I. THE CONSEQUENCES OF SYSTEMATIC ERROR IN ENZYME KINETICS

II. L-TYROSYL-L-TYROSINE DERIVATIVES FOR THE DETECTION OF TRANSPEPTIDATION IN α-CHYMOTRYPSIN-CATALYZED HYDROLYSES

III. THE INTERACTION OF α-METHYL-α-ACYLAMINO ACIDS WITH α-CHYMOTRYPSIN

IV. THE APPARENT IONIZATION CONSTANTS OF A SERIES OF PHENYLALANINE DERIVATIVES

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

1961

ACKNOWLEDGEMENTS

I am particularly indebted to Professor Carl Niemann for his help and guidance toward independent research and for his suggestions of challenging research problems which have given me a wide range of experience.

I am also grateful to the California Institute of Technology for teaching assistantships and tuition fellowships for the 1956-1957, 1957-1958 and 1959-1960 academic years, and for a research fellowship in the summer of 1959; to the U. S. Rubber Company for a fellowship during the 1958-1959 academic year, and to the E. I. duPont de Nemours Company and The Dow Chemical Company for summer grants in 1956, 1957 and 1958.

My colleagues in 262 Crellin have provided many helpful suggestions and a stimulating and congenial atmosphere for which I am grateful. Special thanks go to Drs. Henry Abrash and Abraham Kurtz, who programmed the orthogonal polynomial procedure for the digital computer, thus saving me many hours of calculation.

I would also like to acknowledge the encouragement and support of my parents and my wife, and I dedicate this thesis to them.

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ABSTRACT

The consequences of systematic error in enzyme kinetics were investigated. Systematic error in substrate blank, enzyme blank, velocity determination, substrate concentration and the Beer-Lambert relationship was considered. The advisability of using weighting procedures in the presence of systematic error was questioned.

L-Tyrosyl-L-tyrosine methyl ester, amide, hydrazide and hydroxamide were prepared in order to detect transpeptidation in α-chymotrypsin-catalyzed hydrolyses. The reaction products from the hydrolyses of the corresponding L-tyrosine derivatives were found to contain only negligible amounts of transpeptidation products except for L-tyrosinhydroxamide which gave some L-tyrosyl-L-tyrosine. α- and δ-chymotrypsin were qualitatively the same with respect to these reactions.

The N-acetyl methyl esters of α-methylphenylalanine, α-methyltyrosine and α-methyl-β-(2-naphthyl)-alanine were synthsized and resolved. These esters are good competitive inhibitors of α-chymotrypsin. N-acetyl-(-) α-methyl-β-(2-naphthyl)-alanine is a slowly hydrolyzed substrate of this enzyme. The inactivity of these esters toward α-chymotrypsin-catalyzed hydrolysis is a consequence of their inability to react further after complexing with the enzyme.

The pK'_a values of the α-ammonium groups of D,L-phenylalanine amide, thioamide, amidoxime, hydrazide, methyl ester and hydroxamide were determined. Comparison of these pK'_a values with some for corresponding glycine derivatives shows the former to be 0.59±.04 pK units lower. The infrared spectra of these phenylalanine derivatives were determined in KBr.

PART I

THE CONSEQUENCES OF SYSTEMATIC ERROR IN ENZYME KINETICS

A. PUBLICATION

The pages indicated above contain the text of an article which has been accepted for publication in Biochimica et Biophysica Acta:

"The Consequences of Systematic Errors in Enzyme Kinetics"*

by Harold R. Almond, Jr. and Carl Niemann

*Contribution No. 2560 from the Gates and Crellin Laboratories of Chemistry.

The apparent initial rate of many enzyme catalyzed reactions is given by equation 1,* provided [E] and [S] are varied over a sufficiently limited

$$v = d[P]/dt = - d[S]/dt = k[E][S]/(K + [S]) \quad (1)$$

range and all other reaction parameters are held constant. In this communication we shall be concerned initially with the consequences of a systematic error which will introduce an additional first order term into equation 1 to give equation 2.

$$v = k[E][S]/(K + [S]) + k_A[A] \quad (2)$$

It is assumed in equation 2 that A will yield products which are indistinguishable from those arising from S.

When [E] is invariant, equation 1 is linear and equation 2 non-linear with respect to $1/v$ vs. $1/[S]$, $[S]/v$ vs. $[S]$, or v vs. $v/[S]$ (1). Thus, it might be argued that the presence of a systematic error, of the type contemplated in equation 2, would necessarily be revealed by a non-linear relationship between the two members of any one of the above three pairs of parameters and that the only hazard would be one of confusing a situation described by equation 2 with one devoid of systematic error but characterized by a dependency upon [S] other than that associated with equation 1. However, this view ignores the fact that many enzymatic studies are conducted over such a limited range of

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*Where [E] and [S] are the initial enzyme and substrate concentrations and k and K are the two constants derivable from the dependence of v upon [S] when [E] is invariant. For a discussion of the procedures involved see ref. 1.

[S] that an existing non-linear relationship may not be revealed, with the result that k and K will be evaluated on the basis of an apparent linear relationship which in fact may vary with the relative magnitudes of [S] and K.

To illustrate the kinetic consequences of several of the more common systematic errors equation 2 was evaluated using assumed but reasonable values for the various constants and independent variables and the results so obtained were compared with those arising in the absence of systematic error or errors. The first cases considered were those involving the so-called enzyme and substrate blanks.

It frequently is observed that products indistinguishable from those formed in a total system, otherwise described by equation 1, will arise from either or both E and S when examined separately. In practice the rates of these latter reactions may be individually determined and then subtracted from the rates observed for the total system.* Thus, the term $k_A[A]$ in equation 2 may be specified as in Table I to give equations 2_a to 2_f.** These latter equations were evaluated for

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*For a discussion of the validity of this procedure see ref. 2.

**Although equations 2_a and 2_c may appear to be equivalent to equations 2_b and 2_d it will be shown subsequently that the addition of $\pm k_A[E_T]$ to equation 1 leads to different rate equations than does the addition of $\pm k_{A_2}[E_F]$. This situation arises from the fact that in the former instance it is assumed that all of the enzyme present, $[E_T]$, can give rise to a blank reaction whereas in the latter only the free enzyme, $[E_F]$, can so react. While these pairs of rate equations are different they are kinetically indistinguishable so long as the conditions implicit in equation 1 are maintained.

$K = 5.0 \times 10^{-2}$ M, $k = 2.0 \times 10$ M/min./M, $\pm k_{A_1} = \pm k_{A_2} = \pm 2.0 \times 10^{-1}$ M/min./M, $\pm k_{A_3} = \pm 2.0 \times 10^{-4}$ M/min./M, $[E] = 5.0 \times 10^{-5}$ M and $[S] = 5.0 \times 10^{-4}$ M to 5.0 M. The data so obtained are presented in Figs. Ia, Ib and Ic in the form of v vs. v/[S] plots.

It is evident from Figs. Ia, Ib and Ic that the systematic errors considered in Table I will lead to errors in the direction indicated in Table II. The information summarized in Table II may be interpreted in several ways. First, if a non-linear plot is observed it follows from Table II that the situations described by equations 2_a, 2_b and 2_e will simulate activation by excess substrate, in the sense used by Wolf and Niemann (3), and that of equation 2_f of inhibition by excess substrate.* Alternatively, if an apparent linear relationship is observed, over a narrow range of [S], constants evaluated on the basis of such a relationship will tend to be in error in the directions indicated in Table II. Finally, the data summarized in Table II are useful in identifying those errors which tend to be predominantly additive, e.g., a combination of 2_c and 2_f, or of 2_d and 2_f. There is no combination involving both enzyme and substrate errors that will be generally compensatory because errors

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*See ref. 1, pp. 81-89 for the characteristics of inhibition by excess substrate. Cases 2_e and 2_f are of particular interest because of the requirement of relatively high values of [S] for the demonstration of either activation or inhibition by excess substrate. In these cases it is important that the substrate blank be evaluated with considerable precision because if it is overestimated inhibition by excess substrate will be simulated and if underestimated activation by excess substrate will be mimicked. In equations 2_a and 2_b the dependency of v upon [S] is the same as that encountered in the case of introduction of an endogenous substrate with the enzyme preparation (4). However, in the latter instance the dependency of v upon [E] differs from that of equations 2_a and 2_b.

Table I

Systematic Errors Associated with Enzyme or Substrate Blanks

Eq.	Source of Error	k_A [a]	[A]	Nature of Error
2_a	Enzyme Blank	k_{A_1}	$[E_T]$ [b]	Undercorr. for blank
2_b	" "	k_{A_2}	$[E_F]$ [c]	" " "
2_c	" "	$-k_{A_1}$	$[E_T]$ [b]	Overcorr. for blank
2_d	" "	$-k_{A_2}$	$[E_F]$ [c]	" " "
2_e	Substrate Blank	k_{A_3}	$[S]$ [d]	Undercorr. for blank
2_f	" "	$-k_{A_3}$	$[S]$ [d]	Overcorr. for blank

a. It is assumed that in every case the rate of reaction will be first order in [A].

b. Total enzyme concentration, i.e., $[E_T] = [E]$.

c. Free enzyme concentration, i.e., $[E_F] = [E_T] - [ES]$.

d. Free substrate concentration. However, this will be equivalent to the initial or total substrate concentration, i.e., $[S_T] \doteq [S]$, everywhere except in equation 3 where $[S_T] \doteq [S] + [P]$. The conditions are necessary for the validity of equation 1.

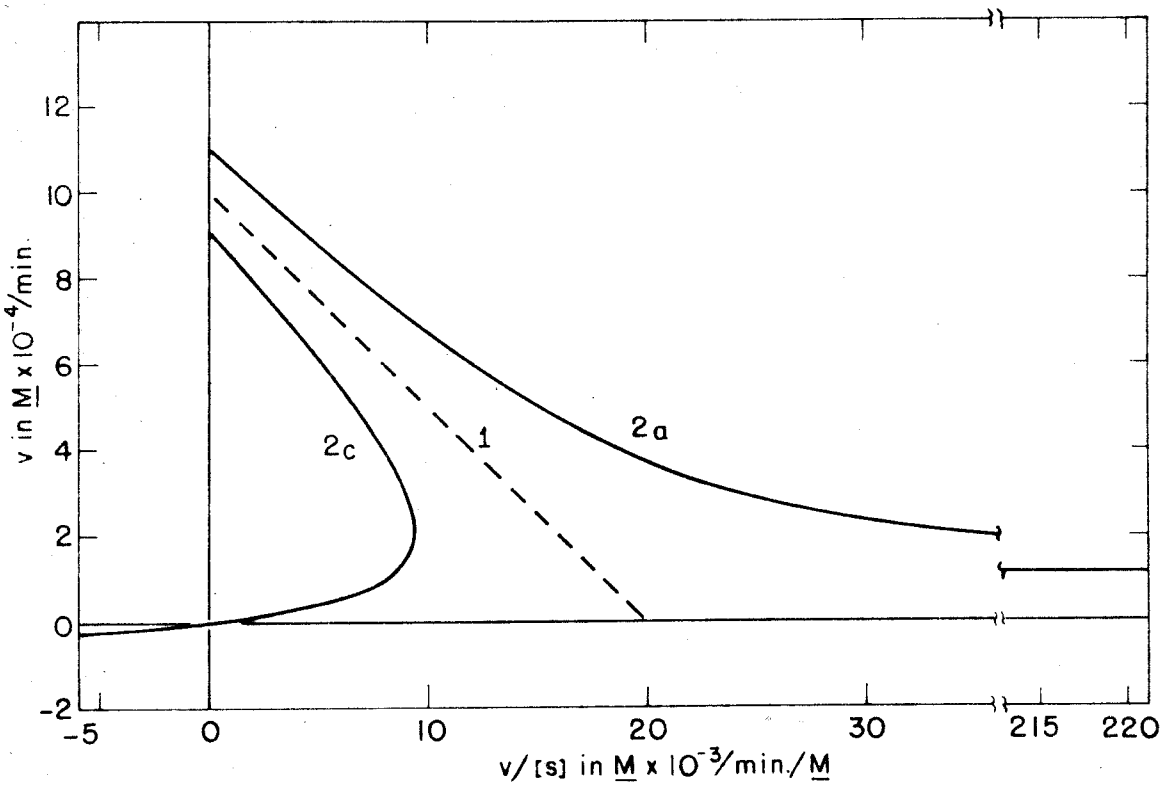

Figure 1a. Systematic error effects on equation 1: equations 2_a and 2_c with $\pm k_{A_1} = \pm 2.0$ M/min./M to exaggerate the effects of the error term. Equations 2_a and 2_c have asymptotes at $v = k_{A_1}[E_T]$ and $v = -k_{A_1}[E_T]$ and intercepts at $v = (k + k_{A_1})[E_T]$ and $v = (k - k_{A_1})[E_T]$ respectively.

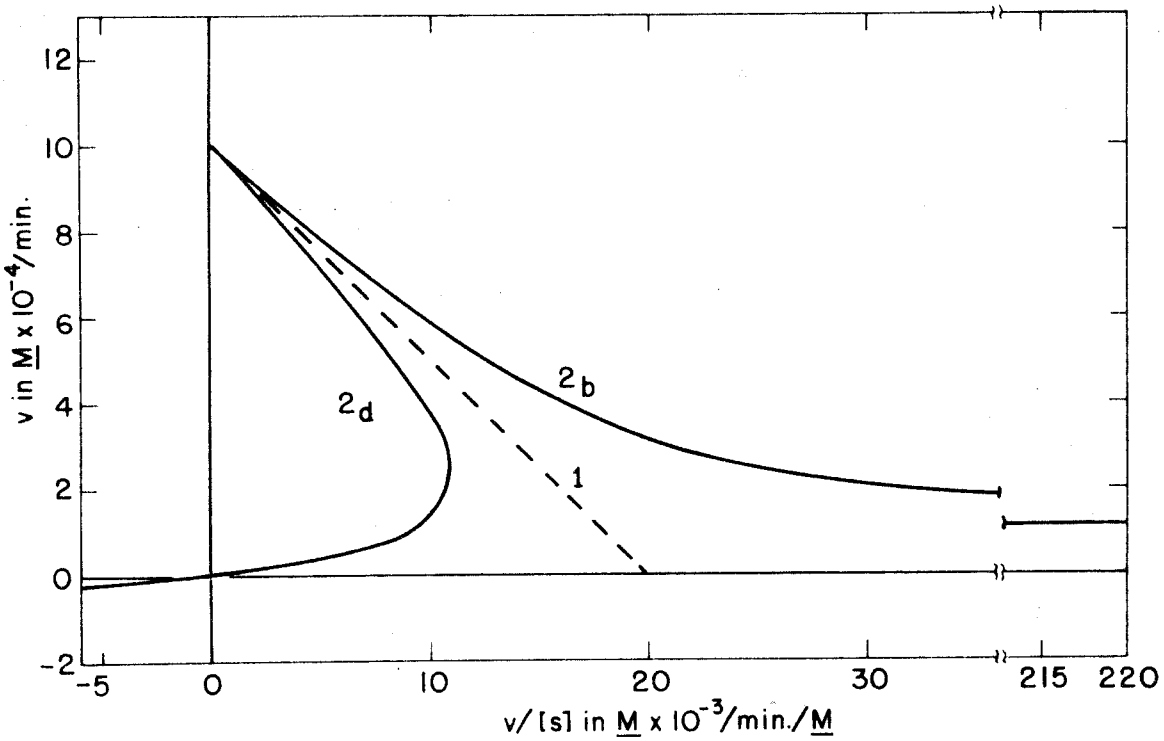

Figure 1b. Systematic error effects on equation 1: equations 2_b and 2_d with $\pm k_{A_2} = \pm 2.0$ M/min./M to exaggerate the effects of the error term. Equations 2_b and 2_d have asymptotes at $v = k_{A_2}[E_T]$ and $v = -k_{A_2}[E_T]$ respectively and a common intercept at $v = k[E_T]$.

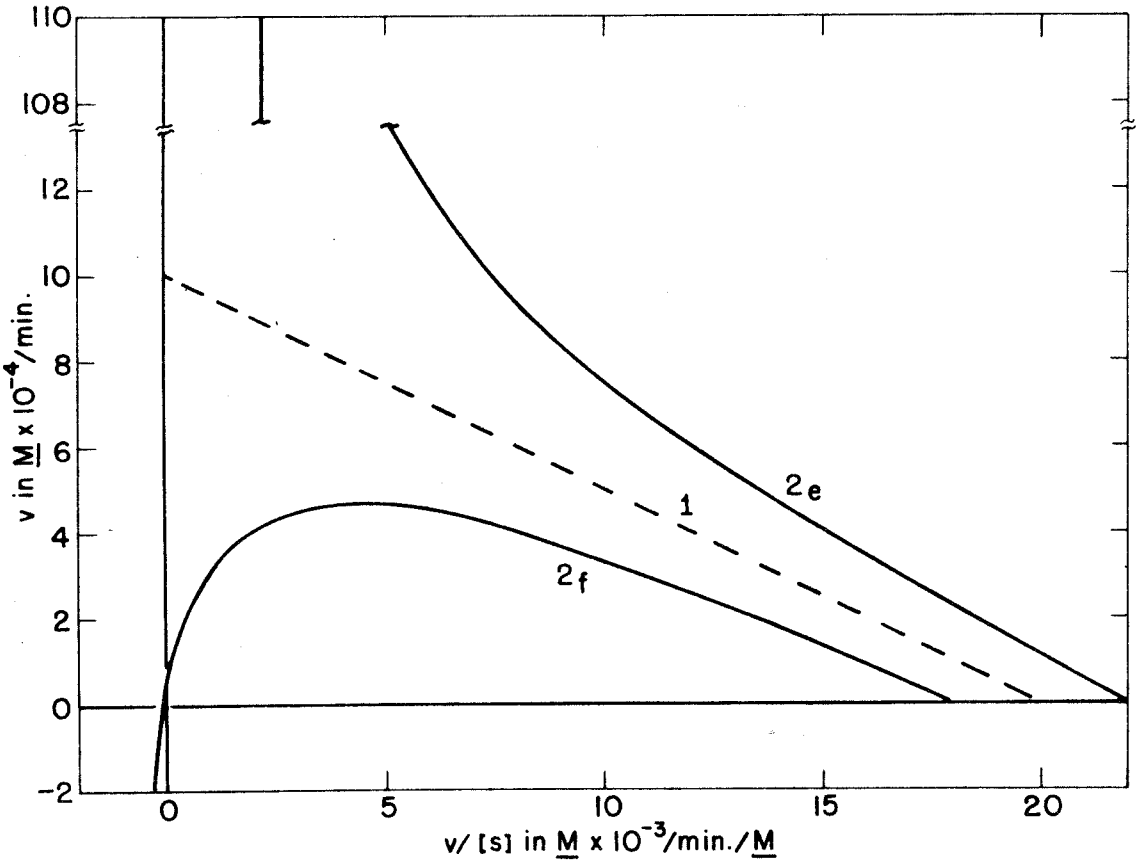

Figure Ic. Systematic error effects on equation 1: equations 2_e and 2_f with $\pm k_{A_3} = \pm 2.0 \times 10^{-3}$ M/min./M to exaggerate the effects of the error term. Equations 2_e and 2_f have asymptotes at $v/[S] = k_{A_3}$ and $v/[S] = -k_{A_3}$ and intercepts at $v/[S] = k_{A_3} + k[E_T]/K$ and $v/[S] = -k_{A_3} + k[E_T]/K$ respectively.

Table II

Relation between True and Apparent Values of K and k

$k_A[A] =$	$k_{A_1}[E_T]$ $(2_a)^c$	$k_{A_2}[E_F]$ $(2_b)^c$	$-k_{A_1}[E_T]$ (2_c)	$-k_{A_2}[E_F]$ (2_d)	$k_{A_3}[S]$ $(2_e)^c$	$-k_{A_3}[S]$ $(2_f)^d$
K'[a]	$< K$	$< K$	$\geqslant K$	$\geqslant K$	$> K$	$< K$
k'[b]	$\lesseqgtr k$	$< k$	$\lesseqgtr k$	$> k$	$> k$	$< k$

a. Apparent value of K.

b. Apparent value of k.

c. Simulates activation by excess substrate.

d. Simulates inhibition by excess substrate.

arising from improper treatment of the enzyme blank will be dominant at low values of $[S]$ and those associated with the substrate blank at high values of $[S]$.

An alternative procedure for interpreting the kinetic consequences of systematic errors of the above kind, which is also useful for assessing the significance level of the individual errors, is based upon transposition of equations 2_a to 2_f to equations $2'_a$ to $2'_f$ inclusive.

$$v[S] + v\,K = (k + k_{A_1})\,[E_T][S] + k_{A_1}\,[E_T]\,K \qquad (2'_a)$$

$$= k[E_T][S] + k_{A_2}\,[E_T]\,K \qquad (2'_b)$$

$$= (k - k_{A_1})\,[E_T]\,[S] - k_{A_1}\,[E_T]\,K \qquad (2'_c)$$

$$= k\,[E_T][S] - k_{A_2}\,[E_T]\,K \qquad (2'_d)$$

$$= (k\,[E_T] + k_{A_3}\,K)\,[S] + k_{A_3}\,[S]^2 \qquad (2'_e)$$

$$= (k\,[E_T] - k_{A_3}\,K)\,[S] - k_{A_3}\,[S]^2 \qquad (2'_f)$$

Examination of these latter equations readily discloses how the various systematic errors interact with k and K. Furthermore, division of both members of equations $2'_a$ to $2'_f$ by $(K + [S])$ reveals that systematic errors of the kind under consideration can be ignored only if $v \gg k_{A_1}\,[E_T]$ or $k_{A_1} \ll k\,([S]/(K + [S]))$ for cases 2_a and 2_c, $v \gg k_{A_2}[E_T]\,(K/(K + [S]))$ or $k_{A_2} \ll k\,([S]/K)$ for cases 2_b and 2_d and $v \gg k_{A_3}[S]$ or $k_{A_3} \ll k\,[E_T]/(K + [S])$ for cases 2_e and 2_f.

It has been suggested upon several occasions that evaluations based upon equation 1 should be weighted to compensate for the altered error function arising from transformation of a hyperbolic to a linear function. (5) While this practice may be justified for data containing only random error the question arises as to its suitability for data containing both random and systematic errors. Values of k and K were obtained from equation 2_a to 2_f for several ten-fold ranges in [S] using both weighted and unweighted linear least-squares fits of lines based upon 1/v vs. 1/[S] plots. It was found that either procedure could lead to values of k and K which were closer to the true values depending upon the particular range of [S] investigated. However, when equations 2_e and 2_f were employed the unweighted fits generally but not invariably led to more accurate values of k and K than did the weighted fits. Thus, it is clear that the practice of weighting to accommodate random error, when both random and systematic errors are present, is no panacea and if used must be used with discretion to avoid causing greater errors in the values of the kinetic constants than would obtain were it not used.

Equation 2 may be extended to accommodate situations involving reversible inhibition by reaction products or added inhibitor (1, 6). In the former instance equation 2 may be modified to give equation 3.

$$v = \left\{ k(K_1/(K_1-K))\,[E][S] \right\} / \left\{ K((K_1+[S_T])/(K_1-K)) + [S] \right\} + k_A[A] \qquad (3)$$

Evaluation of equation 3, with

$k_A[A]$ defined as in Table I, $K_1 = 2K$, K and $0.5K$, $[S_T] = K$ and with the values of k, K, $\pm k_{A_1}$, $\pm k_{A_2}$, $\pm k_{A_3}$ and $[E]$ used previously, was based upon the treatment of Foster and Niemann (7-12). In principle, the integral $\int_o^t$ (eq. 3) dt was evaluated for $k_A[A] = 0$ to obtain values of t for arbitrarily chosen extents of reaction of 20 and 40%. The integral $\int_o^t$ (eq. 3) dt was then evaluated for values of t corresponding to those above to obtain apparent values of $[S']$ that differed from those of $[S]$ because of contributions arising from the term $k_A[A]$. The slopes of $([S_o] - [S_t])/t$ vs. $(\ln([S_o]/[S_t]))/t$ and $([S_o] - [S'_t])/t$ vs. $(\ln([S_o]/[S'_t]))/t$ plots (7-12), which were but slightly curved in the latter instance, were then used to arrive at an estimate of the apparent values of K'_1 relative to those of K_1, for which $k_A[A] = 0$. It was found that for equations 3_a, 3_b, and 3_e $K'_1 > K_1$ and for 3_c, 3_d and 3_f $K'_1 < K_1$. In other words, systematic error arising from no or under-correction of the enzyme or substrate blank reactions could lead one to over-estimate the magnitude of an apparent enzyme-product dissociation constant whereas overcorrection of either of the two blank reactions could lead to the opposite result.

For an added inhibitor, equation 2 may be transformed into equations 4, 5 and 6, which may be associated with "totally competitive," "totally non-competitive"

$$v = \left\{k[E][S]\right\} / \left\{K(1 + [I]/K_2) + [S]\right\} + k_A[A] \qquad (4)$$

$$v = \left\{k[E][S]/(1 + [I]/K_2)\right\} / \left\{K + [S]\right\} + k_A[A] \qquad (5)$$

$$v = \left\{k[E][S]\right\} / \left\{K + [S](1 + [I]/K_2)\right\} + k_A[A] \qquad (6)$$

and "totally uncompetitive" inhibition respectively (1). Equations 4, 5 and 6 were evaluated for the values of k, K, $\pm k_{A_1}$, $\pm k_{A_2}$, $\pm k_{A_3}$, [E] and [S] used previously but with $(1 + [I]/K_2) = 1.0$, 1.1, 2.0 and 10.0 respectively. The data so obtained were presented in 1/v vs. 1/[S] plots to produce six sets of curves, each set consisting of a family of four curves. When examined over a sufficiently wide range of [S] the non-linearity of each curve was readily apparent. However, in contrast to the case involving only [S] as the independent variable, vide ante, it did not appear to be worth while to attempt to identify any of the above curves with those arising in the absence of a systematic error from a dependency upon [S] and [I] other than that assumed in equations 4, 5 and 6 principally because situations involving a simultaneous dependency upon $[S]^n$ and $[I]^m$ are largely unexplored. Instead, attention was directed to the more immediate question as to the possibility of confusing one type of inhibition with another by examining a system over a sufficiently narrow range of [S] as to be led to the conclusion that the dependence of v upon [S] and [I] was linear rather than non-linear. For each family of four curves two or more ranges of [S], each entailing a two-fold variation in [S], were selected as to encompass as large a portion of each curve without leading to negative values of v. Each of the four chords of the line segments associated with a particular range in [S] was extrapolated to its intersection with the other three chords as illustrated in figure II. These points of intersection then were examined to determine their ability to simulate a single point of intersection

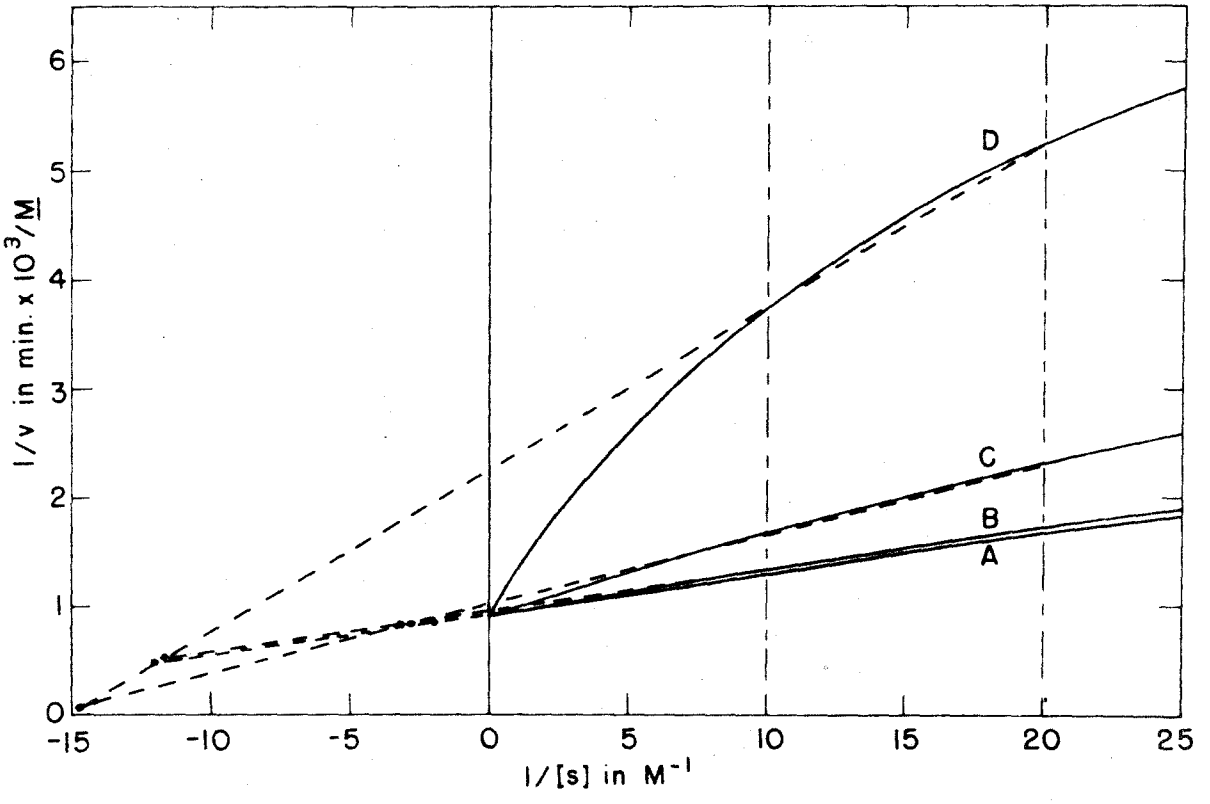

Figure II. Simulation of mixed inhibition by a "Totally Competitive" inhibitor: solid lines, equation 4_a with $k_{A_1} = 2$ M/min./M to exaggerate the effects of the error term and with $(1 + [I]/K_2) = 1$, 1.1, 2 and 10 for curves A, B, C and D respectively; broken lines, extrapolated chords of equation 4_a between $1/[S] = 20$ and 10 M^{-1}.

and to determine their proximity to the $1/v$ and $1/[S]$ axes. This procedure was repeated for all other ranges of $[S]$ and for the other five sets of curves. This information, interpreted in terms of type of inhibition simulated is given in Table III. It is evident from these data that situations interpreted as involving "totally non-competitive" inhibition require careful scrutiny in order to be certain that the type of inhibition and/or the magnitude of the inhibition constants are not a consequence of a systematic error arising from improper treatment of the enzyme or substrate blank reactions of a system which in fact may be "totally competitive" or "totally un-competitive" in basic character.

In the preceding discussion particular attention has been paid to systematic errors arising from enzyme or substrate blank reactions because such reactions are a common feature of many enzyme catalyzed reactions. In particular situations other systematic errors may arise. For example, if the specific substrate or reversible inhibitor is in facile equilibrium with a form, e.g. , a micelle, which interacts with the enzyme to a lesser degree than the monomer, K or K_2 will be overevaluated since the observed values of v will exhibit the same dependencies upon $[S]$ and $[I]$ as in equations 1 and 4 with $k_A[A] = 0$. Alternatively, if a systematic error is present which is proportional to v, i.e., equation 2 with $k_A[A] = k_{A_4} v$, then only the value of k will be influenced and the plots will remain linear. An example of this situation would be a systematic error in the time scale. Similarly, a systematic error in the determination of $[S]$ which is proportional to $[S]$

Table III

Simulation of Alternative Types of Inhibition[a]

Eq. \ $k_A[A]$		$k_{A_1}[E_T]$	$k_{A_2}[E_F]$	$-k_{A_1}[E_T]$	$-k_{A_2}[E_F]$	$k_{A_3}[S]$	$-k_{A_3}[S]$
4	"C"[b]	$M,^{e}(N)^{g}$	M^{e}	M^{e}	M^{e}	M^{e}	$M,^{e}(N)^{g}$
5	"N"[c]	M^{e}	N^{h}	$M,^{e}(C),^{g}(N)^{g}$	N^{h}	$M,^{e}N^{h}$	$M,^{e}N^{h}$
6	"U"[d]	X^{f}	X^{f}	M^{e}	M^{e}	$M,^{e}(N)^{g}$	X^{f}

a. As the significance of the error term $k_A[A]$ diminishes simulation of alternative types of inhibition become less probable. However, even when this element of confusion becomes unimportant errors in the magnitudes of k, K and K_2 may persist.

b. "Totally Competitive" with systematic error.

c. "Totally Non-Competitive" with systematic error.

d. "Totally Un-Competitive" with systematic error.

e. Inhibition of mixed types, i.e., combination of partially competitive and partially non-competitive, etc..

f. No currently recognized type.

g. Fortuitous simulation for a singular range of [S], or more accurately [S]/K.

h. No change in type but only in magnitude of k, K and K_2.

will be reflected only in the value of K. Finally, when a reaction is followed spectrophotometrically by measuring $-d[S]/dt$ or $d[P]/dt$, most departures from the Beer - Lambert relationship are representable by an equation of the form $[C]_{obs.} = [C]_{act.} \pm k_B[C]^n$ which can be differentiated to $d[C]_{obs.}/dt = d[C]_{act.}/dt \pm k_B\, n[C]^{n-1}\, d[C]/dt$. When $n = 1$ the systematic error will be proportional to v and, hence, reflected only in the value of k. Determining $d[P]/dt$ when $n \neq 1$ will introduce no error since $[P] = 0$ at zero time. However, determining $-d[S]/dt$ when $n = 2$ will give the rate expression the form of equation 2_e or 2_f, while other values of n, except $n = 0$, will give somewhat different curves when plotted in the usual manner.

This research was supported in part by a grant from the National Institutes of Health, Public Health Service.

SUMMARY

The consequences of systematic error in enzyme kinetics was considered by introducing a first order error term into the Michaelis-Menten-Briggs-Haldane equation. The situations investigated were those involving over- or undercorrection of an enzyme or substrate blank reaction in the absence and presence of product inhibition and in the presence of a totally competitive, non-competitive or un-competitive inhibitor. In addition, considerations was given to errors arising from a departure from the Beer-Lambert relationship and those proportional to the velocity or substrate concentration. Finally, attention has been called to the questionable validity of using weighting procedures to correct for random error in the presence of systematic error.

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B. APPENDIX

This appendix has been added to fill in the particulars of the calculations mentioned in the preceding article.

The values of $[S]$ and the corresponding velocities calculated from equations 1 and 2 are given in Table IV. Figure Ia, b, c shows the v vs. $v/[S]$ plots for equation 2. The companion plots of $1/v$ vs. $1/[S]$, $[S]/v$ vs. $[S]$ and v vs. $[S]$ are given in figures IIIa, b, c; IVa, b, c and Va, b, c, respectively. In these plots $\pm k_{A1} = \pm k_{A2} = \pm 2.0$ M/min./M and $\pm k_{A3} = \pm 2.0 \times 10^{-3}$ M/min./M to exaggerate the effects of the error term.

The weighting factor, $\omega_i = \sigma^2_{1/v}/\sigma^2_v$, where σ^2 is the variance, used to weight the linear least-squares fitted line to the $1/v$ vs. $1/[S]$ plots of equation 2 is equal to v_i^4 since $\sigma_{1/v}/(1/v_i) = \sigma_v/v_i$. Minimizing the weighted, squared differences between the $(1/v_i)$'s and the regression line, $a + b/S_i$, gives $\Sigma_i \omega_i/v_i = a \Sigma_i \omega_i + b \Sigma_i \omega_i/S_i$ and $\Sigma_i \omega_i/v_i S_i = a \Sigma_i \omega_i/S_i + b \Sigma_i \omega_i/S_i^2$ or upon substitution for ω_i $\Sigma_i v_i^3 = a \Sigma_i v_i^4 + b \Sigma_i v_i^4/S_i$ and $\Sigma_i v_i^3/S_i = a \Sigma_i v_i^4/S_i + b \Sigma_i v_i^4/S_i^2$. The apparent values of k and K calculated from the intercept, a, and the slope, b, obtained from the above equations, as well as the apparent values of k and K calculated from the unweighted least-squares regression lines, are given in Table V.

The integrated form of equation 3 with $k_A[A] = 0$, i.e., $k'[E_T]t = ([S_o] - [S_t]) + K' \ln([S_o]/[S_t])$, where $k' = kK_1/(K_1 - K)$ and

$K' = K(K_1 + [S_o])/(K_1 - K)$, was used to calculate the times, t, required to reach the arbitrarily chosen extents of reaction. $([S_o] - [S_t'])$ was obtained directly from $\int_o^t$ (eq. 3)dt. For equations 3a and 3c

$$([S_o]-[S_t']) = \int_o^t \frac{(k' \pm k_{A1})[E_T][S] \pm k_{A1}K'[E_T]}{K' + [S]}\, dt =$$

$$-\int_{S_o}^{S_t} \frac{(k' \pm k_{A1})[E_T][S] \pm k_{A1}K'[E_T]}{K' + [S]} \times \frac{K' + [S]}{k'[E_T][S]}\, ds =$$

$([S_o] - [S_t])(1 \pm k_{A1}/k') \pm \frac{k_{A1}K'}{k'} \ln([S_o]/[S_t])$ which upon rearrangement gives $[S_t'] = [S_t] \mp ([S_o] - [S_t]) \frac{k_{A1}}{k'} \mp \frac{k_{A1}K'}{k'} \ln([S_o]/[S_t])$.

Similar treatment of equations 3b and 3d and equations 3e and 3f gives

$[S_t'] = [S_t] \mp \frac{k_{A1}K}{k} \ln([S_o]/[S_t])$ and $[S_t'] = [S_t] \mp ([S_o]-[S_t]) \frac{k_{A3}K'}{k'[E_T]} \mp$

$([S_o]^2-[S_t]^2) \frac{k_{A3}}{k'[E_T]}$ respectively. The values of $[S_t]$, t, $([S_o]-[S_t])/t$, $(\ln [S_o]/[S_t])/t$, $[S_t']$, $([S_o]-[S_t'])/t$, $(\ln[S_o]/[S_t])/t$ and the inverse of the slopes calculated for $K_1 = 2K$ and $K_1 = 0.5\,K$ are given in Tables VIa and VIb respectively. The corresponding values obtained when $K_1 = K$ are given in Table VIc, but in this instance equation 3 reduces to

$v = k[E_T][S]/(K + [S_o]) + k_A[A]$. Treatment of this equation in the same manner as above gives $[S_t'] = [S_t] \mp k_{A1}(K + [S_o])(\ln[S_o]/[S_t])/k$, $[S_t'] = [S_t] \mp k_{A2} K (\ln[S_o]/[S_t])/k$ and $[S_t'] = [S_t] \mp k_{A3}([S_o]-[S_t])(K + [S_o])/k[E_T]$ for equations 3a and c, 3b and d, and 3c and f, respectively. Since the slope of the $([S_o]-[S_t])/t$ vs. $(\ln[S_o]/[S_t])/t$ plot equals $-K(K_1 + [S_o])/(K_1 - K)$, then the inverse of an apparent slope greater than the inverse of the actual slope results in an underestimated apparent product-inhibition dissociation constant, i.e., $1/\text{slope}' > 1/\text{slope}$ results in $K_1' < K_1$.

The base lines, where $[I] = 0$, for all types of inhibition will be given by equation 2, for which the values of $1/[S]$ and $1/v_A$ are given in Table VII. The values of $1/v$ calculated from equations 4, 5 and 6 are given in Tables VIII, IX and X, respectively. Besides figure II(VIa), further examples of $1/v$ vs. $1/[S]$ plots which were used to obtain the results given in Table III are shown in figures VIb, c, d, e, f, VIIa, b, c, d, e, f and VIIIa, b, c, d, e, f for "Totally Competitive," "Totally Non-Competitive" and "Totally Un-Competitive" inhibition, respectively. In each figure curves A, B, C and D refer to $(1 + [I]/K_2) = 1$, 1.1, 2 and 10, respectively.

Table IV

Velocities Calculated from Equations 1 and 2.[a]

Equation	1	2a	2a	2b	2b	2c	2c
K_A[b] =	0	0.2	2.0	0.2	2.0	0.2	2.0
[S][c]	v[d]	v[d]	v[d]	v[d]	v[d]	v[d]	v[d]
.0005	.0990	.199	1.10	.198	1.09	-.001	-.901
.001	.1961	.296	1.20	.294	1.18	+.096	-.804
.002	.3846	.485	1.38	.481	1.35	.285	-.615
.005	.9091	1.009	1.91	1.00	1.82	.809	-.091
.01	1.6667	1.767	2.67	1.75	2.50	1.57	+.667
.02	2.857	2.96	3.86	2.93	3.57	2.76	1.86
.05	5.000	5.10	6.00	5.05	5.50	4.90	4.00
.1	6.667	6.77	7.67	6.70	7.00	6.57	5.67
.2	8.000	8.10	9.00	8.02	8.20	7.90	7.00
.5	9.091	9.19	10.09	9.10	9.18	8.99	8.09
1.	9.524	9.62	10.52	9.59	9.57	9.42	8.52
2.	9.756	9.86	10.76	9.76	9.78	9.65	8.76
5.	9.901	10.00	10.90	9.90	9.91	9.80	8.90

Equation	2d	2d	2e	2e	2f	2f
K_A[b]	0.2	2.0	0.2	2.0	0.2	2.0
[S][c]	v[d]	v[d]	v[d]	v[d]	v[d]	v[d]
.0005	0.0	-.891	.100	.109	.098	.089
.001	.098	-.784	.198	.216	.194	.176
.002	.288	-.577	.389	.425	.381	.345
.005	.820	0.0	.919	1.009	.899	.809
.01	1.58	.833	1.69	1.87	1.65	1.47
.02	2.78	2.14	2.90	3.26	2.82	2.46
.05	4.95	4.50	5.10	6.00	4.90	4.00
.1	6.63	6.33	6.87	8.67	6.47	4.67
.2	7.98	7.80	8.40	12.00	7.60	4.00
.5	9.08	9.00	10.09	19.1	8.09	-.91
1.	9.52	9.48	11.52	29.5	7.52	-10.4
2.	9.75	9.73	13.76	49.8	5.76	-30.2
5.	9.90	9.89	19.90	109.9	-.10	-90.1

a. The constants used, except for k_A which are given below, are those given in the text. b. In units of $\underline{M}$/min./$\underline{M}$. c. In units of $\underline{M}$. d. In units of $\underline{M} \times 10^{-4}$/min.

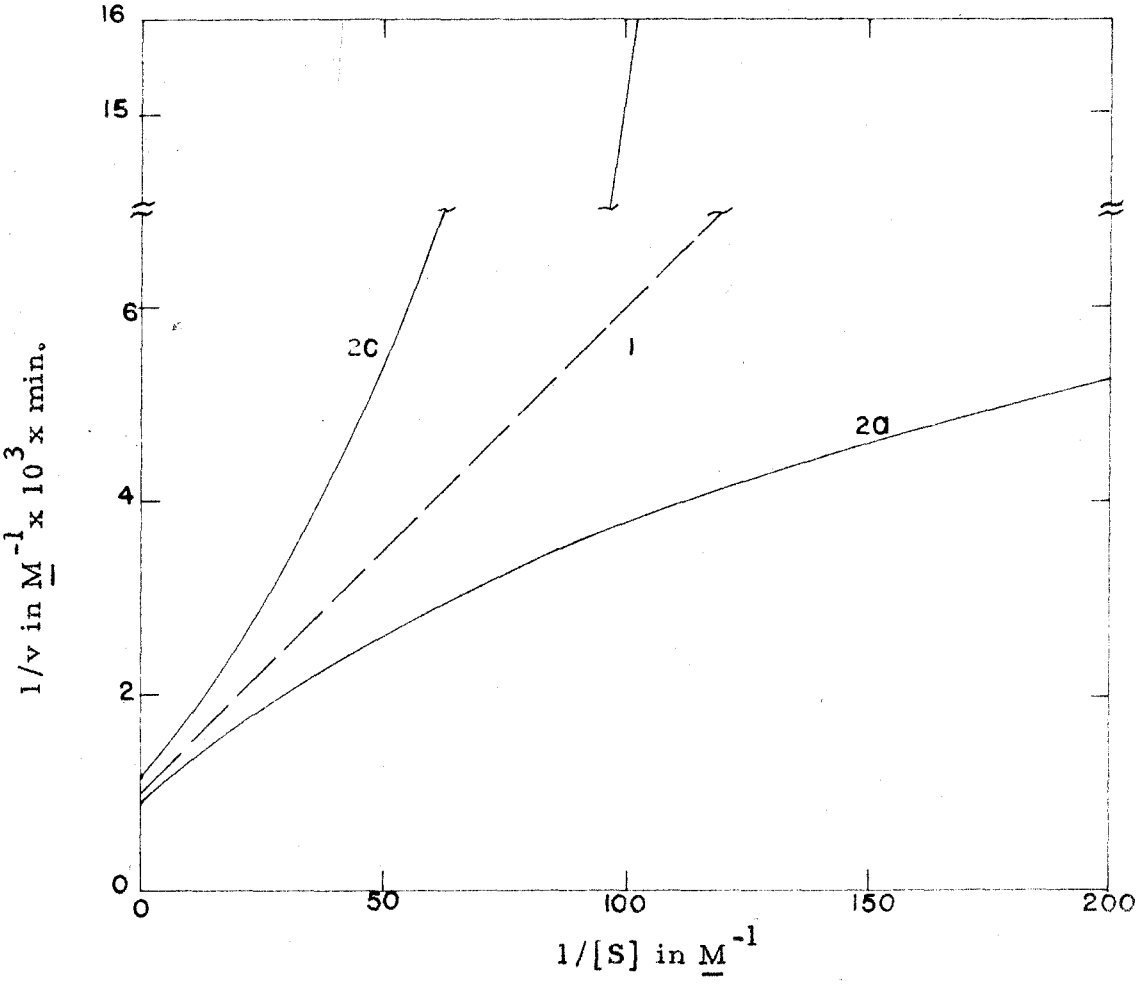

Fig. IIIa. Systematic error effects on equation 1:. equations 2a and 2c which have asymptotes at $1/v = 1/k_{Al}E_T$ and $1/v = -1/k_{Al}E_T$ and intercepts at $1/v = 1/(k + k_{Al})E_T$ and $1/v = 1/(k-k_{Al})E_T$ respectively.

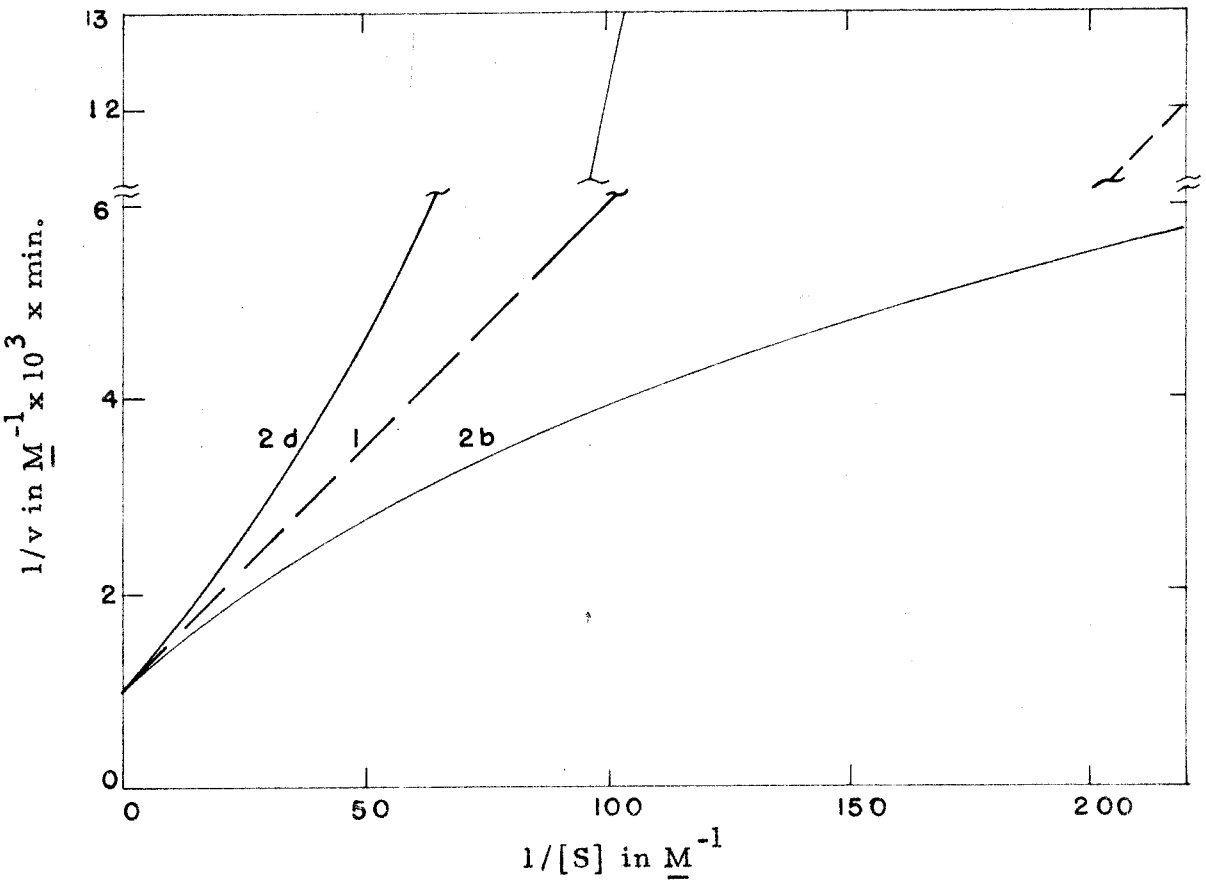

Fig. IIIb. Systematic error effects on equation 1: equations 2b and 2d which have asymptotes at $1/v = 1/k_{A2}E_T$ and $1/v = -1/k_{A2}E_T$ respectively and a common intercept at $1/v = 1/kE_T$.

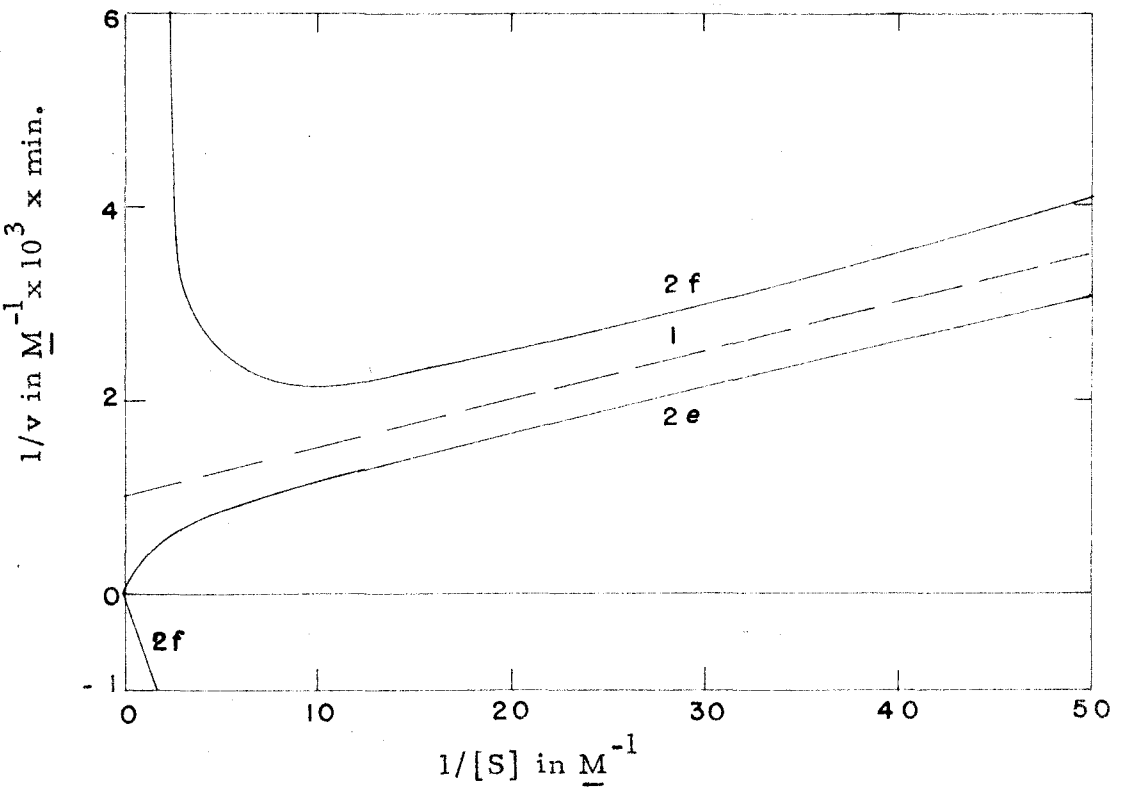

Fig. IIIc. Systematic error effects on equation 1: equations 2e and 2f which have a common intercept at $1/v = 0$.

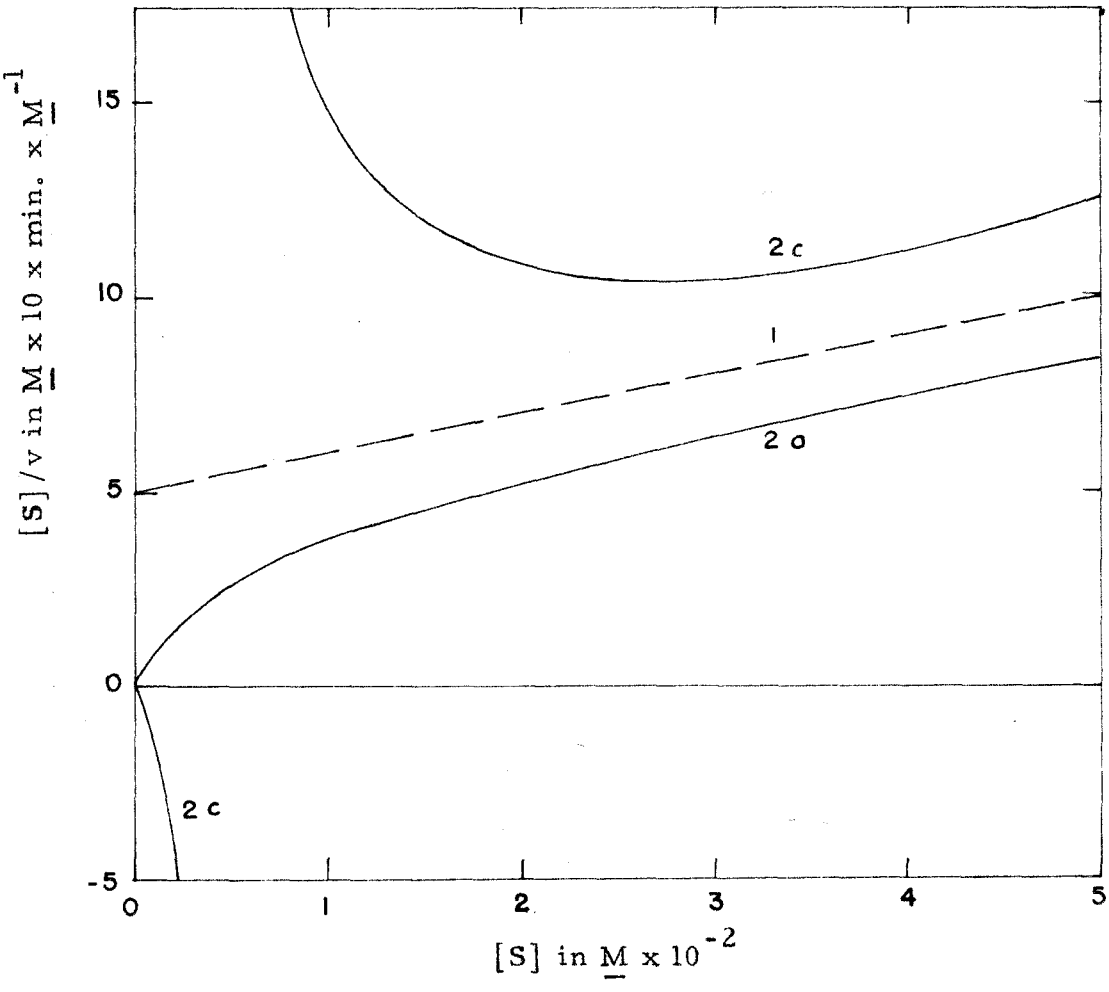

Fig. IVa. Systematic error effects on equation 1: equations 2a and 2c which have a common intercept at $[S]/v = 0$.

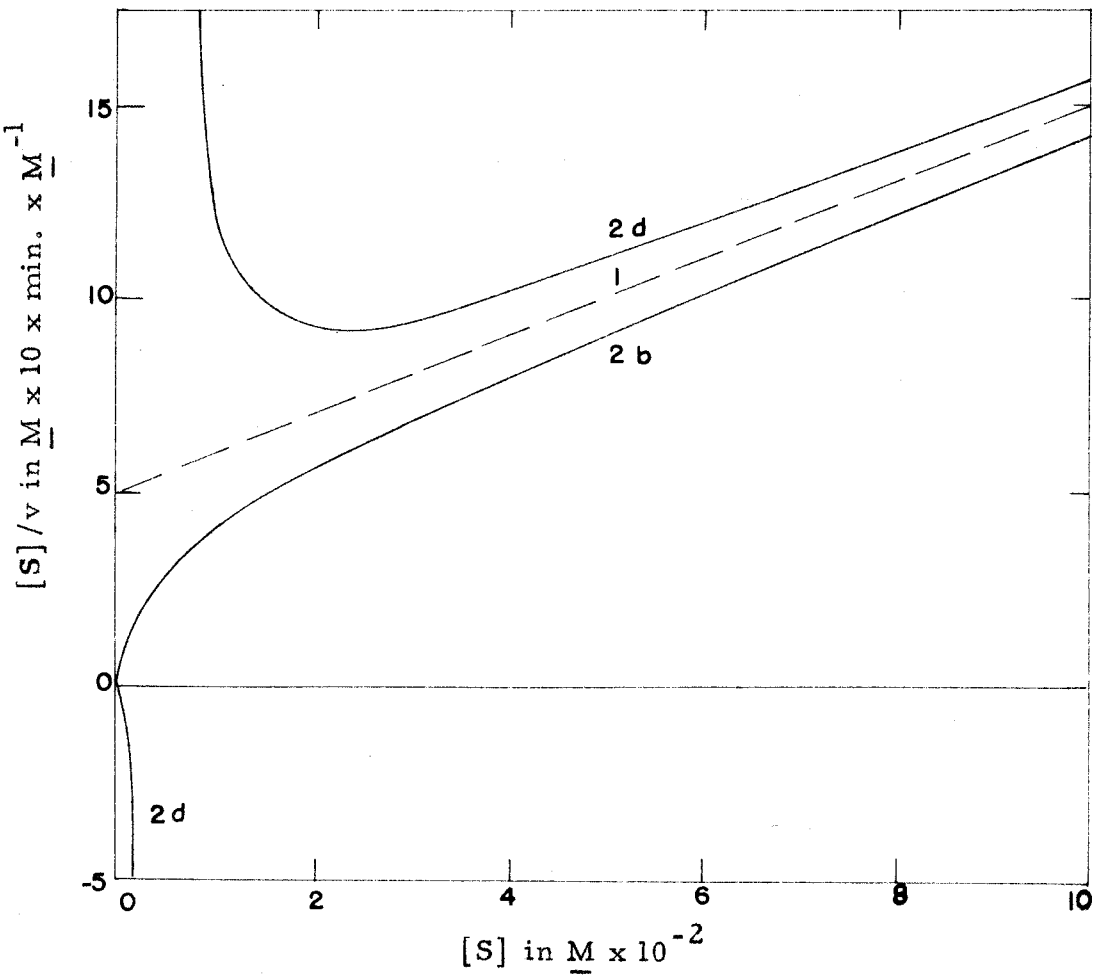

Fig. IVb. Systematic error effects on equation 1: equations 2b and 2d which have a common intercept at $[S]/v = 0$.

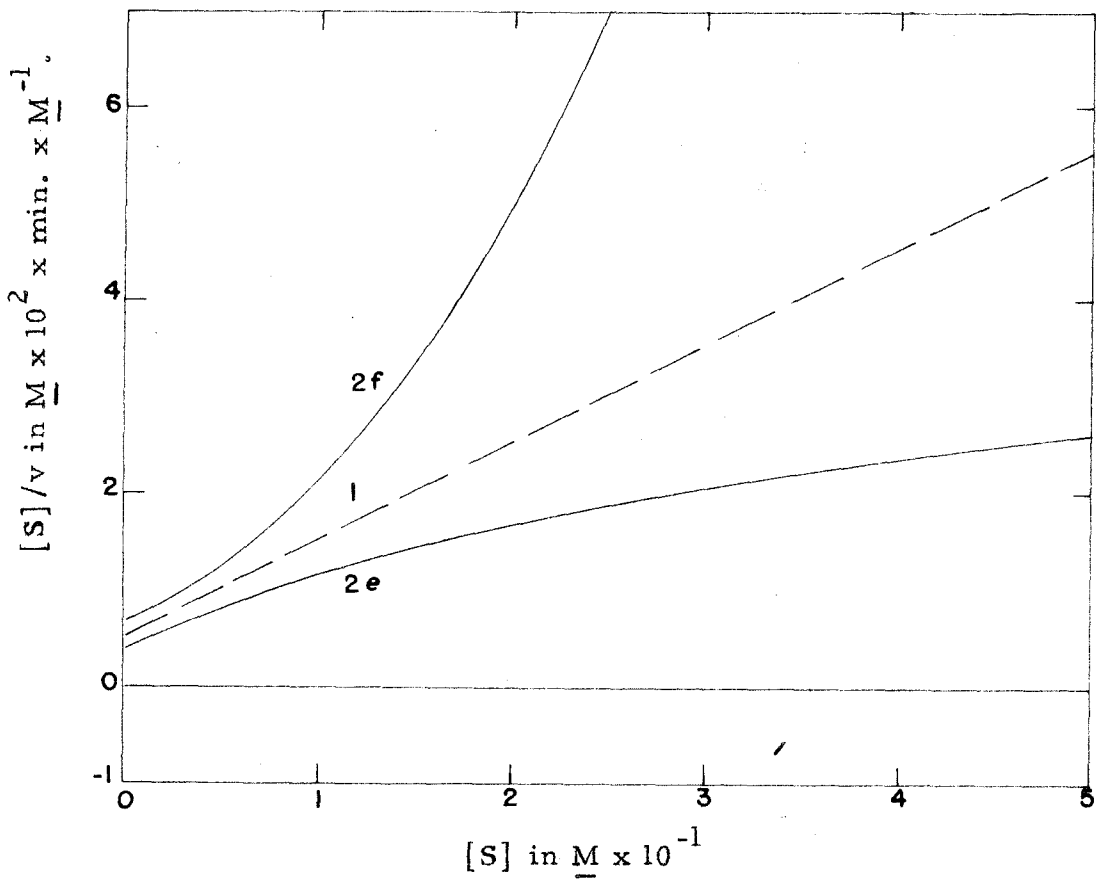

Fig. IVc. Systematic error effects on equation 1: equations 2e and 2f which have asymptotes at $[S]/v = 1/k_{A3}$ and $[S]/v = -1/k_{A3}$ and intercepts at $[S]/v = K/(kE_T + k_{A3}K)$ and $[S]/v = K/(kE_T - k_{A3}K)$ respectively.

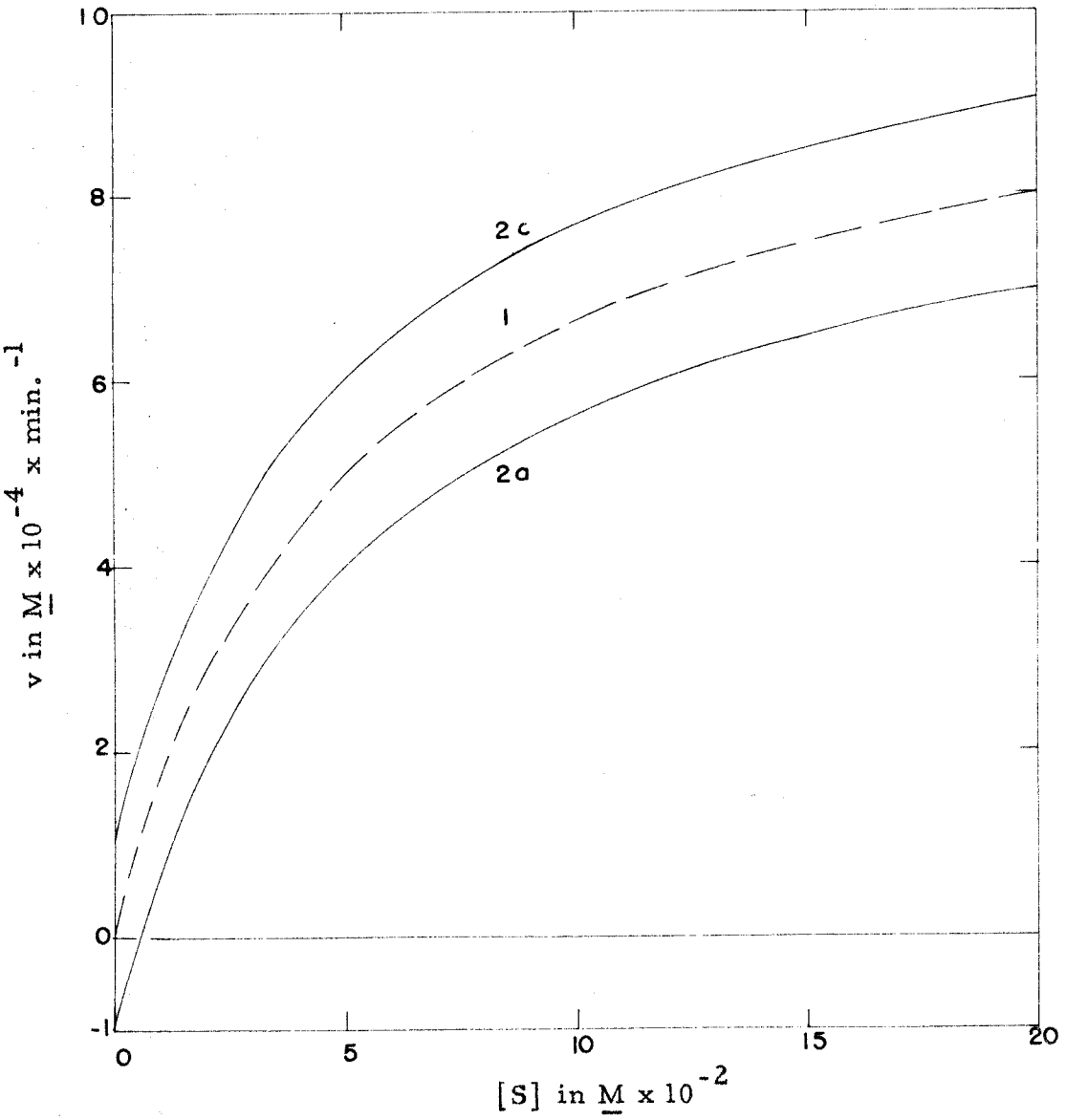

Fig. Va. Systematic error effects on equation 1: equations 2a and 2c.

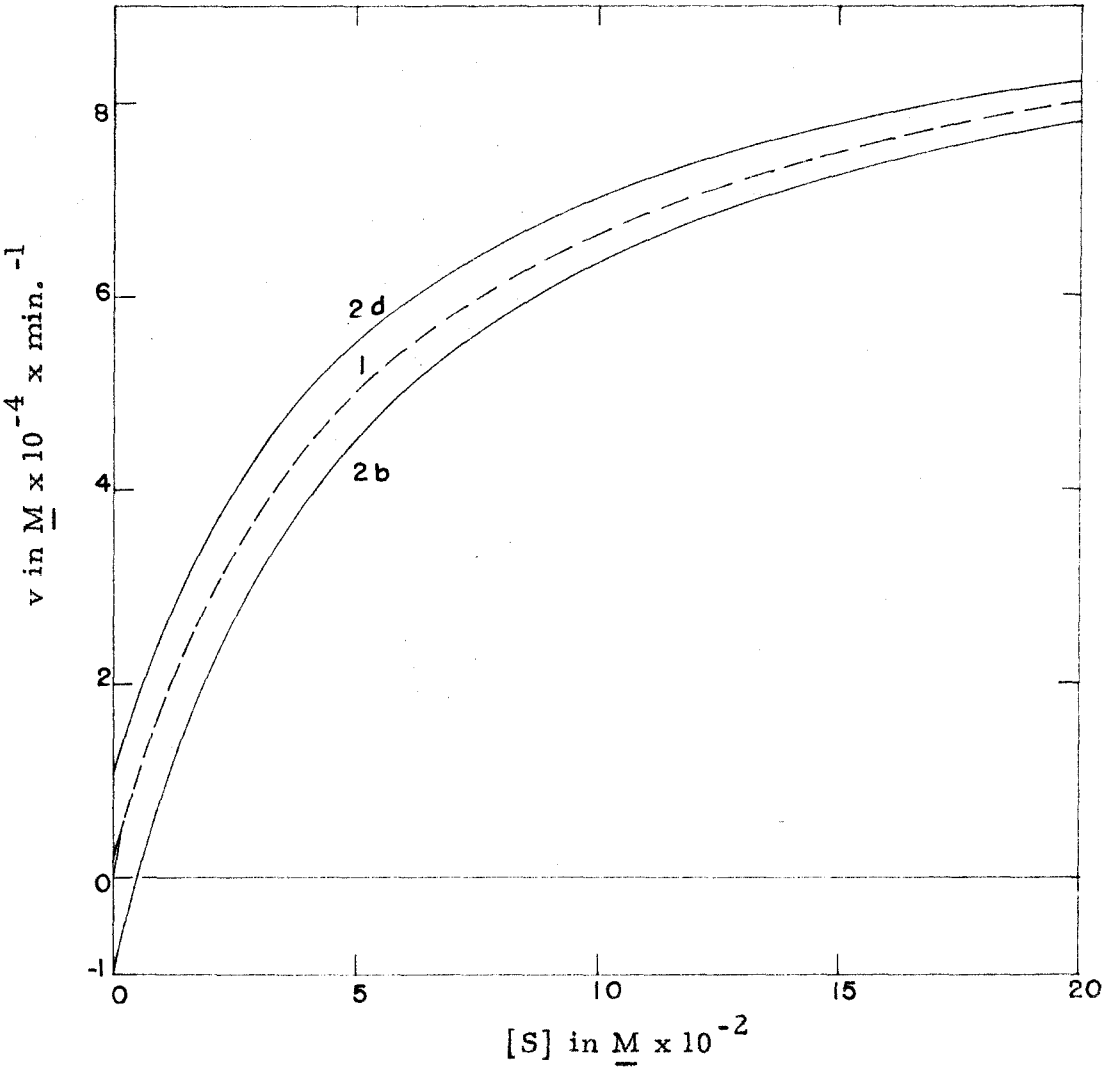

Fig. Vb. Systematic error effects on equation 1: equations 2b and 2d.

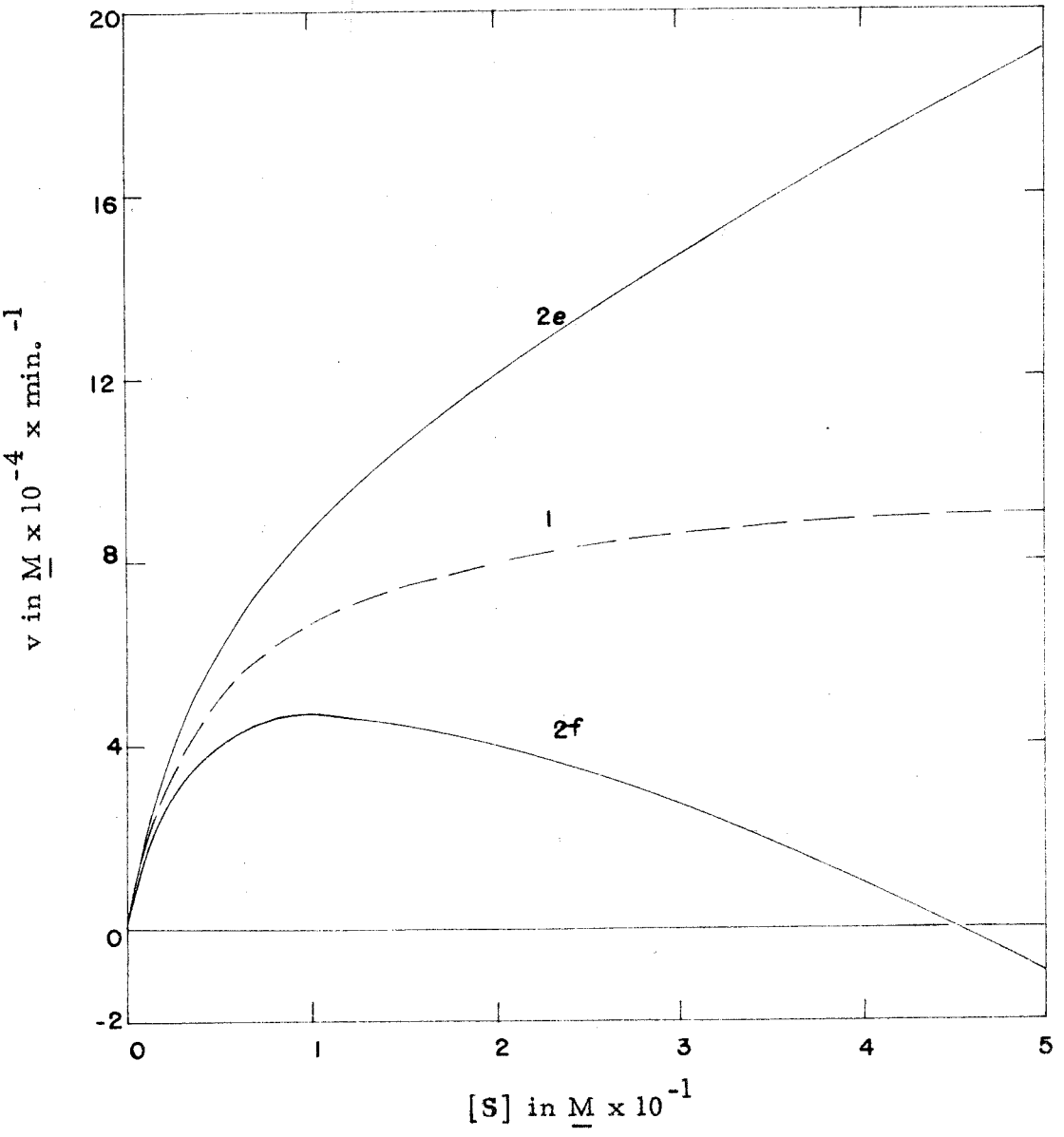

Fig. Vc. Systematic error effects on equation 1: equations 2e and 2f.

Table V

Apparent Values of k and K Calculated from Unweighted and Weighted Linear Least-Squares Fits to Lines Based upon 1/v vs. 1/[S] Plots.[a]

Eq.	$k_A[A]$	1/[S] Range[b]	Apparent k[c]		Apparent K[d]		Better Method
			Unw.	W.	Unw.	W.	
2a	$k_{A_1}[E_T]$	2000-200	2.34	4.79	2.55	6.98	Weighted
		200-20	17.31	19.25	38.12	44.49	Weighted
		20-2	20.17	20.17	48.89	48.95	neither[e]
		2-0.2	20.20	20.20	49.88	49.60	Unweighted[e]
2b	$k_{A2}[E_F]$	2000-200	2.31	4.72	2.52	6.91	Weighted
		200-20	17.10	19.04	37.96	44.40	Weighted
		20-2	19.93	19.98	48.64	48.98	Weighted[e]
2c	$-k_{A1}[E_T]$	200-20	25.99	20.83	75.05	56.16	Weighted
		20-2	19.84	19.82	51.12	51.00	neither[e]
		2-0.2	19.80	19.79	50.48	50.46	neither[e]
2d	$-k_{A2}[E_F]$	200-20	25.33	21.16	71.86	56.73	Weighted[e]
		20-2	20.04	20.02	51.18	51.04	Weighted[e]
		2-0.2	20.00	20.00	50.34	50.58	neither[e]
2e	$k_{A3}[S]$	2000-200	20.46	20.43	50.66	50.58	Weighted[e]
		200-20	20.58	20.61	51.00	51.02	Unweighted[e]
		20-2	22.04	22.48	58.59	62.54	Unweighted
		2-0.2	36.67	43.28	449.	702.	Unweighted
2f	$-k_{A3}[S]$	2000-200	19.66	19.38	49.65	48.88	Unweighted
		200-20	19.44	19.35	49.06	48.73	Unweighted[e]
		20-2	17.98	17.73	40.80	38.38	Unweighted

a. The values for the constants in equation 2 are given in the text.
b. In units of M^{-1}.
c. In units of $\bar{M}$/min./$\bar{M}$; the actual value of k is 20.
d. In units of $\bar{M} \times 10^{-3}$; the actual value of K is 50.
e. The differences between the Unweighted and Weighted values for k and K are less than 1% and may be the result of rounding.

Table VIa

Systematic Error Effects on Product Inhibition

$K_1 = 2K.$ [a]

Equation 3 with $k_A[A] = 0$					
Hydrolysis	S_t [b]	t [c]	$(S_o - S_t)/t$ [d]	$(\ln \frac{S_o}{S_t})/t$ [e]	1/slope [f]
20%	.04	21.7	.461	10.28	
30%	.035	34.3	.438	10.42	-6.7
40%	.03	48.4	.413	10.58	

Equation	Hydrolysis	S'_t [b]	$(S_o - S'_t)/t$ [d]	$(\ln \frac{S_o}{S'_t})/t$ [e]	1/slope' [f]
3a	20%	.03783	.561	12.80	-28
	30%	.03157	.538	13.40	
	40%	.02516	.514	14.09	
3b	20%	.03889	.513	11.47	-16
	30%	.03321	.490	11.85	
	40%	.02744	.457	12.34	
3c	20%	.04217	.361	7.82	5.9
	30%	.03843	.337	7.65	
	40%	.03484	.314	7.42	
3d	20%	.04112	.409	9.11	8.3
	30%	.03679	.385	8.91	
	40%	.03256	.361	8.83	
3e	20%	.03976	.472	2.68	-15
	40%	.02954	.423	3.43	
3f	20%	.04024	.450	4.85	10
	40%	.03046	.404	4.38	

a. The constants used are given in the text, except that $S_o = 0.05$ M, and $\pm k_{A1} = \pm k_{A2} = \pm 2.0$ M/min./M.

b. In units of M.

c. In units of minutes.

d. In units of M x 10^{-3}/min.

e. In units of min.$^{-1}$ x 10^{-3}.

f. In units of M^{-1}.

Table VIb

Systematic Error Effects on Product Inhibition

$K_1 = 0.5\ K.$ [a]

Equation 3 with $k_A[A] = 0$					
Hydrolysis	S_t [b]	t [c]	$(S_o - S_t)/t$ [d]	$(\ln \frac{S_o}{S_t})/t$ [e]	1/slope [f]
20%	.04	23.4	.427	9.53	6.7
40%	.03	56.8	.352	9.03	

Equation	Hydrolysis	S'_t [b]	$(S_o - S'_t)/t$ [d]	$(\ln \frac{S_o}{S'_t})/t$ [e]	1/slope' [f]
3a	20%	.03766	.528	12.08	-8.0
	40%	.02432	.452	12.69	
3b	20%	.03889	.475	10.63	1.3
	40%	.02744	.397	10.53	
3c	20%	.04234	.327	7.11	16
	40%	.03568	.252	5.93	
3d	20%	.04112	.380	8.44	12
	40%	.03256	.307	7.54	
3e	20%	.03988	.433	9.62	6.3
	40%	.02972	.357	9.14	
3f	20%	.04012	.422	9.38	8.9
	40%	.03028	.347	8.71	

a. The constants used are given in the text, except that $S_o = 0.05$ M and $\pm k_{A1} = \pm k_{A2} = \pm 2.0$ M/min./M.

b. In units of M.

c. In units of minutes.

d. In units of M $\times 10^{-3}$/min.

e. In units of $\text{min.}^{-1} \times 10^{-3}$.

f. In units of M^{-1}.

Table VIc

Systematic Error Effects on Product Inhibition

$K_1 = K.^{a}$

Equation 3 with $k_A[A] = 0$					
Hydrolysis	S_t[b]	t[c]	$(S_o - S_t)/t$[d]	$(\ln \frac{S_o}{S_t})/t$[e]	1/slope[f]
20%	.04	22.3	.448	.01	0.0
40%	.03	51.2	.391	.01	

Equation	Hydrolysis	S'_t[b]	$(S_o - S'_t)/t$[d]	$(\ln \frac{S_o}{S'_t})/t$[e]	1/slope'[f]
3a	20%	.03777	.549	12.6	-17
	40%	.02488	.491	13.6	
3b	20%	.03889	.498	11.17	-9.3
	40%	.02744	.441	11.70	
3c	20%	.04223	.349	7.58	12
	40%	.03512	.291	6.91	
3d	20%	.04112	.398	8.86	8.4
	40%	.03256	.341	8.38	
3e	20%	.0398	.457	10.22	-0.5
	40%	.0296	.399	10.25	
3f	20%	.0402	.440	9.78	1.1
	40%	.0304	.383	9.72	

a. The constants used are given in the text, except that $S_o = 0.05$ M and $\pm k_{A1} = \pm k_{A2} = \pm 2.0$ M/min./M.

b. In units of M.

c. In units of minutes.

d. In units of M x 10^{-3}/min.

e. In units of $min.^{-1} \times 10^{-3}$.

f. In units of M^{-1}.

Table VII

$1/v_A$ Calculated from Equation 2.

Equation	2a	2b	2c	2d	2e	2f
$1/[S]^a$	$1/v_A$ [b]	$1/v_A$ [b]	$1/v_A$ [b]	$1/v_A$ [b]	$1/v_A$ [b]	$1/v_A$ [b]
2000	5.02	5.05	---	0.0	10.00	10.20
1000	3.38	3.40	10.41	10.20	5.05	5.15
500	2.06	2.08	3.51	3.47	2.57	2.63
200	.992	1.00	1.24	1.22	1.09	1.11
100	.566	.571	.638	.633	.593	.607
50	.338	.341	.363	.360	.345	.356
20	.196	.198	.204	.202	.196	.204
10	.148	.149	.152	.151	.146	.153
5	.124	.125	.127	.125	.119	.132
2	.109	.110	.111	.110	.099	.124
1	.104	.104	.106	.105	.087	.133
0.5	.102	.103	.104	.103	.073	.174
0.2	.100	.101	.102	.101	.050	---

a. In units of $\underline{M}^{-1}$.

b. In units of min. x $10^4/\underline{M}$.

Table VIII

1/v for a "Totally Competitive" Inhibitor
Calculated from Equation 4.[a]

Equation	(4a)			(4b)		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]$[b]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]
2000	5.26	6.68	9.09	5.55	10.05	50.05
1000	3.59	5.02	8.34	3.73	6.73	33.40
500	2.22	3.38	7.15	2.28	4.08	20.08
200	1.07	1.74	5.02	1.09	1.91	9.18
100	.610	.991	3.38	.619	1.05	4.86
50	.361	.566	2.06	.366	.585	2.54
20	.206	.291	.991	.208	.297	1.09
10	.153	.196	.566	.154	.199	.597
5	.126	.148	.338	.127	.150	.349
2	.110	.119	.196	.111	.120	.200
1	.104	.109	.148	.105	.110	.150
0.5	.102	.104	.123	.103	.105	.125
0.2	.100	.101	.109	.101	.102	.110

Equation	(4c)[d]			(4d)		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]$[b]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]
2000	---	---	---	0.0	0.0	0.0
1000	12.72	---	---	11.20	20.20	100.2
500	3.99	10.41	---	3.80	6.80	33.5
200	1.36	2.66	---	1.33	2.33	11.2
100	.695	1.24	10.41	.684	1.16	5.37
50	.390	.638	3.51	.385	.615	2.67
20	.215	.309	1.24	.212	.303	1.11
10	.157	.204	.638	.156	.201	.603
5	.129	.152	.363	.128	.150	.351
2	.112	.122	.204	.111	.120	.200
1	.107	.111	.152	.106	.110	.150
0.5	.104	.106	.127	.103	.105	.125
0.2	.102	.103	.111	.101	.102	.110

Table VIII (cont'd.)

Equation	(4e)			(4f)[d]		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]$[b]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]
2000	10.98	19.70	90.99	11.22	20.51	111.2
1000	5.54	9.90	45.54	5.66	10.31	55.7
500	2.82	5.00	22.81	2.88	5.21	27.9
200	1.19	2.06	9.17	1.22	2.15	11.2
100	.642	1.08	4.63	.659	1.13	5.68
50	.369	.586	2.34	.381	.615	2.90
20	.206	.291	.991	.215	.309	1.24
10	.150	.192	.536	.160	.208	.682
5	.121	.142	.307	.134	.150	.407
2	.100	.107	.167	.125	.136	.250
1	.087	.090	.115	.134	.141	.214
0.5	.073	.074	.083	.174	.573	.250
0.2	.050	.051	.052	---	---	---

a. The constants used are those given in the text. For values of $1/v_A$, see Table VII.

b. In units of $\underline{M}^{-1}$.

c. In units of min. x $10^4/\underline{M}$.

d. --- indicates negative 1/v values.

Table IX

1/v for a "Totally Non-Competitive" Inhibitor
Calculated from Equation 5. [a]

Equation	(5a)			(5b)		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]$ [b]	$1/v_B$ [c]	$1/v_C$ [c]	$1/v_D$ [c]	$1/v_B$ [c]	$1/v_C$ [c]	$1/v_D$ [c]
2000	5.26	6.69	9.10	5.56	10.10	50.5
1000	3.65	5.05	8.36	3.80	6.80	34.0
500	2.22	3.42	7.22	2.29	4.17	20.8
200	1.08	1.80	5.24	1.10	2.00	10.0
100	.619	1.07	3.75	.628	1.14	5.71
50	.371	.654	2.59	.376	.683	3.41
20	.215	.385	1.67	.218	.396	1.98
10	.162	.291	1.30	.164	.299	1.49
5	.136	.244	1.11	.137	.249	1.25
2	.120	.215	.991	.121	.220	1.10
1	.114	.206	.950	.116	.210	1.05
0.5	.112	.201	.930	.113	.205	1.03
0.2	.110	.198	.917	.111	.202	1.01

Equation	(5c) [d]			(5d)		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]$ [b]	$1/v_B$ [c]	$1/v_C$ [c]	$1/v_D$ [c]	$1/v_B$ [c]	$1/v_C$ [c]	$1/v_D$ [c]
2000	---	---	---	0.0	0.0	0.0
1000	13.48	---	---	11.75	20.40	101.9
500	4.01	10.83	---	8.31	6.93	34.7
200	1.38	2.82	---	1.34	2.44	12.2
100	.707	1.36	15.00	.695	1.36	6.31
50	.400	.753	5.39	.395	.718	3.59
20	.225	.417	2.50	.222	.404	2.02
10	.168	.309	1.77	.166	.302	1.51
5	.140	.256	1.43	.138	.251	1.25
2	.122	.225	1.24	.121	.220	1.10
1	.117	.215	1.17	.116	.210	1.05
0.5	.114	.209	1.14	.113	.205	1.03
0.2	.112	.206	1.12	.111	.202	1.01

Table IX (cont'd.)

Equation	(5e)			(5f)[d]		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]^b$	$1/v_B{}^c$	$1/v_C{}^c$	$1/v_D{}^c$	$1/v_B{}^c$	$1/v_C{}^c$	$1/v_D{}^c$
2000	10.999	19.80	100.0	11.24	20.61	112.4
1000	5.68	10.00	46.27	5.81	10.41	56.8
500	2.83	5.09	23.55	2.89	5.31	29.0
200	1.20	2.15	9.91	1.23	2.25	12.4
100	.651	1.17	5.36	.669	1.23	6.82
50	.379	.681	3.07	.391	.720	4.07
20	.215	.385	1.67	.225	.417	2.50
10	.160	.283	1.15	.171	.319	2.14
5	.130	.227	.833	.146	.278	2.50
2	.108	.180	.524	.138	.282	---
1	.094	.148	.339	.150	.362	---
0.5	.078	.113	.201	.205	1.139	---
0.2	.053	.067	.091	---	---	---

a. The constants used are those given in the text. For values of $1/v_A$, see Table VII.

b. In units of $\underline{M}^{-1}$.

c. In units of min. $\times 10^4/\underline{M}$.

d. --- indicates negative 1/v values.

Table X

1/v for a "Totally Un-Competitive" Inhibitor
Calculated from Equation 6.[a]

Equation	(6a)			(6b)		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]$[b]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]
2000	5.03	5.05	5.24	5.06	5.10	5.50
1000	3.28	3.42	3.75	3.41	3.47	4.00
500	2.07	2.13	2.59	2.09	2.16	2.80
200	.999	1.07	1.67	1.009	1.09	1.82
100	.575	.654	1.30	.581	.667	1.43
50	.347	.431	1.11	.351	.439	1.22
20	.206	.291	.991	.208	.297	1.09
10	.157	.244	.950	.159	.249	1.045
5	.133	.220	.930	.135	.224	1.020
2	.119	.206	.917	.120	.210	1.009
1	.114	.201	.913	.115	.205	1.005
0.5	.111	.198	.911	.113	.202	1.002
0.2	.110	.197	.910	.111	.201	1.001

Equation	(6c)[d]			(6d)		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]$[b]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]
2000	---	---	---	0.0	0.0	0.0
1000	10.45	10.83	15.00	10.22	10.41	12.50
500	3.53	3.70	5.38	3.48	3.60	4.67
200	1.25	1.36	2.50	1.23	1.33	2.22
100	.650	.753	1.76	.642	.734	1.58
50	.373	.471	1.43	.369	.462	1.28
20	.215	.309	1.24	.212	.303	1.111
10	.163	.256	1.17	.161	.251	1.055
5	.137	.230	1.14	.135	.226	1.028
2	.121	.215	1.124	.120	.210	1.011
1	.116	.209	1.117	.115	.205	1.006
0.5	.114	.207	1.114	.113	.203	1.003
0.2	.112	.205	1.112	.111	.201	1.001

Table X (cont'd.)

Equation	(6e)			(6f)[d]		
$(1+[I]/K_2)$	1.1	2	10	1.1	2	10
$1/[S]$[b]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]	$1/v_B$[c]	$1/v_C$[c]	$1/v_D$[c]
2000	10.01	10.10	10.88	10.21	10.31	11.12
1000	5.06	5.15	5.93	5.16	5.25	6.07
500	2.58	2.67	3.45	2.64	2.73	3.55
200	1.10	1.19	1.96	1.12	1.21	2.04
100	.603	.690	1.46	.618	.710	1.55
50	.355	.442	1.19	.365	.458	1.32
20	.206	.291	.991	.215	.309	1.24
10	.155	.238	.868	.165	.263	1.33
5	.128	.206	.727	.143	.247	1.74
2	.107	.174	.502	.136	.266	---
1	.093	.145	.334	.149	.347	---
0.5	.078	.112	.200	.205	1.065	---
0.2	.053	.067	.091	---	---	---

a. The constants used are those given in the text. For values of $1/v_A$, see Table VII.

b. In units of $\underline{M}^{-1}$.

c. In units of min. $\times 10^4/\underline{M}$.

d. --- indicates negative 1/v values.

Fig. VIa. Simulation of mixed inhibition by a "Totally Competitive" inhibitor from equation 4a would be essentially the same as Figure II in the article.

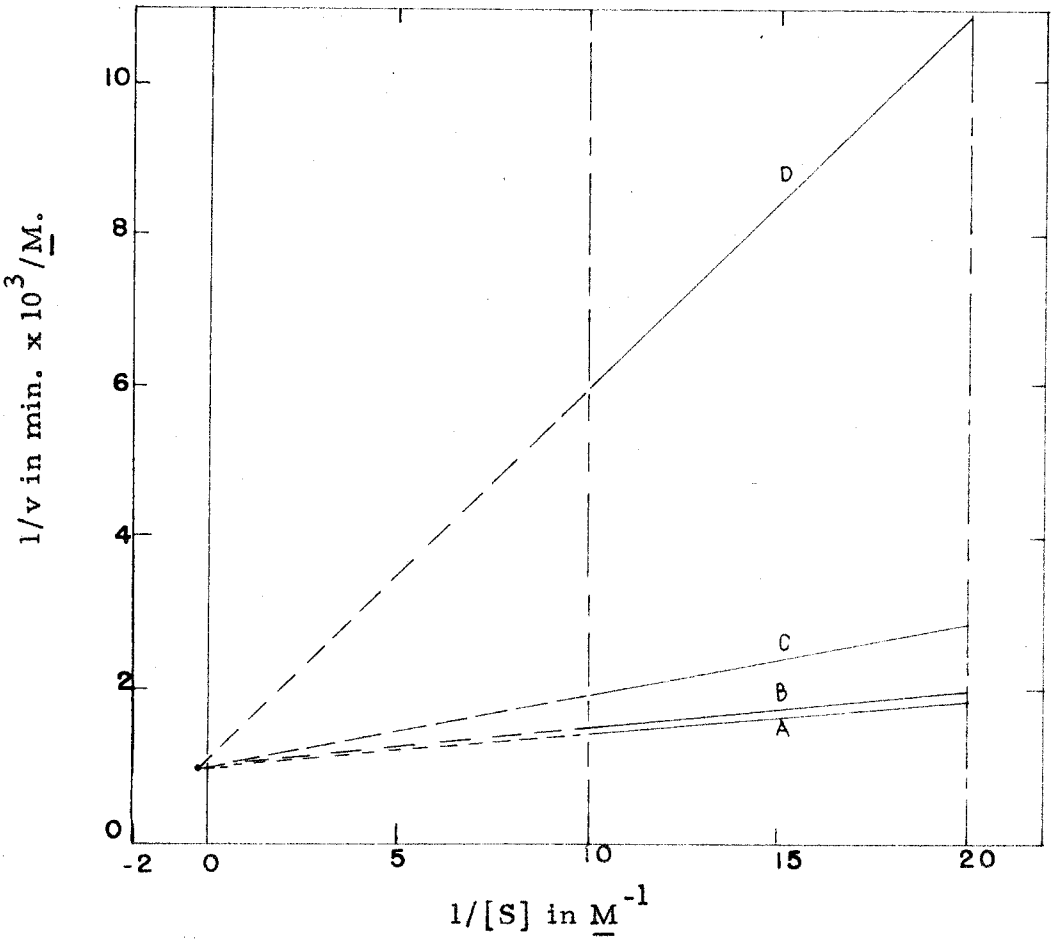

Fig. VIb. Simulation of mixed inhibition by a "Totally Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 4b between $1/[S] = 10$ and 20 M^{-1}.

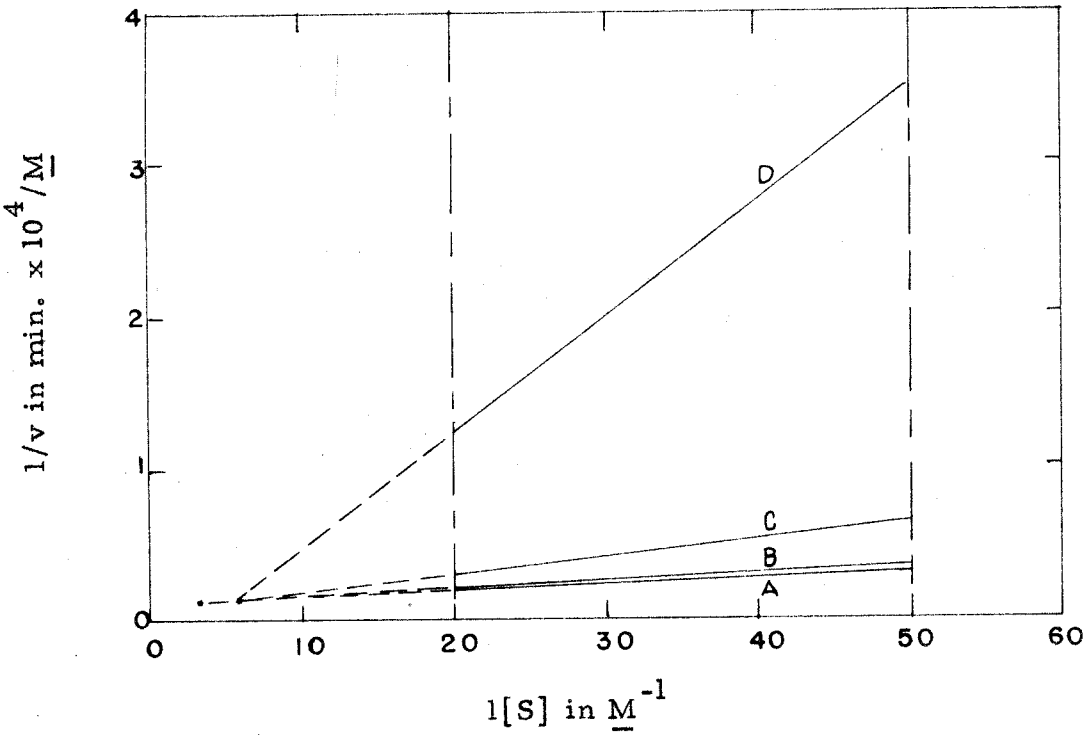

Fig VIc. Simulation of mixed inhibition by a "Totally Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 4c between 1/[S] = 20 and 50 M^{-1}.

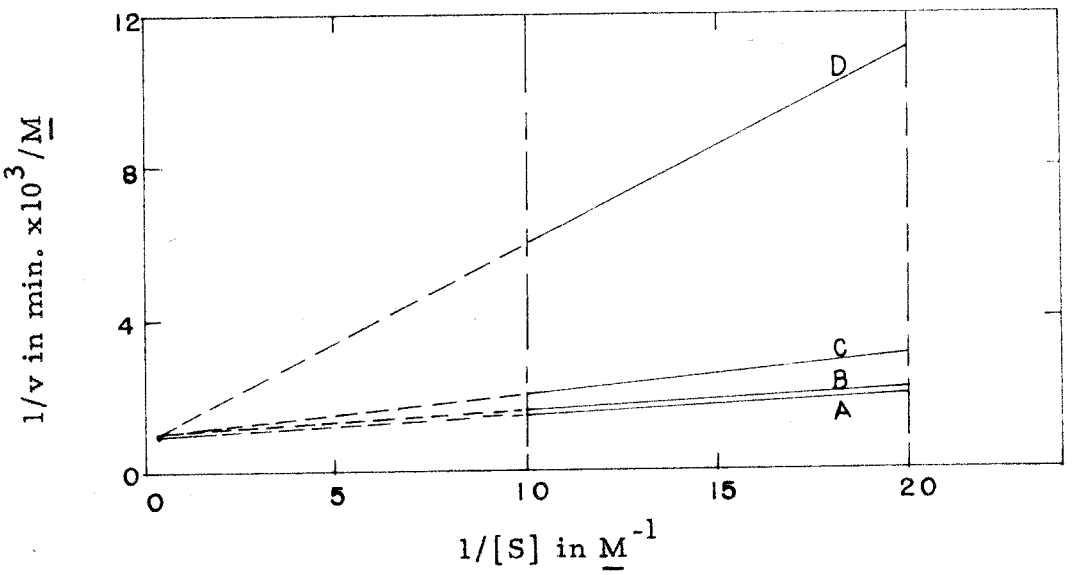

Fig. VId. Simulation of mixed inhibition by a "Totally Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 4d between 1/[S] = 10 and 20 M^{-1}.

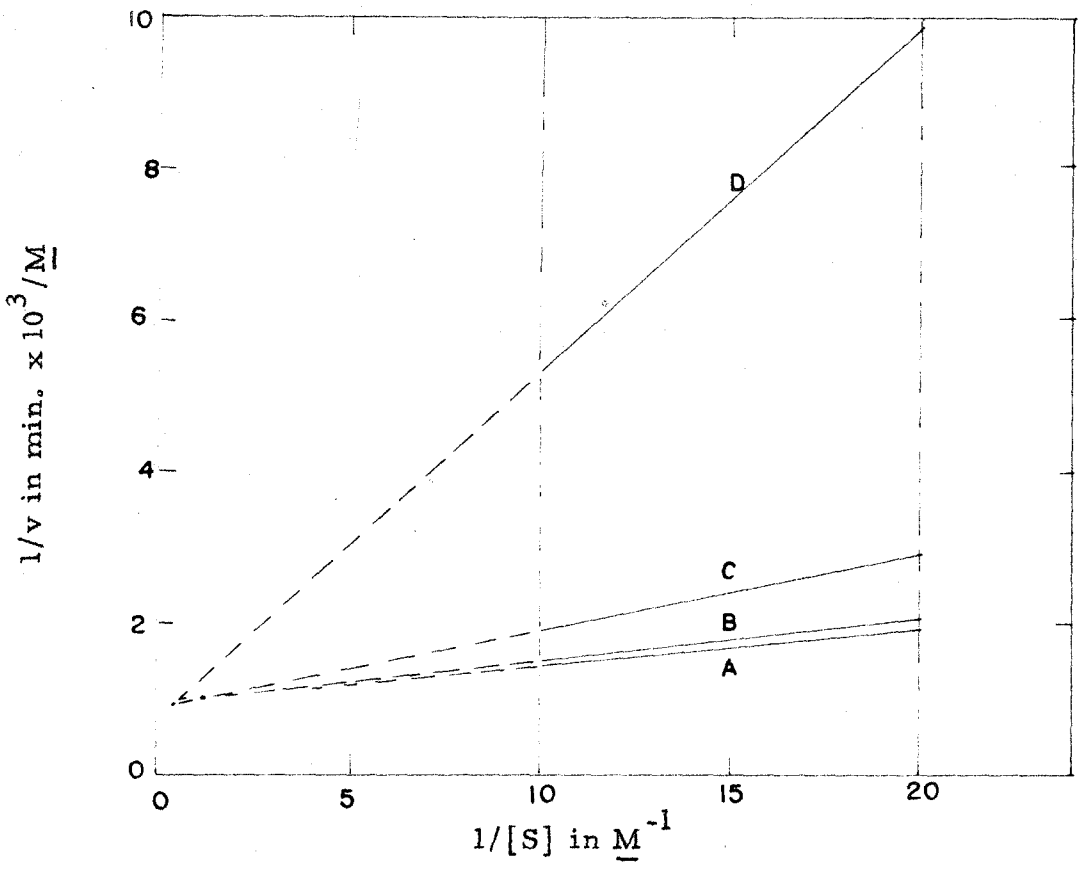

Fig. VIe. Simulation of mixed inhibition by a "Totally Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 4e between 1/[S] = 10 and 20 M^{-1}.

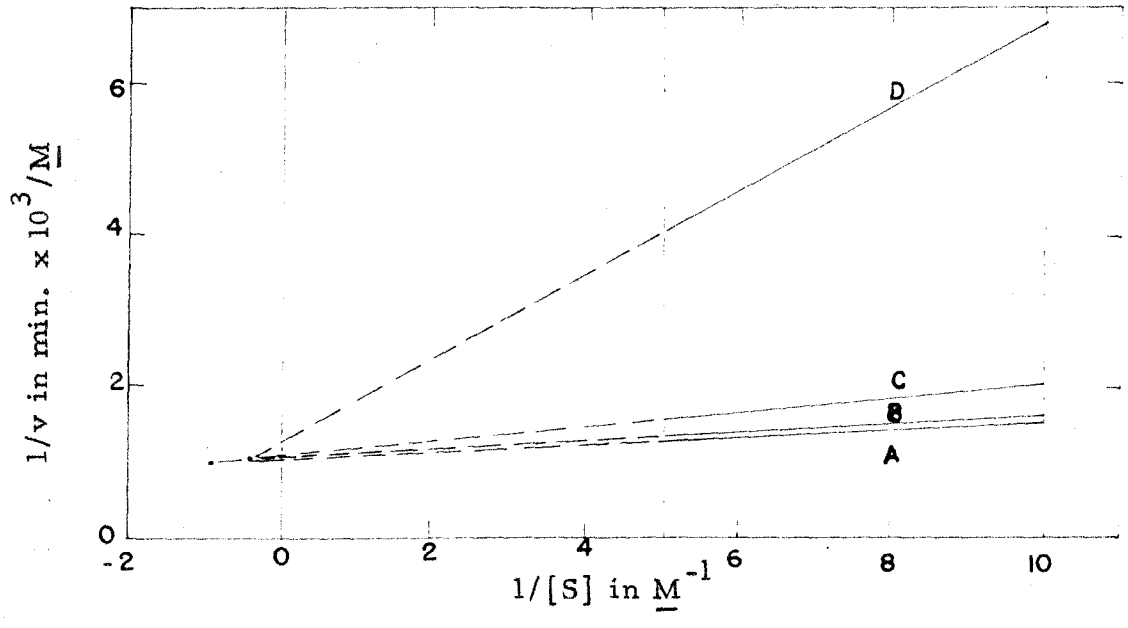

Fig. VIf. Simulation of mixed inhibition by a "Totally Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 4f between 1/[S] = 5 and 10 M^{-1}.

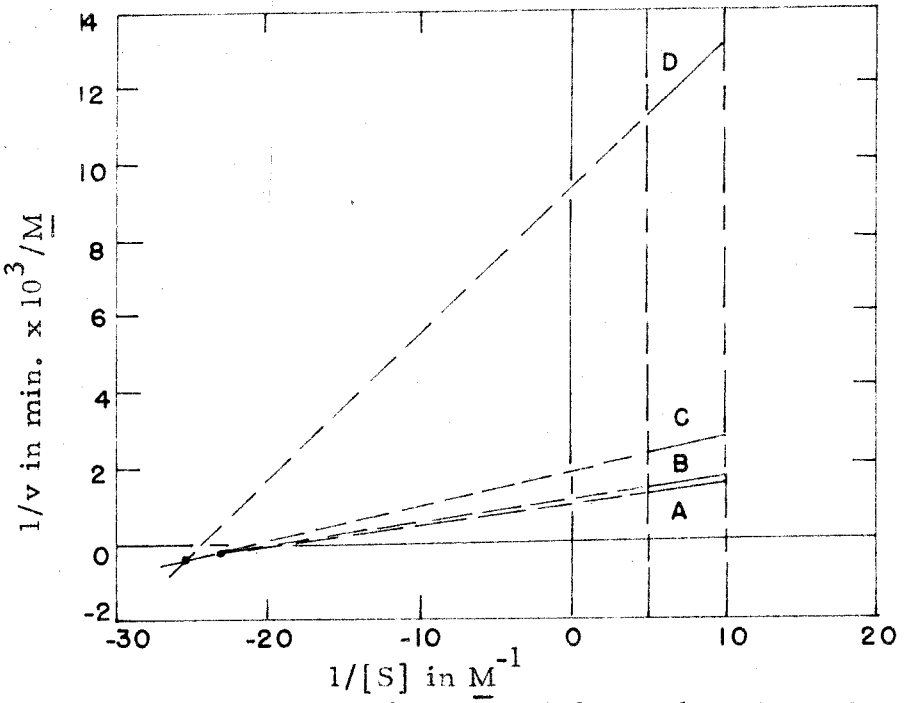

Fig. VIIa. Simulation of mixed inhibition by a "Totally Non-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 5a between $1/[S] = 5$ and 10 M^{-1}.

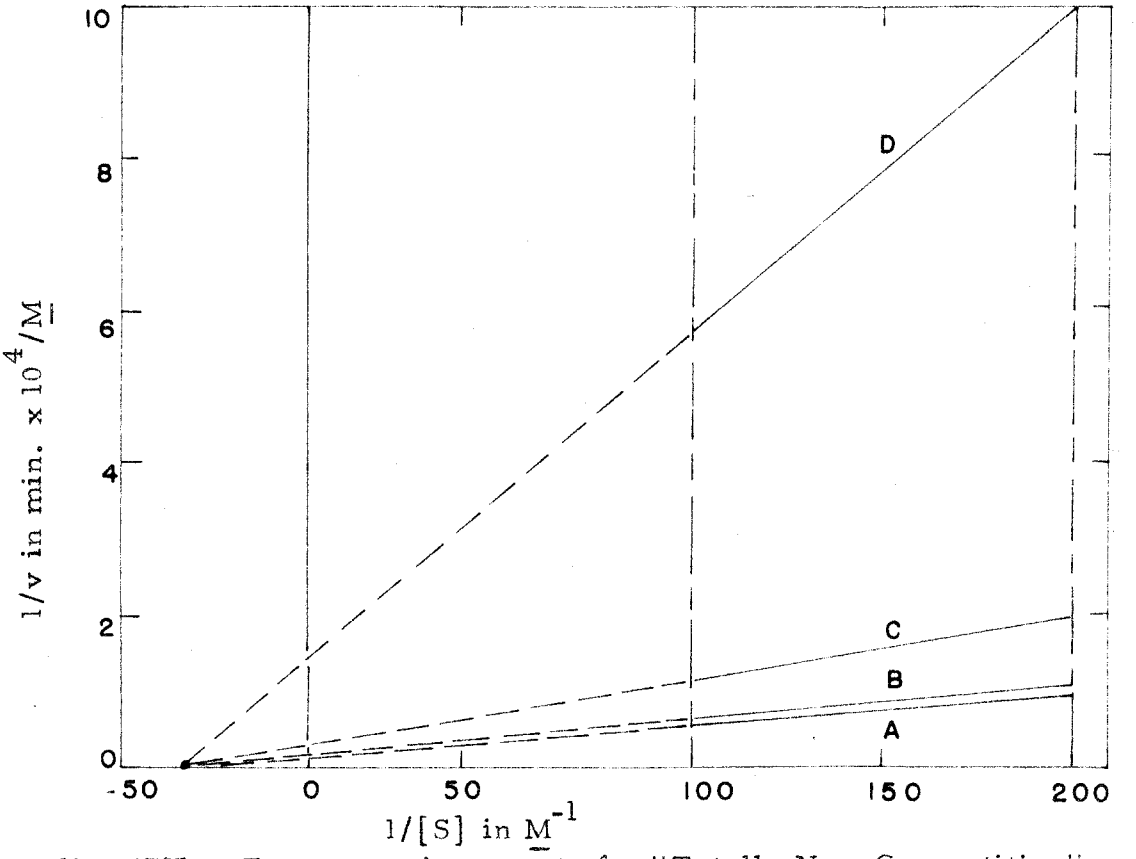

Fig. VIIb. Erroneous intercept of a "Totally Non-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 5b between $1/[S] = 100$ and 200 M^{-1}.

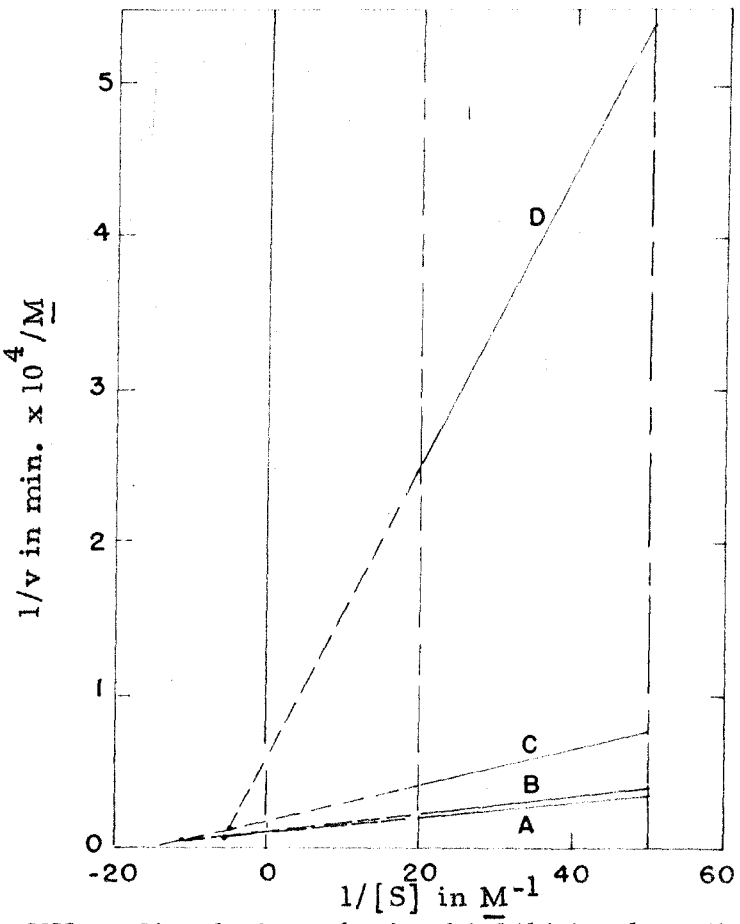

Fig. VIIc. Simulation of mixed inhibition by a "Totally Non-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 5c between 1/[S] = 20 and 50 M^{-1}.

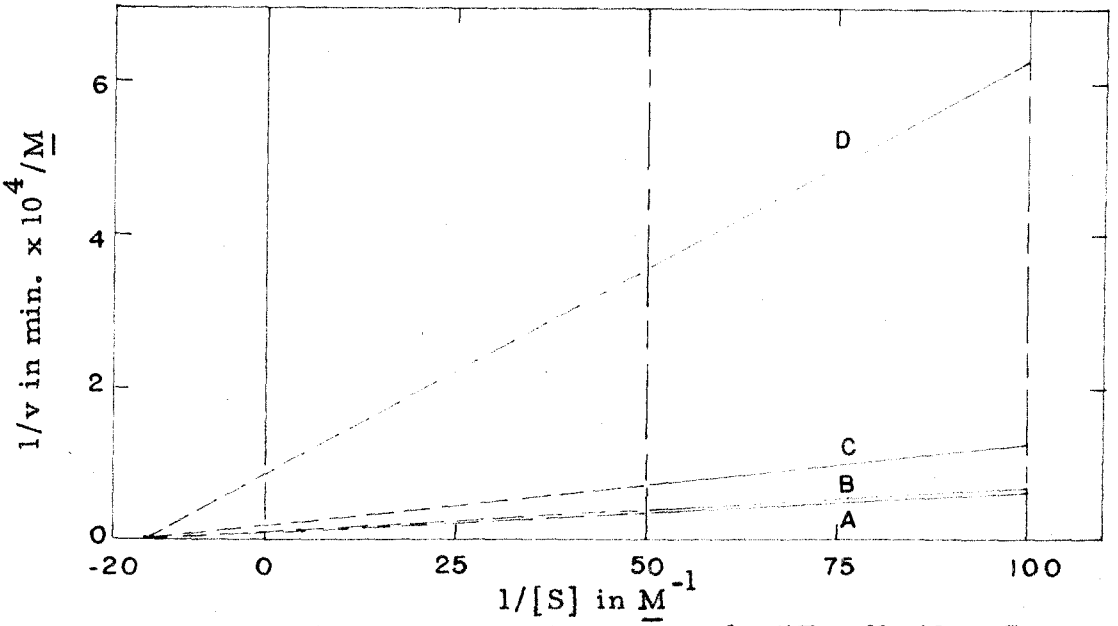

Fig. VIId. Erroneous intercept of a "Totally Non-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 5d between 1/[S] = 50 and 100 M^{-1}.

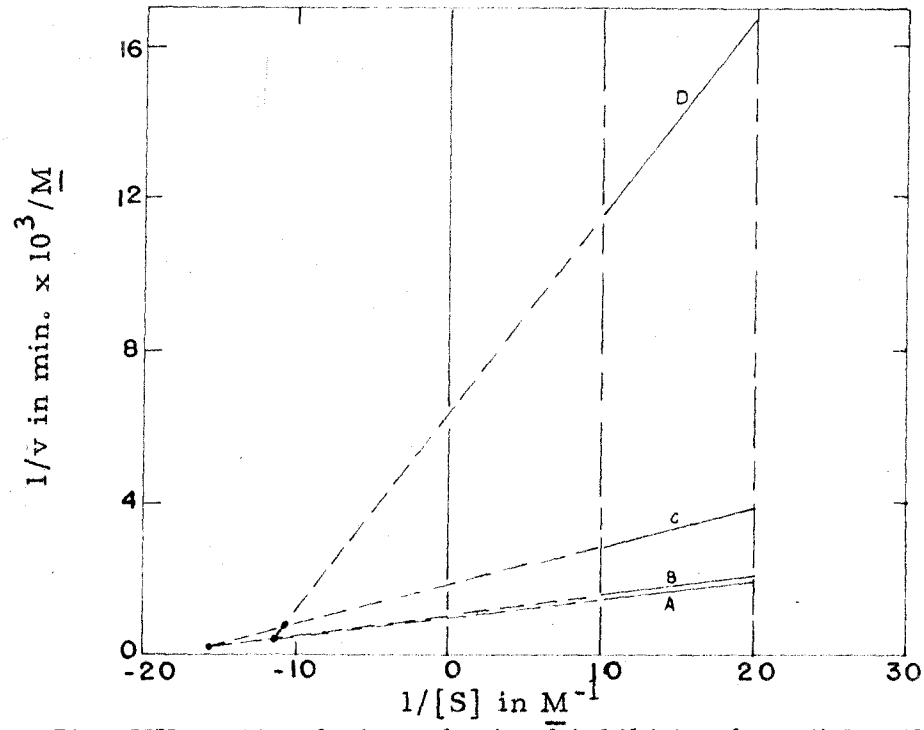

Fig. VIIe. Simulation of mixed inhibition by a "Totally Non-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 5e between 1/[S] = 10 and 20 M^{-1}.

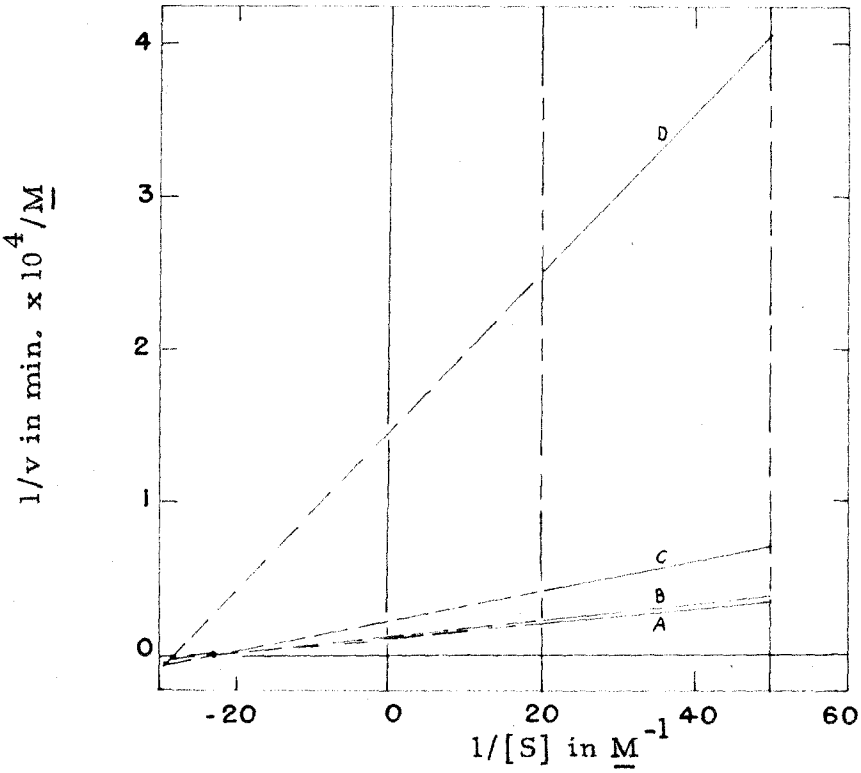

Fig. VIIf. Simulation of mixed inhibition by a "Totally Non-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 5f between 1/[S] = 20 and 50 M^{-1}.

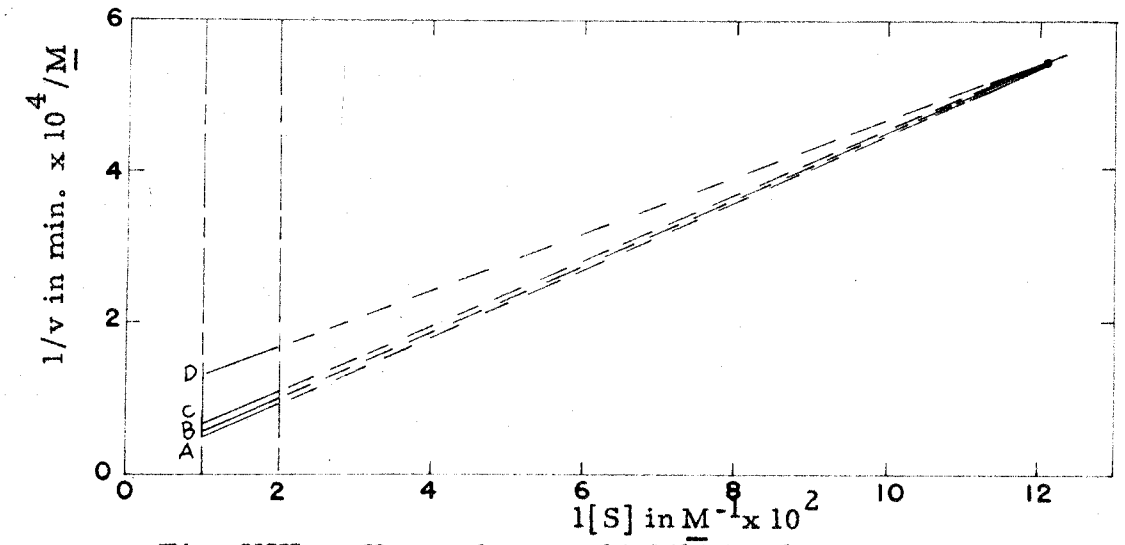

Fig. VIIIa. Unusual type of inhibition by a "Totally Un-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 6a between 1/[S] = 100 and 200 M^{-1}.

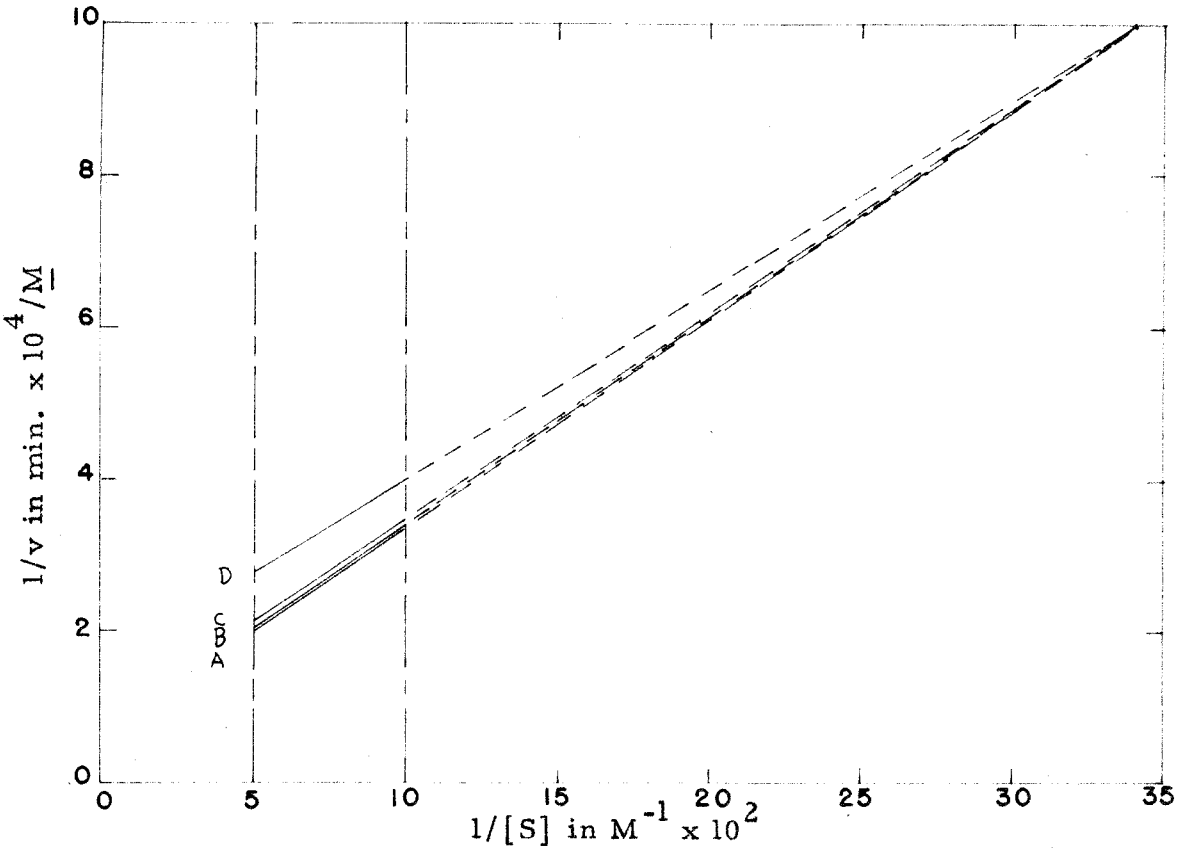

Fig. VIIIb. Unusual type of inhibition by a "Totally Un-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 6b between 1/[S] = 500 and 1000 M^{-1}.

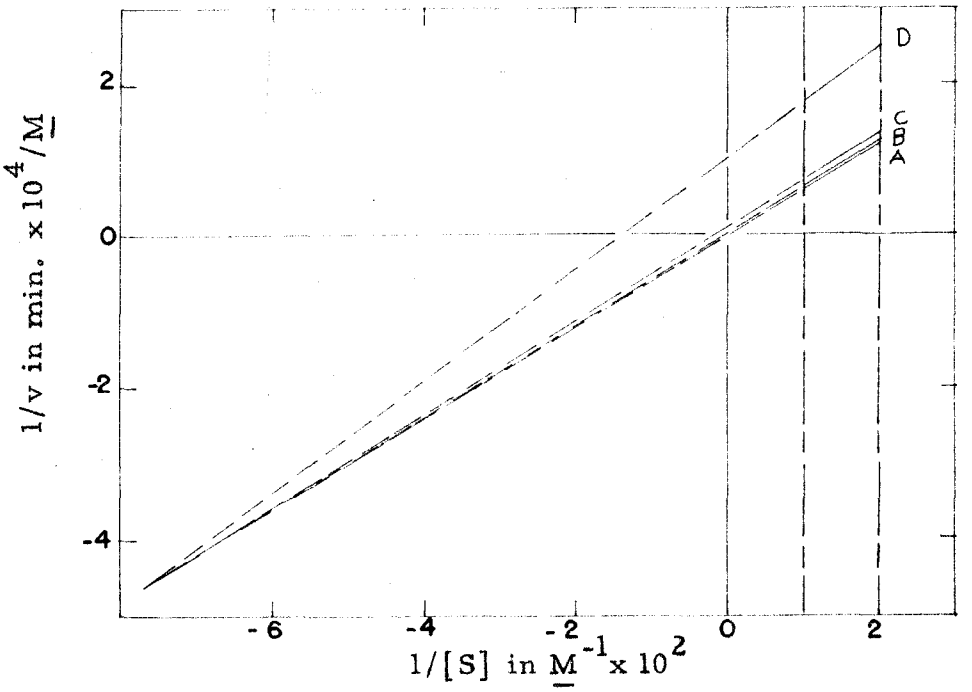

Fig. VIIIc. Simulation of mixed inhibition by a "Totally Un-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 6c between 1/[S] = 100 and 200 M^{-1}.

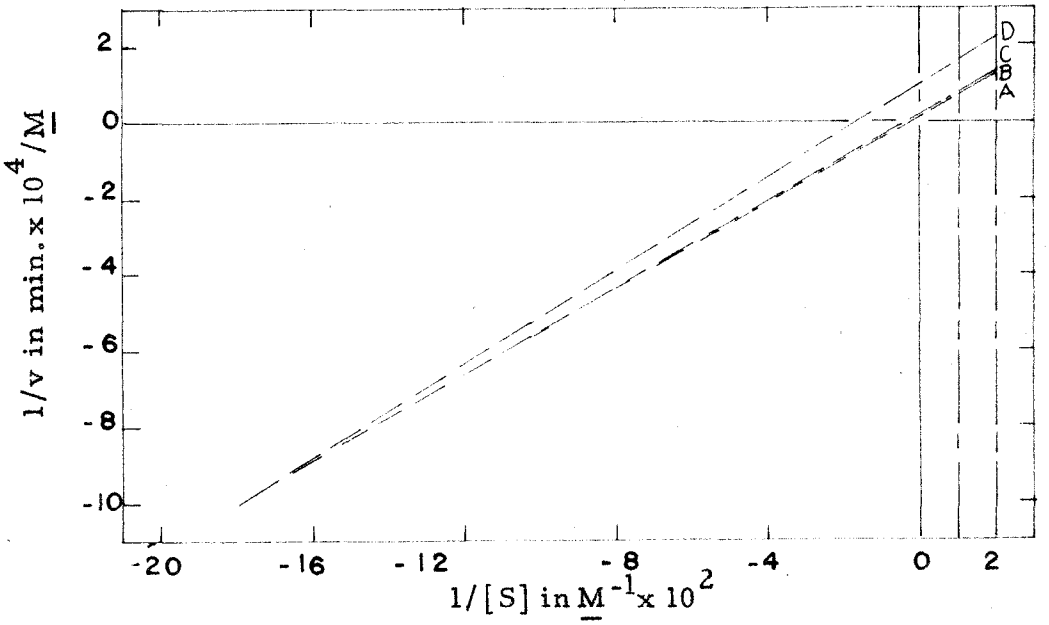

Fig. VIIId. Simulation of mixed inhibition by a "Totally Un-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 6d between 1/[S] = 100 and 200 M^{-1}.

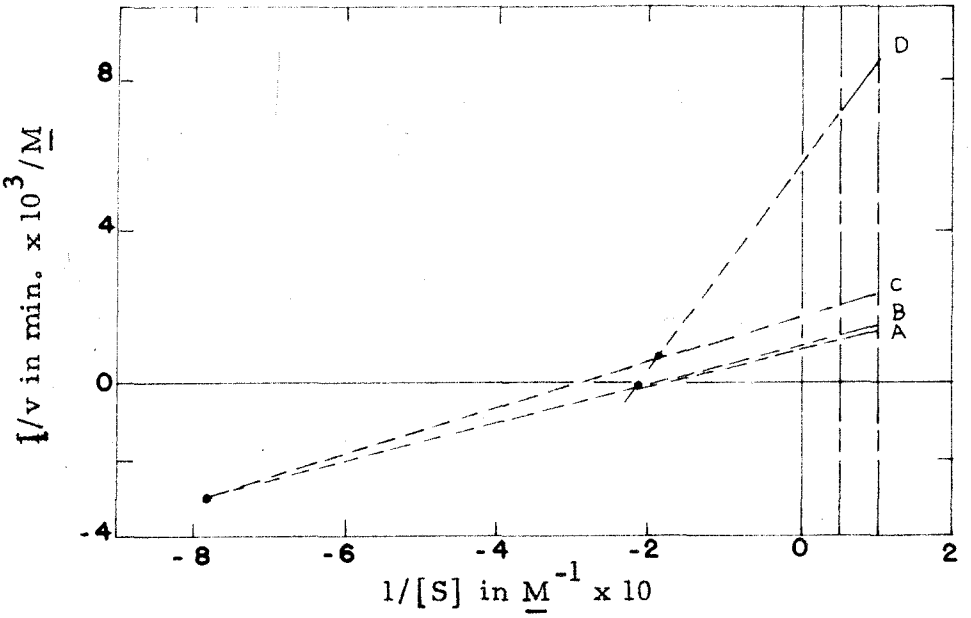

Fig. VIIIe. Simulation of mixed inhibition by a "Totally Un-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 6e between 1/[S] = 5 and 10 M^{-1}.

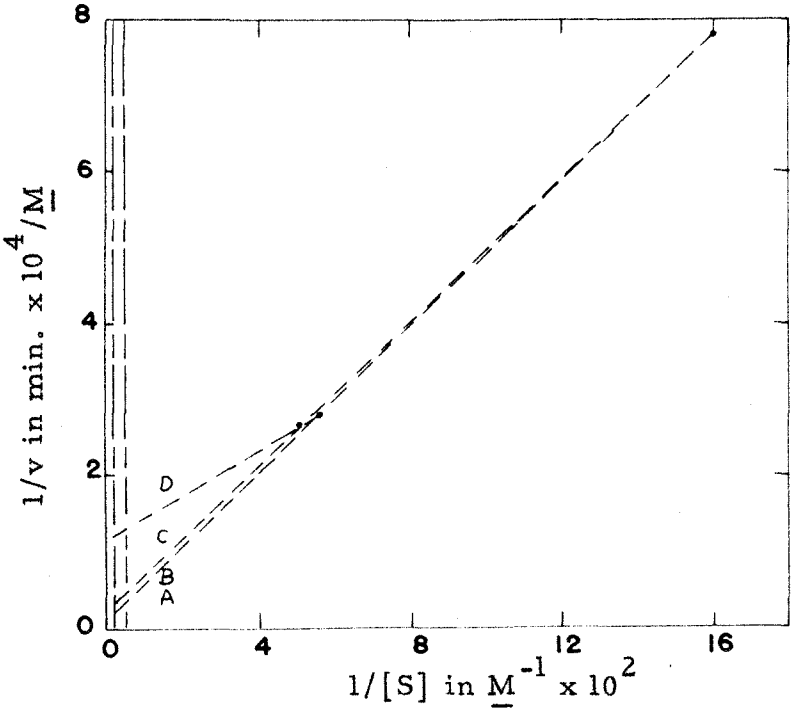

Fig. VIIIf. Unusual type of inhibition by a "Totally Un-Competitive" inhibitor: ---, extrapolated chords; ——, chords of equation 6f between 1/[S] = 20 and 50 M^{-1}.

PART II

L-TYROSYL-L-TYROSINE DERIVATIVES FOR THE DETECTION OF TRANSPEPTIDATION IN α-CHYMOTRYPSIN-CATALYZED HYDROLYSES

A. INTRODUCTION

The velocities of most enzyme-catalyzed reactions are dependent upon the pH of the reaction mixture and diminish rapidly when the medium is too acidic or too basic. Thus, there is a "pH optimum" for which the velocity is a maximum when all other reaction parameters are held constant.

During the course of investigations on the α-chymotrypsin-catalyzed hydrolyses of many N-acyl and non-acylated amino acid derivatives, it became apparent that the non-acylated derivatives exhibited pH optima at significantly lower pH values than the N-acyl derivatives. Examples of pH optima for some tyrosine* derivatives are given in Table XI.

Table XI

The pH Optima for Some L-Tyrosine Derivatives

-CH($CH_2C_6H_4OH$)CO-	$-OC_2H_5$	$-NH_2$	$-NHNH_2$	-NHOH
H_2N-	6.2-7.0 (2,3)	-----	7.05 (1)	6.95 (1)
CH_3CONH-	8.0-8.2 (2,4)	7.90 (1)	7.95 (1)	7.60 (1)
C_6H_5CONH-	7.8[a] (5)	7.8[a] (5)	8.0 (1)	-----
β-(C_5H_4N)CONH-	-----	7.90 (1)	7.80 (1)	-----

a. Measured in 30% methanol.

*Throughout this part of this thesis all amino acids mentioned will be of the L, or natural, configuration unless specifically designated otherwise.

Foster, Jennings and Niemann (6) offered an explanation for this shift of the pH optima with acylation, based upon the assumption that hydrolysis at the amino acid carboxyl function is the predominating reaction. Since the pH optima for almost all uncharged substrates occur at pH 7.9 ±.1,* it can be assumed that the enzyme is protonated at this pH in a manner (6, 8) that is optimal for catalyzing the hydrolysis of these substrates. However, the amino group (pKa' 7.0) of tyrosinhydroxamide would be predominantly unprotonated at pH values greater than seven and would be expected to deactivate the hydrolyzable bond, decreasing the reaction velocity relative to that of the protonated, or acylated, tyrosinhydroxamide. Thus, the enzymatic activity decreases sooner as the pH is increased, shifting the pH optimum to a more acidic region. This argument will hold equally well for the derivatives of tyrosine ethyl ester and hydrazide (see Table XI).

In a review article (9) Schwert suggested that "it seems more probable that the reduction in apparent reaction velocity at higher pH values is attributable to transpeptidation onto the uncharged α-amino group." Support is given this view by some findings (10,11,12) that show that the extent of transpeptidation increases with increasing pH throughout the region of interest, i.e., pH 6 to 8. Thus, there are

---

*The lower pH optimum (pH 7.6) for N-acetyltyrosinhydroxamide can be accounted for (7) by coulombic repulsion between the hydroxamate anion (pKa' 9.0) and an anion at or near the catalytically active site of the enzyme.

three reactions to consider: 1. the hydrolysis of the protonated substrate, 2. the hydrolysis of the unprotonated substrate, 3. transpeptidation onto the unprotonated substrate. As the pH is increased the reactions of the unprotonated substrate, equations 2 and/or 3, will become more important.

$$H_3^{\oplus}NCHRCO\text{-}Y + H_2O \rightarrow H_3^{\oplus}NCHRCO_2^{\ominus} + H^{\oplus} + HY \qquad (1)$$

$$H_2NCHRCO\text{-}Y + H_2O \rightarrow H_3^{\oplus}NCHRCO_2^{\ominus} + HY \qquad (2)$$

$$2\,H_2NCHRCO\text{-}Y \rightarrow H_2NCHRCO\text{-}NHCHRCO\text{-}Y + HY \qquad (3)$$

If the rate of either of these reactions is equal to or greater than that of the protonated substrate, equation 1, the velocity would not be expected to decrease before pH 7.9 is reached, but it would appear to decrease if the liberation of protons was followed. Since the pH optima for the unacylated derivatives fall near pH 7.0 as measured either by the disappearance of substrate or the liberation of products other than acid, the protonated molecules, equation 1, react faster than the unprotonated ones, equations 2 and 3. The explanation of Foster, Jennings and Niemann (6) requires that reaction 2 is slower than reaction 1, and that the transpeptidation reaction, equation 3, is much slower than either hydrolysis reaction; whereas, Schwart's alternative hypothesis (9) requires reaction 3 to have a rate greater than that of reaction 2 but not much less than that of reaction 1. With tyrosine hydroxamide (6) and hydrazide (1) the sum of reactions 1, 2, and 3 was followed,

since the disappearance of hydroxamic acid and the appearance of hydrazine were measured respectively. Thus, in these instances the proposal of Schwert (9) cannot explain the decrease in reactivity with increasing pH without including the conditions that reactions 2 and 3 are slower than reaction 1, the condition upon which Foster, Jennings and Niemann (6) base their hypothesis. However, it would still be useful to know the significance of the transpeptidation reaction under the conditions of the original studies (1, 6).

Goldenberg, Goldenberg and McLaren (13) made the interesting observation that leucine ethyl ester exhibits a pH optimum at pH 6.8 when the liberation of acid is followed potentiometrically and ca. 7.2 when the disappearance of ester is followed colorimetrically. In a review article Halsey, Green and Neurath (14) proposed that this difference in pH optima is probably due to a transpeptidation reaction. However, Lutwack, Mower and Niemann (1) pointed out that hydrolysis of the unprotonated substrate, equation 2, would not be detected by potentiometric titration, thus providing an alternative explanation which predicts the direction of the shift in the pH optimum. Since neither transpeptidation, equation 3, nor hydrolysis of the unprotonated substrate, equation 2, can be detected near pH 7 by observing the liberation of acid, either or both of these reactions could account for the shift in pH optimum. Thus, it would again be of interest to know which of these reactions, equation 2 or 3, is responsible for this effect.

The ability of α-chymotrypsin to catalyze the synthesis of peptides and peptide-like bonds has been well established. Several workers (15-18) found that a protein digest could be resynthesized by chymotrypsin into an insoluble "plastein." Horowitz and Haurowitz (17) concluded that plastein formation probably proceeds by repeated trans-peptidation reactions. Other investigators have synthesized peptides from acylated amino acids and amino acid anilides (19-21) or phenylhydrazine (22) and from amino acid esters and amides (11, 12, 23, 24) using α-chymotrypsin as the catalyst. Some of these reactions exhibit pH optima near pH 7 (15-17, 24), others have pH optima near pH 9 or higher (11, 12, 23); however, Schuller and Niemann (22) observed that several acylated amino acids would not react with phenylhydrazine at pH 7.9, the optimum pH for the hydrolysis of the corresponding amides, but did form the phenylhydrazides at lower pH with pH optima between 5.5 and 6.5.

Fruton, et al., (27, 28) have observed that benzoyltyrosylglycinamide will exchange its glycinamide moiety with N^{15}-glycinamide, in the presence of α-chymotrypsin, but benzoyltyrosinamide only exchanges slightly with $N^{15}H_3$ at the same pH 7.6. They attribute this difference in reactivity to the difference in pK_a' between glycinamide (pK_a' 7.9) and ammonia (pK_a' 9.4).

In order to explain the absence of reactivity of N-alkylphenylalanine ethyl ester with α-chymotrypsin, Kuk-Meiri and Lichtenstein (25)

suggested that all non-acylated amino acid derivatives had to be converted to dipeptides before they could be hydrolyzed. Although Tauber (23) reported that α-chymotrypsin converts phenylalanine ethyl ester into a dipeptide, phenylalanylphenylalanine ethyl ester, with a pH optimum of 8.6, he also noted that this dipeptide ester is not hydrolyzed by α-chymotrypsin at pH 7.7. Since the observed (4, 26) pH optimum for the hydrolysis of phenylalanine ethyl ester occurs at ca. pH 6.4, serious doubt is cast on the hypothesis of Kuk-Meiri and Lichtenstein (25).

A close look at the reaction products of tyrosinamide, as well as some other compounds, was taken by Blau and Waley (29) who chromatographically followed at pH 7.8 the disappearance of tyrosinamide and the appearance of tyrosine and tyrosyltyrosine. Initially more tyrosine than tyrosyltyrosine is liberated; but after a couple of hours this ratio inverts, presumably (29) because of transpeptidation reactions and rapid hydrolysis of a tyrosyltyrosinamide intermediate. However, this latter postulate seems unlikely in view of the work of Tauber (23) mentioned above. Lestrovaya and Mardashev (12) investigated the reaction products of the ethyl esters and amides of phenylalanine and tyrosine more systematically and found that the amount of peptide formed decreased rapidly with decreasing concentration of α-chymotrypsin. The peptide formed was barely detectable at 4 mg. enzyme/ml., a concentration higher than that usually employed in

kinetic studies and five times less than that employed by Blau and Waley (29).

In the studies mentioned above on the α-chymotrypsin-catalyzed formation of peptide-linkages, higher enzyme concentrations, higher substrate concentrations and/or longer incubation periods were used to favor synthesis than are normally employed when determining the kinetic constants of a substrate. One study under the latter conditions was reported in a footnote by McDonald and Balls (30) who found that α-chymotrypsin, tyrosine ethyl ester and n-butanol yielded about equal amounts of tyrosine, butyl tyrosinate and ethyl tyrosinate after one hour at pH 6.2. However, tyrosine and ethyl tyrosinate were the only reaction products in the absence of the butanol, i.e., no peptide was formed unless its paper chromatographic R_f value was the same as that of tyrosine or ethyl tyrosinate.

Although there have been investigations of α-chymotrypsin-catalyzed transpeptidation and transpeptidation-like reactions the variety of conditions used and the wide range (5.5 to 10) of pH optima observed make it impossible to draw any definite conclusions about the extent of transpeptidation reactions under the conditions ordinarily used for evaluating kinetic constants. The studies reported here were undertaken to determine the importance of the transpeptidation reactions under conditions that would allow the questions raised above concerning the shift in pH optima between acylated and nonacylated substrates to be

answered. The approach decided upon was to synthesize tyrosyltyrosine methyl ester, amide, hydrazide and hydroxamide which could then be used as reference compounds for chromatographic examination of the products from the α-chymotrypsin-catalyzed reactions of the corresponding tyrosine derivatives.

B. METHODS AND PROCEDURE

Synthesis

The synthesis of the desired tyrosyltyrosine derivatives should proceed readily from a tyrosyltyrosine ester blocked at its terminal amino group. Bergmann, Zervas, Salzmann and Schleich (31) synthesized O-acetyl-N-carbobenzyloxytyrosyltyrosine ethyl ester in five steps using O-acetyl-N-carbobenzyloxytyrosyl chloride to form the peptide bond. O-Acetyl-N-carbobenzyloxytyrosyltyrosine was prepared by Blau and Waley (29) from the mixed anhydride of O-acetyl-N-carbobenzyloxytyrosine and benzoic acid. Diethyl chlorophosphite was used by Anderson, Welcher and Young to activate either the amino group of ethyl tyrosinate or the carboxyl group of N-carbobenzyloxytyrosine to form N-carbobenzyloxytyrosyltyrosine ethyl ester.

A shorter route to an N-carbobenzyloxytyrosyltyrosine ester appeared to be the synthesis of N,O-dicarbobenzyloxytyrosine (33), conversion to O-carbobenzyloxy-N-carboxytyrosine anhydride (33), and condensation with the free amine of methyl tyrosinate (34). However, the reaction of carbobenzyloxychloride with tyrosine yielded little of the desired N,O-dicarbobenzyloxytyrosine and several fractions with higher melting points and neutralization equivalents. These fractions could not be satisfactorily purified, and this route was abandoned.

In light of the recent work by Goodman and Stueben (35) and Grommers and Arens (36) on salts of carbobenzyloxy amino acids, it appears that a mixture of the sodium salt and free acid precipitated from the acidified solutions. Recently, Katchalski, et al., (37) corrected the earlier procedure (33) for the preparation of N,O-dicarbobenzyloxytyrosine and got much better yields when acylating in less basic solutions. They then used (37) this compound to form N,O-dicarbobenzyloxytyrosyltyrosine ethyl ester, employing isobutyl chloroformate as the coupling agent.

N-Carbobenzyloxytyrosyltyrosine methyl ester was then prepared by the usual azide method of peptide synthesis. The crude N,O-dicarbobenzyloxytyrosine was selectively hydrolyzed in base to give N-carbobenzyloxytyrosine, esterified and converted to the hydrazide (38). Nitrous acid converted this hydrazide to N-carbobenzyloxytyrosinazide which was allowed to react with methyl tyrosinate in ethyl acetate solution (39). The oils and tars that resulted from this reaction were difficult to crystallize and purify, partially because of the ease with which the ester can be hydrolyzed to N-carbobenzyloxytyrosyltyrosine. The direct coupling of N-carbobenzyloxytyrosine with methyl tyrosinate affected by N,N'-dicyclohexylcarbodiimide (40) proved to be a much easier synthesis giving a clean product in good yield.

The removal of the N-carbobenzyloxy group was accomplished most easily by a stream of hydrogen passed over the compound in methanolic hydrogen chloride with a palladium black catalyst. Decarbobenzyloxylation of N-carbobenzyloxytyrosyltyrosine by the method

of Ben-Ishai and Berger (41) with hydrogen bromide in glacial acetic acid gave an oily product that was difficult to purify. Some carbobenzyloxytyrosyltyrosine, obtained as a by-product of the carbodiimide condensation, treated with methanolic hydrogen chloride (42) gave tyrosyltyrosine methyl ester hydrochloride and some carbobenzyloxytyrosyltyrosine methyl ester. However, treatment of carbobenzyloxytyrosyltyrosine methyl ester by this method or Barkdoll and Ross (42) did not produce any tyrosyltyrosine methyl ester.

Carbobenzyloxytyrosyltyrosinamide was prepared by both the azide and N,N'-dicyclohexylcarbodiimide procedures. As before, the latter method proved much more convenient for the isolation of the products. Hydrogenation over palladium black then gave tyrosyltyrosinamide hydrochloride.

Methanolic hydrazine hydrate converted carbobenzyloxytyrosyltyrosine methyl ester to the hydrazide which was then hydrogenated as above to give tyrosyltyrosinhydrazide hydrochloride. Hydroxylamine in methanolic sodium methoxide solution followed by hydrogenation gave tyrosyltyrosinhydroxamide in an analogous manner. An attempt to prepare this compound from tyrosyltyrosine methyl ester by reaction with hydroxylamine in methanolic sodium methoxide yielded only a good crop of tyrosine anhydride.

Some of the tyrosyltyrosine methyl ester was hydrolyzed to give tyrosyltyrosine. However, hydrogenation over palladium black did not reduce carbobenzyloxytyrosyltyrosine. This lack of reactivity

seems reasonable since it is known that carbon dioxide poisons this catalyst.

Enzyme Reactions and Chromatography

The reactions of tyrosine methyl ester, amide, hydrazide and hydroxamide with chymotrypsin were carried out with the highest substrate and enzyme concentrations reported in the literature (1-6, 43) for the determination of their respective kinetic constants and at their pH optima (pH 7.0) in order to maximize, within the limits just stated, the possibility of observing any transpeptidation reactions. Since glycyphenylalaninamide acetate was found (44) to give different products with δ-chymotrypsin, than with α-chymotrypsin, separate experiments were carried out with both α- and δ-chymotrypsin. The substrate solutions in a THAM*-HCl buffer were adjusted to pH 7.0 with 2N HCl before and after the addition of the enzyme stock solution and allowed to stand at room temperature (25-27° C.). The reaction systems investigated are summarized in Table XII.

Samples of 6-24 λ were removed at 0, 1, 2, 4 and 24 hours and chromatographed on Whatman #1 paper with butanol-acetic acid-pyridine-water (30:6:20:24), 2N HCl saturated with butanol, water half-saturated with butanol and/or pyridine-isoamyl alcohol-water (37:43:20) used as the solvent in order to distinguish between tyrosine, tyrosyltyrosine, tyrosine anhydride, the substrate and the corresponding tyrosyltyrosine derivative. The spots were detected by spraying

*Tris-(hydroxymethyl)-aminomethane.

Table XII

Reaction Systems for Chromatography Experiments

No.	Substrate	[S]	Chymo-trypsin	[E][a]	[E][b]	THAM[c]
1	Methyl	.020M	α	.40	.059	.02M
2	Tyrosinate	.020M	α	.004	.00059	.02M
3		.020M	α	.004	.00059	.02M
4		.020M	δ	.004		.02M
5	Tyrosinamide	.024M	α	.22	.032	.02M
6		.024M	δ	.22		.02M
7		.050M	α	1.37	.20	.02M
8		.050M	δ	1.37		.02M
9	Tyrosinhydrazide	.004M	α	1.37	.20	.02M
10		.004M	δ	1.37		.02M
11	Tyrosinhydrox-amide	.013M	α	.68	.10	.20M
12		.013M	δ	.68		.20M

a. Enzyme concentration in mg. protein/ml.

b. Enzyme concentration in mg. protein nitrogen/ml.

c. Concentration of tris-(hydroxymethyl)-aminomethane.

the chromatograms with diazotized sulfanilamide in butanol followed by a half-saturated sodium carbonate solution (45). The chromatograms were then examined under ultraviolet light.

C. RESULTS

The products from the reactions of chymotrypsin with some tyrosine derivatives gave the chromatographic results indicated in Table XIII. Since no difference could be observed between the α- and the δ-chymotrypsin-catalyzed reactions, the data were combined to give the average R_f values and the average extent of the spots presented in Table XIII.

In all cases, the pH did not change more than 0.1 pH unit over a 24 hour period.

Controls

Samples with no enzyme added showed only substrate and some tyrosine when chromatographed. Neither α- nor δ-chymotrypsin stock solutions gave a discrete spot when chromatographed and detected as mentioned above. When samples of tyrosyltyrosinamide of diminishing size were chromatographed, it was found that as little as 0.2 μg. could be detected with the diazotized sulfanilamide reagent.

Methyl Tyrosinate

At the higher enzyme concentration, 4 mg. protein/ml., of experiment 1 (Table XII) the methyl tyrosinate was completely hydrolyzed within five minutes, and tyrosine had precipitated from solution. However, with one hundred times less enzyme the methyl tyrosinate spot

Table XIII

Chromatography of Reaction Products of Tyrosine Derivatives with Chymotrypsin at pH 7.0

Experiments[a]				Reference Compounds	
Substrate[b]	Solvent[c]	Spot[d] Density	R_f and Spot[e] Size	R_f and Spot[e] Size	Compound
Tyr-OCH_3	#1	S	.49 ± .03	.49 ± .02	Tyr
		B	.62 ± .02	.72 ± .02	Tyr-Tyr
		M	.80 ± .03	.79 ± .04	Tyr-OCH_3
				.87 ± .02	Tyr anhydride
				.88 ± .04	Tyr-Tyr-OCH_3
Tyr-NH_2	#1	M	.49 ± .03	.49 ± .02	Tyr
		S	.59 ± .04	.61 ± .02	Tyr-NH_2
		B	.72 ± .03	.72 ± .02	Tyr-Tyr
		B	.75[f] ± .04	.76 ± .03	Tyr-Tyr-NH_2
				.87 ± .02	Tyr anhydride
Tyr-$NHNH_2$	#1	M	.48 ± .02	.49 ± .02	Tyr
				.72 ± .02	Tyr-Tyr
		W	.75 ± .06	.76 ± .08	Tyr-$NHNH_2$
				.87 ± .05	Tyr-Tyr-$NHNH_2$
				.87 ± .02	Tyr anhydride
Tyr-$NHNH_2$	#2			.72 ± .03	Tyr-Tyr-$NHNH_2$
				.73 ± .03	Tyr-Tyr
				.76 ± .02	Tyr anhydride
		W	.79 ± .03	.79 ± .03	Tyr
		W	.78 ± .06	.79 ± .07	Tyr-$NHNH_2$
Tyr-$NHNH_2$	#3	M	.24 ± .02	.25 ± .02	Tyr
		B	.47[g] ± .03	.48 ± .03	Tyr-Tyr
		W	.64 ± .13	.68 ± .13	Tyr-$NHNH_2$
			[illegible] ± .16	.78 ± .16	Tyr-Tyr-$NHNH_2$
Tyr-NHOH	#1	M-S	.50 ± .03	.49 ± .02	Tyr
		M	.71 ± .04	.72 ± .02	Tyr-Tyr
				.78 ± .03	Tyr-Tyr-NHOH
		W	.79 ± .05	.79 ± .11	Tyr-NHOH
				.87 ± .02	Tyr anhydride
Tyr-NHOH	#4			.55 ± .05	Tyr-Tyr-NHOH
		W	.51[h] ± .11	.56 ± .08	Tyr-NHOH
				.65 ± .02	Tyr anhydride
		M	.79 ± .03	.79 ± .04	Tyr
		M	.83 ± .02	.83 ± .03	Tyr-Tyr
Tyr-NHOH	#3	M	.25 ± .02	.25 ± .02	Tyr
		W	.48 ± .03	.48 ± .03	Tyr-Tyr
				.63 ± .03	Tyr-Tyr-NHOH
		M	.75 ± .11	.74 ± .10	Tyr-NHOH

References for Table XIII

a. The reaction mixtures are described in Table XII.

b. The following abbreviations are used: Tyr = tyrosine, Tyr-OCH_3 = methyl tyrosinate, Tyr-NH_2 = tyrosinamide, Tyr-$NHNH_2$ = tyrosinhydrazide, Tyr-NHOH = tyrosinhydroxamide, Tyr-Tyr = tyrosyltyrosine, etc.

c. Solvents: 1. butanol-acetic acid-pyridine-water (30:6:20:24), 2. 2N HCl saturated with butanol, 3. pyridine-isoamyl alcohol-water (37:43:20) and 4. water half-saturated with butanol.

d. Spot densities indicated as S = strong, M = medium, W = weak and B = barely detectable.

e. In R_f units.

f. This spot only appeared on one chromatogram made after 36 hours of reaction.

g. This end of the R_f .64 spot appeared slightly darker in ultraviolet light.

h. The R_f value varied from .65 to .43, and the spot was barely detectable after two hours.

was observed to diminish with time, whereas the tyrosine spot reached a saturation density within one hour, since tyrosine had started to precipitate from solution. This precipitate was filtered, dissolved in water, chromatographed, and found to contain only tyrosine. The spot with R_f .62 (see Table XIII) was barely detectable, $\leq$ 0.2 μg., and did not change its density with time. This spot did not correlate well with any of the reference compounds employed, but it was closest to tyrosyltyrosine, R_f .72. However, the small amount of this compound with R_f .62 and its failure to increase with time indicates that its formation is of negligible importance during the chymotrypsin-catalyzed hydrolysis of methyl tyrosinate.

Tyrosinamide

At the lower substrate and enzyme concentrations of experiments 5 and 6 (Table XII), only tyrosine and tyrosinamide were detected on the chromatograms even after ten hours. However, at .05 M substrate and 1.37 mg. enzyme/ml., experiments 7 and 8, a barely detectable tyrosyltyrosine spot appeared at one hour and slowly increased until it almost equalled at 24 hours and surpassed at 36 hours the density of the tyrosine spot. The hint of a spot at R_f .75 that appeared at 36 hours corresponds to tyrosyltyrosinamide but could be a higher peptide derivative since so much tyrosyltyrosine was available. These results agree with the findings of Blau and Waley (29), but at the concentrations

employed here it can be seen that the formation of tyrosyltyrosine is of negligible importance during the time (< four hours) normally taken for kinetic studies.

Tyrosinhydrazide

When chromatographed with either solvent #1 or #2 (see Table XIII) the reaction products gave only two spots corresponding to tyrosine and tyrosinhydrazide. However, the spot size, or "tailing," of the tyrosinhydrazide spot was so large in both solvents that the presence of tyrosyltyrosine could not be excluded. On the chromatograms developed with 2N HCl saturated with butanol, solvent #2, tyrosine could be seen as a light fluorescent spot within the darker tyrosinhydrazide spot when viewed under ultraviolet light. When developed with solvent #3 (Table XIII) the tyrosinhydrazide spot again had a large spot size; however, the tailing increased after zero time. This additional tail appeared very slightly darker than the rest of the spot under the ultraviolet light, indicating the possible presence of another compound with an R_f value near .47. Thus, a trace of tyrosyltyrosine is again indicated, but, as with methyl tyrosinate, its small concentration and lack of build-up deem it of negligible importance.

Tyrosinhydroxamide

Tyrosine, tyrosinhydroxamide and tyrosyltyrosine were easily discernable on the chromatograms with each of the solvents used. However, the possible presence of tyrosyltyrosinhydroxamide could

only be ruled out when solvent #3 was used as the developer (see Table XIII). The tyrosinhydroxamide was essentially gone at four hours, but the pH did not change significantly due to the high buffer concentration (see Table XII). The tyrosyltyrosine spot was definitely present at fifteen minutes, continued to build up with time being about one-third the size of the tyrosine spot at four hours but had almost disappeared at 24 hours. Thus, a significant proportion, about 1/3, of the reaction of tyrosinhydroxamide with chymotrypsin results in tyrosyltyrosine formation rather than simple hydrolysis.

D. DISCUSSION AND CONCLUSIONS

The results obtained with tyrosine methyl ester, amide and hydrazide are in good agreement with the literature. McDonald and Balls (30) found no peptide formation with methyl tyrosinate under similar conditions, but at high enzyme concentrations Lestrovaya and Mardashev (12) did detect a small amount of peptide.* These latter investigators also found that tyrosinamide produces a significant amount of one peptide* and a trace of a second peptide.** However, the work reported here has shown that under the conditions usually employed for kinetic studies, insignificant amounts of tyrosyltyrosine are produced with tyrosine methyl ester and hydrazide and also with the amide if studied less than four hours.

The possibility of a peptide intermediate during hydrolysis such as that postulated by Kuk-Meiri and Lichtenstein (25) or Blau and Waley (29) should be considered. Tyrosyltyrosine is very slowly hydrolyzed by chymotrypsin as shown by the latter workers (29) and by the tyrosinhydroxamide experiments (Table XII). Since tyrosyltyrosine

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*The R_f value of this peptide corresponds to my tyrosyltyrosine reference sample chromatographed with the same paper and developer.

**This peptide might be tyrosyltyrosyltyrosine, since Blau and Waley (29) found that tyrosyltyrosine produced some of the tripeptide during hydrolysis with chymotrypsin.

was present in trace quantities only during the experiments with tyrosine methyl ester, hydrazide and amide (< four hours), its formation cannot be much faster than its hydrolysis and, consequently, too slow to be of significance with respect to the hydrolysis reaction. Therefore, tyrosyltyrosine can be excluded from consideration as an important intermediate unless significant quantities of this dipeptide are present in the reaction mixture.

The other possibility is that a dipeptide derivative, e.g., tyrosyltyrosinamide, could be an intermediate. Since none of the dipeptide derivatives, corresponding to the four substrates investigated here, were detected in the reaction mixtures, these dipeptide derivatives could only be present in low steady state concentrations. A rough calculation from the data presented by Blau and Waley (29) shows that tyrosyltyrosinamide disappears from solution about sixty times faster than tyrosinamide. However, they (29) demonstrated that tyrosyltyrosinamide gives primarily tyrosinamide and tyrosyltyrosine, in a ratio of about 2 to 1, with little production of tyrosine when incubated with α-chymotrypsin. Therefore, the expected, major degradation product from any peptide derivative is tyrosyltyrosine which should be detectable, for the reasons given above, if this reaction pathway is of any significance. Since neither tyrosyltyrosine nor a tyrosyltyrosine derivative was found in any but trace amounts, it is reasonable to conclude that these peptides are not significant intermediates, and hence, transpeptidation is

of negligible importance during the chymotrypsin-catalyzed hydrolysis of tyrosine methyl ester, amide or hydrazide when studied under the conditions used here (Table XII).

Tyrosinhydroxamide cannot be included with the other three substrates studied, since tyrosyltyrosine was easily detected after 15 minutes and increased in concentration until the tyrosinhydroxamide was exhausted. Again, no other peptide derivative was found. However, the amount of tyrosine was several times* greater than the amount of tyrosyltyrosine, so that the transpeptidation reaction was minor but not negligible, with respect to the hydrolysis reaction.

Tyrosine amide and hydroxamide probably form tyrosyltyrosine in a similar manner but on a different time scale. Blau and Waley (29) proposed equations 4 and 5 to explain the formation of tyrosyltyrosine

$$2\ NH_2CHRCO\text{-}NH_2 \longrightarrow NH_2CHRCO\text{-}NHCHRCO\text{-}NH_2 + NH_3 \qquad (4)^{**}$$

$$NH_2CHRCO\text{-}NHCHRCO\text{-}NH_2 + \overset{\oplus}{N}H_3CHRCO_2^{\ominus} \longrightarrow NH_2CHRCO\text{-}NHCHRCO_2H + NH_2CHRCO\text{-}NH_2 \qquad (5)^{**}$$

and its predominance over tyrosine after several hours. However, the direct participation of tyrosine seems unlikely in light of several investigations (17, 18, 24) which have shown that blocking the carboxyl function, as the ester, amide, etc., greatly enhances the rate of peptide formation

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*Roughly ten times at 15 min. and three times at four hours.

**R = p-hydroxybenzyl-.

from these derivatives both as donors (17,18) and as acceptors (24) of acyl groups. An alternative to reaction 5 would, therefore, be a transpeptidation reaction between tyrosyltyrosinamide and tyrosinamide to give tyrosyltyrosyltyrosinamide plus ammonia with subsequent hydrolysis to tyrosyltyrosine and tyrosinamide. This reaction is analogous to the formation of benzoyltyrosylglycinamide from benzoyltyrosinamide and glycinamide that Fruton, et al., (27, 28) observed.

Since transpeptidation reactions have been shown to be of negligible importance during chymotrypsin-catalyzed hydrolyses of ethyl phenylalaninate* (12) and methyl tyrosinate, it is reasonable to assume that this relationship will also be true for ethyl leucinate. The difference in pH optima for this last substrate with different detection methods as observed by Goldenberg, Goldenberg and McLaren (13) should, therefore, be accounted for by ionization of the substrate as suggested by Lutwack, Mower and Niemann (1) rather than by a transpeptidation reaction as proposed by Halsey, Green and Neurath (14).

In order to explain the differences in the pH optima for the acylated and non-acylated amino acid derivatives, mentioned in the introduction, Foster, Jennings and Niemann (6) proposed that a protonated amino acid derivative, equation 1, would be a better substrate than an unprotonated species, equation 2, and Schwert (9) suggested that a transpeptidation

*Only a trace of peptide was detected using a high enzyme concentration.

reaction, equation 3, was responsible for the difference. Since transpeptidation has been shown to be negligible, or minor with tyrosinhydroxamide, with respect to hydrolysis, the latter hypothesis should be abandoned in favor of the former one. With tyrosinhydroxamide a transpeptidation reaction is probably accounting for a small portion of the decrease in reactivity with increasing pH above 7.0, but the unreactivity of the unprotonated substrate (6) is probably the predominant factor.

Summary

The studies reported here have shown that α- and δ-chymotrypsin are essentially the same with respect to their action on the four substrates studied, that tyrosine methyl ester and hydrazide form only trace amounts of tyrosyltyrosine when hydrolyzed with chymotrypsin, that tyrosinamide does not form significant quantities of tyrosyltyrosine until after four hours of reaction and that tyrosyltyrosine is a minor, but significant product of the action of chymotrypsin on tyrosinhydroxamide. The general conclusion reached is that transpeptidation reactions are of negligible, or minor, importance in the chymotrypsin-catalyzed hydrolyses of tyrosine derivatives under the conditions normally employed for kinetic studies. The implications of this conclusion have been discussed.

E. EXPERIMENTS *

(i) Methyl Tyrosinate

The hydrochloride of this compound was prepared from tyrosine and methanol by the procedure of Brenner and Huber (46) in 95-98% yield, m.p. 187-189°C. The hydrochloride dissolved in a minimum amount of water and brought to pH 8-9 with N NaOH yielded 95% of the chloride free ester, m.p. 134-135°C. Lit. (47) m.p. 135-136°C.

(ii) Ethyl Tyrosinate Hydrochloride

This compound was prepared in the same manner as i in 90-96% yield, m.p. 166-168°C. Lit. (48) m.p. 166°C.

(iii) Tyrosinamide

Aminolysis of methyl tyrosinate gave tyrosinamide in 73% yield, m.p. 154-156°C. Lit. (29, 49), m.p. 153-154°C.

(iv) Tyrosinhydrazide

This compound was prepared from hydrazine hydrate and methyl tyrosinate by the method of Curtius (50), m.p. 193-195°C. Lit. m.p. 195.5° (50); m.p. 193-194°(1).

(v) Tyrosinhydroxamide

Hydroxylamine and methyl tyrosinate yielded 60% of tyrosin-

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*All melting points are corrected to within 2°C. All microanalyses were done by Spang Microanalytical Labs., Ann Arbor, Michigan. Nitrogen was bubbled through all solutions being concentrated or stripped of solvent in order to minimize air oxidation of the tyrosine derivatives.

hydroxamide by the method of Foster, Jennings and Niemann (6). This compound melted from 163-164°C. with decomposition. Lit. m.p. 161-162°C. (dec.).

(vi) Carbobenzyloxy Chloride

A solution of this compound in toluene was prepared from benzyl alcohol and phosgene by the method of Bergmann and Zervas (51). Its concentration was determined by pipetting aliquots of solution into concentrated ammonium hydroxide and filtering, drying and weighing the precipitate of benzyl carbamate.

(vii) N,O-Dicarbobenzyloxytyrosine

Tyrosine was acylated with vi at pH 11 using the method of Katchalski and Sela (33) and yielded ca. 5% of the desired product, m.p. 112-114° C., N.E. 449 (calc. 449.5). Lit. m.p. 117° C. Other fractions melted at 99-105°C., possibly N-carbobenzyloxytyrosine; 236-242°C.;and 124-127° C., N.E. 580.

(viii) N-Carbobenzyloxytyrosine

The fraction from the synthesis of vii which melted at 99-105° C. was dissolved in an excess of 2N NaOH, stood at room temperature 15 minutes, was extracted twice with ether and was acidified with 4 N HCl. The resulting precipitate of viii was recrystallized from dilute sodium acetate solution to give needles which when dried at 56° C. at 1 mm. of Hg over P_2O_5 melted from 91-93° C., N.E. 318 (calc. 315). Lit. (51a) m.p. 101°C.

(ix) N-Carbobenzyloxytyrosine Ethyl Ester

a) From Ethyl Tyrosinate (ii) - Acylation of ii with carbobenzyloxy chloride (vi) by the method of Bergmann and Zervas (51a) gave a 70% yield of this compound (ix), m.p. 78-78.5°C. Lit. m.p. 78° C.

b) From N-Carbobenzyloxytyrosine (viii) - Esterification of viii by the method of Brenner and Huber (46) gave a 75% yield of the ethyl ester (ix), m.p. 75-78° C. Lit. (51a) m.p. 78° C.

(x) N-Carbobenzyloxytyrosinhydrazide

N-Carbobenzyloxytyrosine ethyl ester and hydrazine hydrate gave 80-92% yields of this compound (x) by the method of Harrington and Pitt-Rivers (38), m.p. 221-223° C. after recrystallization from ethanol. Lit. m.p. 220-221° C.

(xi) N-Carbobenzyloxytyrosyltyrosine Methyl Ester

a) Azide Method - This compound (xi) was prepared essentially by the method of Bergmann and Fruton (39). A 10% excess of $NaNO_2$ was added to the hydrazide (x) suspended in ice-cold 2.6 N HCl layered with ethyl acetate. After stirring the mixture one minute, the yellow organic layer containing the azide was separated, washed with cold water, .5 M $NaHCO_3$ and water and added to an ethyl acetate solution of methyl tyrosinate. After this ethyl acetate solution stood overnight at room temperature, it was washed and stripped of solvent in vacuo. The thick syrup, oil or precipitate, m.p. 197-200° C., which resulted

could not be recrystallized from a wide variety of solvents or solvent pairs; acetone-water and acetone-chloroform were the best recrystallization solvent pairs tried. Norite was ineffective whenever employed. The crude product (xi) was obtained in 20-30% yield. Tyrosine and carbobenzyloxytyrosyltyrosine were also isolated from the reaction mixture.

b) Carbodiimide Method - N-Carbobenzyloxytyrosyltyrosine methyl ester (xi) was prepared as described by Sheehan and Hess (40) from equalmolar amounts of carbobenzyloxytyrosine (vii), methyl tyrosine (i) and N,N'-dicyclohexylcarbodiimide (Aldridge Chemical Co.) dissolved in tetrahydrofuran. The solution stood at room temperature 24 hours and was filtered to remove N,N'-dicyclohexylurea, stripped of solvent in vacuo, dissolved in ethyl acetate, washed with 5% acetic acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4, concentrated in vacuo and chilled in an ice bath. The product (xi) was filtered from the solution and dried over P_2O_5, m.p. 174-176° C. Lit. (42) m.p. 174-175°C. Washing the ethyl acetate solution with 2% NaOH instead of 5% $NaHCO_3$ yielded N-carbobenzyloxytyrosyltyrosine, instead of the ester (xi), upon acidification of the basic wash. Purification of this N-carbobenzyloxytyrosyltyrosine by slowly adding a slightly basic solution of this acid to a large excess of cold 4N HCl gave a product that melted from 149-150° C., N.E. 479 (calc. 478.5). Lit. (31) m.p. 148° C.

c) From N-Carbobenzyloxytyrosyltyrosine - A methanol solution of N-carbobenzyloxytyrosyltyrosine (from xi, b) was saturated with anhydrous HCl at 0° C., stored at 4-5° C. two days and then concentrated in vacuo as directed by Barkdoll and Ross (42). Addition of anhydrous ethyl ester caused tyrosyltyrosine methyl ester hydrochloride (xii) to precipitate. This precipitate was filtered, and the filtrate was allowed to evaporate spontaneously leaving a crystalline residue of N-carbobenzyloxytyrosyltyrosine methyl ester (xi) which melted at 175-176° C. after being recrystallized from acetone-water. Lit. (42) m.p. 174-175° C.

(xii) Tyrosyltyrosine Methyl Ester Hydrochloride

a) From N-Carbobenzyloxytyrosyltyrosine - The tyrosyltyrosine methyl ester hydrochloride (xii) that precipitated from methanolic HCl solution (see xi, c) was recrystallized from methanol-ethyl ether, m.p. 210-211° C. Lit. (42) m.p. 210° C.

b) Attempted Synthesis from N-Carbobenzyloxytyrosyltyrosine Methyl Ester (xi) -1) Treatment of xi with methanolic HCl in the same manner as xi, c did not yield any of the desired product (xii). 2) Hydrogenation of xi over palladium black (xxi) in methanolic 0.2 N HCl in a Parr apparatus under 50 psig. hydrogen pressure gave a precipitate, m.p. 274-276° C., upon addition of ether, but none of the dipeptide ester hydrochloride (xii) was isolated.

c) Attempted Synthesis of Tyrosyltyrosine Methyl Ester Hydrobromide - Decarbobenzyloxylation with 1.4 N HBr in glacial acetic acid by the method of Ben-Ishai and Berger (41) gave a 72% yield of the expected CO_2, as measured by displacing water from an inverted graduated cylinder. The black oil that separated upon addition of ether was dissolved in methanolic 1.1 N HBr to reesterify the compound. Addition of ether caused a crude, halogen-free compound to precipitate, m.p. 259-266° C., in about 10% yield. No other products were isolated.

(xiii) N-Carbobenzyloxytyrosyltyrosinamide

a) Azide Method - This compound (xiii) was prepared from N-carbobenzyloxytyrosinhydrazide (x) and tyrosinamide (iii) in the manner described for N-carbobenzyloxytyrosyltyrosine methyl ester (xi, a). The crude, white product (100% recovery) turned yellow upon standing and could not be recrystallized, but only reprecipitated as a tan colored product.

b) Carbodiimide Method - N-Carbobenzyloxytyrosine (viii), tyrosinamide (iii) and N,N′-dicyclohexylcarbodiimide gave N-carbobenzyloxytyrosyltyrosinamide (xiii) in 50% yield by the method of xi, b. The compound (xiii) melted from 187-191° C.

Calculated for $C_{26}H_{27}N_3O_6$ (477.5): C, 65.4; H, 5.7; N, 8.8

Found: C, 65.3; H, 5.9; N, 8.6

(xiv) Tyrosyltyrosinamide Hydrochloride

A suspension of 2.0 g. N-carbobenzyloxytyrosyltyrosinamide (xiii) and .65 g. of palladium black (xxi) in 60 ml. of methanolic .18 N HCl in a 125 ml. Erlenmeyer flask was stirred with a magnetic stirring bar so that the suspension climbed half way up the walls of the flask. After flushing the system with nitrogen, hydrogen was passed over this solution for two hours, and the exit gasses were bubbled through a nearly saturated $Ba(OH)_2$ solution. After 10 to 15 minutes the starting material (xiii) had dissolved, and after one hour essentially no more $BaCO_3$ precipitated. The filtered, dried and weighed precipitate of $BaCO_3$ represented 50-60% of the possible CO_2 evolution. The catalyst was filtered from the solution, and anhydrous ether was added to the filtrate. The resulting precipitate was filtered, recrystallized from methanol-ethyl ether and dried in vacuo over P_2O_5 at 78° C., m.p. 237-240° C.

Calculated for $C_{18}H_{22}N_3O_4Cl$(380): C, 56.9; H, 5.8; N, 11.1

Found: C, 56.3; H, 5.6; N, 10.5

(xv) N-Carbobenzyloxytyrosyltyrosinhydrazide

Refluxing a solution of 2.3 g. of N-carbobenzyloxytyrosyltyrosine methyl ester (4.65 meq.) and .77 ml. of hydrazine hydrate (15.4 meq.) in 50 ml. of methanol for 15 min. and allowing the reaction mixture to stand overnight at room temperature produced clusters of white crystals, 1.3 g., which were filtered from solution. Repeating this procedure on the filtrate yielded a second crop, 1.0 g., m.p. 246-248°C. Lit. (42) m.p. 246° C.

Calculated for $C_{26}H_{28}N_4O_6$(492.5): C, 63.4; H, 5.7; N, 11.4

Found: C, 63.0; H, 5.6; N, 11.3

(xvi) Tyrosyltyrosinhydrazide Dihydrochloride Hydrate

Hydrogenation of xi, c over palladium black as described for xiv gave a 74% yield of $BaCO_3$ and a 77% yield of the desired product, m.p. 222-223° C. after recrystallization from methanol-ether and drying in vacuo over P_2O_5.

Calculated for $C_{18}H_{26}N_4O_6Cl_2$(449): C, 48.1; H, 5.8; N, 12.5

Found: C, 48.4; H, 5.7; N, 12.0

(xvii) N-Carbobenzyloxytyrosyltyrosinhydroxamide

The reaction of hydroxylamine with N-carbobenzyloxytyrosyltyrosine methyl ester in methanol did not proceed at 4° C., under the conditions of Foster, Jennings and Niemann (6), but did proceed after refluxing for 15 minutes and standing two days at room temperature. Removing the solvent in vacuo, dissolving the residue in methanol and adding water gave an oil which gave a white precipitate upon trituration with anhydrous ether. This product (xvii), m.p. 162.5-163.5°C., gave a purple color when in an acidic $FeCl_3$ solution.

(xviii) Tyrosyltyrosinhydroxamide Hydrochloride

Hydrogenation of xvii over palladium black as described for xiv gave a white precipitate upon addition of anhydrous ether to the

reaction mixture. Upon exposure to air the filtered precipitate became a pale green gum which was soluble in water, gave a precipitate with .5 M $AgNO_3$ solution, gave a purple color in acidic $FeCl_3$ solution and gave only one spot when chromatographed and developed with diazotized sulfanilamide (see Table XIII). A sample of the product (xviii) precipitated from methanol-ether charred but did not melt below 300°C.

(xix) Tyrosine Anhydride

During an attempt to prepare tyrosyltyrosinhydroxamide directly from tyrosyltyrosine methyl ester hydrochloride (xii), tyrosine anhydride (xix) was obtained in 70% yield. Hydroxylamine hydrochloride and xii were dissolved separately in methanol and equivalent amounts of methanolic sodium methoxide were added at 0° C. The NaCl precipitate was filtered from the two solutions before they were mixed and stored at 4° C. 18 hours. The resulting precipitate was filtered and dried, m.p. 278-281°C.; $[\alpha]_D^{25}$ -234° ± 4° (C = 1.73% in 1 N NaOH). Lit. (47) m.p. 277-280° C. dec.; $[\alpha]_D$ -223.8° (C = 2.4% in dil. NaOH).

(xx) Tyrosyltyrosine

a) From Tyrosyltyrosine Methyl Ester Hydrochloride (xii) - Alkalizing a dilute aqueous solution of xii to pH 10 and letting it stand at room temperature for one hour hydrolyzed the ester (xii) to tyrosyltyrosine (xx) which precipitated upon neutralization and addition of absolute ethanol to the solution. The product gave only one spot when

chromatographed (see table XIII), and decomposed upon heating, m.p. 300-310° C., $[\alpha]_D^{25}$ -12.1 (C = 1.16% in 1 N HCl). Lit (31) m.p. > 240° C., $[\alpha]_D^{19}$ - 30.1 (H_2O with 1 eq. HCl).

(xxi) Palladium Black

This compound (xxi) was prepared from palladium chloride, formaldehyde and potassium hydroxide by the method of Willstätter and Waldschmidt-Leitz (52) and stored under water. Aliquots were removed as needed and transferred to ethanol by decantation.

Enzymes

The salt-free, bovine α-chymotrypsin was an Armour preparation, lot #T-97207. The salt-free δ-chymotrypsin employed was a Nutritional Biochemicals Corp. preparation, #6714. Stock solutions ten times the concentrations indicated in Table XII were used to make up the reaction mixtures.

Reaction Mixtures and Chromatography

In general, the reaction mixtures were prepared from 8 ml. of substrate stock solution, 1 ml. of THAM stock solution and 1 ml. of enzyme stock solution, all of which were of the appropriate concentrations to give the concentrations indicated in Table XII. The pH was adjusted to pH 7.0 with 2 N HCl using a Leeds and Northrop pH meter both before and after the enzyme stock solution was added to the mixture. The pH was also tested after 24 hours.

At 0, 1, 2, 4 and 24 hours samples of 10, 6, 24 and 10 λ of tyrosine methyl ester, amide, hydrazide and hydroxamide respectively were removed and applied to Watman #1 paper with a 2λ pipette. The paper strips were then developed with the solvents indicated in Table XIII.

The reagent used to spray the chromatograms was prepared by mixing a 1% solution of sulfanilamide in 10% (v/v) HCl and a 5% solution of $NaNO_2$ followed by extraction of the diazotized sulfanilamide into n-butanol (45). After the chromatograms were dry they were sprayed with a half-saturated Na_2CO_3 solution to bring out the peach-colored spots. These spots were also viewed under ultra-violet light.

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*I am indebted to Mr. John E. Baldwin for translating this Russian article for me.

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PART III

THE INTERACTION OF

α-METHYL-α-ACYLAMINO ACIDS

WITH α-CHYMOTRYPSIN

A. INTRODUCTION

During the course of investigations to establish the steric and chemical requirements for substrate and inhibitors of α-chymotrypsin, the question arose as to what effect substituting the α-hydrogen of an amino acid derivative with a relatively bulky α-alkyl group would have.

Manning (1) began the investigation of this problem by preparing D,L-α-methylphenylalanine and D,L-α-methyltyrosine and by attempting to prepare α-methyltryptophan and the α-isopropyl derivatives of these three amino acids. He also found that N-acetyl-O-methyl-D,L-α-methyltyrosine methyl ester was not hydrolyzed by α-chymotrypsin and N-acetyl-D,L-α-methylphenylalanine methyl ester was only slightly hydrolyzed when using prohibitively large amounts of this enzyme (1:1, w/w). O-Methyl-D,L-α-methyltyrosine methyl ester did not react in the presence of "viobin" and papain did not catalyze formation of the toluide of N-acetyl-D,L-α-methylphenylalanine. Thus, Manning (1) was unable to resolve these compounds enzymatically. An attempt to resolve N-acetyl-O-methyl-D,L-α-methyltyrosine as the L-brucine salt by fractional recrystallization was unsuccessful.

However, Manning did establish that the N-acetyl esters of α-methylphenylalanine and α-methyltyrosine are not substrates, or, at best, only very slow substrates of α-chymotrypsin. Other workers

(2-4) have found various D,L-α-methyl-α-amino acid derivatives to be inhibitors of, inert to or weak substrates of a variety of enzyme systems. The slight inhibition of D-amino acid oxidase (2), tyrosine decarboxylase (3) and DOPA* decarboxylase (3) by α-methylphenylalanine, the slight inhibition of these latter two enzymes by α-methyltyrosine and the inertness to tryptophanase (2) and the inhibition of a rat liver suspension (4) and cobra venom (4) by α-methyltryptophan are of special interest to this study.

The question arises as to whether this lack of reactivity is due to failure of the "substrate" to combine with the enzyme or failure of the enzyme-"substrate" complex to react further to give hydrolysis products. These alternatives will be reflected in the kinetic constants, i.e., a high K_S or K_I value and/or a low k_3 value.**

It is, therefore, the intent of the studies reported here to prepare sufficient quantities of the D and L enantiomers of the N-acetyl methyl esters of α-methylphenylalanine, α-methyltyrosine and α-methyltryptophan,*** and to determine the type and extent of their interaction with α-chymotrypsin, i.e., to determine their K_s and k_3 and/or K_I values.

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*3,4-Dihydroxyphenylalanine.

**K_s, K_I and k_3 refer to the enzyme-substrate complex and the enzyme-inhibitor complex dissociation constants and to the rate-limiting forward velocity constant, respectively. These constants are discussed in more detail in Part I of this thesis.

***The N-acetyl-α-methyltryptophan esters were later replaced by the α-methyl-β-(2-naphthyl)-alanine derivatives.

B. METHODS AND PROCEDURE

1. α-Methylphenylalanine and Derivatives

D,L-α-Methylphenylalanine has been synthesized by hydrolysis of 5-methyl-5-benzylhydantoin (5-7), by aminolysis of α-chloro-α-methyl-β-phenylpropionic acid (8) and by a Strecker reaction on phenylacetone (1, 9).

This last method was used and gave 74-90% yields of D,L-α-methylphenylalanine hydrochloride. The conventional Schotten-Baumann (10) acylation gave only a 39% yield of the desired N-acetyl derivative, but reaction with acetic anhydride in pyridine solution gave a 58-65% yield. These latter conditions could be used since the Dakin-West reaction would not be expected to proceed in the absence of an α-hydrogen atom (7).

Since Greenstein (11) had such success at resolving acylamino acid with acylase I, an attempt was made to resolve N-acetyl-D,L-α-methylphenylalanine with this enzyme. However, only 7% hydrolysis, as measured by the Van Slyke method of free amine determination (12), was observed after three days. Fu and Birnbaum (13) have also observed that other α-alkyl amino acids react slowly with acylase I.

Because enzymatic resolution did not seem feasible, N-acetyl-D,L-α-methylphenylalanine was resolved by fractional recrystallization

of its dexedrine salt. After five recrystallizations N-acetyl-(-)-α-methylphenylalanine was recovered from its dexedrine salt and found to have a specific rotation of + 85.7° in water. However, the fraction with the highest negative rotation only had a specific rotation of -15°.

Since both enantiomers of the α-methyl amino acid derivatives were desired, α-phenethylamine was then used as the base, both the L and the D isomers of this amine being available after resolution as the diastereoisomeric d-tartrate salt (14). Fractional recrystallization of the (-)-α-phenethylammonium N-acetyl-α-methylphenylalaninate precipitated from methanol did not resolve the acid. Several other solvents were tried, and acetone was found to give a 55% yield of the levorotatory enantiomer, $[\alpha]_D^{25°}$ - 85.6°, without further recrystallization. More of the dextrorotatory enantiomer was obtained from the salts of the (+)-α-phenethylamine.

These resolved acylamino acids were then esterified with thionyl chloride in methanol.

2. α-Methyltyrosine and Derivatives

D,L-α-Methyltyrosine has been prepared from D,L-α-methylphenylalanine (9) and from p-methoxybenzyl methyl ketone by the Strecker synthesis (1, 6). Manning (1) found the Darzens glycidic ester synthesis the most convenient method for preparing the intermediate ketone.

However, relatively large quantities, 25-50 g., of the

α-methyltyrosine were desired to facilitiate resolution, hence, the less expensive starting material, nitroethane, was coupled with anisaldehyde, reduced with iron in strong hydrochloric acid and hydrolyzed to give p-methoxybenzyl methyl ketone by the method of Hoover and Haas (15). The Strecker reaction on this ketone followed by treatment with 31.4% hydrogen bromide in glacial acetic acid to remove the O-methyl group gave D,L-α-methyltyrosine. Acetylation of this compound to the O,N-diacetyl derivative followed by selective hydrolysis to N-acetyl-D,L-α-methyltyrosine gave a cleaner product than preparing the N-acetyl derivative directly by selective acylation.

Since the resolution of N-acetyl-α-methylphenylalanine with (-)-α-phenethylamine in acetone had been so successful, this system was also used for the resolution of N-acetyl-α-methyltyrosine. However, reforming the salt and reisolating the acid to check for completion of resolution continued to increase the rotation of the acid until this procedure had been repeated about six times. The specific rotation had leveled off at $\pm$ 61°; but the yield was down to about 25 mg. of resolved acylamino acid. This process was repeated with the remaining fractions. Thus, N-acetyl-α-methyltyrosine was resolved by a time consuming "fractional diastereoisomeric saltformation."

These resolved acylamino acids were then esterified to give D- and L-N-acetyl-α-methyltyrosine methyl ester.

3. α-Methyltryptophan

Manning (1) made some preliminary, unsuccessful attempts to synthesize α-methyltryptophan by a Curtius reaction on skatylmethylcyanoacethydrazide which he obtained by condensing gramine with methylcyanoacethydrazide. Two years later Pfister and Leanza (16) reported the synthesis of α-methyltryptophan and its N-acetyl derivatives in 18% yield from 3-indolylacetone via the hydantoin. This ketone was obtained from 3-indolylacetic acid in 25% yield by the method of Brown, Henbest and Jones (17).

Since 25-50 g. of N-acetyl-α-methyltryptophan were desired, the literature preparation (16,17), 4% overall yield, seemed impractical. Attempts were made to condense gramine, gramine methiodide and gramine ethyl iodide with 5-methylhydantoin. Although some amine was liberated from these reaction mixtures nothing but starting material was isolated from the brown tar-like reaction products which were resistant to all attempts at decolorization.

A few attempts were made to synthesize 3-indolylacetone. Indole and chloroacetone reacted in either the presence of sodium methoxide or aluminum chloride, but none of the desired ketone could be isolated from the reaction mixtures. Condensation of gramine with nitroethane gave α-nitro-α-skatylethane, the sodium salt of which was added slowly to an excess of cold acid. The resulting product did not have the expected (17) infrared or ultraviolet spectra of 3-indolylacetone.

Stiles and Finkbeiner (18) recently reported a new synthesis of amino acids from the α-nitro acids which they obtained by reacting a nitro alkane with magnesium methyl carbonate in dimethylformamide. The acidity of the α-hydrogen of the α-nitro acid should allow these compounds to condense quite readily with gramine.

Magnesium α-nitropropionate was prepared by this procedure (18). Extraction of an acidic solution of this chelate at 0° C. gave an ether solution of α-nitropropionic acid which was added directly to a suspension of gramine or gramine methiodide. Some of the magnesium chelate solution was added directly to a gramine methiodide suspension. However, only a little amine liberation was observed, and none of the desired α-nitro-α-skatylpropionic acid was obtained from these attempts. The failure of this method is probably due to extensive decarboxylation of the α-nitro acid. This speculation was supported this year when Finkbeiner (19) reported obtaining a 98% yield of 3-(β-nitroethyl)-indole from alkylation of the magnesium nitroacetate chelate with gramine methiodide.

Recently the synthesis of 5-benzyloxy-α-methyltryptophan in good yield by the condensation of 5-benzyloxygramine with ethyl-α-nitropropionate followed by reduction of the α-nitro group has been reported (20). In light of this work, the best route to α-methyltryptophan now appears to be the formation of ethyl α-nitropropionate by direct esterification (19) of the magnesium chelate formed from

nitroethane and magnesium methyl carbonate, condensation of this ester with gramine (20) and reduction of the α-nitro group to give the desired product.

4. α-Methyl-β-(2-naphthyl)-alanine and Derivatives

Since α-methyltryptophan proved very difficult to obtain and Kurtz (21) had observed that some naphthalene derivatives were better inhibitors of α-chymotrypsin-catalyzed hydrolyses than indole, it was decided to investigate the D and L enantiomers of N-acetyl-α-methyl-β-(2-naphthyl)-alanine methyl ester instead of the corresponding α-methyltryptophan derivatives.

The synthesis and resolution of α-methyl-β-(2-naphthyl)-alanine was carried out in essentially the same manner as for α-methyltyrosine. 2-Naphthaldehyde was condensed with nitroethane, reduced and hydrolyzed to give 2-naphthylacetone. However, the method of Heinzelmann (22), i.e., continuous removal of the water formed during the condensation and no isolation of intermediates, gave a much better yield of the ketone than did the method of Hoover and Haas (15). A Strecker reaction on this ketone followed by Schotten-Baumann acetylation (10) gave N-acetyl-D,L-α-methyl-β-(2-naphthyl)-alanine.

"Fractional diastereoisomeric salt formation" using D- and L-α-phenethylamine for the base gave the resolved N-acetyl-(-)-α-methyl-β-(2-naphthyl)-alanine, $[\alpha]_D$ - 160° in methanol. However, the other enantiomer was not obtained in a pure form, maximum observed $[\alpha]_D$ + 140°.

Some of the original D,L mixture and the (-) enantiomer were esterified to give D,L- and (-)-N-acetyl-α-methyl-β-(2-naphthyl)-alanine methyl ester.

5. Kinetics

Since Manning (1) had been unable to resolve the α-methylphenylalanine and α-methyltyrosine ester derivatives with α-chymotrypsin it was expected that the esters prepared, vide ante, would be inhibitors of this enzyme. These esters were, therefore, evaluated as inhibitors of the α-chymotrypsin-catalyzed hydrolysis of sodium N-acetyl-L-tyrosinhydroxamate, since Kurtz (23) had found that this substrate is hydrolyzed at a rate conveniently followed in the pH-stat (24) and much faster than the substrate and enzyme blanks.

The α-chymotrypsin-catalyzed hydrolyses of sodium N-acetyl-L-tyrosinhydroxamate inhibited by the N-acetyl methyl esters of α-methylphenylalanine, α-methyltyrosine and α-methyl-β-(2-naphthyl)-alanine were carried out in 0.2 M NaCl at 25.0°±1° C. under a flow of nitrogen at pH 7.6, the pH optimum for sodium N-acetyl-L-tyrosinhydroxamate, in the pH-stat. This instrument (24) automatically adds reagent, base, in order to maintain a constant pH in the reaction vessel and provides a continuous recording of the amount of reagent added versus time. The α-chymotrypsin used, Armour lot T97207, was analyzed by micro Kjeldahl distillation and found to contain 14.6% nitrogen. The enzyme concentration in the reaction vessel was 0.02 mg.

protein nitrogen per ml., the substrate concentration was varied from 0.005 to 0.035 M and the two inhibitor concentrations used for each ester were approximately 1/10 and 1/15 saturated solutions since they were prepared from nearly saturated stock solutions.

The initial velocities were obtained from the pH-stat recorded traces by the orthogonal polynomial procedure of Booman and Niemann (25) which was programmed for the Datatron 220 computer by Abrash (26). These velocities were then used to obtain a least-squares fitted line of the data presented as [S][E]/v versus [S] plots. These calculations were also done by the Datatron 220 computer (26).

This Lineweaver-Burk plot (27) for a competitive inhibitor, equation 1, fit the data within experimental error (see Part I, this thesis).

$$\frac{[S][E_o]}{v} = \frac{K_s(1+[I]/K_I)}{k_3} + \frac{[S]}{k_3} \qquad (1)$$

Since only the intercepts change upon changing the inhibitor concentration, the K_I values were obtained from the ratio of the intercepts for the inhibited and non-inhibited runs (26).

Control reactions were also run at higher enzyme and "inhibitor" concentrations with no sodium N-acetyl-L-tyrosinhydroxamate added in order to determine whether any of these "inhibitors" are actually substrates of α-chymotrypsin.

Since only the (-) and D,L esters of N-acetyl-α-methyl-β-(2-naphthyl)-alanine were available, the K_I for the (+) enantiomer was estimated from equation 2, which is easily derived for the case of two

$$\frac{v}{[E_o]} = \frac{k_3[S_1] + k_6[S_2]\, K_{s1}/K_{s2}}{K_{s1}\{1+([S_2]/K_{s2})+([I]/K_I)\} + [S_1]} \qquad (2)$$

competitive substrates and one competitive inhibitor, equations 3, 4 and 5, where $K_{s1} = \frac{k_2 + k_3}{k_1}$, $K_{s2} = \frac{k_5 + k_6}{k_4}$ and $K_I = \frac{k_8}{k_7}$.

$$E + S_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_3} E + P \qquad (3)$$

$$E + S_2 \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES_2 \xrightarrow{k_6} E + P \qquad (4)$$

$$E + I \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EI \qquad (5)$$

Here S_1, S_2 and I refer to sodium N-acetyl-L-tyrosinhydroxamate, (-) and (+) N-acetyl-α-methyl-β-(2-naphthyl)-alanine methyl ester, respectively, and $[S_2] = [I] = \frac{1}{2}$ [D,L-ester]. The control reactions indicate that the second term of equation 2 only contributes 0.1-0.7% to the total velocity, and, therefore, this term was ignored when estimating K_I.

C. RESULTS AND DISCUSSION

The inhibition constants for these esters are given in Table XIV. The large uncertainties in these values are primarily a result of the small percent of inhibition, i.e., $(a'/a) - 1$, which, in turn, is a consequence of the low solubilities of these compounds since the stock solutions used were essentially saturated. These K_I values should, therefore, be looked upon as estimates of the order of magnitude of these constants.

From the control reactions indicated in Table XV it can be seen that only N-acetyl-(-)-α-methyl-β-(2-naphthyl)-alanine methyl ester has a velocity significantly different from that of the enzyme blank. This compound should, therefore, be considered a substrate of α-chymotrypsin with $K_s = .28 \pm .28 \times 10^{-3}$ M and $k_3 = .20 \pm .20 \times 10^{-3}$ M/min./mg. P-N/ml. There is evidence (28) suggesting that the enzyme blank diminishes in the presence of a substrate or inhibitor. Therefore, if the enzyme blank is ignored, k_3 values can be calculated from the velocities for the other esters. These k_3 values indicate the order of magnitude of the largest k_3 value that could be detected by these experiments.

Examination of the velocities, or k_3 values, in Table XV reveals

Table XIV

Inhibition Constants of Several N-Acetyl-α-methyl Amino Acid Esters vs. the α-Chymotrypsin-Catalyzed Hydrolysis of Sodium N-Acetyl-L-Tyrosinhydroxamate at pH 7.60 and 25.0° C. and 0.2 M in NaCl.[a]

N-Acetyl-α-methyl Ester of -	[I][b]	a'/a[c]	K_I[b] ± σ_{K_I}[d]	Weighted[e] Average $K_I \pm \sigma_{K_I}$
(-) phenylalanine	1.182	1.1718	6.9 ± 6.0	8.2 ± 13
	.788	1.0693	11.4 ±23.0	
(+) phenylalanine	1.071	1.1658	6.5 ± 5.8	8.7 ± 29
	.714	1.0387	18.4 ±66.7	
(-) tyrosine	.818	1.0742	11.0 ±17.8	10.3 ± 14
	1.208	1.1231	9.8 ±10.6	
(+) tyrosine	.651	1.4353	1.5 ± .6	1.7 ± .8
	.434	1.2202	2.0 ± 1.2	
(-)-β-(2-naphthyl)--alanine	.0421	1.1331	.32± .29	.28 ± .28
	.0280	1.1136	.25± .26	
D,L-β-(2-naphthyl)--alanine	..0298	1.3680	.081± .037	.082± .04
	.0199	1.2343	.085± .054	
(+)-β-(2-naphthyl)--alanine	----	------	--------	.048[f]± .13
	----	------	--------	

a. [E] = .02 mg. P-N/ml., and [S] = .005 to .035 M.

b. In units of Mx10^{-3}.

c. Ratio of the intercept of inhibited experiment, a', (see equation 1) to intercept for sodium N-acetyl-L-tyrosinhydroxamate, a = 1.4218 min. x mg. P-N/ml.

d. The standard deviation, $\sigma_{KI} = K_I(2\sigma_b/b + \sigma a/a + \sigma a'/a') \frac{a'/a}{[(a'/a)-1)]}$, where b is the common slope of these plots.

e. Weighted in proportion to the % of inhibition, i.e.,

$$w_i = \frac{(a'_i/a) - 1}{\sum_i [(a'_i/a) - 1]}$$

f. Estimated from the results of the D,L and (-)-β-(2-naphthyl)-alanine derivatives, see text.

Table XV

Several N-Acetyl-α-methyl Amino Acid Esters as Substrates for α-Chymotrypsin at pH 7.90 and 25.0°C. and 0.2 M in NaCl.[a]

N-Acetyl-α-methyl Ester of -	[S][b]	v[c]	v_{base}[d]	Upper Limit for k_3[e]
(-) phenylalanine	13.1	.80	.08	9
(+) phenylalanine	10.1	.63	.04	8
(-) tyrosine	4.43	1.09	< .02	25
(-)-β-(2-naphthyl)-alanine	.25	13.4[f] ± .2	< .02	196 ± 198
D,L-(2-naphthyl)-alanine	.36	.85	< .02	7
Enzyme Blank	0	.85		

a. [E] = 0.145 mg. P-N/ml.
b. In units of $\bar{M} \times 10^{-3}$.
c. In units of $\bar{M} \times 10^{-6}$/min.
d. Base catalyzed hydrolysis in units of $\bar{M} \times 10^{-6}$/min.
e. In units of $\bar{M} \times 10^{-6}$/min./mg. P-N/ml. Calculated from v, assuming the K_I values of Table XIV = K_s and assuming the enzyme blank reaction is "negligible."
f. Calculated from a second degree orthogonal polynomial (25) since the recorded trace was curved.

that the levorotatory enantiomers are better "substrates" and the dextrorotatory enantiomers are better "inhibitors" for α-chymotrypsin: the (-)-α-methyl-β-(2-naphthyl)-alanine derivative is a substrate while the (+) enantiomer is an inhibitor since the racemate is hydrolyzed much slower than the (-) enantiomer; the (-)-α-methyltyrosine derivative has a velocity greater than that of the enzyme blank and is probably a very poor substrate; however, not enough of the (+) enantiomer was available for this experiment; and the (-)-α-methylphenylalanine derivative "inhibits" the enzyme blank less than the (+) enantiomer. This analysis is of questionable significance, except for the α-methyl-β-(2-naphthyl)-alanine derivatives; however, it does suggest that the (-) enantiomers belong to the L, or "natural" series of amino acids.

The determination of the absolute configuration of these α-methyl-α-amino acids is encumbered by the failure of their derivatives to react with enzyme in a stereospecific manner. The experiments reported here are the only indications of the absolute configuration of these α-methyl-α-amino acids reported in the literature to date.

Optical rotatory dispersion curves (31, 32) of these α-methyl-α-amino acids or their dithiocarbamates (33) might give another clue to their absolute configuration, especially since the aromatic amino acids have anomalous dispersions (31, 32). However, these measurements would be ambiguous in the absence of other α-methyl-α-amino acids of known configuration for comparison.

The Lutz-Jirgensons rule (29) for determining absolute configuration from the shift in optical rotation between acidic and neutral solutions is of no use here since one of the two exceptions among fifty amino acids (30) is isovaline, the only α-methyl-α-amino acid studied. Winitz, Birnbaum and Greenstein (30) concluded that -

> " the selection of a D- or L- designation for amino acids, in which an alkyl substituent replaces the α-hydrogen, becomes meaningless when based on optical rotation data and emphatically indicates that the interpretation of such data be confined to α-amino acids which contain an α-hydrogen atom."

Fortunately, unambiguous assignments of absolute configuration to these α-methyl-α-amino acid derivatives is not essential to the work reported here; however, some enzymatic evidence is at hand, vide ante.

Comparison of the optical rotations for some aromatic amino acids and the α-methyl analogues listed in Table XVI indicates that the derivatives with the same sign of rotation in the α-methyl series are all of the same absolute configuration, as they are in the normal amino acid series, e.g., N-acetyl-(-)-α-methylphenylalanine is assumed to have the same absolute configuration as N-acetyl-(-)-α-methyltyrosine, etc.

If the sparse evidence that the (-)-α-methyl amino acid derivatives belong to the L series of amino acids is accepted, further speculations can be made concerning the interaction of α-chymotrypsin and these "inhibitors." Of the twenty-three enantiomorphic pairs of substrates and inhibitors that have been studied (38, 39, 43-46), only five have a ratio

Table XVI

Comparison of Specific Rotations and Kinetic Constants of Some Aromatic Amino Acid and α-Methyl Amino Acid Derivatives.

Amino Acid[a] Derivatives	$[\alpha]_D$[b]	K_S or K_I[c]	α-Methyl Amino[a] Acid Derivatives	$[\alpha]_D$[b]	K_s or K_I[c]
Ac-L-Phe	+ 21°		Ac(-)αMe-Phe	-74°	
Ac-L-Phe-Me	+ 19.5°(34)	1.8 (37)	Ac(-)αMe-Phe-Me	-82°	8.2
Ac-D-Phe-Me	- 19.0°(34)	2.0-2.6 (38)	Ac(+)αMe-Phe-Me	+83°	8.7
Ac-L-Tyr	+ 51.5°		Ac(-)αMe-Tyr	-61°	
			Ac(+)αMe-Tyr-Me	+61°	
Ac-L-Tyr-Et	+ 24.6°[d](35)	1.0 (39,40)	Ac(-)αMe-Tyr-Me	-79°	10.3
Ac-D-Tyr-Et	- 24.6°[d](35)	4.0-5.0 (38)	Ac(+)αMe-Tyr-Me	+78°	1.7
Ac-L-Try	+ 20.4°				
Ac-L-Try-Me	+ 11.5° (36)				
Ac-D-Try-Me	- 12.0° (36)	.089 (41)			
Ac-L-Try-Et		.093 (39,40)			
Ac-D-Try-Et		.25 (42)			
			Ac(-)αMe-Nap	-160°	
			Ac(-)αMe-Nap-Me	-206°	.28
			Ac(+)αMe-Nap-Me		.05

a. Ac = N-acetyl, Phe = phenylalanine, Tyr = tyrosine, Try = tryptophan, Nap = β-(2-naphthyl)-alanine, αMe = α-methyl, -Me = methyl ester and -Et = ethyl ester, e.g., Ac(-)αMe-Tyr-Me = N-acetyl-(-)-α-methyltyrosine methyl ester.

b. Measured in methanol.

c. In units of $\bar{M} \times 10^{-3}$.

d. Measured in ethanol.

of K_{SL}/K_{ID} or K_{IL}/K_{ID} less than unity. These five are the D and L enantiomers of N-carbethoxytyrosinamide and N-carbethoxytyrosinmethylamide and the three N-acetyl aromatic acid esters given in Table XVI.

There is reason to believe (36, 43) that the K_s values for most of these compounds are apparent equilibrium constants, i.e., $K_s \doteq k_2/k_1$. However, the aromatic amino acid esters have k_3 values much greater than those for the other compounds considered and apparently anomalous K_{SL}/K_{ID} ratios (< 1). Therefore, the K_s values of these esters may not be equilibrium constants (43). Manning and Niemann (43) elected to defer discussion of these esters for these reasons. In contrast to these "normal" esters, the α-methyl aromatic amino acid esters studied here have very low k_3 values and "normal" K_{IL}/K_{ID} ratios* (≥ 1). These "normal" ratios for the α-methyl derivatives are not unexpected, since they are the ratios of K_I values which can be considered to be apparent equilibrium constants.

Manning (1) proposed that both the L and D enantiomers of a substrate or inhibitor might interact with three or more positions on the enzyme, i.e., R_1, R_2 and R_3 of $R_1R_2CHR_3$ interact with positions ρ_1, ρ_2, and ρ_3 or the enzyme. Since the K_{SL}/K_{ID} ratio is usually greater than unity, vide ante, he expected the D enantiomers to have a greater affinity for the enzyme with their α-hydrogen atoms away

*The K_I values for N-acetyl-(-) and (+)-α-methylphenylalanine methyl esters are essentially the same, i.e., $K_{IL}/K_{ID} \doteq 1$.

from the "bulk" of the enzyme and the L enantiomers to have their α-hydrogen atoms toward, and slightly interacting with, the "bulk" of the enzyme. Manning (1), therefore, expected the K_{ID} values to be little affected by replacing the α-hydrogen with a relatively bulky α-methyl group, but he expected the L α-methyl enantiomers to be "incapable of ES complex formation," thus, precluding hydrolysis but possibly capable of acting as competitive bifunctional inhibitors.

The K_I or K_S values for these N-α-acetamino α-methyl esters are within an order of magnitude of the K_I or K_S values for the corresponding N-α-acetamino esters, Table XVI. These α-methyl derivatives, therefore, have a relatively high affinity for α-chymotrypsin, and their inactivity as substrates is due almost exclusively to their low k_3 values rather than to high K_s values. Thus, Manning's (1) predictions for the D enantiomers have been realized, within an order of magnitude, while those for the L enantiomers have only been realized in part.

D. CONCLUSIONS

It has been shown that the N-acetyl methyl ester derivatives of (-) and (+)-α-methylphenylalanine, (-) and (+)-α-methyltyrosine and (-) and D,L-α-methyl-β-(2-naphthyl)-alanine act as competitive inhibitors of the α-chymotrypsin-catalyzed hydrolysis of sodium N-acetyl-L-tyrosinhydroxamate. N-Acetyl-(-)-α-methyl-β-(2-naphthyl)-alanine methyl ester has also been shown to be a slowly hydrolyzed substrate of α-chymotrypsin, thus providing a clue to the absolute configuration of these compounds. The (-)-α-methyltyrosine and (-)-α-methylphenylalanine N-acetyl esters may also be very poor substrates. The K_I and K_S values for these derivatives are given in Table XIV, and estimates of their maximum k_3 values are given in Table XV.

The inactivity of these esters toward α-chymotrypsin-catalyzed hydrolysis is a consequence of their inability to react further after complexing with the enzyme.

Some of the implications of these results have been discussed.

E. EXPERIMENTS*

(i) D,L-α-Methylphenylalanine

A Strecker reaction (1, 9, 47) on phenylacetone (Eastman, practical) with KCN and NH_4Cl gave 74 to 90% yields of product (i) as the hydrochloride, m.p. 237° C. Lit. (9) m.p. 241-243° C. Saturation of a methanolic solution of this hydrochloride with ammonia produced a precipitate of NH_4Cl which was filtered from solution. The chloride free acid (i) was recovered from the filtrate, m.p. 297° C. Lit. (9) m.p. 294.5-295° C.

(ii) N-Acetyl-D,L-α-methylphenylalanine

a) Schotten-Baumann acylation (10) on i with the pH kept ≥ 11 gave a 39% yield of ii , m.p. 196.5-197.5° C. Lit. (1) m.p. 194.8-197.7° C.

b) Acetylation of i with acetic anhydride in pyridine (48) gave 58 to 65% yields of ii , m.p. 195-197° C.

(iii) N-Acetyl-(-) and (+) -α-methylphenylalanine

a) Attempted Acylase I Resolution (11) - An aqueous suspension of 7 g. ii in 300 ml. water was neutralized to pH 7.0 with LiOH.

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*All melting points are corrected to within ± 2° C. All optical rotations were measured at room temperature, 25° ± 3° C., and have an uncertainty of about ± 2-3%. Microanalyses are by Spang Microanalytical Laboratory, Ann Arbor, Michigan. Almost all concentration of solutions and stripping of solvents was done while sweeping the system with nitrogen to minimize air oxidation of the compounds.

Armour Acylase I, lot 945-40, was added, 0.2 g., and the solution kept in a water bath at 38° C. At various times 0.4 ml. aliquots were removed and analyzed for free amino groups by the Van Slyke procedure (12). After 62 hours 1.18 millimoles of N were detected, implying 7.4% resolution. At 109 hours this had dropped to .33 millimoles of N.

b) Resolution as the Dexedrine Salt - Equivalent amounts of Dexedrine sulfate (Smith, Kline and French Labs., lot 294) and ii were dissolved in aqueous ethanol and an equivalent of 4N NaOH was slowly added to this solution. The resulting precipitate of Na_2SO_4 was filtered off, and the filtrate was concentrated until turbid. The precipitate that formed was then fractionally recrystallized. All salt fractions were dextrorotatory, maximum $[\alpha]_D$ + 17.9°, minimum $[\alpha]_D$ + 8.5°. These salt fractions were dissolved in base (pH > 10), extracted with ether and acidified with concentrated HCl (pH < 2). The recovered acids had specific rotations of + 85.7° and -15° (C° = 1% in water), respectively.

c) Resolution as the (+)- and (-)-α-Phenethylamine Salts - Resolution with (-)-α-phenethylamine in water, isopropanol-isopropyl ether, ethyl acetate and acetone as above (iii, b) omitting recrystallization gave specific rotations of -57°, -58°, -81° and -85.6° (C° = 0.3% in water), respectively, for the acids. The acid from the best solvent, acetone, has $[\alpha]_D$ - 74.3° (C° = 1% in MeOH). (+)-α-Phenethylamine yielded more N-acetyl-(+)-α-methylphenylalanine in a similar manner,

$[\alpha]_D$ + 74.4° (C° = 1% in MeOH).

(iv) N-Acetyl-(-)-α-methylphenylalanine Methyl Ester

Esterification of (-) iii by the method of Brenner and Huber (49) gave the product (iv) which was recrystallized from water, m.p. 82.0-82.5° C., $[\alpha]_D$ - 82.4° (C = 1% in MeOH).

Calculated for $C_{13}H_{17}NO_3$(235): C, 66.4; H, 7.3; N, 6.0.

Found: C, 66.0; H, 7.3; N, 6.1.

(v) N-Acetyl-(+)-α-methylphenylalanine Methyl Ester

Esterification of (+) iii, as above (iv), gave the product, m.p. 77-80° C., $[\alpha]_D$ + 82.9° (C° = 1% in MeOH).

Calculated for $C_{13}H_{17}NO_3$(235): C, 66.4; H, 7.3; N, 6.0.

Found: C, 66.0; H, 7.2; N, 6.0.

(vi) p-Methoxybenzyl Methyl Ketone

Condensing anisaldehyde with nitroethane in ethanol with n-butylamine as the catalyst by the method of Hoover and Haas (15) gave 19-33% yields of (p-methoxyphenyl)-2-nitropropene, m.p. 43.5-45.5° C. Lit. m.p. 43-44° C. Treating this intermediate with iron filings in aqueous HCl (15) gave 45-75% yields of desired ketone (vi), b.p. 121-124° C. at 5-6 mm. Hg., $N_D^{25.2}$ 1.5228. Lit. (15) b.p. 117-122° C. at 5-6 mm. Hg., $N_D^{25.6}$ 1.5223 (1).

(vii) O-Methyl-D,L-α-methyltyrosine Hydrochloride

A Strecker reaction on vi (1) gave the product (vii) in 93% yield, m.p. > 320° C.

(viii) D,L-α-Methyltyrosine

Treatment of vii with 31% HBr in glacial acetic acid at 100° C. by the method of Manning (1) gave the crude product (viii) as the hydrobromide. This product was dissolved in 2N HCl and neutralized to pH 6 with NaOH to precipitate the halide free amino acid (viii) in 60% yield, m.p. 312-314° C. (dec.).

(ix) O,N-Diacetyl-D,L-α-methyltyrosine

Acetylation of viii with acetic anhydride in pyridine (48) gave the desired product, m.p. 222-223° C., N.E. 280 ± 5 (calc. 279).

(x) N-Acetyl-D,L-α-methyltyrosine

a) From O,N-Diacetyl-D,L-α-methyltyrosine (ix) - A solution of ix in 2N NaOH was allowed to stand at room temperature 30 minutes and then acidified to pH 2. The resulting precipitate of the product (x) was filtered, washed with water and recrystallized from water, m.p. 220.0-220.5° C., N.E. 238 ± 4 (calc. 237).

b) From D,L-α-Methyltyrosine (ix) - Schotten-Baumann (10) acylation of ix gave a 45% yield of x after recrystallization from dioxane-water (1:6), m.p. 216.5-218° C. Lit. (1) m.p. 207.5-211.5° C.

(xi) N-Acetyl-(-) and (+)-α-methyltyrosine

Dissolving x in acetone (USP), ca. 1 g. per 100 ml., and adding 0.75 equivalents of (-)-α-phenylethylamine gave a 50% yield (based on the acid, x) of precipitated salt. The salt was filtered and the filtrate evaporated to dryness in vacuo. Each of these fractions was dissolved in base, extracted three times with ether, acidified to pH 2 and filtered. The rotation of the recovered acid was measured and the above process repeated. After about 6 successive salt formations, 20 g. of starting material (x) gave 0.3 g. of N-acetyl-(-)-α-methyltyrosine $[\alpha]_D$ - 61.1° (C = 1-2% in MeOH). Using (+)-α-phenethylamine as the base in a similar fashion gave 0.2 g. of N-acetyl-(+)-α-methyltyrosine, $[\alpha]_D$ - 61.0° (C = 1-2% in MeOH).

(xii) N-Acetyl-(-)-α-methyltyrosine Methyl Ester

Esterification of (-) xi , as for iv, gave the desired product (xii) which was recrystallized from water, m.p. 149-150° C., $[\alpha]_D$ - 78.9° (C = 1% in MeOH).

Calculated for $C_{13}H_{17}NO_4$(251): C, 62.14; H, 6.82; N, 5.57

Found: C, 62.12; H, 6.84; N, 5.52

(xiii) N-Acetyl-(+)-α-methyltyrosine Methyl Ester

Esterification of (+) xi , as for iv, gave 50 mg. of product (xiii) after recrystallization from water, m.p. 137-140° C., $[\alpha]_D$ + 77.9° (C = 1% in MeOH).

(xiv) D,L-5-Methylhydantoin

D,L-Alanine was treated with KCNO in HCl solution by the method of Dakin (50). The product was recrystallized from water, m.p. 139-142° C. Lit. (51) m.p. 140-156° C.

(xv) D,L-5-Methyl-5-skatylhydantoin, Attempted Syntheses

a) A slurry of 5.23 g. of gramine and 3.42 g. of xiv in dry xylene was refluxed 6 hrs. while sweeping dry nitrogen over the solution. An amine was detected in the gasses with moist litmus paper, but only gramine and xiv could be isolated from the reaction mixture.

b) A solution of equivalent amounts of gramine and xiv in ethanol with one equivalent of sodium ethoxide was stirred while an equivalent of ethyl iodide was added (52). Nitrogen was then swept over the solution which was refluxed 6 hours. Amine was again found in the exit gasses. The reaction mixture was stripped of ethanol, dissolved in chloroform and water. The chloroform layer was separated from the water solution, washed with 1N HCl, $NaHCO_3$ and water and stripped of chloroform. Gramine was found among the resulting fractions, but none of the other fractions could be obtained in sufficient purity or amount to be identified as the desired product (xv).

c) Equivalent amounts of sodium metal and xiv were slurried in xylene, nitrogen was passed over the solution and an equivalent of gramine methiodide (xiv) suspended in xylene was added to the slurry.

The exit gasses were bubbled through a benzene solution of picric acid. After 24 hours a small amount of picrate had formed. None of the desired product (xv) could be isolated from the brown reaction mixture, but one fraction did have an ultraviolet spectra essentially the same as that for skatole.

(xvi) Gramine Methiodide

This compound (xvi) was prepared from gramine and methyl iodide by the method of Geissman and Armen (53). The ultraviolet spectra of the product was the same as that of an authentic sample (Union Carbide), λ max. 271, 279 and 287 mμ.

(xvii) 3-Indolylacetone

a) Equivalent amounts of indole, sodium methoxide and chloroacetone in methanol were refluxed one hour. A white precipitate separated almost immediately from the orange-brown solution, but none of the ketone (xvii) could be detected in the reaction mixture.

b) A pale green solution of 11.7 g. indole and 33.4 g. $AlCl_3$ in 100 ml. CS_2 was stirred and cooled in an ice bath. Dropwise addition of 8.1 ml. of chloroacetone caused a vigorous reaction to occur, resulting in a dark red-brown oil which was insoluble in CS_2. However, none of the desired product (xvii) could be isolated from the reaction products.

c) 1-(β-Indolyl)-2-nitropropane was prepared from gramine and nitroethane by the method of Snyder and Katz (54). The crude

product was dissolved in base, washed several times with ether and added dropwise to an excess of HCl at 0° C. The brown precipitate that formed was filtered from the solution, but neither its ultraviolet nor its infrared spectra resembled that of the desired ketone (xvii) (17).

(xviii) Magnesium Methyl Carbonate

This compound was prepared by the method of Stiles and Finkbeiner (18, 55). Methanol was dried over magnesium methoxide, then 250 ml. were added to 24 g. magnesium turnings while cooling in ice. The methanol was then almost completely distilled from the magnesium methoxide. Dimethylformamide, DMF, 100 ml., was added and the resulting solution was saturated with dry CO_2 as rapidly as possible. A short column was added to the reaction vessel which was heated until the vapor temperature reached 110° C. The straw-colored solution was too viscous so 100 ml. of DMF was added and the mixture was resaturated with CO_2. An aliquot was pipetted into an excess of standardized H_2SO_4 which was heated to expel CO_2 and then back titrated with NaOH. The light brown dimethylformamide solution of xviii was 5.81 M in xviii.

(xix) Magnesium 2-Nitro-propionate (18, 55)

The solution of xviii was diluted with DMF to make 200 ml. of 2 M xviii and 7.1 ml. of nitroethane was added. Holding this solution at 50° C. for 5 hours while sweeping with nitrogen gave a clear orange solution of xix. A portion of this solution was slowly poured

into a slurry of 200 g. ice and 160 ml. concentrated HCl layered with 400 ml. ether with considerable evolution of CO_2. The pale yellow ether phase was separated from the aqueous phase, dried over $MgSO_4$ followed by P_2O_5 and stripped of solvent in vacuo at 0° C. The yellow oily residue, 2-nitropropionic acid, was precipitated from ether-carbon disulfide at dry ice temperature but melted when refrigerated at 4° C.

(xx) 2-Skatyl-2-nitropropionic acid, Attempted Syntheses

a) The dried ether solution of 2-nitropropionic acid (cf. xix) was slowly added to a solution of 8.7 g. gramine in 250 ml. xylene at 0° C. while passing nitrogen over the solution. However, the addition of crushed NaOH pellets, heating to 40-50° C. or repeating this experiment in absolute ethanol instead of xylene did not produce any detectable amine in the exist gasses.

b) Addition of a dried ether solution of 2-nitropropionic acid (cf. xix) to gramine methiodide (xvi) as above (xx, a) did liberate some amine, as detected by moist litmus paper. However, none of the desired product could be isolated from the reaction mixture.

c) Some of the DMF solution of xix was added to a suspension of gramine methiodide (xvi) and heated while stirring six hours. Undissolved material was filtered from the dark brown solution. Addition of water to this filtrate produced a tan precipitate which was filtered and triturated with ether. Evaporation of this ether left a

deep red-brown oil which had an ultraviolet spectra similar to that of indole and a strong infrared absorption at 1670 cm^{-1}. However, this small amount of oil could not be purified satisfactorily. None of the other fractions were of interest.

(xxi) 2-Naphthylacetone

This product was prepared from 98 g. of 2-naphthaldehyde (K and K Labs.) and 60 ml. of nitroethane with 22 ml. n-butylamine by the method of Heinzelmann (22). About 90% of the liberated water was trapped from the refluxing solution. After the reduction with iron in HCl the reaction mixture was steam distilled until the distillate was clear (15 l. collected). This distillate was extracted with toluene and then three times with ether, the organic solvents were stripped in vacuo and the residue was rectified. The second fraction (33% yield) had a b.p. 103-118° C. at .45-.55 mm. Hg and formed a white semicarbazone, m.p. 180-190° C., a yellow picrate, m.p. 75-78° C. and an orange 2,4-dinitrophenylhydrazone, m.p. 270-275° C. The literature values are b.p. 130° C. at 1.5 mm. Hg. (56), m.p. 183-184° C. (56), m.p. 79-80° C. (57) and m.p. 172-173° C. (57) respectively.

(xxii) D,L-α-Methyl-β-(2-naphthyl)-alanine

A Stecker reaction (cf. viii) on xxi followed by neutralization of its acid solution gave a 66% yield of the chloride free amino acid (xxii) m.p. 266-271° C.

(xxiii) N-Acetyl-D,L-α-methyl-β-(2-naphthyl)-alanine

Acetylation with acetic anhydride in pyridine (48) followed by treatment with norite and recrystallization from water gave long needles, m.p. 216-217.5° C.

Calculated for $C_{16}H_{17}NO_3$(271): C, 70.8; H, 6.3; N, 5.2

Found: C, 70.6; H, 6.4; N, 5.4

(xxiv) N-Acetyl-(+) and (-)-α-methyl-β-(2-naphthyl)-alanine

Resolution of xxiii as the (-) and (+)-α-phenethylamine salts as described for xi gave the (-) xxiv, $[\alpha]_D$ - 160° (C = 1% in MeOH) after 5 steps. However, the (+) enantiomer fraction only had a specific rotation of + 140° (C = 1% in MeOH) after 7 steps.

(xxv) N-Acetyl-(-)-α-methyl-β-(2-naphthyl)-alanine Methyl Ester

Esterification of 0.2 g. of (-) xxiv as above (iv) gave the ester (xxv), m.p. 127-129° C., $[\alpha]_D$ - 206° (C = .5% in MeOH).

(xxvi) N-Acetyl-D,L-α-methyl-β-(2-naphthyl)-alanine Methyl Ester

Esterification of 1 g. of xxiii as above (iv) gave a good yield of the ester (xxvi), m.p. 127-128° C.

Calculated for $C_{17}H_{19}NO_3$(285): C, 71.56; H, 6.71; N, 4.91

Found: C, 71.53; H, 6.73; N, 5.13

(xxvii) α-Chymotrypsin Nitrogen Analysis*

The α-chymotrypsin used, Armour salt free, bovine, lot #T97207, was analyzed for nitrogen by the micro-Kjeldahl procedure of

*I am indebted to Dr. G. E. Hein, who shared the labor of these analyses.

Redemann (58) and found to contain 14.6 ± .2% nitrogen.

(xxviii) Sodium N-Acetyl-L-tyrosinhydroxamate

This compound was prepared by the method of Kurtz (59), m.p. 189.7-190.0° (dec.), $[\alpha]_D$ + 34.7° (C = 4% in 0.2 N HCl). Lit. (59) m.p. 190.5-191.0° C., $[\alpha]_D$ + 35° (C = 5% in N HCl).

Kinetic Experiments

Stock solutions of 0.05 M substrate (xxviii), .05 M NaCl, 1.65 M NaCl, 1.4 mg. enzyme (xxvii)/ml. and inhibitors (iv, v, xii, xiii, xxv, and xxvi) were prepared in CO_2 free water. The substrate stock solution contained 7.8 ml. of 0.6 N HCl.

The reaction mixtures were prepared from 1 ml. inhibitor, n ml. substrate (n = 1, 2, 3, 5 and 7), 7-n ml. .05 M NaCl, 1 ml. 1.65 M NaCl and 1 ml. enzyme which was added later. The reaction mixture and a portion of the enzyme stock solution were adjusted to pH 7.60 with N NaOH just prior to the addition of the 1 ml. of enzyme stock solution to the reaction mixture in the pH stat. This procedure is essentially the same as that described in more detail by Abrash (60).

The velocities for these runs are given in Table XVII. The data was treated as described under "Methods and Procedure."

Table XVII

Velocities of the α-Chymotrypsin-Catalyzed Hydrolysis of Sodium N-Acetyl-L-tyrosinhydroxamide at pH 7.60 and 25.0° C. and 0.2 M in NaCl, Inhibited by Some N-Acetyl-α-methyl-α-amino Acid Esters.

Inhibitor[a]	[I][b]	$[E_o]$[c]	$[S_o]$[b]	$v_o \pm \sigma_v$[d]	P_m[e]
None		.01956	25.02	205.6± .3	1
"		"	35.02	263.6±4.9	2
"		"	5.00	57.0±1.1	3
"		"	15.01	150.2± .7	2
"		"	10.01	112.8±1.4	2
"		"	25.02	213.2±1.0	1
"		"	10.01	112.9±1.4	2
"		"	15.01	154.2± .5	1
"		"	35.02	260.5±7.2	4
"		"	25.02	236.5±5.5	2
"		"	10.01	115.3±1.7	2
"		"	25.02	214.2±1.0	1
"		"	5.00	59.8± .3	1
"		.02044	34.98	315.6±4.4	3
"		"	5.00	64.2±1.1	3
"		"	24.99	225.4±1.9	2
"		"	9.99	108.7± .7	1
"		.02026	35.03	281.6±3.9	2
"		"	5.00	66.4± .3	2
"		"	25.02	224.9± .8	1
"		"	10.01	124.6±5.1	2
"		.02031	25.03	225.2± .6	1
"		"	5.01	65.0± .7	2
"		"	35.04	272.1± .5	1
"		"	10.01	116.6±1.0	2
"		.02045	25.01	231.6±4.3	2
"		"	5.00	65.5± .7	2
"		"	35.01	297.2±5.7	2
"		"	10.00	123.5±1.5	2
"		.02023	25.04	222.4±1.6	2
"		"	5.01	65.8± .6	2
"		"	35.06	280.5±1.0	2
"		"	10.02	117.2±1.0	2
"		.02046	25.03	216.9±13.7	1
"		"	5.01	66.5± .7	2
"		"	35.04	270.7± .6	1
"		"	10.01	122.1±1.5	2

Table XVII (cont'd.)

Inhibitor[a]	$[I]$[b]	$[E_o]$[c]	$[S_o]$[b]	$v_o \pm \sigma_v$[d]	P_m[e]
Ac(-)α Me Phe-Me	1.182	.02044	4.98	73.2±1.1	4[f]
"	"	"	34.98	253.1±2.8	2
"	"	"	24.99	198.4±3.6	2
"	"	"	14.99	110.6± .6	2[f]
"	"	"	9.99	101.7±1.2	2
"	"	"	34.98	246.9± .7	1
"	"	"	4.98	53.6± .4	1
"	"	"	24.99	213.2±2.2	2
"	"	"	14.99	143.0± .8	1
"	"	"	9.99	105.3± .7	1
"	0.788	"	34.98	277.2±4.3	2
"	"	"	5.00	65.4±1.1	3
"	"	"	24.99	220.8±3.0	2
"	"	"	14.99	147.8± .5	1
"	"	"	9.99	108.2±1.0	1
"	"	"	34.98	263.0± .8	1
"	"	"	4.98	60.9± .6	2
"	"	"	24.99	225.5±3.5	2
"	"	"	14.99	145.2± .5	1
Ac(+)α Me Phe-Me	1.071	.02026	5.00	57.5±1.0	2
"	"	"	35.03	264.1±1.2	1
"	"	"	25.02	217.1±3.6	2
"	"	"	15.01	174.4±3.4	3[f]
"	"	"	10.01	110.1±3.6	2
"	"	"	35.03	274.7±3.8	2
"	"	"	5.01	57.7±9.3	4
"	"	"	25.02	212.6±3.3	2
"	"	"	15.01	137.3± .6	1
"	"	"	10.01	99.4± .7	1
"	0.714	"	35.03	259.9±2.2	1
"	"	"	5.00	60.1± .7	2
"	"	"	25.02	218.1±2.1	1
"	"	"	15.01	164.7±2.9	2
"	"	"	10.01	108.3±2.0	1
"	"	"	35.03	284.7±1.8	2
"	"	"	5.00	66.5±1.1	2
"	"	"	25.02	218.5±2.7	2
"	"	"	15.01	155.8±3.1	2
"	"	"	10.01	106.5±1.3	2

Table XVII (cont'd.)

Inhibitor[a]	[I][b]	[E_o][c]	[S_o][b]	$v_o \pm \sigma_v$[d]	P_m[e]
Ac(-)α Me Tyr-Me	0.818	.02031	5.01	62.1± .5	2
"	"	"	25.03	212.1±1.4	1
"	"	"	35.04	266.6±4.8	2
"	"	"	10.01	107.7± .4	1
"	"	"	15.02	148.3± .5	1
"	"	"	5.01	59.1± .3	1
"	"	"	25.03	216.3±3.0	3
"	"	"	35.04	266.7± .9	1
"	"	"	10.01	111.5± .7	2
"	"	"	15.02	151.3±1.2	2
"	1.208	"	5.01	57.6± .6	2
"	"	"	25.03	224.3±5.1	2
"	"	"	35.04	260.5±1.1	1
"	"	"	10.01	105.6± .4	1
"	"	"	15.02	147.8± .9	2
"	"	"	5.01	57.3± .3	1
"	"	"	25.03	222.9±1.7	2
"	"	"	35.04	263.9±1.1	1
"	"	"	10.01	109.9±3.0	4
"	"	"	15.02	144.5± .5	1
Ac(+)α Me Tyr-Me	0.651	.02045	5.00	45.8± .4	1
"	"	"	25.01	176.5± .8	1
"	"	"	35.01	220.0±1.7	1
"	"	"	10.00	82.5± .3	1
"	"	"	15.01	119.0± .6	1
"	"	"	5.00	48.4± .6	2
"	"	"	25.01	176.4± .6	1
"	"	"	10.00	83.0± .5	1
"	"	"	15.01	125.6±1.4	2
"	0.434	"	5.00	53.8±1.0	2
"	"	"	25.01	191.4± .9	1
"	"	"	35.01	241.6±1.3	1
"	"	"	10.00	98.7± .9	2
"	"	"	15.01	135.9±1.6	2
"	"	"	5.00	53.5±1.4	2
"	"	"	25.01	193.2±1.5	1
"	"	"	35.01	241.4± .5	1
"	"	"	10.00	96.7± .6	2
"	"	"	15.01	135.3±2.0	2

Table XVII (cont'd.)

Inhibitor[a]	[I][b]	$[E_o]$[c]	$[S_o]$[b]	$v_o \pm \sigma_v$[d]	P_m[e]
Ac(-)αMeNap-Me	0.0421	.02023	5.01	57.9± .6	3
"	"	"	25.04	209.4±1.2	1
"	"	"	35.06	265.4±1.0	1
"	"	"	10.02	106.6±2.5	3
"	"	"	15.03	148.2± .3	1
"	"	"	5.01	56.5± .3	1
"	"	"	25.04	215.6±1.0	2
"	"	"	35.06	263.3±1.4	1
"	"	"	10.02	111.1±1.0	2[f]
"	"	"	15.03	145.6± .3	1
"	0.0280	"	5.01	58.7± .2	1
"	"	"	25.04	224.8±3.0	2
"	"	"	35.06	277.6±2.9	2
"	"	"	10.02	115.7±1.5	2[f]
"	"	"	15.03	154.9± .6	2
"	"	"	5.01	59.2± .3	1
"	"	"	25.04	224.9±3.0	2
"	"	"	35.06	275.0±2.9	2
"	"	"	10.02	106.9± .4	1
"	"	"	15.03	150.5± .7	1
Ac-D,L-αMeNap-Me	0.0298	.02046	5.01	48.3± .7	2
"	"	"	25.03	184.8±2.2	2
"	"	"	35.04	219.7±1.3	1
"	"	"	10.01	85.5± .5	1
"	"	"	15.02	128.4±1.2	2
"	"	"	5.01	45.7± .3	1
"	"	"	25.03	183.0± .9	1
"	"	"	35.04	215.5±7.8	1
"	"	"	10.01	90.5±1.8	2
"	"	"	15.02	124.7± .5	2
"	0.0199	"	5.01	52.5± .8	2
"	"	"	25.03	199.9±7.7	1
"	"	"	35.04	233.7±4.0	2
"	"	"	10.01	93.8± .8	1
"	"	"	15.02	132.5±1.0	1
"	"	"	5.01	52.4±1.1	2
"	"	"	25.03	194.6±1.2	1
"	"	"	35.04	240.7±1.5	1
"	"	"	15.02	142.7±2.2	2
"	"	"	10.01	98.4±1.9	2

Table XVII (cont'd.)

a. The abbreviations are those of Table XVI, footnote a.
b. In units of $\underline{M} \times 10^{-3}$.
c. In units of $\overline{mg}$. P-N/ml.
d. Velocities calculated from orthogonal polynomials (26), in units of $\underline{M} \times 10^{-6}$/min.
e. Order of the polynomial used to calculate v_o.
f. These points were eliminated from the calculation of kinetic constants by a 98% confidence limit "t"-test (26),

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PART IV

THE APPARENT IONIZATION CONSTANTS OF A SERIES OF PHENYLALANINE DERIVATIVES

[Reprinted from the Journal of the American Chemical Society, **81**, 2856 (1959).]

[CONTRIBUTION NO. 2414 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Apparent Ionization Constants of a Series of Phenylalanine Derivatives[1]

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RECEIVED NOVEMBER 20, 1958

The apparent ionization constants of the α-ammonium groups present in DL-phenylalanine and its monoprotonated amide, thioamide, amidoxime, hydrazide, methyl ester and hydroxamide have been determined in aqueous solutions at 25.0 ± 0.1° and 0.05, 0.10 and 0.20 M in sodium chloride. The values of $pK^0_{A(NH_3^+)}$ were observed to increase from 6.78 ± 0.03 to 9.15 ± 0.01 in the order $-CONHOH < -CO_2CH_3 \leq -CONHNH_2 \doteq -C(NOH)NH_2 < -CSNH_2 < -CONH_2 \ll -CO_2^-$. Where comparison was possible the phenylalanine derivatives were found to have $pK'_{A(NH_3^+)}$ values that were 0.59 ± 0.04 of a pK unit lower than those of the corresponding glycine derivatives. The infrared spectra of all of the phenylalanine derivatives were determined for the solid in solid potassium bromide.

The use of amino acid derivatives, containing an α-amino or α-ammonium group, and an aromatic side chain, as specific substrates of α-chymotrypsin[3-7] has created a demand for knowledge of the apparent ionization constants of the α-ammonium groups present in these compounds.[7] In addition such data were required for an evaluation of the influence of the aromatic nucleus and the adjacent carboxyl function upon the above ionization constants.

Among the pertinent data that were available at the time this study was initiated were the pK'_A values of the α-ammonium group of glycine, 9.72[8]; alanine, 9.72[8]; phenylalanine, 9.12,[9] 9.13,[8] 9.15[10]; *o*-, *m*- and *p*-fluorophenylalanine, 9.01,[9] 8.98[9] and 9.05[9]; *o*-, *m*- and *p*-chlorophenylalanine, 8.94,[10] 8.91[10] and 8.96[10]; *p*-sulfamylphenylalanine, 8.64[10,11]; tyrosine, 9.11[8,11,12]; tryptophan, 9.39[8]; glycine methyl ester, 7.66[8]; glycine ethyl ester, 7.73[8]; alanine methyl ester, 7.80[8]; leucine methyl ester, 7.63[8]; methyl α-amino-*n*-butyrate, 7.71[8]; tyrosinhydroxamide, 7.0[5,11]; glycinamide, 7.93[8]; tryptophanamide, 7.5[14]; and glycinhydrazide, 7.69.[11,15] In order to provide a more systematic set of data it was decided to examine a series of phenylalanine derivatives in which the nature

(1) Supported in part by a grant from the National Institutes of Health, Public Health Service.

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

(3) E. F. Jansen, M. D. Fellows-Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949); **185**, 209 (1950).

(4) E. F. Jansen, A. L. Curl and A. K. Balls, *ibid.*, **189**, 671 (1951).

(5) R. J. Foster, R. R. Jennings and C. Niemann, THIS JOURNAL, **76**, 3142 (1954).

(6) R. J. Foster and C. Niemann, *ibid.*, **77**, 1886 (1955).

(7) R. Lutwack, H. F. Mower and C. Niemann, *ibid.*, **79**, 2179, 5690 (1957).

(8) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," Reinhold Publ. Corp., New York, N. Y., 1943.

(9) E. L. Bennett and C. Niemann, THIS JOURNAL, **72**, 1804 (1950).

(10) J. C. Nevenzel, W. E. Shelberg and C. Niemann, *ibid.*, **71**, 3024 (1949).

(11) This value refers to the macroscopic ionization constant.

(12) In a recent communication Edsall, Martin and Hollingworth[13] report a value of 9.11 for the macroscopic ionization constant. They also give values for the microscopic constants relative to the ionization of the α-ammonium group for the case where the phenolic hydroxyl group is ionized, 9,70, and where it is not, 9.28.

(13) J. T. Edsall, R. B. Martin and B. R. Hollingworth, *Proc. Natl. Acad. Sci.*, **44**, 505 (1958).

(14) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

(15) C. R. Lindegren and C. Niemann, *ibid.*, **71**, 1504 (1949).

of the carboxyl function was varied so as to include a number of representative types. The compounds chosen for study were phenylalanine, its methyl ester, hydroxamide, amidoxime,[16] amide, thioamide[16] and hydrazide. All of the above compounds, present as their hydrochlorides in aqueous solutions at 25.0 ± 0.1° and 0.05, 0.10 or 0.20 *M* in sodium chloride, were titrated with aqueous sodium hydroxide using an automatic recording titrator.[17]

Fenwick[18] has assumed that a titration curve, *i.e.*, a plot of e.m.f. or *E*, *vs.* volume of added titrant, *V*, is the region of the equivalence point may be represented by equation 1 and the point of inflection, V_i coincident with the equivalence point

$$E = aV^3 + bV^2 + cV + d \qquad (1)$$

by equation 2. Thus, the solution of a set of simultaneous equations based upon equation 1

$$d^2E/dV^2 = 6aV_i + 2b = 0 \qquad (2)$$

and data obtained from the plot in the region of the equivalence point led to values of the coefficients *a* and *b* and by substitution of these values in equation 2 to a value of V_i.[18]

Since the titration curve for a monobasic acid also possesses an inflection point at the half-equivalence point, where $pH = pK'_A$, a procedure similar to that developed by Fenwick[18] may be used to evaluate pK'_A. In the case at hand a third degree orthogonal polynomial was fitted to the trace of the titration curve by the method of least squares using nine equidistant points, *i.e.*, $s = 0,1,2, \ldots 8$, along the *V*-axis so spaced as to encompass an extent of neutralization of *ca.* 70%.[19] In practice the orthogonal polynomials P_m, with coefficients C_m, given in equation 3 were developed from the table given by Milne[20] and

$$P_{0(s)} = 1$$
$$P_{1(s)} = 4 - (s)$$
$$P_{2(s)} = 28-21(s) + 3(s)(s-1)$$
$$P_{3(s)} = 14-21(s) + 7.5(s)(s-1) - 0.833(s)(s-1)(s-2) \qquad (3)$$

$$\tilde{Y} = C'_0 + C'_1(s) + C'_2(s)(s-1) + C'_3(s)(s-1)(s-2) \qquad (4)$$

transformed into equation 4 where $C'_0 = C_0 + 4C_1 + 28C_2 + 14C_3$, $C'_1 = -C_1 - 21C_2 - 21C_3$, $C'_2 = 3C_2 + 7.5C_3$ and $C'_3 = -0.833C_3$. Differentiation of equation 4 leads to equation 5 and to equation 6 for the condition $d^2\tilde{Y}/d\,s^2 = 0$.

$$d^2\tilde{Y}/ds^2 = 2C'_2 + 6C'_3(s-1) \qquad (5)$$

$$s = 1 - (C'_2/3C'_3) = 4 - 1.2\,(C_2/C_3) \qquad (6)$$

Since $C_m = c_m/S_m$, where $S_m = \sum_{s=0}^{8} [P_m(s)]^2$ and $c_m = \sum_{s=0}^{8} P_m(s) \cdot f(s)$, $s = 4 + 0.4286(c_2/c_3)$. The coefficients c_2 and c_3 are readily calculated from the tabulated values of P_m[20] and the observed values of *p*H, *i.e.*, f(*s*). The value of *p*H at the inflection point, pH_i, is then determined by interpolation using the value of *s* obtained as above. If by definition, $K'_A = (H^+)$ [base]/[acid], where parentheses and brackets denote activity and concentration, respectively; the total amount of salt initially added, [NaCl], $= n$; the total amount of acid, ΣR, $= r$; the concentration of added titrant in solution $= B$ and $w = B + [H^+] - [OH^-]$ and if (a) the system is chosen so that $n >> r >> [H^+] - [OH^-]$ and (b) the normality of the added titrant and the total volume of solution are sufficiently great so that $B =$ constant x V it follows that $dK'_A/dB \doteq 0$ and $B \doteq w$. For a monobasic acid, $HR^+ \rightleftarrows H^+ + R$, a treatment similar to that of Auerbach and Smolczyk[21] gives the exact relation $pK'_A = pH - \log (w/(r-w))$. Setting $d^2pH/dw^2 = 0$ gives $W_i = 0.5r$ or $pK'_A = pH_i$.[22]

All of the primary data, *i.e.*, the recorder traces, were evaluated as described above and the results obtained are summarized in Table I. The derivatives listed in this table are arranged in order of increasing acidity of the α-ammonium group, or decreasing basicity of the α-amino group.

Consideration of the pK'_A values of the α-ammonium group of glycine dipolar ion, 9.72,[8] and of phenylalanine dipolar ion, 9.15,[10] would lead one to anticipate lower pK'_A values for the α-ammonium group of carboxyl derivatives of a monoprotonated phenylalanine than those of the corresponding glycine derivatives, particularly since the pK'_A value of β-phenylethylammonium ion, 9.83,[23] is markedly lower than that of methylammonium ion, 10.64,[23] ethylammonium ion, 10.67,[23] or *n*-propylammonium ion, 10.58.[23,24] It will be seen from the data given in Table II that the expected behavior is observed wherever a comparison can be made. It also appears that the difference in the pK'_A values observed for the members of each pair is substantially independent of the nature of the carboxyl function present in a given pair. Thus, one may predict that the pK'_A value of the α-ammonium group in a protonated phenylalanine derivative containing this group will be 0.59 ± 0.04 of a *pK* unit lower than that of the corresponding glycine derivative.[25]

In an earlier communication[7] it was estimated, on the basis of a pK'_A value of 7.3 for the α-ammonium group of monoprotonated phenylalanine methyl ester, and the near equivalence of the macroscopic pK'_A values of the α-ammonium groups of phenylalanine and tyrosine, 9.15 and 9.11, that the pK'_A value of the α-ammonium group of monoproto-

(16) P. E. Peterson and C. Niemann, THIS JOURNAL, **79**, 1389 (1957).

(17) J. B. Neilands and M. D. Cannon, *Anal. Chem.*, **27**, 29 (1955).

(18) F. Fenwick, *Ind. Eng. Chem., Anal. Ed.*, **4**, 144 (1932).

(19) The use of 17 instead of 9 points led to a difference in pK'_A values of less than ±0.003 of a *pK* unit.

(20) W. E. Milne, "Numerical Calculus," Princeton University Press, Princeton, N. J., 1949, pp. 265–271, 375–381.

(21) F. Auerbach and E. Smolczyk, *Z. physik. Chem.*, **110**, 65 (1924).

(22) For a dibasic acid, $H_2R^+ \rightleftarrows H^+ + HR$; $HR \rightleftarrows H^+ + R^-$, it can be shown that $d^2\,pH/dw^2 = 0$ has solutions at $pH_i = (pK'_{A1} + pK'_{A2})/2$, $pK'_{A1} = pH_i - \log [(1 + 8K'_{A2}/h_i)/(1 - K'_{A2}/h_i)]$ and $pK'_{A2} = pH_i - \log [(1 - h_i/K'_{A1})/(1 + 8h_i/K'_{A1})]$ where $h = (H^+)$.

(23) H. C. Brown, D. H. McDaniel and O. Häfliger, in E. A. Braude and F. C. Nachod, "Determination of Organic Structures by Physical Methods," Academic Press, Inc., New York, N. Y., 1955.

(24) In the series $C_6H_5(CH_2)_nNH_3$ and for values of $n = 0$ to 5 the pK'_A values are 4.58, 9.37, 9.83, 10.20, 10.39 and 10.49, respectively,[23] *i.e.*, the influence of the phenyl group is still evident at $n = 5$.

(25) When it is recalled that the difference between the pK'_A value of methylammonium ion and that of β-phenylethylammonium ion is 0.81 of a *pK* unit it will be obvious that this prediction cannot be extrapolated to the extreme case where the carboxyl function is replaced by hydrogen.

TABLE I

APPARENT IONIZATION CONSTANTS OF A SERIES OF PHENYLALANINE DERIVATIVES[a]

Derivative	[NaCl], M	$pK'_{A(NH_3^+)}$ Separate values[b]	$pK'_{A(NH_3^+)}$ Average values[c]	$pK'_{A(NH_3^+)}$ Extrapolated values[d]
DL-Phenylalanine	0.05	9.18		
	.10	9.19		
	.20	9.17 ± .01[e]	9.18 ± 0.01	
DL-Phenylalaninamide	.05	7.30		
	.10	7.31		
	.20	7.35 ± .01[e]	7.33 ± .02	7.22 ± 0.01
DL-Phenylalaninthioamide	.05	7.23		
	.10	7.21		
	.20	7.27 ± .03[f]	7.26 ± .03	7.15 ± .02
DL-Phenylalaninamidoxime	.05	7.15		
	.10	7.16		
	.20	7.18 ± .01[e]	7.17 ± .02[m]	7.06 ± .01[m]
DL-Phenylalaninhydrazide	.05	7.12 ± .02[g]		
	.10	7.15 ± .01[g]		
	.20	7.19 ± .02[h]	7.16 ± .04	7.06 ± .01
DL-Phenylalanine methyl ester	.05	7.06 ± .01[g]		
	.10	7.06 ± .03[g]		
	.20	7.14 ± .03[i]	7.11 ± .04	7.00 ± .03
DL-Phenylalaninhydroxamide[j,k,l]	.05	6.83 ± .01[g]		
	.10	6.88 ± .02[g]		
	.20	6.92 ± .04[h]	6.89 ± .04[m]	6.78 ± .03[m]

[a] In aqueous solutions at 25.0 ± 0.1°. [b] Value may be that of a single determination or the mean of several, in which case the standard deviation is given. [c] Mean of all determinations from 0.05 to 0.20 M NaCl. [d] Value for $\mu = 0$, *i.e.*, $pK_A^0{}_{NH_3^+}$, based upon the simple Debye–Hückel relation and an assumed effective collision diameter of 6 Å.[8] [e] Mean of three determinations. [f] Mean of four determinations. [g] Mean of two determinations. [h] Mean of five determinations. [i] Mean of seven determinations. [j] All values corrected for overlap of ionization of ammonium and hydroxamide groups.[22] [k] pK'_A values for ionization of hydroxamide group: 0.05 M NaCl, 9.14; 0.10 M NaCl, 9.12; 0.20 M NaCl, 9.10 ± 0.01[e]; extrapolated value,[d] 9.22 ± 0.01. [l] Evaluation of primary data by method of J. C. Speakman (*J. Chem. Soc.*, 855 (1940)) with $f_0 = f_1 = f_2 = 1$ to obtain pK'_A values gave $pK'_{A1} = 6.82 \pm 0.03^d$ and $pK'_{A2} = 9.10$. [m] Value given refers to the macroscopic ionization constant.

TABLE II

APPARENT IONIZATION CONSTANTS OF FOUR DERIVATIVES OF GLYCINE AND OF PHENYLALANINE[a]

Derivative	$pK'_{A(NH_3^+)}$ Glycine	$pK'_{A(NH_3^+)}$ Phenylalanine	δ
Acid	9.72	9.15	−0.57
Amide	7.93	7.30	− .63
Hydrazide	7.69	7.12	− .57
Methyl ester	7.66	7.06	− .60

[a] In aqueous solutions at 25.0 ± 0.1° and 0.05 M in sodium chloride.

nated tyrosine methyl ester was *ca.* 7.3. With the downward revision of the pK'_A value of the α-ammonium group of phenylalanine methyl ester the question arises as to the necessity of revising the earlier estimate of the pK'_A value of the analogous tyrosine derivative, bearing in mind that the principal concern with respect to the magnitude of this constant arose from an inquiry as to the relative abundance of the various species that were of importance in systems whose pH varied from *ca.* 6.0 to 7.5.[7] Since these latter conditions limit our interest to the effect of the un-ionized hydroxyl group upon the ionization constant of the α-ammonium group we may compare the value of 9.28, *i.e.*, the microscopic ionization constant of the α-ammonium group in the dipolar ion containing a carboxylate group and an un-ionized phenolic hydroxyl group,[13] with that of the analogous glycine derivative, *i.e.*, 9.72,[8] to arrive at a difference of 0.44 of a pK unit.[26] This latter value can be used to extrapolate the pK'_A value of the α-ammonium group in monoprotonated glycine methyl ester, 7.66, to obtain a value of 7.22 for the tyrosine derivative of interest. The essential agreement of this latter value with that assumed previously[7] does not require significant modification of the argument presented earlier[7] relative to the interpretation of the pH–activity relationship observed with the system α-chymotrypsin–L-tyrosine ethyl ester.

In the preceding discussion our concern has been with the dependence of the pK_A value of the α-ammonium group upon the nature of the α-amino acid side chain. When attention is directed to the dependence of the above constant upon the nature of the carboxyl function it will be seen from the data summarized in Table I that while there is a substantial increase in the acidity of the α-ammonium group when $-CO_2^-$ is replaced by $-CONH_2$, *i.e.*, 1.93 pK_A units, the replacement of the latter function by any one of five other functional derivatives of the carboxyl group results in the extreme case in a further increase of only 0.44 of a pK_A unit. The order of effectiveness of the various carboxyl functions in increasing the acidity of the α-ammonium group, *i.e.*, $-CONOH > -CO_2CH_3 \geq -CONHNH_2 \doteq -C(NOH)NH_2 > -CSNH_2 > -CONH_2 >> -CO_2^-$, is not the one expected if the only factor were the electron deficiency arising from the polarization of the carboxyl carbon

(26) The lesser magnitude of this value relative to that obtaining for the glycine–phenylalanine pair, *i.e.*, 0.59 ± 0.04 implies that replacement of p-hydrogen by a phenolic hydroxyl group causes a decrease in the acid strength of the α-ammonium group, a result which would be expected.[28]

atom. However, it must be noted that with both the hydroxamide and amidoxime the pK_A values refer to macroscopic ionization constants and with the hydroxamide, hydrazide and amidoxime the possibility of intramolecular hydrogen bonding, leading to an increase in the acidity of the α-ammonium group, cannot be excluded. Therefore, if consideration is limited to those derivatives where the above factors cannot intrude, the order observed, with respect to effectiveness in increasing the acidity of the α-ammonium group, *i.e.*, $-CO_2CH_3 > -CSNH_2 > -CONH_2 >> -CO_2^-$, is that expected on the basis of an inductive effect arising from a decreasing electron deficiency at the carboxyl carbon atom.

Since there is relatively little information available with respect to the infrared spectra of carboxyl derivatives of the α-amino acids the spectra of the seven compounds listed in Table I were determined with a sodium chloride prism for the solid in solid potassium bromide. The results are summarized in the Experimental section which follows. As an unsubstituted benzyl group was present in all of the compounds examined it is possible that the maxima observed at 3040 ± 20 (6/7),[27] 2937 ± 30 (6/7), 1615 ± 12 (7/7), 1500 ± 4 (7/7), 1454 ± 7 (7/7), 1155 ± 20 (5/7), 1074 ± 5 (7/7), 1024 ± 9 (7/7), 745 ± 10 (7/7) and 699 ± 2 (7/7) cm.$^{-1}$ are a consequence of the presence of this group.[28] Furthermore, the maxima at 1283 ± 11 (6/7) may be associated with the fact that all of the compounds were α-amino acid derivatives.[28] Finally, it should be noted that the spectra observed for DL-phenylalanine are in substantial agreement with those reported by Wright[29] and thus can serve as a point of reference for the other spectra whose interpretation at the present time is premature because of the absence of information with respect to the spectral behavior of analogous compounds lacking the α-amino group and/or the aromatic side chain.

Experimental[30,31]

DL-Phenylalanine.—A preparation of synthetic DL-phenylalanine (Dow) was recrystallized twice from water and dried *in vacuo* over phosphorus pentoxide.

DL-Phenylalanine Methyl Ester.—Esterification of DL-phenylalanine with methanol and thionyl chloride[32] gave the methyl ester hydrochloride, m.p. 158–159°, after recrystallization from a 3:10 mixture of methanol and ethyl ether; lit.[33] m.p. 158°. The hydrochloride *per se* was used in the determination of the pK'_A value. However, an ethereal solution of the base was prepared, by reaction of an ethereal suspension of the hydrochloride with aqueous sodium bicarbonate, and after the solution had been dried over magnesium sulfate a portion was mixed with potassium bromide and the mixture dried prior to its use for the determination of the infrared spectra.

DL-Phenylalaninamide.—Ammonolysis of an ethereal solution of 3.6 g. of DL-phenylalanine methyl ester, prepared from the hydrochloride by reaction with aqueous sodium bicarbonate, gave 2.3 g. of the amide, m.p. 138–140°, after recrystallization from chloroform; lit.[34] m.p. 138–139°.

DL-Phenylalaninthioamide, m.p. 135–136.3°, was prepared by Peterson and Niemann.[16]

DL-Phenylalaninhydrazide.—A mixture of 1.8 g. of DL-phenylalanine methyl ester and 1 g. of anhydrous hydrazine in 25 ml. of absolute ethanol was heated under refluxing conditions to give 1.1 g. of the hydrazide, m.p. 87.5–89.0°. Recrystallization of this product from a mixture of absolute ethanol and hexane gave the hydrazide, m.p. 88.5–90.0°.

Anal. Calcd. for $C_9H_{13}ON_3$ (179): C, 60.3; H, 7.3; N, 23.5. Found: C, 60.5; H, 7.4; N, 23.4.

DL-Phenylalaninamidoxime (α-amino-β-phenylpropioamidoxime), m.p. 117.5–118.5°, was prepared by Peterson and Niemann.[16]

DL-Phenylalaninhydroxamide.—DL-Phenylalanine methyl ester hydrochloride, 10.8 g., was converted into the hydroxamide as directed by Cunningham, *et al.*,[35] the crude product recrystallized from water and dried *in vacuo* over phosphorus pentoxide, at 56° to give *ca.* 4 g. of the hydroxamide, m.p. 180–181.2°, lit.[35] m.p. 180° dec.

Potentiometric Determination of Apparent Ionization Constants.—All measurements were made with a difunctional recording titrator designed and built by M. D. Cannon, International Instruments Co., Canyon, Calif. The instrument is a modification of that described by Neilands and Cannon[17] and contains the elements essential for either constant or variable pH titrations. In the titrations described herein the instrument was calibrated and set for variable pH titration. When used in this manner the synchronous motor, operated at 6 r.p.m., delivered the titrating fluid at the rate of 30×10^{-3} ml./min. The base employed in these titrations was of high enough concentration as to cause a negligible change in the volume of the solution during the titration. With 0.8534 *N* NaOH the delivery rate corresponds to 25.63 microequivalents per min. The base was delivered to the solution through a Dewitt and Herz Inc. stainless steel hypodermic needle with the tip bent up to limit diffusion of the base into the solution. The cell was of 25-ml. capacity and was enclosed in a thermostated water jacket in order to maintain the temperature at 25.0 ± 0.1°. The solution was vigorously stirred and a CO_2-free atmosphere was maintained by "sweeping" the solution with nitrogen. The pH of the solution was measured using a Beckman no. 4990–29 glass electrode and no. 4970–29 calomel reference electrode in conjunction with a Leeds and Northrup model 7664–41 pH meter. The meter output was coupled with a Leeds and Northrup Speedomax type G recorder to obtain a titration curve directly. In all cases the electrodes were standardized with buffer solution at pH 4, 7 and 10 before and after the titration and correction made for the non-linearity of this calibration. In all cases standard stock solutions were prepared in CO_2-free water as was the base and acid. Ten ml. aliquots of stock solutions of the amino acid derivatives containing approximately 75 microequivalents per 10 ml. were pipetted into the reaction cell, and 10 ml. of the various salt solutions (0.4, 0.2, and 0.1 *M*) were added. One or two ml. of 0.0498 *N* hydrochloric acid was pipetted into the solution and the now acid solution titrated as indicated above. Further experimental details are given in Table I.

Infrared Spectra.—The infrared spectra of the seven compounds listed in Table I were determined with a Perkin–Elmer model 21 spectrophotometer, equipped with a sodium chloride prism, for the solid in solid potassium bromide and were as follows: hydroxamide, 3185(m), 3040(s), 2882(s), 2604(m), 2137(w), 1647(m), 1616(s), 1550(m), 1497(w), 1460(m), 1379(s), 1335(w), 1290(s), 1199(w), 1167(m), 1071(w), 1033(w), 1001(w), 963(w), 912(m), 887(m), 860(w), 771(w), 755(m), 733(w), 697(s), 682(w); methyl ester, 3390(w), 3021(w), 2950(m), 1742(s), 1608(w), 1590(w), 1499(m), 1456(m), 1439(s), 1374(w), 1272(m), 1196(s), 1168(s), 1099(w), 1073(w), 1028(w), 1005(m), 870(w), 835(m), 812(w), 744(s), 701(s); hydrazide, 3344(m), 3289(m), 2994(w), 2915(w), 1678(s), 1656(s), 1618(s), 1515(s), 1497(m), 1458(w), 1443(w), 1397(w), 1282(w), 1071(w), 1029(w), 977(w), 943(w), 925(m), 901(m), 882(w), 753(m), 700(s); amidoxime, 3425(s), 3311(s), 3165(s), 3106(m),

(27) Fraction of compounds examined possessing the specified absorption.

(28) L. J. Bellamy, "Infrared-red Spectra of Complex Molecules," Methuen and Co., London, 1954.

(29) N. Wright, *J. Biol. Chem.*, **127**, 137 (1939).

(30) All melting points are corrected.

(31) Microanalyses by Dr. A. Elek.

(32) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1109 (1953).

(33) "Beilstein," 4th edition, Vol. 14, System 1905, p. 499.

(34) Reference 33, 2nd supplement, p. 606.

(35) K. G. Cunningham, G. T. Newbold, F. S. Spring and J. Stark, *J. Chem. Soc.*, 2091 (1949).

3049(m), 2933(m), 2770(m), 1667(s), 1603(s), 1497(m), 1456(w), 1403(w), 1175(w), 1073(w), 1028(w), 977(w), 912(s), 850(w), 827(w), 748(m), 698(s); thioamide, 3311(s), 3279(s), 3021(m), 2907(m), 2817(m), 1692(w), 1681(w), 1664-(w), 1647(m), 1634(m), 1603(m), 1582(m), 1536(w), 1497-(m), 1464(s), 1458(s), 1362(w), 1311(w), 1272(w), 1205-(w), 1068(w), 1032(w), 1015(w), 987(w), 934(m), 885(m), 754(s), 735(m), 698(s), 676(w), 672(w); amide, 3311(s), 3058(s), 2950(m), 2817(w), 1675(s), 1613(s), 1595(s), 1499-(m), 1451(m), 1416(s), 1361(w), 1335(m), 1302(w), 1284-(w), 1214(w), 1134(m), 1117(m), 1079(m), 1029(w), 1000-(s), 953(s), 928(w), 903(m), 873(w), 849(w), 779(s), 766-(w), 734(s), 700(s); acid, 3448(w), 3040(m), 2967(m), 2710-(m), 2525(m), 2151(m), 1626(s), 1587(s), 1513(s), 1504(s), 1447(m), 1414(s), 1340(m), 1309(s), 1294(m), 1208(w), 1155(w), 1129(w), 1071(w), 1032(w), 984(w), 912(w), 852-(m), 775(w), 745(m), 697(s), 677(w), with all values in cm.$^{-1}$ and with the intensity indicated as strong (s), medium (m) and weak (w).

PASADENA, CALIF.

PROPOSITIONS

1. Florence Fenwick (1) assumed that a titration curve (E.M.F. or pH vs. volume of base added, V) has the form $E = aV^3 + bV^2 + cV + d$ about the end point, thus $\frac{d^2E}{dV^2} = 6aV + 2b = 0$ at the inflection point. The solution of a set of simultaneous equations taken from the curve gives values for a and b from which the end point can be calculated. I propose that this method be applied to the determination of pK'_a values from titration curves (especially from automatic titrations). Use of the orthogonal polynomial rapidly gives "least squares" values for a and b (2).

It is convenient to choose the total concentration of acid (or base) being investigated, r, such that it is negligible compared to the total salt (NaCl) concentration, n, and much greater than the difference $[H]^+ - [OH^-]$ (3). For $K'_a = (H^+)\,[B]\,/[A]$, and $w-b+[H^+]-[OH^-]$ it then follows that $\frac{dK'_a}{dV} \doteq 0$ and $b \doteq w$ (4).

For a monobasic acid ($HR^+ \rightarrow H^+ + R$) we then obtain $pK'_a = pH - \log(w/r-w)$. Setting $\frac{d^2pH}{dw^2} = 0$ gives w_i (inflection) $= \frac{1}{2}r$ or $pK'_a = pH_i$.

For a dibasic acid ($H_2R^+ \rightarrow H^+ + HR$; $HR \rightarrow H^+ + R^-$)

$$pK'_1 = pH - \log\left(\frac{w}{r - w + 2rK'_2/h - wK'_2/h}\right)$$, where $h = (H^+)$, and

$$pK'_2 = pH - \log\left(\frac{w - r + wh/K'_1}{2r - w}\right)$$. Setting $\frac{d^2pH}{dw^2} = 0$ gives solutions at

$$w_i = \frac{1}{2}r\left(\frac{1 + 8K'_2/h}{1 + 2K'_2/h}\right), \quad w_i = \frac{3r}{2}\left(\frac{1}{1 + 2h/K'_1}\right) \quad \text{and} \quad w_i \doteq r$$

from which $pK'_1 = pH_i - \log\left(\frac{1 + 8K'_2/h_i}{1 - K'_2/h_i}\right)$, $pH_i = \frac{pK'_1 + pK'_2}{2}$,

and $pK'_2 = pH_i - \log\left(\frac{1 - h_i/K'_1}{1 + 8h_i/K'_1}\right)$. $pK'_1 = pH_i$ and $pK'_2 = pH_i$

$\pm$ .005 pK units if $K'_1 > 783\ K'_2$.

Although this method is restricted to systems where $[NaCl] >> r >> [H^+] - [OH^-]$ and which give graphically measurable inflections (Auerbach and Smolczyk (3) state that the curve will have an inflection between pK_1 and pK_2 if $K_1 > 16K_2$) it still enjoys the advantages of being independent of volumetric errors, acid and base standardization errors, and inert impurities; and pK'_a values can be easily calculated with a least squares fit of the data.

References

1. F. Fenwick, Ind. & Eng. Chem. Anal. Edn., 4, 144 (1932).

2. Part I, this thesis.

3. ( ) and [ ] denote activities and concentrations respectively. H. T. S. Britton, "Hydrogen Ions," 4th ed., Chapman & Hall Ltd., London, Vol. I, pg. 317 (1955).

4. Here b is the total concentration of added base. The normality of the base added and the total volume of the solution should be large enough to make the volume change during the titration negligible ($b \doteq$ const. x V).

5. F. Auerbach and E. Smolczyk, Z. physikal. Chem., 110, 76 (1924).

2. Triphenyl phosphine has been used to reduce styrene oxide to styrene (1), $\phi_3P + \phi CH\overset{O}{\diagup\diagdown}CH_2 \longrightarrow \phi_3P=O + \phi CH=CH_2$. Hey and Ingold (2) reported that quaternary phosphonium alkoxides thermally decompose to an alkane and tertiary phosphine oxide, $R_4POR' \xrightarrow{\Delta} R_3PO + R\text{-}R'$ indicating that the C-C bond of $>C\overset{O}{\diagup\diagdown}C<$ is not necessary for the reduction. I, therefore, propose that formation of cyclic alkanes should be attempted by reacting triphenyl phosphine with the cyclic ether: $\phi_3P + \underbrace{[O\text{-}[CH_2]_n]} \longrightarrow \phi_3PO + \underbrace{[[CH_2]_n]}$. If no reaction occurs more favorable conditions can be obtained by using:

a) a cyclic ether with an electron withdrawing group on the α-carbon,

b) trialkyl phosphine instead of triphenyl phosphine,

c) episulfides instead of epoxides, ($R_2C\overset{S}{\diagup\diagdown}CR_2$ reacts under milder conditions than does $R_2C\overset{O}{\diagup\diagdown}CR_2$, (3)),

d) phosphine-alkenes instead of triphenyl phosphine (review by Wittig (4)). In the last modification the ring size would not decrease $R_3P=CR'_2 + \underbrace{[O\,[CH_2]_n]} \longrightarrow R_3PO + \underbrace{[[CH_2]_n\text{—}CR'_2]}$.

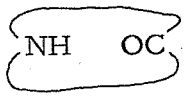
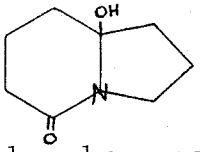
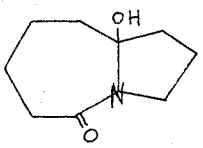
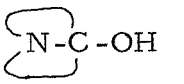
References

1. G. Wittig and W. Haag, Chem. Ber., 88, 1654 (1955).

2. L. Hey and C. K. Ingold, J. Chem. Soc., 531 (1933).

3. C. C. J. Calvenor, W. Davies, and N. S. Heath, ibid., 282 (1949).

4. G. Wittig, Angew. Chem., 68, 505 (1956).

3. Recently several investigators (1-4) have proposed transannular interactions of the cyclol type,  , for cyclic peptides and lactams with more than one peptide linkage. Cohen and Witkop (5) have synthesized "cyclol linkages":  and  . Rydon and Smith (6, 7) have developed a procedure, modified by Reindol and Hoppe (8), for detecting peptides and acylated and some non-acylated amino acids involving a) chlorination (on a chromatogram) and b) treatment with KI plus starch (6), or KI plus o-tolidine or benzidine (7, 8) to give a purple or blue spot. I propose that the number of cyclol linkages could be measured by chlorinating the compound in question and determining the amount of chlorine taken up by the compound by a) quantitative elemental analysis or b) a colorimetric determination based on the above mentioned color reagents. The Cl_2 will react with the -CO-NH- groups but would not be expected to react with the  linkages. Compounds such as ergotamine (4) and those of Cohen and Witkop (5) (see above) should be used as "blanks" to test for rupture of the cyclol linkage under the reaction conditions.

References

1. D. Wrinch, Nature, 179, 536 (1957); 180, 502 (1957).

2. N. I. Gavrilov, et al., Zhur. obshchei Khimii, 24, 563, 1064 (1955); 26, 1779, 2029 (1956).

3. P. H. Hermans, Nature, 177, 127 (1956).

4. A. Stoll, A. Hofmann, and T. Petrzilka, Helv. Chim. Acta, 34, 1544 (1951).

5. L. A. Cohen, B. Witkop, J. Am. Chem. Soc., 77, 6595 (1955).

6. H. N. Rydon and P. W. C. Smith, Nature, 169, 922 (1952).

7. P. W. C. Smith, J. Chem. Soc., 3985 (1957).

8. F. Reindel and W. Hoppe, Chem. Ber., 87, 1103 (1954).

4. A cyclic amino acid derivative with the asymmetric α-carbon in the ring, D-1-keto-3-carbomethoxy-1, 2, 3, 4-tetrahydroisoquinoline, has been shown (1) to be a relatively fast substrate of α-chymotrypsin. α-Methyl-α-acylamino acids, however, are inhibitors or very slow substrates of this enzyme (2). I, therefore, propose that these features be combined in the form of 1-carboalkoxy-1-acylaminocycloalkanes, cyclopropane through cyclohexane, and tested as substrates and inhibitors of α-chymotrypsin. It would be interesting to know whether these cyclic amino acid derivatives would be hydrolyzed in the presence of α-chymotrypsin as with the isoquinoline derivative (1) or whether the α-alkyl group would prohibit hydrolysis even though "tied back" in a ring.

Follow-up experiments could include the corresponding 4, 5-benzo and the 3-phenyl derivatives of these 1-carboalkoxy-1-acylaminocycloalkanes, i.e., analogues of phenylalanine.

References

1. G. E. Hein, R. B. McGriff and C. Niemann, J. Am. Chem. Soc., 82, 1830 (1960).

2. Part III, this thesis.

5. Some correlations have been made between infrared N-H stretching frequencies and pK_a' values for some amines, e.g., A. Bryson (1) correlated the N-H stretch frequencies in $CHCl_3$ of some substituted anilines with their pK_a' values. However, no such correlations could be found between the pK_a' values and the IR spectra (measured in KBr pellets) for the D,L-phenylalanine derivatives studied in Part IV of this thesis.

I propose the pK_a' values of these amino acid derivatives should be compared with their IR spectra in aqueous solution. The N-H stretching frequencies will probably be obscured by the water absorption, but other absorption bands of the amino group should be observable.

The IR spectra of some amino acids in water have been determined (2, 3) using AgCl and BaF_2 cells; however, the polyethylene bags used by Robinson (4) should be more convenient. The trouble in obtaining a uniform sample thickness due to the flexibility of the thin (.002 in.) polyethylene film used for the bags (4) might be overcome by stretching the film over a frame and holding it in with retention rings (see figure 1).

References

1. A. Bryson, J. Am. Chem. Soc., 82, 4858 (1960).

2. R. S. Gore, R. B. Barnes and E. Peterson, Anal. Chem., 21, 382 (1947).

3. F. S. Parker and D. M. Kirschenbaum, Nature, 187, 386 (1960).

4. T. Robinson, ibid., 184 (1959).

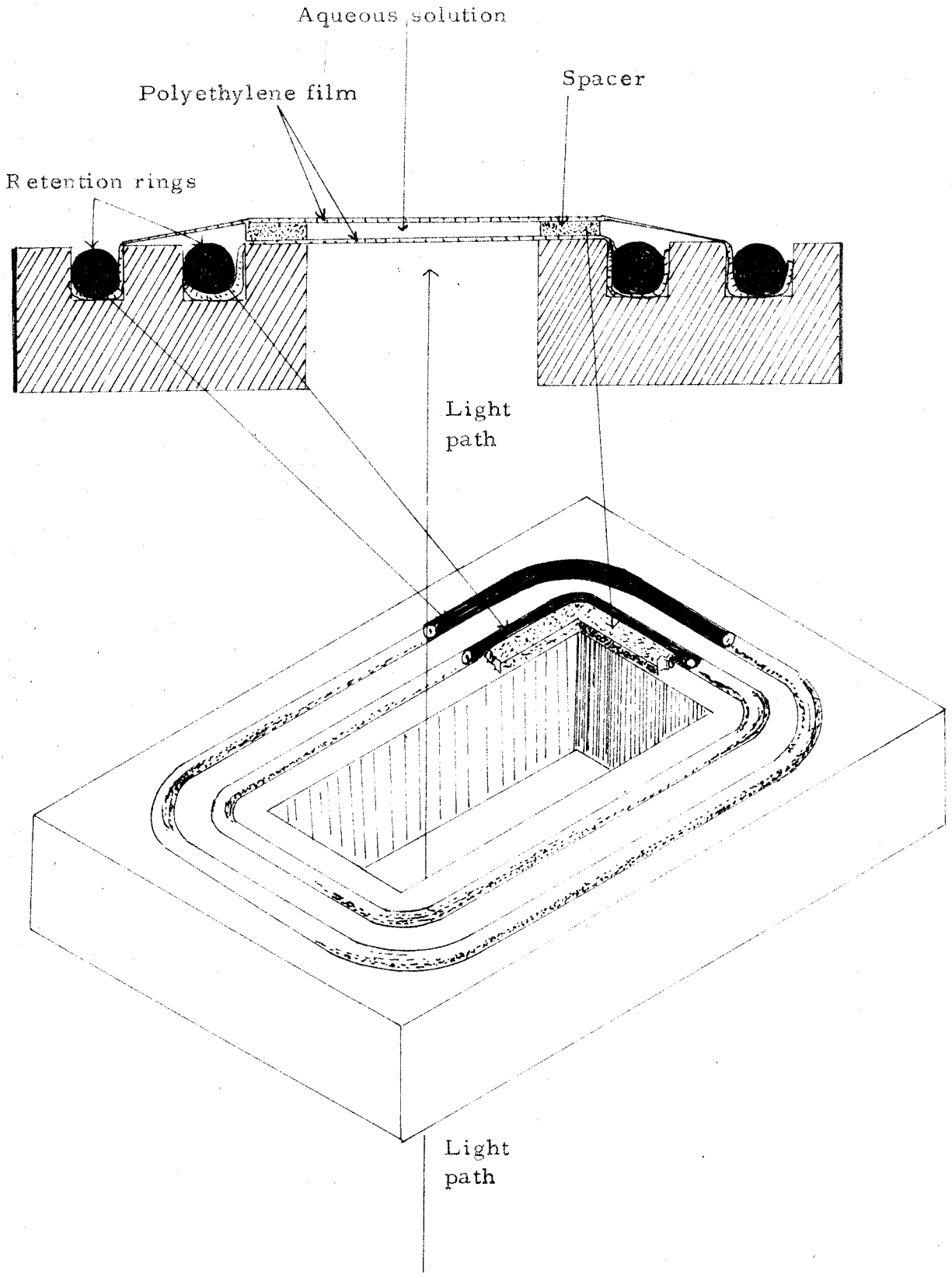


Figure 1. Cross section and isometric perspective of holder for polyethylene film (not drawn to scale).

6. Indole has been shown (1) to inhibit the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide, a trifunctional substrate, in a "competitive" manner, equations 1 and 2.

$$E + S \rightleftharpoons ES \xrightarrow{k_1} E + P \ ; \tag{1}$$

$$E + I \rightleftharpoons EI \tag{2}$$

However, the hydrolysis of methyl hippurate (1, 2), a difunctional substrate, was inhibited in a "mixed" manner, i.e., equation 3 had to be considered.

$$\begin{matrix} ES + I \\ \rightleftharpoons \\ EI + S \end{matrix} ESI \xrightarrow{k_2} E + I + P \tag{3}$$

The system indole-methyl aceturate (and/or ethyl aceturate) - α-chymotrypsin should be very interesting to study. The small size of this substrate may allow the indole to complex more independently, i.e., less "competitively," than it does with methyl hippurate. For the limiting case of a completely independent inhibitor the velocity is independent of the "inhibitor" concentration. However, the methyl and ethyl aceturates apparently form an ES_2 complex (3, 4), equation 4

$$ES + S \rightleftharpoons ES_2 \xrightarrow{k_3} E + P \tag{4}$$

($k_3 = 0$ for ethyl aceturate). If the above prediction is true, indole would be expected to affect the formation of ES very little, i.e., $k_1 \doteq k_2$, but the formation of ES_2 would be "competitively" inhibited, i.e., reaction 3 would compete with reaction 4.

An alternative possibility is that the indole would act as the second substrate molecule activating the hydrolysis, i.e., $k_2 > k_1$. However, this situation seems less likely in light of the work with methyl hippurate, $k_2 \doteq \frac{1}{2}k_1$ (1, 2).

References

1. H. T. Huang and C. Niemann, J. Am. Chem. Soc., 75, 1395 (1953).

2. T. H. Applewhite, R. B. Martin and C. Niemann, ibid., 80, 1457 (1958).

3. J. P. Wolf, III and C. Niemann, ibid., 81, 1012 (1959).

4. J. P. Wolf, III, Ph. D. thesis, California Institute of Technology, 1959, Part II.

7. With high α-chymotrypsin concentrations L-tyrosyl-L-tyrosinamide, TTA, is hydrolyzed to L-tyrosinamide, TA, and L-tyrosyl-L-tyrosine, TT, (ca. 2:1) but L-tyrosine, T, is formed much more slowly (1). Blau and Waley (1) also observed that TA is hydrolyzed more slowly to T with formation of TT and that this TT concentration eventually surpasses that of T. These authors (1) postulated equations 1 to 5 to explain these observations (A = ammonia). Equation 3 is used to

$$TTA \longrightarrow T + TA \quad (1)$$

$$TTA \longrightarrow TT + A \quad (2)$$

$$TTA + T \longrightarrow TT + TA \quad (3)$$

$$TA \longrightarrow T + A \quad (4)$$

$$2TA \longrightarrow TTA + A \quad (5)$$

rationalize the formation of "extra" amounts of TT. However, the direct incorporation of tyrosine seems unlikely since it has been shown (2-4) that tyrosine esters and amides are incorporated into peptides much more rapidly. One alternative to reaction 3 would be equation 6 (5).

$$TTA + TA \longrightarrow TTTA + A \longrightarrow TT + TA + A \quad (6)$$

Incubation of TTA and labeled tyrosine, T*, and also TA and T* with α-chymotrypsin and separation of the reaction products could decide between these alternatives (equation 3 or 6). If only the T fraction has activity, reaction 3 can be excluded.

If the TT fraction becomes labeled, peptide end group analysis to decide which T residue is labeled (T*T or TT*) would further

elucidate the mechanism of this transpeptidation. An experiment using T*TA would be interesting in this respect in that the T* moiety should be found in the TT fraction.

References

1. K. Blau and S. G. Waley, Biochem. J., 57, 538 (1954).

2. J. Horowitz and F. Haurowitz, Biochem. Biophys. Acta, 33, 231 (1959).

3. J. Horowitz and F. Haurowitz, J. Am. Chem. Soc., 77, 3138 (1955).

4. J. T. Kaganova and V. N. Orehovich, Doklady Akad. Navk. S.S.S.R., 95, 1259 (1954); Chem. Abstr., 48, 10078 i (1954).

5. Part II, this thesis.

8. The "mechanism" of detoxication is known (1) for many aniline derivatives containing an acidic function, e.g., p-aminobenzoic acid is excreted as p-acetamidobenzoic acid, p-aminohippuric acid and p-aminobenzoylglucuronide (2, 3).

Landsteiner (4) and co-workers have shown that aniline derivatives, when diazotized and coupled to a native protein, e.g., casein, form "azoproteins" which act as antigens, and that the original aniline derivatives act as simple haptens. Thus, p-aminobenzoic acid can be used to form an azoprotein which can be used to immunize an animal, e.g., a rabbit. The antiserum from this animal will form a precipitate with the azoprotein, but this precipitate will not form in the presence of the hapten, p-aminobenzoic acid.

The interplay between these two types of defense mechanisms of the body could be investigated by: (a) injecting p-aminobenzoic acid into rabbits and determining the amounts of the detoxication products; (b) immunizing these animals against an azoprotein formed from p-aminobenzoic acid; and (c) again injecting p-aminobenzoic acid and determining the detoxication products and the activity of the serum from these rabbits. These experiments would show whether or not: (a) the antiserum in vivo can be made ineffective against precipitation with antigen, and (b) the immunized animal has an altered detoxication mechanism.

Further information might be gained by using labeled p-aminobenzoic acid and sacrificing some of the animals at various times to

check for localization of the reactants.

References

1. R. T. Williams, "Detoxication Mechanisms," 2nd ed., John Wiley and Sons, Inc., N. Y., 1959.

2. Ref. 1, pp. 449-450.

3. P. R. Venkataraman, A. Venkataraman and H. B. Lewis, Arch. Biochem., 26, 173 (1950).

4. K. Landsteiner, "The Specificity of Serological Reactions," Harvard Univ. Press, Cambridge, Mass., 1945.

9. Current investigations (1) have shown that α-chymotrypsin can exhibit a stereospecific preference for the ester moiety of N-acylglycine sec-butyl ester, i.e., the racemic ester is almost 50% hydrolyzed at one rate and then the remaining ester is hydrolyzed at a slower rate. It would, therefore, be interesting to study the steric requirements of this second site of antipodal specificity of α-chymotrypsin.

Comparing the hydrolysis rates of a series of peptides such as the N-acetyl amides of L-tyrosyl-D,L or D- and L-alanine, tyrosylleucine, tyrosyltyrosine, etc., would be very informative in this respect. However, amides are relatively slow substrates and their hydrolysis cannot be followed accurately in the pH stat (2). Therefore, I further propose that the α-hydroxy analogues of the second amino acid moiety of these peptides, e.g., $CH_3CO\text{-}NHCH(CH_2C_6H_5OH)CO\text{-}OCHRCONH_2$, would be more convenient to study, being faster substrates with their hydrolyses easily followed in the pH stat.

References

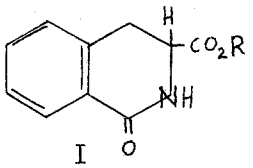
1. Current investigations of Drs. G. E. Hein and R. Wallace.

2. A. N. Kurtz, Ph. D. Thesis, California Institute of Technology, 1960, Part IV.

10. The reversal of antipodal specificity observed (1) for the α-chymotrypsin-catalyzed hydrolysis of 1-keto-3-carbomethoxy-1, 2, 3, 4-tetrahydroisoquinoline, I, may be a result of the rigid configuration imposed on the heterocyclic ring by the benzo and amide groups. On this basis, structures II and III should also exhibit this reversal of antipodal specificity. However, the saturated lactams, IV and V, may be "flexible" enough to act as "normal" substrates of α-chymotrypsin.

The N-acetyl ester of leucine-3-ene, VII, would have some of the structural rigidity of I, but it would have many more degrees of freedom.

The decahydroisoquinoline derivatives, VI, would be very interesting to study in this respect and also to establish some of the steric factors involved. Unfortunately, the separation of and assignment of configuration to the eight possible isomers of VI would be a difficult task.



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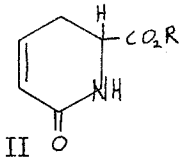

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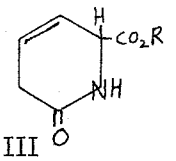

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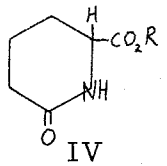

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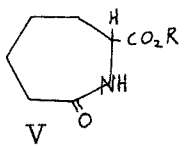

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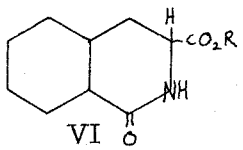

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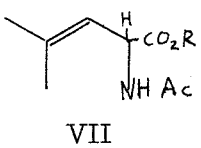

VII

Reference

1. G. E. Hein, R. B. McGriff and C. Niemann, J. Am. Chem. Soc., 82, 1830 (1960).