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Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1960

ACKNOWLEDGEMENTS

I am grateful to Professor Carl Niemann for suggesting a variety of research problems that challenged my ingenuity and ability. I must particularly acknowledge the freedom he gave to me to explore those facets of the suggested problems that were of greatest interest to me.

I am also indebted to George T. Felbeck who induced me to come to the California Institute of Technology; his encouragement provided me with the will to succeed in the face of discouraging odds. I am also grateful to the Union Carbide Corporation for the leave of absence that enabled me to complete my studies.

Tuition scholarships provided by the California Institute of Technology for the years 1957-1959 are also acknowledged, as well as the E. I. duPont de Nemours Company grants for the summers of 1957 and 1958.

I thank Professor John D. Roberts, Professor Richard M. Badger, Dr. Edwin R. Buchman and Professor George S. Hammond for their committee service.

This Thesis is dedicated to my wife, Rose, and to my two sons, Peter and Daniel. I am proud of the patience and understanding which my family showed during the past three years.

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ABSTRACT

Substitution of a nitrogen atom in place of the C-H group that occurs at the asymmetric center of typical substrates of alpha-chymotrypsin results in the complete loss of the ability of the enzyme to catalyze the hydrolysis of the carboethoxy or carboxamido group. However, the nitrogen-substituted analogs, which otherwise possess all of the remaining significant structural entities of typical substrates, function as inhibitors of alpha-chymotrypsin catalyzed hydrolyses but in general show less affinity for the enzyme than do the analogous C-H containing substrates.

It has been found that 1-acetyl-2-[L-tyrosyl] hydrazine is a reversible inhibitor of alpha-chymotrypsin catalyzed hydrolyses with unusual affinity for the enzyme. At pH 7.9 the enzyme inhibitor constant was found to have a value of $K_I = 0.074 \times 10^{-3}$ M, which shows that this substance associates reversibly with alpha-chymotrypsin at the active site to a greater extent than any other known substrate or reversible inhibitor.

Preliminary results, based upon inhibition studies, indicate that alpha-chymotrypsin possesses a greater affinity for the naphthalene function than for the indole function.

A study of the applicability of the pH-stat to alpha-chymotrypsin catalyzed hydrolyses that produce a buffer showed that the reliability was a function of the buffer capacity. Amides gave poor results; hydrazides gave good or fair results, one of which contradicted a literature value, and a hydroxamide gave excellent results.

PART I

STRUCTURAL ANALOGS OF TYPICAL SUBSTRATES OF ALPHA-CHYMOTRYPSIN

A. Introduction

A recognized feature of enzyme-catalyzed reactions is their specificity towards certain substrates and inhibitors. Alpha-chymotrypsin is no exception to this rule and workers in this field use the expression "typical substrate" to imply that a structural specificity exists between enzyme and substrate. Substances that combine with the enzyme but are incapable of producing hydrolysis products are termed inhibitors or activators according to their kinetic behavior in a system containing enzyme and a substrate. Due to the experimental difficulties associated with direct measurements of association between enzyme and other substances workers in enzyme chemistry have accepted the suggestion of Henri (1) and Michaelis and Menten (2) that evidence for the existence of intermediates may be obtained kinetically from situations described by the representation given in equation 1.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products} \qquad (1)$$

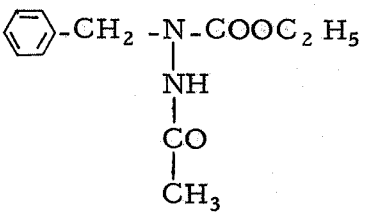
where the forward velocity is given by k_3 [ES].

In this Thesis this same "kinetic" approach is accepted and the behavior of alpha-chymotrypsin with substrates, inhibitors and activators is explained in terms of probable intermediate complexes.

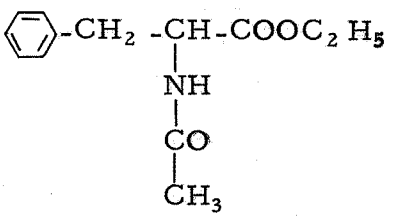
B. A Structural Analog of Acetyl-DL-Phenylalanine Ethyl Ester

The purpose of this study was to study the behavior of alpha-chymotrypsin with a molecule that possessed the structural features of a "typical" substrate, namely a benzyl side-chain, an acetamido group, and a carbethoxy function arranged about a nitrogen atom, rather than an asymmetric carbon atom. It was hoped that such a study would reveal the influence of the C-H center on the two kinetic parameters, K_s and k_3.

The analog chosen for this study was ethyl 1-acetyl-2-benzyl carbazate (I) the structural analog of acetyl-DL-phenylalanine ethyl ester (II).



(I)



(II)

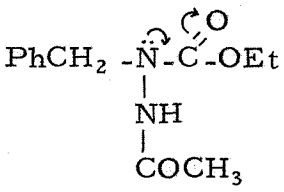
In order to make the study more comprehensive a number of ester and amide analogs of acetylglycine and acetylphenylalanine and related derivatives were investigated. Results of the latter study are given in Part I.C of the Thesis; however, the major effort of this part of the Thesis was concerned with the analog (I) in a quantitative manner, the study of the remainder of the analogs being of a more qualitative nature.

The preparation of analog (I) is given in detail in the Experimental-Synthesis section and the kinetic behavior is described in detail in this section. The behavior of (I) in aqueous systems in the absence of enzyme was also studied in order to determine whether the compound was susceptible to non-enzymatic hydrolysis. The second-order basic hydrolysis constant of the analog (I) was found to be 3.42 ± 0.04 l.mol.$^{-1}$min.$^{-1}$ over the pH range 7.50-9.00, and when compared with the constant for acetyl-DL-phenylalanine ethyl ester, which was found to be 17.7 ± 1.6 l.mol.$^{-1}$min.$^{-1}$, it was evident that analog (I) was more resistant to basic hydrolysis.

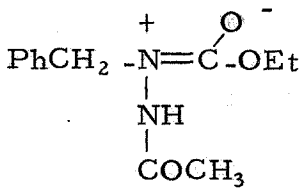
Incubation of the analog with alpha-chymotrypsin at pH 8 for one week showed that (I) was not a substrate since it was recovered in 94% yield from the reaction mixture. However, it was found that (I) could function as an inhibitor in the alpha-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-valine methyl ester. Accordingly, a comprehensive inhibition study was undertaken, the results of which make up the larger part of Part I of this Thesis.

The behavior of (I), ethyl 1-acetyl-2-benzyl carbazate in the system alpha-chymotrypsin plus chloroacetyl-L-valine methyl ester was not totally competitive. However, a competitive constant, $K_I = 19 \pm 1 \times 10^{-3}$ M, was calculated by a statistical analysis of the data.

The absence of enzyme-catalyzed hydrolysis of the analog (I) as well as the decreased reactivity towards basic hydrolysis may be due in part to the reduction of the electrophilic character of the carbonyl carbon atom by virtue of the donation of a pair of electrons by the nitrogen in the 2-position to that carbon atom:



(I)



(Ia)

Since a requirement for hydrolytic cleavage of esters in either the non-enzymatic or the enzymatic case is the attack by water at the electrophilic carbon, the lack of reactivity of (Ia) to hydrolysis may be due to depolarization of the carbonyl group located at the susceptible -OEt bond. However, it does not seem reasonable that this effect would cause k_3 to become zero; instead one would expect a decrease in k_3. Furthermore, if the donor characteristics of the -OEt group contribute to the resonance structure of (I), then even in the EI complex a water molecule cannot compete successfully with the donor groups already in close proximity to the carbonyl carbon.

An alternate explanation for the observed result that $k_3 = 0$ for the analog is that a potential substrate can associate with the active site of the enzyme in a multiplicity of modes of combination,

only one of which may be favorable for hydrolysis. Kinetic measurements of K_s or K_I are composite with respect to the possibility of modes of combination; however, the electronic structure or the configuration of the analog may reduce the number of modes of combination.

The fact that carbazates are inhibitors rather than substrates for alpha-chymotrypsin-catalyzed hydrolyses has one point in its favor. Briggs and Haldane (3) developed the steady state treatment of equation 1 which led to $K_s = (k_2 + k_3)/k_1$, a composite kinetic constant, rather than the earlier affinity constant of Michaelis and Menten (1), where $K_s = k_2/k_1$. Accordingly, it must be recognized that K_s obtained by the kinetic treatment of enzyme situations may represent anything from the equilibrium constant $K_s = ([E][S])/ES$ to an assemblage of kinetic constants.

When a substance functions as an inhibitor in the sense of equation 2,

$$E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI \qquad (2)$$

the enzyme-inhibitor dissociation constant, $K_I = k_5/k_4$ is a true equilibrium constant, $K_I = ([E][I])/EI$. However, direct evidence for the existence of EI complexes, just as with ES complexes, is also lacking. In the few cases where spectrochemical evidence supported the hypothesis of an intermediate complex, i.e.,

Chance's (4) work on catalase, and Vallee, Coombs and Williams (7) on insulin, a metal ligand, iron in the former and zinc in the latter, was associated with the enzyme enabling these authors to obtain absorption spectra characteristic of metal-chelate complexes. Further support for the occurrence of EI complexes has come from the equilibrium dialysis method of determining the number of active sites per molecule of enzyme (48, 49).

Accepting the value of K_I obtained by kinetic measurements as being identical with the dissociation constant of the EI complex, one is in a position to compare structural parameters on the basis of a single number, K_I, rather than K_s which may have within it a number of kinetic constants, hence the advantage of employing inhibitors for the elucidation of the active site in alpha-chymotrypsin.

Huang and Niemann (5, 6) advanced the hypothesis that the active site in alpha-chymotrypsin was trifunctional and that one could assign complementary binding sites between the enzyme and a trifunctional substrate or inhibitor. The structures of substrates and inhibitors were represented by the symbols $R_2 CH_2 R_1 R_3$, where R_2 is the amino acid side chain, R_1 the nitrogen-containing group, and R_3 the potentially hydrolyzable function. Support for this hypothesis came from the observation that indole, a monofunctional inhibitor containing the elements of only R_2 behaved in a strictly competitive manner with nicotinyl-L-tryptophanamide, a trifunctional

substrate of the $R_2 CH_2 R_1 R_3$ type, whereas with methyl hippurate, a bifunctional substrate of the $R_1 CH_2 R_3$ variety, indole could also function in a non-competitive manner because of the formation of ESI ternary complexes.

Since this analog study includes other members of the phenylalanine and glycine series, a discussion of the significance of the structural parameters in the case of the analogs is deferred to section C of Part I of this Thesis. In this section of the Thesis the results of the quantitative study of the inhibition characteristics of (I), ethyl 1-acetyl-2-benzyl carbazate are presented since the elucidation of those characteristics is based upon a rigorous analysis of the kinetic data that were obtained.

Kinetic Inhibition Study

Experiments were carried out at pH 7.90 $\pm$ 0.01, NaCl = 0.100 M at 25.0°C in the constant pH apparatus of Neilands and Cannon (8). The experimental technique has been described by Applewhite, Martin and Niemann (9) and was followed without modification. Four experiments were carried out with chloroacetyl-L-valine methyl ester as the substrate at four different concentrations of the inhibitor, i.e., $[I]_o$ = 0, 4.41×10^{-3}M, 8.83×10^{-3}M and 18.1×10^{-3}M. Substrate concentrations were varied from $[S]_o$ = 4.50×10^{-3}M to 56.0×10^{-3}M at constant inhibitor levels and a

constant enzyme concentration, $[E]_o$ = 0.1464 mg. PN/ml. was employed throughout.

The data were obtained as continuous recorder traces versus time and corresponded to the volume of standardized base required to maintain the pH constant to within $\pm$ 0.01 pH unit. The data are given in Tables II-V in terms of initial velocities and the results are summarized in Table I. Lineweaver-Burk (10) plots of $[S]_o[E]_o/v_o$ versus $1/[S]_o$ for the four levels of inhibition are shown in Figure 1. Under the conditions specified above it is concluded that the enzyme-inhibitor dissociation constant $K_I = 20 \pm 4 \times 10^{-3}$M assuming that the inhibition is totally competitive. That the inhibition may not be totally competitive is shown by the downward trend in apparent k_3, an observation that requires further explanation.

Computation of the Initial Velocities

It was observed that all of the recorder traces obtained in the inhibited runs curved downwards. When this situation arises, the initial velocities may be computed by the orthogonal polynomial procedure of Booman and Niemann (11) which may be used to compute the initial velocities without making any assumptions as to the cause of the curvature. It was also observed that the recorder traces obtained during the first minute of operation indicated a

significantly higher rate of base addition than during the remainder of the ten minute run, despite the fact that the pH was within $\pm$ 0.01 of the control point. Use of the data starting with zero time therefore resulted in large deviations in the calculated apparent K'_s and k'_3 values, i.e., to the extent of $\pm$ 21% of the K'_s value and $\pm$ 19% of the k'_3 values. When, however, the time coordinate was displaced by one minute and the orthogonal polynomial procedure was applied to the time interval 1-9 minutes, and the initial velocity was taken as the first derivative at t = 1 min., i.e., v_1, then it was found that the precision of the computed K'_s and k'_3 values was increased to $\pm$ 9% and $\pm$ 7% respectively. It is acknowledged that the procedure of computing the velocities at t = 1 min. is subject to some error but in none of the traces did the curvature exceed 1% per minute; therefore, a maximum error of 1% is made by assuming $v_1 \doteq v_0$. Since the precision of the constants K'_s and k'_3 is rarely better than $\pm$ 10% and $\pm$ 5% respectively, it is believed that the method of displacing the time coordinate does not add significantly to the total error.

Computations for v_o and v_1 were carried out with the Datatron 205 digital computer*, and the values of v_o and v_1 were corrected

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*Appendix I.

for enzyme and substrate blanks by the method of Martin and Niemann (12).

Competitive Inhibition Constant - Approximate Method

The system, enzyme-substrate-competitive inhibitor and hydrolysis products may be described by equations 3 and 4,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P_1 + P_2 \ldots \quad (3)$$

$$E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI \quad (4)$$

Steady-state approximation leads to equation 5, when it is assumed

$$-\left(\frac{dS}{dt}\right) = \frac{k_3 [E]_o [S]}{K_s\left(1 + \frac{[I]}{K_I}\right) + [S]} \quad (5)$$

that $\frac{d}{dt}[S] \gg \frac{d(ES)}{dt}$ and $\frac{d}{dt}[S] \gg \frac{d(EI)}{dt}$, $[S]$ and $[I] \gg [E]$

a steady state is rapidly attained, and $-\frac{d}{dt}[S]$ describes the forward velocity of enzymatic hydrolysis of a substrate in the presence of a competitive inhibitor. Under initial conditions, assuming that a steady state has been achieved, the velocity may be replaced by $v \doteq v_o \doteq -\frac{d}{dt}[S]$, defining time = 0 as the time at which steady-state has been attained. Since experience with alpha-chymotrypsin has shown that an induction period is absent, i.e., a rapid steady-state is attained and that the extent of conversion of substrate to products is essentially zero prior to the attainment of the steady-state, v of equation 5 may be replaced by v_o, $[S]$ by $[S]_o$ and $[I]$ by $[I]_o$ leading to equation 6.

$$v_o = \frac{k_3[E]_o[S]_o}{K_s\left(1 + \frac{[I]_o}{K_I}\right) + [S]_o} \qquad (6)$$

where $K_s = \frac{k_2 + k_3}{k_1}$, and $K_I = \frac{k_5}{k_4}$

Equation 6 is in the form suitable for a Lineweaver-Burk (10) plot after rearrangement to equation 7,

$$\frac{1}{v_o} = \frac{K'_s}{k_3[E]_o} \cdot \frac{1}{[S]_o} + \frac{1}{k_3[E]_o} \qquad (7)$$

where $K'_s = K_s\left(1 + \frac{[I]_o}{K_I}\right)$

K_I may be calculated at various $[I]_o$ levels since k_3 and K_s may be separately evaluated at $[I]_o = 0$ with the same substrate. For the case at hand it was found that the constants evaluated at v_1 were more precise and reasonable than those obtained at v_o. The values of k'_3 obtained from the v_1 data at the four inhibitor levels of $[I]_o = 0$, 4.41×10^{-3}M, 8.83×10^{-3}M and 18.1×10^{-3}M were $k'_3 = 1.65 \pm 0.09 \times 10^{-3}$ a, $1.55 \pm 0.11 \times 10^{-3}$ a, $1.42 \pm 0.13 \times 10^{-3}$ a, and $1.49 \pm 0.13 \times 10^{-3}$ a respectively. The plots of $[S]_o[E]_o/v_1$ versus $[S]_o$ shown in figure 1 were reasonably linear with the precision of the data falling off as $[I]_o$ increased. The least squares lines for all four inhibitor levels visually appeared to be parallel, a necessary condition for competitive inhibition; however, mathematically they were not parallel as revealed by the downward trend in k'_3. A plot

a. The units of k_3 and k'_3 are M/min. mg. PN/ml. in this Thesis, unless otherwise indicated.

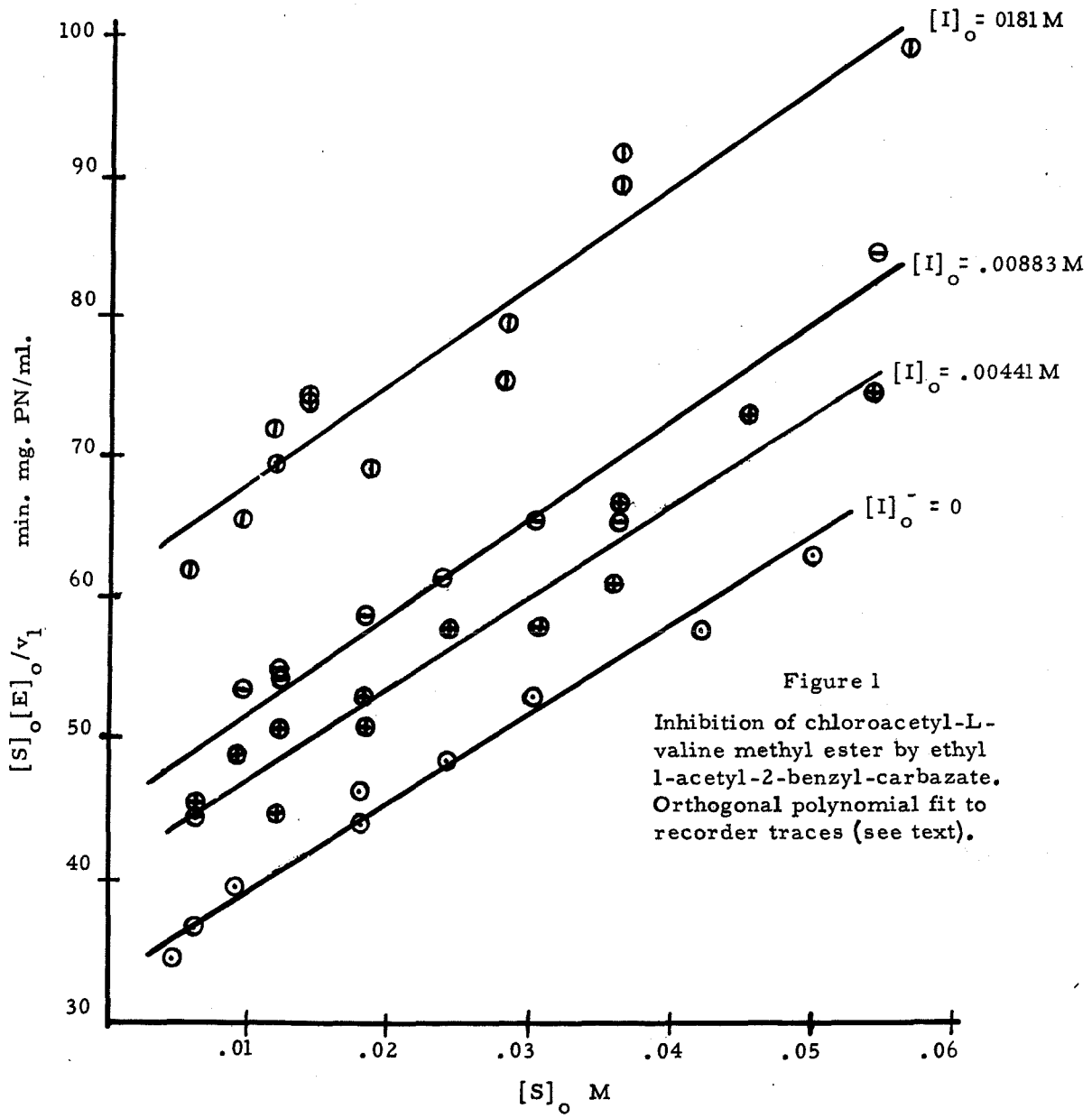


Figure 1

Inhibition of chloroacetyl-L-valine methyl ester by ethyl 1-acetyl-2-benzyl-carbazate. Orthogonal polynomial fit to recorder traces (see text).

of $1/v_I$ versus $1/[S]_o$ for each case gave four lines which visually appeared to intersect at a common ordinate intercept (also a test for competitive inhibition). Therefore, it was believed that the system could be treated approximately as being totally competitive by assuming a mean value of k_3' for all four experiments neglecting the downward trend in k_3'. When this was done, a value for $K_I = 20 \pm 4 \times 10^{-3}$ M was obtained. The v_0 and v_I data are given in Tables II-V. Computed K_s' and k_3' values are also given in the tables. Table I summarizes the computed K_s' values obtained at each level of inhibitor concentration. From the mean k_3' of the four inhibitor levels, K_s'', (K_s' corrected to total competitive inhibition) was obtained for each inhibitor level including $[I]_o = 0$ by computing a new intercept for each of the $[S]_o[E]_o/v_o$ vs. $[S]_o$ plots, where the slopes of all of the individual $[I]_i$ plots took the same value, $1/k_3'$. The corrected values of K_s', K_s'', are given in Table I together with the estimates of K_I'', the apparent total competitive inhibition constant, computed from the K_s'' values.

Competitive Inhibition Constant - Exact Method

The values of K_s and k_3 for chloroacetyl-L-valine methyl ester were first determined by Applewhite, Waite and Niemann (13): $K_s = 47.1 \pm 0.8 \times 10^{-3}$ M and $k_3 = 1.755 \pm 0.028 \times 10^{-3}$ M. These authors used the orthogonal polynomial procedure for the estimation

Table I

Inhibition by Ethyl 1-Acetyl-2-Benzyl Carbazate of the alpha-Chymotrypsin Catalyzed Hydrolysis of Chloroacetyl-L-Valine Methyl Ester at pH 7.90 and 25.0° C.

Inhibition Constant Corrected to Competitive Inhibition

$[I]_o$[a]	K'_s[a,b]	K''_s[a,c]
v_o orthogonal polynomial		
0	28.4 ± 6.1	37.4 ± 10.8
4.41	55.2 ± 4.0	58.5 ± 5.3
8.83	78 ± 20	75 ± 28
18.1	122 ± 37	97 ± 46
		Mean K''_I = 9.3 ± 10.8[a,d]
		Mean k_3 = 1.75 ± .47[e]
v_1 orthogonal polynomial		
0	46.9 ± 2.9	43.2 ± 2.9
4.41	55.3 ± 4.8	53.8 ± 5.5
8.83	56.5 ± 5.5	60 ± 6.8
18.1	83 ± 7.7	85 ± 9.2
		Mean K''_I = 19.8 ± 5.8[a,d]
		Mean k_3 = 1.54 ± .14[e]

a. In units of 10^{-3}M. b. In units of 10^{-3}M, K'_s = measured. c. K''_s = corrected to competitive inhibition. d. K''_I = corrected to competitive inhibition. e. In units of M/min. mg. protein nitrogen/ml.

Table II

alpha-Chymotrypsin Catalyzed Hydrolysis of Chloroacetyl-L-Valine Methyl Ester

pH = 7.90, 25.0°C, $[E]_o$ = 0.1464 mg. PN/ml., NaCl = 0.100 M
Notebook Ref. 934-20

$[S]_o$[a]	v_o[b]	$\pm\sigma v_o$[b]	P_m[c]	v_1[b]	$\pm\sigma v_1$[b]	P_m[c]
42	1.214	.010	2	1.170	.003	1
30	.978	.013	2	.920	.013	3
24	.850	.011	2	.815	.005	2
18	.711	.011	2	.680	.009	2
18	.665	.0084	2	.643	.003	1
12	.517	.0084	2	.491	.005	2
9	.572	.017	4	.383	.004	2
6	.280	.0032	2	.273	.008	2
4.5	.360	.020	5	.225	.003	3

K'_s[a] = $28.4 \pm 6.1 \times 10^{-3}$ M[e] $\quad 46.9 \pm 2.9 \times 10^{-3}$ M[e]

k'_3 = $1.33 \pm .22 \times 10^{-3}$ [d, e] $\quad 1.65 \pm .09 \times 10^{-3}$ [d, e]

a. In units of 10^{-3} M
b. In units of 10^{-4} M/min. corrected for blanks
c. Order of polynomial, $t_{.10}$ test (11)
d. In units of M/min. mg. protein nitrogen/ml.
e. Evaluated by method of least squares

Table III

Inhibition by Ethyl 1-Acetyl-2-Benzyl-Carbazate of the alpha-Chymotrypsin Catalyzed Hydrolysis of Chloroacetyl-L-Valine Methyl Ester

pH = 7.90, 25.0°C; $[E]_o$ = 0.1464 mg. PN/ml.; NaCl = 0.100 M
Notebook Ref. 934-22
$[I]_o = 4.41 \times 10^{-3}$M

$[S]_o$[a]	v_o[b]	$\pm\sigma v_o$[b]	P_m[c]	v_1[b]	$\pm\sigma v_1$[b]	P_m[c]
54	1.168	.011	3	1.138	.012	3
45	1.028	.008	3	.971	.002	1
36	.987	.014	3	.857	.004	1
36	.901	.009	2	.946	.011	3
30	.866	.007	3	.833	.007	3
24	.681	.008	2	.669	.005	2
18	.578	.006	3	.551	.006	3
18	.610	.005	3	.576	.004	3
12	.408	.009	3	.386	.003	2
12	.425	.010	3	.447	.011	4
9	.315	.005	3	.305	.005	3
6	.239	.006	3	.218	.003	3

$K'_s = 55.2 \pm 4.0 \times 10^{-3}$M[e] $\quad 55.3 \pm 4.8 \times 10^{-3}$M[e]

$k'_3 = 1.65 \pm .10 \times 10^{-3}$ [d, e] $\quad 1.55 \pm .11 \times 10^{-3}$ [d, e]

a. In units of 10^{-3}M
b. In units of 10^{-4}M/min. corrected for blanks
c. Order of polynomial, t.10 test (11)
d. In units of M/min. mg. protein nitrogen/ml.
e. Evaluated by method of least squares

Table IV

Inhibition by Ethyl 1-Acetyl-2-Benzyl Carbazate of the alpha-Chymotrypsin Catalyzed Hydrolysis of Chloroacetyl-L-Valine Methyl Ester at pH 7.90 and 25.0°C.

$[E]_o$ = 0.1464 mg. protein nitrogen per ml., NaCl = 0.100 M
Notebook Ref. 934-21

$[I]_o = 8.83 \times 10^{-3}$ M

$[S]_o$ [a]	v_o [b]	$\pm\sigma v_o$ [b]	P_m [c]	v_1 [b]	$\pm\sigma v_1$ [b]	P_m [c]
54	0.952	.002	1	0.994	.002	1
36	.877	.003	3	.876	.010	4
30	.762	.009	3	.724	.010	3
24	.683	.017	4	.623	.007	3
18	.507	.011	3	.493	.005	2
18	.453	.003	2	.491	.003	2
12	.375	.016	3	.353	.006	2
12	.368	.006	4	.361	.004	3
9	.236	.003	2	.272	.003	2
6	.156	.003	2	.223	.004	4

$K'_s = 78 \pm 20 \times 10^{-3}$ M [e]

$k_3' = 1.81 \pm .43 \times 10^{-3}$ [d, e]

$K'_s = 56.5 \pm 5.5 \times 10^{-3}$ M [e]

$k_3' = 1.42 \pm .13 \times 10^{-3}$ [d, e]

a. In units of 10^{-3}M. b. In units of 10^{-4}M/min. corrected for blanks. c. Order of polynomial, $t_{.10}$ test (11). d. In units of M/min. mg. protein nitrogen/ml. e. Evaluated by method of least squares.

Table V

Inhibition by Ethyl 1-Acetyl-2-Benzyl Carbazate of the alpha-Chymotrypsin Catalyzed Hydrolysis of Chloroacetyl-L-Valine Methyl Ester at pH 7.90 and 25.0°C.

$[E]_o$ = 0.1464 mg. protein nitrogen per ml., NaCl = 0.100 M
Notebook Ref. 934-26

$[I]_o = 18.1 \times 10^{-3}$M

$[S]_o$ [a]	v_o [b]	$\pm \sigma v_o$ [b]	P_m [c]	v_1 [b]	$\pm \sigma v_1$ [b]	P_m [c]
56	1.020	0.018	4	0.873	0.008	3
36	.701	.009	3	.610	.005	3
36	.633	.005	2	.625	.004	2
28	.704	.013	5	.583	.003	3
28	.607	.013	3	.551	.010	2
18	.459	.004	4	.412	.005	4
14	.356	.006	4	.297	.002	2
14	.370	.006	4	.298	.002	2
11.2	.271	.005	4	.245	.003	3
11.2	.244	.001	5	.255	.002	3
9	.196	.001	2	.218	.003	4
5.6	.133	.002	2	.144	.003	3

$K'_s = 122 \pm 37 \times 10^{-3}$M [e]

$k'_3 = 2.21 \pm .65 \times 10^{-3}$ [d, e]

$K'_s = 83 \pm 7.7 \times 10^{-3}$M [e]

$k'_3 = 1.49 \pm .13 \times 10^{-3}$ [d, e]

a. In units of 10^{-3}M. b. In units of 10^{-4}M/min. corrected for blanks. c. Order of polynomial, $t_{.10}$ test (11). d. In units of M/min. mg. protein nitrogen/ml. e. Evaluated by method of least squares.

of initial velocities" except in those cases where the recorder traces of extent of reaction vs. time were essentially linear throughout their course." Since bias may enter into such a mixed procedure it was believed in the present work that the results would be of greater statistical significance if the orthogonal polynomial procedure, which incorporates a statistical "t" test for the order of the polynomial selected, were used throughout. In the present work 38 out of 43 runs were represented by polynomials of order $\gg 1$ for the 1 to 9 minute interval at a significance level of $t_{.10}$; hence, if bias exists in the use of the mixed procedure of Applewhite, Waite and Niemann, such bias should be absent in the present work by virtue of the use of the orthogonal polynomial procedure for all of the runs.

The larger limits of precision encountered in this Thesis may be due to the arbitrary selection of a $t_{.10}$ significance level and in part by the assumption that an operational steady-state was achieved at initial conditions. The $t_{.10}$ level was arrived at by experience with systems going to substantial conversion of substrate, where curvature is to be expected, whereas in the present system conversions rarely exceeded 10% and curvature was not to be expected. A possible way out of this dilemma would be to use a $t_{.01}$ level which would favor linear dP/dt traces.

That the orthogonal polynomial method may give large errors can be shown by a comparison of duplicate runs as shown in

Table VI. The two runs at $[S]_o$ = 0.0360 M had nearly identical traces, especially with respect to the areas under the traces; nevertheless, a P_1 level was selected in one instance and a P_3 level in the duplicate run giving v_1 = 0.898 and 0.987 respectively. Numerous examples of such deviations are apparent from even a cursory examination of Tables II-V.

It became apparent that a solution to this problem could be reached by the use of statistical procedures for the evaluation of each initial velocity obtained by the orthogonal polynomial procedure. Furthermore, inspection of figure 1 indicated that the initial velocities obtained at low substrate concentrations were often higher than those expected from the remaining data. Since errors in v_1 are absolute with respect to substrate concentration a weighting procedure would be advantageous. Hearon et al. (24) have discussed the merits of weighting initial velocities when the velocities are subject to random error. However, in the case at hand errors are biased by the use of the orthogonal procedure and errors may not be random.

As an alternative procedure for testing the reliability of individual initial velocities statistical procedures (14) were employed for rejecting data by two methods. The first method recognizes the fact that as $[S]_o$ approaches zero Michaelis-Menten kinetics approach first order kinetics; hence values of initial velocities obtained at low substrate concentrations are less reliable for determining the

Table VI

Comparison of Selection of v_1 by Orthogonal Polynomial Method

Inhibited Run Notebook Ref. 934-22

time min.	$[S]_o$ 36	36	18	18	12	12
	Scale Readings					
1	.75	.75	.48	.48	.71	.66
2	1.45	1.47	.91	.92	1.32	1.34
3	2.12	2.15	1.34	1.35	1.95	1.96
4	2.80	2.82	1.74	1.77	2.58	2.56
5	3.43	3.46	2.15	2.17	3.16	3.16
6	4.09	4.13	2.55	2.57	3.75	3.74
7	4.72	4.77	2.96	2.97	4.35	4.36
8	5.39	5.43	3.36	3.37	4.93	4.94
9	6.06	6.09	3.76	3.77	5.52	5.52
Polynomial	v_1[a] at each polynomial level					
P_1	.898	.904	.557	.558	.409	.411
P_2	.920	.938	.577	.590	.426	.434
P_3	.977	.987	.591	.617	.431	.455
P_4	.966	1.008	.603	.616	.421	.487
$t_{.10}$	P_1	P_3	P_3	P_3	P_2	P_4
v_1[c] $t_{.10}$	.898[a]	.987[a]	.591[a]	.617[a]	.386[c]	.447[c]
Significance levels	$(J)/(K)$[b]					
P_1	119	104	126	79	112	75
P_2	.95	1.66	2.9	3.0	3.2	2.0
P_3	2.3	2.4	1.3	3.6	.45	1.1
P_4	.2	0.6	.65	.03	.52	1.6

Footnotes to Table VI:

a. v_1 uncorrected for enzyme and substrate blanks. In units of 10^{-4} M/min.
b. (J)/(K) refers to ratio of t_{n-m}(calc)/t_{n-m}(.10 level)(11).
c. Corrected for blanks.

constants than are the velocities obtained at higher substrate concentrations. The second method assumes that all the inherent errors of the experiment are associated with the ordinate $[S]_o[E]_o/v_1$ and that the basis for rejection should be a function of the difference between the observed ordinate value and the ordinate value calculated from the least squares solution of the slope and intercept. These procedures and the results obtained therefrom are described below:

Data Rejection by Method 1

For each individual experimental point a new slope was computed between the limits K_s'/k_3' and that point. From this new slope a new value k_{3i}' was calculated and compared with the k_3' value derived from all of the points. The assembled k_{3i}' values were then compared by the analysis of variances at a 99% significance level for rejection. By selecting a t test value for a 99% confidence limit only those runs that were in serious error were made evident. Following this step K_s' and k_3' were recalculated using the acceptable data. The following summary showed that in three out of four cases the lowest $[S]_o$ runs were rejected and in the examples of duplicate runs cited in Table VI the run at $[S]_o$ = 12* was rejected where the orthogonal polynomial procedure selected a fourth order polynomial. Recalculated values of k_3' showed a non-competitive trend which was more consistent than that noted earlier.

* In this Thesis, [S], [I], K_s, and K_I are expressed in units of 10^{-3} M.

Table	$[S]_o$ [a]	v_I [b]	Recalculated K_s' [a]	Recalculated k_3' [c]
II	4.5	0.225	50.6±2.9	1.74±0.09
III	12.0	0.447	59.2±4.8	1.61±0.12
IV	6.0	0.223	63.1±5.7	1.53±0.13
V	5.6	0.114	82.0±9.9	1.45±0.16

Data Rejection by Method 2

The differencesbetween the measured $[S]_o[E]_o/v_I$ and the $[S]_o[E]_o/v_I$ calculated from the slope of the least squares line based on all of the data were computed and compared by the analysis of variances to a 99.5% confidence level. After rejection of the data not passing the test K_s' and k_3' were recalculated with the results as shown below:

Table	$[S]_o$ [a]	v_I [b]	Recalculated K_s' [a]	Recalculated k_3'
II	4.5	0.225	50.6±2.9	1.74±.09
III	12.0	0.447	59.2±4.8	1.61±.12
IV	6.0	0.223		
	36.0	0.876	58.6±2.4	1.48±.05
V	5.6	0.114	82.0±9.9	1.45±.16

Data rejection by Method 2 is slightly more rigorous than Method 1 which accounts for the rejection of $[S]_o$ = 36.0 from Table IV. The conclusion reached previously that there exists a

a. In units of 10^{-3} M. b. In units of 10^{-4} M. c. In units of 10^{-3} M/min. mg. PN/ml.

small but detectable non-competitive contribution is not contradicted by Method 2.

Assumption of Linear dP/dt Traces

Further support for the non-competitive trend was obtained by assuming that the orthogonal polynomial was biased in favor of non-linear traces occurring during the first minute or two of the kinetic run. The data obtained during the one to nine minute interval were used to calculate an initial velocity by fitting to a first order equation by the method of least squares. The justification for this procedure was presented previously, viz. apparent linearity after the second minute. The agreement between duplicate runs averaged 1.3% by this method which was far better than the results achieved by the orthogonal polynomial procedure.

The values for the kinetic constants obtained by this method were k_3^1 = 1.85 ± .07, 1.59 ± .03, 1.51 ± .05, 1.37 ± .05 x 10^{-3} M/min. mg. PN/ml.; K_s^1 = 56.7 ± 2.4, 60.0 ± 1.1, 65.1 ± 2.2, 83.8 ± 3.4 x 10^{-3} M at $[I]_o$ = 0, 4.41, 8.83 and 18.1 x 10^{-3} M respectively. The average precisions for k_3^1 were 3.0% and for K_s^1 3.6% with all data passing a high significance level for acceptance. The initial velocities are given in Table VII; the data are plotted in figure 2 as $[S]_o[E]_o/v_o$ vs. $[S]_o$ from which relationship the constants k_3^1 and K_s^1 were computed by the method of least squares.

Table VII

Kinetics of the Inhibition of Chloroacetyl-L-Valine Methyl Ester by Ethyl-1-Acetyl-2-Benzyl Carbazate. Assumption of Linear Fit to Recorder Traces.

$[I]_o = 0$		$[I]_o = 4.41$[a]		$[I]_o = 8.83$[a]		$[I]_o = 18.1$[a]	
$[S]_o$[a]	v_o[b]	$[S]_o$[a]	v_o[b]	$[S]_o$[a]	$[v_o]$[b]	$[S]_o$[a]	$[v_o]$[b]
42	1.172	54	1.104	54	1.001	56	.805
30	.923	45	.984	36	.795	36	.605
24	.803	36	.873	30	.680	36	.599
18	.651	36	.878	24	.601	28	.514
18	.645	30	.779	18	.483	28	.502
12	.463	24	.669	18	.476	18	.357
9	.366	18	.541	12	.354	14	.287
6	.265	18	.546	12	.343	14	.285
4.5	.205	12	.385	9	.265	11.2	.240
		12	.388	6	.184	11.2	.238
		9	.297			9	.186
		6	.212			6	.130
k_3'[c]	$1.85 \pm .07$		$1.59 \pm .03$		$1.51 \pm .05$		$1.37 \pm .05$
K_s'[a]	56.7 ± 2.4		60.0 ± 1.1		65.1 ± 2.2		83.8 ± 3.4

a. 10^{-3} M.

b. Units 10^{-4} M/min. mg. PN/ml.

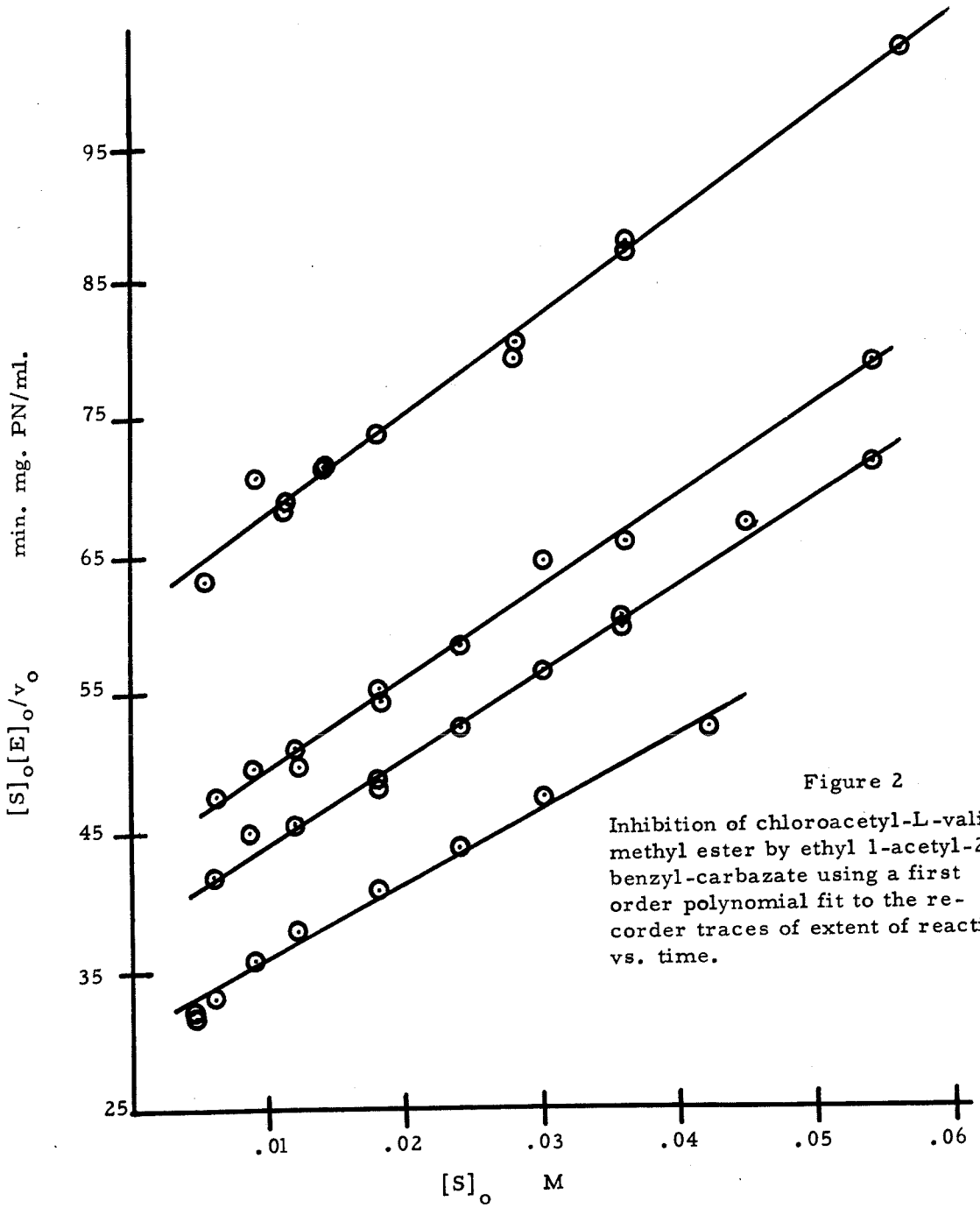


Figure 2

Inhibition of chloroacetyl-L-valine methyl ester by ethyl 1-acetyl-2-benzyl-carbazate using a first order polynomial fit to the recorder traces of extent of reactions vs. time.

Ethyl 1-Acetyl-2-Benzyl Carbazate as a Mixed Competitive and Non-Competitive Inhibitor

A downward trend in k_3' and increasing trend in K_s' with increasing inhibitor concentration has been recognized in enzyme kinetics as a mixed competitive and non-competitive situation arising from ternary complexes (6) or as Blum (25) pointed out, from an enzyme that can exist in two or more conformations that are a function of the inhibitor (or any modifier). Since chloroacetyl-L-valine methyl ester may hardly be called a "typical substrate," nor for that matter can the inhibitor under discussion be called a "typical inhibitor," the results of this experiment may be discussed in terms of ESI complexes.

Before proceeding with a mixed competitive-non-competitive analysis it was necessary to test the significance of the downward drift in k_3'. This was done at a 95% chi square level from which it was concluded that no differences in precision existed amongst the four sets of inhibitor concentrations. Following this analysis a 95% F test for homogeneity of the four k_3' values was carried out with the result that to a 95% confidence level the four sets gave different values of k_3' that were significant. Admitting that the differences are small, i.e., over the range of $k_3' = 1.74$ to 1.45×10^{-3} the conclusion that non-competitive inhibition was present is taken with some reservation. Since statistical significance was shown, an

estimate of the non-competitive contribution was deemed necessary.

Huang and Niemann (6) developed the kinetics for a non-competitive situation arising from a monofunctional inhibitor, indole, and a bifunctional substrate, methyl hippurate. These authors justified non-competitive kinetics on the basis of the "three center hypothesis" wherein a R_1 or R_2 monofunctional inhibitor occupies only one of the three complementary ρ_1, ρ_2, ρ_3 enzyme binding sites. It is easy enough to reject non-competitive interaction at the active site for typical trifunctional substrates and inhibitors, neither of which conditions may be satisfied here. However, interaction at a site adjacent to the active site or at a site that changes the configuration at the active site cannot be so summarily rejected. A discussion of these factors is deferred until the results of the non-competitive treatment are presented.

Mixed Competitive and
Non-Competitive Inhibition

In this development it is assumed that two sites of interaction are available on the enzyme. The first site, designated the "active site," is the locus for hydrolytic reactions, competition for which by the inhibitor results in a competitive contribution to the overall inhibition. The second site, designated the "inactive site," is the locus for association between enzyme and substrate or inhibitor. Occupancy of the "inactive site" by inhibitor may reduce

the rate of reaction of substrate at the active site by partially displacing the R_3 hydrolyzable group. In order to simplify the kinetic development, EI'I and ES'S complexes are not considered as they are not likely to be encountered with the substrate concentrations used in these studies. The symbols I', S' indicate association at the "inactive site" whereas the unprimed symbols I and S are used to designate association at the "active site." The development of this situation is given in Appendix II.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

$$E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI$$

$$E + S \underset{k_9}{\overset{k_8}{\rightleftharpoons}} ES'$$

$$E + I \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EI'$$

$$ES' + I \underset{k_{11}}{\overset{k_{10}}{\rightleftharpoons}} ES'I \not\longrightarrow P$$

$$EI' + S \underset{k_{13}}{\overset{k_{12}}{\rightleftharpoons}} EI'S \xrightarrow{k_{14}} EI' + P$$

$$ES + I \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} EI'S \xrightarrow{k_{14}} EI' + P$$

$$EI + S \underset{k_{18}}{\overset{k_{17}}{\rightleftharpoons}} ES'I \not\longrightarrow P$$

It is shown in Appendix II, that under Zone A (15, 16) conditions, where I EI, ES'I, EI'S and S ES, ES'I, EI'S that the reciprocal

of the forward velocity may be expressed by equation 8.

$$\frac{1}{v} = \frac{1}{[S]}\left[\frac{K'_S}{V'}\left(1 + \frac{[I]}{K_I}\right)\left(\frac{1}{1 + \frac{rK_S[I]}{C_\beta}}\right)\right] + \left[\frac{1}{V'} + \frac{K'_S[I]}{V'}\left(\frac{1}{C_\alpha} + \frac{1}{C_\beta}\right)\left(\frac{1}{1 + \frac{rK_S[I]}{C_\beta}}\right)\right] \quad (8)$$

where,

$$K_S = \frac{k_2 + k_3}{k_1} \qquad K_I = k_5/k_4 \qquad K_{I.1} = k_7/k_6$$

$$K_\alpha = k_9/k_8 \qquad K_{I.2} = k_{11}/k_{10} \qquad K_{I.3} = k_{18}/k_{17}$$

$$K_{S.1} = \frac{k_{13} + k_{14}}{k_{12}}$$

$$K_{S.2} = \frac{k_{14} + k_{16}}{k_{15}} \qquad \frac{1}{V'} = \frac{1}{V}\left(1 + \frac{K_S}{K_\alpha}\right)$$

$$\frac{K_S}{V} = \frac{K'_S}{V'} \qquad V' = k'_3[E]_o \qquad r = k_{14}/k_3$$

and,

$$v = k_3[ES] + k_{14}[EI'S] = k_3([ES] + [EI'S]).$$

Equation 8 is in the form suitable for plotting $1/v_o$ versus $1/[S]_o$ where $[I] = [I]_o$. It is apparent that K'_S/V', the slope of equation 8, is increased by the proportionality

$$\left(1 + \frac{[I]}{K_I}\right)\left(\frac{1}{1 + \frac{rK_S[I]}{C_\beta}}\right),$$

and the intercept $1/V'$ is increased by the proportionality

$$\left(1 + K'_S[I]\right)\left(\frac{1}{C_\alpha} + \frac{1}{C_\beta}\right)\left(\frac{1}{1 + \frac{rK_S[I]}{C_\beta}}\right)$$

From equation 8 it is apparent that the slope increases by the factor $(1 + [I]/K_I)$ and decreases by the factor $(1/(1+rK_s[I]/C_\beta))$ as [I] increases. It was found that the slopes obtained from $1/v_o$ vs. $1/[S]_o$ plots increased linearly with $[I]_o$ (figure 3) indicating that $[I]_o/K_I \gg rK_s[I]_o/C_\beta$. Similarly it was found that the intercepts increased linearly in the region $[I]_o = 0$ to 8.83×10^{-3}M although a slight downward drift in intercept with increasing $[I]_o$ was apparent (figure 4). This situation was not quite tenable with the assumption that $rK_s/C_\beta = 0$. However, since a prior knowledge of K_I was not at hand the various constants could not be separated.

As a first approximation for K_I one may assume that $[I]_o/K_I \gg rK_s/C_\beta$ since the plots of slope versus $[I]_o$ were linear. The value for K_I thus obtained was $20 \pm 1 \times 10^{-3}$M from the orthogonal polynomial procedure estimates of initial velocities. Setting as the upper limit for rK_s/C_β the precision of the K_I value (5%) one arrives at an upper limit for $rK_s/C_\beta = 2.5$ with a lower limit = 0. In the absence of an independent measurement of K_I in a truly competitive situation one cannot say that the rate of conversion of the ternary complex EI'S is finite. However, from the upper limit of $rK_s/C_\beta = 2.5$, limits for the quantity $K_s(1/C_\alpha + 1/C_\beta)$ may be set at 10.4 to 13.4. As a further condition for non-negative values for the latter quantity it is seen that r, the ratio k_{14}/k_3, which is the ratio of the forward velocity constant for the ternary [EI'S] complexes to

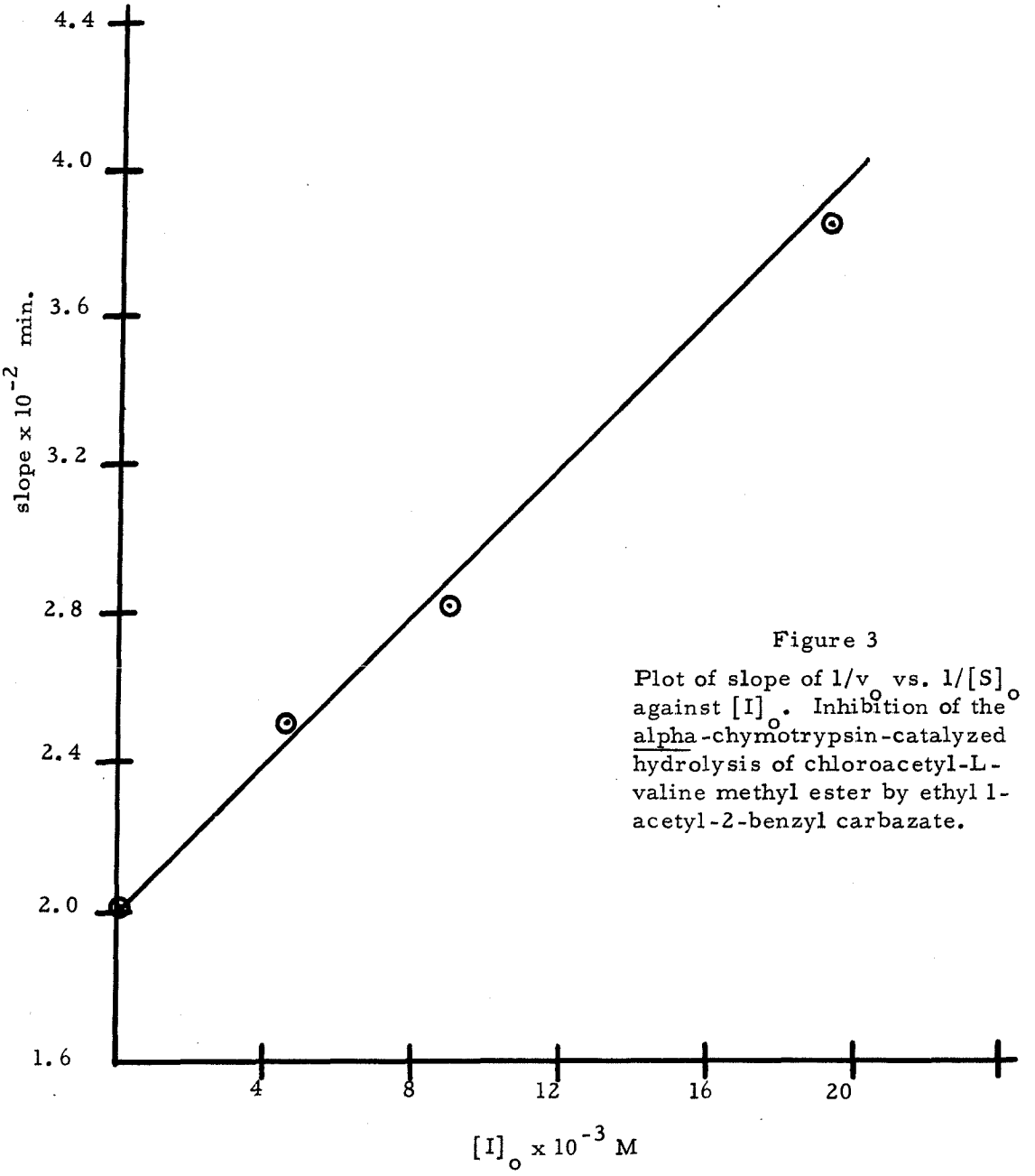


Figure 3

Plot of slope of $1/v_o$ vs. $1/[S]_o$ against $[I]_o$. Inhibition of the alpha-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-valine methyl ester by ethyl 1-acetyl-2-benzyl carbazate.

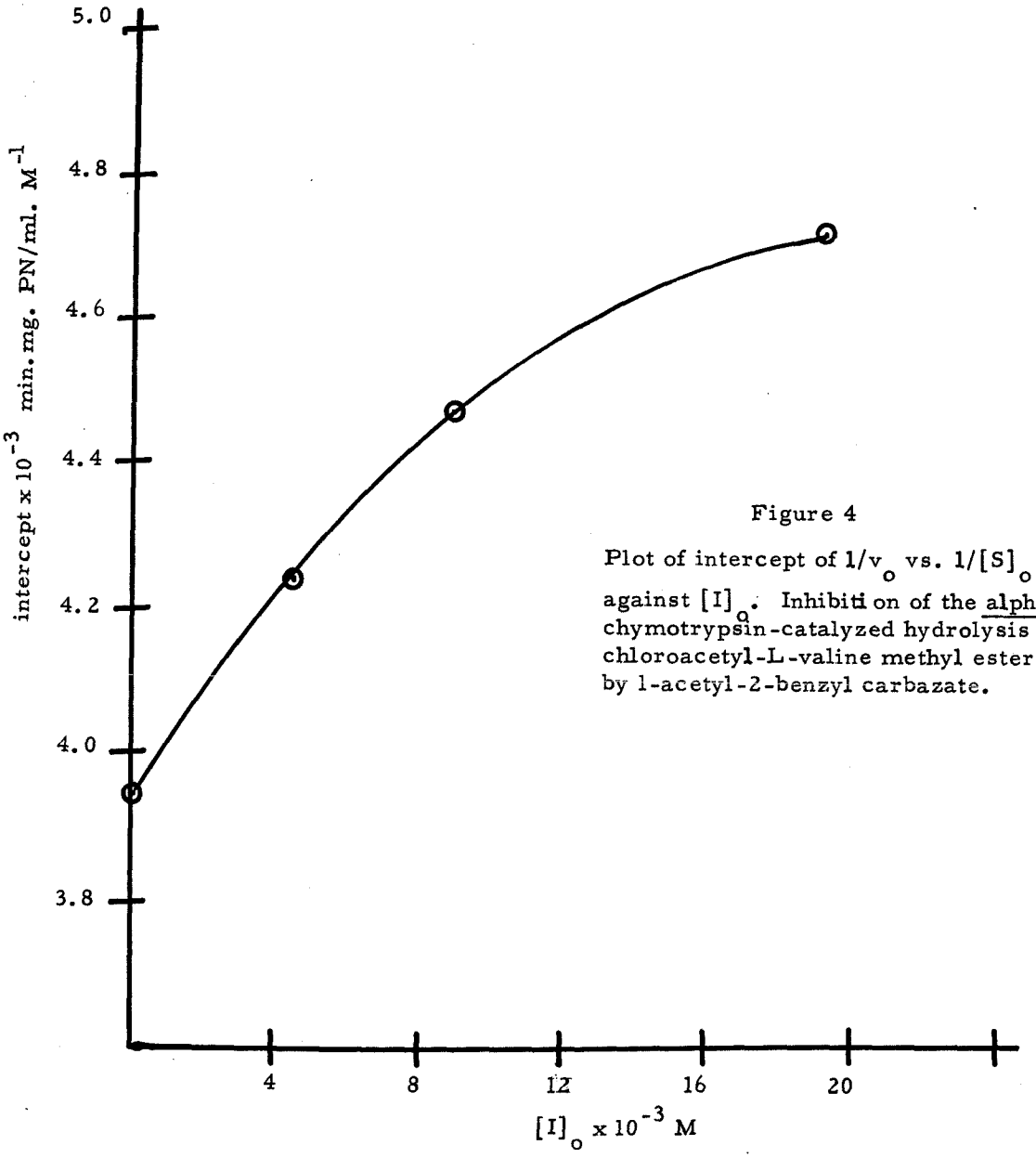


Figure 4

Plot of intercept of $1/v_o$ vs. $1/[S]_o$ against $[I]_o$. Inhibition of the alpha-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-valine methyl ester by 1-acetyl-2-benzyl carbazate.

products and the forward velocity constant for the binary [ES] complexes, need be no larger than 0.25 nor no less than 0.15.

Similar computations based on the results of initial velocities obtained by the method of linear traces described previously gave $K_I = 19 \pm 1 \times 10^{-3}$M with even greater significance attached to the premise that $[I]/K_I \gg r/C_\beta$. In the absence of independent methods for evaluating $1/C_\alpha$ which is related to the ability to form ES'I complexes one cannot say with any degree of certainty that the contribution of the rK_s/C_β term is small because of a low rate of conversion of EI'S to products or to an unfavorable EI'S affinity constant. It is possible that ternary interactions of this inhibitor with enzyme and a substrate such as methyl aceturate would lead to a more favorable non-competitive situation from which the constants C_α, C_β, K_s may be separated.

The assumption of a rate = 0 for conversion of the EI'S ternary complex to products is certainly acceptable since it would imply that four association centers are available at the active site or that overlapping between the active site and an adjacent site may occur. That this notion may be more acceptable than may at first appear is that the behavior of ester substrates is remarkably different from amide substrates, i.e., $K_{s_L} > K_{I_D}$ for amides and $K_{I_D} > K_{s_L}$ for esters (26). These results might be explained by one

orientation favoring L-esters, another orientation favoring L-amides, and a single orientation for both D-esters and D-amides from a selection of four centers.

Also, if r = 0, then $K_{s.1}$ and $K_{s.2}$ are true equilibrium constants since k_{14} would equal zero. Since the affinity of the enzyme for ternary complexes is a function of both the substrate and the inhibitor, the analog inhibitor should be evaluated against the substrate to which it is analogous. Thus, the present inhibitor under discussion, ethyl 1-acetyl-2-benzyl carbazate, should be compared to the D- and L- ethyl esters of N-acetyl-phenylalanine. Since the latter substrate is hydrolyzed very rapidly in the presence of alpha-chymotrypsin it is difficult to study it under Zone A (15, 16) conditions. A particularly simple system to study in the pH-stat would be the D- and L- methyl esters of N-acetyl-valine with methyl 1-acetyl-2-isopropyl carbazate as the analog. Another system worth investigating would contain as the analog inhibitor 1-acetyl-2-benzyl-semicarbazide evaluated against acetyl-L-phenylalaninamide. This latter system has the advantage of using a substrate that may be considered more typical.

The value of the competitive inhibition constant K_I for acetyl-D-phenylalanine ethyl ester has not been reported in the literature. However, the methyl ester has been reported to have $K_I = 2.2 \pm 0.4 \times 10^{-3}$M (17). If as a first approximation one draws

an analogy to the homologous series of acetyl-D-tryptophan esters (18) then K_{I_L} ethyl/K_{I_D} methyl $\doteq$ 3. This analogy to the acetyl-D-tryptophan homologous ester series is taken with some reservation because Hal Waite (unpublished observations) has observed that K_s and k_3 were nearly equivalent for the methyl and ethyl esters of chloroacetyl-L-valine. The present state of knowledge of the effect of the -OR group of esters on K_s and k_3 for substrates and inhibitors of alpha-chymotrypsin catalyzed hydrolyses does not justify the indicated relationship that the affinity of enzyme for ethyl esters is less than for methyl esters. J. P. Wolf III (23) also observed that K_s ethyl/K_s methyl $\doteq$ 3 for the two aceturic esters; hence it can be stated for the present at least that the affinity of enzyme for an ethyl ester is not greater than for a methyl ester. Therefore, an estimate of K_I for acetyl-D-phenylalanine ethyl ester falls in the range of 3 to 6 x 10^{-3}M, so that the analog inhibitor does not bind to enzyme as well as its related D-ethyl ester.

K_{I_L} for acetyl-L-phenylalanmethylamide has been reported to be ca. 25 x 10^{-3}M (20); for acetyl-L-phenylalaninamide, K_s = 31 x 10^{-3}M; for the corresponding D-enantiomorphs K_I = 5.9 x 10^{-3}M and 12 x 10^{-3}M respectively. It would appear then that the carbazate analog resembles the L-amides in the ability to associate with alpha-chymotrypsin. A summary of the known or estimated constants for these related compounds is given in Table VIII.

Table VIII

Comparison of Enzyme-Substrate and Enzyme-Inhibitor Dissociation Constants

Acetyl Phenylalanine Series

Compound	K_{s_L}	K_{I_L}	K_{I_D}	K_I	Ref.
I N-Acetyl-L-phenylalanine methyl ester	n. m.				
II N-Acetyl-L-phenylalanine ethyl ester	n. m.				
III N-Acetyl-L-phenylalanine amide	31				(19)
IV N-Acetyl-L-phenylalanine methyl amide		25			(20)
V N-Acetyl-D-phenylalanine methyl ester			2.2		(17)
VI N-Acetyl-D-phenylalanine ethyl ester			3-6(est.)		--
VII N-Acetyl-D-phenylalanine amide			12		(21)
VIII N-Acetyl-D-phenylalanine methyl amide			5.9		(20)
IX Ethyl-1-acetyl-2-benzyl-carbazate (analog of II)				20	--

In order to obtain additional evidence for relationships between substrates and analogs a qualitative study of similar phenylalanine and glycine analogs was undertaken and is reported in the next section of the Thesis.

C. Nitrogen Analogs of Phenylalanine and Glycine

Analogs of the ethyl esters and amides of acetylglycine and acetylphenylalanine were prepared and evaluated as inhibitors against acetyl-L-valine methyl ester. In addition, several intermediates of the phenylalanine analogs possessing only two of the three functionalities, i.e., 1-acetyl-2-benzylhydrazine, and two cyclic intermediates analogous to azlactones were also evaluated.

In order to cover more ground in this survey experiments were conducted in a more qualitative manner than those of the preceding section. The assumptions made were that all inhibitors were competitive and that when organic solvents were used for those inhibitors too insoluble in water to measure in a pure water system an apparent K_s for the substrate-solvent-water could be employed for comparison with the measured K'_s for the inhibitor-substrate-solvent-water system. Justification for this latter approximation was based upon preliminary results that indicated that dioxane and acetone behaved in an apparent competitive manner with acetyl-L-valine methyl ester. Applewhite, Martin and Niemann (9) also found that dioxane and acetone exerted an effect on K_s of methyl hippurate in a competitive manner (as a first approximation). In several cases, where solubility in water permitted, K_I was determined in water, 5% aqueous dioxane, and 20% aqueous acetone.

Without exception, inhibitor constants were not significantly different. The results of this study are presented in Table IX.

Kinetic Measurements and Calculations

Equation 9 describes the kinetics of a doubly competitive situation, from which the relationships given in equation 10 may be used to calculate K_I.

$$\frac{1}{v_o} = \frac{K_s}{V}\left(1 + \frac{[I_1]_o}{K_{I_1}} + \frac{[I_2]_o}{K_{I_2}}\right) \tag{9}$$

$$K'_s = K_s\left(1 + \frac{[I_1]_o}{K_{I_1}}\right) \quad ; \quad K_{I_2} = \frac{K_s[I_2]_o}{K''_s - K'_s} \tag{10}$$

Where K_s refers to the apparent enzyme-substrate constant measured in the absence of inhibitors, K'_s is the apparent constant determined in the presence of organic solvent, and K''_s is the apparent constant of the substrate-solvent-inhibitor system.

That this approximate method gives reasonable results may be seen from the relative independence of K_I upon solvent. Thus, K_I for 1-acetyl-2-benzylhydrazine ranged from 10 to 15 x 10^{-3}M and quinoline from 0.6 to 0.9 x 10^{-3}M for the three solvents water, acetone and dioxane. Benzyl urethane and 2-methyl-4-benzyl-1,3,4-oxadiazolone-5 essentially gave the same value of K_I, 6 x 10^{-3}M and 3 x 10^{-3}M, respectively, for both 5% dioxane and 20% acetone.

Table IX

Enzyme-Inhibitor Dissociation Constants of Nitrogen Analogs of Amino Acid Substrates Measured Against Acetyl-L-Valine Methyl Ester at pH 7.90

25.0°C, NaCl = 0.100 M. $[S]_o$ = 20×10^{-3}M and 40×10^{-3}M

$[E]_o$ = 0.1464 mg. protein nitrogen/ml.[c]

Key	Compound	K_s[a]	$[I]_o$[a]	K_I[a]	Solvent	Ref.
	Acetyl-L-phenylalanine ethyl ester	n.m.				
	Acetyl-D-phenylalanine ethyl ester			3-6(est.)		
I	Ethyl 1-acetyl-2-benzyl-carbazate		8	20	water	b
	Acetyl-L-phenylalanin-amide	31			water	(19)
	Acetyl-D-phenylalanin-amide			12	water	(21)
VI	1-Acetyl-2-benzyl semi-carbazide		8	30	water	b
	Acetyl glycine ethyl ester	96			water	(23)
VIII	Ethyl 1-acetyl carbazate		500	330	water	b
IX	Aceturamide		40	340	water	b
VII	1-Acetyl semicarbazide		200	1200	water	b
	Methyl hydrocinnamate	0.2				
	Methyl N-phenyl glycine ester	0.3			water	(23)
XI	Benzyl urethane		20	6	5% dioxane	b
	" "			6	20% acetone	b

Table IX (cont'd.)

Key	Compound	K_s [a]	$[I]_o$ [a]	K_I [a]	Solvent	Ref.
III	1-Acetyl-2-benzylhydrazine		10	12	water	b
	" "		20	10	20% acetone	b
	" "		20	15	5% dioxane	b
X	(N-β-phenyl-ethyl)-acetamide		20	7	water	b
	Quinoline		2.5	0.6	water	b
	"		1.25	0.7	5% dioxane	b
	"		1.25	0.9	20% acetone	b
IV	2-methyl-4-benzyl-1, 3, 4-oxadiazolone-5		10	3	5% dioxane	b
	2-methyl-4-benzyl-1, 3, 4-oxadiazolone-5		10	3	20% acetone	b
V	2-methyl-(4H)-1, 3, 4-oxadiazolone-5		10	280	water	b

a. In units of 10^{-3}M.
b. Measured in this Thesis.
c. For an assumed molecular weight of 22,000, nitrogen content of 16.0%, Armour Lot No. 283.

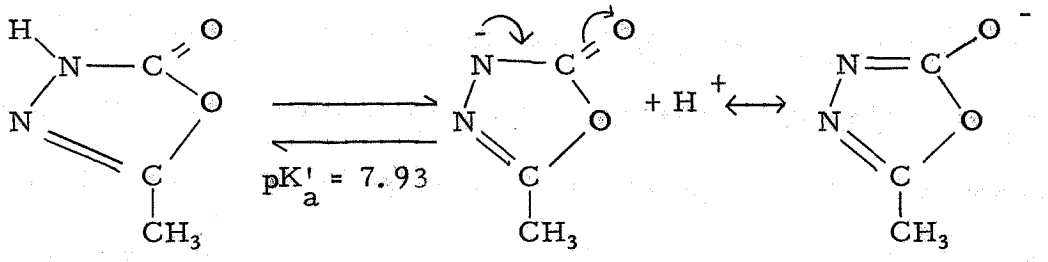
Two runs were made for each inhibitor at $[S]_o$ for the reference substrate acetyl-L-valine methyl ester equal to 40×10^{-3}M and 20×10^{-3}M at pH 7.90, 25.0°C, and NaCl = 0.100 M. The reference points for K'_s and k'_3 were determined separately and are given below:

Solvent	K'_s [a]	k'_3 [b]	Ref.
Water (K_s)	111 ± 6	2.20 ± 0.04	-----
5% Dioxane[d]	490 ± 76	2.2[c]	-----
20% Acetone[d]	450 ± 85	2.2[c]	-----
Water (K_s)	108 ± 9	2.2	(22)
Water (K_s)	125.6 ± 3.4	2.33 ± 0.06	(13)

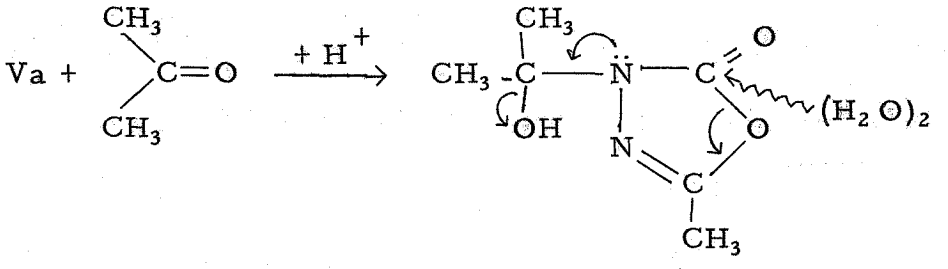
a. In units of 10^{-3}M. b. In units of 10^{-3}M/min. mg. PN/ml., assuming a nitrogen content of 16.0%. Enzyme, Armour Lot No. 283. c. Corrected for competitive inhibition in 2nd and 3rd cases. d. Vol/vol %.

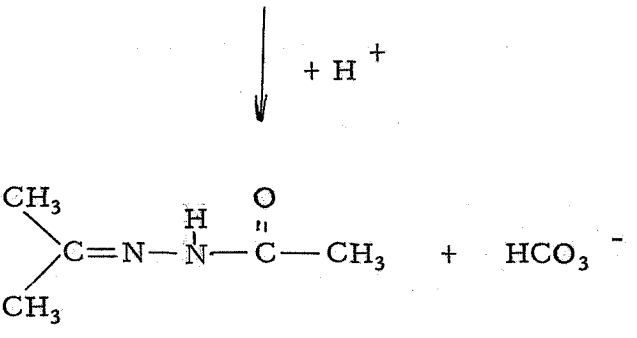
Ethyl 1-acetyl carbazate was found to have a K_I value of 330×10^{-3}M, which value is approximately three times the K_s value reported for acetyl glycine ethyl ester ($K_s = 96 \times 10^{-3}$M).

2-Methyl-1, 3, 4-oxadiazolone-5 gave a value of $K_I = 280 \times 10^{-3}$M in water. When the latter compound was tested in the presence of acetone, hydrogen ion was produced at a rate in excess of the uninhibited substrate enzymatic rate. Although the isolation of products from this latter reaction was not attempted it is likely that hydrazone formation was taking place with release of bicarbonate ion. In view of the additional fact that 2-methyl-1, 3, 4-oxadiazolone-5 was found to be an acid with $pK'_a = 7.93 \pm 0.02$ the likelihood of hydrazone formation is increased. The breakdown of this cyclic compound may follow the following scheme:



V Va





Isopropylidene-acethydrazone

(1-acetyl-2-isopropylidene-hydrazine)

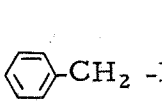
This situation is analogous to the Erlenmeyer azlactone condensation for the synthesis of acylated dehydroamino acids from glycine derivatives but goes under milder conditions than the C-C condensations because of the stability of the cyclic anion and because of the driving force accompanying the formation of bicarbonate anion at pH 7.9.

None of the other compounds studied in this series exhibited interaction with acetone or dioxane under the conditions of the experiment. In addition, none of them showed detectable blank reactions in the absence of enzyme, and in the presence of enzyme all of these compounds decreased the normal enzyme blank.

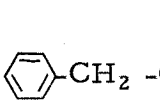
Analog-Enzyme-Inhibitor Dissociation Constants

It was pointed out previously in Part IB of this Thesis that ethyl 1-acetyl-2-benzyl carbazate gave a K_I value of 20×10^{-3}M, which was the mean of K_s and K_I for L- and D-acetylphenylalaninamide, but was close to K_{I_L} for acetyl-L-phenylalanmethylamide. K_I for 1-acetyl-2-benzyl semicarbazide, the analog of acetylphenylalaninamide, was 30×10^{-3}M. Therefore, replacement of the carbethoxy group in the analog by a carboxamido group increased K_I by 50% which is reasonable with the expected contribution of a second nitrogen's electron pair.

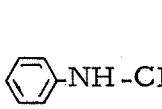
Benzyl urethane, which may be considered to be the analog of ethyl hydrocinnamate or ethyl N-phenyl-glycine ester:



Benzyl urethane



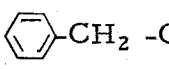
Ethyl hydrocinnamate



Ethyl N-phenyl-glycine ester

gave a K_I value of 6×10^{-3} M. Since K_s for only the methyl esters of hydrocinnamic acid and N-phenyl glycine were available for comparison one would expect an increase in K_s by a factor of 2 to 3 in going from a methyl to an ethyl ester, and an increase in K_s by a factor of 2 to 5 in going from a carbethoxy to a carboxamido group. Hence the value of $K_I = 6 \times 10^{-3}$ M for benzyl urethane appears reasonable.

K_I values for uncharged bifunctional inhibitors containing a phenyl and a carboxamido group such as acetanilide, benzamide, hippurylamide, phenylacetamide, γ-phenylpropionamide, and γ-phenylbutyramide (17) fall in the range 7 to 15×10^{-3} M; therefore, the value for 1-acetyl-2-benzylhydrazine, $K_I = 12 \times 10^{-3}$ M (water) also is reasonable. K_I for (N-β-phenylethyl)-acetamide.



, to which 1-acetyl-2-benzylhydrazine is strictly analogous, was also measured and found to be 7×10^{-3} M.

Huang and Niemann (18) in the tryptophane series observed that K_I increased in the series indole, acetyltryptamine, acetyl-D-tryptophanamide, $K_I = 0.7 \times 10^{-3}$M, 1.8×10^{-3}M and 2.3×10^{-3}M respectively. However, they also observed that acetyl-D-tryptophan methyl ester had a K_I of 0.089×10^{-3}M, far lower than even indole. Although benzylhydrazine, the first member of the analogous series, has not been measured, the increase in K_I when going from 1-acetyl-2-benzylhydrazine < ethyl 1-acetyl-2-benzyl carbazate < 1-acetyl-2-benzyl-semicarbazide and the series (N- β -phenylethyl)-acetamide < acetyl-D-phenylalanamide confirms the conclusion in the analog series that similar structural factors are involved as well as the hypothesis that the alpha-nitrogen in the analog donates an electron-pair to the carbonyl carbon of the carbethoxy group causing that group to behave like a carboxamido group rather than like an ester function.

Of all the inhibitors studied, 2-methyl-4-benzyl-1, 3, 4-oxadiazolone-5 gave the lowest K_I value, i.e., 3×10^{-3}M. It is tempting to speculate on the possibility that such a cyclic structure analogous to the azlactones may arise in the transition state of the ES complex in the stage following the loss of an -OR group from an ester substrate. It is well known (27) that azlactones are rapidly attacked by water at room temperatures so that one may speculate that the following sequence takes place:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_4} ES_2 + ROH$$

$$ES_2 + HOH \xrightarrow{k_5} E + P$$

where ES_1 represents a complex of enzyme and substrate and ES_2 is the enzyme-azlactone complex. The forward velocity constant k_3' as usually written for the Michaelis-Menten scheme would then equal $k_4 k_5 / (k_4 + k_5)$ and it is conceivable therefore that k_3' for an ester substrate may be much larger than k_3' for an amide substrate for which an azlactone-like intermediate would be unlikely. The lack of association of 2-methyl-1, 3, 4-oxadiazolone-5 with the enzyme, where $K_I = 280 \times 10^{-3} M$, appears to stand in contradiction to the azlactone-intermediate hypothesis; however, it should be recalled that the latter cyclic inhibitor, which lacks the benzyl in the 4-position, was an acid of $pK_a' = 7.93$ so that the anionic form would be repelled by an anionic site on the enzyme and the protonated form by virtue of a lowered hydrogen-bonding ability of structure Va. A rationalization of the high affinity of the analog containing the 4-benzyl substituent could then be made on the basis of an inductive effect by the benzyl group coupled with the affinity of an enzyme center for the aromatic sidechain.

In order to obtain some experimental justification for the plausibility of an azlactone intermediate an attempt was made to

measure the second-order basic hydrolysis constant of 2-phenyl-4-benzyl-oxazolone at pH 7.9. Although extensive hydrolysis of this substance occurred before it could be injected into the system, an estimate for the constant gave a value of 10^4 l mol^{-1} min^{-1}. From the literature values for benzoyl-L-phenylalanine ethyl ester (27) $K_s = 5.7 \times 10^{-3}$M, $k_3 = 390 \times 10^{-3}$M/min. mg. PN/ml. one may estimate that the enzymatic hydrolysis of the latter ester substrate would proceed at a rate only an order of magnitude greater at $[E]_o = [OH^-] = 7.94 \times 10^{-7}$ M than would the basic hydrolysis of the corresponding azlactone. One might argue then that the enzymatic hydrolysis rate should not exceed the basic hydrolysis rate of the azlactone; however, the transition state for the enzyme-azlactone complex may be much more favorable for attack by HOH or OH^- than the transition state for the azlactone alone. The existence of an enzyme-azlactone intermediate arising from ester substrates cannot therefore be completely excluded.

In contrast to the hydrolytic instability of the azlactones it has been found that the second-order basic hydrolysis constant for 2-methyl-4-benzyl-1, 3, 4-oxadiazolone-5 was 0.62 l mol^{-1} min^{-1} so that replacement of the 4-carbon in azlactones by a nitrogen atom imparts unusual stability to the ring.

Glycine Analogs

Ethyl 1-acetyl carbazate, the analog of acetyl glycine ethyl ester, gave a value of $K_I = 330 \times 10^{-3}$ M which is approximately three times the value of K_s reported for acetyl glycine ethyl ester, $K_s = 96 \times 10^{-3}$ M (23). Since a similar relationship was observed in the phenylalanine series, i.e. K_I (analog)/K_s (substrate) $\doteq$ 3 to 6, it seems likely that the increasing negative charge on the carbonyl oxygen, i.e. $-\ddot{N}-C=O \leftrightarrow -\overset{\oplus}{N}=C-\overset{\ominus}{O}$, is responsible for the lower affinity of the ester analogs for the enzyme.

1-Acetyl semicarbazide, the analog of aceturamide, gave a value of $K_I = 1200 \times 10^{-3}$ M, whereas aceturamide gave a value of $K_I = 340 \times 10^{-3}$ M, therefore, the absence of an aromatic side chain appears to operate in favor of an increased electronegativity of the carbonyl oxygen situated at the potentially hydrolyzable function.

On the basis of the observed results with the phenylalanine and glycine analogs it is concluded that an alpha-hydrogen on a potential substrate or inhibitor is not a necessary requirement for association to alpha-chymotrypsin and that the lesser affinity for the nitrogen analog may be ascribed to electrostatic repulsion between the carbonyl oxygen residing at the potentially hydrolyzable function and an anionic site on the enzyme. It also seems reasonable to conclude that the nitrogen analog-enzyme complex is not hydrolyzed due to the decreased polarity of the carbon at the same carbonyl function.

D. EXPERIMENTAL-SYNTHESIS

Ethyl 1-acetyl-2-benzyl carbazate (I)

This compound does not appear in the literature. Ronco and Erlenmeyer (28) prepared ethyl 2-benzyl carbazate which could yield I by simple acetylation. However, their sequence: hydrazine → ethyl carbazate → monohydrazone of diacetyl and ethyl carbazate → ethyl 1-diacetylidene-2-benzyl carbazate → ethyl 2-benzyl carbazate did not look very attractive due to an overall yield of 15%. Two alternative methods were investigated: A, the sequence acethydrazide → 1-acetyl-2-benzylidene-hydrazine → 1-acetyl-2-benzylhydrazine → I, and: B, benzylamine → benzylurethane → N-nitrosobenzylurethane → ethyl 2-benzyl carbazate → I. Of the two methods, A gave the desired product whereas various reduction procedures failed for the attempted reduction of N-nitroso-benzylurethane to ethyl 2-benzyl carbazate in high yield.

Method A:

$CH_3CONHNH_2$ +  → $CH_3CONHN{=}CH$- 

1-acetyl-2-benzalhydrazone(II)

II + Pd-H_2 → $CH_3CONHNHCH_2$- 

N'-benzylacethydrazide(III)

III + Cl-COOEt →  -CH_2-N(-COOEt)-HN-$COCH_3$

ethyl 1-acetyl-2-benzyl carbazate (I)

Acethydrazide

88 grams (1 mole) of ethyl acetate, reagent grade, 50.3 grams (1 mole) of hydrazine hydrate (99-100% grade), and 50 ml. of absolute ethyl alcohol were refluxed for 20 hours. The product was stripped in vacuo and recrystallized twice from ether-chloroform to give the hygroscopic product in 65% yield. M.p. 64-66° C. Lit. m.p. 67° C. (29).

1-Acetyl-2-benzylidene-hydrazine II

Acethydrazide, 37 grams (0.5 mole) was dissolved in 100 ml. water. Benzaldehyde, redistilled, 53 grams (0.5 mole) was added in small portions with vigorous shaking and cooling in ice water over a period of one-half hour. The crude reaction mixture was stored at 5° C. for two days, then filtered and washed with water. The dried product was washed with ethyl ether to remove the yellow benzalazine impurity yielding a colorless crude product that was recrystallized from aqueous ethyl alcohol in 66% yield. M.p. 139-141° C. (corr.). Lit. m.p. 134° C. (30).

1-Acetyl-2-benzylhydrazine III

10.0 grams (0.062 mole) of II were dissolved in 50 ml. absolute ethyl alcohol and shaken with 0.25 grams of palladium black at 50 psi hydrogen in a Parr apparatus for 24 hours. The crude

mixture was filtered and the solvent removed in vacuo at 25° C. The crude product had a m.p. of 78-81° C. The crude product was dissolved in 100 ml. of water and the pH of the solution was adjusted to pH 5. The aqueous solution was then extracted with four 100 ml. portions of ether, the ether extract dried with anhydrous sodium sulfate, filtered and vacuum stripped to give a product with a m.p. 80-82° C.(corr.), in 74% yield. The latter product was then recrystallized from anhydrous ether to give the desired product, III in 43% yield, m.p. 81-82° C. The infra-red spectrum of a 0.46 M solution of III in $CHCl_3$ showed peaks at 3425 and 3289 $cm.^{-1}$ (N-H), a strong C=O at 1667 $cm.^{-1}$ (Amide I) and a shoulder at 1548 $cm.^{-1}$ (Amide II). Principal peaks also occurred at 1495, 1456, 1370 and 1277 $cm.^{-1}$.

Analysis: Calculated: C, 65.8; H, 7.4; N, 17.1.

$C_9H_{12}N_2O$(164) Found: C, 65.9; H, 7.4; N, 16.9.

Early attempts to carry out the above reduction gave low melting products (70-80° C.) which could not be purified by ether recrystallization. The impurity was found to be acethydrazide which exhibits an Amide II band at 1659 $cm.^{-1}$, and all impure preparations of compound III showed the presence of the peak at 1659 $cm.^{-1}$. Acethydrazide was removed from these products by ether extraction from an aqueous solution that had been acidified to pH 5. The acethydrazide impurity resulted from the palladium-catalyzed hydrogenolysis of the C=N bond in 1-acetyl-benzalhydrazone. Schlogl (31)

found that hydrogenolysis of the C=N bond of compound II proceeded to the extent of 95% with a 10% Pd-C catalyst; therefore, the success of the reduction described previously may be ascribed to the selectivity of the Pd catalyst.

Derivatives of III

1-Acetyl-2-p-tosyl-benzylhydrazine: m.p. 190-191° C.(corr.)

1-Acetyl-2-benzyl-4-phenyl semicarbazide: 4.2 grams of III were dissolved in 30 ml. triethylamine plus 30 ml. anhydrous benzene. Phenylisocyanate, 3.2 grams, redistilled, were added rapidly and the solution heated on the steam bath for 1 hour. The insoluble product was filtered and washed with pentane; crude m.p. 179.5-181.5° C. (corr.). After one recrystallization from chloroform the product was obtained in 46% yield, m.p. 183.0-183.7° C.(corr.).

Analysis: Calculated: C, 67.8; H, 6.0; N, 14.8.

$C_{16}H_{17}N_3O_2$ (283) Found: C, 67.7; H, 6.2; N, 14.7.

Derivative of Acethydrazide

1-Acetyl-4-phenyl semicarbazide: 4.1 grams of acethydrazide were dissolved in 75 ml. anhydrous benzene. 6.7 grams redistilled phenylisocyanate in 35 ml. anhydrous benzene was added slowly with stirring. The mixture was refluxed for 1.5 hours with stirring. The thick slurry that formed was filtered hot and washed with hexane. The crude product was first recrystallized from 95% ethyl alcohol,

m. p. 168-169° C (corr.). A second recrystallization from hexane: ethyl alcohol (4:1) gave 30% of the analytically pure product, m. p. 174.7-175.2° C (corr.). Lit. (33) m. p. 169° C.

Analysis:	Calculated:	C, 56.0; H, 5.7; N, 21.8.
$C_9H_{11}N_3O_2$ (193)	Found:	C, 55.7; H, 5.8; N, 22.0.

Palladium Catalyst

This catalyst is essentially that of Wilstätter and Waldschmidt-Leitz (32). Palladium chloride, 10 grams, was dissolved in 150 ml. distilled water containing 5 ml. conc. HCl and 71 ml. formaldehyde (Merck 36-38%). The reaction mixture was placed in a bath at -10° C and with vigorous stirring 142 ml. of 50% KOH were added at a maximum temperature of 3° C. The reaction mixture was then heated to 60° C and stirred for 15 minutes after which time the mixture was cooled to room temperature and washed with distilled water by decantation to neutrality and to the absence of chloride ion. The catalyst was stored under water. Aliquots of the catalyst suspension in water gave the required amount of catalyst for any particular run.

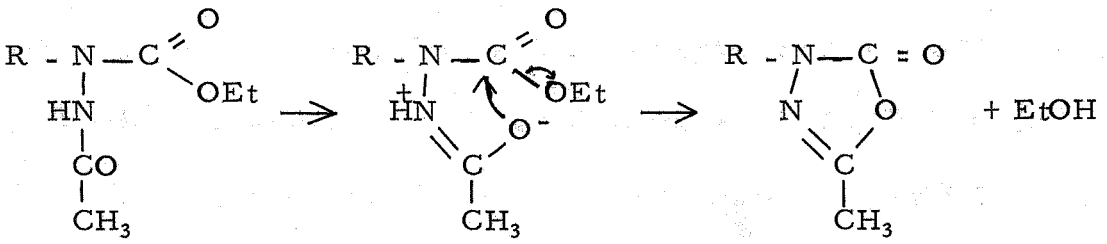
Ethyl 1-acetyl-2-benzyl carbazate I

1-Acetyl-2-benzylhdrazine, 16.4 grams (0.1 mole) were dissolved in 75 ml. chloroform (Reagent grade). Triethylamine, 10.2 grams (0.1 mole)-sodium distilled, was then added and the solution cooled

to 0° C. To the latter solution, 10.9 grams (0.1 mole) ethylchloroformate dissolved in 25 ml. chloroform (Reagent grade) were added over a period of one-half hour. The resulting solution was kept at 0° C for one-half hour, then kept at room temperature for 16 hours. The chloroform solution of the product was washed with 60 ml. 2N HCl, 60 ml. saturated aqueous $NaHCO_3$, and twice with 100 ml. portions of water. The chloroform layer was then dried with anhydrous sodium sulfate and treated in the cold with Norite. The filtered chloroform solution was then evaporated in vacuo at 25° C to remove the solvent. The product, a viscous oil, was transferred to a high vacuum system and degassed at 25° C at a pressure below 10^{-4} mm. for about 16 hours. The final product, a viscous colorless oil, resisted all attempts at crystallization. $n_D^{25} = 1.5173$. This product lacked the cyclic C=O band at 1795 $cm.^{-1}$, but showed peaks at 3268 $cm.^{-1}$ (N-H), and a split C=O at 1718 and 1689 $cm.^{-1}$ in CCl_4. The yield was 91%.

Attempted distillation of this carbazate at 0.1 mm. at a maximum pot temperature of 125° C resulted in cyclization to 2-methyl-4-benzyl-1, 3, 4-oxadiazolone-5, a colorless oil, $n_D^{25} = 1.5335$, identical with the preparation given below. Pure ethyl alcohol was isolated from the cold trap during this distillation. The thermal cyclization of 1-acyl carbazates was first noted by Rupe and Gebhardt (34), who obtained 2-methyl-4-phenyl-1, 3, 4-oxadiazolone-5 upon attempted

distillation of ethyl 1-acetyl-2-phenyl carbazate, a reaction that is probably general:



Proof of the structures of ethyl 1-acetyl-2-benzyl carbazate, I, and 2-methyl-4-benzyl-1, 3, 4-oxadiazolone-5, IV, rests on the elementary analyses, infra-red spectra, refractive index and the synthesis of IV by an alternate method as described below. The data are given subsequent to the presentation of the alternate synthesis of IV.

2-Methyl-4-benzyl-1, 3, 4-oxadiazolone-5. IV

This compound does not appear in the literature but numerous examples of the reaction of phosgene with acid hydrazides to the 1, 3, 4-oxadiazolone-5 ring system have been given by Lieser and Nischk (35) and Dornow and Bruncken (36). The procedure given here is substantially that of Lieser and Nischk.

1-Acetyl-2-benzylhydrazine, 16.4 grams (0.1 mole), were dissolved in 200 ml. chlorobenzene. Phosgene was passed into the refluxing solution at a rapid rate, concurrent with a small stream of dry nitrogen. After 1 hour the absorption of phosgene appeared to be

complete. After cooling, the solvent layer was washed with 100 ml. 5% $NaHCO_3$ and then twice with water. The dried chlorobenzene was stripped in vacuo and the crude product distilled in a Holtzman column. Four fractions $b_{0.25}$ = 112° C were collected in 82% yield. The refractive index of all of the fractions were ±0.0001 of each other. The properties of I and IV are summarized in Table X.

The principal distinguishing feature of the cyclic compound IV is the appearance of the infra-red spectrum. N-H is absent and the C=O shifts to 1795 $cm.^{-1}$, which is characteristic of a 5-membered lactam. Sharp bands at 1642 $cm.^{-1}$ and at 1314 $cm.^{-1}$ appeared only in the cyclic compound and could be ascribed to C=N and C-O-C stretching vibrations. The pattern of the three bands that appear in IV at 1437, 1393 and 1314 $cm.^{-1}$ also is maintained in compound V described below which has the same ring system and only differs in the substitution of H for benzyl in the 4-position. Compound I, the open-chain molecule, showed three strong bands at 1441, 1408 and 1379 $cm.^{-1}$; however, the C-O-C stretching vibration that appeared in the two cyclic derivatives at 1314 and 1321 $cm.^{-1}$ is conspicuously absent from the open-chain I. All three compounds, I, IV, and V, showed the symmetrical $C-CH_3$ CH deformations at 1379 $cm.^{-1}$ for I, and 1390, 1393 $cm.^{-1}$ for IV and V respectively.

The increase in refractive index when going from the open molecule I, n_D^{25} = 1.5173, to IV, the cyclic molecule where

Table X

Properties of Compounds I and IV

I: Ethyl 1-acetyl-2-benzyl carbazate

IV: 2-Methyl-4-benzyl-1, 3, 4-oxadiazolone-5

	I		IV	
Elementary Analysis	$C_{12}H_{16}N_2O_3$ (236)		$C_{10}H_{10}N_2O_2$ (190)	
	calc.	found	calc.	found
carbon	61.0	61.0	63.2	63.4
hydrogen	6.8	7.0	5.3	5.3
nitrogen	11.9	12.2	14.7	14.5
Infra-red (CCl_4) conc.	0.024 M		0.040 M	
N-H stretch	3268 $cm.^{-1}$		absent	
C=O stretch	1718 1689		1795 $cm.^{-1}$	
C=N stretch	absent		1642	
C-O-C stretch	absent		1314	
$C-CH_3$ C-H def. symm.	1379		1393	
$C-CH_3$ C-H def. asymm.	1441		1437	
Refractive Index n_D^{25}	1.5173		1.5337	
Density d_4^{25}	-----		1.192	
Solubility				
water	0.05 M		0.002 M	
$CHCl_3$, CCl_4, acetone, alcohol	sol.		sol.	

n_D^{25} = 1.5337 is also acceptable. Compound IV obtained by the reaction of phosgene with 1-acetyl-2-benzylhydrazine was identical to the distillate obtained during the distillation of compound I.

2-Methyl-4-H-1,3,4-oxadiazolone-5. V

This compound was reported by Dornow and Bruncken (36). Acethydrazide, 74 grams (1 mole) was dissolved in 500 ml. water. Phosgene was passed into the solution without cooling below 25° C. until a total pickup of 75 grams of phosgene had taken place. After allowing the crude reaction mixture to stand at room temperatures for 2 days it was neutralized with sodium bicarbonate. Water was stripped in vacuo with steam bath heating and the crude solid mixture was extracted with benzene in a Soxhlet apparatus. From the benzene extract there crystallized out 25.2 grams (25% yield) of the desired product. M.p. 110-111.5° C. (corr.). Lit. m.p. 112° C.(36). The product is quite soluble in water and is an acid of pK_a' 7.93±0.02. Solubility in CCl_4 was limited but the solubility in $CHCl_3$ was sufficient for determination of the infra-red spectrum. N-H: 3472, 3268 $cm.^{-1}$; C=O: 1786 $cm.^{-1}$; C=N: 1650 $cm.^{-1}$; $C-CH_3$ CH def. asymm.: 1435 $cm.^{-1}$; $C-CH_3$ CH symm.: 1390 $cm.^{-1}$; C-O-C stretch 1321 $cm.^{-1}$; 935 $cm.^{-1}$ a very strong band that may be associated with N-N stretching (37, 38, 39).

Analysis: Calculated: C, 36.0; H, 4.0; N, 28.0.

$C_3H_4N_2O_2$ (100) Found: C, 36.0; H, 4.2; N, 28.0.

1-Acetyl-2-benzyl semicarbazide. VI

1-Acetyl-2-benzylhydrazine, 4.9 g. (.03 mole) and 3.63 grams (.0345 mole) nitrourea (40) were dissolved in 50 ml. absolute ethyl alcohol and heated on the steam bath for 22 hours under reflux. The solvent was stripped in vacuo and the product was recrystallized from chloroform, then from ethyl alcohol-benzene-hexane to give a 47% yield of VI. M.p. 153.5-154.5° C (corr.). This compound is moderately soluble in water.

Analysis: Calculated: C, 58.0; H, 6.3; N, 20.3.

$C_{10}H_{13}N_3O_2$ (207) Found: C, 57.8; H, 6.5; N, 20.0.

1-Acetyl semicarbazide. VII

Acethydrazide and nitrourea were reacted in the same manner as compound VI. The product was very soluble in water, M.p. 166.0-166.5° C. Lit. 165° C. (41).

Analysis: Calculated: C, 30.8; H, 6.0; N, 35.9.

$C_3H_7N_3O_2$ (117) Found: C, 31.0; H, 5.9; N, 36.0.

Ethyl 1-acetyl carbazate. VIII

Ethyl carbazate was prepared by the method of Diels (42), the hydrazinolysis of ethyl carbonate. Ethyl 1-acetyl carbazate,

VIII, was prepared by the Schotten-Baumann method with ethyl carbazate and acetic anhydride. M.p. 89-90° C (corr.). Lit. m.p. 81° C (50).

Analysis:	Calculated:	C, 41.09; H, 6.90; N, 19.17.
$C_5H_{10}N_2O_3$ (146)	Found:	C, 41.04; H, 6.84; N, 19.20.

Aceturamide. IX

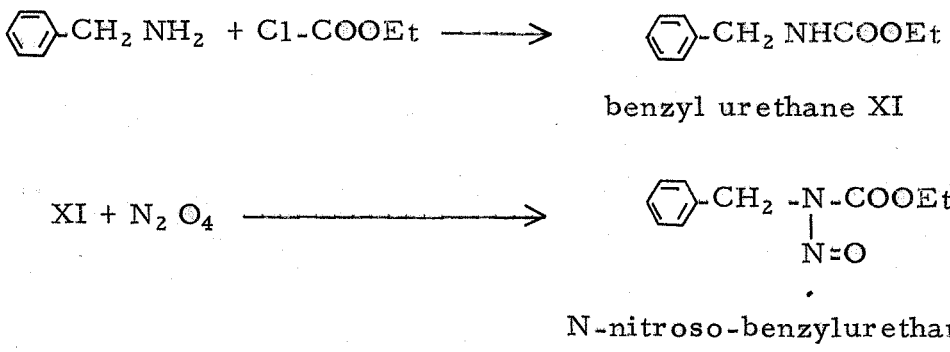
Ammonolysis of ethyl aceturate in methanol gave aceturamide, m.p. 138.0-139.5° C (corr.). Lit. m.p. 137° C.

N-β-Phenylethyl)-acetamide. X

β-Phenylethyl amine was acetylated with acetic anhydride under Schotten-Baumann conditions to give X. M.p. 54-55° C (corr.). Lit. m.p. 45° C (43).

Method B. For ethyl 1-acetyl-2-benzyl carbazate

The attempted sequence was the following:



All of the above steps were uneventful until the final attempted conversion of XII to XIII. Both palladium-hydrogen or platinum-hydrogen cleaved off the nitroso group under the mild conditions of reduction 50 psi H_2, absolute ethyl alcohol, 25°C. Benzyl urethane was obtained in greater than 90% yield from these reductive cleavages. Zinc-acetic acid reduction, which has been used successfully for the reduction of secondary nitroso amines to hydrazines (44) gave a high yield of a single product that did not resemble the desired compound XIII or its 1-acetyl derivative which would be compound I. The zinc-acetic acid reduction product of XII corresponded to a coupling between benzyl urethane XI and XII via a -CO- group.

*Direct synthesis to XIII not accomplished.

Benzyl urethane. XI

Redistilled benzylamine, 96.4 grams (0.9 mole) in 300 ml. ether was shaken with 40 grams NaOH in 200 ml. water while small portions of an ether solution of ethyl chloroformate (97.5 grams in 100 ml. ether) were added with cooling. The ether layer was separated, dried with anhydrous sodium sulfate, and the ether stripped. The crude product was distilled at 17 mm. to yield XI in 75% yield, m.p. 44° C. Lit. m.p. 44° C (45).

N-nitroso-benzylurethane. XII

Into a 1 liter, 3-neck flask were put 33 grams (0.4 mole) of sodium acetate (anhydrous) and 250 ml. CCl_4. The flask was cooled with powdered dry ice while 18.4 grams (0.2 mole) NO_2 were passed into the system. The above mixture was warmed to 0° C and a solution of benzyl urethane, 24.1 grams (0.135 mole) in 300 ml. CCl_4, was added with stirring over a period of 20 minutes. The crude mixture was then poured over crushed water ice, and extracted with ether. The combined ether-CCl_4 layers were washed once with 200 ml. of 10% Na_2CO_3 and with two portions of water. The solvent layer was then dried with anhydrous sodium sulfate and stripped in vacuo at a maximum pot temperature of 40° C. The crude product was further stripped at 25° C at 0.1 mm. to yield XII as an amber-colored liquid in 94% yield. $n_D^{25} = 1.5166$, $d_4^{25} = 1.1500$. This product

is a powerful skin vesicant and must be handled with care. The infra-red spectrum of XII showed that N-H was absent, a sharp increase in the intensity of the 1379 cm.$^{-1}$ peak (weak in benzyl urethane) and a new strong band appeared at 1348 cm.$^{-1}$ which may be due to the -N-N=O vibration (38).

Attempted Reduction of XII to ethyl 2-benzyl carbazate XIII by Zn-HAc.

N-Nitroso-benzylurethane, 41.6 grams (0.2 mole), 55.8 grams powdered zinc (0.85 g-atom), 82 ml. water, and 85 ml. glacial acetic acid (0.96 mole) were stirred together with cooling to 10° C for 20 minutes. Stirring was continued for 3-1/2 hours at 25° C, then for 1 hour on the steam bath. The crude reaction mixture was partitioned with 300 ml. ether and filtered. The ether layer was then washed with 100 ml. of 10% NaOH and with 100 ml. of water. After stripping off the ether the crude product was distilled and gave a product $b_{0.2}$ = 106° C in 85% yield, n_D^{25} = 1.5189, presumed to be (XIIIa).

Analysis: Calculated: C, 63.2; H, 6.8; N, 10.5.

$C_{21}H_{25}N_3O_5$ (399) Found: C, 63.6; H, 7.4; N, 11.7.

The infrared spectrum in CCl_4 (0.19 M) N-H: 3448, 3344 cm.$^{-1}$; C=O: 1733, 1709 cm.$^{-1}$; Amide II - 2°: 1515 cm.$^{-1}$; C-CH_3 C-H def : 1446, 1381 cm.$^{-1}$; C-O: 1227 cm.$^{-1}$.

Cleavage of XIIIa with conc. HCl gave an oil, XIII, in 98% yield, $b_{0.5}$ 93-95° C, n_D^{25} = 1.5214. XIII had an active hydrazine group and gave a benzalhydrazone, m.p. 66°-67°C.(corr.).

Analysis: Calculated: C, 61.8; H, 7.3; N, 14.4.

$C_{10}H_{14}N_2O_2$ (194) Found: C, 61.7; H, 7.2; N, 15.0.

Ethyl 1-benzylidene-2-benzyl carbazate M.p. 66°-67° C.(corr.).

Analysis: Calculated: C, 72.32; H, 6.43; N, 9.92.

$C_{17}H_{18}N_2O_2$ (282) Found: C, 72.57; H, 6.49; N, 9.85.

The IR spectrum of XIII in CCl_4 showed a single N-H at 3344 cm.$^{-1}$, a single C=O at 1709 cm.$^{-1}$, and a strong Amide II-1° at 1629 cm.$^{-1}$. Ronco and Erlenmeyer (28) gave a m.p. of 64-65° C for the benzalhydrazone of ethyl 2-benzyl carbazate, hence it is believed that XIII was the desired product, ethyl 2-benzyl carbazate.

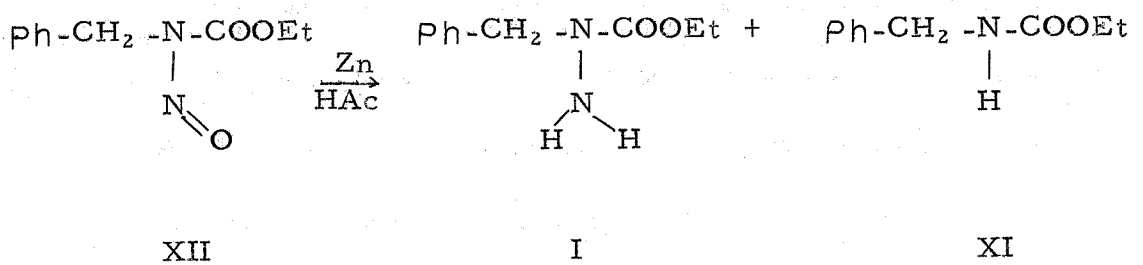
In addition, benzyl urethane in 19% yield was recovered from the HCl cleavage of XIIIa, indicating that this fragment was present in XIIIa.

Oxidation of XIIIa with alkaline permanganate gave benzoic acid in 75% yield and an aryl ketone $C_{16}H_{21}O_6$ or $C_8H_{10}O_3$, XIIIc, m.p. 145-146° C. Compound XIIIc was not identified.

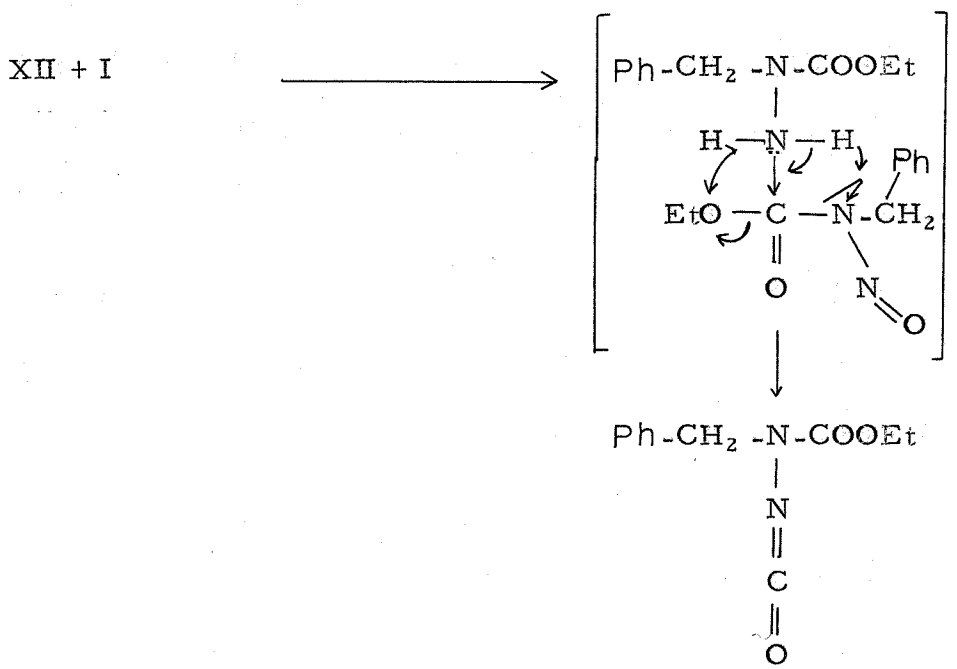
In view of the fact that XIIIa gave positive Tollens and Benedict tests and XIII, the acid hydrolysis product was an active hydrazine, it is concluded that during the zinc-acetic acid reduction of N-nitroso

benzyl-urethane fragmentation and coupling to XIIIa took place via an isocyanate intermediate. It is noted that Rodionov and Kiseleva (47) obtained 2-phenyl-1, 3, 4-oxadiazolone-5 in 40% yield upon doing a Hofmann rearrangement with benzoyl-urea.

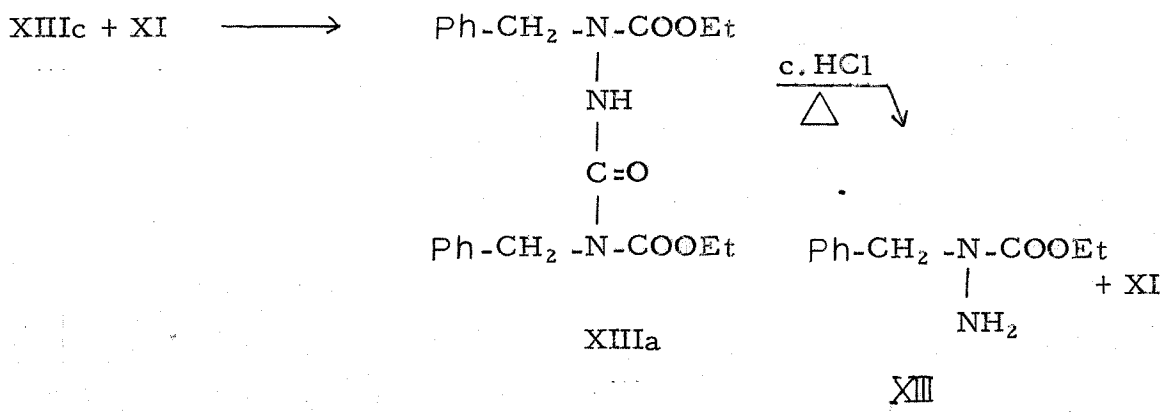
It is believed that the structure of XIIIa, the main product from the reduction of N-nitroso-benzyl-urethane is represented by the following structure and arises by the sequence shown on the following page.



XII I XI



XIIIc



XIIIa XIII

Stability of Ethyl 1-Acetyl-2-Benzyl Carbazate (I) in the Presence of alpha-Chymotrypsin

3.06 grams (.01295 mole) of I and 0.275 grams alpha-chymotrypsin were dissolved in approximately 230 ml. of CO_2-free water and the pH of the solution adjusted to 7.95 by the addition of 0.1 N NaOH. The resulting solution was adjusted to a volume of 250 ml. and the entire system was thermostated at 25° C with gentle stirring. The pH was held at 7.95 by the addition of 0.1 N NaOH. After 70 minutes, 2.35 of 0.1 N NaOH had been added; after 275 minutes a total of 3.50 ml. of 0.1 N NaOH had been added; after 16-1/2 hours a total of 4.00 ml. of 0.1 N NaOH had been added, after which no further quantities of NaOH were required for a period of one week. The total of NaOH added therefore corresponded to a maximum of 1.3% hydrolysis of (I); however, this figure included the enzyme blank for which a measurement had not been made over such a long period of time.

At the end of one week the entire reaction system was extracted with three 100 ml. portions of carbon tetrachloride and three 100 ml. portions of ethyl ether. The combined organic layers were evaporated in vacuo at 25° C. A yield of 2.88 grams (94%) of residue was obtained as a viscous, non-crystallizable oil having a refractive index n_D^{25} = 1.5131 and an infra-red spectrum (CCl_4) identical to starting substrate.

Second-Order Basic Hydrolysis Constants

During the course of these investigations it often became necessary to measure the second-order basic hydrolysis constants of substrates and inhibitors in order to apply proper blanks to the enzymatic hydrolyses. These constants were determined on the pH-stat in the following manner:

Experimental:

A 0.040 M stock solution of substrate (or inhibitor) was prepared in CO_2 -free water. Aliquots of the stock solution were transferred to the reaction system which was also 0.1 M in NaCl. The final test solution had a volume of 10 ml. The hydrolysis reaction was followed at constant pH by the addition of standardized 0.0107 N NaOH, at a temperature of 25.0 $\pm$ .1° C. The reaction system was swept by a stream of CO_2 -free nitrogen. The following schedule of test runs was made for each substrate over a period of 8 minutes. The recorder traces were linear in all cases and the computed velocities of the hydrolyses were obtained graphically from the recorder traces.

Schedule for Second-Order Basic Hydrolysis Constants

Substrate conc. [M]	pH
.024	7.50
.024	7.90
.024	8.50
.036	8.50
.004	9.00
.016	9.00
.028	9.00
.024	9.00

Calculations

The second-order basic hydrolysis of an ester substrate may be expressed as follows:

$$R\text{-}COOR' + [OH^-] \longrightarrow RCOO^- + R'OH$$

and, $$v = k_B [RCOOR'][OH^-] \qquad (11)$$

where v = the velocity of the reaction in units of moles-liter^{-1}min.$^{-1}$ of base added,

k_B = the second-order basic hydrolysis constant in units of ℓ-mol^{-1}-min.$^{-1}$

and [RCOOR'] and [OH$^-$] are expressed in molarity units.

Accordingly, a plot of v versus the product [RCOOR'][OH$^-$] should be linear with a slope equal to k_B. Computed values of k_B were obtained from a least squares fit to the relationship expressed by equation (11) and were computed on the Datatron 205 using program code 100-001 (Kurtz). In all cases, plots of v versus the product [RCOOR'][OH$^-$] were linear to ±10%. The values of k_B obtained for all substrates and inhibitors during the course of these investigations are given in Table XI.

Table XI

Second-Order Basic Hydrolysis Constants (k_B)

Compound	k_B ($\ell.\text{-mol.}^{-1}\text{min.}^{-1}$)
Chloroacety-L-valine methyl ester	12.6 ± 0.3
Ethyl 1-Acetyl-2-benzyl carbazate	3.42 ± 0.04
2-Methyl, 4-benzyl, 1, 3, 4-oxadiazolone-5 (in 10% acetone)	0.62 ± 0.08
N-Acetyl-DL-phenylalanine ethyl ester	17.7 ± 1.6
α-Methyl-N-acetyl-DL-phenylalanine methyl ester	0.31 ± 0.05
Methyl hippurate	129 ± 4

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PART II

1-ACETYL-2-[L-TYROSYL] HYDRAZINE: AN INHIBITOR OF ALPHA-CHYMOTRYPSIN

A. Introduction: The Transformation of N-Acetyl-L-Tyrosinhydrazide into an Inhibitor

MacAllister and Niemann (1) were the first to point out that hydrazides of certain α-N-acylated amino acids could function as substrates for alpha-chymotrypsin, in particular, α-N-nicotinyl-L-tyrosinhydrazide. Subsequent investigations (2, 3) have confirmed the initial observation of MacAllister and Niemann and have showed that hydrazides represented but another example of the general structural requirements for alpha-chymotrypsin substration or inhibition. The effect of the aryl side-chain was similar to amides and hydroxamides, e.g., for α-N-acetyl-D-tryptophanhydrazide, $K_I = 0.8 \times 10^{-3}$ M (4) and for α-N-acetyl-D-tyrosinhydrazide $K_I = 7.5 \times 10^{-3}$ M wherein the affinity of alpha-chymotrypsin for the indole nucleus is better than for the phenol nucleus. Similarly, the effect of acyl substitution on the alpha-amino function paralleled the experience with other typical substrates, e.g., K_S increased in the L-tyrosinhydrazide series $\phi CO- < Cl_2CHCO- < H- <$ nicotinyl-$<$ $HCO- < CH_3CO- < (CH_3)_3CCO-$ which is similar to the L-tyrosinamide series where K_s increased in the order $\phi CO- < HCO- <$ nicotinyl, $ClCH_2CO- < CH_3CO-$ (5).

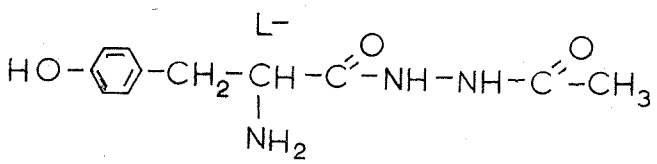
However, a comparison of the values of k_3 for the tyrosinamides gives the relationship isonicotinyl- $>$ nicotinyl $>$

$ClCH_2CO-$ = $\phi CO-$ > F_3CCO- = CH_3CO- > carbethoxy- > formyl, whereas for the tyrosinhdrazides, k_3 decreases in the order CH_3CO- > nicotinyl > Cl_2CHO- > $\phi CO-$ > $(CH_3)_3CCO-$ > formyl-> H-. The relationship amongst the values of k_3 for the hydrazides is clearly different from that of the values of K_s particularly with respect to the position of N-acetyl-L-tyrosinhydrazide. The latter has the largest value of k_3 for any of the hydrazides, whereas α-N-acetyl-L-tyrosinamide occupies a position more or less median with respect to the range of k_3 for acylated tyrosinamides, i.e., 0.45 to 6.4×10^{-3} M/min.-mg. PN/ml. Therefore, the conclusion that the N-acyl moiety has the same influence in the L-tyrosinamide series that it has in the L-tyrosinhydrazide would be true for K_s but not for k_3, thus possibly revealing the composite nature of k_3.

During a study of the applicability of the pH-stat (Part IV of this Thesis) to substrates leading to internal buffering action it was observed that stock solutions of acetyl-L-tyrosinhydrazide (I) were stable in water at 95-100°C. for reasonable periods of time, e.g. 1-3 hours. However, when stock solutions were prepared in the presence of strong acids, where the substrate was present as the protonated species α-N-acetyl-L-tyrosin-$NHNH_3^+$, a new species was formed that was no longer a substrate; on the contrary, this species possessed extremely powerful reversible inhibition characteristics toward alpha-chymotrypsin. It was observed experimentally that a

.05 M solution of α-N-acetyl-L-tyrosin-$NHNH_3^+Cl^-$ maintained at 95-100° C. exhibited two parallel phenomena: first, the inhibition of the alpha-chymotrypsin catalyzed hydrolysis of acetyl-L-valine methyl ester by an aliquot of the solution increased regularly with time, to a level where $K_I = .1 \times 10^{-3}$ M, and second, potentiometric titration showed that the concentration of the strongly acid, acetyl-L-tyrosin-$NHNH_3^+$ decreased while a new acid species, RNH_3^+ of pKa' = 7, made its appearance simultaneously. The reaction appeared to be stoichiometric with respect to the original hydrazide species.

In Part II of this Thesis, there is reported the isolation, properties and proof of structure of this new inhibitor which is believed to be 1-acetyl-2-[L-tyrosyl] hydrazine II.*



1-acetyl-2-[L-tyrosyl] hydrazine (II)

*The nomenclature for diacyl hydrazides corresponds to the convention followed in the Journal of the American Chemical Society and Chemical Abstracts, namely, as N^1- and N^2- derivatives of hydrazine.

Kinetics of the Formation of 1-Acetyl-2-[L-tyrosyl] hydrazine II.

The transformation of α-N-acetyl-L-tyrosinhydrazide to 1-acetyl-2-[L-tyrosyl] hydrazine (II) was followed enzymatically and potentiometrically. Experimental details are given in Part II-E. A solution .05 M in α-N-acetyl-L-tyrosinhydrazide and .05 M in HCl was maintained at 96 ± 1° C. Aliquots of the reaction mixture were removed at various time intervals and titrated potentiometrically with 0.125 N NaOH in the pH-stat. From the titration curves, the titer due to the appearance of the stronger base inhibitor II was calculated. Similarly, aliquots were assayed for inhibitor content using the system alpha-chymotrypsin-acetyl-L-valine methyl ester with $[S]_o$ = .0400 M and $[E]_o$ = .1464 mg. PN/ml. The size of the aliquot that was used corresponded to a maximum $[I]_o$ = 0.001 M, e.g. at t = 0 minutes the concentration of α-N-acetyl-L-tyrosinhydrazide corresponded to a concentration of 0.001 M and at $t = \infty$, assuming 1:1 stoichiometry for the formation of inhibitor, the concentration of inhibitor would correspond to $[I]_o$ = 0.001 M in the enzyme + acetyl-L-valine methyl ester + inhibitor system.

It was found that in the region 0-80% conversion, the appearance of a base of pKa' = 7 followed first-order kinetics, with k = 0.028 $min.^{-1}$ and in the region 0-65% conversion, the appearance of

an inhibitor with $K_{I_L} = 0.083 \times 10^{-4}$ M was also first-order with $k = 0.025$ min.$^{-1}$. When the conversion exceeded 65% the rate of inhibitor formation decreased, when measured by the enzyme assay, whereas the appearance of base was still reasonably first order. The order of the reaction with respect to H^+ was not investigated.

The results of this study are given in Table XII and plotted in figure 5 as $\ln[S]_t$ vs. t. A similar study with α-N-acetyl-D-tyrosinhydrazide gave similar results when followed potentiometrically; however, the product, 1-acetyl-2-[D-tyrosyl] hydrazine, was an inhibitor with far less affinity for enzyme, i.e., $K_{I_D} = 5 \times 10^{-3}$M. Subsequent studies also showed that L-tyrosinhydrazide was present during the original transformation to L-inhibitor II, arising from the deacylation of either (or both) of the α-N-acetyl-L-tyrosinhydrazide (I) or 1-acetyl-2-[L-tyrosyl] hydrazine (II). Since Edward, Hutchison and Meacock (6) found that below 2.5 M HCl acethydrazide was hydrolyzed at a slower rate than acetamide, whereas the reverse occurred at HCl $>$ 2.5 M, the view is favored that the hydrolysis of an acetyl group proceeds at a faster rate from α-N-acetyl-L-tyrosinhydrazide than from 1-acetyl-2-[L-tyrosyl] hydrazine.

Other pertinent observations were that the reverse reaction, L-tyrosinhydrazide plus acetic acid to the inhibitor II, under conditions equivalent to the original transformation, proceeded to the extent of only 6% after four hours at 95° C. This conclusion was

Table XII

Kinetics of the Conversion of α-N-Acetyl-L-tyrosin-hydrazide to 1-Acetyl-2-[L-tyrosyl] hydrazine at 96°C.

$[S]_o$ = 0.0508 M

Added (H^+) = 0.052 M

Time (min.)	Potentiometric Titration		Enzyme Assay[b]		
	$[S]_t$[a,c]	$\ln[S]_t$	$[S]_t$[a,c]	$\ln[S]_t$	v_o[d]
0	(5.08)	-2.98	(5.08)	-2.98	-
8	3.73	-3.29	3.85	-3.26	0.276
15	3.09	-3.48	3.13	-3.46	0.191
30	2.09	-3.87	2.23	-3.80	0.141
45	1.32	-4.33	1.74	-4.05	0.123
60	0.89	-4.72	1.85	-3.99	0.126
75	0.67	-5.01	1.60	-4.14	0.119
90	----------		1.54	-4.17	0.117
105	0.34	-5.68	----------		0.116
122	0.11	-6.81	1.13	-4.48	0.106
150	0.06	-7.42	----------		0.119
180	0.00	-----	----------		0.104

$k = 0.028$ min.$^{-1}$ (0 - 98% conv.)

$k = 0.025$ min.$^{-1}$ (0-66% conv.)

a. Conc. of α-N-acetyl-L-tyrosinhydrazide. Units of 10^{-2}M.

b. Assayed at $[E]_o$ = 0.1464 mg. PN/ml., acetyl-L-valine methyl ester = 0.0400 M, pH = 7.90, NaCl = 0.100 M, 25° C.

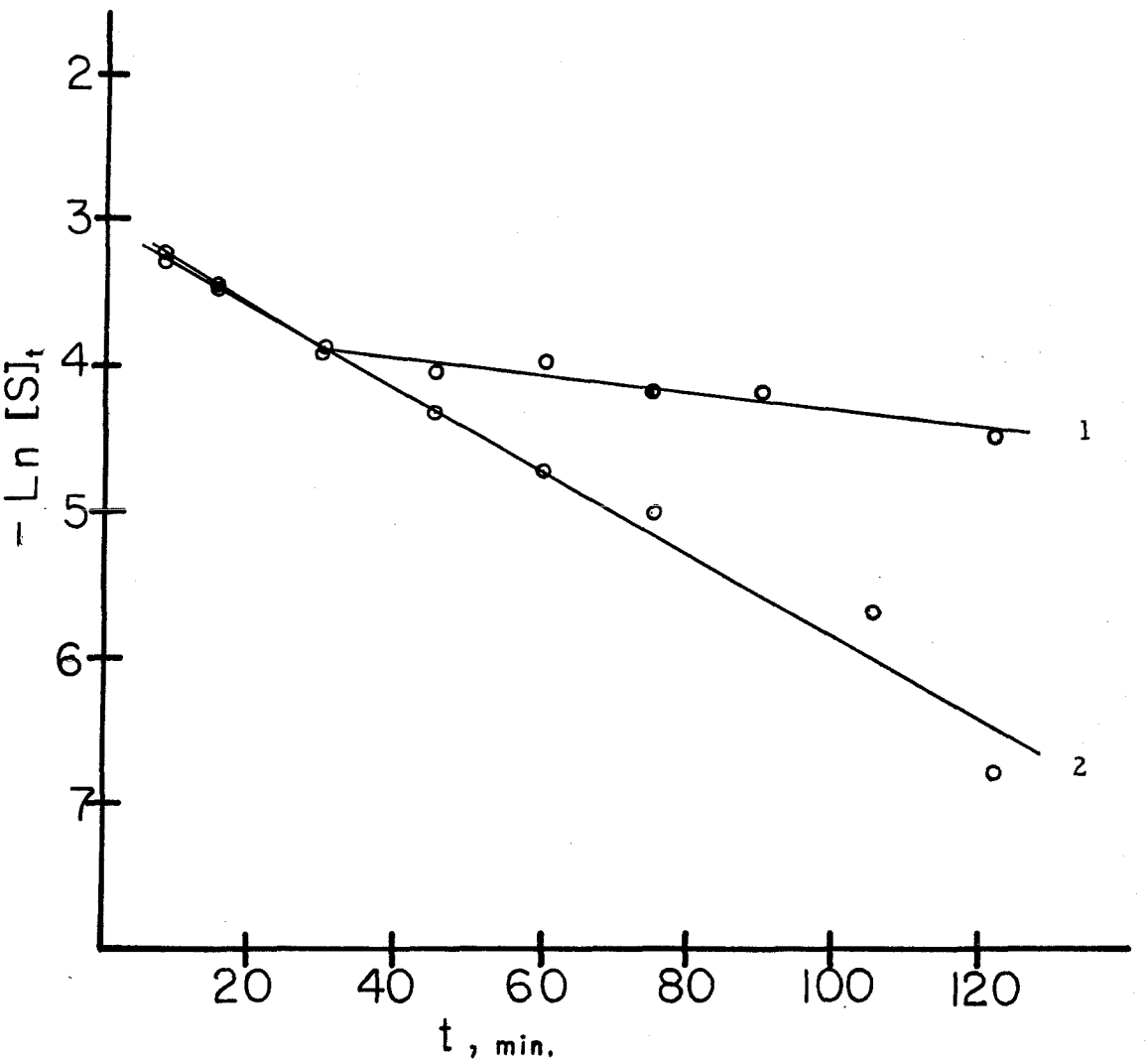
c. Calculated as explained in text. Units 10^{-4}M/min.

d. Units of 10^{-4} M/min. Uncorrected for blanks.

Figure 5

Kinetics of the conversion of N-acetyl-L-tyrosinhydrazide (I) to 1-acetyl-2-[L-tyrosyl]hydrazine (II) in aqueous HCl at 96° C.

$(S)_{t=0}$ = 0.0508 M, HCl = 0.052 M



Curve 1 - Enzyme assay of (II)

Curve 2 - Potentiometric determination of total bases of pKa' ≐ 7

reached in an experiment in which an aqueous solution, .05 M with respect to each of L-tyrosinhydrazide, HCl and acetic acid, was incubated at 95° C. for four hours. The concentration of the aforementioned species would then correspond to the hypothetical situation where α-N-acetyl-L-tyrosinhydrazide (I) was de-acylated by an acid-catalyzed hydrolysis in a fast reaction, with the subsequent acylation of the 1-N hydrazine by acetic acid. For compound II to form via this path, k_2 would of necessity be $> k$, or k_o, or k_3, since it had been observed (enzyme assay criterion) that II formed rapidly. However, the conversion to II occurred to a maximum extent of 6% as measured by enzyme assay. Therefore, it is concluded that $k_2 \ll k_o, k_I$ as expected for a first-order mechanism compared to a second-order mechanism. The experimental data are given in Section F.

When β-naphthalene sulfonic acid was added to the equilibrated (3 hours) crude reaction mixture obtained from acid and 1-N-acetyl-L-tyrosinhydrazide, despite the fact that enzymatic assay showed a content of 85%, a 39% yield of L-tyrosinhydrazide·2 naphthalene sulfonate V was obtained. The latter salt, V, showed two end points by potentiometric titration and corresponded to a molecular weight of 630 ± 2, i.e., L-tyrosinhydrazide dinaphthalenesulfonate salt ($pKa_1' = 3.2$, $pKa_2' = 7.12 \pm .02$). The expected range for the pKa of the α-ammonium group is

reported to be 7.4 ± 0.4; hence pKa_1' may be assigned to the hydrazine-N^1-amino and pKa_2' to the α-amino group (34, 20). It is believed that the reaction scheme shown in figure 6 explains the results that were obtained.

The reaction is represented as a series of equilibria that are displaced toward the protonated species 1-acetyl-2-[L-tyrosyl] hydrazine IIa and L-tyrosinhydrazide III. α-N-Acetyl-L-tyrosinhydrazide I is presumed to go through a cyclic intermediate VI which may or may not lose a molecule of water to form a 1, 2, 4-triazolone-6, a ring structure that has been reported in the literature (7, 8). Referring to figure 6, it would be expected that $k_3 \ll k_o$ or k_1 since the protonated species of I, I_a, would not be a favorable species for acid-catalyzed hydrolysis due to mutual charge repulsion. The measured first-order constant, k = .025 to .028 $min.^{-1}$, is of the proper order of magnitude for an intramolecular reaction involving cleavage at -CO--NH-. Bender (9) reported a value of k = .014 $min.^{-1}$ for the intramolecular hydrolysis of phthalamic acid at 47.3°C; by extrapolation to 95°C., k for the latter hydrolysis may be calculated to be ca. 1.1 $min.^{-1}$.* The acyl migration reaction reported in this Thesis may be slower because the concentration of the nucleophilic species I is reduced by protonation.

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* $\Delta H^{\ddagger}$ = 20.7 kcal./mole, $\Delta S^{\ddagger}$ = -12.4 e.u. (9).

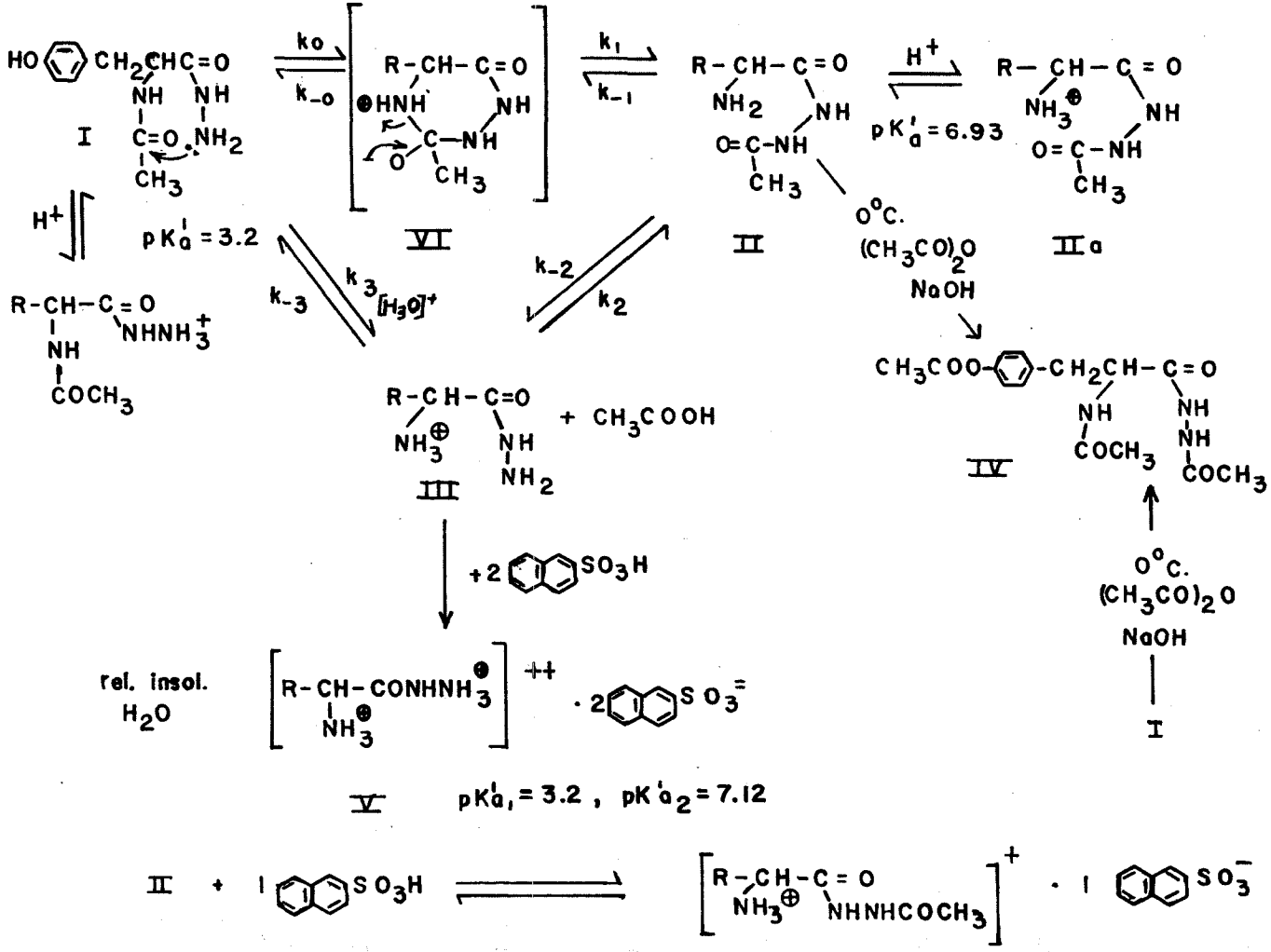


Figure 6 Acetyl-L-Tyrosinhydrazide Reactions in Acid at 96°C.

The pKa' for N-acetyl-L-tyrosinhydrazide was found to be equal to 3.2; therefore, the concentration of non-protonated species I was about one-fourth of the protonated species I_a which may account in part for the lower rate if the formation of VI proceeds by nucleophilic attack of the N^1-nitrogen of the hydrazide on the acetyl carbon of I as indicated in figure 6 with a shift of a proton to the alpha-nitrogen, the latter possessing the positive charge since the alpha-nitrogen would be expected to be more basic than either of the hydrazine nitrogens. VI then changes to II of which the protonated species II_a is the dominant species for a base of pKa' = 6.93, under the conditions of the experiment. The overall favorable energy of the system would then be derived from the large enthalpy change (10-12 kcal./mole)(10) accompanying protonation of an amino group. In the absence of information relative to the dependence of the rate of the acyl migration on the concentration of H^+, another mechanism must be considered in which the reaction proceeds via intramolecular transfer of a proton from the 1-N hydrazine nitrogen to the α-N nitrogen. A study of the pH dependence of the rate, with isotopic labelling of the acetyl group, would assist in the elucidation of the mechanism.

The isolation of the dinaphthalene sulfonate salt of L-tyrosinhydrazide (V) in 39% yield from the equilibrated crude reaction mixture that had a minimum of 80% II by enzyme assay may be

explained as a displacement of the equilibria from $II_a \rightarrow VI \rightarrow I \rightarrow V$, the reaction proceeding in the indicated direction due to the relative insolubility of V in water.

Additional support for the rearrangement of I to II was obtained after isolation of II as the free base; it was found that acetylation of either I or II with a slight excess of acetic anhydride and sodium hydroxide at 0° C. gave the same compound IV, 1-acetyl-2-[O-acetyl-α-N-acetyl-L-tyrosyl] hydrazine in crude yields of 90%. The mild conditions of the latter acetylation decrease the probability of displacement of II to I when coupled with the observed stability of II in alkaline solutions.

The solubility of L-tyrosinhydrazide dinaphthalene sulfonate in water exceeded 0.1 M at 25° C. As a consequence of this property the conversion of I → II was possible with β-naphthalene sulfonic acid acting as the proton donating species. When the latter conversion was carried out in a manner analogous to the conversion with HCl and I, there was obtained 1-acetyl-2-[L-tyrosyl] hydrazine-mononaphthalene sulfonate salt VII in 45% yield, soluble in water and ethyl alcohol but recrystallizable from isopropyl alcohol. The pKa' value of VII was found to equal 6.93 and the equivalent weight found to be 440 (theory 445). Accordingly, the pathway leading to complete de-acylation via precipitation of L-tyrosinhydrazide dinaphthalene sulfonate (V) may be blocked if the solubility product of V is not exceeded.

Proof of Structure of 1-Acetyl-2-[L-tyrosyl] hydrazine (II)

The isolation of 1-acetyl-2-[L-tyrosyl] hydrazine II as the free base in analytical purity was achieved only after much difficulty. It was found to be quite soluble in water, methanol and ethanol. Its moderate solubility in isopropyl alcohol permitted recrystallization from that solvent in reasonable yields but five recrystallizations were required to get it to an analytically pure state. When pure, II was found to be slightly hygroscopic and insoluble in all of the common organic solvents except the lower alcohols. Aqueous solutions of II were weakly basic (pH $\doteq$ 8.) gave a positive Ninhydrin test (red color) and failed to react with ϕCHO. As with the crude reaction product discussed previously attempts to precipitate a naphthalene sulfonate of isolated (II) resulted in de-acetylation with the formation of L-tyrosinhydrazide. 2 naphthalene sulfonate.

The pKa' of isolated II was found to be 6.93 $\pm$ .02 and when evaluated as a competitive inhibitor against alpha-chymotrypsin and acetyl-L-valine methyl ester II gave a value of $K_I = 0.074 \times 10^{-3}$ M. These values are comparable to those for the crude reaction mixture, where values of pKa' = 7.0 and $K_I = 0.09 \times 10^{-3}$ M values were obtained. Since subsequent experiments showed that of all the possible species present in the crude reaction mixture, viz. figure 6, K_{I_L} of compound II $< K_s$ or K_I of any other possible component of the

mixture by a factor of at least 25 so that the K_{I_L} found for the crude reaction mixture represented a concentration equilibrium level of compound II in the range of 80-85%.

The ultraviolet and infra-red spectra of II were also observed. The ultraviolet spectra of II in acid and base were examined and compared with α-N-acetyl-L-tyrosinhydrazide (I). In 0.02 M HCl, I and II were exactly equivalent showing λ max. 276 (ϵ = 1450)* λmax. (infl.) 281 (ϵ = 1250), and λmax. 224 (ϵ = 9500). In 0.01 M NaOH I gave peaks at λ max. 294 (ϵ = 5600), and λmax. 240 (ϵ = 25,000); II in base gave peaks at λmax. 294 (ϵ = 2500), and λ max. 242 (ϵ = 21,000). These changes in the ultraviolet spectra show that the only differences between I and II are due to changes in the acidity of the phenolic -OH group, the decrease in intensity of the anionic λ max. 294 peak when going from I → II shows that the phenolic group in II is a weaker acid than the corresponding group in I and may be ascribed to a decreased inductive effect of the $-CO-CH_3$ function when that group lies at a greater distance from the phenolic side-chain group as in II. Accordingly, the ultraviolet spectra show that a significant measure of confidence is attached to the idea that only minor structural changes accompanied the rearrangement of I → II.

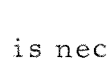
The infra-red spectra of I and II further confirmed these changes. The spectra of I and II were determined in Nujol mulls.

*Wavelengths in millimicrons.

I, the original hydrazide gave maxima at 3268, 1664, 1613, 1513, 1299, 1271 and 1242 cm.$^{-1}$. The rearranged compound II gave maxima at 3333(sh), 3215(s), 1664(sh weak), 1616, 1585, 1515, 1299(weak) and 1258 cm.$^{-1}$. In addition, II mulled in hexachlorobutadiene-1,3 gave a peak at 1481 cm.$^{-1}$ not obtainable in Nujol. The principal differencesbetween I and II were that the 1664 cm.$^{-1}$ (C = 0) absorption was strong in I and very weak in II, a new band appeared at 1585 cm.$^{-1}$ in II, and the paired bands 1271, 1242 cm.$^{-1}$ in I characteristic of alpha-acylamido amino acid derivatives (12) were replaced by a single band at 1258 cm.$^{-1}$. The persistence of the 1613-1616 cm.$^{-1}$ bands in I and II shows the presence of $-NH_2$ N-H deformation in both compounds. The shift of the N-H stretching mode from 3268 cm.$^{-1}$ in I to 3215 cm.$^{-1}$ in II may mean that II exists as a cyclic cis structure in the solid state. Hinman (13) reported the Nujol-mull spectra of 1-benzyl-2-dimethyl hydrazine which gave two principal peaks at 1635 and 1538 cm.$^{-1}$ whereas Randall et al. (16) gave 1695(sh), 1595, and 1534 cm.$^{-1}$ for 1,2-diacetylhydrazine in the carbonyl region. The alteration of the 1664 cm.$^{-1}$ peak in I to a shoulder in II may then be taken as some support for a diacyl hydrazide structure in II.

In order to extend the scope of the rearrangement of α-N-acetyl to 1-acetyl amino acid hydrazines two experiments were carried out with α-N-trimethylacetyl-L-tyrosinhydrazide and α-N-acetyl glycylhydrazide under conditions analogous to the I - II transformation,

i.e., 0.05 M HCl and 0.05 M substrate at 95° C. Potentiometric titrations and alpha-chymotrypsin + acetyl-L-valine methyl ester assays were conducted as before. After 3 hours at 95° C., α-N-trimethylacetyl-L-tyrosinhydrazide was converted to the extent of only 17% to a base of $pKa' = 7$. Enzyme assay of the crude reaction product led to an estimate for the competitive inhibition constant $K_I = 3 \times 10^{-3}$ M. These results were of considerable interest because first, the steric effect of the t-butyl group appeared to support the proposed mechanism of intramolecular nucleophilic attack by the 1-N-hydrazine nitrogen, resulting in a low conversion, and second, the t-butyl group apparently provided steric interference to the formation of the EI complex giving a relatively large value for K_I. Research into this system must certainly be continued for the indicated results are only of a preliminary nature.

The results obtained with α-N-acetylglycylhydrazide were also of some consequence, although necessarily of a preliminary nature. Conversion to a base of $pKa' = 7.5$ approximated 86% as measured potentiometrically; however, the product did not exhibit even a trace of inhibition in the enzyme assay. One may conclude that the group HO-  -CH_2- is necessary to the formation of EI complexes of low K_I values when involving N^1-acetyl hydrazides. A similar separation of the affinity constants by several orders of magnitude is noted

for α-N-acetyl-L-tyrosin ethyl ester, $K_s = 0.7 \times 10^{-3}$ M (14) and ethyl aceturate, $K_s = 93 \times 10^{-3}$ M (15), whereas only one order of magnitude separates K_s for α-N-acetyl-L-tyrosinamide, $K_s = 32 \times 10^{-3}$ M (5), and aceturamide, $K_I = 340 \times 10^{-3}$ M (loc. cit. Part I).

The importance of an alpha-amino group to the functioning of 1-acetyl-2-[L-tyrosyl] hydrazine as an inhibitor was also shown in a preliminary experiment with the triacetyl derivative IV, triacetyl-L-tyrosinhydrazide. It will be recalled that 1-acetyl-2-[O-acetyl-α-N-acetyl-L-tyrosyl] hydrazine (IV) was obtained by treatment of both α-N-acetyl-L-tyrosinhydrazide or 1-acetyl-2-[L-tyrosyl] hydrazine with acetic anhydride in the presence of NaOH. It was observed that the triacetyl compound IV was a substrate for alpha-chymotrypsin giving a rate of hydrolysis about 50% higher than α-N-acetyl-L-tyrosinhydrazide.

Stability of 1-Acetyl-2-[L-tyrosyl] hydrazine II in Aqueous Solutions.

An aqueous solution of II has a pH ≐ 8. It was also found that the inhibitory capacity of a 0.005 M solution of II was constant when heated at 95° C. for 4-1/2 hours in water. This result shows that the reverse reaction II → I (or hydrolytic cleavage of the CH_3CO- group) does not occur to any measurable extent under these conditions; therefore, it is somewhat surprising that aqueous solutions of I are stable at 95° C. at pH 4-5. However, it has also been observed that

aqueous solutions of I buffered to pH ≐ 8 give an immediate red Ninhydrin test (characteristic of II) upon gentle warming. Therefore, it may be concluded that the acyl transfer may occur in the presence of alkali as well as acid, the driving force for the reaction under acid conditions being the protonation of the alpha-amino group as postulated previously and under basic conditions the driving force may be provided by solvent stabilization of the alpha-amino basic group in II. The latter rationalization would also explain the failure to obtain the reverse reaction II → I at pH ≐ 8.

Summary of Ninhydrin Tests.

The Ninhydrin test for a free alpha-amino group has given favorable results in this study. The behavior of the various derivatives encountered in this work gave diagnostic evidence for the presence of major products. Thus, α-N-acetyl-L-tyrosinhydrazide (I) gave a pale yellow color, 1-acetyl-2-[L-tyrosyl] hydrazine (II) gave a red color, L-tyrosinhydrazide a deep purple color, and L-tyrosine gave the characteristic azure blue color; however, quantitative spectrophotometric investigations were not carried out.

B. Inhibition of the alpha-Chymotrypsin-Catalyzed Hydrolysis of Acetyl-L-Valine Methyl Ester by 1-Acetyl-2-[L-tyrosyl]hydrazine II.

The behavior of 1-acetyl-2-[L-tyrosyl] hydrazine (II) as an inhibitor was investigated at pH = 7.90, NaCl = 0.100 M and at 25.0° C. in a system containing alpha-chymotrypsin and acetyl-L-valine methyl ester. Two concentrations of inhibitor were employed, $[I]_o = 0.237 \times 10^{-3}$ M and 0.474×10^{-3} M. The enzyme concentration used was $[E]_o = 0.1464$ mg. PN/ml. throughout and the inhibitor characteristics of II were observed by varying the concentration of the substrate, acetyl-L-valine methyl ester, from $[S]_o = .0150$ M to $[S]_o = .140$ M. Experiments were conducted in the pH-stat in the manner described previously.

The recorder traces resulting from the addition of base to the system were linear over the time interval 1-9 minutes; accordingly, the initial velocities were estimated from the fitting of a straight line to the 1-9 minute interval. The initial velocities thus obtained are recorded in Table XIII. Plots of $1/v_o$ vs. $1/[S]_o$ and $[S]_o[E]_o/v_o$ vs. $[S]_o$ (Figure 7) were linear; however, the inhibitor characteristics were recognizable as being a case of mixed competitive - non-competitive inhibition, i.e., K_s' increased in the presence of inhibitor but k_3' also decreased with increasing inhibitor concentration. At

Table XIII

Inhibition of the alpha-Chymotrypsin-Catalyzed Hydrolysis of Acetyl-L-valine Methyl Ester by 1-Acetyl-2-[L-Tyrosyl] hydrazine

pH = 7.90, 25.0° C.; $[E]_o$ = 0.1464 mg. PN/ml.; NaCl = 0.100 M

Notebook Ref. 934-88, -89

$[I]_o = 0.227 \times 10^{-3}$ M			$[I]_o = 0.454 \times 10^{-3}$ M		
$[S]_o$ [a]	v_o [b]	$\frac{[S]_o[E]_o}{v_o}$ [c]	$[S]_o$ [a]	v_o [b]	$\frac{[S]_o[E]_o}{v_o}$ [c]
140	.676	303	70	.263	390
100	.523	280	70	.267	384
70	.425	241	50	.195	375
70	.410	250	50	.195	375
50	.305	240	40	.159	368
50	.302	242	40	.157	373
40	.260	225	30	.129	340
40	.267	219	30	.128	343
30	.184	239	20	.089	328
30	.201	219	20	.086	340
20	.140	209	15	.066	334
15	.104	211	15	.068	322

K_s'' [a]	267 ± 24	273 ± 31
k_3'' [d]	1.34 ± .12	0.87 ± 0.09
$K_s''/k_3''[E]_o$	1360 min.	2140 min.
$1/k_3'[E]_o$	5100 min./M	7850 min./M

a. Units of 10^{-3} M. b. Units of 10^{-4} M/min. c. Units of min.-mg. PN/ml. d. Units of M/min.-mg. PN/ml.

For acetyl-L-valine methyl ester K_s' = 111 x 10^{-3} M.

k_3' = 2.15 x 10^{-3} M/min.-mg. PN/ml.

Table XIII (continued)

K_s'' and k_3'' estimated from L. S. fit to the relationship

$$\frac{[S]_o[E]_o}{v_o} = \frac{K_s''}{k_3''} + \frac{[S]_o}{k_3''}$$

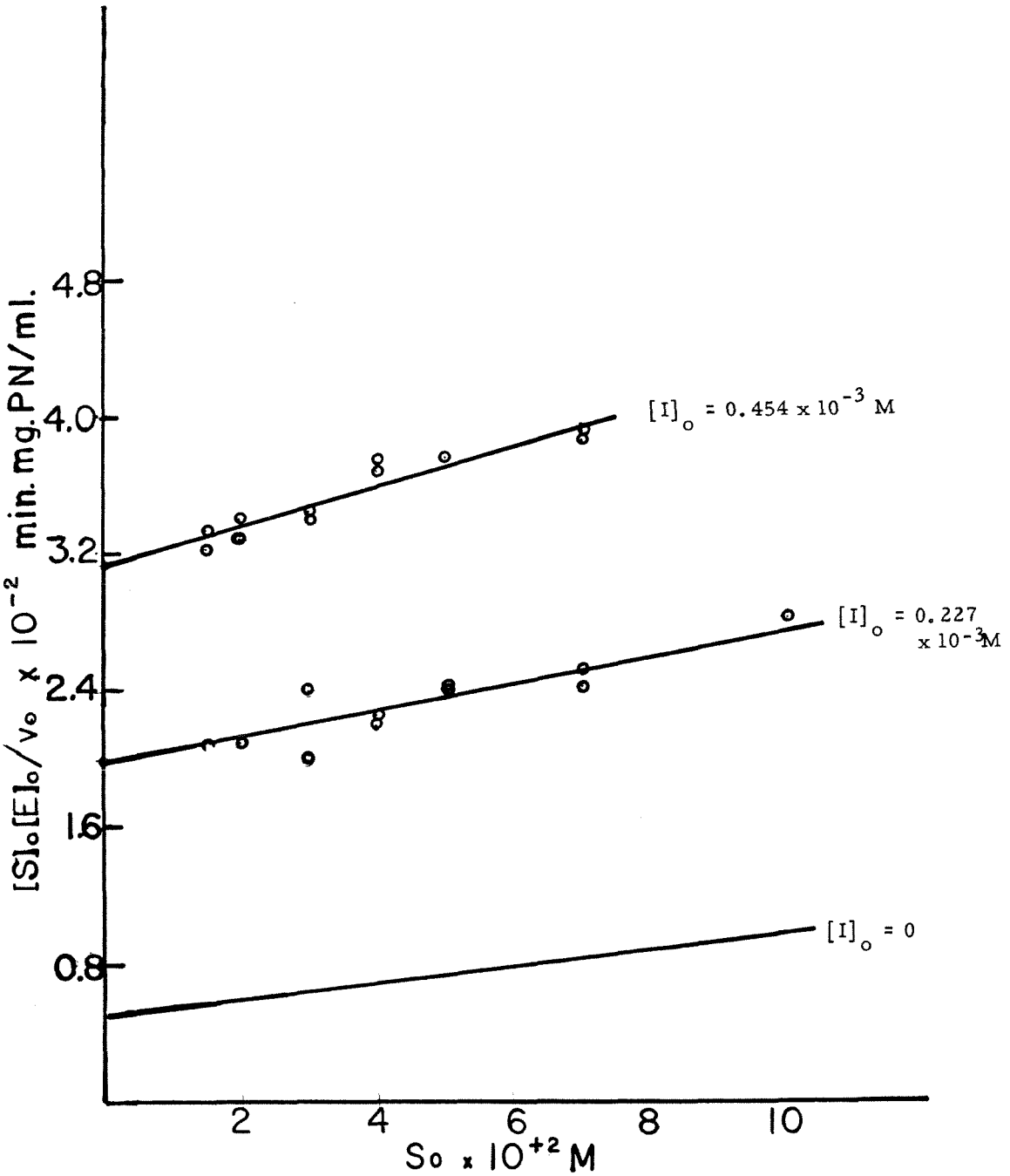


Figure 7 - Inhibition of Acetyl-L-Valine Methyl Ester by 1-Acetyl-2-[L-tyrosyl]hydrazine at pH = 7.90

$[I]_o = 0$, $K'_s = 111 \times 10^{-3}$ M, $k'_3 = 2.15 \times 10^{-3}$; at $[I]_o = 0.237 \times 10^{-3}$M, $K''_s = 267 \times 10^{-3}$ M, $k''_3 = 1.34 \times 10^{-3}$ and at $[I]_o = 0.474 \times 10^{-3}$ M, $K''_s = 273 \times 10^{-3}$ M, $k''_3 = 0.87 \times 10^{-3}$. Mixed competitive-non-competitive inhibition was discussed in Part I of this Thesis in connection with the possibility of ternary ES'I and EI'S complexes arising from the system enzyme plus chloroacetyl-L-valine methyl ester and ethyl 1-acetyl-2-benzyl carbazate. The decrease of the value of k''_3 in the present system under discussion appears to be of even greater significance for the hypothesis that valine substrates may yield ternary complexes even with trifunctional inhibitors. That the observed deviations in k''_3 reported so far in this Thesis are not due to some systematic error is shown in Section C of Part II of this Thesis wherein it is shown that 1-acetyl-2-[L-tyrosyl] hydrazine behaves in a totally competitive manner towards N-acetyl-L-tyrosin-hydroxamide.

The competitive inhibition constant, K_I, was evaluated from equation 8, Part I. It was pointed out in Part I that a plot of the slope of a $1/v_o$ vs. $1/[S]_o$ plot vs. $[I]_o$ will show a negative deviation from linearity when the term rK_s/C_β cannot be neglected with respect to the order of magnitude of $1/K_I$. From the three experimentally observed slopes of the plots of $1/v_o$ vs. $1/[S]_o$ numerical analysis gave $K_I = 0.074 \pm 0.014 \times 10^{-3}$ M and $rK_s/C_\beta = 650$ M^{-1}. Similarly,

the relationship between the intercepts of the $1/v_o$ vs. $1/[S]_o$ plot and $[I]_o$ yielded for the constant $(\frac{1}{C_\alpha} + \frac{1}{C_\beta})$ values of 0.33 or $0.44 \times 10^5\ M^{-2}$. In the absence of an independent measurement for K_I in a totally competitive system, the composite constants $r K_s/C_\beta$ and $(\frac{1}{C_\alpha} + \frac{1}{C_\beta})$ cannot be separated so that estimates as to the relative significance of the C_α and C_β terms and of r, the relative rate of breakdown of ternary EI'S complexes compared to binary ES complexes, cannot be justifiably made.

C. Inhibition of the alpha-Chymotrypsin-Catalyzed Hydrolysis of N-Acetyl-L-Tyrosinhydroxamide by 1-Acetyl-2-[L-Tyrosyl]hydrazine.

The anticipated use of 1-acetyl-2-[L-tyrosyl] hydrazine (II) as an inhibitor for the alpha-chymotrypsin catalyzed hydrolysis of L-tyrosine methyl ester (pH optimum 6.7)* made it necessary to measure K_I' for (II) at a pH = 6.7. Accordingly, II was evaluated as an inhibitor against acetyl-L-tyrosinhydroxamide at pH = 6.7. The pH optimum of the latter substrate (30) occurs at pH = 7.6; therefore, some indication of the effect of pH on K_s' and k_3' for the substrate as well as the effect of pH on the enzyme inhibitor dissociation constant became available from this study. The results indicated below should only be considered to be preliminary since but two experiments were carried out with six different substrate concentrations at $[I]_o = 0$ and $[I]_o = 0.237 \times 10^{-3}$ M, at NaCl = 0.100 M, pH = 6.70, 25.0° C., $[E]_o = 0.1464$ mg. PN/ml.

At $[I]_o = 0$, $[S]_o$ was varied from 7.5 to 40×10^{-3} M (6 concentrations) and at $[I]_o = 0.237 \times 10^{-3}$ M, $[S]_o$ was varied from 7.5 to 35×10^{-3} M (6 concentrations). The recorder traces of extent of conversion versus time were non-linear and initial velocities were evaluated by the orthogonal polynomial procedure (loc. cit.). Enzyme and substrate blanks were essentially absent at this pH. The

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* Unpublished results of A. N. Kurtz.

constants K_s' and k_3'' were evaluated by a least squares fit to the relationship:

$$\frac{[S]_o[E]_o}{v_o} = \frac{K_s'}{k_3''} + \frac{[S]_o}{k_3''} \qquad (12)$$

The data are recorded in Table14; the plots of equation 12 are given in figure 8. The apparent velocity constant k_3'' was corrected for the buffering effect of the liberated hydroxylamine as shown in Part IV of this Thesis, i.e.,

$$k_3' = k_3''\left(1 + \frac{[NH_3OH^+]}{[NH_2OH]}\right) \qquad pK_a' = 5.99$$

At $[I]_o = 0$, the value of K_s' was $65.2 \pm 3.9 \times 10^{-3}$ M, $k_3'' = 17.6 \pm 1.0 \times 10^{-3}$ M/min.-mg. PN/ml. and $k_3' = 21.0 \pm 1.2 \times 10^{-3}$ M/min.-mg. PN/ml.

Jennings (29) obtained values of K_s' and k_3' for this substrate as a function of pH but at an ionic strength equal to 0.3 M. At pH 6.6 Jennings found K_s' to have a value of $59 \pm 5 \times 10^{-3}$ M and $k_3' = 22.7$; at pH = 6.95 the value of K_s' was found to be equal to 44 ± 4 and $k_3' = 25.7$. The effect of ionic strength on the constants K_s' and k_3' will be shown in Part IV of this Thesis; differences between Jennings' constants and the constants reported here may be ascribed to the effect of ionic strength.

At $[I]_o = 0.237 \times 10^{-3}$ M, the value of K_s' was $117 \pm 14 \times 10^{-3}$M,

Table XIV

The Inhibition of N-Acetyl-L-tyrosinhydroxamide by 1-Acetyl-2-[L-tyrosyl] hydrazine at pH = 6.70

$[E]_o$ = 0.1464 mg. PN/ml., NaCl = 0.100 M, 25.0° C.

$[I]_o = 0$			$[I]_o = 0.237 \times 10^{-3}$ M		
$[S]_o$ [a]	v_o [b]	P_m [c]	$[S]_o$ [a]	v_o [b]	P_m [c]
40	.988	2	35	.533	2
25	.699	2	25	.391	2
20	.616	2	20	.329	2
15	.476	2	15	.260	2
10	.342	2	10	.177	2
7.5	.270	3	7.5	.140	2

K_s' [a] = 65.2 ± 3.9 | K_s' [a] = 117 ± 14

k_3'' [d] = 17.6 ± 1.0 | k_3'' [d] = 15.5 ± 1.9

k_3' [d e] = 21.0 ± 1.2 | k_3' [d e] = 18.6 ± 2.2

$$K_I' \text{ [a]} = 0.22 \pm .03$$

a. Units of 10^{-3} M.

b. Units of 10^{-3} M/min.

c. Order of orthogonal polynomial.

d. Units of M/min.-mg. PN/ml.

e. $k_3' = k_3''\left(1 + \frac{[NH_3OH]^+}{[NH_2OH]}\right) = 1.195\, k_3''$.

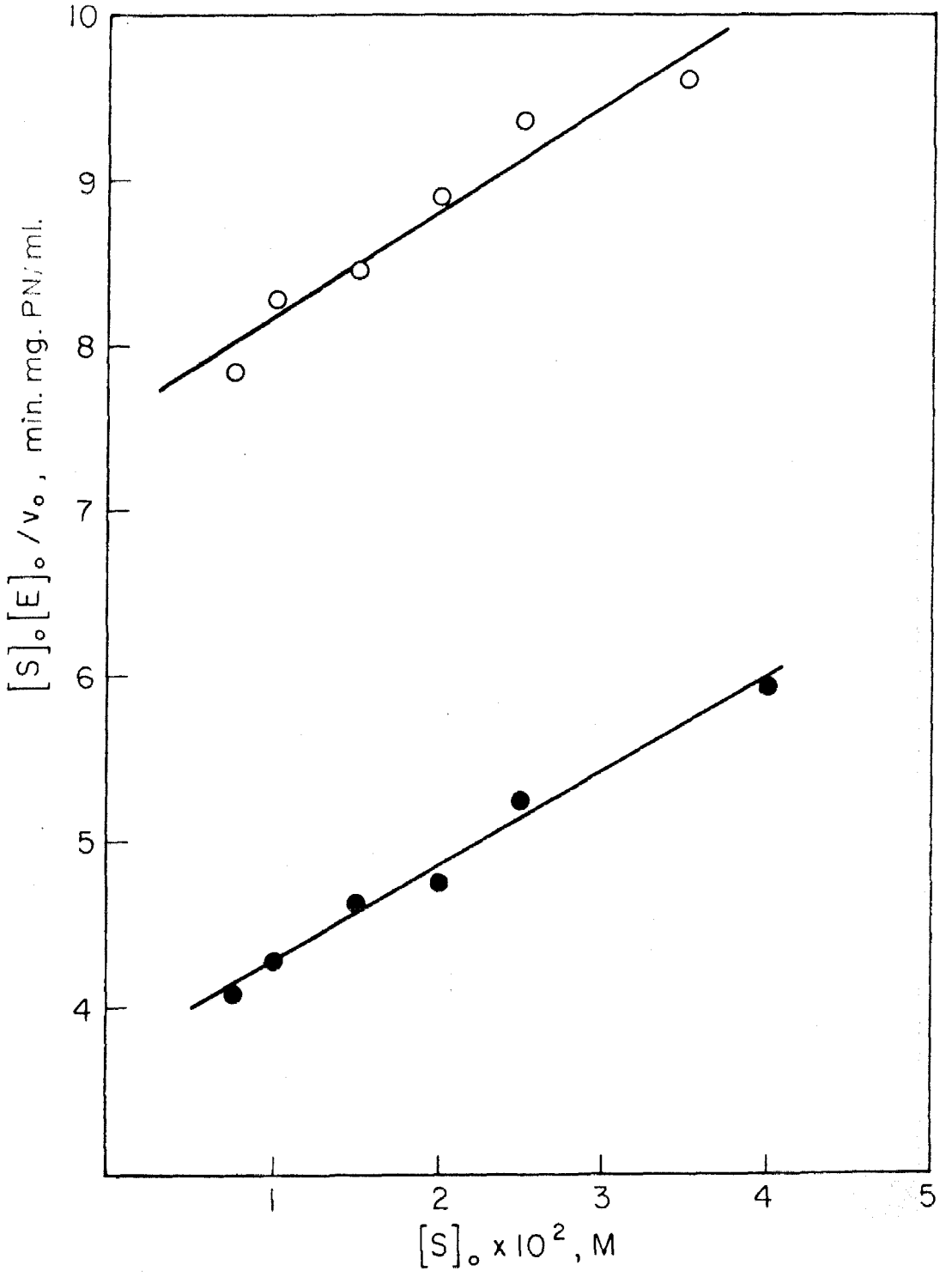

Figure 8. The Inhibition of N-Acetyl-L-tyrosinhydrazide by 1-Acetyl-2-[L-tyrosyl] hydrazine at pH = 6.70, NaCl = 0.100 M, 25.0°C. $[E]_o$ = 0.1464 mg. PN/ml.

$k_3'' = 15.5 \pm 1.9 \times 10^{-3}$ M/min.-mg. PN/ml. and $k_3' = 18.6 \pm 2.2 \times 10^{-3}$ M/min.-mg. PN/ml. The apparent difference in k_3' between the case $[I]_o = 0$ and $[I]_o = 0.237 \times 10^{-3}$ M is not significant, considering the experimental error. Therefore, the apparent enzyme-inhibitor constant was calculated on a total competitive basis corrected to k_3' for $[I]_o = 0$. The value of K_I thus obtained was $0.22 \pm 0.03 \times 10^{-3}$ M.

It is interesting to note that K_I' for this inhibitor increased markedly as the pH decreased in contrast to D-tryptophanamide and tryptamine (19). The change in K_I' at pH = 7.9 ($K_I' = 0.074 \times 10^{-3}$ M) to pH = 6.70 ($K_I' = 0.22 \times 10^{-3}$ M) may appear to be correlated, within experimental error, to the decreasing concentration of the unprotonated species of inhibitor II as the pH was lowered. From the known value of pKa' for inhibitor II, pKa' = 6.93, the ratio of protonated to unprotonated species at pH = 7.90 is 0.107 and at pH = 6.70, the ratio is 1.7. From the latter relationships, if it is assumed that the enzyme lacks affinity for the protonated species, corrected values of K_I' were calculated to be 0.058×10^{-3} M and 0.076×10^{-3} M at pH = 7.90 and 6.70 respectively, where the latter values of K_I' now relate only to the unprotonated species. Therefore, it would appear that within the limits of experimental error the change in apparent K_I from $K_I = 0.074 \times 10^{-3}$ M at pH 7.90 to

$K_I = 0.22 \times 10^{-3}$ M at pH 6.70 is ascribable to the hypothesis that the enzyme possesses affinity only for the uncharged, unprotonated inhibitor species. However, it will he shown later that the correlation may be fortuitous because it is known that the enzyme contains an ionizable group of pKa 7 to 8 so that the relative abundance of the protonated to non-protonated species at the active site must also be taken into account.

The pH dependence of K_s and k_3 for N-acetyl-L-tyrosinhydroxamide may also be obtained from the experiment where $[I]_o = 0$ at pH 6.70 since K_s and k_3 have been evaluated at pH = 7.6 (5). The effect of ionic strength on the alpha-chymotrypsin catalyzed hydrolysis of N-acetyl-L-tyrosinhydroxamide is presented in Part IV of this Thesis, the latter affording a more suitable basis for comparison due to the known effects of ionic strength on the velocities of hydrolyses catalyzed by this enzyme.

At pH 7.60 values of $K'_s = 47 \times 10^{-3}$ M and $k'_3 = 31 \times 10^{-3}$ M/min.-mg. PN/ml. were obtained at NaCl = 0.100 M. When compared with the values obtained at pH = 6.70, $K'_s = 65 \times 10^{-3}$ M and $k'_3 = 21 \times 10^{-3}$ M/min.-mg. PN/ml. it is apparent that the decrease in the velocity of the reaction on the acid side of the pH optimum (pH = 7.60) is reflected both in an increased K'_s (lower affinity) and a decreased k'_3. Since this substrate may for all practical purposes

be considered to exist as the un-ionized species in the pH interval 6.7 - 7.6 (pKa' for the hydroxamic acid group ca. 9) the change in K'_s and k'_3 with decreasing pH may reflect the decrease of a favorable mode of combination between enzyme and substrate that occurs as the negative charge of the enzyme decreases at a nucleophilic site. Comparative values of the constants at the two pH's are given in Table XV. The decrease in k'_3 and increase in K'_s are in agreement with the work of Jennings (29) when interpolationsfor the effect of ionic strength are made.

Discussion:

The problem of the interaction of alpha-chymotrypsin with L-substrates that exist in both the protonated and non-protonated forms, e.g., L-tyrosinamide and L-tyrosinhydroxamide is of some consequence because such situations may be encountered in the alpha-chymotrypsin degradation of proteins when susceptible groups occur on the free-amino end of the protein. The pH optima of such basic amide-type substrates always occur at a lower pH than the corresponding optima of their corresponding N-acylated derivatives (17). In addition, the slower enzyme-catalyzed hydrolysis of the non-acylated substrate has been found to be reflected principally in a lower k_3, rather than in K_s. The decreased activity of the free-

Table XV

The Effect of pH on the alpha-Chymotrypsin-Catalyzed Hydrolysis of N-Acetyl-L-tyrosinhydroxamide

pH	$[E]_o$ [e]	Ionic Strength NaCl M	Ionic Strength (buffer) M	K'_s [a]	k'_3 [b]	Ref.
6.70	.146	0.100	---	65±4	21±1.2	c
7.60	.0146	0.100	---	47±4	31±2	c
7.6	.0208	---	0.3	50±5	32±3	(30)
7.6	.0297	---	0.3	40±5	32±3	(30)
7.65	.0201	---	0.5	42±2	34±2	(22)
7.6	.0208-.0297	---	0.3-0.5	43±4	33±3	(5) d
6.6	.040	---	0.3	59±5	22.7	(29)
6.95	.040	---	0.3	44±4	25.7	(29)

a. Units of 10^{-3} M.

b. Units of 10^{-3} M/min.-mg. PN/ml.

c. This Thesis

d. Preferred values.

e. Units of mg. PN/ml.

amino containing substrate has been attributed (17) to the donor characteristics of the amino group resulting in decreased polarity of the carbonyl group adjacent to the susceptible bond. However, the enzyme may associate with only one or the other of the protonated or unprotonated substrate species. If the marked decrease of k_3 for the non-acylated species were due to the association of enzyme only with the unprotonated substrate species then for a substrate having an ionizable group of pKa' = 7 the correct value for $[S]_o$ would be = 1/2 that of the expected $[S]_o$ resulting in a lower value for K_s (1/2 apparent K_s) than that calculated from the total $[S]_o$. The apparent K_s for L-tyrosinhydroxamide and the apparent K_s for acetyl-L-tyrosinhydroxamide are reported to be 41 and 45 x 10^{-3} M respectively (17), whereas L-tyrosinhydrazide and acetyl-L-tyrosinhydrazide have K_s values of 6 and 22 x 10^{-3} M respectively (18). Such an hypothesis would not necessarily lead to a reversal of the expected $K_{s_L} > K_{I_D}$ relationship that has been found to describe amide substrates since a similar argument may be invoked for the inhibitors, i.e., D-tyrosinhydroxamide for which K_I = 40 x 10^{-3} M.

The problem outlined above also exists for ionizable D-inhibitors or ionizable inhibitors in general. For anionic inhibitors the increase in K_I with decreasing pH has been attributed (19) to electrostatic repulsion of a negatively charged site on the enzyme that is largely unprotonated at pH = 7.9 and largely protonated at pH = 6.9. However, when amino groups are present such as in

tryptamine and D-tryptophanamide, K_I was found to be relatively invariant from pH 6.9 - 7.9 even though the ratio of the species $-NH_3^+/-NH_2$ varies over an order of magnitude. Obviously then, information is needed as to the relative variation of K_s and k_3 with pH for L-substrates and non-acylated L-substrates in particular. Cunningham and Brown (14) reported only a slight increase in K_s for acetyl-L-tryptophan ethyl ester over the pH range 6-8, the principal effect on the velocity being ascribed to k_3. Manning (2) also observed a slight increase in K_s for N-carboethoxy-L-tyrosinamide over the pH range 7.85 to 8.25. As a first approximation, N-acylated L-substrates would not be expected to show large variations in K_s in the region of the pH optimum.

The effect of pH on the kinetic constants K_s and k_3 for non-acylated L-substrates of alpha-chymotrypsin has not been reported in the literature.* Balls and co-workers (23, 24, 25) employed L-tyrosine ethyl ester as a substrate for alpha-chymotrypsin and modified alpha-chymotrypsins; however, their study was limited to the location of the pH-optimum which they reported to be pH = 6.25. The latter value was claimed to be in error (26) as a consequence of the neglect of the pKa' of L-tyrosine ethyl ester. A statement appears in the literature (26) that measurements of the velocities of

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* Jennings (29) determined K_s and k_3 for L-tyrosinhydroxamide over the pH range 6 to 8. K_s appeared to minimize and k_3 to maximize at pH ca. 7.

hydrolyses at constant pH, using the technique of the addition of base to maintain a constant pH, give only the extent of reaction of the protonated substrate species, i.e., the acid-base equilibrium of the substrate supplies both forms to the enzyme so that 100% conversion of the substrate occurs regardless of whether the enzyme profers protonated or unprotonated species. However, the kinetics do not reveal a preference for one of the species by enzyme. Professor Niemann has suggested that a comparison of the pH-stat method with a chemical method, i.e., hydroxylamine for an ester (27), would enable a decision to be reached as to which one or both of the species associates with enzyme.

The observation that K_I for 1-acetyl-2-[L-tyrosyl]hydrazine (II) varied with pH in a manner that could be explained by the association of unprotonated II with enzyme appears to contradict the hypothesis that the enzyme favors protonated substrate, a hypothesis that has been offered to explain the lowered pH optima of non-acylated substrates (17), and the effect of pH on K_s and k_3 for L-tyrosinhydroxamide (29). The correlation of K_I with pKa' of the inhibitor II may be fortuitous because the extent of protonation of the active site of the enzyme decreases significantly over the pH range 6.7 - 7.6, the correlation arising as a consequence of the near equivalence of the pKa' of the active site and the pKa' of the inhibitor II (i.e., pKa' = 6.9).

If the decrease in the value of the apparent K_I as the pH was increased from 6.7 to 7.6 be ascribed to the relative concentration of protonated-unprotonated species of both the enzyme and inhibitor, then it follows that the change in K_I with pH may also be explained by the presence at the active site of a group with a pKa' = 6.8 to 8. The change in K_I may then be only an apparent one. In the absence of a separate estimate of any of the enzyme species at both pH's, the pKa' of the enzyme cannot be fixed closer than the limits given. Accordingly, the suggestion that the enzyme associates only with protonated inhibitor is much more acceptable than the suggestion that the enzyme associates with unprotonated inhibitor. Association of the enzyme with unprotonated species could only give the correlation indicated previously if a further restriction were implied, namely, that the pKa' of the active site was outside of the range 6-8, a conclusion that stands in contradiction to the known facts. Furthermore, Jennings (29) showed that variations in K_s and k_3 with pH could be explained by the presence, at the active site of the enzyme, of two ionizable groups of pKa' = 6.9 and 8.5, when acetyl-L-tyrosinhydroxamide was used as a substrate.

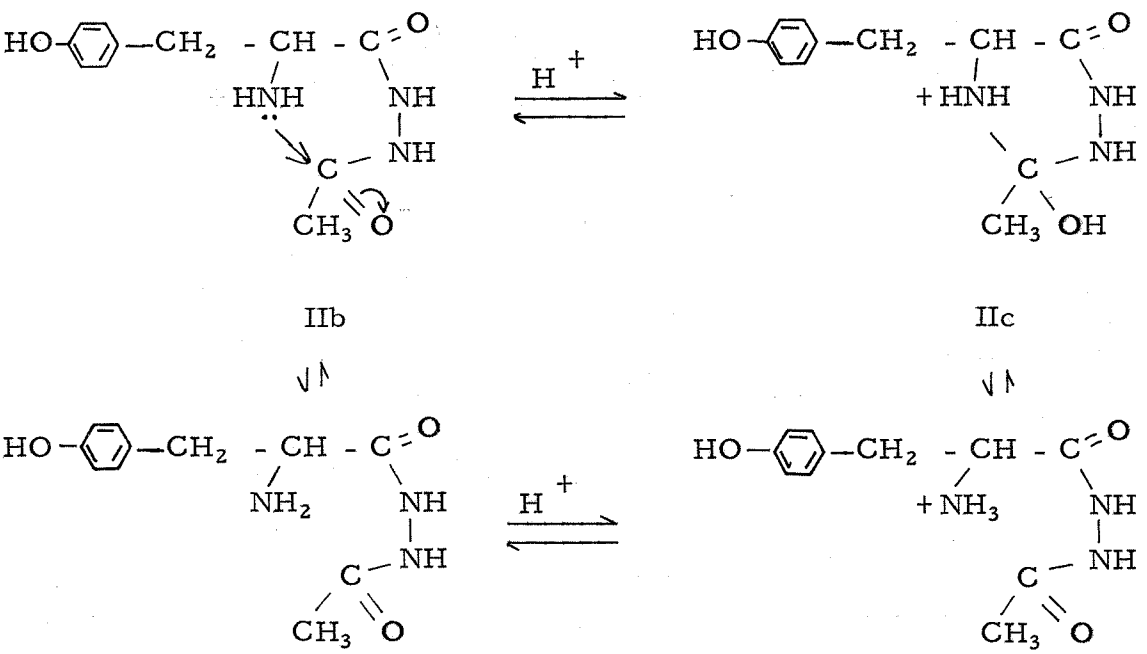
Significance of the Unusual Affinity of Enzyme for Inhibitor II.

The competitive inhibition constant for 1-acetyl-2-[L-tyrosyl] hydrazine, $K_{I_L} = 0.074 \times 10^{-3}$ M at pH = 7.90 is two orders of magnitude lower than any known acetyl tyrosine amide-type L-substrate and a full order of magnitude lower than the reported value for acetyl-L-tyrosine ethyl ester ($K_s = 0.7 \times 10^{-3}$ M)(14). Furthermore, inhibitor II combines reversibly with alpha-chymotrypsin to a greater extent than any known substance. Clearly, then the structural features of the active site as well as the structure of the inhibitor must account for this result.

The enzyme-inhibitor constant corresponds to $-\Delta F^\circ = 5.6$ kcal./mole at 25° C which is still within the range of energies associated with H-bonding and charge interactions. The gain in entropy associated with the positively charged inhibitor complexing with the negatively charged enzyme site must be offset by a decreasing negative enthalpy for desolvation. It is suggested that when the solvated, charged enzyme and inhibitor molecules come together, de-solvation of water molecules occurs with a gain in entropy so that ΔF° is still quite negative.

It is evident from the low value of K_I that the failure of inhibitor II to show substrate properties cannot be ascribed to lack of formation of an enzyme intermediate. Therefore, an explanation

for $k_3 = 0$ for the inhibitor is needed. The addition of bulk to the R' leaving group of the carbonyl hydrolyzable function, i.e., R-C(=O)-R', does not appear to cause $k_3 \rightarrow 0$, thus k_3 for the N-acetyl-tyrosine series, R' = -NHOH, $-CH_2$-COOEt, $-NH_2$, $-NHNH_2$ are reported to be 33×10^{-3}, 2.5×10^{-3}, 2.4×10^{-3}, and 0.93×10^{-3} (5, 20, 28). It will be recalled that the infra-red spectrum of inhibitor II in the solid state showed strong N-H stretching absorption at 3215 cm.$^{-1}$. The shift of the N-H stretching band to lower frequencies taken with the decreased intensity of the C=O band at 1664 cm.$^{-1}$ may be indicative of the preference of inhibitor II for an intramolecular cis structure, IIc:



IIb ⇌ IIc

II ⇌ IIa

In association with enzyme, the protonated cis structure IIc and the leaving group, due to the steric restriction of the cis configuration, may no longer be in juxtaposition to the electrophilic enzyme site needed for the rupture of the $-C\overset{\overset{O}{\|}}{\cdots}N-$ bond. In fact, the inhibitor may locate to the enzyme in a mode of combination that is very favorable for association, i.e., unstrained conformation, but unfavorable for hydrolysis. However, this mode in all likelihood is probably a mode favored by L-rather than D-enantiomorphs which would account for the value of K_{I_D} for 1-acetyl-2-[D-tyrosyl]hydrazine being equal to 5×10^{-3} M, i.e., $K_{I_L} \ll K_{I_D}$.

D. Experimental Synthesis

N-Acetyl-L-Tyrosinhydrazide (I)

This compound was prepared by the method of Hogness and Niemann (30).

M.p. 232-233°C. (corr.); α_D^{25} +44° (c, 0.36% in water)

Lit. m.p. 227-228° C.; α_D^{25} +44° (c, 0.4% in water) (2)

All preparations of I used in this Thesis melted at 231-233° C.(corr.); therefore it is believed that the older values of the m.p. are too low.

1-Acetyl-2-[L-Tyrosyl]hydrazine (II)

Six grams (.025 mole) α-N-acetyl-L-tyrosinhydrazide were dissolved in 500 ml. of 0.05 M aqueous HCl at 95° C. The solution was stirred and maintained at 95-98° C. for 3 hours, then stripped in vacuo on a steam bath. The crude product, a viscous oil, was further stripped in vacuo for 1/2 hour at 25° C. The latter product was then dissolved in 50 ml. methyl alcohol and the exact equivalent of 1 M NaOMe in methyl alcohol was added. After 1 hour, sodium chloride was filtered off and the methyl alcohol solution of the free base was vacuum stripped and recrystallized from methyl alcohol plus isopropyl ether to give 2.5 grams (45%) of m.p. 120-140° C. After two recrystallizations from acetonitrile and two recrystallizations from isopropyl alcohol + Norite, 0.71 g. (13%) of the product was obtained as hygroscopic, colorless, microscopic needles,

m.p. 138-146° C., soluble in water, methyl alcohol and ethyl alcohol. Insoluble $CHCl_3$, ether and all other common organic solvents.

α_D^{25} +63° ± 2° (c, 1.1% in water). Ninhydrin positive, red color. Potentiometric titration of the free base versus aqueous hydrochloric acid gave pKa' = 6.93 ± .02 with a single end point.

Analysis: Calculated: C, 55.7; H, 6.4; N, 17.7.

$C_{11}H_{15}N_3O_3$ (237) Found: C, 55.4; H, 6.4; N, 18.0.

1-Acetyl-2-[D-Tyrosyl]hydrazine (XI)

1-Acetyl-2-[D-tyrosyl]hydrazine was prepared by the same procedure described for the preparation of the L-enantiomorph. The final product was obtained in 26% crude yield; recrystallized from isopropyl alcohol three times gave a constant m.p. and rotation.

M.p. 143-144° C. (corr.); α_D^{25} - 60° (c, 1.1% in water)

Analysis: Calculated: C, 55.7; H, 6.4; N, 17.7.

$C_{11}H_{15}N_3O_3$ (237) Found: C, 55.5; H, 6.5; N, 17.6.

1-Acetyl-2-[L-Tyrosyl]hydrazine -β- Naphthalenesulfonate Salt (VII)

The isolation of 1-acetyl-2-[L-tyrosyl]hydrazine as the β-naphthalene sulfonate salt appears to be the most convenient method of preparation of the inhibitor. However, it must be noted that β-naphthalene sulfonate anion is a competitive inhibitor for alpha-

chymotrypsin (see Part III).

α-N-Acetyl-L-tyrosinhydrazide, 4.74 g. (0.020 mole), and 4.53 g. (0.020 mole) β-naphthalene sulfonic acid monohydrate (Eastman Kodak No. 897, recrystallized from isopropyl alcohol) were dissolved in 200 ml. water and stirred at 98° C. for three hours. The hot solution was treated with Norite and filtered. After concentration of the volume to 50 ml. by vacuum stripping, the product still failed to crystallize out at 5° C. The crude product was stripped to dryness and recrystallized from isopropyl alcohol to give 4.0 g. (45%), m.p. 138-140° C. Two additional recrystallizations from isopropyl alcohol gave the product in analytical purity, m.p. 147-149° C. (corr.) (softens 130° C.). α_D^{25} + 56° (c, 2.07% in water). Potentiometric titration showed a single end-point and gave an equivalent weight of 440 (theory 445) after correcting for the phenolic tyrosine group; $pKa' = 6.93 \pm .02$.

Analysis:	Calculated:	C, 56.62; H, 5.20; N, 9.43.
$C_{21}H_{23}N_3O_6S$(445)	Found:	C, 56.60; H, 5.14; N, 9.36.

L-Tyrosinhydrazide di-β-Naphthalene Sulfonate Salt (V)

A crude preparation of 1-acetyl-2-[L-tyrosyl]hydrazine as the free base was carried out in the manner described above. A solution of 6 grams of the crude product was dissolved in 100 ml. water. Enzyme assay of the latter solution against acetyl-L-valine

methyl ester showed that the solution was approximately 0.22 M with respect to 1-acetyl-2-[L-tyrosyl]hydrazine. 3 g. (0.045 mole) of β-naphthalene sulfonic acid monohydrate were added to 50 ml. of the 0.22 M solution of the crude inhibitor and the mixture heated on the steam bath for one hour whereupon complete solution was effected. After treatment with Norite, filtering and cooling, the product crystallized out.

Yield, 2.0 grams (41%). Recrystallized from water, m.p. 267-270° C. (corr.), α_D^{25} + 24° (c, 1.6% in dimethylformamide).

Potentiometric titration of the salt with NaOH gave two end-points, the first one being exactly equivalent to the second (after correcting for the phenolic tyrosyl group). Molecular weight by titration, 630 ± 2 (theory 611) pKa_1' = 3.2 pKa_2' = 7.12 ± .02. The molecular weight found titrimetrically corresponded to a mono-hydrate. This product was identical to the dinaphthalene sulfonate salt of L-tyrosinhydrazide prepared separately (i.e., criteria of mixed m.p. and titration).

A deep purple color was obtained with Ninhydrin upon buffering the solution of the salt with sodium bicarbonate and heating.

The formation of the L-tyrosinhydrazide dinaphthalene sulfonate rather than 1-acetyl-2-[L-tyrosyl]hydrazine mononaphthalene sulfonate has been explained in the text.

1-Acetyl-2-[O-Acetyl-α-N-acetyl-L-tyrosyl]hydrazine (IV)

A. From N-acetyl-L-tyrosinhydrazide:

A solution of 4.74 g. (0.02 mole) α-N-acetyl-L-tyrosinhydrazide in 50 ml. water containing 0.040 mole sodium hydroxide was treated with 4.10 g. (0.040 mole) acetic anhydride at 0° C. with good stirring for one hour. The product separated out as a thick slurry in a crude yield of 90%. The crude product was recrystallized from water and treated with Norite to give the final product in analytical purity. Colorless, large needles, m.p. 219-220° C. (corr.) (m.p. 214-215° C. (corr.) when vacuum dried over P_2O_5 at 77° C.). α_D^{25} + 26° (c, 0.5% DMF) . Ninhydrin test was negative.

Analysis:	Calculated:	C, 56.06; H, 5.96; N, 13.08.
$C_{15}H_{19}N_3O_5$ (321)	Found:	C, 56.04; H, 6.13; N, 13.11.

B. From 1-acetyl-2-[L-tyrosyl]hydrazine:

Fifty ml. of a crude aqueous preparation of 0.22 M (by enzyme assay) 1-acetyl-2-[L-tyrosyl]hydrazine were treated with 20 ml. of 2N NaOH and 4 ml. acetic anhydride at 0° C. Crude product separated out in 90% yield and was recrystallized from water to give a product identical with the triacetyl-L-tyrosinhydrazide described in A above. M.p. 219-220° C. (corr.); mixed m.p. with IV A. 219-220° C. (corr.).

1-Acetyl-2-[α-N-Acetyl-L-tyrosyl]hydrazine(VIII)

Compound IV, 1.35 grams was dissolved in 10 ml. of 2N aqueous sodium hydroxide. The solution was left standing at room temperature for one hour, then cooled and the pH adjusted to 3 with conc. HCl. After 2 days at 5° C. the solution deposited 1.05 grams of crystals that were filtered and washed with a few ml. of cold water, m.p. 228-229° C. (corr.). The product was recrystallized from aqueous isopropyl alcohol, m.p. 238°C (corr.), α_D^{25} + 18 ± 1° (c, 1.1% in water). This product depressed the melting points of α-N-acetyl-L-tyrosinhydrazide, and 1-acetyl-2-[O-acetyl-α-N-acetyl-L-tyrosyl]hydrazine (IV).

Analysis: Calculated: C, 55.90; H, 6.14; N, 15.05.

$C_{13}H_{17}N_3O_4$ (279) Found: C, 56.01; H, 6.10; N, 14.94.

Acetyl-L-Valine Methyl Ester (IX)

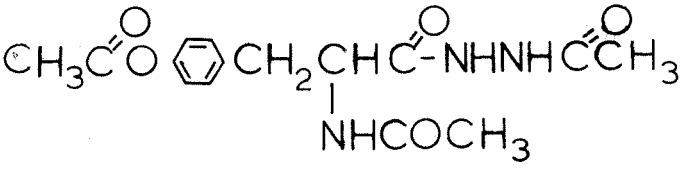
Preparation has been described previously (31).

Sodium Acetyl-L-tyrosinhydroxamate (X)

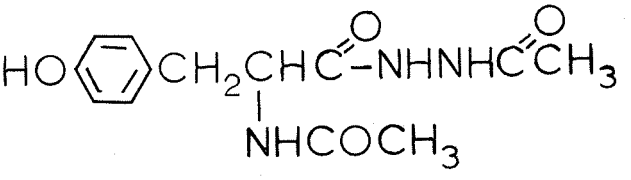
The preparation of acetyl-L-tyrosinhydroxamide has been described by Hogness and Niemann (30). In their preparation, the sodium salt of acetyl-L-tyrosinhydroxamide was isolated prior to the conversion of the latter to N-acetyl-L-tyrosinhydroxamide. In the following procedure, sodium N-acetyl-L-tyrosinhydroxamate was prepared in a similar manner.

Sixty ml. of 3.5 M sodium methoxide in methyl alcohol were added to 12.2 g. (0.175 mole) hydroxylamine hydrochloride in 70 ml. methyl alcohol at 40° C., stirred for 1/2 hour, then filtered. To the above filtrate, 10.4 g. (0.044 mole) N-acetyl-L-tyrosine methyl ester and 14 ml. of 3.5 M sodium methoxide in methyl alcohol were added at 25° C. The resulting solution was stored at 5° C. for 3 days. The crude crop of crystals were filtered and washed with 30 ml. methyl alcohol. Drying in vacuo gave 7.1 g. (62%) of crude product, m.p. 189.0-189.5° C. (corr.)(dec.). Recrystallized from aqueous methyl alcohol, m.p. 190.5-191.0° C. (corr.)(dec.), α_D^{25} + 35° (c, 5% in 0.21 M HCl). Lit. m.p. 182-183° C. (crude product)(dec.). The rotation of the free acid was given in the literature as +38.3° (c, 5% in water)(30). The rotation of the sodium salt in 0.21 M HCl given above corresponds exactly to that of the free acid when corrections are made for the differences in concentration of the solutions whose rotations were measured. Potentiometric titration of the sodium salt gave an equivalent weight of 262 (theory 260).

Analysis:	Calculated:	C, 50.8; H, 5.0; N, 10.8.
$C_{11}H_{13}N_2O_4Na$(260)	Found:	C, 50.7; H, 4.8; N, 10.8.



1-Acetyl-2-[O-acetyl-α-N-acetyl-L-tyrosyl] hydrazine (IV)



1-Acetyl-2-[α-N-acetyl-L-tyrosyl] hydrazine (VIII)

E. Experimental - Kinetics

Enzymatic experiments were carried out in water in the pH-stat in the usual manner. The other conditions were constant pH, 25.0° C., constant ionic strength and a total reaction volume of 10 ml. The titration cell was swept by a stream of CO_2 -free nitrogen. Inhibition characteristics were determined against acetyl-L-valine methyl ester or N-acetyl-L-tyrosinhydroxamide as indicated below.

1-Acetyl-2-[L-tyrosyl]hydrazine (II) versus Acetyl-L-Valine Methyl Ester at pH = 7.90

Two experiments, each at a constant $[I]_o = 0.237 \times 10^{-3}$ M and $[I]_o = 0.474 \times 10^{-3}$ M were carried out. The kinetic constants k_3' and K_s' (i.e., apparent values) were determined at pH = 7.90 versus acetyl-L-valine methyl ester by varying the substrate concentration at constant inhibitor concentration, constant ionic strength (NaCl = 0.100 M) and $[E]_o = 0.1464$ mg. PN/ml. (II) behaved in a mixed competitive-non-competitive manner towards acetyl-L-valine methyl ester. The recorder traces were linear after t = 1 or 2 minutes. $K_I = 0.074 \pm .003 \times 10^{-3}$ M. The results were given in Table XIII . Notebook Ref. 934-87, 88, 89.

1-Acetyl-2-[L-tyrosyl]hydrazine (II) versus N-Acetyl-L-tyrosinhydroxamide (X)* at pH = 6.70

Inhibitor concentration (II) was maintained at 0.237×10^{-3} M, NaCl = 0.100 M, pH = 6.70 ± .01 and $[E]_o$ = 0.1464 mg. PN/ml. N-Acetyl-L-tyrosinhydroxamide as the sodium salt was used as the substrate by removing aliquots from a stock solution 0.0500 M in each of substrate salt and HCl. Substrate concentration was varied from $[S]_o$ = 7.5 to 35×10^{-3} M at six $[S]_o$ concentrations; the reaction solution was adjusted to 0.100 M NaCl by the addition of the calculated amount of aqueous NaCl solutions required to yield at t = 0 min. an ionic strength of 0.100 M.

The recorder traces of extent of conversion versus time were non-linear. Initial velocities were computed by the orthogonal polynomial method (33).

The inhibited run was compared to the same substrate under identical conditions but with $[I]_o = 0$. The apparent kinetic constants k_3' and K_s' were obtained from a least squares solution of equation 13

$$\frac{[S]_o[E]_o}{v_o} = \frac{K_s'}{k_3'} + \frac{1}{k_3'} \cdot [S]_0 \qquad (13)$$

where, $K_s' = K_s \left(1 + \frac{[I]_o}{K_I}\right)$

*. Stock solution of the sodium salt. At pH = 6.70 (X) exists principally as free N-acetyl-L-tyrosinhydroxamide (30).

The apparent k_3' values were corrected for the buffer effect due to hydroxylamine by equation 14 as shown in Part IV of this Thesis.

$$k_3 = k_3' \left(1 + \frac{\overset{+}{NH_3}OH}{NH_2 OH}\right) \qquad (14)$$

The ratio $\overset{+}{NH_3}OH/NH_2 OH$ at pH = 6.70 was calculated from the measured pKa' of hydroxylamine at μ = 0.1 M NaCl. Under the conditions specified above, and with the limited number of experimental points taken, inhibitor II behaved in a totally competitive manner towards N-acetyl-L-tyrosinhydroxamide. $K_I = 0.22 \times 10^{-3}$ M (at pH = 6.70 $\pm$ .01). (Note: pH 6.70 is not the optimum pH for this substrate. This pH was selected because of the contemplated use of inhibitor II with L-tyrosin methyl ester as a substrate.) The data are recorded in Table XIV.

Notebook ref. 934-99

1-Acetyl-2-[D-tyrosyl]hydrazine (XI) versus Acetyl-L-Valine Methyl Ester at pH = 7.90

An approximate competitive constant was obtained in the manner described for the nitrogen structural analogs of phenylalanine and glycine of Part I of this Thesis. At $[I]_o$ = 0.020 M, $[S]_o$ = 0.040 M, NaCl = 0.100 M, $[E]_o$ = 0.1464 mg. PN/ml., the value of K_I was 5.0×10^{-3} M. For $[I]_o$ = 0.0100 M, $[S]_o$ = 0.040 M, NaCl =

0.100 M, $[E]_o$ = 0.1464 mg. PN/ml., the value of K_I was 5.1×10^{-3} M. Enzymatic assay of the crude conversion product of N-acetyl-D-tyrosinhydrazide with HCl at 98° C. gave a similar value of K_I.

Notebook ref. 934-111, runs 2, 3

1-Acetyl-2-[L-tyrosyl]hydrazine-β-Naphthalene Sulfonate Salt VII versus Acetyl-L-Valine Methyl Ester at pH = 7.90

Two experiments at $[I]_o = 4.62 \times 10^{-4}$ M and 9.24×10^{-4} M, $[S]_o$ = 0.0400 M, NaCl = 0.100 M, pH = 7.90 gave $K_I = 0.080 \pm .006 \times 10^{-3}$ M. For the calculation of K_I of VII the system was treated as one containing enzyme-substrate plus two competitive inhibitors by the use of equation 15 , where K'_s has the usual Michaelis-Menten significance.

$$K'_s = K_s \left(1 + \frac{[I_1]_o}{K_{I_1}} + \frac{[I_2]_o}{K_{I_2}} \right) \qquad (15)$$

The value of K_{I_2}, the enzyme inhibitor constant for β-naphthalene-sulfonate anion at pH = 7.9 was taken to be 1.84×10^{-3} M. (See Part III this Thesis).

Notebook ref. 934-92, runs 1, 2

L-Tyrosinhydrazide versus Acetyl-L-Valine Methyl Ester at pH = 7.90

L-Tyrosinhydrazide is a slow substrate for alpha-chymotrypsin with a reported $k_3 = 0.03 \times 10^{-3}$ M/min.-mg. PN/ml. (3); therefore, it was treated as a competitive inhibitor for acetyl-L-valine methyl ester ($k_3 = 2.15 \times 10^{-3}$ M/min.-mg. PN/ml.). An approximate value for the apparent K_I was obtained, $K'_{I_L} = 7 \pm 0.5 \times 10^{-3}$ M (Lit. $K_s = 6 \pm 4 \times 10^{-3}$ M (3)). Measurements were made at $[I]_o = 8 \times 10^{-3}$ M and 4×10^{-3} M, $[S]_o = 0.0400$ M.

Notebook ref. 934-91, runs 13, 14

L-Tyrosinhydrazide-dinaphthalene Sulfonate Salt (V) versus Acetyl-L-Valine Methyl Ester at pH = 7.90

Eight runs were made at one inhibitor concentration such that the final concentration of L-tyrosinhydrazide was 0.183×10^{-3} M and that of β-naphthalene sulfonate anion was 0.355×10^{-3} M. $[S]_o$ was varied from 7.5 to 35×10^{-3} M. The apparent k'_3 value was $2.1 \pm 0.1 \times 10^{-3}$ M/min.-mg. PN/ml. and $K'_s = 0.148 \pm .007$ M so that the system behaved in a competitive manner towards acetyl-L-valine methyl ester. From K'_s of equation 15 and an assumed value of $K_{I_L} = 7 \times 10^{-3}$ M for L-tyrosinhydrazide the value of K'_I

for β-naphthalene sulfonate anion was computed to be $1.2 \pm 0.2 \times 10^{-3}$ M. The latter value was lower than the separately measured enzyme inhibitor constant for β-naphthalene sulfonate anion (See Part III) for which the value of K_I was known to be 1.84×10^{-3} M. The low value of K_I and the subsequent poor precision is attributed to the low $[I]_o/K_I$ values used in this experiment.

Notebook ref. 934-91, runs 1 to 8

1-Acetyl-2-[O-Acetyl-α-N-Acetyl-L-tyrosyl]hydrazine (IV) versus Acetyl-L-Valine Methyl Ester at pH = 7.90

Compound IV behaved as a substrate for alpha-chymotrypsin. IV at a concentration of 4×10^{-3} M, with $[E]_o$ = 0.1464 mg. PN/ml. gave an initial velocity, $v_o = 0.154 \times 10^{-4}$ M/min. (uncorr. for blanks) at pH = 7.90, NaCl = 0.100 M.

Compound IV treated approximately as a competitive inhibitor towards acetyl-L-valine methyl ester gave an apparent K_I' value of 3×10^{-3} M. $[I]_o = 1 \times 10^{-3}$ M, $[S]_o$ = .040 M, pH 7.90, NaCl = 0.100 M, measured $v_o = 0.704 \times 10^{-4}$ M/min.

The behavior of IV as a substrate was not further investigated; it is not known whether the enzymatic hydrolysis was confined to one or more of the three acetyl groups. IV should be an interesting compound for study as a substrate for alpha-chymotrypsin.

Notebook ref. 934-90, runs 8, 9

Kinetics of the Conversion of N-Acetyl-L-tyrosinhydrazide (I) to 1-Acetyl-2-[L-tyrosyl]hydrazine (II)

N-Acetyl-L-tyrosinhydrazide (I), 1.20 g. (0.0508 mole) was added with stirring to 95 ml. of distilled water in a flask equipped for reflux. The temperature of the system was adjusted to 96 ± 1° C. and 5 ml. of 1.04 M HCl were added rapidly. 5 ml. aliquots of the reaction mixture were removed at t = 8, 15, 30, 45, 60, 75, 90, 122, 150 and 180 min. with quenching in ice water. 1 ml. aliquots, undiluted, were titrated potentiometrically in the pH-stat versus 0.214 N NaOH. The titration curves showed two breaks, the first corresponded to an acid of pKa' = 3.2, the second to an acid of pKa' = 7; the appearance of product was taken as the difference between the two end-points. First order kinetics were observed up to 80% conversion.

For enzymatic assay, the original aliquots taken at the times indicated were diluted five-fold (i.e., maximum $[I]_o$ stock = 0.01 M). One ml. of the diluted aliquots were assayed with alpha-chymotrypsin at $[E]_o$ = 0.1464 mg. PN/ml. and acetyl-L-valine methyl ester at $[S]_o$ = 0.0400 M, NaCl = 0.100 M, pH = 7.90 ± .01, 25.0° C. The apparent concentration of inhibitor (II) was calculated from equation

using an assumed K_I value of 0.083×10^{-3} M, neglecting the contributions of any other species that may have been present since

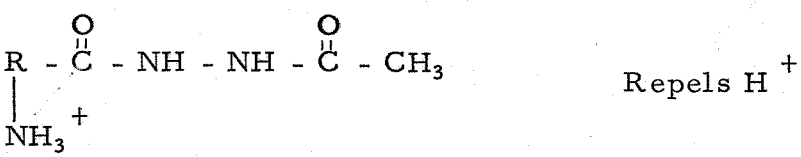
K_s for the latter were >> K_I for compound (II). First-order kinetics were observed up to 65% conversion. The slopes of the enzymatic first-order plots were lower than the slope of the titrimetric first-order plot as expected for the concurrent production of L-tyrosinhydrazide which would make a contribution to the slope of the titrimetric plot but not to the enzymatic plot.

The first-order constant for the enzymatic assay was 0.025 $min.^{-1}$ and 0.028 $min.^{-1}$ for the titrimetric first-order constant. The results are given in Table XII and first-order plots in figure 5. Hydrazine was also found amongst the products of the reaction. Hydrazine was determined spectrophotometrically as the p-dimethylaminobenzalazine (32). The per cent conversion to hydrazine versus time is shown below:

t (min.)	% conversion
8	0.28
30	0.52
75	0.68
122	0.76
180	0.82

The rapid decrease in hydrazine formation with time showed that 1-acetyl-2-[L-tyrosyl]hydrazine did not hydrolyze (i.e., deacylation) under the conditions of the experiment. The stability of II when protonated may be a consequence of mutual charge repulsion between the $-NH_3^+$ protonated base and H^+ necessary for the

acid-catalyzed hydrolysis of either of the acyl hydrazide bonds, i.e.,



Notebook ref. 934-75, 800-97

Reverse Reaction Studies

L-Tyrosine with Acethydrazide and alpha-Chymotrypsin

Incubation of L-tyrosine with enzyme and acethydrazide at 25° C. for 1 hour at pH 5.8 and at pH 7.9 did not result in the appearance of a species of low K_I, i.e., 1-acetyl-2-[L-tyrosyl]hydrazine. Therefore, it is unlikely that $k_3 = 0$ for inhibitor II as a consequence of a rapid recombination of L-tyrosine with acethydrazide at the active site.

L-Tyrosinhydrazide with HCl and Acetic Acid

To 100 ml. of 0.05 M HCl were added 0.975 g. (0.005 mole) L-tyrosinhydrazide and 0.30 g. (0.005 mole) glacial acetic acid. The resulting solution, in a flask equipped with reflux condenser, was placed in a steam bath (t = 95° C.) for four hours, then quenched in an ice bath. The concentration of inhibitor II, 1-acetyl-2-[L-tyrosyl]hydrazine, was estimated as follows:

An aliquot (0.4 ml.) of the crude reaction mixture was assayed for inhibitor II content versus acetyl-L-valine methyl ester in the manner described in Part I of this Thesis for the nitrogen analogs of phenylalanine and glycine. The estimate of the concentration of inhibitor II was made in an approximate manner by assuming that L-tyrosinhydrazide functioned as a competitive inhibitor in this system and calculations of $[I]_o$ were carried out with the aid of equation 15 in a manner analogous to the kinetic treatment described previously for 1-acetyl-2-[L-tyrosyl]hydrazine mono-naphthalene sulfonate (page 131). A value of $v_o = 0.653 \times 10^{-4}$ M min.$^{-1}$ was obtained, from which $K'_s = 0.148$ M. The assumption of a value for $K_I = 0.083 \times 10^{-4}$ M gave $[I]_o = 0.113 \times 10^{-3}$ M, equal to a maximum conversion of 5.6% since the sum of the concentrations of L-tyrosinhydrazide and inhibitor II equalled 0.002 M in the system being assayed.

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PART III

BINUCLEAR AROMATICS AS INHIBITORS OF ALPHA-CHYMOTRYPSIN-CATALYZED HYDROLYSES

A. β-Naphthalene Sulfonic Acid, a Competitive Inhibitor of alpha-Chymotrypsin-Catalyzed Hydrolyses.

The isolation of 1-acetyl-2-[L-tyrosyl] hydrazide as the β-naphthalene sulfonate salt made it necessary to separately evaluate the inhibitor characteristics (if any) of β-naphthalene sulfonic acid(I). An inhibition study was carried out with (I), using acetyl-L-valine methyl ester as the substrate.

The results of the study reported here were obtained at $[I]_o$ = 0.00158 M; $[S]_o$ was varied from 0.0100 M to 0.0700 M, pH = 7.90, NaCl = 0.100 M and $[E]_o$ = 0.1464 mg. PN/ml. Kinetic runs were made in the usual manner in the pH-stat. Recorder traces were linear and initial velocities were taken as the slope of the recorder trace. The data are given in Table XVI and plotted in figure 9. The constants K_S' and k_3' were obtained in the usual manner from the slope and intercept of the $[S]_o[E]_o/v_o$ versus $[S]_o$ plot. At $[I]_o$ = 0.00158 M, k_3' was found to be $2.25 \pm .19 \times 10^{-3}$ M/min.-mg. PN/ml. and $K_S' = 206 \pm 18 \times 10^{-3}$ M. The value of k_3' was in experimental agreement with k_3 for acetyl-L-valine methyl ester for which k_3 = $2.15 \pm 0.09 \times 10^{-3}$ M/min.-mg. PN/ml. (Part II of this Thesis); therefore, β-naphthalene sulfonate anion (the only species present at pH = 7.90) behaves in a totally competitive manner towards alpha-chymotrypsin.

Table XVI

The Inhibition of the alpha-Chymotrypsin-Catalyzed Hydrolysis of Acetyl-L-valine Methyl Ester by β-Naphthalene Sulfonate Anion[a]

Notebook ref. 934-87, runs 7,13-17
934-88, runs 16,17

$[S]_o$[b]	v_o[c]	$\frac{[S]_o[E]_o}{v_o}$[d]
70	0.829	123.6
50	0.646	113.3
40	0.519	112.8
30	0.418	105.1
20	0.292	100.3
15	0.227	96.7
10	0.148	98.9

$k_3' = 2.25 \pm 0.19 \times 10^{-3}$ M/min.-mg. PN/ml.

$K_S' = 206 \pm 18 \times 10^{-3}$ M.

$K_I = 1.84 \pm 0.27 \times 10^{-3}$ M.

a. $[I]_o = 1.58 \times 10^{-3}$ M, pH = 7.90, 25.0° C, NaCl = 0.100 M, $[E]_o = 0.1464$ mg. PN/ml.

b. Units of 10^{-3} M. c. Units of 10^{-4} M/min.

d. Units of min.-mg. PN/ml.

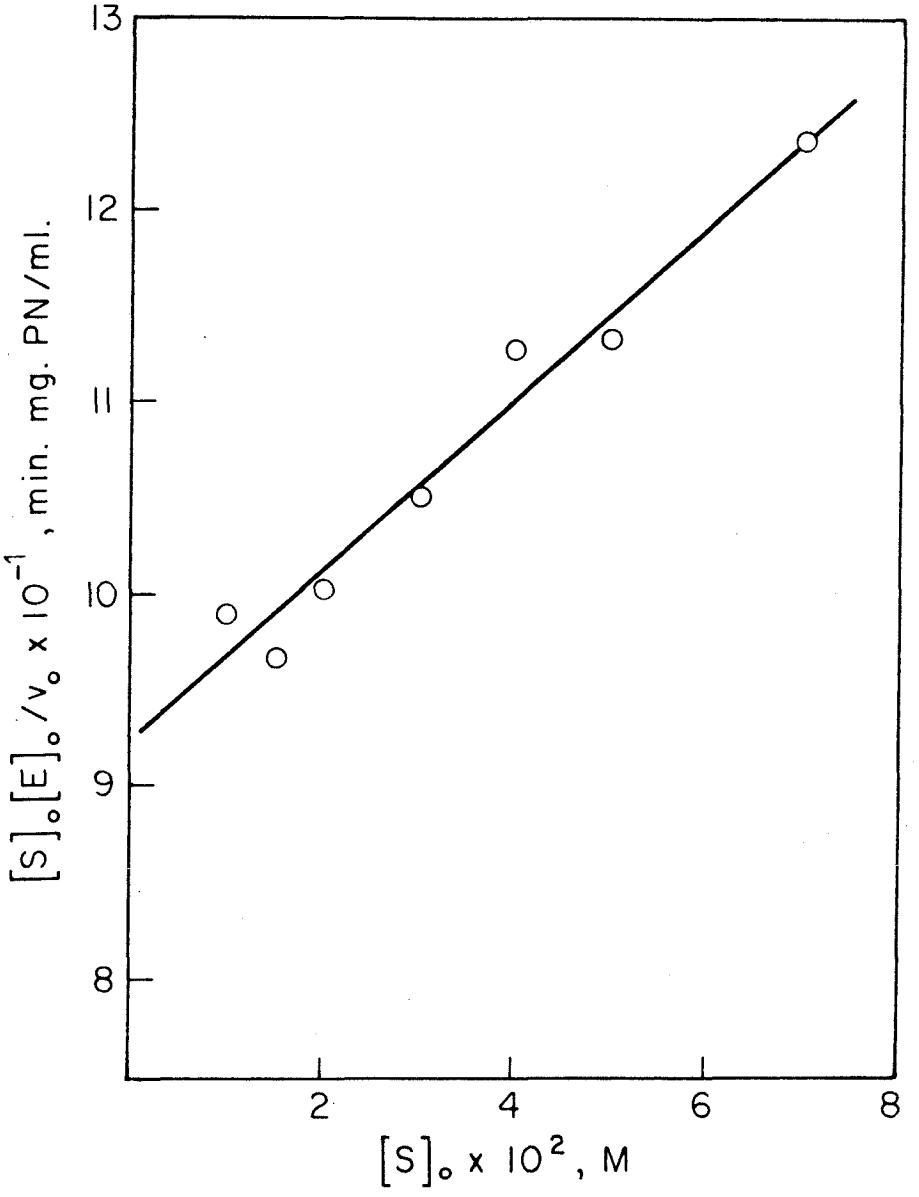


Figure 9. The Inhibition of Acetyl-L-Valine Methyl Ester by β-Naphthalene sulfonate at pH = 7.90, 25.0°C, NaCl = 1.00 M, $[E]_o$ = 0.1464 mg. PN/ml.

From equation 17 , the inhibition constant was calculated to be $K_I = 1.84 \pm 0.27 \times 10^{-3}$ M.

$$K_I = \frac{K_S ([I]_o)}{K'_S - K_S} \qquad (17)$$

B. The Competitive (Assumed) Inhibitor Constants of Naphthalene and Related Aromatic Compounds. Preliminary Results

The observation that β-napthalene sulfonate anion behaved in a totally competitive manner in the system alpha-chymotrypsin + acetyl-L-valine methyl ester permitted a rapid survey of the competitive inhibition constants of various naphthalene derivatives to be made. The scope of the survey was broadened by the inclusion of nitrogen containing bicyclic aromatics such as quinoline and benzimidazole.

Approximate competitive inhibition constants were obtained from the initial velocities obtained either at one inhibitor concentration with $[S]_o$ = 0.020 and 0.040 M or at one substrate concentration, $[S]_o$ = 0.040 M and two inhibitor concentrations, $[I]_o$ = value shown in Table XVII and $\frac{1}{2}[I]_o$ value shown in Table XVII . The results are given in Table XVII . The inhibition constants, K_I, were calculated from equation 18.

$$K_I = \frac{v_o K_S [I]_o}{k_3 [E]_o [S]_o - v_o (K_S + [S]_o)} \qquad (18)$$

where v_o is the initial velocity measured in the presence of inhibitor, corrected for blanks; $k_3 = 2.15 \times 10^{-3}$ M/min.-mg. PN/ml. and $K_S = 111 \times 10^{-3}$ M for acetyl-L-valine methyl ester; $[E]_o = 0.1464$ mg. PN/ml.

Discussion:

The results given in Table XVII show that alpha-chymotrypsin exhibits unusual affinity for binuclear aromatics. K_I* decreased significantly from 1.84 to 0.20 in the series β-naphthalene sulfonate > β-naphthoate > β-naphthylamine = α-naphthylamine > α-naphthol. The larger values for the anions are explained on the basis of a mutual negative charge repulsion between an enzyme site and the anionic sulfonate or carboxylate similar to the results obtained with indole-containing anions (4). It would be predicted that K_I for the anionic inhibitors would decrease about an order of magnitude at one pH unit lower (i.e., 6.9), hence the value of $K_I = 0.2 - 0.3$ obtained for α-naphthol and the two naphthylamines may be considered to be near the lower limits of K_I for the naphthalene nucleus.

* In this discussion K_I values are given in units of 10^{-3} M.

Table XVII

Approximate Competitive (Assumed) Inhibition Constants of Bicyclic Aromatics[a]

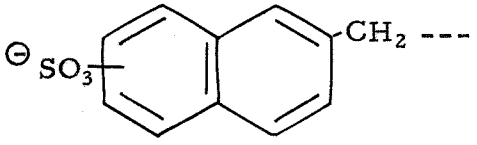
Compound	$[I]_o$[b]	K_I[b]	Ref.[c]
alpha-naphthol	2	0.20	c
alpha-naphthylamine	2	0.29	c
beta-naphthylamine	0.93	0.26	c
beta-naphthoate	6.5	1.4	c
beta-naphthalene sulfonate	1.58	1.84	c
2,7-dinaphthalene sulfonate	40	400	c
quinoline	0.89	0.6	c
quinoxaline	10	5	c
1-ethylquinolinium iodide	20	43	c
benzimidazole	2	3	c
imidazole	50	45	c
alpha-naphthyl propionate	-	3.8	(1)
alpha-naphthyl methyl malonate	-	55	(1)
benzenesulfonate	10	71	c
pyridine		50	(2)
benzoate		150	(3)
phenol		7	(2)

a. Measured against acetyl-L-valine methyl ester, $[S]_o$ = 0.020 M or 0.040 M, $[E]_o$ = 0.1464 mg. PN/ml., Lot No. T-97207, pH = 7.90, NaCl = 0.100 M, 25.0° C.

b. Units of 10^{-3} M. c. This Thesis.

The conclusion that K_I for the naphthalene nucleus should have a value = 0.2 is of great interest because it is much lower than indole, K_I = 0.7, the side-chain group that heretofore has shown the greatest affinity for alpha-chymotrypsin both as free indole and in indole-containing substrates and inhibitors. Accordingly, the study of naphthalene-analogs of amino-acid substrates of alpha-chymotrypsin should be commenced even though problems of insolubility may be anticipated.

The absence of inhibition by 2,7-dinaphthalene sulfonate, K_I = 400, is also of unusual interest because it appears to indicate that bonding to the naphthalene nucleus is effectively prevented by the presentation of a formal negative charge to the enzyme regardless of the orientation of the nucleus to the active site. Therefore, one would predict that sulfonated naphthalene substrates of the type



would not be substrates or inhibitors.

α-Naphthylpropionate has been reported to be a competitive inhibitor in the literature (1). It has also been used in equilibrium dialysis studies (4); however, these previous workers overlooked the fact that naphthalene-containing compounds show better affinity for alpha-chymotrypsin than do indole-containing compounds. For

example, Neurath and Gladner gave K_I = 3.8 for α-naphthylpropionate at pH = 7.8. The analogous indole compound, β-(β-indole)-propionate has a reported value of K_I = 11-15 at pH = 7.9; therefore, the use of naphthalene analogs should have been proposed by these authors.

As a result of these studies, it was predicted, and confirmed, that binuclear N-containing substances would also show good affinity towards alpha-chymotrypsin. It has been found that quinoline, with K_I = 0.6, is almost identical to indole, K_I = 0.7. Also benzimidazole has a reasonably low value, K_I = 3; however, quinoxaline gave a value of K_I nearly an order of magnitude greater than quinoline. 1-Ethyl quinolinium iodide was an even poorer inhibitor with K_I = 43; however, speculation as to whether the effect due to the latter is caused by the positively charged nitrogen or the bulky ethyl group is premature.

The employment of nitrogen binuclear aromatics as inhibitors also opens up the study of fast substrates such as the tyrosine and tryptophan esters, since their relatively large solubility, e.g. quinoline = 0.2 M and quinoxaline (very soluble in water) would permit large values of the ratio $[I]_o / K_I$ to be experimentally achieved; hence fast substrates could be evaluated under conditions that approach zone A, although the anticipated relationship $K_S' >> [S]_o$ may exclude the experimental separation of the constants K_S and k_3, but should still yield good values for k_3 / K_S, the pseudo-first order constant.

The compounds reported in Table XVII were commercial products of reagent or pure grade. β-Naphthalene sulfonic acid monohydrate was recrystallized from isopropyl alcohol; α- and β-naphthylamine were recrystallized as the hydrochlorides; quinoline was distilled, a middle cut being taken.

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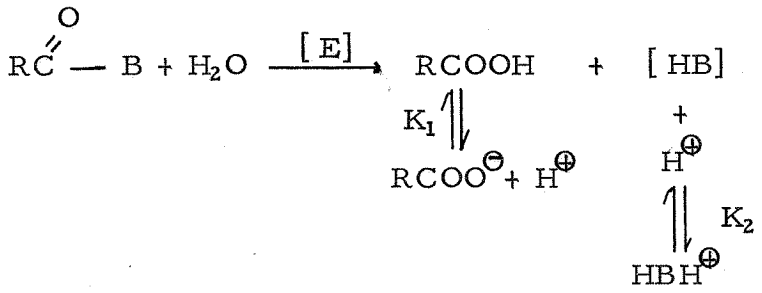
PART IV

APPLICABILITY OF THE pH-STAT TO ALPHA-CHYMOTRYPSIN CATALYZED HYDROLYSES THAT PRODUCE A BUFFER

A. Introduction

In part IV of this Thesis we are concerned with alpha-chymotrypsin catalyzed hydrolyses that yield a product having the characteristics of a buffer. A general formulation for the case where the substrate is not a buffer would be given by equation 19



(19)

where B_1 the leaving group may be $-NH_2$, $-OR'$, $-NHOH$, $-NHNH_2$, or $-NHR''$ for an amide, ester, hydroxamide, hydrazide or peptide, representing the range of substrates normally encountered in alpha-chymotrypsin catalyzed hydrolyses.

In the formulation presented in equation 19 , the leaving group B is a very strong base, hence as a product it always exists as the species BH, since the dissociation constant K_3 for the reaction: $BH \overset{K_3}{\rightleftharpoons} B^{\ominus} + H^{\oplus}$ is of the order of magnitude $\leq K_w$ (K_w = dissociation constant for $H_2O \rightleftharpoons H^+ + OH^{\ominus}$). Therefore, in the systems usually studied in the pH-Stat, i. e. $pH < 10$, we may neglect the effect of K_3 on any of the acid-base equilibria and need only consider formulation 19 . Also, for the case at hand, the dissociation constant K_1, for the carboxylic acid produced in the hydrolytic reaction should exceed $[H^+]$ of the system by at least two orders of magnitude, i. e. $pK_1 \leq (pH - 2)$ for the condition that better than 99% of the carboxylic function be titratable. The latter condition is usually fulfilled in studies

of alpha-chymotrypsin catalyzed hydrolyses of amino acid derivatives since the values of K_1 for the latter are such that $pK_1 \leq (pH - 2)$ where pH refers to the operating constant pH of the hydrolyses usually studied in the range of pH 6 to 9.

Hence, for all practical purposes we need only consider the effect of the buffering capacity of $HBH^{\oplus} \rightleftarrows HB + H^{\oplus}$, or K_2, for the range of pH 6 to 9 normally encountered in studies of the alpha-chymotrypsin catalyzed hydrolyses of acylated amino acid substrates.

The problem that arises during kinetic studies of enzyme catalyzed hydrolyses (alpha-chymotrypsin in particular) is two-fold: first, one needs to know whether the stoichiometry, i. e. the addition of standardized base, is qualified by the presence of the buffer, and second, whether the Michaelis-Menten constants, K_s and k_3, are related to the buffer dissociation constant K_1. The problem of stoichiometry may be resolved rather simply. For the condition that $pK_1 \ll pH$ and $pK_1 \ll pK_2$ we may look upon the species $[HB]$ as one capable of removing $[H^+]$ from the system, i. e. $[HB]$ behaves as an internal base, lessening the requirement for a constant pH that external $OH^{\ominus}$ be added to the system in order to neutralize the $H^{\oplus}$ resulting from the dissociation of the RCOOH molecule. In the absence of secondary reactions, product balance is such that:

$$[RCOO^{\ominus}] = [HB] + [HBH]^{\oplus}$$

and the observed or apparent concentration of product at time t, $[RCOO^{\ominus}]_t'$, must be corrected to the true concentration of product $[RCOO^{\ominus}]_t$ by equation 20 to compensate for the loss of $[H^+]$ to the

buffer [HB].

$$[RCOO^{\ominus}]_t = [RCOO^{\ominus}]_t' \left(1 + \frac{[HBH]^+}{[HB]}\right) \qquad (20)$$

In effect then, the presence of a buffer product causes the apparent extent of conversion of a substrate to be less than the true extent of conversion by the factor $\left(1 + \frac{[HBH]^+}{[HB]}\right)$. This buffer effect explains the failure to observe 100% stoichiometry in the enzymatic cleavage of a single peptide bond in a protein, e. g. Otteson (1) observed 90% stoichiometry during the subtilisin-catalyzed conversion of ovalbumin to plakalbumin at constant pH = 8 . If a peptide bond were split in the latter conversion then knowledge of the pKa' of the free amino-end of the new peptide and application of equation 20 would correct the apparent stoichiometry. It is also apparent that this method may be used to determine pK_2' for the case of the production of a single buffer group. Since the pH-Stat, or other constant pH systems are now being employed for the study of enzyme-catalyzed peptide cleavage (1 to 5) care must be exercised in the interpretation of the data.

The second qualification, namely, the elucidation of the effect of K_2 on the Michaelis-Menten constants may also be considered on the basis of the above formulation, however, with the added assumption that all ionization equilibria are achieved rapidly, i. e. the equilibrium expressed by K_2 does not affect the steady-state. The latter assumption is a reasonable one and is actually implicit even for the case where HB is not a buffer due to the great excess of water that is always present in these systems.

Application of the Michaelis-Menten scheme to the buffer case considered here is presented in detail in Appendix III. The development is complex but the results are simple. It is shown in Appendix III that the presence of the buffer affects only the value of k_3', the apparent forward velocity constant that is obtained from the customary plots of $[S]_o[E]_o/v_o$ vs $[S]_o$ (or any equivalent plot) when v_o is taken to be the measured initial velocity. The value of K_s', the apparent enzymesubstrate constant, is independent of the presence of the buffer at the substrate concentrations usually encountered. It was found that k_3, the true velocity constant, was related to k_3' the apparent velocity constant, by equation 21

$$k_3 = k_3' \left(1 + \frac{[HBH]^+}{[HB]} \right) \qquad (21)$$

This theoretical development was confirmed by the determination of K_s and k_3 in the pH-Stat for a variety of tyrosine substrates possessing the buffer, B, groups -NHOH, $-NHNH_2$ and $-NH_2$ exemplifying a range of pK_2' of 5.99 to 9.24. Since the values of K_s and k_3 for the substrates chosen were already known, having been measured by independent methods, confirmation for the buffer method arises as a consequence of the agreement between the known values and the buffer experimental values.

B. Kinetics-Experimental

The experimental method employed in this study is exactly equivalent to the pH-Stat method of determining K_s and k_3 in the absence of a buffer described previously in this Thesis. The computation of the apparent constant K_s' and k_3' from equation 22 is also carried out as described previously.

$$\frac{[S]_o[E]_o}{v_o} = \frac{K_s'}{k_s'} + \frac{[S]_o}{k_3'} \qquad (22)$$

However, it should be noted that the apparent initial velocity, v_o', is a direct function of the apparent k_3' which in turn is always lower than k_3, the true constant, by the factor $1/(1 + [HBH]^+/[HB])$. Accordingly, measured initial velocities are lower than in the absence of the buffer by the same factor, hence, in order to get measured velocities $> 0.1 \times 10^{-4}$ M/min. substrate concentration and/or enzyme concentration must be increased as the basicity of [BH] increases, i.e. as the factor $\left(1 + \frac{[HBH]^+}{[HB]}\right)$ increases. It is wise to accept 0.1×10^{-4} M/min. as a lower limit to an acceptable measured initial velocity because enzyme blanks are of the order of magnitude of v blank $\doteq 0.01 - 0.1 \times 10^{-4}$ M/min-mgPN/ml.

Effect of Ionic Strength on pK_2'

The buffer method also requires that the ratio $\frac{[HBH]^+}{[HB]}$ be known with great accuracy, especially as to the effect of ionic strength on pK_2'. The latter were determined in the pH-Stat by the $\frac{1}{2}\, pK_a'$ method as a function of ionic strength; the results are recorded in Table XVIII.

Table XVIII

The Effect of Ionic Strength on pKa ' of Several Buffers [a, b], at 25.0°C.

Buffer: $NH_3 + H^+ \rightleftarrows [NH_4]^+$

NaCl M	pKa ' (= pK_2')
≤0.002	9.20
0.46	9.26
0.92	9.27
1.84	9.24

$\overline{pK_2'} = 9.24 \pm .02$

Lit. $pK_o' = 9.25$ (7)

Buffer: $NH_2\text{-}NH_2 + H^+ \rightleftarrows [NH_2\text{-}NH_3]^+$

≤0.02	8.10
0.10	8.10
1.00	8.11

$\overline{pK_2'} = 8.10 \pm .01$

Lit. pK_o' (20°C) = 8.12 (8)

Buffer: $NH_2OH + H^+ \rightleftarrows [NH_3OH^+]$

NaCl M	pKa ' (= pK_2')
≤0.002	5.99
0.032	6.04
0.064	6.03
0.128	5.98
0.256	6.01
0.460	6.04
0.92	6.00
1.38	6.01
1.84	5.93
2.30	5.97

$\overline{pK_2'} = 5.99 \pm 0.02$

Lit. $pKa_o' = 5.96$ (6)

Table XVIII (Contd.)

Buffer:	$CH_3ONH_2 + H^+ \rightleftharpoons [CH_3ONH_3]^+$	
0.002	4.59	
0.032	4.55	
0.064	4.58	
0.128	4.59	$\overline{pK_2'} = 4.61 \pm .03$
0.256	4.62	
0.46	4.70	
1.38	4.62	
1.84	4.62	
2.30	4.64	

a. The precision of a pair of measurements was found to be $\pm$ 0.01 pKa ' unit.

b. All bases were added to the system as the hydrochlorides; aqueous NaCl was added to bring the ionic strength to the value indicated and the volume of the system adjusted to 10 ml. In each case the solutions were 0.002 M with respect to the buffer and were titrated with 0.0536 M NaOH in the pH-Stat.

The precision of any pair of measurements was found to be $\pm$ 0.01 pKa' unit; however, some scatter in the data was also evident. Therefore if this method is eventually developed to a point where a deviation of $\pm$ 0.03 pKa' units can cause large errors in the correction factors in comparison to the kinetic experimental error of measuring initial velocities then the pKa' values should be determined by a more accurate method. However, it is also evident that a precision of $\pm$ 0.03 pK_2' units only introduces a maximum error of $\pm$ 4% for the case where $pH = pK_2'$, i.e. the ratio $1 + \frac{[HBH]^+}{[HB]}$ for that case varies from 2.07 to 1.93 ($\Delta = \pm$ 4%). Since the error due to the uncertainty in measured pK_2' increases as the value of pK_2' exceeds the value of the pH, the ionic strength effects on pK_2' shown in Table XVIII may be neglected for all cases where $pK_2' < pH$ and an average pK_2' may be used. For those buffers having $pK_2' > pH$, pK_2' must be known to a greater precision than $\pm$ 0.03 in order to reduce the over-all error. The base methoxyamine was also included in this study in anticipation of its use in substrates. The value of pK_2' was 4.61 $\pm$ 0.03 for the range of NaCl = 0.002 to 2.30 M, therefore the methoxy group shows a base-weakening effect on the basicity of the amino group.

Enzyme Catalyzed Hydrolyses

The following tyrosine substrates, representing the range of product buffers of pK_2' = 5.99 to 9.24 were evaluated by the method previously discussed. Since the precision of the method is a function of the buffer capacity the results will be discussed according to buffer types, i.e. amides, hydrazides and hydroxamide

for which pK_2' decreases in the order 9.24, 8.10, 5.99.

Amides

N-Acetyl-L-tyrosinamide and N-nicotinyl-L-tyrosinamide were evaluated at pH = 7.90, 25.0°C, $[E]_o$ = 0.293 mg. PN/ml. at two ionic strengths, NaCl = 0.116 M and 1.93 M. The runs were characterized by a sharp drop in the pH of the system to ca. pH = 6 to 7 when the 1 ml of pH = 7.95 enzyme stock solution was added to the substrate system in the pH-Stat. The pH was rapidly adjusted to pH = 7.90 by the rapid addition of 1 N NaOH. In all cases, at t = 1 minute the operating pH of 7.90 had been reestablished. It was observed that the amount of 1 N NaOH required to reestablish the pH was roughly proportional to $[S]_o$, however, a quantitative relationship could not be established.

The recorder traces were sharply curved in all cases as would be expected for reactions proceeding to a large extent of conversion. An estimate of the initial velocity was made at t = 1 min., i.e. v_1, from the data obtained at t = 1 to 9 minutes by the use of the 17 point orthogonal polynomial procedure* in the manner described in Part I of this Thesis. Enzyme blanks were high in these runs due to the high concentration of enzyme employed and gave values of $v_E = 0.14 \pm 0.02 \times 10^{-4}$ M/min. at NaCl = 0.116 M and $v_E = 0.067 \pm 0.001 \times 10^{-4}$ M/min. at NaCl = 0.93 M. Substrate blanks were absent and the enzyme blanks were treated by the method of Martin and Niemann (9).

*Appendix I, code 200-001

In the subsequent discussion only the values of k_3, the apparent k_3' corrected for the buffer effect, are given. The apparent k_3' as well as other pertinent data and results are given in Tables XIX and XX.

Results

Acetyl-L-tyrosinamide at NaCl = 1.93 M gave the value of $k_3 = 7.2 \pm 3.2$,* $K_s = 44 \pm 23$. Nicotinyl-L-tyrosinamide at NaCl = 0.116 M gave the values of $k_3 = 4.6 \pm 1.3$ and $K_s = 16 \pm 6$; at NaCl = 1.93 M, $k_3 = 6.0 \pm 0.6$, $K_s = 2.3 \pm 1$. The literature values for acetyl-L-tyrosinamide are $k_3 = 2.4$, $K_s = 32$ at 0.02 M THAM and for nicotinyl-L-tyrosinamide $k_3 = 5.0$, $K_s = 12$ at 0.02 M THAM.** Since neither one of these substrates has been previously evaluated at varying ionic strengths, K_s and k_3 are not strictly comparable, for the present study and the literature values, however, it is noted that for nicotinyl-L-tyrosinamide the values of k_3 are in excellent agreement with NaCl = 0.116 M and THAM = 0.02 M; also, the values of K_s agree within experimental error. The results obtained with nicotinyl-L-tyrosinamide stand in sharp contrast to the results of a study of the affect of ionic strength on K_s and k_3 for chloroacetyl-L-tyrosinamide (10). With the latter substrate it was found that K_s was independent of ionic strength up to 1.5 M NaCl or KCl; the increase in the rates of the enzymatic hydrolyses was reflected completely in the increase of k_3.

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*All values of k_3 in units of 10^{-3} M/min.-mg. PN/ml., and K_s in units of 10^{-3} M will be used throughout Part IV.

**Tris-(hydroxymethyl)-aminomethane hydrochloric acid buffer.

Table XIX

Acetyl-L-Tyrosinamide Evaluated as a Substrate in the pH-Stat at pH = 7.90, 25.0°C, $[E]_o$ = 0.293 mg. PN/ml.

Notebook Ref. 934-64

NaCl = 1.93 M

$[S]_o$ [a]	v_1 [b]	P_m [c]
28.9	0.370	4
28.9	0.374	4
22.5	0.337	4
22.5	0.265	4
16.0	0.250	3
16.0	0.154[e]	2
9.63	0.168	3
9.63	0.154	3
6.42	0.117	4
6.42	0.139	4

k_3' [c] = 0.31 ± .14

k_3 [c,d] = 7.2 ± 3.2

K_s' [a] = 44 ± 23

a. Units of 10^{-3} M

b. 17 point orthogonal polynomial method, v_1 = initial velocity at t = 1 min., $t_{.10}$ level. Units of 10^{-4} M/min., corrected for enzyme blank.

c. Units of 10^{-3} M/min.-mg. PN/ml.

d. $k_3 = k_3' (1 + \frac{NH_4^+}{NH_3}) = 23k_3'$

e. Without this point, k_3' [c] = 0.287 ± 0.059; k_3 [c,d] = 6.6 ± 1.3; K_s' [a] = 38.5 ± 8.8

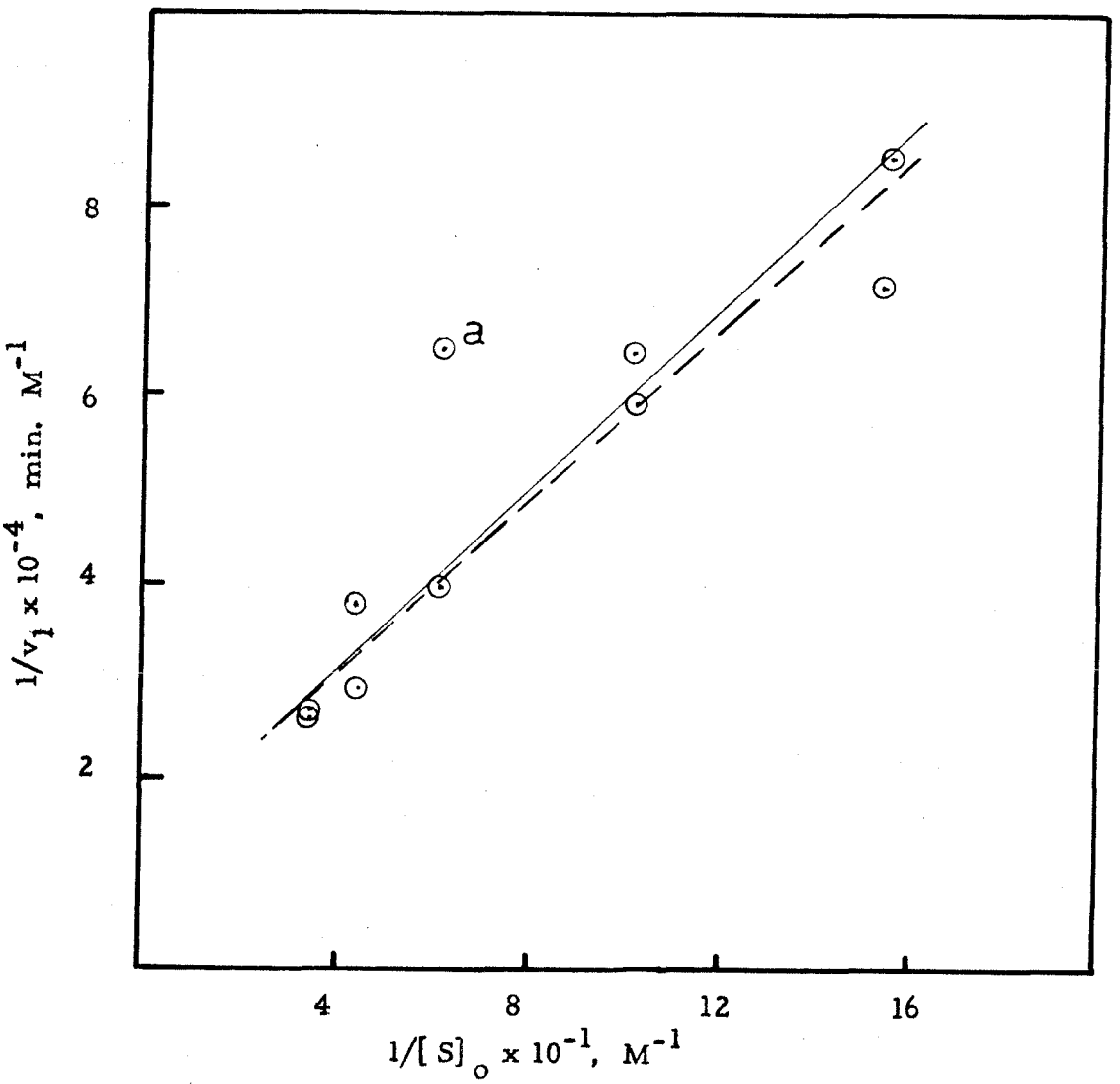

Figure 10 . The alpha-Chymotrypsin-Catalyzed Hydrolysis of Acetyl-L-tyrosinamide in the pH-stat. pH = 7.90, 25.0°C, $[E]_o$ = 0.293 mg. PN/ml, NaCl = 1.93 M. a. Dashed least squares without this point.

Table XX

Nicotinyl-L-Tyrosinamide Evaluated as a Substrate in the pH-Stat.

$[E]_o$ = 0.293 mg-PN/ml; pH = 7.90, 25.0°C.

Notebook Ref. 934-46, -65

NaCl = 0.116 M			NaCl = 1.93 M		
$[S]_o$ [a]	v_1 [b]	P_m [c]	$[S]_o$ [a]	v_1 [b]	P_m [c]
18	0.275	3	14.4	0.589	2
18	0.315	3	14.4	0.650	3
12	0.247	2	11.6	0.704	3
9	0.198	3	11.6	0.550	2
9	0.188	2	8.66	0.637	4
6	0.152	2	8.66	0.566	4
6	0.102	1	5.78	0.688	3
3	0.129	4	5.78	0.613	5
3	0.091	2	5.78	0.444	3
			2.89	0.371	3
			2.89	0.344	3
k_3' [c] = 0.202 ± .055			k_3' [c] = 0.262 ± 0.026		
k_3' [c,d] = 4.6 ± 1.3			k_3 [c,d] = 6.0 ± 0.6		
K_s' [a] = 16.1 ± 5.7			K_s' [a] = 2.3 ± 1.0		

a. Units of 10^{-3} M/min.-mg. PN/ml.

b. 17 point orthogonal polynomial method, v_1 = initial velocity at t = 1 min., $t_{.10}$ level. Units of 10^{-4}M/min., corrected for enzyme blank.

c. Units of 10^{-3} M/min-mg PN/ml.

d. $k_3 = k_3' \left(1 + \frac{NH_4^+}{NH_3}\right) = 23k_3'$

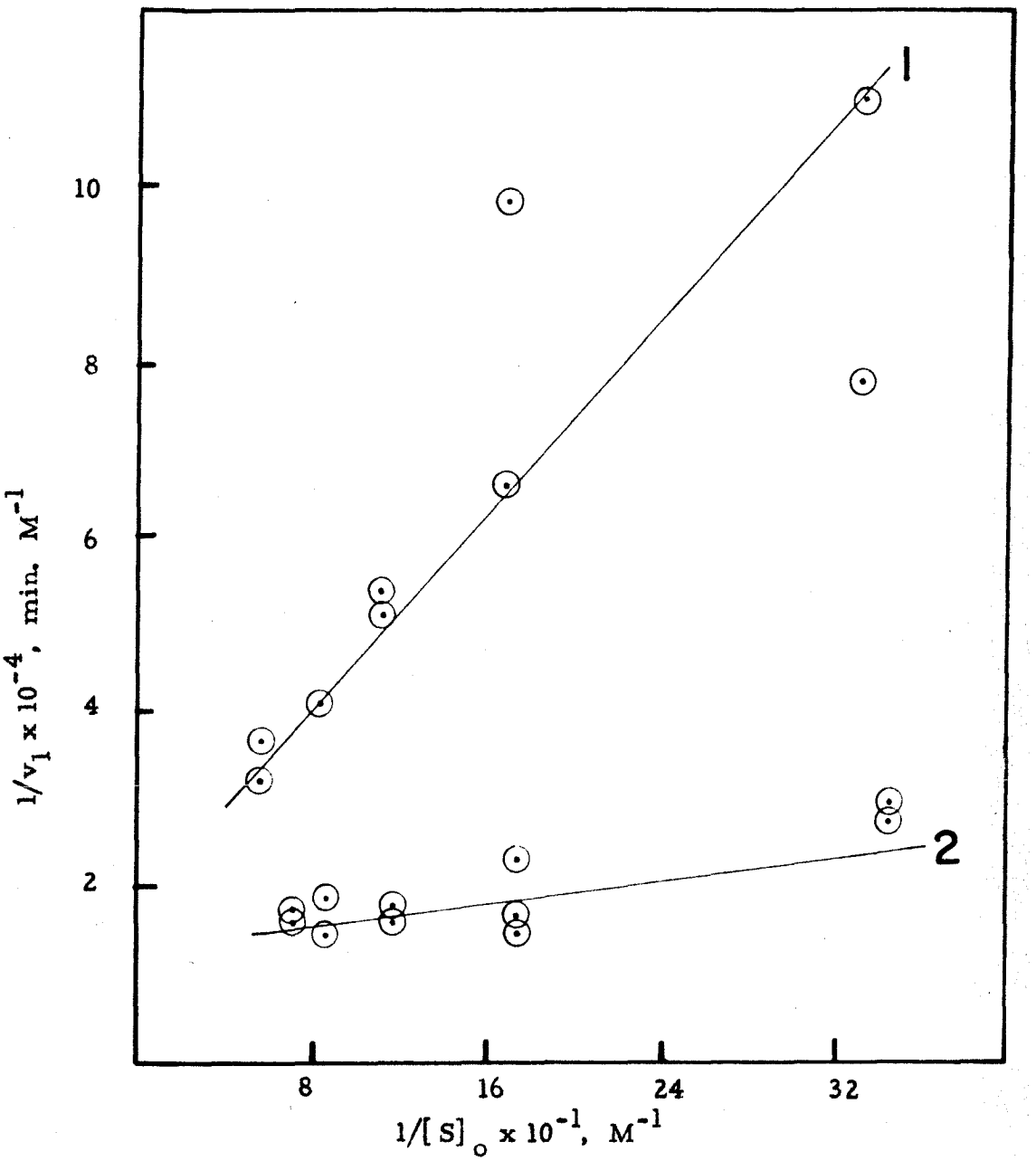

Figure 11 . The alpha-Chymotrypsin-Catalyzed Hydrolysis of Nicotinyl-L-tyrosinamide in the pH-stat at pH = 7.90, 25°C, $[E]_o$ = 0.293 mg. PN/ml. Curve 1: NaCl = 0.116 M; Curve 2: NaCl = 1.93 M.

The results obtained with acetyl-L-tyrosinamide at ionic strength NaCl = 0.116 M were not acceptable since the measured apparent velocities were of the order of the magnitude of the enzyme blank. At the higher ionic strength the results were more reasonable but precisions of the order of $\pm$ 50% were obtained. This poor result does not reflect a poor reliability of the buffer method; on the contrary, since the apparent measured initial velocities were similar in order of magnitude to the enzyme blank, one can only say that any experimental procedure that would give velocities in the range of the enzyme blank should be considered to be unreliable. It should also be noted that the results at high ionic strength, where the enzyme blank has a lower value, do not necessarily improve in precision, therefore, the use of amides in the pH-Stat should be limited to exploratory studies.

Nicotinyl-L-tyrosinamide gave values of k_3 and K_s comparable to the literature values, however, the reproducibility of duplicate runs was very poor due to the difficulty of establishing the initial steady state at high enzyme concentrations.

It is possible that the use of integrated equations could improve the results, however, in the latter case, some sort of graphical correction to the first few minutes would still need to be applied.

Hydrazides

N-Acetyl-L-tyrosinhydrazide and N-trimethylacetyl-L-tyrosinhydrazide were evaluated in the same manner that was described for the amides. Two runs were made for each substrate

at two ionic strengths, 0.100 M NaCl and 1.93 M NaCl.

N-Acetyl-L-tyrosinhydrazide

N-Acetyl-L-tyrosinhydrazide gave recorder traces that were essentially linear after the first two minutes, therefore, a first order equation was fit to the data obtained during the 2 to 10 minute interval, the slopes of these straight lines were then taken to be equal to the initial velocities. The kinetic constants k_3' and K_s' were calculated by equation 22 in the usual manner. At NaCl = 0.100 M a value of $k_3 = 1.0 \pm 0.2$ and $K_s' = 23.3 \pm 4.2 \times 10^{-3}$ M was obtained. At NaCl = 1.93 M, a value of $k_3 = 2.4 \pm 0.2$ and $K_s' = 18.6 \pm 1.4 \times 10^{-3}$ M, with $[S]_o = 2.89$ to 11.6×10^{-3} M at NaCl = 1.93 M, and $[S]_o = 3.0$ to 12.0×10^{-3} M at NaCl = 0.100 M. All experiments were carried out at a single enzyme concentration, $[E]_o = 0.1464$ mg. PN/ml. The enzyme blanks were $v_E = 0.025 \times 10^{-4}$ M/min. and 0.019×10^{-4} M/min. at NaCl = 0.100 M and 1.93 M respectively.

The literature values of k_3 and K_s for this substrate were originally evaluated (11) at $k_3 = 0.7 \pm 0.2$ and $K_s = 22 \pm 8 \times 10^{-3}$ M in 0.02 M THAM. In a subsequent work (12) revised values of $k_3 = 1.1 \pm 0.2$ and $K_s = 29.5 \pm 6.0 \times 10^{-3}$ M were obtained by an improved procedure. The values of k_3 and K_s reported in this Thesis agree with the latest values within the experimental error of the latter. However, it was observed in this Thesis that K_s' decreased slightly (but not very significantly) as the ionic strength increased which may account in part for the higher value of $K_s' = 29.5 \pm 6$ reported in the literature (12). It is concluded that the pH-Stat buffer correction method described here

is quite suitable for this substrate. The data are given in Table XXI and plotted in figure 12.

N-Trimethylacetyl-L-tyrosinhydrazide was evaluated at NaCl = 0.100 M and NaCl = 0.92 M at pH = 7.90 but could not be evaluated at an ionic strength of 1.9 M NaCl due to "salting-out" of the substrate. This substrate is soluble in water to the extent of 0.25 M therefore its behavior was investigated over a wide range of substrate concentrations, from 10 to 200 x 10^{-3} M at NaCl = 0.100 M and from 10 to 150 x 10^{-3} M at NaCl = 0.92 M. Recorder traces of extent of conversion versus time were erratic within the first 3 minutes due to difficulties in re-establishing the operating pH after the addition of enzyme; however, the traces were reasonably linear beyond t = 3 minutes. The data are given in Table XXII and plotted in figure 13.

The constants k_3 and K_s' were evaluated over the entire substrate concentration range. At NaCl = 0.1 M values of k_3 = 1.8 $\pm$ 0.1 and K_s' = 152 $\pm$ 10 x 10^{-3} M were obtained. At NaCl = 0.92 M values of k_3 = 2.0 $\pm$ 0.2 and K_s' = 104 $\pm$ 15 x 10^{-3} were obtained.

The literature values (11) for this substrate at 0.02 M THAM and pH = 7.8 for the range $[S]_o$ = 0.49 to 10.48 x 10^{-3} M, are k_3 = 0.4 $\pm$ 0.2 and K_s = 45 $\pm$ 20. The same reference gives for the same $[S]_o$ = 2.99 to 10.48 x 10^{-3} M, k_3 = 0.4 $\pm$ 0.02 and K_s = 40 $\pm$ 20. The reason for the lack of agreement between the two methods is not obvious. Some of the velocities measured in the pH-Stat were not very reproducible, others, viz. the points at NaCl = 0.92M and $[S]_o$ = 0.125 and 0.15 M were distinctly in error with respect to the remaining data. The constants k_3 and K_s'

Table XXI

The alpha-Chymotrypsin-Catalyzed Hydrolysis of N-Acetyl-L-Tyrosinhydrazide in the pH-Stat at pH = 7.90, 25.0°C.

$[E]_o$ = 0.1464 mg-PN/ml. Lot No. 283

NaCl = 0.100 M				NaCl = 1.93 M			
$[S]_o$ [a]	v_o [b]	$\frac{1}{v_o}$ [c]	$\frac{1}{[S]_o}$	$[S]_o$ [a]	v_o [b]	$\frac{1}{v_o}$ [c]	$\frac{1}{[S]_o}$
12.0	0.205	4.88	83.3	11.6	0.530	1.89	86.2
12.0	0.204	4.90	83.3	11.6	0.538	1.86	86.2
9.0	0.152	6.58	111	8.67	0.438	2.28	115
9.0	0.174	5.75	111	8.67	0.448	2.23	115
7.5	0.136	7.35	133	7.22	0.377	2.65	139
7.5	0.138	7.25	133	7.22	0.393	2.54	139
6.0	0.126	7.94	167	5.78	0.314	3.18	173
6.0	0.109	9.17	167	5.78	0.312	3.21	173
4.5	0.091	11.0	222	4.33	0.265	3.77	231
4.5	0.097	10.3	222	4.33	0.272	3.68	231
3.0	0.070	14.3	333	2.89	0.189	5.29	346
3.0	0.069	14.5	333	2.79	0.186	5.38	346

k_3' [e] = 0.390 ± 0.066 — k_3' [e] = 0.936 ± .042

k_3 [e,f] = 1.0 ± 0.2 — k_3 [e,f] = 2.41 ± 0.11

K_s' [a] = 23.3 ± 4.2 — K_s' [a] = 18.6 ± 0.9

a. Units of 10^{-3}M

b. Units of 10^{-4} M/min. obtained from a first order least squares fit of the 2 - 10 min. data.

c. Units of $10^4 M^{-1}$

d. Units of M^{-1}

e. Units of 10^{-3}M/min-mg. PN/ml

f. k_3 obtained from the following:

$$k_3 = k_3' \left(1 + \frac{NH_2\ NH_3^+}{NH_2\ NH_2}\right) = 2.58\ k_3'$$

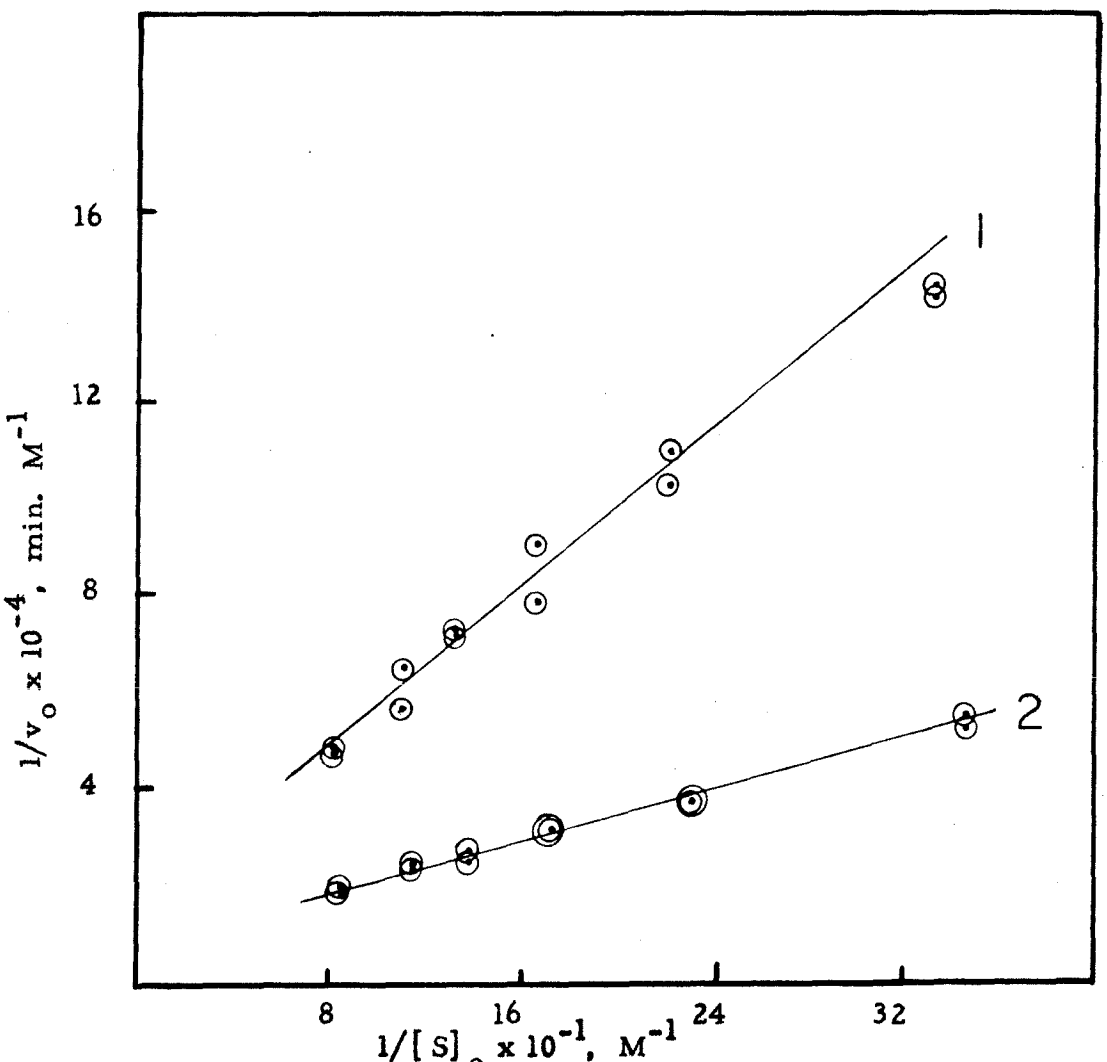

Figure 12 . The alpha-Chymotrypsin-Catalyzed Hydrolysis of Acetyl-L-tyrosinhydrazide in the pH-stat at pH = 7.90, 25°C, $[E]_o$ = 0.1464 mg. PN/ml. Curve 1: NaCl = 0.10 M; Curve 2: NaCl = 1.93 M.

Table XXII

The alpha-Chymotrypsin-Catalyzed Hydrolysis of N-Trimethylacetyl-L-Tyrosinhydrazide in the pH-Stat at pH = 7.90, 25.0°C.

$[E]_o$ = 0.1464 mg.-PN/ml. Lot No. 283. Lot No. 283

Notebook Ref. 934-81, 82, 83, 84

NaCl = 0.100 M			NaCl = 0.92 M		
$[S]_o$ [a]	v_o [b]	$\frac{[S]_o[E]_o}{v_o}$ [c]	$[S]_o$ [a]	v_o [b]	$\frac{[S]_o[E]_o}{v_o}$ [c]
200	0.625	4.69	150	0.758	2.90
200	0.600	4.88	125	0.687	2.66
150	0.501	4.38	125	0.715	2.56
150	0.497	4.42	100	0.477	3.04
100	0.395	3.71	100	0.498	2.94
100	0.390	3.75	75	0.439	2.50
75	0.368	2.98	75	0.432	2.54
75	0.372	2.95	50	0.336	2.18
50	0.257	2.85	50	0.352	2.08
50	0.327	2.24	35	0.278	1.85
35	0.178	2.88	35	0.268	1.91
35	0.175	2.93	25	0.223	1.64
25	0.143	2.56	20	0.186	1.58
25	0.139	2.63	20	0.214	1.37
20	0.130	2.25	15	0.163	1.34
20	0.126	2.33	15	0.163	1.34
15	0.099	2.22	10	0.130	1.13
15	0.095	2.31			
10	0.064	2.29			
10	0.064	2.29			

Table XXII (Cont'd.)

$k_3'^{d}$ = 0.71 ± 0.05	$k_3'^{d}$ = 0.78 ± 0.09
$k_3^{d,e}$ = 1.8 ± 0.1	$k_3^{d,e}$ = 2.0 ± 0.2
$K_s'^{a}$ = 152 ± 10	$K_s'^{a}$ = 104 ± 15

For $[S]_o^{a,f}$ = 10 - 100

$k_3'^{d}$ = 0.51 ± 0.02

$k_3^{d,e}$ = 1.3 ± 0.05

$K_s'^{a}$ = 56 ± 3

a. Units of 10^{-3} M.

b. Units of 10^{-4} M. (First order fit to 3 - 10 min. data.)

c. Units of 10^{-2} min. mg.-PN/ml.

d. Units of 10^{-3} M/min. mg.-PN/ml.

e. k_3 obtained from $k_3 = 2.58\ k_3'$.

f. Dotted line of figure.

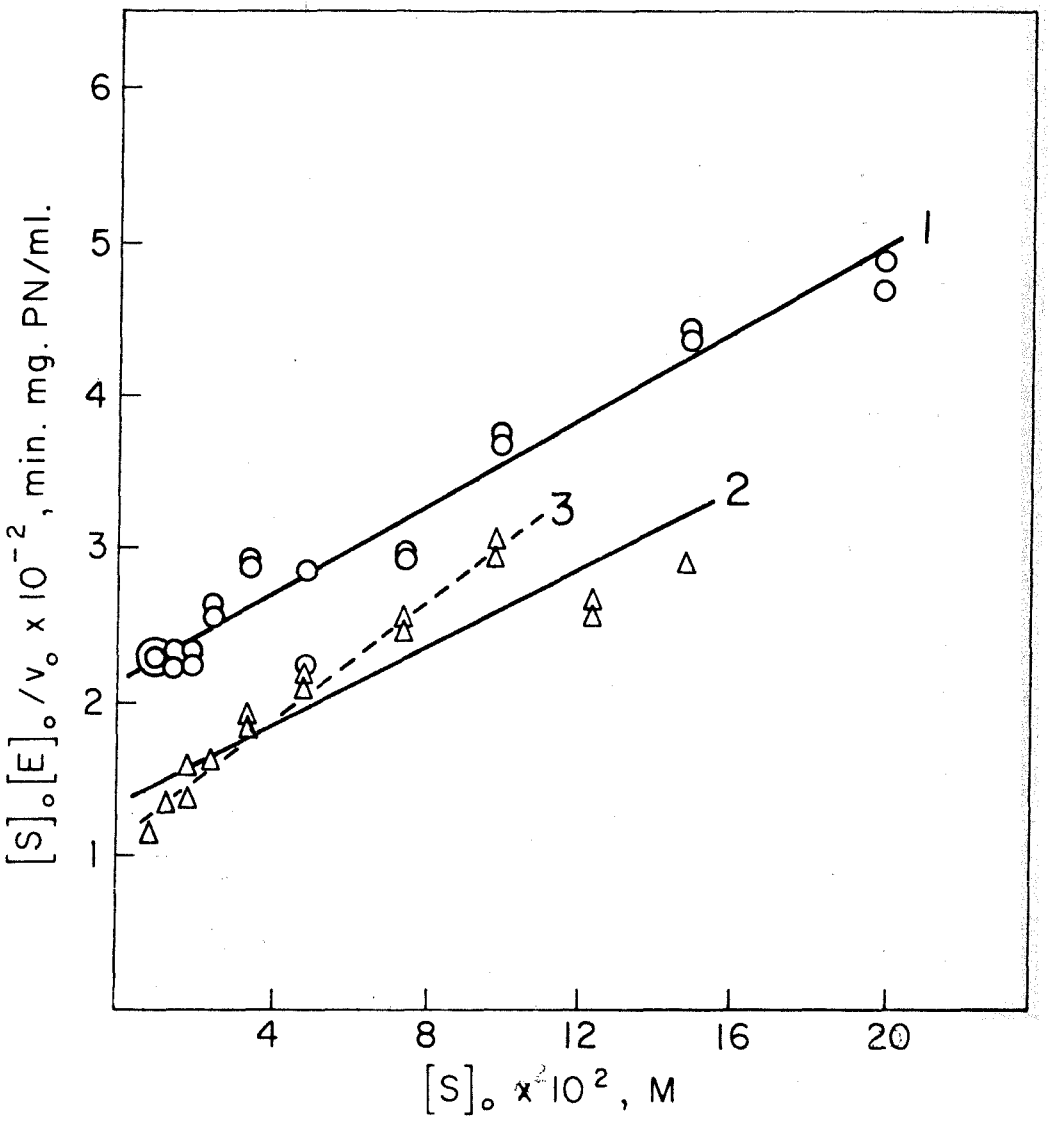

Figure 13. The alpha-Chymotrypsin-Catalyzed Hydrolysis of Trimethylacetyl-L-tyrosinhydrazide in the pH-stat at pH = 7.90, 25°C, $[E]_o$ = 0.1464 mg. PN/ml. Curve 1: NaCl = 0.10 M; Curve 2: NaCl = 0.92 M, $[S]_o$ = 10-150 x 10^{-3} M; Curve 3: NaCl = 0.92 M, $[S]_o$ = 10-100 x 10^{-3} M.

were re-calculated in the latter run for the range $[S]_o$ = 10 to 100 x 10^{-3} M with a significant increase in precision, giving k_3 = 1.3 ± 0.05 and K_s' = 56 ± 3 x 10^{-3} M.

In the absence of repeat experiments by both the "buffer" and the "azine" methods with the same lot of substrate a final decision cannot be made as to the general reliability of hydrazide substrates by the pH-Stat product buffer method.

Hydroxamide

N-Acetyl-L-tyrosinhydroxamide was found to be an excellent substrate for the pH-Stat due to the large difference between the pH of the optimum, pH = 7.60 and pK_2' of the buffer hydroxylamine. The effect of ionic strength was studied over the range of NaCl = 0.040 to 2.0 M. This substrate was used as the sodium salt in the manner described in Part II.

The pH optimum was found to be 7.60 in the pH-stat, in agreement with the literature (13, 14), therefore all experiments were conducted at pH = 7.60 ± 0.1. Recorder traces of extent of conversion versus time showed a slight curvature (possibly due to a slight inhibitory action of hydroxlyamine (13)) and were accordingly evaluated by the 9 point orthogonal polynomial method at a $t_{.10}$ significance level. In common with previous experience with tyrosine substrates the recorder traces within the first minute of operation were unreliable, therefore initial velocities were taken at t = 1 minute. The results are presented in Tables XXIII to XXIV

Table XXIII

The Effect of Ionic Strength on the alpha-Chymotrypsin Catalyzed Hydrolysis of acetyl-L-Tyrosinhydroxamide at pH = 7.60, 25.0°C[c, d]

Ionic Strength NaCl		k_3[a]	K_s[b]
M	activity		
0.040	0.033	29.2 ± 1.8	51.4 ± 3.6
0.200	0.148	31.4 ± 2.4	46.2 ± 3.8
0.500	0.343	36.6 ± 3.2	38.3 ± 3.9
1.00	0.670	32.6 ± 2.5	26.7 ± 2.7
2.00	1.39	30.7 ± 1.0	15.1 ± 0.8

a. Units of 10^{-3} M/min. mg. PN/ml

b. Units of 10^{-3} M

c. $[E]_o$ = 0.0209 mg. PN/ml., Lot No. 283.

d. Notebook Ref. 934-51, 58, 59, 55, 52.

Table XXIV

The alpha-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-Tyrosinhydroxamide in the pH-Stat at pH = 7.60, 25.0°C

$[E]_o$ = 0.0209 mg. PN/ml, Lot No. 283

NaCl = 0.040 M			NaCl = 0.200 M			NaCl = 0.500 M		
$[S]_o$	v_1 [b]	P_m [c]	$[S]_o$ [a]	v_1 [b]	P_m [c]	$[S]_o$ [a]	v_1 [b]	P_m [c]
40	0.280	2	40	0.278	1	40	0.348	2
40	0.246	1	40	0.305	2	40	0.401	2
			40	0.307	2			
30	0.220	1				30	0.334	1
30	0.218	1	30	0.240	1	30	0.330	2
			30	0.260	2			
20	0.163	1	30	0.261	2	20	0.256	1
20	0.163	1				20	0.275	2
			20	0.169	1			
10	0.0963	2	20	0.210	3	10	0.163	2
10	0.0934	1	20	0.200	3	10	0.155	1
7.5	0.0771	2	10	0.115	2	7.5	0.130	3
7.5	0.0771	2	10	0.110	1	7.5	0.126	2
			10	0.112	2			
5.0	0.0536	2				5.0	0.0814	2
5.0	0.0533	2	7.5	0.0886	2	5.0	0.0756	3
			7.5	0.0898	2			
			7.5	0.0867	2			
			5.0	0.0629	2			
			5.0	0.0714	4			
			5.0	0.0587	1			

k_3' [d] = 28.4 ± 1.8 k_3' [d] = 30.6 ± 2.3 k_3' [d] = 35.7 ± 3.1

Table XXIV (Cont'd)

NaCl = 1.00 M			NaCl = 2.00 M		
$[S]_o$ [a]	v_1 [b]	P_m [c]	$[S]_o$ [a]	v_1 [b]	P_m [c]
40	0.394	1	40	0.429	1
40	0.407	3	40	0.452	2
			40	0.460	1
30	0.349	1	30	0.429	3
30	0.346	3	30	0.419	3
			30	0.432	2
20	0.254	3			
20	0.327	2	20	0.329	2
			20	0.356	2
10	0.196	2	20	0.380	2
10	0.186	2			
			10	0.263	2
7.5	0.143	2	10	0.235	3
7.5	0.159	2	10	0.253	2
5.0	0.091	2			
5.0	0.106	2	7.5	0.189	3
			7.5	0.225	3
			7.5	0.206	2
			5.0	0.159	3
			5.0	0.148	2
			5.0	0.154	2
k_3 ' [d] = 31.8 ± 2.4			k_3 ' [d] = 29.9 ± 0.9		

Footnotes to Table XXIV

a. Units of 10^{-3} M

b. Units of 10^{-3} M/min.

c. Order of 9 point orthogonal polynomial

d. Units of 10^{-3} M/min.-mg. PN/ml.

e. Corrected by: $k_3 = k_3' (1 + .025) = 1.025\, k_3'$

Note: The values of k_3 and K_s' are given in Table XXIII.

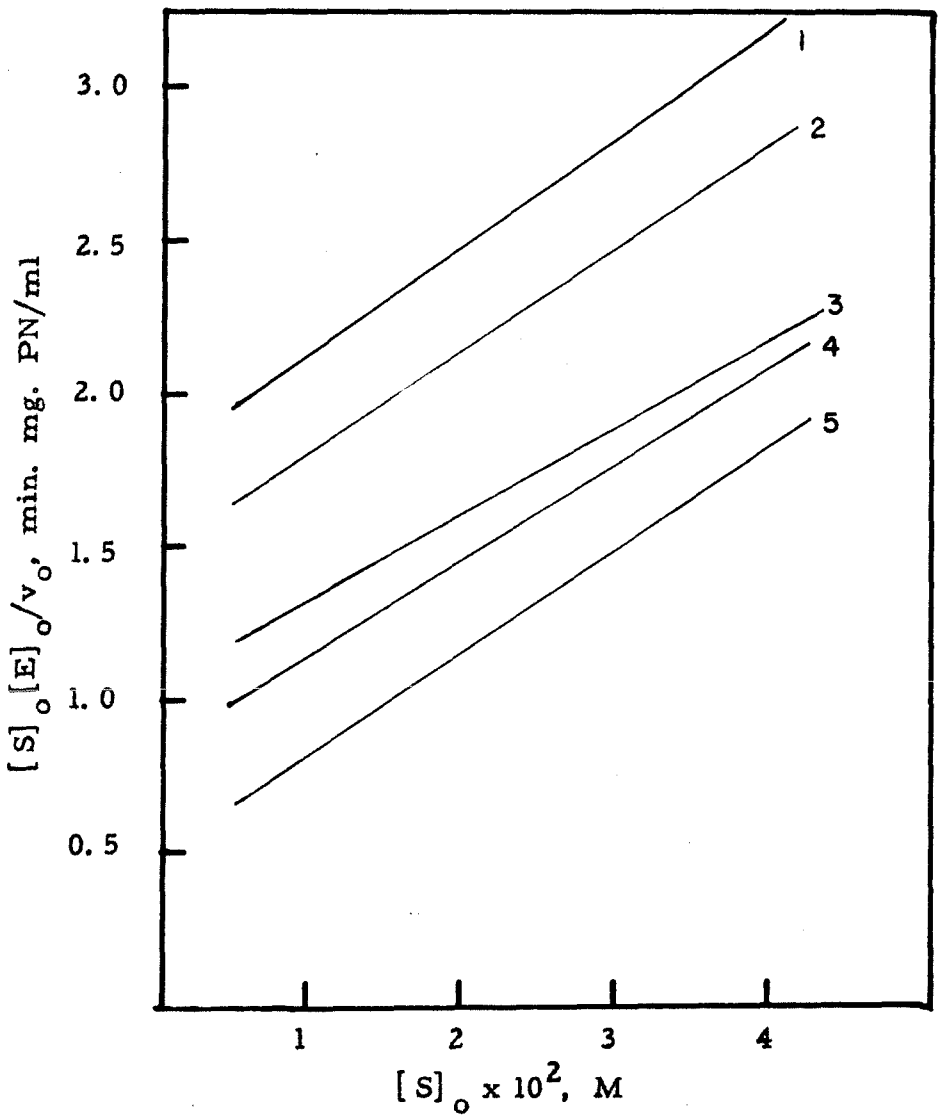

Figure 14. The Effect of Ionic Strength on the alpha-Chymotrypsin-Catalyzed hydrolysis of Acetyl-L-tyrosinhydroxamide in the pH-stat at pH = 7.60, 25°C, $[E]_o$ = 0.0209 mg PN/ml. Curve 1: NaCl = 0.04 M; Curve 2: NaCl = 0.20 M; Curve 3: NaCl = 0.50 M; Curve 4: NaCl = 1.0 M; Curve 5: NaCl = 2.0 M.

It was found that k_3 appeared to exhibit a maximum at 0.500 M NaCl; the values of k_3 at 0.040 M, 0.20 M, 0.50 M, 1.00 M and 2.00 M NaCl were $k_3 = 29.4 \pm 1.8$, 31.7 ± 2.4, 27.0 ± 3.2, 32.9 ± 2.5, and 31.0 ± 1.0 respectively. Due to the limits of precision encountered in this work, the attainment of a maximum for k_3 at 0.50 M NaCl does not have much significance. Within the limits of precision encountered in this work it can be stated that k_3 is independent of ionic strength in the range of 0.040 to 2.00 M NaCl. The mean value of $k_3 = 32.4 \pm 2.8$.

The value of K_s was found to be very dependent on ionic strength, the relation being, $\log K_s = -(1.283 \pm 0.0008) - (0.273 \pm 0.007)(M_{NaCl})$. The dependence of K_s and the independence of k_3 on ionic strength is opposite in behavior to chloroacetyl-L-tyrosinamide (10) and nicotinyl-L-tyrosinhydrazide (15) where K_s was found to be independent of ionic strength and k_3 was found to increase with ionic strength. The results with acetyl-L-tyrosinhydroxamide are similar to the results obtained with acetyl-L-valine methyl ester-NaCl or $MgCl_2$ (16) insofar as the dependence of K_s upon ionic strength is concerned although the latter two examples also showed a small increase in k_3 with ionic strength.

The results obtained with acetyl-L-tyrosinhydroxamide were examined with respect to the activation mechanism discussed by Martin and Niemann (16) and Segal, Kachmar and Boyer (17). None of the proposed mechanisms appeared to explain the independence of k_3 and dependence of K_s upon ionic strength. Since all of these mechanisms were treated as "specific activation", it is concluded

that the activation is a case of "non-specific activation" of enzyme by salts, i.e. $E + nA \rightleftharpoons EA_n$. For such a mechanism it would be expected that k_3 would be independent of ionic strength since in effect the salt may be said to alter the affinity of enzyme for the substrate, i.e. changes in the modes of combination with substrate, none of which lead to a more favorable transition state for hydrolysis.

The results are given in Table XXIV and plotted as $[S]_o [E]_o/v_o$ vs $[S]_o$ in figure 14. Since the recorder traces were nearly linear, the data were re-calculated on the basis of a first-order fit to the traces for the 1-9 minute interval. The results in terms of K_s and k_3 were not significantly different from the results obtained by the orthogonal polynomial procedure. The "linear fit" results are presented in Table XXV.

A summary of all of the experiments conducted in this survey is given in Table XXVI.

Table XXV

The alpha-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-Tyrosinhydroxamide in the pH-Stat at pH = 7.60. Linear Assumption of Recorder Traces.

NaCl		k_3 [a]	K_s [b]
M	activity		
0.040	0.033	29.4 ± 1.1	53.9 ± 2.2
0.200	0.148	32.2 ± 1.4	50.4 ± 2.3
0.500	0.343	42.8 ± 3.0	49.9 ± 3.8
1.00	0.670	36.6 ± 2.5	33.5 ± 2.7
2.00	1.39	32.1 ± 0.9	17.8 ± 0.8

a. Units of 10^{-3} M/min-mg PN/ml

b. Units of 10^{-3} M

Table XXVI

Summary of Kinetic Constants

The alpha-Chymotrypsin-Catalyzed Hydrolysis of Substrates that Produce an Internal Buffer

Results Obtained in the pH-Stat at 25.0°C

Substrate	$[S]_o$[a]	NaCl M	pH	$[E]_o$[b]	k_3[c]	K_s[d]
Acetyl-L-tyrosinamide	6.42-28.9	1.93	7.90	0.293	7.2 ± 3.2	44 ± 23
" (Lit.)[e]	10-30	0.02[f]	7.9	0.146	2.4 ± 0.3	32 ± 4
Nicotinyl-L-tyrosinamide	3-18	0.116	7.90	0.293	4.6 ± 1.3	16 ± 6
"	2.9-14.4	1.93	7.90	0.293	6.0 ± 0.6	2.3 ± 1.0
" (Lit.)[e]	5-20	0.02[f]	7.8	0.049	5.0 ± 1.0	12 ± 3
Acetyl-L-tyrosinhydrazide	3-12	0.10	7.90	0.146	1.0 ± 0.2	23 ± 4
"	2.8-11.6	1.93	7.90	0.146	2.4 ± 0.1	19 ± 1
" (Lit.)[g]	4.5-120	0.02[f]	7.9	0.145	1.1 ± 0.2	29.5 ± 6
Trimethylacetyl-L-tryosinhydrazide	10-200	0.10	7.90	0.146	1.8 ± 0.1	152 ± 10
"	10-150	0.92	7.90	0.146	2.0 ± 0.2	104 ± 15
"	10-100	0.92	7.90	0.146	1.3 ± 0.05	56 ± 3
" (Lit)[h]	0.49-10.5	0.02	7.8	0.145	0.4 ± 0.2	45 ± 20

Table XXVI (Cont'd)

Acetyl-L-tyrosinhydroxamide	5-40	0.04	7.60	0.0209	29.2 ± 1.8	51.4 ± 3.6
"	5-40	2.00	7.60	0.0209	30.7 ± 1.0	15.1 ± 0.8
" (Lit.)[i]	3-70	0.02	7.60	0.04	35.2	47 ± 3

a. Units of 10^{-3} M.

b. Units of mg. PN/ml.

c. Units of 10^{-3} M/min.-mg. PN/ml.

d. Units of 10^{-3} M.

e. Reference 18.

f. Measured in 0.02 M THAM.

g. Reference 12.

h. Reference 11.

i. Reference 19.

C. The Apparent Adsorption of Trimethylacetyl-L-Tyrosinhydrazide by alpha-Chymotrypsin

When the 1 ml aliquot of enyzme stock solution of pH = 7.90 was added to the 9 ml of reaction system containing water, trimethylacetyl-L-tyrosinhydrazide and sodium chloride at pH = 7.90, a sharp drop in pH occurred that was proportional to the concentration of substrate. For the kinetic runs described previously the pH was rapidly re-equilibrated to pH = 7.90 by the manual addition of 1.07 N NaOH from a calibrated micro-syringe, an operation that took about 15-30 seconds.

Initial and final readings on the micro-syringe gave the equivalents of base needed to re-establish the pH. From the latter value there was subtracted the amount of acid that was produced by the hydrolytic reaction during the interval of the 1.07 N NaOH addition. At NaCl = 0.1 M, it was found that 13 protons were liberated per mole of enzyme when $[S]_o = 0.025$ M. The release of protons per mole of enzyme increased to 82 with $[S]_o = 0.2$ M. The data could be expressed by a Langmuir adsorption isotherm by plotting $[E]_o / \Delta[H^{\oplus}]$ versus $1/[S]_o$ giving an intercept (by extrapolation) equivalent to 245 protons per mole of enzyme at saturation and a slope equal to 1.8×10^{-3} M^{-1}.

Since alpha-chymotrypsin can supply only about 10 protons at pH = 7.9 from the free basic groups of the histidines, arginines and lysines that are present in the molecule, it is believed that the protons arise by adsorption of the substrate as the anionic tyrosyl species (i.e. phenolate anion). Further support for this hypothesis

rests on the observation of a similar release of protons when alpha-chymotrypsin was placed in solution with any tyrosine substrate examined in this Thesis. The magnitude of this phenomenon observed with trimethylacetyl-L-tyrosinhydrazide was due to the experimental ability to achieve high substrate concentrations. The data are given in Table XXVII.

Table XXVII

Proton Release when alpha-Chymotrypsin Associates with Trimethylacetyl-L-tyrosinhydrazide[d]

$[S]_o$ [a]	$\Delta[H^+]$ [b]	$\frac{\Delta[H^+]}{[E]_o}$ [c]
200	3.32	80
200	3.47	83
150	2.38	57
150	2.87	69
100	1.77	42
100	1.81	43
75	1.42	34
50	1.06	25
25	0.54	13

For Langmuir absorption isotherm plot:

ordinate = $[E]_o/\Delta[H]^+$ abscissa = $1/[S]_o$

ordinate intercept = 245 of protons released per molecule of enzyme at saturation

slope = 1.8×10^{-3} M^{-1}

a. $[S]_o$ = units of 10^{-3} M of trimethylacetyl-L-tyrosinhydrazide

b. Units of 10^{-3} M, protons liberated, mole per liter of reaction system.

c. Dimensionless = M/M.

d. $[E]_o = 4.15 \times 10^{-5}$ M for an assumed MW = 22,000, Lot No. 283, pH = 7.90, NaCl = 0.100 M, 25.0°C.

D. Substrates

All of the substrates used in this study have been described previously. The preparations used in this study had the following properties:

Compound	m. p. °C (corr.)	α_D^{25}	Acknowledged Source
Acetyl-L-tyrosinamide	226-228°	+52 (c, 0.8% H_2O)	1
Nicotinyl-L-tyrosinamide	225-226.5°	-38°(c, 1% CH_3OH)	2
Acetyl-L-tyrosinhydrazide	232-234°	+44°(c, 0.355% H_2O)	--
Trimethylacetyl-L-tyrosinhydrazide	181-182°	+28 (c, 2% H_2O)	3*
Sodium acetyl-L tyrosinhydroxamate	190.5-191.0°	+35°(c, 5% in 0.21M HCl)	--

1. Peter Rony
2. John Francis
3. Richard McGriff*

*The sample prepared by Mr. McGriff melted at 181-2°C (corr.). A sample that had been prepared by H. F. Mower melted at 181-2°C (corr.) lit. (17) m. p. 179-180°C. Mixed m. p. - McGriff and Mower = 181-2°C (corr.). The melting points and rotations were performed by Mr. McGriff.

PART IV

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PART V

APPENDICES

APPENDIX I

Datatron 205 Programs for the Solution of Enzyme Kinetic Constants.

Code No.	Known		Calculates
100-002:	$[S]_o$, v_o* $[E]_o$		k_3', K_S'
100-004:	y_i, x_i		slope, intercept
100-008:	$[S]_o$, $[P]_t$, t_{min}	A.	slope and intercept of $\frac{1}{t}([S]_o - [S]_t)$ vs. $\frac{1}{t}\ln\left(\frac{[S]_o}{[S]_t}\right)$
		B.	slope and intercept of $\ln [S]_t$ vs. t
200-001:	(orthogonal polynomial 9 points) $[S]_o$, $[P]_t$, t = 0 - 9 minutes		v_o, v_1 and the corresponding k_3' and K_S'
200-002:	(orthogonal polynomial 17 points) $[S]_o$, $[P]_t$, t = 0 - 9 minutes		v_o, v_1 and the corresponding k_3' and K_S'

*Corrected for enzyme and substrate blanks.

APPENDIX II

"Kinetic Development for an Enzyme with Two Sites, Only One of Which is Capable of Yielding Products."

It is assumed that two interaction sites are available on the enzyme. The first site, designated the "active site", is the locus for hydrolytic reactions, competition for which by the inhibitor results in a competitive contribution to the overall inhibition.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \longrightarrow E + P$$

$$E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI$$

$$E + S \underset{k_9}{\overset{k_8}{\rightleftharpoons}} ES'$$

$$E + I \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EI'$$

$$ES' + I \underset{k_{11}}{\overset{k_{10}}{\rightleftharpoons}} ES'I \not\longrightarrow P$$

$$EI' + S \underset{k_{13}}{\overset{k_{12}}{\rightleftharpoons}} EI'S \xrightarrow{k_{14}} EI' + P$$

$$ES + I \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} EI'S \xrightarrow{k_{14}} EI' + P$$

$$EI + S \underset{k_{18}}{\overset{k_{17}}{\rightleftharpoons}} ES'I \not\longrightarrow P$$

Under Zone A conditions (15, 16) where $I \gg EI$, $ES'I$, $EI'S$ and $S \gg ES$, $ES'I$, $EI'S$ where

$$K_s = \frac{k_2 + k_3}{k_1} \qquad K_I = k_5 / k_4 \qquad K_{I.1} = k_7 / k_6$$

$$K_6 = k_9 / k_8 \qquad K_{I.2} = k_{11} / k_{10} \qquad K_{I.3} = k_{18} / k_{17}$$

$$K_{s.1} = \frac{k_{13} + k_{14}}{k_{12}}$$

$$K_{s.2} = \frac{k_{14} + k_{16}}{k_{15}} \qquad \frac{1}{V'} = \frac{1}{V}\left(1 + \frac{K_s}{K_6}\right)$$

$$\frac{K_s}{V} = \frac{K'_s}{V'} \qquad V' = k'_3 [E]_o \qquad r = k_{14} / k_3$$

The forward velocity, $v = k_3[ES] + k_{14}[EI'S]$ (9)

$$\frac{d}{dt}[EI'S] = k_{12}[EI'][S] - (k_{13}+k_{14})[EI'S] + k_{15}[ES][I] - k_{16}[EI'S] \quad (10)$$

$$\frac{d}{dt}[ES] = k_1[E][S] - (k_2+k_3)[ES] - k_{15}[ES][I] + k_{16}[EI'S) \quad (11)$$

Under Zone A conditions, where $[S]_o \gg$ ES, EI'S, ES'I, $[S]_o = [S]$; $[I]_o \gg$ EI, EI'S, ES'I, $[I]_o = [I]$. With the simplifying assumption that $K_s = \frac{[E][S]}{[ES]}$ under steady-state conditions, $\frac{d}{dt}[EI'S] = 0$ and equation 2 becomes, upon solving for [EI'S]

$$[EI'S] = \frac{k_{12}[EI'][S] + k_{15}[ES][I]}{(k_{13}+ k_{14} + k_{16})} \quad (12)$$

Also, under steady-state conditions, $\frac{d}{dt}[ES'I] = 0$

$$\frac{d}{dt}[ES'I] = k_{10}[ES'][I] + k_{17}[EI][S] - (k_{11} + k_{18})[ES'I) = 0 \quad (13)$$

$$[ES'I] = \frac{k_{10}[ES'][I] + k_{17}[EI][S]}{k_{11} + k_{18}} \quad (14)$$

Since $[ES'] = \frac{K_s[ES]}{K_\alpha}$

and $[EI] = \frac{K_s[ES][I]}{K_I[S]}$ from the definitions of equilibrium

constants

$$[ES'I] = \left(\left(\frac{k_{10}[I][K_S][ES]}{K_\alpha} \right) + \left(\frac{k_{17}K_S[ES][I]}{K_I} \right) \right) \left(\frac{1}{k_{11} + k_{18}} \right) \quad (15)$$

Letting $\frac{1}{C_a} = \left(\frac{k_{10}}{K} + \frac{k_{17}}{K_I} \right) \left(\frac{1}{k_{11} + k_{18}} \right)$ (16)

$$[ES'I] = \frac{K_S[ES][I]}{C_a} \quad (17)$$

From (12) and the definition of equilibrium constants:

$$[ES'I] = \left(\frac{k_{12}[ES][I]K_S}{K_{I.1}} + k_{15}[ES][I] \right) \left(\frac{1}{k_{13} + k_{14} + k_{16}} \right) \quad (18)$$

Letting $\frac{1}{C_\beta} = \left(\frac{k_{12}}{K_{I.1}} + \frac{k_{15}}{K_s} \right) \left(\frac{1}{k_{13} + k_{14} + k_{16}} \right)$ (19)

$$[ES'I] = \frac{K_S[ES][I]}{C_\beta} \tag{20}$$

$$E_o = E + ES + EI + ES' + EI' + ES'I + EI'S \tag{21}$$

$$[E]_o = \frac{K_S[ES]}{[S]} + [ES] + \frac{K_S[ES][I]}{K_I[S]} + \frac{K_S[ES]}{K_\sigma} + \frac{K_S[ES][I]}{K_{I.1}[S]} + \frac{K_S[ES][I]}{C_\alpha} + \frac{K_S[ES][I]}{C_\beta} \tag{22}$$

Rearranging:

$$[ES] = \frac{[E]_o[S]}{K_S\left(1 + \frac{[I]}{K_I} + \frac{[I]}{K_{I.1}}\right) + [S]\left(1 + \frac{K_S}{K_\sigma} + K_S[I]\left(\frac{1}{C_\alpha} + \frac{1}{C_\beta}\right)\right)} \tag{23}$$

Since $v = k_3[ES] + k_{14}[EI'S]$

let $r = \frac{k_{14}}{k_3}$; $V = k_3[E]_o$

$$v = k_3([ES] + r[EI'S]) \tag{24}$$

and, $$v = \frac{k_3[E]_o[S]}{K_S\left(1 + \frac{[I]}{K_I} + \frac{[I]}{K_{I.1}}\right) + [S]\left(1 + \frac{K_S}{K_\sigma} + K_S[I]\left(\frac{1}{C_\alpha} + \frac{1}{C_\beta}\right)\right)} + \left(\frac{r k_3 K_S[I]}{C_\beta}\right)\left(\frac{[E]_o[S]}{K_S\left(1 + \frac{[I]}{K_I} + \frac{[I]}{K_{I.1}}\right) + [S]\left(1 + \frac{K_S}{K_\sigma} + K_S[I]\left(\frac{1}{C_\alpha} + \frac{1}{C_\beta}\right)\right)}\right) \tag{25}$$

$$\frac{V}{v} = \frac{1}{[S]}\left\{K_S\left(1+\frac{[I]}{K_I}+\frac{[I]}{K_{I.1}}\right)\left(\frac{1}{1+\frac{rK_S[I]}{C_\beta}}\right)\right\} +$$

$$+\left(1+\frac{K_S}{K_\ell}+K_S[I]\left(\frac{1}{C_\alpha}+\frac{1}{C_\beta}\right)\right)\left(\frac{1}{1+\frac{rK_S[I]}{C_\beta}}\right) \qquad (26)$$

Since it is likely that the affinity of I for the inactive site is less than for the active site, $K_{I.1} >> K_I$, so that

$$\frac{1}{v} = \frac{1}{[S]}\left\{\frac{K_S}{V}\left(1+\frac{[I]}{K_I}\right)\left(\frac{1}{1+\frac{rK_S[I]}{C_\beta}}\right)\right\} + \frac{1}{V}\left(1+\frac{K_S}{K_\ell}+K_S[I]\left(\frac{1}{C_\alpha}+\frac{1}{C_\beta}\right)\right)\cdot$$

$$\cdot\left(\frac{1}{1+\frac{rK_S[I]}{C_\beta}}\right) \qquad (27)$$

Also, $\frac{1}{V'} = \frac{1}{V}\left(1+\frac{K_S}{K_\ell}\right)$; $\frac{K_S}{V} = \frac{K'_S}{V'}$; $V' = k'_3[E]_o$

leading to equation 28 (= equation 8 of text)

$$\frac{1}{v} = \frac{1}{[S]}\left\{\frac{K'_S}{V'}\left(1+\frac{[I]}{K_I}\right)\left(\frac{1}{1+\frac{rK_S[I]}{C_\beta}}\right)\right\} + \frac{1}{V'}\left(1+K'_S[I]\left(\frac{1}{C_\alpha}+\frac{1}{C_\beta}\right)\right)\cdot$$

$$\cdot\left(\frac{1}{1+\frac{rK_S[I]}{C_\beta}}\right) \qquad (28)$$

APPENDIX III

Michaelis-Menten Kinetics In the Presence of a Product That is a Buffer

$$E + [SB] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ESB] \xrightarrow{k_3} E + P + HB + H_2B^{\oplus} \quad (1)$$

Material Balance:

$$B = [HB] + [H_2B]^{\oplus} = P \quad (2)$$

Material Balance:

$$[ESB] + H_2O \rightarrow E + P^{\ominus} + [HB] + [H_2B]^{+} + H_o^{\oplus} \quad (3)$$

Titrimeter Feeds $OH^{\ominus}$:

$$H^{\oplus} + OH^{\ominus} = H_2O \quad (4)$$

$$\frac{d}{dt}[P^{\ominus}] = \frac{d}{dt}[H_o^{\oplus}] + \frac{d}{dt}[H_2B]^{\oplus} \quad (5)$$

equivalent to

$$v = v'(\text{apparent}) + H^{\oplus} \text{ lost to buffer}$$

From the acid-base equilibrium,

$$[H_2B]^{+} \qquad [HB] + H^{\oplus}$$

$$\log \frac{[H_2B]^{+}}{[HB]} = pKa' - pH \quad (6)$$

$$\frac{d}{dt}[ESB] = k_1[SB][E] - k_2[ESB] - k_3[ESB] = 0 \quad (7)$$

$$E = E_o - [ESB] \quad (8)$$

Substitution of (8) into (7) leads to (9)

$$[ESB] = \frac{k_1[SB][E]_o}{(k_2+k_3)+k_1[SB]} = \frac{[SB][E]_o}{(\frac{k_2+k_3}{k_1})+[SB]} \quad (9)$$

$$v = \frac{d}{dt}[P^{\ominus}] = k_3[ESB] = \frac{k_3[SB][E]_o}{K_s + [SB]} \tag{10}$$

By material balance:

$$v = \frac{d}{dt}[P^{\ominus}] = \frac{d}{dt}[HB] + \frac{d}{dt}[H_2B]^{\oplus} \tag{11}$$

Therefore, since $\frac{d}{dt}[HB] = \frac{d}{dt}[H_o^{\oplus}] = v'_o$

$$v = v' + \frac{d}{dt}[H_2B]^{\oplus} \tag{12}$$

Since at constant pH, $\log \frac{[H_2B]^+}{[HB]} = pKa' - pH = z$

$$\frac{d}{dt}[H_2B]^{\oplus} = \frac{e^{(2.303z + \ln[HB])}}{[HB]} \frac{d}{dt}[HB] \tag{13}$$

Substituting (13) into (12) leads to (14)

$$v = v' + \frac{e^{(2.303z + \ln[HB])}}{[HB]} \frac{d}{dt}[HB] \tag{14}$$

However, by material balance

$$\frac{d}{dt}[HB] \equiv v'$$

Therefore,

$$v = v' + \frac{e^{(2.303z + \ln[HB])}}{[HB]} (v') \tag{15}$$

However, the following is evident,

$$e^{(2.303z + \ln[HB])} = \frac{[H_2B]^{\oplus}}{[HB]}[HB] \tag{16}$$

Substituting (16) into (15)

$$v = v' + \frac{\frac{[H_2B]^+[HB]v'}{[HB]}}{[HB]} \tag{17}$$

or

$$v = v' \left(1 + \frac{[H_2B]^+}{[HB]} \right) \tag{18}$$

Substituting (18) into (10)

$$v' \left(1 + \frac{[H_2B]^+}{[HB]} \right) = \frac{k_3[SB][E]_o}{K_s + [SB]} \tag{19}$$

Let $R = \left(1 + \frac{[H_2B]^+}{[HB]} \right)$

$$v' = \frac{\frac{k_3}{R}[SB][E]_o}{K_s + [SB]} \tag{20}$$

Since $[SB] \gg [EBS]$, substitute $[SB]_o = [SB]$. Leading to (21) at initial conditions

$$v'_o = \frac{\frac{k_3}{R}[SB]_o[E]_o}{K'_s + [SB]_o} \tag{21}$$

Note: $[SB]_o = [S]_o$ in usual M-M formulation

v'_o = the apparent initial velocity estimated in the usual manner

$\frac{k_3}{R}$ = the apparent constant, k'_3, obtained by the usual reciprocal plots of M-M equations

Conclusion: $k_3 = R \cdot k'_3$

$K_s = K'_s$

PART VI

PROPOSITIONS

PROPOSITIONS

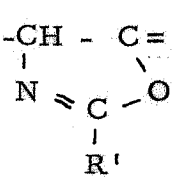
1. Linear polyimides are potentially available from the acid-catalyzed reaction between divinyl esters and diamides, a reaction that has been reported for monofunctional amides and vinyl or isopropenyl esters (1). When the amides are of a primary nature, the linear polyimide, which contains the -CO-NH-CO- repeating unit, should be capable of cross-linking with an excess of the divinyl ester since it is known that N-acylation at an imide-nitrogen also takes place with vinyl esters.

2. It is proposed that the reaction of primary amides with phosphorous oxychloride in the presence of pyridine to product a nitrile occurs by electrophilic attack on the oxygen of the amide group (2). Evidence for the attack on oxygen is the formation of two isomeric products, $C_{16}H_{14}N_2O$, when N-benzoyl-DL-phenylalaninamide is treated with $POCl_3$-pyridine at 0° C. One of the products corresponds to N-benzoyl-DL-phenylalanine nitrile which could arise by attack of the $(POCl_2)^+$ cation on the oxygen of the primary amide group. The isomeric product, which shows a carbonyl infra-red frequency consistent with a five-membered lactone, corresponds to 2-phenyl-4-benzyl-imidazolone-5. The latter product could result from the equally probable attack of the $(POCl_2)^+$ cation on the oxygen of the alpha-acylamide oxygen.

3. A convenient method for the preparation of deutero-substituted amides involves the palladium-deuterium catalyzed cleavage of N-nitrosoamides. Although it is known that the proton in a secondary amide will exchange with the deuterium in deuterium oxide in the presence of acid, the latter method requires a large excess of heavy water over the amide in order to obtain completely N-deuterated amide. The method outlined in this proposition should produce a pure N-deuterated amide and would require only stoichiometric amounts of deuterium gas. The N-deuterated amide should be useful in mechanistic studies and possible isotope effects accompanying the N-nitrosation of secondary amides.

4. A mixed-anhydride mechanism has been proposed for the aryl-sulfite esterification of carbobenzoxyamino acids (3). Support for this mechanism comes from the fact that the reaction proceeds with retention of configuration for carbobenzoxy derivatives and with racemization for N-acetyl-L-phenylalanine.

5. Alpha-acylamide nitriles fail to show a nitrile band in the infrared. It is proposed that alpha-acylamido nitriles exist as the cyclic oxazolimine-5 structure,



a structure which would account for the absence of an infra-red active nitrile band as well as the observed failure of these "nitriles" to yield thioamides when subjected to the attack of hydrosulfide anion, a reaction which is known to proceed with facility for monofunctional nitriles.

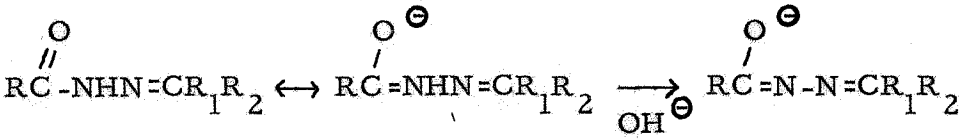
6. A method for the determination of the terminal carboxyl amino acid in proteins is proposed. It has been observed that in the presence of aqueous acid an acetyl group on the alpha-nitrogen of an acylated amino acid hydrazide transfers intramolecularly to the free $-NH_2$ group of the hydrazide. The resulting 1-acetyl-2-[amino acid acyl] hydrazine appears to be stable in acid media hence conversion of a protein terminal carboxyl to a terminal hydrazide followed by hot aqueous acid treatment should result first in a rapid acyl migration to the $-NH_2$ of the hydrazine followed by the slower acid-catalyzed cleavage of the protein acyl amide nitrogen bonds. Amongst the hydrolytic fragments there should be found the diacyl hydrazine corresponding to the two carboxyl terminal amino acids of the protein. Alternatively, the terminal carboxyl amino acid may be released after the first acyl transfer by oxidative cleavage of the hydrazine function.

7. It has been observed (4) that alpha-methyl substitution on an ester substrate of alpha-chymotrypsin resulted in the complete loss of the ability of the enzyme to catalyze the hydrolysis of the ester function. The effect is believed to be a case of steric interference by the methyl group to the formation of an ES complex or to an ES mode of combination that is unfavorable for hydrolysis. It is predicted that ethyl alpha-acetamido-alpha-cyano propionate, $CH_3C(CN)(NHCOCH_3)COOEt$, will be a good substrate of alpha-chymotrypsin because that ester is in a configuration favorable to the formation of an ES complex that will be subject to enzymatic hydrolysis. The favorable configuration arises as a consequence of an intramolecular association between the oxygen of the alpha-amide group and the carbon of the nitrile group in the manner proposed in proposition No. 5.

8. It is proposed that the ionic state of the active site of alpha-chymotrypsin may be elucidated by a study of the effect of pH on the enzyme-inhibitor constant of 1-acetyl-2-[L-tyrosyl] hydrazine.

9. Additional evidence for restricted rotation about the carbonyl C-N bond of amides may be obtained from the pH-sensitivity of acyl hydrazones. Restricted rotation about the carbonyl C-N

bond in acyl hydrazones could arise from structure II. In the presence of base, structure III may predominate, which structure is analogous to ketazines which are known to absorb strongly in the ultraviolet.



I II III

10. The decomposition of N-nitrosoamides, $R\text{-}\underset{\underset{NO}{|}}{N}\text{-}COCH_3$ (I) in the presence of base leads to olefins and esters with the elimination of nitrogen (5). It has been proposed (5) that ester formation occurs by an intramolecular and an intermolecular mechanism since acyl exchange occurs in the presence of added acylate anion. It is now proposed that all of the results may be explained on the basis of a diazomethane intermediate since N-nitroso-N-t-butyl acetamide does not yield t-butyl acetate under the conditions of the experiment but instead yields butenes approximating in composition the butenes that arise from a t-butyl carbonium ion. Since the t-butyl nitrosoamide cannot form a stable diazomethane derivative (by virtue of the absence of a proton on the tertiary carbon) the t-butyldiazo cation loses nitrogen with the formation of a t-butyl carbonium ion, the latter yielding butenes. When the R group has a proton on the carbon alpha to the amide

nitrogen, that proton may leave in the presence of base resulting in a stable diazomethane derivative. The experimental observations of racemization, inversion, and retention of configuration about the carbon alpha to the amide nitrogen may be explained as due to intramolecular and intermolecular attack of acylate anion on the diazomethane-carboxylate ion pair as well as attack of the diazomethane derivative upon the carboxylic acid species. The presence of the diazomethane intermediate should become evident if the reactions are studied in the presence of ketones or aldehydes.

11. Carbon-13 distribution in fossil fuels derived from plants has failed to show a correlation between isotopic composition and metamorphic rank. It is predicted that the petrographic component of these fuels known as attritus will all have the same carbon isotopic composition.

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