonated species, is without effect even when present in concentrations which are considerably greater than those of the specific substrate. Hence, K_P is equal to K_{P_1} in these instances. Thus with knowledge of the K_{P_1} value of the carboxylate ion of the acylated alpha-amino acid, and the K_S and k_3 values of the corresponding specific substrate, it is possible to describe, within the limits of experimental error, the alpha-chymotrypsin catalyzed hydrolysis of a number of specific substrates over a substantial portion of the reaction in terms of equation (33) (7, 10, 11, 13).

The procedure described by Foster and Niemann (7) may be extended to accommodate the presence of an added competitive

inhibitor and to evaluate K_I, the corresponding enzyme-inhibitor dissociation constant. The integrated rate equation for this situation is:

$$k_3 [E] t = (1 - K_S/K_P)([S]_o - [S]_t) + K_S(1 + [S]_o/K_P + [I]/K_I) \ln [S]_o/[S]_t. \quad (34)$$

In this instance the slopes of the lines through the experimental points are equal to $-K_S(K_P + [S]_o + [I] K_P/K_I)/(K_P - K_S)$, and the slope of the line drawn through the intersections of these lines with those of slope $[S]_o$ drawn through the origin is $-K_S(1 + [I]/K_I$. The ordinate intercept of the latter line is $k_3 [E]$ and its abscissa intercept is $k_3 [E]/K_S(1 + [I]/K_I)$.

In the following discussions, care has been taken to specify, as completely as possible, the reaction conditions which were employed for each particular set of experiments, and in every case attention has been directed to those parameters, i.e., $[E]$, E'_S, E'_I, S'_S, and I'_I that must be maintained within certain limits (8, 9, 14) in order to satisfy the assumptions inherent in the various treatments.

THE KINETICS OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE IN THE PRESENCE OF THAM-HCl BUFFER

Data and Results

The present experiments were all performed at 25° and pH 7.9 ± 0.1 in aqueous solutions 0.02 M with respect to the THAM component of a THAM-HCl buffer employing the ninhydrin method of analysis. Except as noted, the enzyme concentration corresponded to 0.200 mg. protein-nitrogen/ml. of reaction mixture.

Care has been taken to assure, as completely as possible, that the enzyme reactions were carried out in such a manner as to maintain zone A conditions (8, 9). Evaluation of the experimental data which are presented in Table XI and Fig. 3 according to the procedure described by Foster and Niemann (7) gave the following kinetic constants for the specific substrate acetyl-L-tyrosinamide:

$$K_S = 34 \pm 2 \times 10^{-3}\ M$$

$$k_3 = 2.5 \pm 0.1 \times 10^{-3}\ M/\text{min./mg. protein-nitrogen/ml.}$$

$$K_{P_1} = 80 \pm 20 \times 10^{-3}\ M.$$

The above K_S, k_3, and K_{P_1} values were obtained by a least squares treatment of the data from ten experiments at values of $[S]_o$

ranging from 5 X 10^{-3} M to 45 X 10^{-3} M, i.e., $S'_S = [S]/K_S$ was between the limits of 0.146 and 1.28.

In the least squares treatment employing the equation $y = a + bx$, the value of $a = V$ was 0.503 ± 0.025 X 10^{-3} M/min. and that of $b = -K_S$ was - 34 ± 2 X 10^{-3} M. The limits express the calculated probable error in the constants.

For those experiments which were conducted at enzyme concentrations other than 0.200 mg. protein-nitrogen/ml. (see Tables VIII - XII), initial velocity values were extrapolated to their respective values at an enzyme concentration corresponding to 0.200 mg. protein-nitrogen/ml., these data combined with the data presented above, obtained at $[E]$ = 0.200 mg. protein-nitrogen/ml., and evaluated by the method of least squares. This procedure gave the following kinetic constants for the specific substrate acetyl-L-tyrosinamide:

$$K_S = 34 \pm 4 \times 10^{-3} \text{ M}$$

$$k_3 = 2.5 \pm 0.3 \times 10^{-3} \text{ M/min./mg. protein-nitrogen/ml.}$$

$$K_{P_1} = 80 \pm 20 \times 10^{-3} \text{ M.}$$

The above K_S, k_3, and K_{P_1} values were obtained from a total of thirty-three experiments conducted at values of $[S]_o$ ranging from 3 X 10^{-3} M to 45 X 10^{-3} M, i.e., S'_S was between the limits of 0.088 and 1.28. In the least squares treatment employing the

equation $y = a + bx$, the value of $a = V$ was $0.505 \pm 0.06 \times 10^{-3}$ M/min., and that of $b = -K_S$ was $-34 \pm 4 \times 10^{-3}$ M. The limits express the probable error in the constants.

There is reason to believe (15-17) that at concentrations of the order of 10^{-5} M or less, alpha-chymotrypsin is present in aqueous solutions essentially in the form of the monomer. The assumption has been made that the molecular weight of monomeric alpha-chymotrypsin is 22,000, and for the purposes of calculation that its nitrogen content is 16 % (14). On this basis a solution which contains 0.100 mg. protein-nitrogen/ml. is equal to 2.84×10^{-5} M in this enzyme. Thus, in the above experiments, it may be noted that $[E]$ was between the limits of 0.76×10^{-5} M and 6.3×10^{-5} M, and $E'_S = [E]/K_S$ was between the limits of 0.022×10^{-2} and 0.185×10^{-2}, and at no time exceeded 0.0019, a value far below the maximum permissible value of 0.1 associated with observations which may be in error by ± 10 per cent.

Table II presents a comparison of the three constants K_S, k_3, and K_{P_1} for the specific substrate acetyl-L-tyrosinamide obtained by the author with those constants obtained by Thomas, MacAllister, and Niemann (10, 14). In view of the fact that the values presented in Table II were obtained from independent investigations in which different enzyme preparations and analytical methods were used, the agreement appears to be excellent.

Table II

KINETIC CONSTANTS FOR THE SYSTEM alpha-CHYMOTRYPSIN-ACETYL-L-TYROSINAMIDE

Investigator	K_S [a]	k_3 [b]	K_{P_1} [a]
Bernhard	34 ± 4	2.5 ± 0.3	80 ± 20
Niemann et al. [c]	32 ± 4	2.4 ± 0.3	110 ± 30

a., In units of 10^{-3} M at 25° and pH 7.9 in aqueous solutions 0.02 M in the THAM component of a THAM-HCl buffer.

b., In units of 10^{-3} M/min./mg. protein-nitrogen/ml.

c., Cf. refs. (10, 14).

IN THE PRESENCE OF PHOSPHATE BUFFERS

The pH-Activity Relationship for Acetyl-L-tyrosinamide

The pH optima for the alpha-chymotrypsin catalyzed hydrolysis of all previously investigated acyl derivatives of L-tyrosinamide have been found to be within the region of pH 7.75 to 8.0. Thomas, MacAllister, and Niemann (10) conducted experiments which determined the pH optimum for the system acetyl-L-tyrosinamide, $[S]_o = 20 \times 10^{-3}$ M; $[E] = 0.125$ mg. protein-nitrogen/ml.; and 0.02 M THAM-HCl buffer. They reported the optimum at pH 7.9 ± 0.1 at 25°. They also conducted similar experiments using a 0.02 M ethylenediamine-hydrochloric acid buffer. These latter experiments also gave a pH optimum of 7.9 ± 0.1 at 25°.

In view of the fact that phosphate buffers have been known to produce results which are not strictly comparable in all cases with THAM-HCl buffers (2), it was deemed desirable to investigate the pH-activity relationship for acetyl-L-tyrosinamide in phosphate buffer solutions.

The pH-activity relationship for the system acetyl-L-tyrosinamide, $[S]_o = 5 \times 10^{-3}$ M; $[E] = 0.200$ mg. protein-nitrogen/ml.; in aqueous solutions at 25° was determined by performing a number of hydrolysis experiments buffered at the desired pH with a 0.02 M sodium phosphate buffer solution. In employing this buffer

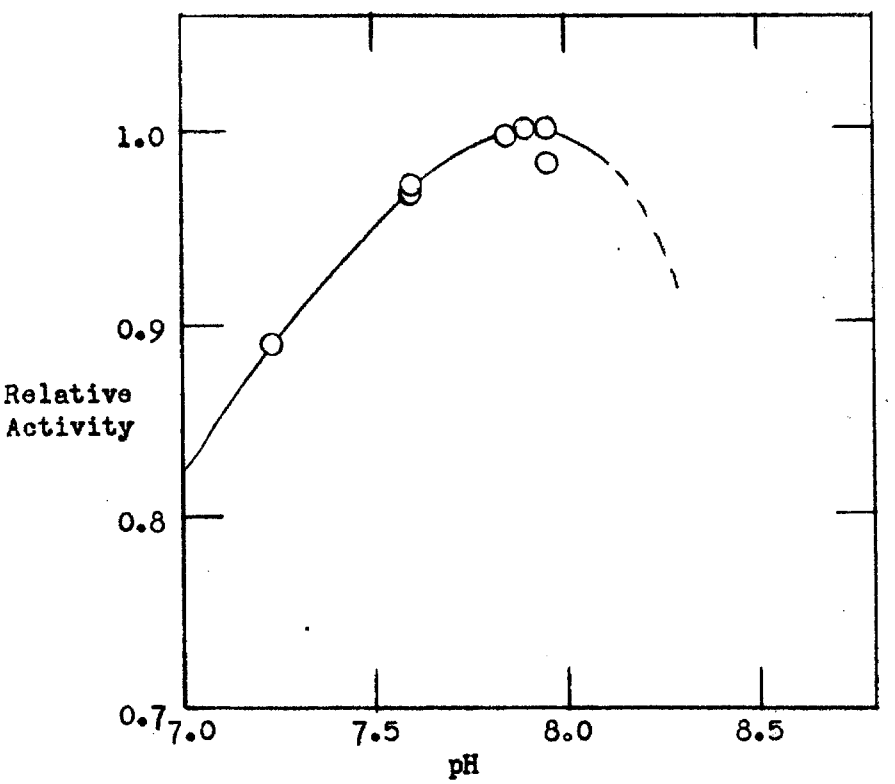
it was only possible to work over a rather limited range of pH. It was found that considerable difficulty was encountered in maintaining a constant pH above 7.95 with this low a concentration of phosphate buffer.

The procedure was to determine the extent of hydrolysis by the colorimetric ninhydrin method described in Part II of this thesis. In Fig. 1 the relative activity[1] is plotted versus the pH of the reaction media. The experimental data are presented in Table XIII. The pH-activity curve for the substrate, acetyl-L-tyrosinamide, in the presence of 0.02 M sodium phosphate buffer is identical, within the limits of experimental error, to those curves obtained in 0.02 M THAM-HCl buffer and in 0.02 M ethylenediamine-hydrochloric acid buffer (10). Thus for the experimental conditions described above, the pH optimum was found to be 7.9 ± 0.1 at 25°.

Data and Results

The following experiments were performed at 25° and pH 7.9 ± 0.1 in aqueous solutions 0.1 M with respect to phosphorus present as phosphate of a sodium phosphate buffer, and, except as noted, an enzyme concentration corresponding to 0.200 mg. protein-

[1] As measured by the extent of hydrolysis after 110 minutes; thus, the greatest degree of hydrolysis equaled a relative activity of 1.0, and the other degrees of hydrolysis were assigned proportionate values.



pH-Activity Curve

Fig.1 Acetyl-L-tyrosinamide, $[S]_o = 5 \times 10^{-3}$ M; $[E] = 0.200$ mg. protein-nitrogen/ml.; 0.02 M phosphate buffer; $T = 25^{\circ}$.

nitrogen/ml. of reaction solution. The ninhydrin method of analysis was employed.

Again, as indicated previously, care has been taken to assure, as completely as possible, that the enzyme reactions were carried out in such a manner as to maintain zone A conditions (8, 9). Evaluation of the primary data presented in Table XIV according to the method described by Foster and Niemann (7) gave the following values for the specific substrate acetyl-L-tyrosinamide:

$$K_S = 32 \pm 2 \times 10^{-3} \underline{M}$$

$$k_3 = 3.3 \pm 0.2 \times 10^{-3} \underline{M}/\text{min./mg. protein-nitrogen/ml.}$$

$$K_{P_1} = 70 \pm 20 \times 10^{-3} \underline{M}.$$

The line whose slope equals - K_S that best fitted the experimental data was obtained by inspection, and the limits indicated are the maximum observable limits of error in the constants.

The above constants were evaluated in systems such that $S'_S = 0.0625$ to 1.25 and $E'_S = 0.185 \times 10^{-2}$.

Effect of Buffer Cations

In order to determine the effect of buffer cations upon the course of the hydrolytic reaction, experiments were performed at 25° and pH 7.9 ± 0.1 in aqueous solutions 0.1 M with respect to phosphorus present as phosphate of a potassium phosphate buffer, and, except as noted, with an enzyme concentration corresponding

to 0.200 mg. protein-nitrogen/ml. of reaction solution. The ninhydrin method of analysis was employed. Evaluation of the primary data presented in Table XV and Fig. 4 by the method of Foster and Niemann (7) gave the following values for the specific substrate acetyl-L-tyrosinamide:

$$K_S = 33 \pm 2 \times 10^{-3} \underline{M}$$

$$k_3 = 3.25 \pm 0.15 \times 10^{-3} \underline{M}/\text{min.}/\text{mg. protein-nitrogen/ml.}$$

$$K_{P_1} = 80 \pm 20 \times 10^{-3} \underline{M}$$

and

$$S'_S = 0.11 - 1.25$$

$$E'_S = 0.185 \times 10^{-2}.$$

The line whose slope equals - K_S that best fitted the experimental data was obtained by inspection, and the limits indicated are the maximum observable limits of error in the constants.

Thus, in the reaction systems described above employing 0.1 M phosphate buffers, there is no detectable difference, within the limits of experimental error, between the effect of sodium or potassium buffer cations upon the course of the alpha-chymotrypsin catalyzed hydrolysis of the specific substrate acetyl-L-tyrosinamide, under the conditions described above, as reflected by the kinetic constants K_S, k_3, and K_{P_1}, or by changes in the initial velocities,

i.e., v_o values, of the individual reactions.

Table III presents a comparison of the three kinetic constants K_S, k_3, and K_{P_1} for the specific substrate acetyl-L-tyrosinamide obtained by the author from kinetic experiments conducted in the presence of both 0.1 M potassium and 0.1 M sodium phosphate buffers with those constants obtained by Neurath and coworkers (18-21) in 0.1 M phosphate buffer.

Once again it may be noted that the values presented in Table III were obtained from independent investigations in which widely different enzyme preparations and analytical methods were used. In view of these facts, the values presented above show good agreement.

It may be noted in the above experiments that S'_S was between the limits of 0.0625 and 1.25, and E'_S was equal to 0.185×10^{-2}, thus meeting the conditions set for zone A (8, 9).

Table III

KINETIC CONSTANTS FOR THE SYSTEM alpha-CHYMOTRYPSIN-ACETYL-L-TYROSINAMIDE

Investigator	Buffer Cation	K_S [a]	k_3 [b]	K_{P_1} [a]
Bernhard	Na	32 ± 2 [c]	3.3 ± 0.2 [c]	70 ± 20 [c]
Bernhard	K	33 ± 2 [c]	3.25 ± 0.15 [c]	80 ± 20 [c]
Neurath et al.	- [d]	23 [cde]	2.7 [cde]	-
	- [d]	27 [cdf]	3.0 [cdf]	-
	- [d]	29 [cdg]	3.1 [cdg]	-
	- [d]	32.6 [cdh]	2.7 [cdh]	-
Niemann et al.	-	32 ± 4 [i]	2.4 ± 0.3 [i]	110 ± 30 [i]

a., In units of 10^{-3} M.

b., In units of 10^{-3} M/min./mg. protein-nitrogen/ml.

c., At 25° and pH 7.9 ± 0.1 in aqueous systems 0.1 M in phosphate buffer.

d., Unspecified buffer cation.

e., Cf. ref. (18).

f., Cf. ref. (19).

g., Cf. ref. (20).

h., Cf. ref. (21).

i., At 25° and pH 7.9 in aqueous systems 0.02 M in the THAM component of a THAM-HCl buffer.

EFFECT OF PHOSPHATE BUFFER CONCENTRATION AND IONIC STRENGTH

Data and Results

The following experiments were performed at 25° and pH 7.9 ± 0.1 in aqueous solutions and in the presence of various amounts of phosphate buffers. The specific substrate was acetyl-L-tyrosinamide. The enzyme preparation employed was a fresh sample of the salt-free type with a concentration corresponding to 0.200 mg. protein-nitrogen/ml. The same precautions, as noted above, were taken to assure adherence to zone A conditions (8, 9). The ninhydrin method was employed for all determinations. The experimental data evaluated by the method described by Foster and Niemann (7) gave the following kinetic constants:

Acetyl-L-tyrosinamide

The experimental data are presented in Table XVI. Sodium phosphate buffer concentration = 0.2 M

$$K_S = 30 \pm 2 \times 10^{-3} \text{ M}$$

$$k_3 = 3.85 \pm 0.3 \times 10^{-3} \text{ M/min./mg. protein-nitrogen/ml.}$$

$$K_{P_1} = 82 \pm 20 \times 10^{-3} \text{ M}$$

and

$[E] = 5.7 \times 10^{-5}$ M

$S'_S = 0.167 - 1.33$

$E'_S = 0.19 \times 10^{-2}$

Acetyl-L-tyrosinamide

The experimental data are presented in Table XVII.

Sodium phosphate buffer concentration = 0.4 M

$K_S = 31 \pm 3 \times 10^{-3}$ M

$k_3 = 4.95 \pm 0.3 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml.

$K_{P_1} = 76 \pm 20 \times 10^{-3}$ M

and

$[E] = 5.7 \times 10^{-5}$ M

$S'_S = 0.161 - 1.29$

$E'_S = 0.18 \times 10^{-2}$.

For the above two cases, the line whose slope equals - K_S that best fitted the experimental data was obtained by inspection, and the limits indicated are the maximum observable limits of error in the constants.

Discussion

It should be noted from an examination of Table III that although quite good agreement exists between the various K_S and

K_{P_1} values which were determined from experiments performed in the presence of both buffer media, i.e., THAM-HCl and phosphate, there is an apparent lack of agreement, well outside of the limits of experimental error, between the values of k_3 evaluated in the presence of THAM-HCl buffers and those evaluated in the presence of phosphate buffers.

Since the studies in the presence of phosphate buffers were conducted with molar concentrations of the buffer five times greater than those conducted in the presence of THAM-HCl buffers, it appeared reasonable to assume that changes in buffer concentration, or possibly ionic strength, could have an appreciable effect upon the values of k_3. Indeed Jandorf (22) and Shine and Niemann (12) reported that added magnesium sulfate increased the esterase and amidase activity of the enzyme alpha-chymotrypsin. The former reported that the effect can be duplicated with other salts, while no increase in esterase activity was found in the presence of non-electrolytes. In this connection Shine (23) has demonstrated that for the system alpha-chymotrypsin-chloroacetyl-L-tyrosinamide-0.02 M THAM-HCl buffer, added sodium chloride and potassium chloride increase the velocity of the hydrolytic reaction, and Niemann (24) has shown from Shine's data that plots of ln v_o increase linearly with $M^{\frac{1}{2}}$, where M is the concentration of added

salt expressed in moles[1].

The author examined reaction systems employing various concentrations of phosphate buffers, i.e., 0.1, 0.2, and 0.4 M[2], and found that the values of the kinetic constants K_S and K_{P_1} do not appear to differ significantly, within the limits of experimental error, from those values of the two constants evaluated in systems employing a 0.02 M THAM-HCl buffer.

Reference to Table IV indicates that the values of the kinetic constant k_3, evaluated in the presence of phosphate buffers, do differ significantly from the value of k_3 evaluated in the presence of a 0.02 M THAM-HCl buffer.

Apparently the value of k_3 increases with increasing phosphate buffer concentration and/or with increasing ionic strength. Numerous plots, employing the experimental data presented in Table IV, were made of the various relationships that may possibly exist between the constant k_3 and ionic strength, e.g., log k_3 versus the square root of the ionic strength of the reaction media, etc., and between k_3 and phosphate concentration. These, however,

---

[1] Shine has also demonstrated that several non-electrolytes, e.g., sucrose and glucose, are capable of increasing the velocity of the hydrolytic reaction, at least in the case of the specific substrate, chloroacetyl-L-tyrosinamide.

[2] Upper limit of buffer concentrations examined because of buffer component solubility.

failed to disclose any precise linear relationship between k_3, or a function thereof, and buffer concentration, or ionic strength, or a function thereof.

The ionic strength of each of the reaction mixtures was calculated on the basis of the concentration of buffer species present[1], and all other ions in the reaction medium. The following equation was employed:

$$\mu = \frac{1}{2} \sum_i m_i z_i^2$$

where μ is the ionic strength, m_i the molality of the ion, and z_i the valence or charge of the ion.

Presented in Table IV is a summary of all of the kinetic constants, i.e., K_S, k_3, and K_{P_1} values, for the specific substrate acetyl-L-tyrosinamide evaluated in the presence of a 0.02 M THAM-HCl buffer and in the presence of phosphate buffers of increasing concentrations.

---

[1] Employing the following dissociation constants for phosphoric acid: $k_1 = 1.1 \times 10^{-2}$, $k_2 = 7.5 \times 10^{-8}$, and $k_3 = 4.8 \times 10^{-13}$, (25).

Table IV

KINETIC CONSTANTS FOR ACETYL-L-TYROSINAMIDE

Buffer	K_S [a]	K_{P_1} [a]	k_3 [b]	μ [c]	log k_3	$\mu^{\frac{1}{2}}$
0.02 M THAM-HCl	34 ± 4	80 ± 20	2.5 ± 0.3	0.01	0.398	0.10
0.1 M Phosphate[d]	33 ± 2	80 ± 20	3.25 ± 0.15	0.27	0.512	0.52
0.1 M Phosphate[e]	32 ± 2	70 ± 20	3.3 ± 0.2	0.27	0.518	0.52
0.2 M Phosphate [e]	30 ± 2	82 ± 20	3.85 ± 0.3	0.54	0.585	0.735
0.4 M Phosphate[e]	31 ± 3	76 ± 20	4.95 ± 0.3	1.08	0.695	1.04

a., In units of 10^{-3} M at 25° and pH 7.9 ± 0.1 in aqueous systems.

b., In units of 10^{-3} M/min./mg. protein-nitrogen/ml.

c., Molal.

d., Potassium salt employed.

e., Sodium salt employed

THE KINETICS OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TRYPTOPHANAMIDE

To preclude the possibility that the effects described above were caused in some manner by the precise nature of the specific substrate, an examination was made of the effect of phosphate buffer upon another specific substrate of alpha-chymotrypsin, i. e., acetyl-L-tryptophanamide.

Data and Results

The following experiments were performed at 25° and pH 7.9 ± 0.1 in aqueous solutions 0.1 M in potassium phosphate buffer, and with an enzyme concentration corresponding to 0.222 mg. protein-nitrogen/ml. The ninhydrin method of analysis was employed. Again care was taken to ensure, as completely as possible, that the enzyme reactions were carried out in such a manner as to maintain zone A conditions (8, 9). The primary data, presented in Table XVIII and Fig. 5, were evaluated by the method of Foster and Niemann (7). The line whose slope equals - K_S that best fitted the experimental data was obtained by inspection, and the limits indicated represent the maximum observable limits of error in the constants described below. The following values were obtained for the kinetic constants of the specific substrate acetyl-L-tryptophanamide:

$$K_S = 5.7 \pm 0.3 \times 10^{-3} \underline{M}$$

$$k_3 = 0.68 \pm 0.02 \times 10^{-3} \underline{M}/\text{min./mg. protein-nitrogen/ml.}$$

$$K_{P_1} = 7.3 \pm 0.3 \times 10^{-3} \underline{M}.$$

The above kinetic constants were evaluated in systems such that S'_S was between the limits of 1.75 and 7.05, and E'_S was equal to 0.11×10^{-2}. Thus, these values were well within the limits imposed by zone A conditions (8, 9).

The kinetic constants for the specific substrate acetyl-L-tryptophanamide have been determined at 25° and pH 7.9 in aqueous systems 0.02 M in the THAM component of a THAM-HCl buffer and their values reported by Huang and Niemann (11, 14). They are as follows:

$$K_S = 5.0 \pm 0.5 \times 10^{-3} \underline{M}$$

$$k_3 = 0.55 \pm 0.1 \times 10^{-3} \underline{M}/\text{min./ mg. protein-nitrogen/ml.}$$

$$K_{P_1} = 8.0 \pm 1.0 \times 10^{-3} \underline{M}.$$

It may be observed that the constants K_S and K_{P_1}, as before, appear to be relatively insensitive to changes in buffer species and/or ionic strength. However, a pronounced increase may be noted in the value of k_3 in changing from 0.02 M THAM-HCl buffer to 0.1 M potassium phosphate buffer. These data would tend to indicate that the increase in the values of the kinetic constant k_3 observed for the case of the

specific substrate acetyl-L-tyrosinamide when this constant was evaluated in the presence of phosphate buffers of increasing concentrations is part of a more general phenomenon associated in some manner with the buffer system and/or the ionic strength of the reaction media and is thus not limited to a particular specific substrate.

Again attention is directed to those parameters S'_S and E'_S which were in accord with those conditions specified for zone A behavior (8, 9).

THE ENZYME-INHIBITOR DISSOCIATION CONSTANTS FOR SOME COMPETITIVE INHIBITORS OF alpha-CHYMOTRYPSIN

With the demonstration that the ninhydrin method of analysis was capable of furnishing an accurate and reproducible means of following the kinetics of the alpha-chymotrypsin catalyzed hydrolysis of alpha-acylamino acid amides, and with the establishment of the effects of phosphate buffer systems upon the course of the hydrolytic reaction as reflected by the kinetic constants K_S, k_3, and K_{P_1}, an investigation of the enzyme-inhibitor dissociation constants for several competitive inhibitors of the enzyme was initiated.

IN THE PRESENCE OF THAM-HCl BUFFER

Data and Results

The following experiments were conducted at 25° and pH 7.9 in aqueous solutions 0.02 M with respect to the THAM component of a THAM-HCl buffer. In all cases the specific substrate employed for these evaluations of the enzyme-inhibitor dissociation constants was acetyl-L-tyrosinamide. The enzyme concentration corresponded to 0.200 mg. protein-nitrogen/ml. of reaction mixture. In each experiment the pH of the medium remained within the desired range, i.e., 7.9 ± 0.1, even when the inhibitors containing the carboxylate group were introduced in the form of their potassium salts.

Evaluation of the experimental data was made by the method described by Foster and Niemann (7). In all cases the ninhydrin method of analysis was employed. Care has been taken to assure, as completely as possible, that the enzyme reactions were performed in such a manner as to maintain zone A conditions (8, 9). The line whose slope equals $- K_S(1 + [I]/K_I)$ that best fitted the experimental data was obtained by inspection, and the limits indicated are the maximum observable limits of error in the constants. For the evaluation of K_I, a K_S value of 34×10^{-3} M was employed.

beta-(beta-Indole)-propionate

This inhibitor was introduced in the form of its potassium salt. The experimental data are presented in Table XIX.

$K_I = 12 \pm 1.5 \times 10^{-3}$ M $E_I' = 0.475 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $S_S' = 0.625 - 1.25$

$E_S' = 0.17 \times 10^{-2}$ $I_I' = 3.3$

beta-(beta-Indole)-propionamide

The experimental data are presented in Table XX and Fig. 6.

$K_I = 1.9 \pm 0.3 \times 10^{-3}$ M $E_I' = 3.0 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $S_S' = 0.625 - 1.25$

$E_S' = 0.17 \times 10^{-2}$ $I_I' = 0.79$

Phenylacetate

This inhibitor was introduced as its potassium salt. The experimental data are presented in Table XXI and Fig. 7

$K_I = 192 \pm 15 \times 10^{-3}$ M $E'_I = 0.03 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $I'_I = 0.78$

Phenylacetamide

The experimental data are presented in Table XXII.

$K_I = 11 \pm 1 \times 10^{-3}$ M $E'_I = 0.518 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $I'_I = 0.90$

Tryptamine[1]

This compound was introduced in the form of its hydrochloride, and it was found that in the concentrations used the final pH of the system remained in the desired region, i.e., pH 7.9 ± 0.1, without further adjustment. The experimental data are presented in Table XXIII.

---

[1] Since this primary amine was studied at pH 7.9 ± 0.1, it is probable that the inhibitor actually evaluated was the corresponding cation.

$K_I = 1.6 \pm 0.3 \times 10^{-3}$ M $E'_I = 3.6 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $I'_I = 6.25$

Acetyl-L-tyrosinate

For this case $K_I = K_{P_1}$. The experimental data are presented in Tables X-XII and Figs. 2 and 3.

$K_I = 80 \pm 20 \times 10^{-3}$ M $E'_I = 0.0095 - 0.079 \times 10^{-2}$

$[E] = 0.76 - 6.3 \times 10^{-5}$ M $S'_S = 0.11 - 1.31$

$E'_S = 0.024 - 0.185 \times 10^{-2}$

IN THE PRESENCE OF PHOSPHATE BUFFER

Data and Results

The following experiments were performed at 25° and pH 7.9 ± 0.1 in aqueous solutions 0.1 M in potassium phosphate buffer, and except as noted, with acetyl-L-tyrosinamide as the specific substrate and with an enzyme concentration corresponding to 0.200 mg. protein-nitrogen/ml. In each experiment the pH of the medium remained within the desired range, i.e., 7.9 ± 0.1, even when the inhibitors containing a carboxylate group were introduced in the form of their potassium salts. Evaluation of the experimental data was made by the Foster-Niemann method (7), and in all cases the ninhydrin method of analysis was employed. The line whose slope equals

$- K_S(1 + [I]/K_I)$ that best fitted the experimental data was obtained by inspection, and the limits indicated are the maximum observable limits of error in the constants. For the evaluation of K_I, a K_S value of 32×10^{-3} M was employed. As before, care has been taken to assure, as completely as possible, adherence to zone A conditions (8, 9).

beta-(beta-Indole)-propionate

This inhibitor was introduced as its potassium salt. The experimental data are presented in Table XXIV.

$K_I = 4.0 \pm 0.4 \times 10^{-3}$ M $\quad E'_I = 1.42 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $\quad S'_S = 0.156 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $\quad I'_I = 2.5$

beta-(beta-Indole)-propionamide

The experimental data are presented in Table XXV and Fig. 8.

$K_I = 1.8 \pm 0.2 \times 10^{-3}$ M $\quad E'_I = 3.17 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $\quad S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $\quad I'_I = 0.83$

Phenylacetate

This inhibitor was introduced in the form of its potassium

salt. The experimental data are presented in Table XXVI.

$K_I = 60 \pm 5 \times 10^{-3}$ M $E'_I = 0.095 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $I'_I = 0.83$

Phenylacetamide

The experimental data are presented in Table XXVII.

$K_I = 11 \pm 1 \times 10^{-3}$ M $E'_I = 0.52 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $S'_S = 0.156 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $I'_I = 0.90$

Tryptamine[1]

This compound was introduced in the form of its hydrochloride, and it was found that in the concentrations used the final pH of the system remained in the region desired, i.e., 7.9 ± 0.1, without further adjustment. The enzyme concentration corresponded to 0.222 mg. protein-nitrogen/ml. for these experiments. The experimental data are presented in Table XXVIII and Fig. 9.

$K_I = 1.4 \pm 0.3 \times 10^{-3}$ M $E'_I = 4.5 \times 10^{-2}$

$[E] = 6.3 \times 10^{-5}$ M $S'_S = 0.156 - 1.25$

$E'_S = 0.197 \times 10^{-2}$ $I'_I = 3.6$

[1]Since this primary amine was studied at pH 7.9 ± 0.1, it is probable that the inhibitor actually evaluated was the corresponding cation.

Acetyl-L-tyrosinate

For this case $K_I = K_{P_1}$. The experimental data are presented in Tables XIV, XV, and Fig. 4.

$K_I = 80 \pm 20 \times 10^{-3}$ M $\qquad E'_I = 0.0713 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $\qquad S'_S = 0.156 - 1.25$

$E'_S = 0.17 \times 10^{-2}$

Acetyl-L-tryptophanate

For this case $K_I = K_{P_1}$. The enzyme concentration corresponded to 0.222 mg. protein-nitrogen/ml. for these experiments. The experimental data are presented in Table XVIII and Fig. 5.

$K_I = 7.3 \pm 0.3 \times 10^{-3}$ M $\qquad E'_I = 0.86 \times 10^{-2}$

$[E] = 6.3 \times 10^{-5}$ M $\qquad S'_S = 1.75 - 7.0$

$E'_S = 1.1 \times 10^{-2}$

Discussion

A summary of the enzyme-inhibitor dissociation constants of the above competitive inhibitors of the enzyme alpha-chymotrypsin evaluated in the presence of both buffer systems, i.e., THAM-HCl and phosphate, is presented in Table V. The values of K_I, i.e., the enzyme-inhibitor dissociation constants, obtained by Huang and Niemann (2, 14) in the presence of 0.02 M THAM-HCl buffer and those K_I values obtained by Neurath and Gladner (1, 2) in the presence

Table V

ENZYME-INHIBITOR DISSOCIATION CONSTANTS

Inhibitor	K_I[a] In 0.02 M THAM-HCl		In 0.1 M Phosphate	
	H and N[b]	Bernhard	Bernhard	N and G[c]
beta-(beta-Indole)-propionate	15 ± 3	12 ± 1.5	4.0 ± 0.4	2.5
Phenylacetate	200 ± 50	192 ± 15	60 ± 5	42
beta-(beta-Indole)-propionamide	2.3 ± 0.4	1.9 ± 0.3	1.8 ± 0.2	
Phenylacetamide	15 ± 3	11 ± 1	11 ± 1	
Tryptamine	2.3 ± 0.4	1.6 ± 0.3	1.4 ± 0.3	
Acetyl-L-tyrosinate	110 ± 30	80 ± 20	80 ± 20	
Acetyl-L-tryptophanate	8.0 ± 1.0	-	7.3 ± 0.3	

a., In units of 10^{-3} M. See text for explanation.

b., Cf. refs. (2, 14).

c., Cf. refs. (1, 2).

of 0.1 M phosphate buffer are included for purposes of comparison. It may be observed from these data that there is no significant difference in the values of K_I, within the limits of experimental error, for the uncharged, bifunctional competitive inhibitor beta-(beta-indole)-propionamide when evaluated against a common substrate, acetyl-L-tyrosinamide, in the presence of a 0.02 M THAM-HCl buffer or in the presence of 0.1 M phosphate buffer. The same conclusions may be drawn for the other uncharged, bifunctional, competitive inhibitor investigated phenylacetamide, and the cationic, bifunctional, competitive inhibitor tryptamine.

For the anionic, trifunctional, competitive inhibitors acetyl-L-tyrosinate and acetyl-L-tryptophanate, where $K_I = K_{P_1}$, there also is no apparent change in the values of the enzyme-inhibitor dissociation constants, i.e., K_I values, when these two inhibitors were evaluated in either of the two buffer media. Thus, for the cases described above, K_I does not appear to be markedly sensitive to changes in ion species, buffer concentration, or ionic strength.

For the anionic, bifunctional, competitive inhibitors beta-(beta-indole)-propionate and phenylacetate, a change in buffer species and concentration from 0.02 M THAM-HCl to 0.1 M phosphate changes the apparent K_I values quite markedly, i.e., by a factor of three to four times. Thus, the same competitive inhibitor

of the enzyme alpha-chymotrypsin is apparently more effective, i.e., has a smaller enzyme-inhibitor dissociation constant, in the presence of a 0.1 M phosphate buffer than in the presence of a 0.02 M THAM-HCl buffer.

EFFECT OF IONIC ENVIRONMENT

Since the concentrations of the buffer species for the two systems described above, i.e., 0.02 M THAM-HCl and 0.1 M phosphate buffers, were quite different, it was of considerable interest to determine whether the change in the enzyme-inhibitor dissociation constants for the anionic, bifunctional, competitive inhibitors beta-(beta-indole)-propionate and phenylacetate was due to concentration effects, ionic strength effects, specific ion effects, or to some combination of these effects. A number of experiments were designed to investigate these effects separately, holding two of the factors constant and varying the third. A series of experiments were performed in the presence of potassium phosphate buffers of various concentrations and various ionic strengths, and another series was performed in the presence of various concentrations of potassium phosphate buffer at a uniform ionic strength, i.e., 0.2 M. The pH and the ionic strength of these latter experiments were maintained at a constant level by the addition of suitable quantities of 1.0 M THAM-HCl buffer solution. Still additional experiments were performed at various ionic strengths with no phosphate buffer present.

The ionic strengths were calculated on the basis of the concentration of buffer species present, the concentration of ionizable inhibitors, and the concentrations of added salts if present. The accuracy of the values reported for the ionic strenghts of the reaction media is ± 5 %.

Data and Results

The following experiments were conducted in aqueous solutions at 25° and pH 7.9 ± 0.1, and except as noted, with an enzyme concentration corresponding to 0.200 mg. protein-nitrogen/ ml. of reaction mixture. In all cases the specific substrate was acetyl-L-tyrosinamide, and the potassium salts of the inhibitors and buffers were employed. In each experiment the pH of the medium remained within the desired range, i.e., pH 7.9 ± 0.1, when the inhibitors containing a carboxylate group were introduced in the form of their potassium salts. Evaluation of the experimental data was made by the method described by Foster and Niemann (7). The line whose slope equals $- K_S(1 + [I]/K_I)$ that best fitted the experimental data was obtained by inspection, and the limits indicated are the maximum observable limits of error in the constants. For the evaluation of K_I in all of the following experiments, a K_S value of 32×10^{-3} M was employed. In all cases the ninhydrin method of analysis was employed. Care has been taken to assure, as completely as possible, that the enzyme reactions were performed in such a

manner as to maintain zone A conditions (8, 9).

The symbol μ_o is used to indicate the ionic strength of the reaction medium at the beginning of the reaction.

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXIX.

Phosphate concentration = 0.0 M

THAM-HCl buffer concentration = 0.4 M

μ_o = 0.2 molal

K_I = $7.6 \pm 0.4 \times 10^{-3}$ M $\quad E'_I = 0.75 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $\quad S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $\quad I'_I = 1.32$

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXX.

Phosphate concentration = 0.0001 M

THAM-HCl buffer concentration = 0.4 M

μ_o = 0.2 molal

K_I = $7.5 \pm 0.5 \times 10^{-3}$ M $\quad E'_I = 0.76 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $\quad S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $\quad I'_I = 1.33$

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXXI and Fig. 10.

Phosphate concentration = 0.001 M

THAM-HCl buffer concentration = 0.4 M

μ_o = 0.2 molal

$K_I = 8.0 \pm 0.3 \times 10^{-3}$ M $\quad E'_I = 0.71 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $\quad S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $\quad I'_I = 1.25$

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXXII.

Phosphate concentration = 0.01 M

THAM-HCl buffer concentration = 0.27 M

μ_o = 0.17 molal

$K_I = 7.6 \pm 0.4 \times 10^{-3}$ M $\quad E'_I = 0.75 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $\quad S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $\quad I'_I = 1.32$

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXXIII.

Phosphate concentration = 0.04 M

THAM-HCl buffer concentration = 0.2 M

μ_o = 0.2 molal

K_I = 6.6 ± 0.4 X 10^{-3} M $\quad E'_I$ = 0.86 X 10^{-2}

[E] = 5.7 X 10^{-5} M $\quad S'_S$ = 0.625 - 1.25

E'_S = 0.17 X 10^{-2} $\quad I'_I$ = 1.52

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXXIV and Fig. 11.

Phosphate buffer concentration = 0.0725 M

THAM-HCl buffer concentration = 0.0 M

μ_o = 0.2 molal

K_I = 5.1 ± 0.3 X 10^{-3} M $\quad E'_I$ = 1.12 X 10^{-2}

[E] = 5.7 X 10^{-5} M $\quad S'_S$ = 0.625 - 1.25

E'_S = 0.17 X 10^{-2} $\quad I'_I$ = 1.96

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXIV.

Phosphate buffer concentration = 0.1 M

μ_o = 0.27 molal

K_I = 4.0 ± 0.4 X 10^{-3} M $\quad E'_I$ = 1.43 X 10^{-2}

[E] = 5.7 X 10^{-5} M $\quad S'_S$ = 0.156 - 1.25

E'_S = 0.17 X 10^{-2} $\quad I'_I$ = 2.5

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXXV. These experiments were performed with an enzyme concentration corresponding to 0.222 mg. protein-nitrogen/ml. of reaction solution.

Phosphate buffer concentration = 0.2 M

μ_o = 0.54 molal

$K_I = 3.2 \pm 0.5 \times 10^{-3}$ M $\quad E'_I = 1.97 \times 10^{-2}$

$[E] = 6.3 \times 10^{-5}$ M $\quad S'_S = 0.156 - 1.25$

$E'_S = 0.197 \times 10^{-2}$ $\quad I'_I = 3.1$

beta-(beta-Indole)-propionate

The experimental data are presented in Table XXXVI.

Phosphate buffer concentration = 0.4 M

μ_o = 1.08 molal

$K_I = 2.5 \pm 0.3 \times 10^{-3}$ M $\quad E'_I = 2.28 \times 10^{-2}$

$[E] = 5.7 \times 10^{-5}$ M $\quad S'_S = 0.625 - 1.25$

$E'_S = 0.17 \times 10^{-2}$ $\quad I'_I = 4.0$

beta-(beta-Indole)-propionate

These experiments were performed in the presence of a THAM-HCl buffer with added sodium chloride. The experimental data are presented in Table XXXVII.

THAM-HCl buffer concentration = 0.4 M

Sodium chloride concentration = 0.4 M

μ_o = 0.6 molal

K_I = 4.7 ± 0.4 X 10^{-3} M $\quad$ E'_I = 1.2 X 10^{-2}

$[E]$ = 5.7 X 10^{-5} M $\quad$ S'_S = 0.625 - 1.25

E'_S = 0.17 X 10^{-2} $\quad$ I'_I = 2.1

Phenylacetate

These experiments were performed in the presence of THAM-HCl buffer with no added phosphate present. The experimental data are presented in Table XXXVIII and Fig. 12.

THAM-HCl buffer concentration = 0.3 M

μ_o = 0.3 molal

K_I = 110 ± 10 X 10^{-3} M $\quad$ E'_I = 0.052 X 10^{-2}

$[E]$ = 5.7 X 10^{-5} M $\quad$ S'_S = 0.625 - 1.25

E'_S = 0.17 X 10^{-2} $\quad$ I'_I = 1.36

It will be noted from the above data that in every case $[E]$ was of the order of 10^{-5} M, $E'_S = [E]/K_S$ was less than 0.2 X 10^{-2}, $E'_I = [E]/K_I$ less than 2.3 X 10^{-2}, and $S'_S = [S]/K_S$ between the limits of 0.16 and 1.25. Thus, in all of the experiments described above, the conditions were such as to insure the presence of substantially monomeric alpha-chymotrypsin, the maintenance of zone A conditions

with respect to both the specific substrate and the competitive inhibitor (8, 9, 14), and the attainment of values of S'_S between the limits necessary for the application of equation (33) in the form of $([S]_o - [S]_t)/t$ versus $\ln([S]_o/[S]_t)/t$ plots (7) within the limits of experimental error. It is appropriate to recall that for an experimental error of $\pm$ 5 %, the permissible limits of S'_S are 0.05 to 20, and for an error of 10 %, 0.1 to 10 (14). The values of $I'_I = [I]/K_I$ are greater than 1.25. It may be seen from equation (34) that for a value of I'_I of 0.1 and an experimental error of $\pm$ 10 %, the term $K_S(1 + [I]/K_I)$ is equal to K_S within the limits of error. Since, in the evaluation of K_I, the probable experimental error is likely to be at least twice this value, it is obvious that values of I'_I must exceed 0.2 for K_I values to be of significance, and if they are to be estimated with any reasonable degree of accuracy, the value of I'_I should be of the order of 1.0 or greater; such conditions were thus fulfilled in all cases.

Discussion

A summary of the enzyme-inhibitor dissociation constants presented in the section above and the systems employed for their evaluation is given in Table VI. A consideration of this table indicates that there are at least two factors which appear to have a marked influence upon the values of K_I for the anionic, bifunctional, competitive inhibitors described herein, one factor being that of ionic

Table VI

THE EFFECT OF IONIC ENVIRONMENT UPON THE ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF ANIONIC BIFUNCTIONAL COMPETITIVE INHIBITORS

Inhibitor	THAM-HCl Buffer Conc.[a]	Phosphate Buffer Conc.[a]	μ_o[b]	K_I[c]	$-\Delta F^{od}$
beta-(beta-Indole)-propionate	0.02	0.00	0.05	12 ± 1.5	2640
	0.4	0.00	0.2	7.6 ± 0.4	2920
	0.4	0.0001	0.2	7.5 ± 0.5	2910
	0.4	0.001	0.2	8.0 ± 0.3	2880
	0.27	0.01	0.17	7.6 ± 0.4	2920
	0.2	0.04	0.2	6.6 ± 0.4	2990
	0.00	0.0725	0.2	5.1 ± 0.3	3140
	0.00	0.1	0.28	4.0 ± 0.4	3290
	0.00	0.2	0.55	3.2 ± 0.5	3420
	0.00	0.4	1.09	2.5 ± 0.3	3570
	0.4	0.00	0.6[e]	4.7 ± 0.4	3190
Phenylacetate	0.02	0.00	0.16	192 ± 15	985
	0.3	0.00	0.3[f]	110 ± 10	1320
	0.00	0.1	0.3[f]	60 ± 5	1670

a., Molar.

(continued on next page)

Table VI (cont.)

b., Molal.

c., In units of 10^{-3} M in aqueous solutions at 25^{o} and pH 7.9 ± 0.1.

d., In cal. per mole to the nearest 10 cal.

e., 0.4 M sodium chloride present.

f., 0.15 M in phenylacetate, i.e., inhibitor.

strength and the other of ion species. Whereas, solutions of relatively high ionic strength produce a measurable decrease in the enzyme-inhibitor dissociation constants for anionic, bifunctional, competitive inhibitors of the enzyme, phosphate buffers produce a still greater decrease in the values of K_I at comparable ionic strengths. In passing from a system in which the ionic strength was maintained at 0.05 molal and in the presence of a 0.02 M THAM-HCl buffer to one in which the ionic strength was maintained at 0.28 molal, and in the presence of a 0.1 M phosphate buffer, the K_I value of the anionic, bifunctional, competitive inhibitor beta-(beta-indole)-propionate decreased from $12 \pm 1.5 \times 10^{-3}$ M to $4.0 \pm 0.4 \times 10^{-3}$ M (a 66 per cent decrease), corresponding to a decrease in $-\Delta F^{o}$ of approximately 650 cal. per mole. Similarly, in passing from a system in which the ionic strength was maintained at 0.16 molal and in the presence of a 0.02 M THAM-HCl buffer to one in which the ionic strength was maintained at 0.3 molal and in the presence of a 0.1 M phosphate buffer, the K_I value of the anionic, bifunctional, competitive inhibitor phenylacetate decreased from $192 \pm 15 \times 10^{-3}$ M to $60 \pm 5 \times 10^{-3}$ M (a 69 per cent decrease), corresponding to a decrease in $-\Delta F^{o}$ of approximately 685 cal. per mole. Apparently the change in ionic species and strength produces a regular effect upon the system regardless of the specific nature of the anionic, bifunctional, competitive inhibitor, at least for the

inhibitors described in Table VI.

It is more instructive to examine the two effects separately. It may be seen that when the enzyme-inhibitor dissociation constant for phenylacetate was evaluated in systems in which no phosphate was present and with an ionic strength corresponding to 0.16 molal, the K_I value was found to be $192 \pm 15 \times 10^{-3}$ M. When K_I was evaluated in systems in which no phosphate was present, and the ionic strength was maintained at 0.3 molal, the value of K_I had decreased to $110 \pm 10 \times 10^{-3}$ M, a 43 per cent change. An examination of the $-\Delta F^{\circ}$ values presented in Table VI shows that this decrease in the value of K_I corresponds to a decrease in $-\Delta F^{\circ}$ of 335 cal. per mole. When this inhibitor was evaluated in the presence of a 0.1 M phosphate buffer and in solutions in which the ionic strength was maintained at 0.3 molal, the value of K_I was found to be $60 \pm 5 \times 10^{-3}$ M, a 69 per cent change; this corresponds to a decrease in $-\Delta F^{\circ}$ of 685 cal. per mole from those systems in which no phosphate buffer was present and in which the ionic strength was maintained at 0.16 molal. Thus, the addition of 0.1 M phosphate buffer to the system effected a decrease in $-\Delta F^{\circ}$, in addition to that caused by ionic strength effects, of 350 cal. per mole.

There is reason to believe that there is a negative charge situated at or near the catalytically active site of alpha-chymotrypsin (26). An increase in the ionic strength of the reaction medium tends

to increase the dielectric constant of that medium and hence tends to reduce the coulombic repulsion exerted between two like charges. Thus, the approach of a negatively charged inhibitor ion is facilitated by an increase in the ionic strength. Since combination is enhanced, the value of K_I is reduced.

Ionic strength effects play but a part in the inhibition processes described herein. A possible description of the inhibition process for the case of anionic, bifunctional, competitive inhibitors in the absence of phosphate may be that there is reasonably effective R_2-ρ_2 interaction at the catalytically active site of the enzyme, and that when R_3 equals a carboxylate moiety, the R_3-ρ_3 interaction is quite small (2). Thus, the <u>principal</u> attractive forces producing combination between enzyme and inhibitor result from one center binding. Consider the case of inhibition by an anionic, bifunctional, competitive inhibitor in the presence of phosphate. If one envisions a patch of the enzyme's surface surrounding the catalytically active site, one can conceive of an additional site or area near the ρ_3 center in this patch which is receptive to interaction or some sort of combination with phosphate[1]. Upon the approach of the negatively charged

[1] The term "phosphate" is used here in its broadest generic sense. It is quite possible that either $HPO_4^=$ or $H_2PO_4^-$, present in a ratio of approximately 6:1, or a combination of the two species may be the active entity in question since they are both present in amounts considerably in excess of the concentration of enzyme, i.e., <u>ca.</u> 10^{-5} <u>M</u>. Regrettably, isolation of one of these species is of <u>course</u> impossible if one is to maintain the pH of the reaction medium within the proper limits, i.e., pH 7.9 ± 0.1, for the examination of the kinetic reactions.

inhibitor ion, R_2-ρ_2 interaction takes place and the R_3 group, i.e., a carboxylate group, of the inhibitor molecule attempts to make its usual weak R_3-ρ_3 interaction. The presence of phosphate near the ρ_3 center of the enzyme provides a strong negative charge which repels the carboxyl group and forces it from its normal mode of combination into an alternate mode of combination wherein R_3 may contribute significantly to the total binding. Thus phosphate supplies the energy required to consummate an alternate mode of combination which is prohibited to the anionic, bifunctional, competitive inhibitor in the absence of phosphate. Apparently this alternate mode of combination is unavailable for anionic, trifunctional, competitive inhibitors, e.g., acetyl-L-tyrosinate, and thus these compounds are unaffected by the presence of phosphate in the reaction media.

It is possible to derive an expression for enzyme-substrate-buffer ion and enzyme-inhibitor-buffer ion dissociation constants and to estimate to some extent the possible interaction between the buffer ions, the enzyme, the substrate, and the inhibitor. If in addition to the basic Michaelis-Menten mechanism for the reaction of enzyme, E, and substrate, S, to yield products, P , and of enzyme and inhibitor, I, to yield the unproductive species, EI, a component, B, of the buffer (other than hydrogen or hydroxyl ions) is also bound at another site at which it affects the kinetic constants, the reaction scheme presented in Fig. 2 appears reasonable. This

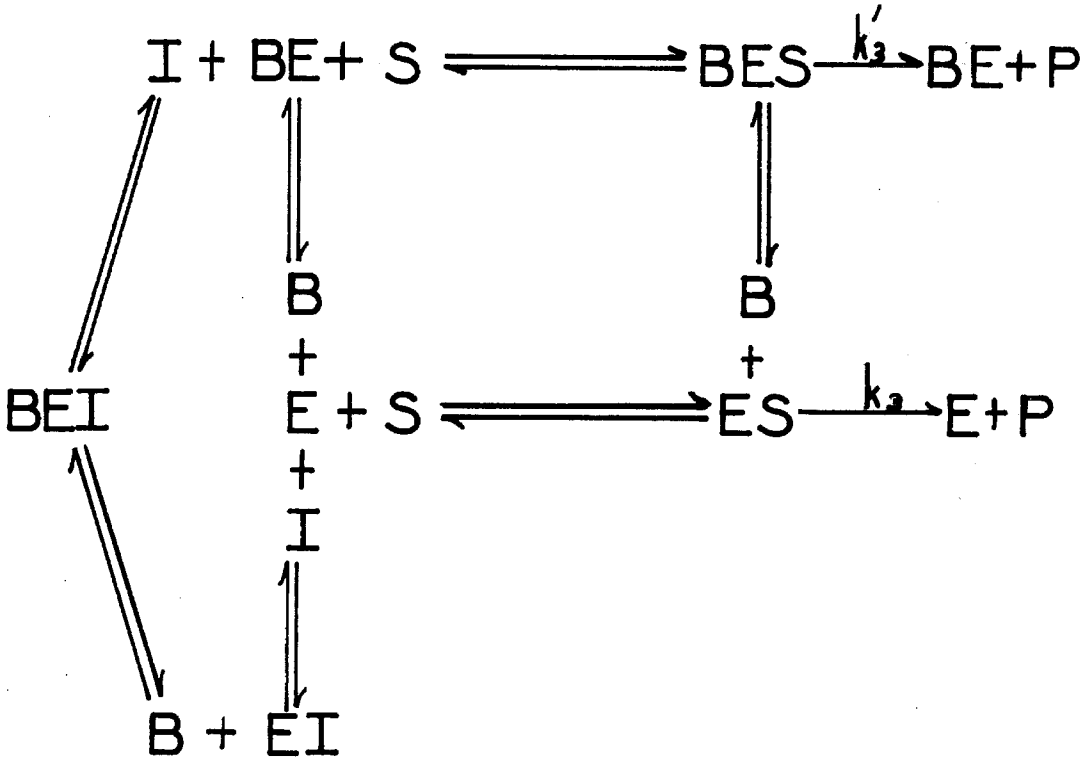


Fig. 2

scheme graphically presents the possible interplay between the various components of the reaction mixture. For the derivation of a mathematical relationship between these reactions, it is preferable to separate them thus:

$$E_f + S \rightleftharpoons ES \xrightarrow{k_3} E_f + P_1 \qquad \text{(I)}$$

$$E_f + I \rightleftharpoons EI \qquad \text{(II)}$$

$$B + E_f \rightleftharpoons BE^1 \qquad \text{(III)}$$

$$B + ES \rightleftharpoons BES \xrightarrow{k_3'} BE + P_1 \qquad \text{(IV)}$$

$$BE + S \rightleftharpoons BES \xrightarrow{k_3'} BE + P_1 \qquad \text{(V)}$$

$$BE + I \rightleftharpoons BEI \qquad \text{(VI)}$$

$$EI + B \rightleftharpoons BEI \qquad \text{(VII)}$$

It is unnecessary to consider such equations as $E + B \rightleftharpoons EB$ since this implies competitive inhibition by B, and as noted above,

---

[1] The substance written to the right of E in a complex is presumed to be bound at the catalytically active site of the enzyme, while a substance written to the left of E is bound at a neighboring site at which it has an effect on the catalytically active site, or at least on the kinetic constant.

for the case where B is phosphate, the rate of the hydrolytic reaction is not decreased. It is also appropriate to recall that for the case where B is phosphate, the constant K_S is essentially independent of the ionic strength and of the phosphate concentration of the reaction medium.

It will be assumed that in all derivations, the equilibria for which the following dissociation constants are indicated are adjusted rapidly in comparison to the rate of appearance of product, that $[S] \gg [E]$ and $[B] > [E]$, that $[S_f] \doteq [S]$, $[I_f] \doteq [I]$, $[B_f] \doteq [B]$, that $d[ES]/dt \doteq 0$, and that $d[BES]/dt \doteq 0$. Let $[E]$ be the total enzyme concentration, $[E_f]$ be the free enzyme concentration,

$$K_S = [E_f][S]/[ES]$$

$$K_I^{ol} = [E_f][I]/[EI]$$

$$K_{BE} = [B][E_f]/[BE]$$

$$K_{BES} = [B][ES]/[BES]$$

---

[1] The superscript, o, indicates that this K_I is to be evaluated in systems in which no B is present, but in which the ionic strength is maintained at a level equivalent to those systems in which K_I's for the same inhibitor are evaluated in the presence of varying amounts of B.

$$K_{SBE} = [BE][S]/[BES]$$

$$K_{BEI} = [BE][I]/[BEI]$$

and $$K_{EIB} = [EI][B]/[BEI] .$$

Thus $[E_f] = [ES]\, K_S/[S] =$

$$([E] - [ES] - [EI] - [BE] - [BES] - [BEI]) \qquad \text{(VIII)}$$

$$[EI] = [E_f][I]/\, K_I^o = [ES][I]\, K_S/K_I^o\, [S]$$

$$[BE] = [B][E_f]\,/K_{BE} = [B][ES]\, K_S/K_{BE}[S]$$

$$[BES] = [B][ES]/K_{BES} = [BE][S]/K_{SBE}$$

$$[BES] = [B][S][E_f]/K_S\, K_{BES} = [B][S][E_f]/K_{BE}\, K_{SBE}$$

Thus $K_S\, K_{BES} = K_{BE}\, K_{SBE}$

$$[BEI] = [BE][I]\,/K_{BEI} = [EI][B]/K_{EIB}$$

$$[BEI] = [B][I][E_f]/K_{BE}\, K_{BEI} = [B][I][E_f]/K_I^o\, K_{EIB}$$

and thus,

$$K_I^o K_{EIB} = K_{BE} K_{BEI}$$

$$K_{BE} = K_S K_{BES}/K_{SBE} = K_I^o K_{EIB}/K_{BEI} \qquad \text{(IX)}$$

Substituting in equation (VIII):

$$[ES] K_S/[S] = ([E] - [ES] - [ES][I] K_S/K_I^o[S] - [ES][B] K_S/K_{BE}[S] - [ES][B]/K_{BES} - [ES][B][I] K_S/K_I^o K_{EIB}[S]).$$

Transposing, factoring out, and solving for ES one obtains:

$$[ES] = \frac{[E]}{1 + K_S/[S]\left[1 + [I]/K_I^o + [B]/K_{BE} + [B][I]/K_{EIB} K_I^o\right] + [B]/K_{BES}}$$

Since $v = k_3 [ES] + k_3' [BES] = k_3 [ES] + k_3' [ES][B]/K_{BES}$

thus $v = (k_3 + k_3' [B]/K_{BES}) [ES]$

or

$$v = \frac{(k_3 + k_3'[B]/K_{BES}) [E]}{1 + K_S/[S]\left[1 + [I]/K_I^o + [B]/K_{BE} + [B][I]/K_{EIB} K_I^o\right] + [B]/K_{BES}} \qquad \text{(X)}$$

One may also solve for K_{BE} and K_{EIB} which yield the following relationships:

$$K_{BE} = \frac{[B]}{\left[([E]/v)(k_3+k'_3[B]/K_{BES})-1-[B]/K_{BES}\right]\left[[S]/K_S\right]-1-[I]/K_I^o-[B][I]/K_{EIB}K_I^o} \quad \text{(XI)}$$

$$K_{EIB} = \frac{[B][I]}{K_I^o\left\{\left[([E]/v)(k_3+k'_3[B]/K_{BES})-1-[B]/K_{BES}\right]\left[[S]/K_S\right]-1-[I]/K_I^o-[B]/K_{BE}\right\}} \quad \text{(XII)}$$

Since, as stated above, values of K_S are unaffected by the presence of B in the reaction media, where B is phosphate, one is led to two conclusions concerning the system. Either K_{BE} and K_{BES} are infinitely large as compared to K_S and K_I, and hence there is little or no BE and BES formed, or K_S is equal to K_{SBE} and there is effectively no difference between the path by way of ES to products and BES to products. If one examines the latter case and assumes that K_S is equal to K_{SBE} and k_3 is equal to k'_3[1], then from equation (IX) K_{BE} is equal to K_{BES}. Substituting K_{BE} for K_{BES} in equation (XI) and solving for K_{BE} one obtains the following equation:

[1] There is at present no information available as to the effect of varying B upon k_3 at constant ionic strength.

$$K_{BE} = \frac{[B]\left[k_3 - (v/[E])([K_S/[S]) + 1\right]}{-k_3+(v/[E])\left[1+(K_S/[S])(1+[I]/K_I^o+[B][I]/K_{EIB}\ K_I^o)\right]} \qquad \text{(XIII)}$$

An approximate evaluation of this expression, insofar as the experimental data permitted, indicates that values of K_{BE} will be extremely large as compared to values of K_I and K_S. Thus the two conclusions concerning the system have the same net effect upon K_{BE} and K_{BES}, i.e., that they are extremely large and can be ignored for the case at hand.

It is thus possible to simplify equation (X), i.e., the velocity expression obtained employing all seven equilibria, to the following:

$$v = \frac{k_3 [E]}{1 + (K_S/[S])(1 + [I]/K_I^o + [I][B]/K_I^o\ K_{EIB})} \qquad \text{(XIV)}$$

Solving for K_{EIB}:

$$K_{EIB} = \frac{[B]}{(K_I^o/[I])\left\{\left[(k_3[E][S]/v) - [S]\right](1/K_S) - 1 - [I]/K_I^o\right\}} \qquad \text{(XV)}$$

Employing equation (XV) for the system alpha-chymotrypsin-acetyl-L-tyrosinamide-beta-(beta-indole)-propionate and where B equals phosphate, K_{EIB} was evaluated as $150 \pm 50 \times 10^{-3}$ M. It is

impossible with the present accuracy of the kinetic experiments employing the methods described herein (see Part II of this thesis) to calculate meaningful values for K_{EIB} at low concentrations of B, i.e., less than or equal to 0.01 M phosphate, for the systems described herein, i.e., B equals phosphate, or a species thereof, I equals beta-(beta-indole)-propionate, and S equals acetyl-L-tyrosinamide, since the small differences in velocity values which occur at these values of B cannot be accurately determined because the differences are well within the limits of experimental error.

It will be noted that the value for K_{EIB} is quite large as compared to values for K_I. Consequently, contributions of BEI from the equilibrium expressed in equation (VI), i.e., $EI + B \rightleftharpoons BEI$, are quite small at low values of B. This is in agreement with the experimental observations presented in Table VI.

It is now possible to relate changes in the values of K_I to changes in the concentration of B. In the absence of inhibition by one of the hydrolysis products, i.e., in the case for initial rates, the velocity expression for an inhibited reaction reduces to

$$v_o = k_3 [E][S]_o / K_S(1 + [I]/K_I) + [S]_o \qquad \text{(XVI)}$$

(see Part I, page 11 of this thesis). If one multiplies the numerator and denominator of equation (XIV) by $[S]$, one obtains the following:

$$v = k_3[E][S]/K_S\,(1 + [I]/K_I^o + [I][B]/K_I^o\,K_{EIB}) + [S] \qquad \text{(XVII)}$$

Equating equations (XVI) and (XVII) and solving for K_I:

$$K_I = K_I^o/1 + [B]/K_{EIB} \qquad \text{(XVIII)}.$$

Equation (XVIII) yields values of K_I which agree well with experimentally determined values of K_I. Table VII presents a summary of the experimentally determined values for K_I for the anionic, bifunctional, competitive inhibitors beta-(beta-indole)-propionate and phenylacetate and compares these values of K_I to those calculated by means of equation (XVIII). It will be noted that in the extreme, agreement of the calculated values for K_I with the experimentally determined values for K_I is within 17 per cent. This is within the limits of experimental error for many published values of K_I (14).

Thus it may be seen for the case at hand that the formulations derived in this section satisfactorily describe, within the limits of experimental error, the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide by anionic, bifunctional, competitive inhibitors at pH 7.9 and 25°.

The author would like to express his appreciation to Robert Bock, Ralph Lutwack, and Myron Arcand for the many helpful suggestions and criticisms offered in the preparation of this portion of the thesis.

Table VII

ENZYME-INHIBITOR DISSOCIATION CONSTANTS

AT pH 7.9 ± 0.1 AND 25°

Inhibitor	[B][a]	K_I(Exptl.)[b]	K_I(Calcd.)[bc]
beta-(beta-Indole)-propionate	0.00	7.6 ± 0.4	7.6
	0.0001	7.5 ± 0.5	7.6
	0.001	8.0 ± 0.3	7.6
	0.01	7.6 ± 0.4	7.1
	0.04	6.6 ± 0.4	6.0
	0.0725	5.1 ± 0.3	5.1
	0.1	4.0 ± 0.4	4.5
	0.2	3.2 ± 0.5	3.3
	0.4	2.5 ± 0.3	2.1
Phenylacetate	0.1	60 ± 5	65

a., Where B is equal to the total phosphate concentration in moles.

b., In units of 10^{-3} M.

c., Calculated by means of equation (XVIII).

EXPERIMENTAL (27, 28)

Acetyl-L-tryptophanamide (I). - This material was prepared as directed by Huang and Niemann (11), clusters of fine needles, m. p. 192-193°; $[\alpha]_D^{23}$ + 20 ± 1° (c 2 % in methanol). Lit. (11), m. p. 192-193°; $[\alpha]_D^{25}$ + 20 ± 1° (c 2 % in methanol).

Acetyl-L-tyrosine (II). - II, colorless rods, m. p. 151-153°; $[\alpha]_D^{25}$ + 47.4° (c 2 % in water) was prepared as directed by du Vigneaud and Meyer (29); lit. (29), m. p. 152-154°; $[\alpha]_D^{26}$ + 47.5°.

Acetyl-L-tyrosinamide (III). - This material was prepared as directed by Thomas, MacAllister, and Niemann (10), colorless needles, m. p. 226-228°; $[\alpha]_D^{24}$ + 51.8° (c 0.8 % in water); lit. (10, 21), m. p. 222-224°; $[\alpha]_D^{25}$ + 49.7° (c 0.8 % in water).

beta-(beta-Indole)-propionic Acid (IV). - IV, colorless needles, m. p. 133-134°, was an Eastman Kodak Co. reagent grade product (Cat. No. 2530) which was recrystallized twice from a water-methanol mixture; lit. (2), m. p. 133-134°.

beta-(beta-Indole)-propionamide (V). - This material was prepared as described by Huang and Niemann (2), fine, stunted,

colorless, needles, m.p. 205-207°; lit. (2), m.p. 204-205°.

Phenylacetic Acid (VI). - This material, shiny platelets, m.p. 77-78°, was an Eastman Kodak Co. reagent grade product (Cat. No. 574) which was recrystallized three times from an ethanol-water mixture; lit. (2), m.p. 76-77°.

Phenylacetamide (VII). - VII, short, colorless needles, m.p. 157-158°, was prepared as described by Huang and Niemann (2); lit. (2), m.p. 157-158°.

Tryptamine Hydrochloride (VIII). - VIII, short, dense, colorless prisms, m.p. 250-251°, was an Eastman Kodak Co. reagent grade product (Cat. No. 2028) which was recrystallized twice from 5 N hydrochloric acid; lit. (30), m.p. 250-251°.

THAM-HCl Buffer Solutions. - Technical tris-(hydroxymethyl)-aminomethane was treated with decolorizing carbon and recrystallized three times from distilled water to give large, colorless, rod-like crystals, m.p. 169-169.5°. A stock solution, 0.20 M with respect to the amine component, was prepared by the addition of sufficient 1 N hydrochloric acid to an aqueous solution of the amine to give a solution of pH 8.03 at 25° after the stock solution was made up to volume. This stock solution was used in all of the studies employing 0.02 M THAM-HCl buffer and conducted

at pH 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 pH 7.90 ± 0.05 at 25°. Other stock solutions were prepared for studies employing this buffer at higher concentrations, and in these cases a suitable allowance was made for a decrease in pH upon a 1:10 dilution.

Phosphate Buffer Solutions. - A stock solution, 1.0 M with respect to phosphorus present as phosphate, was prepared by the addition of sufficient 5 N aqueous potassium hydroxide solution to an aqueous solution of reagent grade potassium dihydrogen phosphate to give a solution of pH 8.05 at 25° after the stock solution was made up to volume. This stock solution was used in all studies employing 0.1 M potassium phosphate buffer and conducted at pH 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 pH 7.9 ± 0.05 at 25°. Other stock solutions were prepared for studies employing this buffer at different concentrations, and in these instances, a suitable allowance was made for a decrease in pH upon a 1:10 dilution.

For experiments conducted in the presence of sodium phosphate buffers, the above procedure was followed with the exceptions that sodium dihydrogen phosphate was substituted for the potassium dihydrogen phosphate and sodium hydroxide was

substituted for the potassium hydroxide.

Enzyme Solutions. - Crystalline alpha-chymotrypsin containing magnesium sulfate (Armour, Lot No. 10705), and crystalline alpha-chymotrypsin, salt-free (Armour, Lot No. 00592) were used in these studies. Enzyme preparation Lot No. 10705 initially contained 13.3 per cent nitrogen. Analysis of this preparation after eleven months showed it to contain 11.9 per cent nitrogen[1]. Subsequent experiments employing this lot of enzyme were adjusted to compensate for this change in the nitrogen content. Enzyme preparation Lot No. 00592 contained 14.8 per cent nitrogen. Enzyme stock solutions were prepared daily, brought to 25°, and used immediately. The protein-nitrogen content of the enzyme was determined as described in Part I of this thesis.

Enzyme Experiments. - The substrate, or substrate and inhibitor, was dissolved, with the aid of heat if necessary, in 3 to 7 ml. of distilled water contained in a 10-ml. G.S. volumetric flask; the appropriate buffer solution was then added, and the clear solution placed in a 25.0 ± 0.05° bath for twenty to twenty-five minutes. At minus twenty seconds from time zero, the volumetric flask was withdrawn from the bath, and at time zero 1.0 ml. of

---

[1] Caused by the absorption of moisture.

enzyme solution was added, and the solution made up to volume with distilled water. The flask was stoppered, gently inverted twelve to fourteen times to insure adequate mixing, returned to the bath, and 1.0-ml. aliquots withdrawn at convenient intervals and delivered into a series of 10-ml. G.S. volumetric flasks each containing approximately 8.5 ml. of 0.2 M citrate buffer solution (pH 4.8 ± 0.1)[1]. As each aliquot was added, the solution was immediately made up to volume with the citrate buffer solution, the flask stoppered and gently inverted ten to twelve times to insure adequate mixing of the contents. After completion of the kinetic experiment, a 1.0-ml. aliquot of this solution was added to 1.0 ml. of ninhydrin reagent[2] in a five inch pyrex test tube, the tube shaken gently by hand for ten seconds to insure thorough mixing of the contents, and covered by an aluminum cap[3]. The tubes containing the quenched reaction mixture and the ninhydrin solution were placed in a water bath maintained at 100° for twenty minutes to develop the characteristic blue-violet color. After exactly twenty minutes, the tubes were removed, 5.0 ml. of the diluent solution[2] added to each tube, and the tubes gently inverted eight to ten times to mix the contents. Samples of these solutions were then placed in

[1] See Part II of this thesis for the preparation of this reagent.

[2] For the preparation of this reagent see Part II of this thesis.

[3] For a description of this cap see Part II of this thesis.

1.0 cm. corex absorption cells and the optical density of the solutions measured in a model B Beckman spectrophotometer.

The following data have been determined according to the procedure described in this part of the thesis.

$[E]$ = alpha-chymotrypsin concentration in mg. protein-nitrogen/ml. of reaction mixture

$[I]$ = added inhibitor concentration in units of 10^{-3} M

$[S]_o$ = initial substrate concentration in units of 10^{-3} M

t = time in minutes

O.D. = optical density

$[S]_t$ = substrate concentration at time t in units of 10^{-3} M

Blank = the optical density of a sample containing all of the components of the reaction mixture with the exception of the specific substrate

N(10) = the optical density of a sample containing all of the components of the reaction mixture with the exception of the specific substrate, but containing added ammonium chloride in the amount indicated in the parentheses, in units of 10^{-3} M

v_o = corrected initial velocity in units of 10^{-3} M/min.

Table VIII

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.0266 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	v_o
5	2	.020	0.0080
	10	.013	
	20	.032	
	30	.055	
	40	.131	
	50	.080	
	60	.124	
10	2	.006	0.0140
	10	.025	
	20	.076	
	30	.122	
	40	.068	
	50	.172	
	60	.224	
Dilution 1:10			Blank = 0.121
10	2	.044	0.0163
	10	.056	
	21	.070	
	30	.090	
	40	.101	
	50	.136	
	60	.122	
Dilution 1:10			Blank = 0.087

These data are illustrative of preliminary experiments.

Table IX

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.133 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_t$	$\ln [S]_t$	v_o
3	2	.315	---	---	---	0.0268
	10	.410	.170	2.830	1.040	
	20	.523	.415	2.585	0.950	
	30	.618	.605	2.395	0.873	
	41	.732	.855	2.145	0.763	
	50	.819	1.025	1.975	0.680	
	60	.901	1.200	1.800	0.588	
Dilution 1:5		Blank = 0.329		N(3) = 1.754		
3	10	.442	.245	2.755	1.014	0.0256
	20	.557	.480	2.520	0.924	
	30	.639	.650	2.350	0.854	
	40	.744	.865	2.135	0.758	
	50	.842	1.065	1.935	0.660	
	60	.915	1.220	1.780	0.577	
Dilution 1:5		Blank = 0.325		N(3) = 1.780		
3	2	.406	.150	2.850	1.047	0.0234
	10	.449	.245	2.755	1.013	
	20	.548	.445	2.555	0.938	
	30	.660	.670	2.330	0.846	
	40	.742	.845	2.155	0.767	
	50	.810	.995	2.005	0.695	
	60	.907	1.190	1.810	0.593	
5	2	.365	.070	4.930	1.595	0.0388
	10	.512	.365	4.635	1.533	
	20	.683	.720	4.280	1.454	
	30	.834	1.040	3.960	1.376	
	40	.988	1.350	3.650	1.295	
	50	1.116	1.620	3.380	1.218	
	60	1.250	1.900	3.100	1.131	
Dilution 1:5		Blank = 0.330		N(2) = 1.300		

Table IX (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_t$	$\ln [S]_t$	v_o
5	2	.340	.030	4.970	1.603	0.0406
	10	.499	.360	4.640	1.535	
	20	.659	.695	4.305	1.460	
	30	.848	1.085	3.915	1.365	
	40	.993	1.380	3.620	1.286	
	50	1.123	1.645	3.355	1.210	
	60	1.270	1.950	3.050	1.115	
Dilution 1:5		Blank = 0.325		N(3) = 1.780		
5	2	.349	.045	4.955	1.600	0.0430
	10	.512	.390	4.610	1.528	
	20	.692	.765	4.235	1.443	
	30	.870	1.140	3.860	1.350	
	40	1.010	1.455	3.545	1.265	
	50	1.138	1.700	3.300	1.193	
	60	1.290	2.200	2.800	1.030	
Dilution 1:5		Blank = 0.329		N(3) = 1.754		
10	2	.308	.310	9.690	2.271	0.0752
	10	.424	.810	9.190	2.218	
	20	.594	1.510	8.490	2.139	
	30	.743	2.130	7.870	2.063	
	40	.875	2.680	7.320	1.991	
	50	1.025	3.300	6.700	1.902	
	60	1.129	3.720	6.280	1.837	
10	2	.292	.260	9.740	2.276	0.0751
	10	.433	.840	9.160	2.216	
	20	.623	1.630	8.370	2.125	
	30	.762	2.210	7.790	2.053	
	40	.904	2.800	7.200	1.974	
	50	1.020	3.260	6.740	1.908	
	60	1.152	3.820	6.180	1.821	

Table IX (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_t$	$\ln [S]_t$	v_o
10	2	.305	.310	9.69	2.271	0.0751
	10	.450	.910	9.09	2.207	
	20	.593	1.500	8.50	2.140	
	30	.772	2.250	7.75	2.048	
	40	.882	2.710	7.29	1.987	
	50	1.000	3.190	6.81	1.918	
	60	1.121	3.690	6.31	1.842	
Dilution 1:10		Blank = 0.230		N(6) =	1.675	
10	2	.227	.002	9.99	2.302	0.0788
	10	.376	.680	9.32	2.232	
	20	.543	1.350	8.65	2.157	
	30	.723	2.100	7.90	2.067	
	40	.865	2.680	7.32	1.990	
	50	1.010	3.240	6.76	1.911	
	60	1.132	3.760	6.24	1.831	
10	2	.224	.001	9.99	2.302	0.0747
	10	.375	.670	9.33	2.233	
	20	.542	1.330	8.67	2.160	
	30	.694	1.980	8.02	2.082	
	40	.857	2.640	7.36	1.996	
	50	.966	3.080	6.92	1.934	
	60	1.093	3.600	6.40	1.856	
Dilution 1:10		Blank = 0.214		N(5) =	1.426	

Table X

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.179 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_{t/t}$	$\ln [S]_o/[S]_{t/t}$
5	30	.695	1.36	0.0453	0.0105
	40	.807	1.76	0.0440	0.0108
	50	.905	2.12	0.0424	0.0110
	60	1.014	2.50	0.0417	0.01155
	80	1.146	2.98	0.0372	0.0113
	95	1.246	3.34	0.0351	0.0116
	110	1.329	3.62	0.0329	0.0117
Dilution 1:10			Blank = 0.314		N(4) = 1.432
5	15	.198	.69	0.0460	0.0099
	20	.212	.98	0.0490	0.01088
	40	.250	1.65	0.0412	0.01000
	60	.285	2.26	0.0377	0.01002
	80	.306	2.64	0.0330	0.00940
	95	.321	2.91	0.0306	0.00917
	110	.346	3.36	0.0305	0.0101
Dilution 1:50			Blank = 0.159		N(4) = .382
10	30	1.045	2.62	0.0873	0.0101
	40	1.257	3.36	0.0840	0.01025
	50	1.431	4.00	0.0800	0.0102
	60	1.597	4.58	0.0764	0.0102
	80	1.861	5.53	0.0691	0.01005
	95	2.025	6.13	0.0646	0.0100
	110	2.185	6.69	0.0608	0.01005
Dilution 1:10			Blank = 0.314		N(4) = 1.432
10	15	.231	1.29	0.0860	0.00926
	20	.248	1.62	0.0810	0.00878
	40	.322	2.92	0.0730	0.00863
	60	.380	3.97	0.0662	0.00844
	80	.443	5.08	0.0635	0.00887
	95	.479	5.74	0.0604	0.00900
	110	.508	6.25	0.0568	0.00892
Dilution 1:50			Blank = 0.159		N(4) = .382

Table X (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
30	20	.380	3.80	0.190	0.0673
	25	.438	4.90	0.196	0.0709
	50	.651	8.80	0.176	0.0695
	75	.846	12.35	0.165	0.0707
	100	1.010	15.35	0.153	0.0715
	125	1.170	18.25	0.146	0.0750
	150	1.280	20.25	0.135	0.0750
40	20	.426	4.70	0.235	0.0625
	25	.498	6.00	0.240	0.0652
	50	.776	11.05	0.221	0.0647
	75	1.026	15.65	0.209	0.0663
	100	1.232	19.40	0.194	0.0664
	125	1.436	23.10	0.185	0.0688
	150	1.596	26.05	0.174	0.0703

Dilution 1:50 Blank = 0.172 N(20) = 1.268

Table XI

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.200 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t / t$	$\ln [S]_o / [S]_t / t$
5	6	.352	.29	0.048	0.00973
	20	.544	1.10	0.055	0.01235
	40	.718	1.84	0.046	0.01143
	60	.902	2.61	0.044	0.0123
	80	1.056	3.27	0.041	0.01328
	100	1.127	3.58	0.036	0.0126
	122	1.302	4.32	0.035	0.0165
Dilution 1:10			Blank = 0.285	N(6) = 1.691	
5	10	.384	.37	0.037	0.0077
	20	.504	.86	0.043	0.00945
	40	.719	1.76	0.044	0.01086
	60	.881	2.43	0.041	0.0111
	80	1.015	3.00	0.038	0.01145
	95	1.085	3.29	0.035	0.0113
	110	1.153	3.58	0.033	0.01143
10	10	.508	.88	0.088	0.0094
	20	.749	1.89	0.095	0.0105
	40	1.128	3.46	0.087	0.01058
	60	1.450	4.80	0.080	0.0109
	80	1.699	5.85	0.073	0.0110
	95	1.830	6.40	0.067	0.01074
	110	1.974	7.02	0.064	0.0110
Dilution 1:10			Blank = 0.288	N(6) = 1.735	
15	20	.422	2.675	0.134	0.0098
	25	.491	3.400	0.136	0.0103
	50	.753	6.125	0.123	0.0105
	75	.978	8.450	0.113	0.0110
	100	1.140	10.125	0.101	0.0112
	125	1.279	11.575	0.093	0.0118
	150	1.381	12.625	0.084	0.0123
Dilution 1:25			Blank = 0.161	N(10) = 1.126	

Table XI (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	6	.275	1.35	0.225	0.0116
	25	.576	4.50	0.180	0.0102
	50	.980	8.70	0.174	0.0114
	75	1.180	10.78	0.144	0.0103
	100	1.400	13.10	0.131	0.0106
	126	1.570	14.89	0.118	0.0108
	150	1.648	15.71	0.105	0.0103
Dilution 1:25			Blank = 0.145	N(16) = 1.677	
25	20	.562	4.125	0.206	0.00904
	25	.642	4.950	0.198	0.00883
	50	1.048	9.150	0.183	0.00912
	75	1.379	12.600	0.168	0.00935
	100	1.666	15.600	0.156	0.00978
	125	1.870	17.750	0.142	0.00990
	150	2.075	19.925	0.133	0.01060
Dilution 1:25			Blank = 0.161	N(10) = 1.126	
30	15	.296	3.40	0.227	0.00807
	30	.453	6.70	0.224	0.00846
	60	.722	12.30	0.205	0.00881
	90	.932	16.70	0.186	0.00904
	121	1.120	20.50	0.170	0.00949
	150	1.190	22.00	0.147	0.00882
	165	1.250	23.25	0.141	0.00904
Dilution 1:50			Blank = 0.135	N(32) = 1.670	
35	20	.398	4.65	0.233	0.00713
	25	.461	5.80	0.232	0.00726
	50	.722	10.80	0.216	0.00738
	75	.941	14.90	0.199	0.00739
	100	1.129	18.45	0.185	0.00749
	125	1.277	21.25	0.170	0.00747
	150	1.441	24.35	0.162	0.00794
Dilution 1:50			Blank = 0.151	N(30) = 1.740	

Table XI (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
40	15	.331	4.10	0.273	0.00719
	30	.518	8.00	0.267	0.00743
	60	.848	14.90	0.249	0.00775
	90	1.126	20.65	0.230	0.00805
	121	1.395	26.25	0.217	0.00882
	150	1.493	28.30	0.189	0.00819
	180	1.610	30.70	0.171	0.00811
Dilution 1:50			Blank = 0.135	N(32) = 1.670	
45	20	.448	5.60	0.280	0.00664
	25	.504	6.65	0.266	0.00638
	50	.816	12.55	0.251	0.00654
	75	1.091	17.70	0.236	0.00666
	100	1.329	22.25	0.223	0.00681
	125	1.552	26.45	0.212	0.00708
	150	1.710	29.45	0.196	0.00708
Dilution 1:50			Blank = 0.151	N(30) = 1.740	

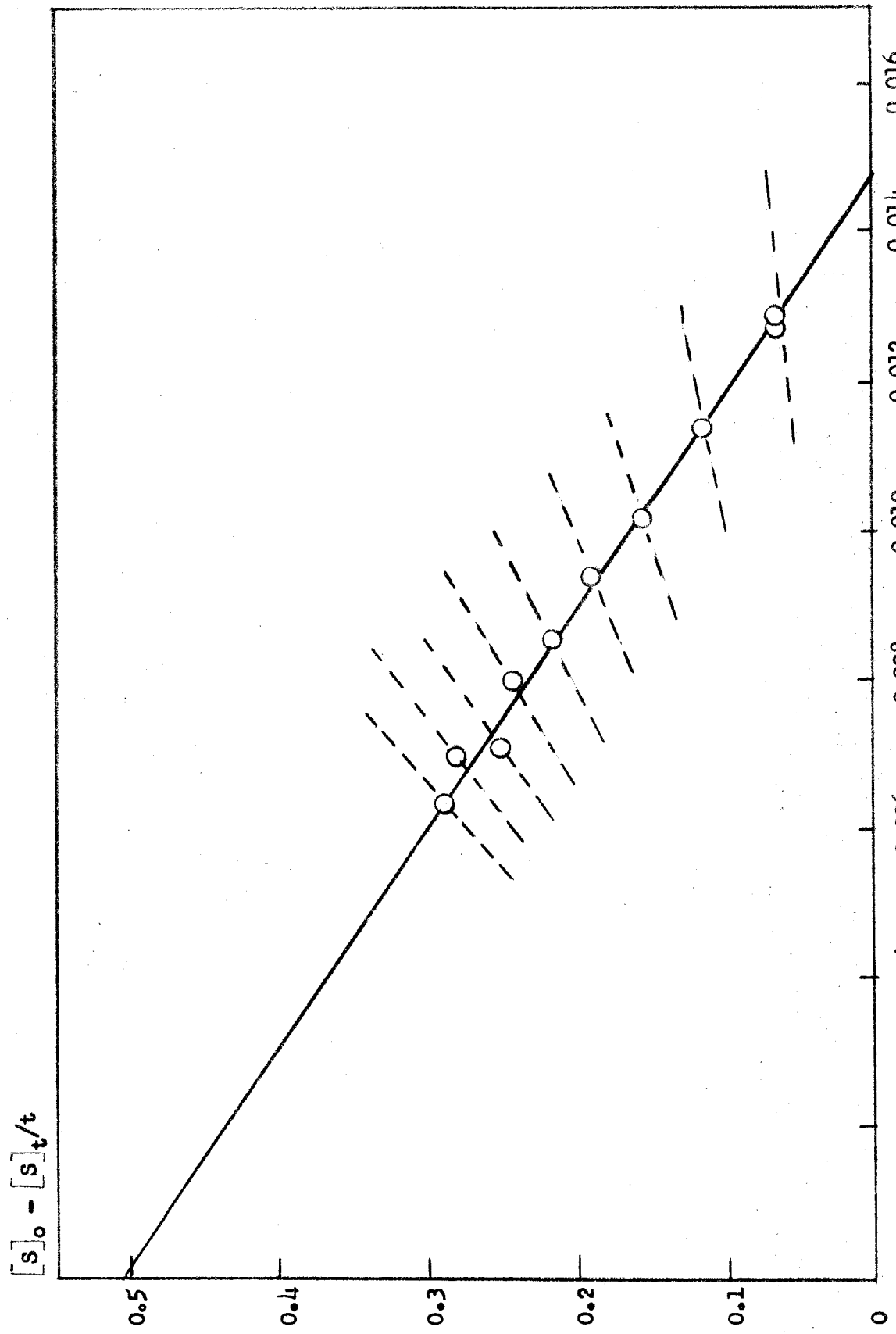


Fig. 3 Foster-Niemann Plot of the Data Presented in Table XI

Table XII

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.222 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
10	20	.195	2.15	0.107	0.0121
	41	.263	3.75	0.0915	0.0114
	60	.312	4.85	0.0809	0.0111
	80	.360	6.00	0.0750	0.0114
	95	.400	7.00	0.0737	0.0127
	110	.410	7.20	0.0655	0.0116
Dilution 1:50			Blank = 0.105[1]	N(20) = 0.948[1]	
10	20	.326	1.90	0.0950	0.0106
	40	.421	3.75	0.0938	0.0117
	60	.490	5.10	0.0851	0.0119
	80	.536	5.95	0.0743	0.0113
	95	.577	6.75	0.0710	0.0118
	110	.601	7.20	0.0655	0.0116
Dilution 1:50			Blank = 0.223[2]	N(20) = 1.262[2]	

[1]Employing 0.50 ml. ninhydrin reagent.
[2]Employing 2.0 ml. ninhydrin reagent.

Table XIII

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT 25°

$[E]$ = 0.200 Buffer = 0.02 M Phosphate (Na)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$	pH
5	10	.491	.52	0.052	0.0107	7.25
	20	.626	1.02	0.051	0.0114	
	40	.845	1.83	0.046	0.0114	
	60	1.056	2.61	0.044	0.0123	
	80	1.190	3.10	0.039	0.0121	
	95	1.270	3.39	0.036	0.0119	
	110	1.359	3.72	0.034	0.0124	
Dilution = 1:10			Blank = 0.350		N(4) = 1.435	
5	10	.585	.62	0.062	0.0131	7.60
	20	.720	1.11	0.056	0.0125	
	40	.995	2.13	0.053	0.0139	
	60	1.184	2.83	0.047	0.0139	
	80	1.368	3.49	0.044	0.0149	
	95	1.456	3.82	0.040	0.0152	
	110	1.518	4.05	0.037	0.0151	
Dilution = 1:10			Blank = 0.419		N(6) = 2.044	
5	10	.728	.77	0.077	0.0166	7.60
	20	.880	1.35	0.068	0.0157	
	40	1.100	2.16	0.054	0.0141	
	60	1.310	2.94	0.049	0.0148	
	80	1.460	3.49	0.044	0.0150	
	95	1.540	3.78	0.040	0.0148	
	110	1.610	4.05	0.037	0.0151	
10	10	.831	1.17	0.117	0.0122	7.60
	20	1.135	2.29	0.115	0.0130	
	40	1.579	3.84	0.096	0.0105	
	60	1.950	5.31	0.088	0.0126	
	80	2.280	6.53	0.082	0.0132	
	95	2.5	---	---	----	
	110	2.5	---	---	----	
Dilution 1:10			Blank = 0.517		N(6) = 2.138	

Table XIII (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln[S]_o/[S]_t/t$	pH
5	10	.540	.48	0.048	0.0140	7.80
	20	.679	.97	0.049	0.0108	
	40	.955	1.94	0.049	0.0122	
	60	1.160	2.66	0.044	0.0127	
	80	1.319	3.23	0.040	0.0129	
	95	1.390	3.47	0.037	0.0125	
	110	1.469	3.75	0.034	0.0126	
10	10	.695	1.02	0.102	0.0104	7.80
	20	.993	2.07	0.104	0.0116	
	40	1.480	3.79	0.095	0.0119	
	60	1.822	5.00	0.083	0.0115	
	80	2.095	5.96	0.075	0.0113	
	95	2.265	6.56	0.069	0.0112	
	110	2.360	6.90	0.063	0.0105	
Dilution 1:10			Blank = 0.399		N(6) = 2.170	
5	10	.574	.57	0.057	0.0122	7.85
	20	.725	1.13	0.057	0.0128	
	40	.998	2.14	0.054	0.0140	
	60	1.194	2.86	0.048	0.0142	
	80	1.365	3.48	0.044	0.0149	
	95	1.455	3.83	0.040	0.0153	
	110	1.525	4.18	0.038	0.0164	
Dilution 1:10			Blank = 0.419		N(6) = 2.044	
5	10	.514	.60	0.060	0.0128	7.95
	20	.674	1.20	0.060	0.0137	
	40	.841	1.81	0.045	0.0112	
	60	1.137	2.91	0.049	0.0109	
	80	1.281	3.44	0.043	0.0146	
	95	1.373	3.78	0.040	0.0149	
	110	1.461	4.11	0.037	0.0157	
Dilution 1:10			Blank = 0.350		N(4) = 1.435	

Table XIV

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.200 Buffer = 0.1 M Phosphate (Na)

$[S]_o$	t	O. D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	10	.518	.71	0.071	0.0152
	20	.709	1.39	0.070	0.0163
	40	1.033	2.56	0.064	0.0179
	60	1.254	3.34	0.056	0.0183
	80	1.402	3.86	0.048	0.0185
	95	1.480	4.14	0.044	0.0185
	110	1.552	4.40	0.040	0.0192
10	10	.706	1.41	0.141	0.0151
	20	1.051	2.61	0.131	0.0152
	40	1.608	4.59	0.115	0.0154
	60	2.000	5.98	0.099	0.0152
	80	2.350	7.19	0.090	0.0159
	95	2.495	7.72	0.081	0.0156
	110	2.620	8.16	0.074	0.0154
Dilution 1:10			Blank = 0.309	N(4) = 1.440	
30	20	.446	5.50	0.275	0.0101
	25	.527	6.95	0.278	0.0105
	50	.864	12.90	0.258	0.0113
	75	1.132	17.70	0.236	0.0119
	100	1.300	20.70	0.207	0.0117
	125	1.462	23.65	0.189	0.0124
	150	1.585	25.75	0.071	0.0130
40	20	.516	6.75	0.337	0.0092
	25	.601	8.25	0.330	0.0093
	50	1.006	15.45	0.309	0.0098
	75	1.322	21.10	0.281	0.0100
	100	1.573	25.55	0.256	0.0102
	125	1.754	28.85	0.230	0.0104
	150	1.933	32.10	0.214	0.0108
Dilution 1:50			Blank = 0.139	N(30) = 1.825	

Table XV

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.200 Buffer = 0.1 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	10	.525	0.74	0.074	0.0158
	20	.716	1.41	0.071	0.0165
	40	1.026	2.53	0.063	0.0175
	60	1.243	3.32	0.055	0.0182
	80	1.389	3.82	0.048	0.0181
	95	1.462	4.10	0.043	0.0181
	110	1.533	4.35	0.040	0.0186
10	10	.710	1.39	0.139	0.0149
	20	1.054	2.64	0.132	0.0153
	40	1.609	4.61	0.115	0.0154
	60	1.979	5.94	0.099	0.0150
	80	2.310	7.15	0.0894	0.0157
	95	2.460	7.69	0.0809	0.0154
	110	2.580	8.12	0.0737	0.0152
Dilution 1:10			Blank = 0.314	N(4) = 1.445	
20	20	.741	4.38	0.219	0.0123
	25	.872	5.54	0.222	0.0130
	50	1.324	9.62	0.193	0.0131
	75	1.690	12.88	0.172	0.0138
	100	1.892	14.92	0.149	0.0137
	125	2.105	16.61	0.133	0.0142
	150	2.230	17.73	0.118	0.0145
Dilution 1:25			Blank = 0.248	N(16) = 2.033	
30	19	.460	5.75	0.303	0.0113
	25	.545	7.25	0.290	0.0111
	50	.860	12.85	0.257	0.0112
	75	1.156	18.15	0.242	0.0125
	100	1.309	20.85	0.229	0.0119
	125	1.452	23.35	0.187	0.0121
	150	1.580	25.10	0.171	0.0130
Dilution 1:50			Blank = 0.136	N(30) = 1.822	

Table XV (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
35	15	.429	4.85	0.323	0.0099
	20	.518	6.45	0.322	0.0102
	40	.819	11.75	0.294	0.0102
	60	1.074	16.30	0.272	0.0104
	80	1.302	20.25	0.256	0.0108
	100	1.483	23.34	0.233	0.0110
	120	1.624	25.93	0.216	0.0113
Dilution 1:50		Blank = 0.157		N(30) = 1.845	
40	20	.516	6.70	0.335	0.0091
	25	.608	8.35	0.334	0.0094
	50	1.016	15.65	0.313	0.0099
	75	1.328	21.20	0.283	0.0101
	100	1.570	25.50	0.255	0.0102
	125	1.746	28.65	0.229	0.0101
	150	1.932	32.00	0.214	0.0107
Dilution 1:50		Blank = 0.136		N(30) = 1.822	

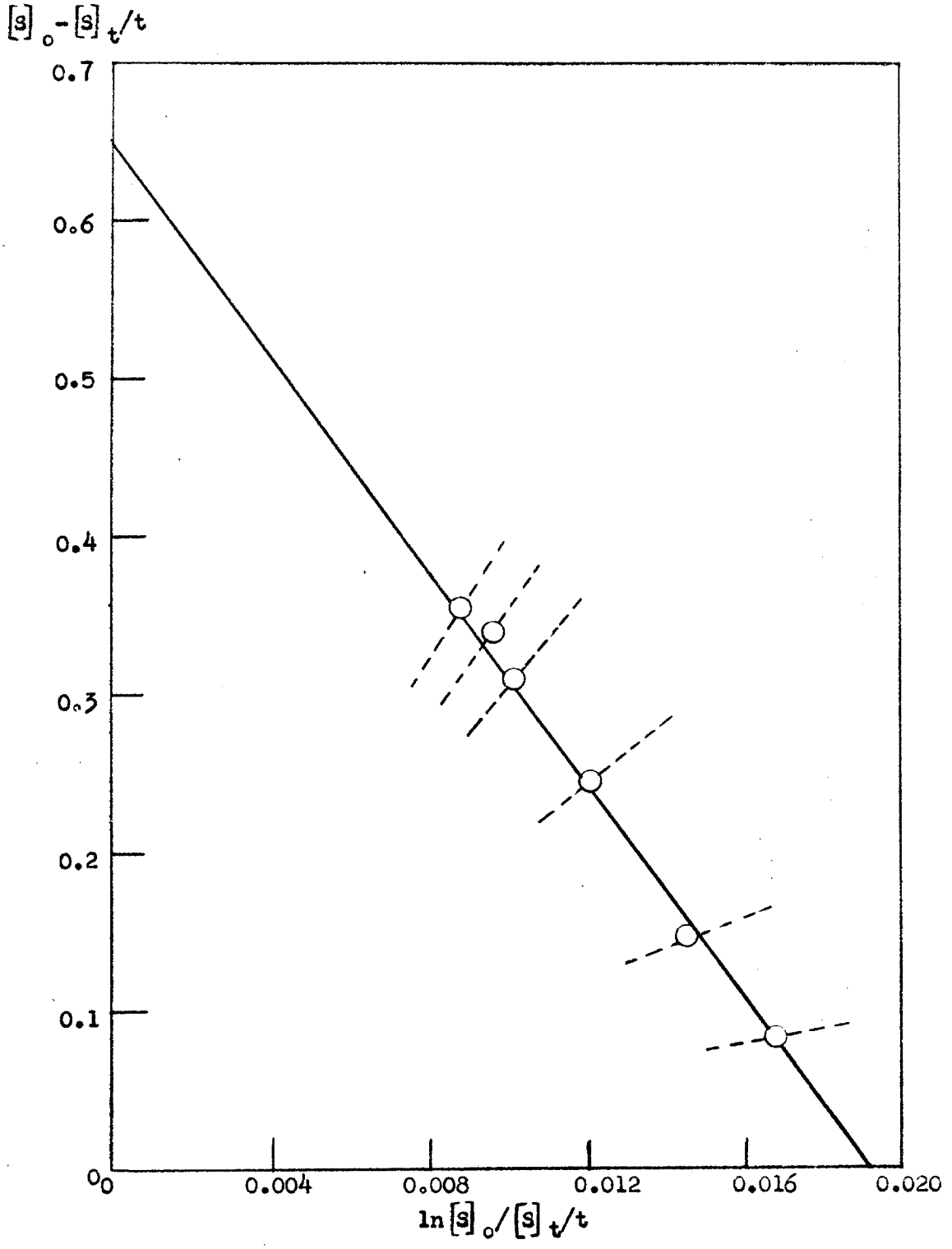


Fig. 4 Foster-Niemann Plot of Data Presented In Table XV

Table XVI

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.200 Buffer = 0.2 M Phosphate (Na)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	10	.567	0.88	0.088	0.0192
	20	.787	1.68	0.084	0.0202
	40	1.085	2.74	0.069	0.0199
	60	1.272	3.42	0.057	0.0192
	80	1.415	3.93	0.049	0.0193
	95	1.480	4.15	0.044	0.0187
	110	1.536	4.36	0.040	0.0187
Dilution 1:10		Blank = 0.314		N(4) = 1.435	
5	10	.581	0.92	0.092	0.0199
	20	.795	1.69	0.085	0.0206
	40	1.156	2.99	0.075	0.0228
	60	1.368	3.74	0.062	0.0230
	80	1.507	4.24	0.053	0.0236
	95	1.562	4.45	0.047	0.0232
	110	1.621	4.66	0.042	0.0244
10	10	.827	1.82	0.182	0.0199
	20	1.242	3.31	0.166	0.0199
	40	1.903	5.68	0.142	0.0209
	60	2.320	7.18	0.120	0.0211
	80	2.620	8.27	0.103	0.0219
	95	2.730	8.66	0.091	0.0212
	110	2.825	9.00	0.082	0.0209
Dilution 1:10		Blank = 0.320		N(4) = 1.439	
10	10	.810	1.75	0.175	0.0192
	20	1.225	3.25	0.162	0.0197
	40	1.855	5.51	0.138	0.0199
	60	2.280	7.04	0.117	0.0203
	80	2.530	7.95	0.099	0.0198
	95	2.720	8.63	0.091	0.0209
	110	2.790	8.88	0.081	0.0199
Dilution 1:10		Blank = 0.314		N(4) = 1.435	

Table XVI (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
30	10	.394	3.85	0.385	0.0139
	25	.676	8.85	0.354	0.0140
	50	1.035	15.20	0.304	0.0142
	75	1.300	19.85	0.265	0.0144
	100	1.495	23.40	0.234	0.0151
	125	1.616	25.50	0.204	0.0151
	150	1.700	27.00	0.180	0.0153
40	10	.416	4.20	0.420	0.0113
	25	.759	10.30	0.412	0.0118
	50	1.219	18.40	0.368	0.0123
	75	1.570	24.70	0.329	0.0128
	100	1.850	29.65	0.297	0.0135
	125	2.061	33.45	0.268	0.0148
	150	2.140	34.85	0.232	0.0137

Dilution 1:50 Blank = 0.180 N(28) = 1.755

Table XVII

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE AT pH 7.9 AND 25°

[E] = 0.200 Buffer = 0.4 M Phosphate (Na)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	10	.686	1.14	0.114	0.0258
	15	.860	1.75	0.117	0.0287
	30	1.170	2.86	0.095	0.0283
	45	1.411	3.72	0.083	0.0303
	60	1.550	4.22	0.070	0.0309
	75	1.626	4.48	0.060	0.0302
	90	1.725	4.84	0.054	0.0383
10	10	.973	2.16	0.216	0.0243
	15	1.250	3.15	0.210	0.0252
	30	1.920	5.54	0.185	0.0268
	45	2.340	7.03	0.156	0.0270
	60	2.650	8.14	0.135	0.0280
	75	2.855	8.87	0.118	0.0290
	90	2.960	9.24	0.103	0.0286
Dilution 1:10			Blank = 0.365	N(4) = 1.500	
30	10	.439	4.70	0.470	0.0170
	15	.534	6.40	0.427	0.0159
	30	.865	12.20	0.406	0.0174
	45	1.107	16.40	0.365	0.0175
	60	1.318	20.00	0.334	0.0183
	75	1.466	22.70	0.303	0.0188
	90	1.580	24.65	0.274	0.0192
40	10	.446	4.85	0.485	0.0131
	15	.594	7.50	0.500	0.0138
	30	.958	13.80	0.460	0.0141
	45	1.256	19.05	0.424	0.0144
	60	1.515	23.50	0.392	0.0148
	75	1.719	27.05	0.361	0.0150
	90	1.850	29.30	0.326	0.0147
Dilution 1:50			Blank = 0.168	N(16) = 1.088	

Table XVIII

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TRYPTOPHANAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.222 Buffer = 0.1 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
10	20	.419	1.750	0.088	0.0096
	25	.462	2.175	0.087	0.0098
	50	.664	3.950	0.079	0.0101
	75	.818	5.375	0.072	0.0103
	100	.946	6.550	0.066	0.0106
	125	1.029	7.275	0.058	0.0104
	150	1.112	8.050	0.054	0.0109
Dilution 1:25			Blank = 0.228	N(10) = 1.332	
15	20	.462	2.175	0.1088	0.00784
	25	.504	2.550	0.1020	0.00746
	50	.748	4.775	0.096	0.00767
	75	.930	6.450	0.086	0.00749
	100	1.105	8.050	0.081	0.00768
	125	1.237	9.225	0.074	0.00763
	150	1.383	10.575	0.071	0.00814
Dilution 1:25			Blank = 0.227	N(16) = 1.976	
15	20	.464	2.100	0.105	0.00750
	25	.511	2.55	0.102	0.00746
	50	.754	4.775	0.096	0.00767
	75	.943	6.475	0.086	0.00753
	100	1.121	8.100	0.081	0.00776
	125	1.250	9.275	0.074	0.00770
	150	1.391	10.575	0.071	0.00813
20	20	.479	2.225	0.111	0.00590
	25	.540	2.800	0.112	0.00603
	50	.809	5.250	0.105	0.00609
	75	1.044	7.425	0.099	0.00618
	100	1.242	9.200	0.092	0.00616
	125	1.428	10.900	0.087	0.00629
	150	1.578	12.250	0.082	0.00632
Dilution 1:25			Blank = 0.233	N(16) = 1.985	

Table XVIII (cont.)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	.460	2.150	0.108	0.00568
	25	.529	2.775	0.111	0.00596
	50	.800	5.250	0.105	0.00609
	75	1.034	7.425	0.099	0.00621
	100	1.206	8.950	0.090	0.00593
	125	1.388	10.625	0.085	0.00606
	150	1.522	11.875	0.079	0.00600
Dilution 1:25			Blank = 0.227	N(16) = 1.976	
25	20	.488	2.375	0.119	0.00498
	25	.560	3.025	0.121	0.00517
	50	.857	5.700	0.114	0.00517
	75	1.106	7.950	0.106	0.00510
	100	1.350	10.175	0.102	0.00522
	125	1.551	12.000	0.096	0.00524
	150	1.751	13.775	0.092	0.00534
Dilution 1:25			Blank = 0.228	N(10) = 1.332	
30	20	.319	2.40	0.120	0.00417
	25	.354	3.10	0.124	0.00435
	50	.512	5.95	0.119	0.00441
	75	.660	8.60	0.115	0.00450
	100	.784	10.85	0.109	0.00450
	125	.898	12.90	0.103	0.00449
	150	1.009	14.95	0.100	0.00460
40	20	.330	2.60	0.130	0.00335
	25	.362	3.25	0.130	0.00338
	50	.531	6.30	0.126	0.00343
	75	.688	9.10	0.121	0.00344
	100	.836	11.80	0.118	0.00350
	125	.964	14.15	0.113	0.00349
	150	1.109	16.75	0.112	0.00362
Dilution 1:50			Blank = 0.185	N(30) = 1.835	

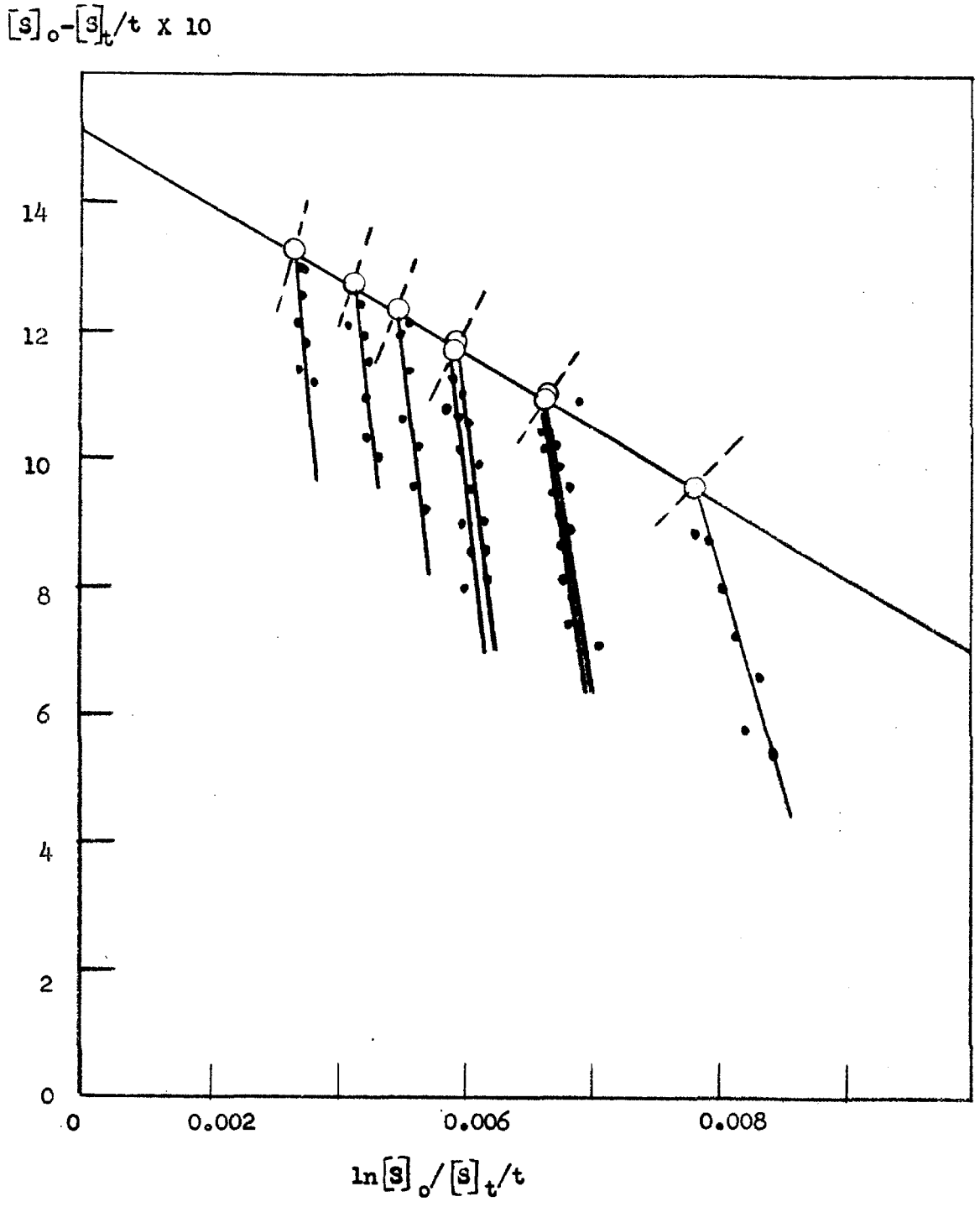


Fig. 5 Foster-Niemann Plot of Data Presented In Table XVIII

Table XIX

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 40.0 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	.282	1.15	0.575	0.00296
	25	.317	1.45	0.580	0.00301
	50	.466	2.85	0.570	0.00307
	75	.591	4.00	0.533	0.00298
	100	.710	5.10	0.510	0.00294
	125	.835	6.25	0.500	0.00299
	150	.948	7.30	0.487	0.00303
25	20	.310	1.40	0.700	0.00288
	25	.349	1.75	0.700	0.00290
	50	.549	3.60	0.720	0.00296
	75	.694	4.95	0.660	0.00294
	100	.835	6.25	0.625	0.00288
	125	.970	7.50	0.600	0.00285
	150	1.116	8.85	0.590	0.00291
Dilution 1:25			Blank = 0.158	N(10) = 1.239	
30	20	.209	1.50	0.0750	0.00256
	25	.236	1.95	0.0780	0.00268
	50	.331	3.75	0.0750	0.00267
	75	.411	5.25	0.0700	0.00256
	100	.503	6.90	0.0690	0.00262
	125	.576	8.30	0.0664	0.00259
	150	.648	9.65	0.0643	0.00259
40	20	.240	2.05	0.1025	0.00263
	25	.267	2.55	0.1020	0.00263
	50	.385	4.75	0.0950	0.00252
	75	.491	6.70	0.0893	0.00245
	100	.586	8.45	0.0840	0.00237
	125	.684	10.30	0.0824	0.00238
	150	.778	12.00	0.0800	0.00238
Dilution 1:50			Blank = 0.130	N(30) = 1.748	

Table XX

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 1.5 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	.465	2.525	0.126	0.00673
	25	.631	3.125	0.125	0.00679
	50	.829	5.825	0.117	0.00688
	75	1.076	8.050	0.107	0.00687
	100	1.297	10.050	0.101	0.00698
	125	1.490	11.775	0.094	0.00711
	150	1.651	13.250	0.088	0.00724
25	20	.516	2.975	0.149	0.00633
	25	.589	3.625	0.145	0.00660
	50	.941	6.875	0.138	0.00664
	75	1.260	9.700	0.129	0.00655
	100	1.530	12.150	0.122	0.00666
	125	1.759	14.225	0.114	0.00672
	150	1.902	16.000	0.107	0.00680
Dilution 1:25			Blank = 0.188	N(10) = 1.292	
30	20	.323	3.35	0.168	0.00593
	25	.370	4.20	0.168	0.00604
	50	.571	7.85	0.157	0.00612
	75	.747	11.10	0.148	0.00616
	100	.909	14.00	0.140	0.00629
	125	1.042	16.45	0.131	0.00635
	150	1.158	18.55	0.124	0.00642
40	20	.364	4.10	0.205	0.00539
	25	.422	5.10	0.204	0.00545
	50	.661	9.50	0.190	0.00542
	75	.888	13.65	0.182	0.00557
	100	1.088	17.30	0.173	0.00566
	125	1.267	20.60	0.165	0.00578
	150	1.410	23.20	0.155	0.00578
Dilution 1:50			Blank = 0.141	N(30) = 1.789	

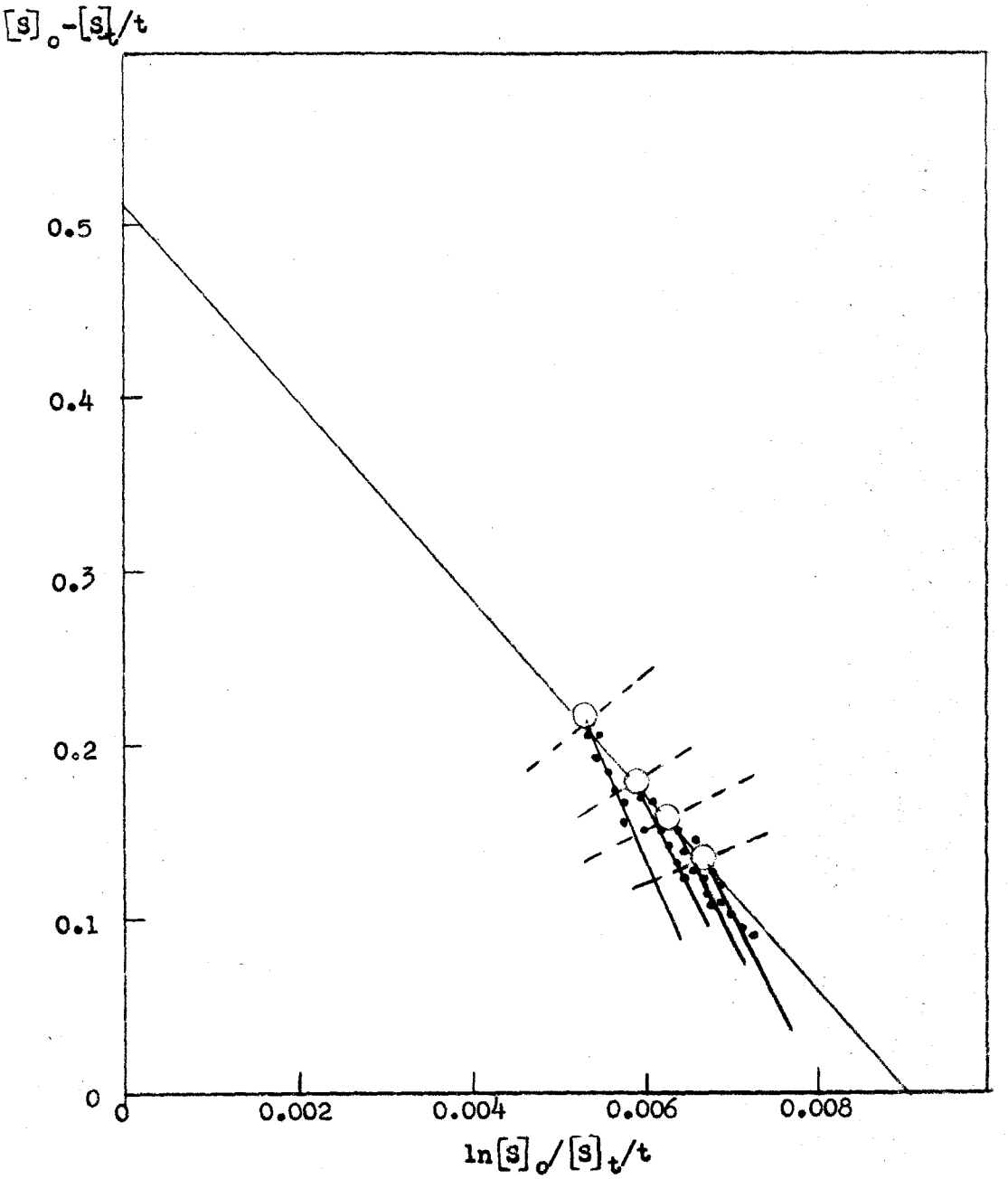


Fig. 6 Foster-Niemann Plot of the Data Presented in Table XX

Table XXI

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY PHENYLACETATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 150.0 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	.423	2.350	0.118	0.00624
	25	.494	2.950	0.118	0.00638
	50	.771	5.425	0.109	0.00632
	75	1.010	7.550	0.101	0.00632
	100	1.211	9.325	0.093	0.00628
	125	1.388	10.950	0.088	0.00634
	150	1.534	12.250	0.082	0.00632
25	20	.471	2.750	0.138	0.00584
	25	.546	3.425	0.137	0.00590
	50	.887	6.450	0.129	0.00597
	75	1.162	8.900	0.119	0.00588
	100	1.412	11.150	0.112	0.00590
	125	1.625	13.075	0.105	0.00592
	150	1.814	14.750	0.098	0.00594
Dilution 1:25			Blank = 0.163	N(10) = 1.286	
30	20	.310	3.20	0.160	0.00562
	25	.352	3.95	0.158	0.00566
	50	.538	7.25	0.145	0.00554
	75	.706	10.25	0.137	0.00558
	100	.854	12.85	0.129	0.00559
	125	.968	14.95	0.120	0.00551
	150	1.090	17.05	0.114	0.00560
40	20	.348	3.85	0.193	0.00506
	25	.388	4.60	0.184	0.00488
	50	.621	8.75	0.175	0.00494
	75	.829	12.45	0.166	0.00497
	100	1.011	15.70	0.157	0.00499
	125	1.160	18.30	0.146	0.00488
	150	1.312	21.10	0.141	0.00499
Dilution 1:50			Blank = 0.131	N(30) = 1.808	

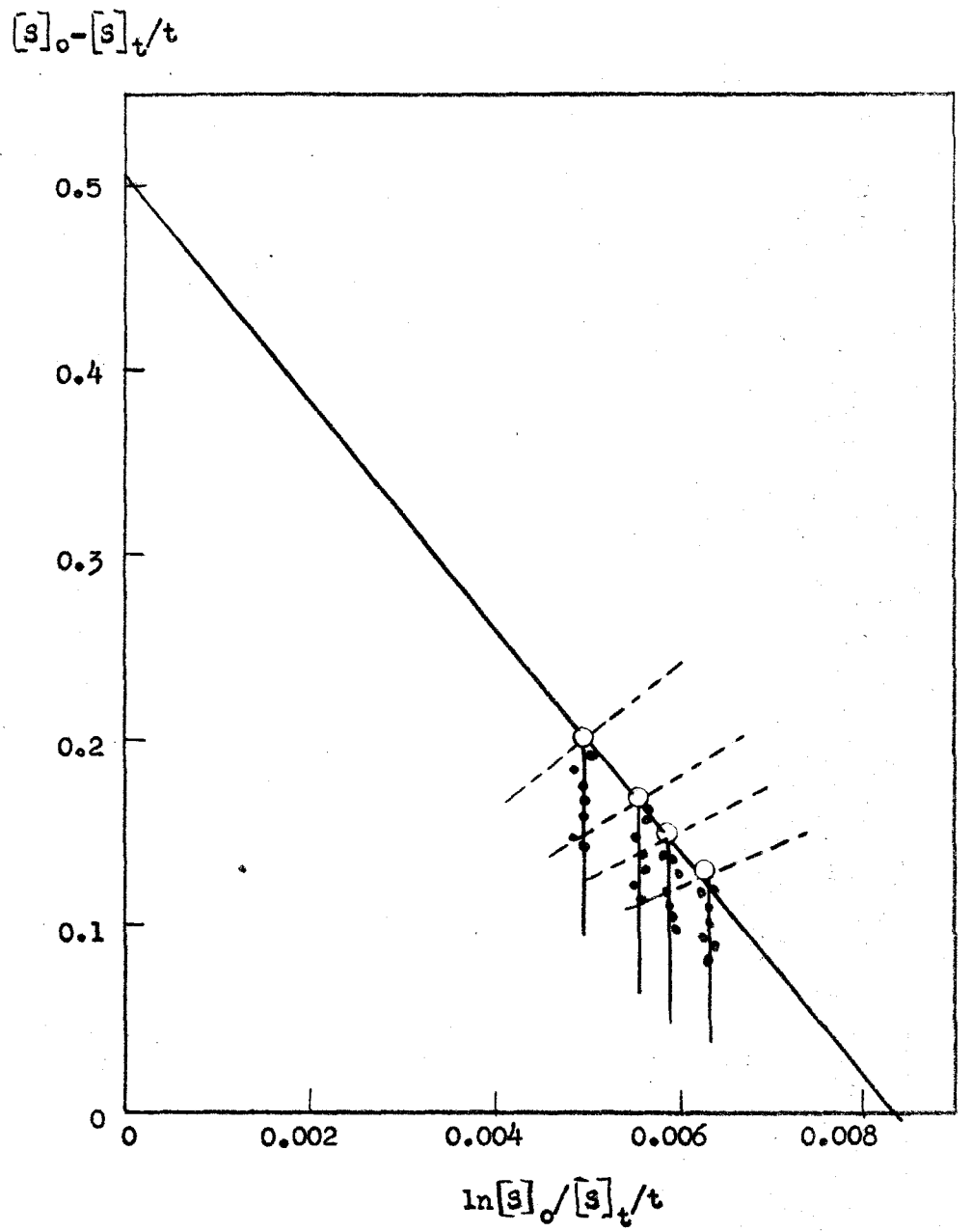


Fig. 7 Foster-Niemann Plot of the Data Presented in Table XXI

Table XXII

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY PHENYLACETAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	.376	2.200	0.110	0.00581
	25	.424	2.700	0.108	0.00579
	50	.648	5.050	0.101	0.00583
	75	.839	7.000	0.093	0.00574
	100	1.018	8.850	0.089	0.00586
	125	1.173	10.450	0.084	0.00592
	150	1.292	11.700	0.078	0.00585
25	20	.407	2.550	0.128	0.00540
	25	.474	3.200	0.128	0.00544
	50	.742	6.025	0.120	0.00553
	75	.977	8.450	0.113	0.00549
	100	1.183	10.575	0.106	0.00551
	125	1.367	12.475	0.100	0.00553
	150	1.544	14.325	0.096	0.00562
Dilution 1:25			Blank = 0.162	N(10) = 1.127	
30	20	.308	2.95	0.148	0.00516
	25.5	.346	3.70	0.145	0.00527
	50	.517	6.90	0.138	0.00523
	75	.669	9.75	0.130	0.00524
	100	.799	12.20	0.122	0.00522
	125	.922	14.55	0.116	0.00531
	150	1.031	16.55	0.110	0.00535
40	20	.342	3.65	0.183	0.00476
	25	.400	4.70	0.188	0.00499
	50	.618	8.80	0.176	0.00497
	75	.826	12.75	0.170	0.00513
	100	1.008	16.15	0.162	0.00516
	125	1.143	18.65	0.149	0.00503
	150	1.288	21.35	0.142	0.00509
Dilution 1:50			Blank = 0.148	N(30) = 1.745	

Table XXIII

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY TRYPTAMINE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.02 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln[S]_o/[S]_t/t$
20	20	.520	0.70	0.0350	0.00185
	25	.541	0.90	0.0360	0.00185
	50	.639	1.80	0.0360	0.00187
	75	.726	2.60	0.0347	0.00184
	100	.808	3.35	0.0335	0.00184
	125	.892	4.15	0.0332	0.00185
	150	.969	4.85	0.0323	0.00186
25	20	.542	0.90	0.0450	0.00182
	25	.567	1.10	0.0440	0.00183
	50	.677	2.15	0.0430	0.00181
	75	.796	3.20	0.0427	0.00183
	100	.892	4.15	0.0415	0.00182
	125	.998	5.10	0.0408	0.00182
	150	1.090	5.95	0.0397	0.00181
Dilution 1:25			Blank = 0.443	N(10) = 1.526	
30	20	.403	1.10	0.0550	0.00187
	25	.405	1.15	0.0460	0.00157
	50	.474	2.40	0.0480	0.00167
	75	.544	3.70	0.0493	0.00176
	100	.601	4.75	0.0475	0.00172
	125	.650	5.65	0.0452	0.00167
	150	.711	6.80	0.0453	0.00175
40	20	.419	1.35	0.0675	0.00172
	25	.434	1.65	0.0660	0.00168
	50	.522	3.30	0.0660	0.00172
	75	.605	4.80	0.0640	0.00171
	100	.684	6.30	0.0630	0.00174
	125	.759	7.70	0.0616	0.00171
	150	.829	8.95	0.0597	0.00169
Dilution 1:50			Blank = 0.341	N(30) = 1.961	

Table XXIV

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.1 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	14	.384	0.35	0.0250	0.00516
	20	.422	0.48	0.0240	0.00503
	40	.544	0.93	0.0230	0.00515
	60	.650	1.32	0.0220	0.00512
	80	.768	1.74	0.0218	0.00534
	95	.835	1.99	0.0209	0.00534
	110	.903	2.24	0.0204	0.00541
10	15	.497	0.76	0.0506	0.00525
	20	.568	1.02	0.0510	0.00536
	40	.809	1.90	0.0475	0.00527
	60	1.036	2.73	0.0455	0.00531
	80	1.237	3.45	0.0432	0.00530
	95	1.384	3.98	0.0418	0.00534
	110	1.492	4.37	0.0398	0.00522
Dilution 1:10			Blank = 0.289	N(4) = 1.390	
30	19	.354	3.30	0.174	0.00615
	25	.368	3.60	0.144	0.00510
	50	.521	6.35	0.127	0.00475
	75	.662	8.95	0.119	0.00472
	100	.788	11.25	0.113	0.00470
	126	.897	13.20	0.105	0.00460
	150	1.013	15.40	0.103	0.00480
40	20	.358	3.40	0.170	0.00444
	25	.425	4.65	0.186	0.00496
	50	.624	8.25	0.165	0.00463
	75	.791	11.30	0.151	0.00443
	100	.948	14.15	0.142	0.00436
	125	1.080	16.60	0.133	0.00429
	150	1.200	18.75	0.125	0.00421
Dilution 1:50			Blank = 0.170	N(30) = 1.810	

Table XXV

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 1.50 Buffer = 0.1 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	.540	3.100	0.155	0.00841
	25	.626	3.850	0.154	0.00854
	50	.974	7.000	0.140	0.00863
	75	1.263	9.625	0.128	0.00874
	100	1.502	11.800	0.118	0.00890
	125	1.683	13.425	0.107	0.00891
	150	1.843	14.875	0.099	0.00910
25	20	.604	3.675	0.184	0.00792
	25	.706	4.575	0.183	0.00808
	50	1.122	8.325	0.167	0.00809
	75	1.472	11.525	0.154	0.00825
	100	1.762	14.150	0.142	0.00834
	125	2.012	16.425	0.131	0.00854
	150	2.208	18.200	0.121	0.00867
Dilution 1:25			Blank = 0.199	N(10) = 1.306	
30	20	.380	4.15	0.208	0.00744
	25	.439	5.25	0.210	0.00768
	50	.676	9.55	0.191	0.00767
	76	.898	13.35	0.176	0.00775
	100	1.060	16.50	0.165	0.00798
	125	1.206	19.15	0.153	0.00814
	150	1.318	21.20	0.141	0.00817
40	20	.442	5.30	0.265	0.00711
	25	.498	6.30	0.252	0.00685
	50	.808	11.90	0.238	0.00705
	75	1.073	16.75	0.223	0.00724
	100	1.313	21.10	0.211	0.00750
	125	1.486	24.30	0.194	0.00748
	150	1.650	27.30	0.182	0.00765
Dilution 1:50			Blank = 0.152	N(30) = 1.802	

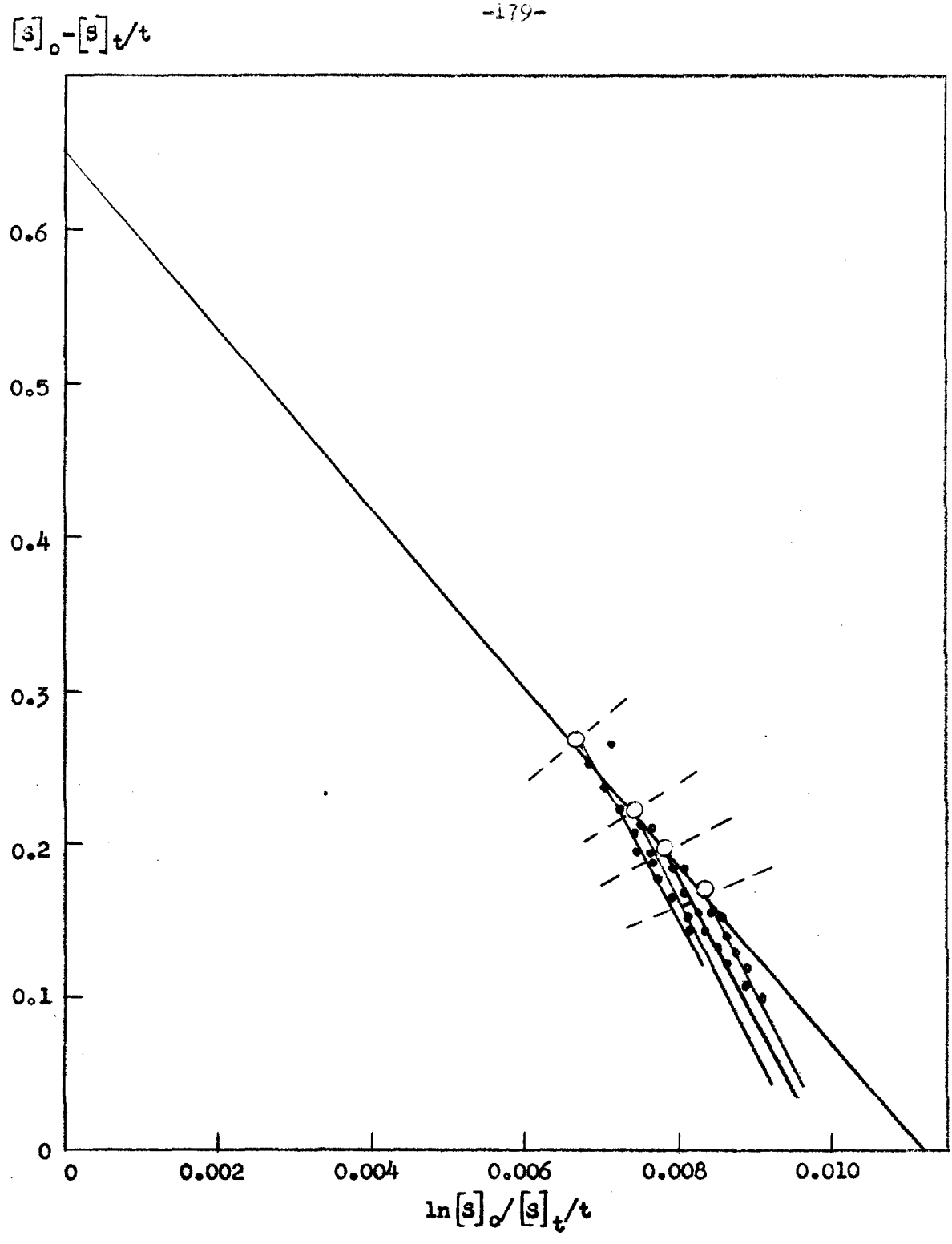


Fig. 8 Foster-Niemann Plot of the Data Presented in Table XXV

Table XXVI

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY PHENYLACETATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 50.0 Buffer = 0.1 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	.552	3.150	0.158	0.00857
	25	.644	3.950	0.158	0.00880
	50	.993	7.175	0.144	0.00889
	75	1.296	9.900	0.132	0.00911
	100	1.540	12.125	0.121	0.00932
	125	1.748	14.000	0.112	0.00963
	150	1.887	15.300	0.102	0.00966
25	20	.620	3.750	0.188	0.00811
	25	.725	4.700	0.188	0.00830
	50	1.160	8.675	0.174	0.00851
	75	1.539	12.125	0.162	0.00885
	100	1.846	14.925	0.149	0.00909
	125	2.113	17.300	0.138	0.00942
	150	2.295	18.950	0.126	0.00947
Dilution 1:25			Blank = 0.203	N(10) = 1.307	
30	20	.391	4.25	0.213	0.00764
	25	.449	5.30	0.212	0.00778
	50	.702	9.85	0.197	0.00796
	75	.938	14.15	0.189	0.00851
	100	1.126	17.55	0.176	0.00880
	125	1.278	20.20	0.162	0.00894
	150	1.379	22.10	0.147	0.00898
40	20	.434	5.00	0.250	0.00667
	25	.515	6.45	0.258	0.00702
	50	.818	11.95	0.239	0.00710
	75	1.104	17.10	0.228	0.00744
	100	1.343	21.50	0.215	0.00771
	125	1.545	25.15	0.201	0.00792
	150	1.690	27.75	0.185	0.00789
Dilution 1:50			Blank = 0.159	N(30) = 1.812	

Table XXVII

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY PHENYLACETAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.1 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	14	.468	0.62	0.044	0.00946
	20	.538	0.86	0.043	0.00935
	40	.717	1.51	0.038	0.00897
	60	.889	2.13	0.036	0.00926
	80	1.028	2.64	0.033	0.00938
	95	1.120	2.97	0.031	0.00947
	110	1.190	3.23	0.029	0.00944
10	15	.640	1.24	0.0826	0.00883
	20	.753	1.65	0.0825	0.00903
	40	1.121	2.97	0.0742	0.00881
	60	1.429	4.09	0.0683	0.00878
	80	1.694	5.05	0.0631	0.00878
	95	1.834	5.56	0.0586	0.00854
	110	1.999	6.13	0.0557	0.00865
Dilution 1:10			Blank = 0.299	N(4) = 1.406	
30	20	.360	3.85	0.192	0.00686
	25	.412	4.80	0.192	0.00696
	50	.629	8.65	0.173	0.00680
	75	.818	12.00	0.160	0.00680
	100	1.007	15.35	0.154	0.00715
	125.5	1.141	17.80	0.142	0.00716
	150	1.265	20.00	0.133	0.00733
40	20	.418	4.85	0.242	0.00645
	25	.480	6.00	0.240	0.00647
	50	.767	11.10	0.222	0.00650
	75	1.016	15.60	0.208	0.00659
	100	1.243	19.65	0.197	0.00676
	125	1.410	22.60	0.181	0.00665
	150	1.566	25.35	0.169	0.00647
Dilution 1:50			Blank = 0.141	N(30) = 1.822	

Table XXVIII

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY TRYPTAMINE AT pH 7.9 AND 25°

$[E]$ = 0.222 $[I]$ = 5.0 Buffer = 0.1 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	14	1.014	0.32	0.0229	0.00470
	20	1.068	0.50	0.0250	0.00526
	40	1.165	0.86	0.0215	0.00472
	60	1.260	1.20	0.0200	0.00457
	80	1.350	1.54	0.0193	0.00468
	95	1.422	1.81	0.0191	0.00473
	110	1.481	2.02	0.0184	0.00470
10	15	1.126	0.72	0.0480	0.00500
	20	1.178	0.91	0.0456	0.00476
	40	1.381	1.64	0.0410	0.00447
	60	1.566	2.33	0.0388	0.00442
	80	1.740	2.96	0.0370	0.00438
	95	1.838	3.32	0.0349	0.00425
	110	1.963	3.78	0.0344	0.00432
Dilution 1:10			Blank = 0.927	N(4) = 2.024	
30	20	0.415	2.60	0.130	0.00453
	25	0.436	2.95	0.118	0.00414
	50	0.580	5.65	0.113	0.00417
	75	0.723	8.30	0.111	0.00430
	100	0.830	10.25	0.102	0.00417
	125.5	0.930	12.10	0.0964	0.00411
	150	1.042	14.20	0.0946	0.00426
40	20	0.441	3.05	0.153	0.00398
	25	0.480	3.80	0.152	0.00398
	50	0.666	7.20	0.144	0.00398
	75	0.840	10.40	0.139	0.00401
	100	0.989	13.20	0.132	0.00400
	125	1.115	15.45	0.124	0.00391
	150	1.243	17.85	0.119	0.00393
Dilution 1:50			Blank = 0.273	N(30) = 1.899	

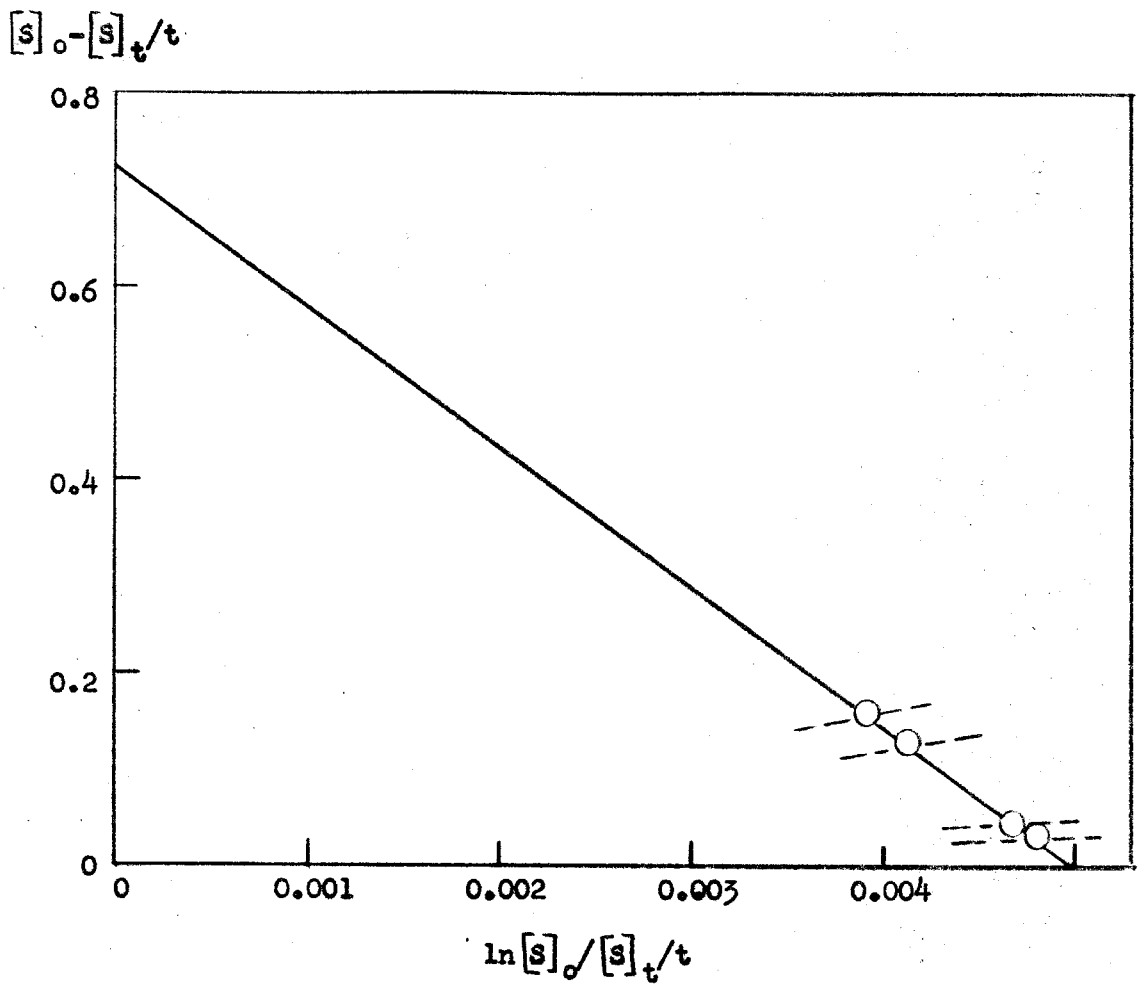


Fig. 9 Foster-Niemann Plot of the Data Presented in Table XXVIII

Table XXIX

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.4 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	0.430	2.300	0.115	0.00611
	25	0.492	2.850	0.114	0.00614
	50	0.761	5.325	0.107	0.00619
	75	0.974	7.275	0.097	0.00603
	100	1.180	9.175	0.092	0.00614
	125	1.351	10.725	0.086	0.00615
	150	1.490	12.000	0.080	0.00611
25	20	0.478	2.700	0.135	0.00572
	25	0.549	3.375	0.135	0.00580
	50	0.844	6.100	0.122	0.00560
	75	1.136	8.750	0.117	0.00574
	100	1.377	10.950	0.110	0.00576
	125	1.576	12.775	0.102	0.00572
	150	1.754	14.375	0.096	0.00570
Dilution 1:25			Blank = 0.187	N(10) = 1.270	
30	20	0.311	3.10	0.155	0.00543
	25	0.354	3.85	0.154	0.00548
	50	0.540	7.25	0.145	0.00554
	75	0.698	10.20	0.136	0.00554
	100	0.822	12.50	0.125	0.00539
	125	0.953	14.95	0.120	0.00553
	150	1.060	16.90	0.113	0.00553
40	20	0.352	3.75	0.188	0.00492
	25	0.391	4.50	0.180	0.00499
	50	0.614	8.70	0.174	0.00492
	75	0.782	11.75	0.157	0.00464
	100	0.979	15.40	0.154	0.00486
	125	1.140	18.35	0.147	0.00493
	150	1.273	20.85	0.139	0.00491
Dilution 1:50			Blank = 0.152	N(30) = 1.760	

Table XXX

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.4 M THAM-HCl and 0.0001 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	0.422	2.300	0.115	0.00611
	25	0.482	2.825	0.113	0.00607
	50	0.753	5.250	0.105	0.00609
	75	0.986	7.350	0.098	0.00612
	100	1.172	9.050	0.091	0.00603
	125	1.355	10.650	0.085	0.00601
	150	1.511	12.050	0.080	0.00614
25	20	0.472	2.750	0.138	0.00582
	25	0.538	3.325	0.133	0.00569
	50	0.862	6.250	0.125	0.00575
	75	1.143	8.800	0.117	0.00578
	100	1.344	11.000	0.110	0.00580
	125	1.609	12.925	0.103	0.00582
	150	1.780	14.475	0.097	0.00577
Dilution 1:25			Blank = 0.168	N(10) = 1.282	
30	20	0.299	3.00	0.150	0.00526
	25	0.342	3.70	0.148	0.00527
	50	0.517	6.80	0.136	0.00514
	75	0.670	9.55	0.127	0.00512
	100	0.811	12.10	0.121	0.00516
	125	0.942	14.50	0.116	0.00527
	150	1.056	16.50	0.110	0.00532
40	20	0.352	3.95	0.198	0.00522
	25	0.395	4.70	0.188	0.00499
	50	0.625	8.80	0.176	0.00496
	75	0.832	12.50	0.167	0.00500
	100	1.011	15.70	0.157	0.00498
	125	1.149	18.15	0.145	0.00484
	150	1.320	21.25	0.142	0.00505
Dilution 1:50			Blank = 0.135	N(30) = 1.805	

Table XXXI

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.4 M THAM-HCl and 0.001 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	0.474	2.375	0.119	0.00633
	25	0.546	3.000	0.120	0.00648
	50	0.818	5.425	0.109	0.00632
	75	1.061	7.575	0.101	0.00635
	100	1.259	9.350	0.094	0.00630
	125	1.449	11.050	0.088	0.00637
	150	1.601	12.425	0.083	0.00647
25	20	0.529	2.850	0.143	0.00607
	25	0.600	3.500	0.140	0.00604
	50	0.931	6.450	0.129	0.00597
	75	1.221	9.025	0.120	0.00596
	100	1.469	11.250	0.113	0.00598
	125	1.704	13.350	0.107	0.00611
	150	1.874	14.875	0.099	0.00602
Dilution 1:25			Blank = 0.209	N(10) = 1.330	
30	20	0.368	3.50	0.175	0.00620
	25	0.402	4.10	0.164	0.00587
	50	0.586	7.45	0.149	0.00569
	75	0.752	10.45	0.139	0.00572
	100	0.906	13.20	0.132	0.00580
	125	1.041	15.65	0.125	0.00590
	150	1.131	17.25	0.115	0.00570
40	20	0.391	3.90	0.195	0.00512
	25	0.450	5.00	0.200	0.00534
	50	0.674	9.05	0.181	0.00512
	75	0.833	12.80	0.171	0.00514
	100	1.068	16.05	0.161	0.00513
	125	1.234	19.10	0.153	0.00519
	150	1.380	21.75	0.145	0.00522
Dilution 1:50			Blank = 0.174	N(30) = 1.838	

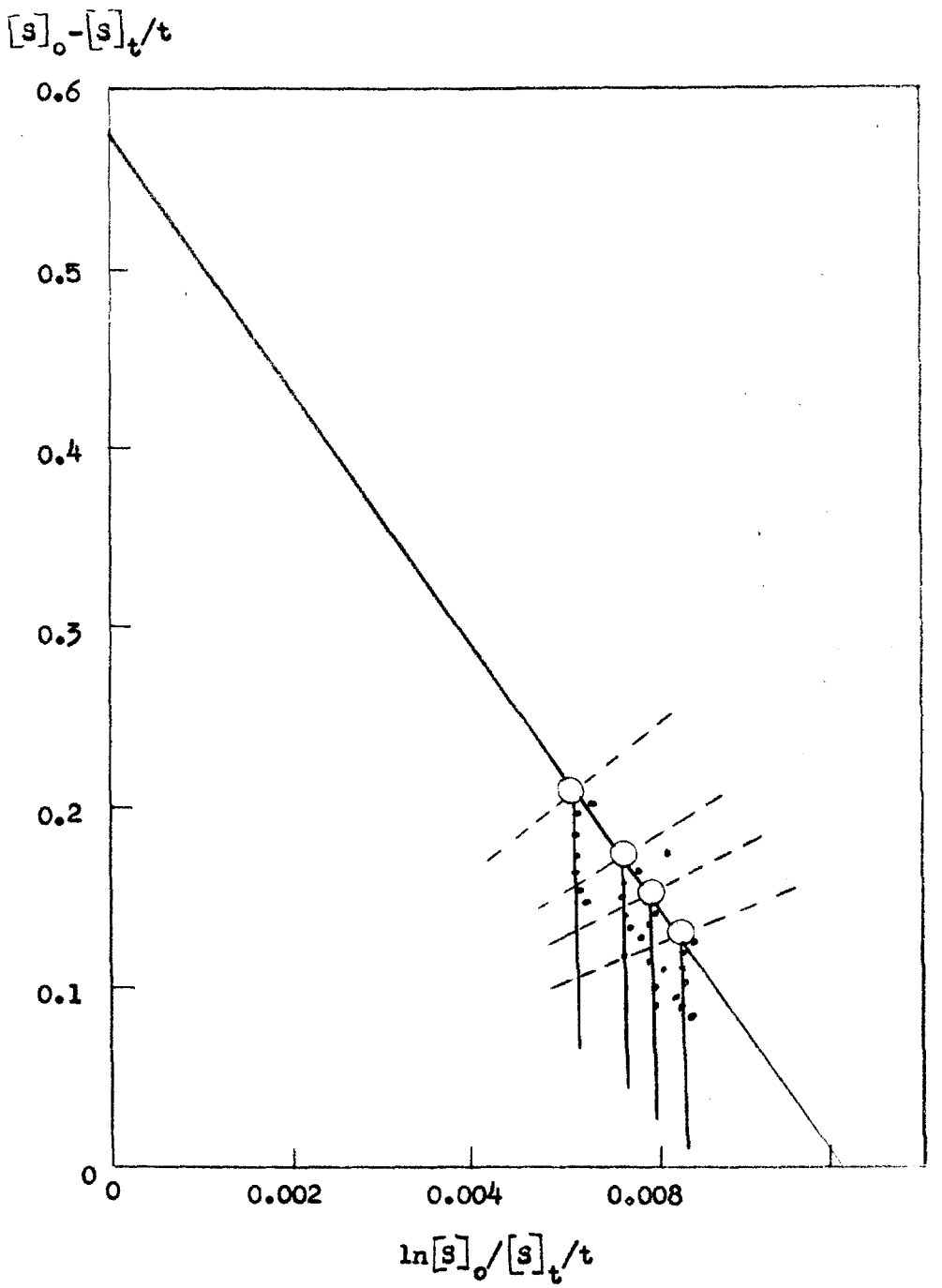


Fig. 10 Foster-Niemann Plot of the Data Presented in Table XXXI

Table XXXII

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.27 M THAM-HCl and 0.01 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t / t$	$\ln [S]_o / [S]_t / t$
20	20	0.449	2.25	0.113	0.00597
	25	0.494	2.70	0.108	0.00580
	50	0.771	5.15	0.103	0.00596
	75	1.003	7.20	0.096	0.00595
	100	1.209	9.05	0.091	0.00603
	125	1.367	10.45	0.084	0.00592
	150	1.520	11.85	0.079	0.00598
25	20	0.502	2.75	0.138	0.00584
	25	0.563	3.30	0.132	0.00566
	50	0.895	6.25	0.125	0.00574
	75	1.158	8.60	0.115	0.00562
	100	1.408	10.85	0.109	0.00569
	125	1.622	12.75	0.102	0.00571
	150	1.793	14.30	0.095	0.00566
Dilution 1:25			Blank = 0.196	N(10) = 1.315	
30	20	0.335	3.10	0.155	0.00544
	25	0.368	3.65	0.146	0.00518
	50	0.548	6.95	0.139	0.00526
	75	0.709	9.80	0.131	0.00527
	100	0.869	12.70	0.127	0.00550
	125	0.890	14.70	0.118	0.00539
	150	1.096	16.80	0.112	0.00547
40	20	0.390	4.05	0.203	0.00534
	25	0.429	4.75	0.190	0.00507
	50	0.642	8.60	0.172	0.00484
	75	0.856	12.50	0.167	0.00499
	100	1.044	15.85	0.159	0.00504
	125	1.209	18.80	0.150	0.00507
	150	1.339	21.15	0.141	0.00503
Dilution 1:50			Blank = 0.172	N(30) = 1.825	

Table XXXIII

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.2 M THAM-HCl and 0.04 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	0.428	2.200	0.110	0.00582
	25	0.484	2.700	0.108	0.00580
	50	0.739	5.025	0.101	0.00578
	75	0.954	6.975	0.093	0.00572
	100	1.146	8.725	0.087	0.00573
	125	1.322	10.350	0.083	0.00582
	150	1.460	11.600	0.077	0.00578
25	20	0.478	2.650	0.133	0.00561
	25	0.547	3.275	0.131	0.00562
	50	0.846	6.025	0.121	0.00551
	75	1.113	8.450	0.113	0.00551
	100	1.341	10.525	0.105	0.00546
	125	1.549	12.425	0.099	0.00549
	150	1.718	13.950	0.093	0.00544
Dilution 1:25		Blank = 0.183		N(10) = 1.286	
30	20	0.280	2.55	0.128	0.00518
	25	0.336	3.60	0.144	0.00510
	50	0.512	6.85	0.137	0.00518
	75	0.676	9.75	0.130	0.00524
	100	0.800	12.05	0.121	0.00514
	125	0.909	14.05	0.112	0.00506
	150	0.995	15.65	0.104	0.00493
40	20	0.349	3.80	0.190	0.00498
	25	0.390	4.60	0.184	0.00489
	50	0.605	8.45	0.169	0.00475
	75	0.821	12.45	0.166	0.00497
	100	0.971	15.20	0.152	0.00478
	125	1.099	17.55	0.140	0.00462
	150	1.271	20.70	0.138	0.00486
Dilution 1:50		Blank = 0.141		N(30) = 1.779	

Table XXXIV

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.0725 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	0.350	1.950	0.098	0.00512
	25	0.398	2.400	0.096	0.00509
	50	0.629	4.525	0.091	0.00510
	75	0.832	6.375	0.085	0.00512
	100	1.008	7.950	0.080	0.00506
	125	1.164	9.425	0.075	0.00510
	150	1.310	10.750	0.072	0.00514
25	20	0.394	2.375	0.119	0.00497
	25	0.447	2.850	0.114	0.00485
	50	0.727	5.425	0.109	0.00489
	75	0.980	7.700	0.103	0.00491
	100	1.198	9.700	0.097	0.00491
	125	1.378	11.375	0.091	0.00486
	150	1.557	13.025	0.087	0.00491
Dilution 1:25			Blank = 0.134	N(10) = 1.226	
30	20	0.267	2.70	0.135	0.00471
	25	0.311	3.50	0.140	0.00495
	50	0.460	6.20	0.124	0.00464
	75	0.602	8.85	0.118	0.00467
	100	0.736	11.30	0.113	0.00472
	125	0.841	13.20	0.106	0.00464
	150	0.950	15.25	0.102	0.00474
40	20	0.303	3.35	0.168	0.00436
	25	0.344	4.10	0.164	0.00432
	50	0.554	7.95	0.159	0.00443
	75	0.738	11.30	0.151	0.00443
	100	0.901	14.30	0.143	0.00442
	125	1.026	16.65	0.133	0.00430
	150	1.161	19.10	0.127	0.00433
Dilution 1:50			Blank = 0.121	N(30) = 1.750	

Table XXXV

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

[E] = 0.222 [I] = 10.0 Buffer = 0.2 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	14	0.441	0.42	0.0300	0.00627
	20	0.471	0.52	0.0260	0.00544
	40	0.633	1.12	0.0280	0.00634
	60	0.762	1.57	0.0262	0.00628
	80	0.861	1.92	0.0240	0.00607
	95	0.960	2.26	0.0238	0.00633
	110	1.026	2.50	0.0227	0.00630
10	15	0.546	0.78	0.0520	0.00544
	20	0.626	1.07	0.0535	0.00611
	40	0.910	2.08	0.0520	0.00584
	60	1.152	2.95	0.0492	0.00585
	80	1.408	3.85	0.0482	0.00608
	95	1.524	4.26	0.0449	0.00585
	110	1.666	4.76	0.0433	0.00585
Dilution 1:10			Blank = 0.324	N(4) = 1.451	
30	20	0.299	3.20	0.160	0.00564
	25	0.330	3.75	0.150	0.00526
	50	0.516	7.15	0.143	0.00542
	75	0.679	10.10	0.135	0.00547
	100	0.836	12.95	0.129	0.00564
	125	0.950	15.05	0.120	0.00547
	150	1.074	17.30	0.115	0.00573
40	20	0.338	3.90	0.195	0.00513
	25	0.384	4.75	0.190	0.00507
	50	0.619	9.00	0.180	0.00511
	75	0.843	13.10	0.175	0.00529
	100	1.031	16.50	0.165	0.00532
	125	1.174	19.10	0.153	0.00519
	150	1.320	21.75	0.145	0.00523
Dilution 1:50			Blank = 0.122	N(30) = 1.774	

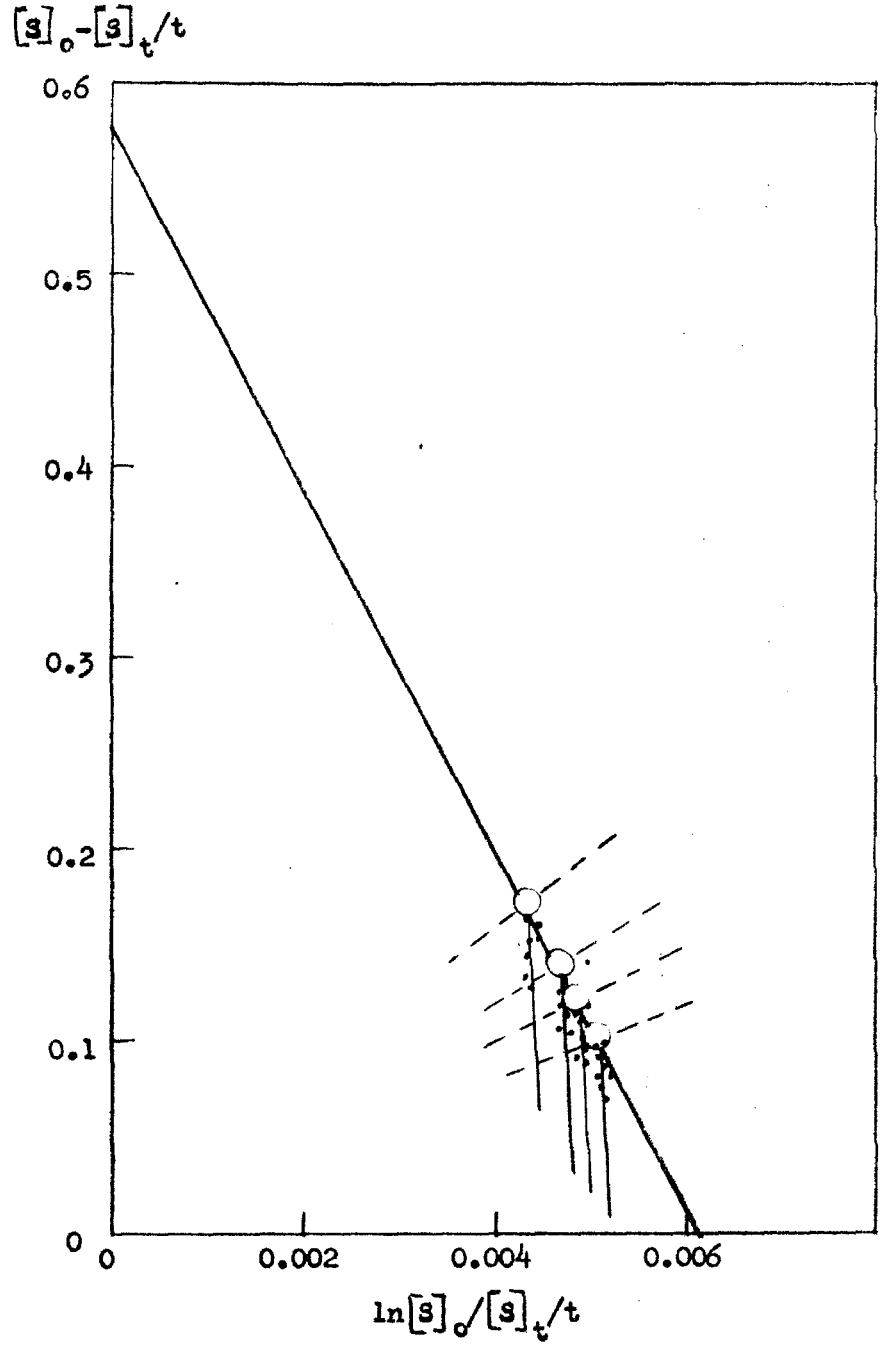


Fig. 11 Foster-Niemann Plot of the Data Presented in Table XXXIV

Table XXXVI

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.4 M Phosphate (K)

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	0.473	2.200	0.110	0.00580
	25	0.524	2.675	0.107	0.00576
	50	0.791	5.050	0.101	0.00582
	75	1.012	7.050	0.094	0.00579
	100	1.207	8.750	0.088	0.00575
	125	1.418	10.650	0.085	0.00581
	150	1.526	11.625	0.078	0.00580
25	20	0.520	2.625	0.131	0.00553
	25	0.586	3.200	0.128	0.00549
	50	0.899	6.000	0.120	0.00549
	75	1.182	8.550	0.114	0.00558
	100	1.413	10.600	0.106	0.00552
	125	1.622	12.500	0.100	0.00554
	150	1.795	14.050	0.094	0.00550
Dilution 1:25			Blank = 0.224	N(10) = 1.345	
30	20	0.368	3.10	0.155	0.00545
	25	0.405	3.80	0.152	0.00542
	50	0.594	7.15	0.143	0.00544
	75	0.769	10.30	0.137	0.00561
	100	0.898	12.65	0.127	0.00547
	125	1.016	14.75	0.118	0.00542
	150	1.159	17.30	0.115	0.00572
40	20	0.408	3.80	0.190	0.00498
	25	0.455	4.70	0.188	0.00499
	50	0.684	8.80	0.176	0.00497
	75	0.887	12.40	0.165	0.00494
	100	1.075	15.80	0.158	0.00503
	125	1.243	18.85	0.151	0.00509
	150	1.376	21.20	0.141	0.00504
Dilution 1:50			Blank = 0.194	N(30) = 1.872	

Table XXXVII

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY beta-(beta-INDOLE)-PROPIONATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 10.0 Buffer = 0.4 M THAM-HCl with 0.4 M NaCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
20	20	0.448	2.400	0.120	0.00637
	25	0.519	3.075	0.123	0.00668
	50	0.805	5.675	0.114	0.00668
	75	1.075	8.125	0.108	0.00694
	100	1.274	9.950	0.100	0.00688
	125	1.443	11.475	0.092	0.00682
	150	1.624	13.125	0.088	0.00711
25	20	0.516	3.050	0.153	0.00650
	25	0.606	3.850	0.154	0.00668
	50	0.955	7.025	0.141	0.00660
	75	1.255	9.750	0.130	0.00659
	100	1.556	12.475	0.125	0.00681
	125	1.754	14.300	0.114	0.00677
	150	1.909	15.675	0.105	0.00658
Dilution 1:25			Blank = 0.185	N(15) = 1.833	
30	20	0.332	3.55	0.178	0.00627
	25	0.381	4.40	0.176	0.00634
	50	0.589	8.15	0.163	0.00634
	75	0.761	11.25	0.150	0.00627
	100	0.913	14.05	0.141	0.00632
	125	1.054	16.55	0.132	0.00642
	150	1.157	18.40	0.123	0.00634
40	20	0.378	4.35	0.218	0.00575
	25	0.440	5.45	0.218	0.00586
	50	0.698	10.10	0.202	0.00582
	75	0.928	14.30	0.191	0.00589
	100	1.100	17.35	0.174	0.00568
	125	1.285	20.75	0.166	0.00585
	150	1.439	23.55	0.157	0.00593
Dilution 1:50			Blank = 0.140	N(30) = 1.795	

Table XXXVIII

INHIBITION OF THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY PHENYLACETATE AT pH 7.9 AND 25°

$[E]$ = 0.200 $[I]$ = 150 Buffer = 0.3 M THAM-HCl

$[S]_o$	t	O.D.	$[S]_o - [S]_t$	$[S]_o - [S]_t/t$	$\ln[S]_o/[S]_t/t$
20	20	0.496	2.575	0.129	0.00690
	25	0.570	3.250	0.130	0.00709
	50	0.861	5.900	0.118	0.00699
	75	1.181	8.200	0.109	0.00704
	100	1.331	10.150	0.102	0.00708
	125	1.511	11.775	0.094	0.00712
	150	1.670	13.200	0.088	0.00720
25	20	0.554	3.100	0.155	0.00660
	25	0.637	3.825	0.153	0.00664
	50	0.991	7.075	0.142	0.00666
	75	1.229	9.850	0.131	0.00668
	100	1.562	12.225	0.122	0.00671
	125	1.768	14.100	0.113	0.00663
	150	1.962	15.875	0.106	0.00671
Dilution 1:25			Blank = 0.209	N(10) = 1.315	
30	20	0.363	3.55	0.178	0.00629
	25	0.412	4.40	0.176	0.00633
	50	0.619	8.15	0.163	0.00634
	75	0.797	11.40	0.152	0.00637
	100	0.950	14.20	0.142	0.00641
	125	1.079	16.50	0.132	0.00638
	150	1.192	18.55	0.124	0.00642
40	20	0.409	4.35	0.218	0.00574
	25	0.463	5.35	0.214	0.00573
	50	0.726	10.10	0.202	0.00582
	75	0.940	14.05	0.187	0.00576
	100	1.139	17.60	0.176	0.00580
	125	1.304	20.65	0.165	0.00580
	150	1.447	23.20	0.155	0.00578
Dilution 1:50			Blank = 0.165	N(30) = 1.819	

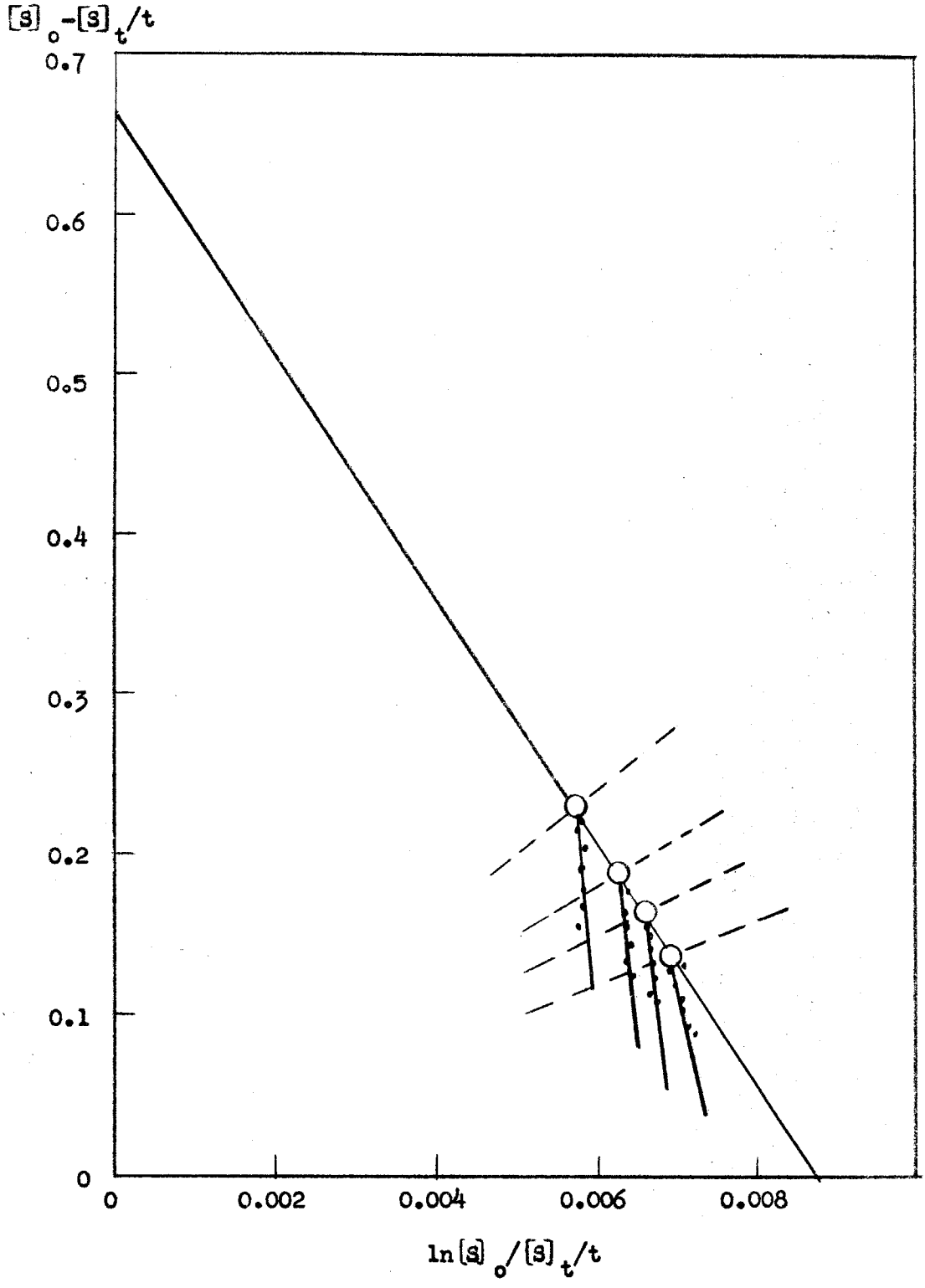


Fig. 12 Foster-Niemann Plot of the Data Presented in Table XXXVIII

REFERENCES

(1) Neurath, H., and Gladner, J.A., J. Biol. Chem., 188, 407 (1951).

(2) Huang, H. T., and Niemann, C., J. A. C. S., 74, 5963 (1952).

(3) Jennings, R. R., and Niemann, C., ibid., 75, 4687 (1953).

(4) Eadie, G. S., J. Biol. Chem., 146, 85 (1942).

(5) Hofstee, H. J., Science, 116, 329 (1952).

(6) Eadie, G. S., ibid., 116, 688 (1952).

(7) Foster, R. J., and Niemann, C., Proc. Nat. Acad. Sci., 39, 999 (1953).

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

(9) Goldstein, A., ibid., 27, 529 (1944).

(10) Thomas, D. W., MacAllister, R. V., and Niemann, C., J. A. C. S., 73, 1548 (1951).

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

(12) Shine, H. J., and Niemann, C., ibid., 74, 97 (1952).

(13) Huang, H. T., MacAllister, R. V., Thomas, D. W., and Niemann, C., ibid., 73, 3221 (1951).

(14) Foster, R. J., and Niemann, C., ibid., 77, 0000 (1955).

(15) Schwert, G. W., J. Biol. Chem., 179, 655 (1949).

(16) Schwert, G. W., and Kaufman, S., ibid., 190, 807 (1951).

(17) Smith, E. L., Brown, D. M., and Laskowski, M., ibid., 191, 639 (1951).

(18) Kaufman, S., and Neurath, H., Arch. Biochem., 21, 245 (1949).

(19) Schwert, G. W., and Kaufman, S., J. Biol. Chem., 180, 517 (1949).

(20) Kaufman, S., and Neurath, H., ibid., 181, 623 (1949).

(21) Kaufman, S., and Neurath, H., ibid., 180, 181 (1949).

(22) Jandorf, B. J., Fed. Proc., 9, 186 (1950).

(23) Shine, H. J., Unpublished results.

(24) Niemann, C., Unpublished results.

(25) Handbook of Chemistry and Physics, Chemical Rubber Pub. Co., Cleveland, 1954.

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

(27) All melting point values are corrected.

(28) Microanalyses by Dr. A. Elek.

(29) du Vigneaud, V., and Meyer, C. E., J. Biol. Chem., 98, 295 (1932).

(30) Huang, H. T., and Niemann, C., J. A. C. S., 74, 105 (1952).

PART IV

THE ABSENCE OF WALL EFFECTS IN A TYPICAL alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS

ABSENCE OF WALL EFFECTS IN A TYPICAL alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS

In the past (1, 2) it has been tacitly assumed that alpha-chymotrypsin catalyzed hydrolyses, which are conducted under the conditions ordinarily used in in vitro studies of the mode of action of this enzyme, proceed entirely in solution and that wall effects, arising from the interaction of the reactants with the walls of the container, are unimportant. However, it appears that no one has ever determined whether or not the above assumption is a valid one. Therefore, in this investigation, a representative alpha-chymotrypsin catalyzed hydrolysis was examined with respect to possible wall effects arising from the nature and surface area of the container.

Data and Results

The initial velocities were determined, by the method of Jennings and Niemann (3) from both zero and first order plots, for the alpha-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosin-hydroxamide (2, 4, 5), in aqueous solutions at 25° and pH 7.6 and 0.3 M in the THAM[1] component of a THAM-HCl buffer, under conditions where the enzyme concentration was maintained at 0.0266 mg. protein-nitrogen/ml., i.e., ca. 0.76×10^{-5} M[2], the initial specific

[1] Tris-(hydroxymethyl)-aminomethane.

[2] Based upon a molecular weight of 22,000 and a nitrogen content of 16.0 % for monomeric alpha-chymotrypsin (2).

substrate concentration at 10 X 10^{-3} M, and where only the nature or the surface area of the container was varied. It will be seen from the data presented in Table I, for experiments 1 to 3 inclusive, that there is no significant difference in the initial velocities when either Pyrex or Kimble glass or polyethylene containers of equivalent surface area were employed. Furthermore, the addition of powdered Kimble glass to either the Kimble or Pyrex glass containers, or powdered Pyrex glass to the Pyrex container, cf., Table I, experiments 4 to 8 inclusive, was without effect even though the increase in surface area was of the order of twentyfold in the extreme cases.

Therefore, it may be concluded from the results of this study that, for the case at hand, wall effects are experimentally unimportant under the conditions which are generally employed in in vitro studies with alpha-chymotrypsin, and that it is reasonable to assume that in all alpha-chymotrypsin catalyzed reactions which are studied under these conditions, the reaction can be postulated as proceeding in solution insofar as can be determined within the limits of experimental error.

The average of the eight values of v_o which are given in Table I, i.e., 0.158 X 10^{-3} M/min., may be compared with the value of 0.166 ± 0.028 X 10^{-3} M/min. calculated on the basis of $[E]$ = 0.0266 mg. protein-nitrogen/ml., $[S]_o$ = 10 X 10^{-3} M,

$K_S = 43 \pm 4 \times 10^{-3}$ M and $k_3 = 33 \pm 3 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. (2). The fact that these two values agree, within the limits of experimental error, can be taken as evidence of the consistency of the values of v_o reported in this communication with those determined earlier (2, 4, 5).

Table I

alpha-CHYMOTRYPSIN CATALYZED HYDROLYSES OF ACETYL-L-TYROSINHYDROXAMIDE[a]

Expt. No.	Nature of Vessel	Powd. Glass Nature	Added Weight[b]	Total Area[c]	v_o[d]
1	Pyrex Glass	---	--	22	0.160
2	Kimble Glass	---	--	22	0.159
3	Polyethylene	---	--	22	0.156
4	Kimble Glass	Kimble[e]	0.30	83	0.153
5	" "	"	1.50	412	0.157
6	" "	Pyrex[f]	0.20	61	0.160
7	Pyrex Glass	"	0.27	81	0.157
8	" "	"	1.33	406	0.157

Average of all values 0.157

a., In aqueous solutions at 25° and pH 7.62 and 0.3 M in the THAM component of a THAM-HCl buffer, $[E]$ = 0.0266 mg. protein-nitrogen/ml., $[S]_o = 10 \times 10^{-3}$ M.

b., In g.

c., In units of $cm.^2$.

d., In units of 10^{-3} M/min.

e., 150-200 mesh, density 2.5 $g./cm.^3$.

f., 150-200 mesh, density 2.25 $g./cm.^3$.

EXPERIMENTAL

Containers. - The Pyrex and Kimble glass containers used in this study were standard 10-ml. G.S. volumetric flasks which had been cleaned with hot water containing a detergent and then thoroughly washed with distilled water. The polyethylene container was a 60 ml. screw cap bottle which had been treated similarly.

Powdered Glass. - Pyrex and Kimble glass tubing was ground in an iron mortar and pestle, the product which passed a 150 mesh screen collected on a 200 mesh screen, digested with concentrated hydrochloric acid for 48 hours at 25-30°, thoroughly washed with distilled water, and dried at 145° for 2 hours. In the calculation of surface areas an average diameter of 0.0088 cm. was assumed. The density of Pyrex glass was taken to be 2.25 g./cm.^{3} and that of the Kimble glass 2.5g./cm.^{3}.

Enzyme and Specific Substrate. - The alpha-chymotrypsin was an Armour preparation Lot No. 10705. The acetyl-L-tyrosinhydroxamide, m.p. ca. 140° with decomposition, $[\alpha]_D^{25} = 37.0°$ (c 5 % in water), was prepared essentially as described by Hogness and Niemann (4).

Enzyme Experiments. - All reactions were conducted as

described by Hogness and Niemann (4) except that the improved analytical procedure described by Foster, Jennings, and Niemann (6) was substituted for the one used earlier (4). In every case plots of both $([S]_o - [S]_t)$ versus t and $\ln [S]_o/[S]_t$ versus t were made and then corrected as described by Jennings and Niemann (3) using a value of $K_S = 43 \times 10^{-3}$ M. The values of v_o given in table I are the averages of those obtained from the corrected zero and first order plots (presented in table III) which in no case differed by more than $\pm$ 0.005 X 10^{-3} M/min.

Contribution No. 1929 from the
Gates and Crellin Laboratories of Chemistry
California Institute of Technology
Pasadena 4, California

SUPPLEMENT

Because of the necessary brevity of published communications, the bulk of the experimental data has been omitted from the preceding communication. In order to present a complete exposition of the problem, the following pages contain these experimental data.

The legend of symbols is as follows:

t = time in minutes

O. D. = optical density

Table II

KINETIC DATA

$[S]_o$ = 10 X 10^{-3} M Acetyl-L-tyrosinhydroxamide

$[E]$ = 0.0266 mg. protein-nitrogen/ml.

Buffer = 0.3 M THAM-HCl

T = 25° pH 7.6 ± 0.2

Expt. No.	t	O.D.	ln O.D.
1	2	1.31	+0.270
	7	1.21	+0.191
	14	1.066	+0.064
	21	0.960	-0.041
	28	0.845	-0.168
	35	0.750	-0.288
	42	0.661	-0.414
2	2	1.303	+0.262
	8	1.178	+0.165
	16	1.037	+0.037
	24	0.900	-0.105
	32	0.790	-0.236
	40	0.681	-0.384
	48	0.596	-0.518
3	2	1.320	+0.278
	7	1.230	+0.207
	14	1.095	+0.091
	21	0.970	-0.030
	28	0.870	-0.139
	35	0.768	-0.264
	42	0.686	-0.377

Table II (cont.)

Expt. No.	t	O. D.	ln O. D.
4	2	1.322	+0.279
	7	1.230	+0.207
	14	1.100	+0.094
	21	0.987	-0.013
	28	0.874	-0.135
	35	0.779	-0.250
	42	0.692	-0.368
5	3	1.32	+0.277
	7	1.245	+0.219
	14	1.113	+0.106
	21	0.995	-0.005
	28	0.880	-0.128
	35	0.782	-0.246
	42	0.693	-0.367
6	3.5	1.30	+0.262
	7	1.22	+0.198
	14	1.094	+0.086
	21	0.958	-0.043
	28	0.861	-0.150
	35	0.762	-0.271
	42	0.668	-0.403
7	2	1.328	+0.283
	7	1.229	+0.206
	14	1.084	+0.081
	21	0.978	-0.022
	28	0.867	-0.143
	35	0.767	-0.265
	42	0.684	-0.380
8	4	1.294	0.257
	7	1.220	0.199
	14	1.088	0.083
	21	0.979	-0.021
	28	0.860	-0.150
	35	0.762	-0.272
	42	0.678	-0.389

Table III

SUMMARY OF INITIAL VELOCITIES

Expt. No.	Corrected Zero Order Initial Velocities[a]	Corrected First Order Initial Velocities[a]
1	0.158	0.161
2	0.157	0.160
3	0.154	0.158
4	0.151	0.154
5	0.154	0.159
6	0.158	0.162
7	0.154	0.159
8	0.156	0.158

a., In units of 10^{-3} M/min. in aqueous solutions at 25^{o} and pH 7.62, 0.3 M in the THAM component of a THAM-HCl buffer, $[E]$ = 0.0266 mg. protein-nitrogen/ml., $[S]_o$ = 10 X 10^{-3} M.

REFERENCES

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

(2) Foster, R. J., and Niemann, C., J. A. C. S., 77, 0000 (1955).

(3) Jennings, R. R., and Niemann, C., ibid., 75, 4687 (1953).

(4) Hogness, D. S., and Niemann, C., ibid., 75, 884 (1953).

(5) Foster, R. J., and Niemann, C., Proc. Nat. Acad. Sci., 39, 999 (1953).

(6) Foster, R. J., Jennings, R. R., and Niemann, C., J. A. C. S., 76, 3142 (1954).

PART V

DILATOMETRIC INVESTIGATIONS

DILATOMETRIC INVESTIGATIONS

The dilatometer affords a means of measuring positive or negative changes in the volume of a reaction mixture. The procedure most commonly employed is that of confining the reaction solution in a closed vessel fitted with a calibrated capillary tube. The rise or fall of the meniscus in the capillary tube can be correlated with changes in volume, and since volume changes can be related to the amounts of various species present in the reaction medium, a direct measurement of the extent of a reaction is permitted.

The volume changes accompanying chemical reactions in liquid systems have been studied quite extensively. In the realm of enzyme chemistry, van't Hoff (1) was one of the earliest to invoke the aid of the dilatometer for the study of the enzymatic synthesis of glucosides. Galeotti (2) carried out dilatometric investigations of the inversion of sucrose, the saponification of ethyl acetate, and the hydrolysis of proteins by acids and in some cases by enzymes. Later (3) he extended his researches to a study of the conditions of the synthesis of simple esters and fats brought about by pancreatic extracts.

The possibility of employing the instrument for the study of enzyme reactions was re-examined and extended by Sreenivasaya and Sastri (4), who have shown that the kinetics of the action of many

enzymes on their respective substrates could be dilatometrically followed (5). Weber (6) and later Rona and Sreenivasaya et al. (7-10) continued investigations employing dilatometers and these prepared the way for the extensive studies undertaken by Linderstrøm-Lang and his group (11-14).

From the data available in the literature (6-14) it is apparent that the contraction in the volume of a reaction solution accompanying the hydrolysis of proteins and related substances in aqueous solutions of about pH 7 is very great as compared to that observed in say the case of carbohydrate hydrolysis (12, 13). A plausible explanation for this may be found in the increase in the number of electrical charges which accompanies the scission of the peptide bond. The splitting of glycylglycine, a very simple peptide, at near neutral pH, for example, involves the disappearance of a dipeptide dipole and the formation of two amino acid dipoles, e. g.,

$$\overset{+}{N}H_3\text{-}CH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}\overset{-}{C}OO + H_2O \longrightarrow 2\overset{+}{N}H_3\text{-}CH_2\text{-}\overset{-}{C}OO$$

i. e., two new charges have thus appeared which will be surrounded by water molecules and consequently give rise to a contraction in the volume of the solution. This contraction has been named electrostriction. Linderstrøm-Lang and Jacobsen (12) consider the volume change accompanying the actual hydrolysis of a protein or peptide-like substance as being small in comparison with the volume change

brought about by electrostriction.

This part of the thesis is devoted to a description of some preliminary studies which were made to determine the applicability of the dilatometric method to the study of the alpha-chymotrypsin catalyzed hydrolysis of alpha-acylamino acid amides.

The primary problem in designing a dilatometer for use in following the course of an enzyme catalyzed hydrolysis is that the enzyme solution must be kept separate from the substrate solution until the investigator desires to initiate the hydrolytic reaction; yet the addition of enzyme to substrate must be both convenient and rapid. To meet these needs, fourteen distinct types of dilatometers, differing in numerous structural and operational features, were tested under varying experimental conditions. Several representative examples of these devices are shown in Figs. 1-3. Fig. 1 shows a simple two bulb dilatometer with filling cap and detachable capillary tube. The substrate solution was introduced into the large bulb, and the enzyme solution was introduced into the small bulb. An inert solvent, e.g., toluene, was then used to fill the remainder of the dilatometer. The dilatometer was then fixed to a metal frame mounted on a pivot which was capable of being turned so that the whole frame tilted in such a manner as to permit the enzyme solution to flow into the main bulb containing the substrate solution when the experimenter desired to start the reaction. This device suffered from several faults. First,

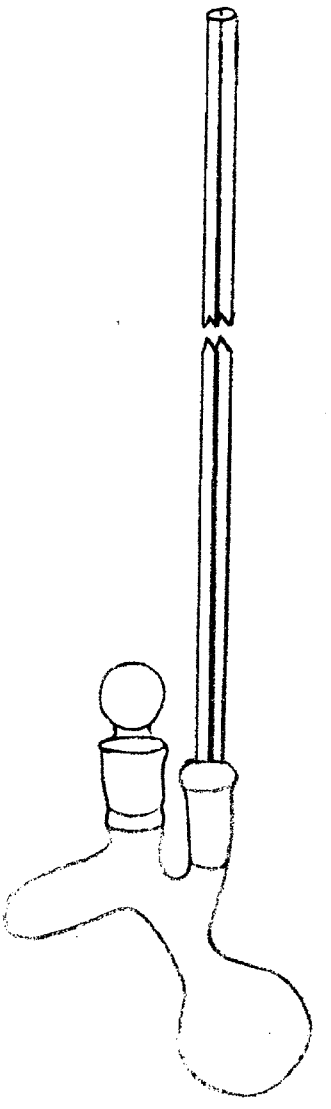


Fig. 1 Two Bulb Dilatometer

some small leakage was always encountered at the joints. Second, there was no efficient way to mix adequately the enzyme and the substrate solutions after the introduction of the enzyme solution into the large bulb of the dilatometer.

Two types of dam dilatometers are presented in Fig. 2. The substrate was placed in the main body of the dilatometer and a rubber dam was fitted over the filling aperture. The enzyme solution was introduced into the dilatometer by means of a hypodermic syringe whose needle was passed through the rubber dam. This device suffered from several disadvantages also. Frequently air bubbles were trapped in the arms of the dilatometer making accurate readings impossible. Again, as before, there was no way to mix adequately the enzyme solution with the substrate solution. In addition there was leakage around the dam during the introduction of the enzyme solution because of the pressure increase inside the device.

A dilatometer design evolved[1] (see Fig. 3) which eliminated all joints, stopcocks, and openings which were directly in contact with, or in the path of the solutions. Thus, there was no leakage problem. The enzyme solution could be conveniently introduced, and thoroughly mixed with the substrate solution during the initial stage of operation. All air was driven from the dilatometer

[1] Some features in the design were originated by Benford and Ingold (15).

before any readings were made so that no bubbles accumulated in the capillary tubes. Finally the need for an inert solvent was eliminated.

Results and Discussion

Dilatometric measurements demand the maintenance of fairly steady temperatures whose limits of variation largely determine the sensitiveness of the instrument. All of the measurements described herein have been conducted with a thermostat capable of maintaining temperatures within a variation of 0.005°.

During the operation of the dilatometer care was exercised to exclude, insofar as possible, various heating effects such as the heat of solution and heat of reaction. In the present investigation, a solution containing substrate and buffer was introduced into the mixing cylinder, B, of the dilatometer (see Fig. 3), and the entire system brought to thermal equilibrium by immersion in a large constant temperature water bath regulated to maintain a temperature of $25.000 \pm 0.005^{\circ}$. The crystalline enzyme preparation was dissolved in the appropriate buffer solution and brought to thermal equilibrium at 25° by immersion in the water bath.

To initiate the hydrolytic reaction, 1.0 ml. of the enzyme-buffer solution was added to the substrate-buffer solution in the dilatometer, and the solutions thoroughly mixed while the dilatometer remained in the constant temperature water bath. Thus

it was felt that the heats of dilution of the substrate, enzyme, and buffer would be minimized to the point at which their effects upon the reaction would be negligible. It was also assumed that in such dilute solutions as were employed in this study, the heat of reaction would have a negligible effect upon the change in volume of the reaction mixture. In addition, the large constant temperature water bath would undoubtedly dissipate any moderate heat effects occurring during the course of the reaction. In order to compensate for temperature effects in readings of l_t, the dilatometer reading at time t, a second dilatometer, identical to the one in which the hydrolytic reaction was being conducted, was filled with a solution containing all of the components of the reaction solution with the exception of the specific substrate, and this dilatometer was suspended in the large constant temperature water bath. This device served as a sensitive thermometer, readily responding to the smallest temperature variations of the thermostat. Thus adjustments were made for any changes in the volume of the reaction solution not directly due to the enzymatic reaction.

The assumption was made that the decrease in volume of the reaction mixture was directly proportional to the amount of hydrolysis of the substrate, and consequently, a function of the concentration of the substrate.

Extrapolation of a plot of the sum of the levels of the

menisci at time t, l_t, versus time to zero time gave the initial menisci readings at time zero, l_o. For convenience, a linear measurement was made since l_t is directly proportional to the volume of the reaction mixture at time t. Readings at "infinite" time, l_{oo}, were made by observing values of l_t until no further change in the readings was obtained. The total change in length, L_T, a function of the total volume change, is then simply $l_o - l_{oo}$; this corresponds to 100 per cent hydrolysis. It is then possible to determine the amount of hydrolysis per unit time as follows:

$$\% \text{ hydrolysis} = (l_o - l_t)/(l_o - l_{oo}) = \Delta l_t/L_T$$

Thus, $[S]_t = [S]_o(1 - \Delta l_t/L_T)$. With this information one may then employ the procedure described by Foster and Niemann (16) to obtain K_S, k_3, and K_{P_1}.

The present experiments were all performed at 25° and pH 7.9 ± 0.1 in aqueous solutions 0.02 M with respect to the THAM[1] component of a THAM-HCl buffer, and with an enzyme concentration corresponding to 0.099 mg. protein-nitrogen/ml. The specific substrate employed was nicotinyl-L-tryptophanamide. The primary data presented in Table I and Fig. 4 were evaluated by the method described by Foster and Niemann (16). The line whose slope equals

---

[1] Tris-(hydroxymethyl)-aminomethane.

$-K_S$ that best fitted the experimental data was obtained by inspection, and the limits indicated represent the maximum observable limits of error in the constants described below. The following values were obtained for the kinetic constants of the specific substrate nicotinyl-L-tryptophanamide employing the dilatometric procedure:

$$K_S = 2.7 \pm 1.5 \times 10^{-3} \underline{M}$$

$$k_3 = 1.5 \pm 0.4 \times 10^{-3} \underline{M}/\text{min./mg. protein-nitrogen/ml.}$$

$$K_{P_1} = \text{Indeterminate}$$

Values of K_P are obtained by means of the following expression:

$$m = \frac{-K_S (K_P + [S]_o)}{(K_P - K_S)}$$

where m is equal to the slope of the line connecting the experimental points. If one rearranges the above equation thusly,

$$m = \frac{-K_S \left(1 + \frac{[S]_o}{K_P}\right)}{\left(1 - \frac{K_S}{K_P}\right)}$$

it may be seen that when $K_P \gg [S]_o$ and $K_P \gg K_S$, m will

approach a value equal to $-K_S$. For the case at hand the values of m are as follows, -1.02, -1.60, -1.70, -2.20, -2.64, and -3.22 X 10^{-3} M which appear to support this contention. Thus ad hoc, K_P cannot be accurately determined with the data at hand.

The above constants were evaluated in systems such that S'_S was between the limits of 1.85 and 3.70, and E'_S was equal to 1.04 X 10^{-2}. Thus these values are well within the limits imposed by zone A conditions (17, 18).

The kinetic constants for the specific substrate nicotinyl-L-tryptophanamide have been determined at 25° and pH 7.9 in aqueous systems 0.02 M in the THAM component of a THAM-HCl buffer, and their values reported by Niemann and coworkers (19, 20). They are as follows:

$$K_S = 2.5 \pm 0.2 \times 10^{-3} \text{ M}$$

$$k_3 = 1.5 \pm 0.2 \times 10^{-3} \text{ M/min./mg. protein-nitrogen/ml.}$$

$$K_{P_1} = 15 \pm 5 \times 10^{-3} \text{ M.}$$

Although there is good agreement between the kinetic constants obtained by the author employing the dilatometric technique and those obtained by Niemann et al. (19, 20), it should be noted that the limits of error in the constants determined by the dilatometric method are quite large as compared to those obtained by

Niemann et al.

The volume decrease for the case at hand is quite small, i.e., ca. 0.0014 ml./10 X 10^{-3} M in substrate, and errors were undoubtedly introduced by assuming that the measuring capillaries, H, (see Fig. 3) had bores of uniform diameter. The method is extremely sensitive to small errors in l_t and L_T, and no doubt all of these factors contributed to the spread in the limits of error for the kinetic constants.

EXPERIMENTAL (21, 22)

Substrate

Nicotinyl-L-tryptophanamide (I). - This compound, soft, long needles, m.p. 180-181°, was prepared as directed by Iselin, Huang, MacAllister, and Niemann (23). When a concentrated aqueous solution of nicotinyl-L-tryptophanamide was quickly cooled, the compound crystallized in clusters of short, thick, needles, m.p. 190.5-191.5°. This latter product when recrystallized from dilute aqueous solutions gave nicotinyl-L-tryptophanamide, soft, long needles, m.p. 180-181°; $[\alpha]_D^{24}$ - 34.5 ± 1° (c 2 % in methanol); lit. (19), m.p. 180-181°; $[\alpha]_D^{24}$ - 34.5 (c 2 % in methanol).

Anal. Calcd. for $C_{17}H_{16}O_2N_4$ (308); C, 66.23; H, 5.23; N, 18.17. Found: C, 66.27; H, 5.31; N, 18.11.

Buffer Solutions

Buffer solutions were prepared as described in Part III, p. 141 of this thesis.

Enzyme Solutions

Crystalline alpha-chymotrypsin containing magnesium sulfate (Armour Lot No. 90402) was employed in these studies. The chymotrypsin preparation contained 10.4 % N. Stock solutions of the enzyme were prepared daily, brought to 25°, and used immediately.

Description of the Dilatometer

The dilatometer employed in the kinetic studies described herein was designated model XIV modification 3. Reference to Fig. 3 indicates the salient features of this instrument. Pressure cap A is a standard taper joint, size 19/22. The mixing chamber, B, is a cylinder 2 cm. in diameter and 10 cm. in length. The capillaries designated H are both 7 cm. in length with bore diameter of 0.3 mm. The reaction chamber, E, is a flattened cylinder of ca. 17 ml.[1] capacity. The numbered scale, I, is of glass graduated in millimeters. Plug D is a 10/30 standard taper joint, and outlet G is a standard micro stopcock. All connecting lines, J, are of capillary tubing with a bore diameter of 1 mm. The entire dilatometer has an overall length of approximately 25 cm.

Procedure

The substrate was placed in a 25-ml. G.S. volumetric flask, and ca. 15 ml. of distilled water added. Heat was applied to effect solution; 2.5 ml. of THAM-HCl buffer solution, 0.2 M with respect to the THAM component, then added, and the stoppered flask placed in a constant temperature water bath maintained at $25.0 \pm 0.1^{\circ}$ for thirty minutes. After the contents of the flask had reached 25°, the solution was made up to volume with distilled

[1] The precise value of this capacity varied from instrument to instrument, and was determined for each dilatometer.

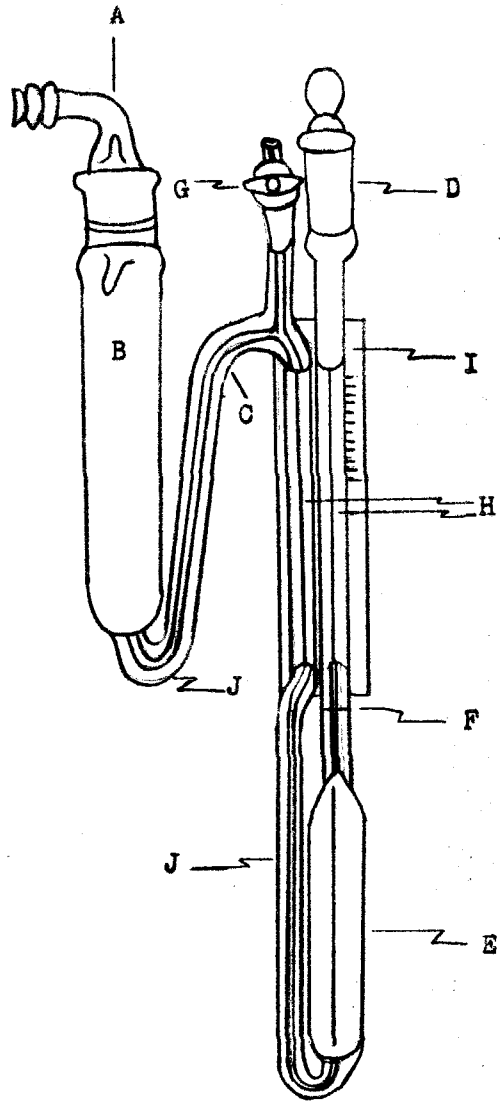


Fig. 3 Dilatometer Mark XIV mod. 3
(See text for explanation)

water which was also kept at 25°. The flask was stoppered and inverted gently 12 to 14 times to insure adequate mixing. A 20.0-ml. aliquot of this substrate-buffer solution was then delivered into mixing chamber B, see Fig. 3, of the dilatometer with stopcock G closed and plug D in place. Cap A was then placed on the mixing cylinder to protect the contents from foreign material. The dilatometer and its contents was then placed in a 37 liter constant temperature water bath heated by means of a minimum lag infrared heat unit[1] and regulated to maintain a temperature of 25.000 ± 0.005°.

The appropriate amount of solid enzyme was weighed into a 5-ml. G.S. volumetric flask, and 0.5 ml. of THAM-HCl buffer solution, 0.2 M with respect to the THAM component, then added. The solution was made up to volume with distilled water, the flask stoppered, and inverted gently 10 to 12 times to insure thorough mixing of the contents. The clear enzyme-buffer solution was then placed in a 25° constant temperature water bath.

After a period of thirty to forty minutes, cap A of the dilatometer was removed from the mixing chamber, and a 1.0-ml. aliquot of the enzyme solution was delivered into the chamber. A stop watch was started, and the solutions thoroughly mixed with the

---

[1]Available from the Arthur H. Thomas Co.

aid of a very thin glass stirring rod with a flattened end. Cap D was removed, and a low pressure air line (ca. 3-5 lbs./in.^{2}) affixed to cap A. Air pressure was employed to force the contents of cylinder B into chamber E. The use of air pressure enabled quite rapid filling of the remainder of the dilatometer. When the fluid level reached the top of chamber E, the air line was disconnected. When the meniscus reached the line at point F, stopcock G was opened. Thus both capillaries, H, were partially filled and their tops open to the atmosphere. Readings[1] were then made of the heights of both menisci at selected time intervals.

---

[1] It was found convenient to use a large three power magnifying glass affixed to the outside of the constant temperature bath to measure the levels of the menisci.

Table I

THE alpha-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF NICOTINYL-L-TRYPTOPHANAMIDE AT pH 7.9 AND 25°

$[E]$ = 0.099 mg. protein-nitrogen/ml.

Buffer = 0.02 M THAM-HCl

$[S]_o$[a]	t[b]	Δl_t[c]	$[S]_t$[a]	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	10	1.4	3.94	0.106	0.0238
	16	2.2	3.34	0.104	0.0252
	18	2.5	3.11	0.105	0.0264
	21	2.9	2.81	0.104	0.0274
	24	3.3	2.50	0.104	0.0289
	30	3.8	2.12	0.096	0.0286
	36	4.4	1.67	0.093	0.0304
	43	5.0	1.21	0.088	0.0330
	49	5.5	0.84	0.085	0.0364
L_T = 6.6					
5	10	0.7	4.42	0.058	0.0123
	14	1.1	4.09	0.065	0.0143
	21	1.6	3.79	0.058	0.0132
	26	2.0	3.34	0.064	0.0155
	30	2.3	3.09	0.064	0.0161
	42	3.2	2.34	0.063	0.0181
	46	3.5	2.09	0.063	0.0190
	76	4.5	1.25	0.049	0.0183
L_T = 6.0					

[a] In units of 10^{-3} M
[b] In minutes
[c] In mm.

Table I (cont.)

$[S]_o$	t	Δl_t	$[S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
5	11	1.2	4.00	0.090	0.0203
	15	1.7	3.59	0.094	0.0221
	21	2.4	3.00	0.095	0.0244
	25	2.8	2.67	0.093	0.0251
	27	3.1	2.42	0.096	0.0268
	30	3.5	2.09	0.097	0.0291
	34	3.9	1.75	0.096	0.0308
	36	4.1	1.59	0.095	0.0318
	40	4.5	1.25	0.094	0.0347
L_T = 6.0					
8	10	1.7	6.89	0.111	0.0149
	15	2.3	6.49	0.101	0.0140
	18	2.7	6.23	0.098	0.0139
	20	3.1	5.97	0.102	0.0146
	25	3.9	5.44	0.102	0.0155
	30	4.6	4.98	0.101	0.0158
	36	5.7	4.26	0.104	0.0175
	40	6.0	4.06	0.099	0.0170
	45	6.7	3.61	0.098	0.0177
	50	7.4	3.14	0.097	0.0187
	65	9.0	2.10	0.091	0.0206
L_T = 12.2					
10	15	3.4	8.20	0.120	0.0133
	17	3.8	7.99	0.118	0.0132
	20	4.4	7.67	0.117	0.0133
	24	5.4	7.14	0.119	0.0140
	28	6.3	6.67	0.119	0.0144
	32	7.2	6.19	0.119	0.0150
	40	8.7	5.40	0.115	0.0154
	53	11.3	4.02	0.113	0.0172
	64	13.0	3.12	0.108	0.0182
	80	15.3	1.90	0.101	0.0208
	125	18.4	0.26	0.078	0.0292
L_T = 18.9					

Table I (cont.)

$[S]_o$	t	Δl_t	$[S]_t$	$[S]_o - [S]_t/t$	$\ln [S]_o/[S]_t/t$
10	15	3.5	8.15	0.123	0.0136
	17	3.9	7.94	0.121	0.0136
	20	4.6	7.57	0.122	0.0139
	25	5.7	6.98	0.121	0.0144
	28	6.5	6.56	0.123	0.0150
	30	6.9	6.35	0.122	0.0151
	34	7.6	5.98	0.118	0.0151
	40	8.8	5.34	0.117	0.0157
	54	11.4	3.97	0.112	0.0171
	85	15.7	1.69	0.098	0.0209
	124	18.2	0.37	0.078	0.0266
L_T =	18.9				

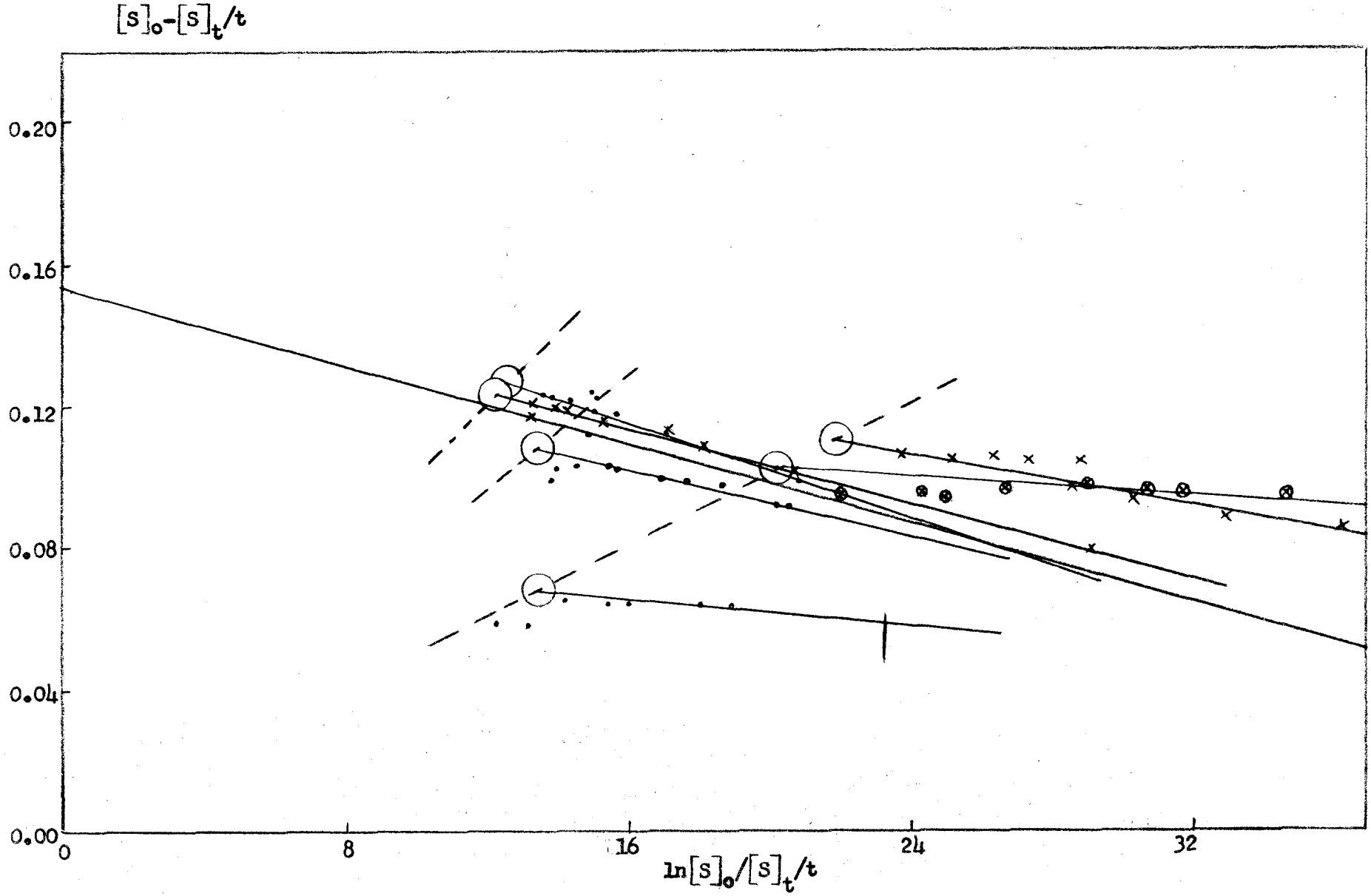


Fig. 4 Foster-Niemann Plot of the Data Presented in Table I

REFERENCES

(1) van't Hoff, J.H., Sitzgsber. preuss. Akad. Wiss., 34, 963 (1910).

(2) Galeotti, G., Z. physik. Chem., 76, 105 (1911).

(3) Galeotti, G., ibid., 80, 241 (1912).

(4) Sreenivasaya, M., and Sastri, B.N., Biochem. J., 23, 975 (1929).

(5) Sreenivasaya, M., and Sreerangachar, H.B., J. Indian Inst. Sci., 15A, 17 (1935).

(6) Weber, H.H., Biochem. Z., 218, 1 (1930).

(7) Rona, P., and Fischgold, H., ibid., 261, 66; 366 (1933).

(8) Sreenivasaya, M., Sastri, B.N., and Sreerangachar, H.B., Biochem. J., 28, 351 (1934).

(9) Sreenivasaya, M., and Sreerangachar, H.B., ibid., 28, 1219 (1934).

(10) Sreenivasaya, M., and Bhagvat, K., Ergebn. Enzymforsch., 6, 234 (1937).

(11) Linderstrøm-Lang, K., and Lanz, H., Compt. rend. Lab. Carlsberg, Ser. chim., 21, 315 (1942).

(12) Linderstrøm-Lang, K., and Jacobsen, C.F., ibid., 24, 1 (1941).

(13) Linderstrøm-Lang, K., and Jacobsen, C.F., Enzymologia, 10, 97 (1941).

(14) Jacobsen, C.F., ibid., 24, 281 (1943).

(15) Benford, G.A., and Ingold, C.K., J. Chem. Soc., 1938, 929.

(16) Foster, R.J., and Niemann, C., Proc. Nat. Acad. Sci., 39, 999 (1953).

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

(18) Goldstein, A., ibid., 27, 529 (1944).

(19) Huang, H. T., and Niemann, C., J. A. C. S., 73, 1541 (1951).

(20) Foster, R. J., and Niemann, C., ibid., 77, 0000 (1955).

(21) All melting points are corrected.

(22) Microanalysis by Dr. A. Elek.

(23) Iselin, B. M., Huang, H. T., MacAllister, R. V., and Niemann, C., J. A. C. S., 72, 1729 (1950).

PROPOSITIONS

PROPOSITIONS

1. From consideration of rate equations for enzyme catalyzed hydrolysis reactions, it seems reasonable to assume that the apparent K_I of a DL-inhibitor should be equal to $2 K_{I_1} K_{I_2} / K_{I_1} + K_{I_2}$. On this basis it is suggested that the apparent K_I value for benzoyl-DL-phenylalanine reported by Kaufman and Neurath may be in error.

Kaufman, S., and Neurath, H., J. Biol. Chem., 181, 623 (1949).

2. Assuming that an additional site, or bonding area, is utilized in the combination of anionic, bifunctional, competitive inhibitors of alpha-chymotrypsin with the enzyme in the presence of phosphate buffers at pH values in the region of 7.9, it would be of interest to: a. Examine synthetic reactions in the presence of enzyme and phosphate buffer and determine if this new bonding area can play a role in, and affect the pH optima of these reactions. b. Determine the effect of varying B on k_3' at constant ionic strength. This would add considerable information concerning the actual hydrolytic process.

This thesis, Part III, pp. 128 - 139.

3. The extent of O^{18} exchange between H_2O^{18} and the carboxyl oxygen of various amino acid derivatives in the presence of alpha-chymotrypsin should be investigated with a view to obtaining

correlation between structural modifications of the substrate, the kinetic constants, i.e., K_S, k_3, K_P, and K_I, and these exchanges.

4. A novel method for the synthesis of functional derivatives of amino acids and of peptide bonds is proposed which involves the formation of symmetrical amino acid anhydrides by means of alkoxyacetylenes. A mechanism for this reaction is suggested.

Arens, J.F., and Doornbos, T., Rec. trav. chim., 74, 79 (1955).

5. Formation of 1, 3-indanediones would make excellent derivatives for many compounds with active alpha-hydrogens.

Kilgore et al, Ind. Eng. Chem., 34, 494 (1942).

6. The compound 2-ketoquinuclidine should exhibit steric inhibition to amide resonance.

a. A possible synthesis of this compound is proposed.

b. I propose that the kinetics of its hydrolysis be studied.

c. It is predicted that the amide will hydrolyze at a rapid rate as compared to "normal" amides.

d. The amide should exhibit marked basic properties in contrast to "normal" amides.

Holley, Science, 117, 23 (1953).

7. Evidence has been presented which indicates that in peas, an

oxidation product of IAA contains an indole ring and is not indole-3-aldehyde. Still others have reported as many as six oxidation products of IAA in plants. It is proposed that this oxidation proceeds via N-hydroxylation and subsequent ring opening to give a hydroxamic acid.

Manning, D. T., Ph. D. Thesis, Calif. Inst. of Tech., 1955.
Houff, et al., J. A. C. S., 76, 5654 (1954).
Stutz, R., Fed. Proc., 14, 288 (1955).

8. The state of auxin in the intact plant has been a matter of considerable interest for a number of years. In an effort to elucidate the linkages or interactions involved between so called "bound auxin" and the plant constituents, oxidized or acetylated proteolytic enzymes may prove to be of value. These enzymes tend to retain moderate esterase activity but greatly reduced proteinase activity. Thus they might serve to distinguish between ester and peptide linkages involved in auxin binding.

Jansen et al., J. Biol. Chem., 189, 671 (1951).

9. I propose that the herbicide 3-(p-chlorophenyl)-1, 1-dimethylurea (CMU) may act as a citrulline antagonist and thus block the formation of arginine in the plant. It would be of interest to administer arginine concomitantly with CMU and ascertain whether the toxicity is mitigated. It is further proposed that omega-N, N-dimethylcitrulline may possess herbicidal properties.

Bucha et al., Science, 114, 493 (1951).

10. A number of desert plants excrete substances from their roots which ward off the encroachment of other plants. I propose the name alexophytic compounds be used to describe these substances. This word stems from the Greek root alexein, to ward off.

11. To date there has been no report of the identification or isolation of arsenic chlorofluorides. The reaction of ammonium fluoride, a reagent which usually effects monofluorination, with $AsCl_3$ yields only AsF_3. I propose that these mixed halides exist in a redistribution reaction equilibrium, and that spectral studies should reveal their existence.