I. The Alpha-Chymotrypsin-Catalyzed and Base-Catalyzed Hydrolysis of Some Substituted Glycine Esters and Related Compounds.

II. The Nature and Properties of the Enzyme-Substrate Complex Produced Under Conditions of Very High Substrate Concentrations.

III. A Hypothesis Concerning Alpha-Chymotrypsin-Substrate Complexes.

Thesis by

John P. Wolf III

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Abstract

A series of substituted glycine esters have been evaluated as substrates for α-chymotrypsin. The second order base-catalyzed hydrolysis constants for these compounds have also been determined.

Four substrates for α-chymotrypsin have been found to exhibit unusual kinetic behavior at very high concentrations in the presence of α-chymotrypsin. It has been shown that these data can be the result of two molecules of substrate combining with each enzyme active site.

A hypothesis concerning the enzyme-substrate complex is offered which helps to correlate the data given in this work.

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Introduction

The work contained in this thesis was originally undertaken to determine whether or not the velocity of the α-chymotrypsin-catalyzed hydrolysis of an α-acylamido ester was a function of the acid strength of the acid corresponding to the acylating group. At the same time, it was anticipated that by varying the size and nature of the acylamido moiety over very wide ranges one could appraise, at least qualitatively, the steric and electronic requirements of the active site of the enzyme. With this kind of information one might gain an insight as to the nature of the enzyme-substrate complex.

Part I

THE ALPHA-CHYMOTRYPSIN-CATALYZED AND BASE-CATALYZED HYDROLYSIS OF SOME SUBSTITUTED GLYCINE ESTERS AND RELATED COMPOUNDS.

Catalysis by α-Chymotrypsin

Since the apparatus and techniques used in this study have been extensively described and discussed in the literature (1, 2) only a brief description will be given here. All kinetic experiments were conducted in a pH stat (1). This is a machine which adds titrant to a reacting system such that a constant pH is maintained throughout the course of the reaction. The amount of titrant added is plotted as a function of time on an automatic recorder and this trace is the primary datum from such a machine.

For a system wherein acidic products are formed with time and base is used as the titrant in the pH stat and the system is constrained to remain at a pH greater by at least two pH units than the pK_a of any of the reaction products the tangent to the trace so obtained is immediately dP/dt. For the cases at hand which obey Michaelis-Menten kinetics (3, 4) one may, by using such data, evaluate those constants which when used with the appropriate rate equation will quantitatively describe the experimental facts.

Care was taken in this study to insure, as much as possible, that those constants which were evaluated should be strictly comparable. To this end all experiments were conducted under identical conditions; e.g., methanol was the alcohol moiety in all esters, concentrations of added salts and enzyme were held constant with the substrate being varied over approximately the same concentration

ranges and the temperature and the pH of the systems were identical in all cases. By these measures it was thought possible to obviate complications such as wall effects (5), enzyme dimerization (6) and steric contributions to the velocity from the alcohol moiety interacting with the enzyme.

Base-catalyzed hydrolysis

The reason for evaluating the constants of the base-catalyzed hydrolysis of the esters contained in the work was to afford a basis for the comparison, in a relative manner, with that constant associated with the decomposition of the enzyme-substrate complex and at the same time one would have a reasonably accurate correction to apply to the enzyme-catalyzed hydrolysis such that the constants of the latter could be determined as unambiguously as possible.

It was assumed, on the basis of modern theories of base-catalyzed ester hydrolysis (7), that the reaction would be first order with respect to both ester and hydroxyl ion concentrations. To test this hypothesis the ester and hydroxyl ion concentrations were varied in a non-uniform manner and the resulting observed velocities plotted as a function of the product $[S][OH]$.*** In all cases the results were linear well within experimental error and it was shown that over the concentration ranges investigated the velocity of the base-catalyzed hydrolysis of these esters could be adequately described by an equation of the form

$$v = k_B [S] [OH] . \qquad (1)$$

The experimental k_B values are given in table III for those compounds considered in this work. The experimental details are discussed in the following section of this thesis.

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* The symbol S will, in all cases, denote ester.

** Brackets will be used to denote molar concentrations throughout this thesis.

The enzyme blank

It is an experimental fact that if one dissolves crystalline α-chymotrypsin in distilled water the resulting solution will be distinctly acidic (8). Furthermore, if the pH of this solution is raised to above 6.6 and constrained to remain at the particular pH, as with a pH stat, one finds that base will have to be added with time (9). This reaction which consumes bases with time when only enzyme is in solution is defined as the enzyme blank.

The nature of the reaction which is releasing acid (or consuming base) with time is not known. It has been shown that small amounts of ninhydrin positive compounds are formed during the course of the enzyme blank. It was estimated that they could not account for more than ca. 10% of the base taken up and therefore are not considered to be of major importance in this reaction (10). However, it has been shown that if the enzyme blank arises from the hydrolysis of an artifact contained in the crystalline enzyme preparation, and is catalyzed by the enzyme, then one can correct for it in a straightforward manner (9).

Figure 1 shows the effects of various additives on the magnitude and character of the enzyme blank reaction. Added salt or an inhibitor of the enzyme both diminish the amount of base taken up at a given time. However, they do not alter the shape of the base versus time trace. The reaction in the presence of substrate is distinctly different in that dP/dt is a constant after approximately the first minute for the system shown.

On comparing curves A, B and C of figure 1 one can readily see that curve A is of different character than the others. Moreover, the amount of base taken up by A at ten minutes is only ca. 20% over that by B and ca. 50% that by C. The substrate used in this plot was methyl chloroaceturate with a K_s of 69.10^{-3} M* while the inhibitor was indole with a K_I of $0.8 \pm 0.2 \cdot 10^{-3}$ M (11). If the blank were due only to free enzyme as has been suggested as a possibility (9) then one should expect that it, in the presence of the substrate, would be slightly larger than C. But, in the presence of substrate, one obtains a trace which is, after the first minute, linear.

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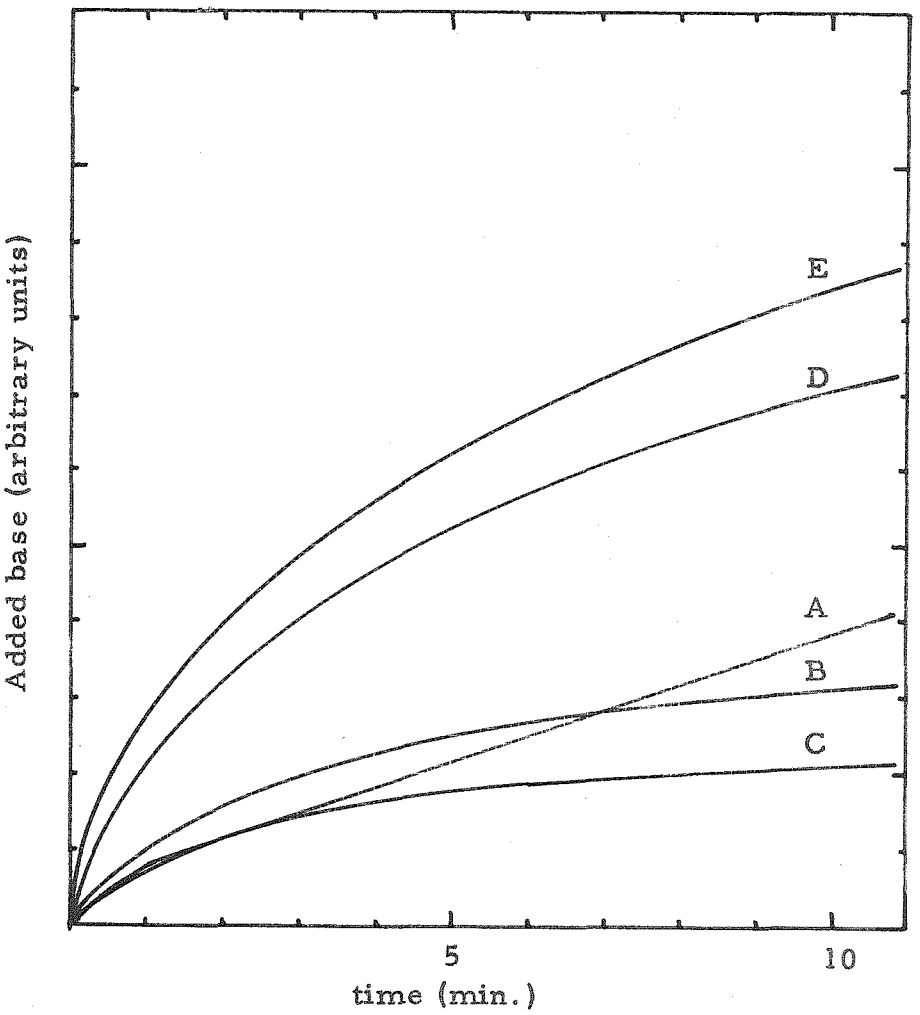
* See table III.

Figure 1

Enzyme Blank
Base added versus time*



A. Enzyme + 0.5 M NaCl + $5.0 \cdot 10^{-3}$M substrate
B. Enzyme + 0.5 M NaCl
C. Enzyme + 0.5 M NaCl + $8.5 \cdot 10^{-4}$M inhibitor
D. Enzyme + $8.5 \cdot 10^{-4}$M inhibitor
E. Enzyme only

*All systems at pH 7.90 ± 0.03, 25.0 ± 0.1 °C, contain 0.996 mg. protein-nitrogen per ml. (Armour * Co. crystalline bovine α-chymotrypsin, Lot #283) and titrated under an atmosphere of nitrogen.

Therefore, one may conclude from this data that the enzyme blank is greatly reduced in the presence of a substrate of the enzyme.

The extent to which the blank has been depressed presently cannot be assessed with accuracy. Qualitatively, it seems doubtful that the resulting blank accounts for more than ca. 10% of the base taken up when substrate concentrations are greater than $5.0 \cdot 10^{-3}$ M. On this basis, the enzyme blank has been completely neglected in the calculations of all the constants determined for the α-chymotrypsin-catalyzed reactions in this work as only those substrates affording linear dP/dt traces at velocities less than 10^{-5} M min^{-1} have been included.

The substrate blank

If one considers a system constrained to remain above pH 7 containing α-chymotrypsin and a substrate of the ester type and $[S] \gg [E]$ it does not seem unreasonable to assume that the ester may undergo normal base-catalyzed hydrolysis if it is not associated with the enzyme. Since the $[S] / [E]$ ratios are, in the majority of cases in this work, greater than 100 it has been considered adequate to reduce the velocity observed in the presence of enzyme by that velocity found with substrate alone at the same pH, salt content, concentration and temperature in order to obtain that portion due only to the enzyme-catalyzed reaction.

As a test of the validity of this procedure, diisopropyl-phosphoryl chymotrypsin, which has been shown to be catalytically inactive (12), was added to a system containing methyl succinimido-acetate at pH 7.90 ± 0.03. Table I contains the data obtained from that series of experiments. It can be seen that the variation in the observed velocity as the concentration of protein was increased 5 fold is well within the limit of experimental error. Therefore, one may conclude that the base-catalyzed hydrolysis of a substrate is unaffected by the amount of catalytically inactive protein in the system.

Table 1

The Base-Catalyzed Hydrolysis of Methyl Succinimidoacetate in the Presence of Diisopropylphosphoryl Chymotrypsin

Exp. no.	DFP[a]	E_o[b]	S_o[c]	v[d]
1	2	0.0	1.162	6.00
2	2	6.4	1.162	5.78
3	2	9.6	1.162	6.29
4	2	12.8	1.162	6.23
5	2	16.0	1.162	6.38
6	2	32.0	1.162	5.87
			ave:	6.09 ± 0.20

a) Diisopropylphosphorofluoridate.
b) In units of 10^{-2} mg. protein-nitrogen per ml.
c) In units of 10^{-2} M.
d) In units of 10^{-6} M min.$^{-1}$

The evaluation of data

If one considers a system which can be represented by the following equation,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (2)$$

it can be shown (4), that the rate equation for the system is

$$v_o = k_3 [E_o][S_o] / (K_s + [S_o]) \qquad (3)$$

where

$$K_s = (k_2 + k_3)/k_1 \qquad (4)$$

and the quantities with subscript "$_o$" are those obtaining at the initiation of the reaction. It can be seen that equation 3 can be rearranged to at least two forms which permit the evaluation of K_s and k_3 if one measures v_o, $[S_o]$ and $[E_o]$; viz.,

$$[S_o]/v_o = [S_o]/k_3[E_o] + K_s/k_3[E_o] \text{ (ref. 13)} \qquad (5)$$

and

$$v_o = -K_s v_o/[S_o] + k_3[E_o] \qquad \text{(ref. 14)} \qquad (6)$$

While K_s is seen formally to be a complex constant it may be considered as related to the dissociation constant for the enzyme-substrate complex and if $k_2 \gg k_3$ that relation approaches an identity.

The procedures for obtaining K_s and k_3 and the second-order base-catalyzed reaction constant k_B are illustrated in Table II and figure 2 for methyl p-aminohippurate. The results for all of the compounds investigated are given in Table III and all pertinent data are given in appendices A and B.

Equation 3 predicts that the relative magnitudes of $[S_o]$ and K_s should have a profound influence on the dependence of v'* with respect to $[S_o]$. That is, when $[S_o] \ll K_s$ a plot of v' versus $[S_o]$ should be linear and when $[S_o] \gg K_s$ v' will be independent of $[S_o]$. The rearranged forms of equation 3 given by equations 5 and 6 require that when $[S_o] \approx K_s$ plots of $[S_o]/v_o$ versus $[S_o]$ or v_o versus $v_o/[S_o]$ should be linear. The data of methyl p-aminohippurate shown in figure 2 demonstrate the latter case.

The applicability of equation 6 over large ranges of $[S_o]$ was investigated in the instances where the substrate was methyl mesylurate or N-acetyl-l-alanine methyl ester. Figures 3 and 4 show that equation 6 adequately describes the experimental data over a 60 fold substrate concentration range.

Three cases were found where the dependence of v_i'** on $[S_o]_i$ was linear. The compounds were ethyl aceturate, methyl-l-pyroglutamate and methyl ethoxyaceturate. The v_i' versus $[S_o]_i$

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*See footnotes to table II.

**v' is the observed velocity corrected for the base-catalyzed substrate blank. See table II.

Table II

Catalyzed Hydrolyses of Methyl p-Aminohippurate

Base catalysis[a]

Exp. no.	NaOH[b]	$[S_o]$[c]	pH[d]	$[OH^-]$[e]	v_{total}[f]	$[S][OH^-]$[g]
1	1.222	5.00	7.90	7.94	1.19	3.96
2	1.222	5.00	8.50	31.63	2.71	15.81
3	1.222	12.50	8.50	31.63	5.43	39.51
4	1.222	7.50	9.00	100.0	9.98	75.00
5	1.222	10.00	9.00	100.0	12.79	100.0

$k_B = 121 \pm 1\ \underline{M}^{-1}min^{-1}$
calculated by least squares fit to equation 1.

α-Chymotrypsin catalysis[h]

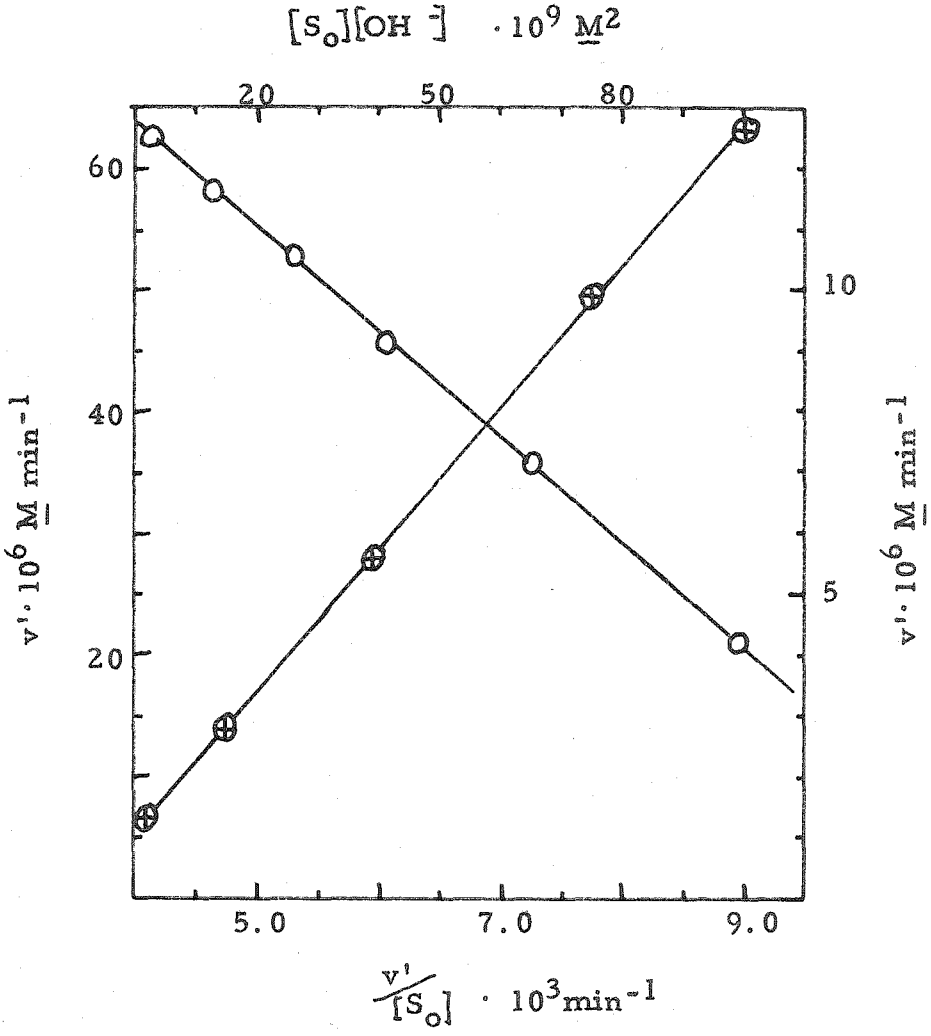
Exp. no.	NaOH[b]	$[E_o]$[i]	$[S_o]$[c]	v_t[f]	v_{blank}[f]	v'[f]	$\frac{v'}{[S_o]}$[j]	% hydrolysis
1	1.222	1.023	2.50	22.91	0.59	22.32	8.93	7.3
2	6.110	1.023	5.00	37.59	1.19	36.40	7.28	
3	6.110	1.023	7.50	47.26	1.78	45.48	6.06	
4	6.110	1.023	10.00	55.21	2.37	52.84	5.28	
5	6.110	1.023	12.50	61.10	2.96	58.14	4.65	
6	6.110	1.023	15.00	66.50	4.55	61.95	4.13	3.5

$K_s = 8.29 \pm 0.09 \cdot 10^{-3}\underline{M}$
$k_3 = 5.89 \pm 0.04 \cdot 10^{-4}\underline{M}\ min^{-1}$(mg. protein-nitrogen per ml.)$^{-1}$
calculated by least squares fit to equation 5.

a) The experimental conditions were as follows. The system was made up to a volume of 10.0 ml., 0.5 $\underline{M}$ in sodium chloride, held at a temperature of 25.00 ± 0.03°C. and titrated under an atmosphere of nitrogen.
b) Titrant strength given in units of $10^{-2}\underline{M}$.
c) In units of $10^{-3}M$.
d) In units of pH ± 0.03.
e) In units of $10^{-7}M$.
f) In units of $10^{-6}\underline{M}min^{-1}$. All v(s) (v', v_t, v_b) are initial velocities.
g) In units of $10^{-9}\underline{M}^2$.
h) The experimental conditions are as follows. The system was made up to a volume of 10.0 ml., 0.5 $\underline{M}$ in sodium chloride, pH of 7.90 ± 0.01, held at a temperature of 25.00 ± 0.03°C. and titrated under an atmosphere of nitrogen. The amount of enzyme (Armour & Co. crystalline bovine α-chymotrypsin, lot no. 283) was held constant, in all cases, at $1.600 \pm 0.050 \cdot 10^{-1}$ mg. protein-nitrogen per ml.
i) In units of mg. E per ml. To convert to mg. protein-nitrogen per ml., one has but to multiply mg. E per ml. by 0.160.
j) In units of $10^{-3}min^{-1}$.

Figure 2

Base- and Enzyme-Catalyzed Hydrolyses of Methyl p-Aminohippurate



○ Enzymic hydrolysis, left and bottom scales

⊕ Base-catalyzed hydrolysis, right and upper scales

Table III

Collected Kinetic Constants*

Compound	K_s [a]	k_3 [b]	$\frac{k_3}{K_s}$ [c]	k_B [d]
Methyl aceturate	3.07±0.59	1.95±0.27	6.35	160±4
Methyl propionurate	3.80±0.62	2.61±0.38	6.87	139±2
Methyl isobutyrurate	4.54±0.45	2.93±0.27	6.45	117±2
Methyl isovalurate	2.40±0.16	3.56±0.18	1.44	79.7±3.8
Methyl pivalurate	not a substrate			89.5±0.8
Methyl phenaceturate	0.785±0.05	0.88±0.03	11.2	150±3
Methyl chloroaceturate	6.87±0.10	8.03±0.11	11.7	224±3
Methyl dichloroaceturate	2.18±0.14	3.34±0.16	15.3	237±3
Methyl trichloroaceturate	not a substrate			230±8
Methyl ethoxyaceturate	first order in $[S_o]$		2.32	302±8
Methyl thioethoxyaceturate	1.04±0.01	0.78±0.03	7.50	128±1
Methyl nicotinurate	3.13±0.04	66.9±0.7	214	126±3
Methyl isonicotinurate	1.77±0.04	23.1±0.1	130	179±2
Methyl picolinurate [e]	too fast to follow			190±1
Methyl hippurate (2)	0.755±0.03	29.1±0.5	355	
Methyl p-aminohippurate	0.829±0.009	5.89±0.04	71.0	121±1
Methyl 2-furourate	1.96±0.07	141±4	719	166±1
Methyl mesylurate	8.46±0.10	1.72±1.2	20.3	316±5
Methyl 3-indolylurate	0.137±0.014	8.06±0.37	588	126±3
Methyl succinimidoacetate	not a substrate			369±7
Methyl carboethoxyamidoacetate	6.5±1.2	19±3	34.2	127±2
Methyl l-pyroglutamate	first order in $[S_o]$		15.9	1440±30
Methyl α-acetamidoisobutyrate	not a substrate			f
Methyl β-acetamidopropionate	not a substrate			26.1±0.7

Table III (cont'd.)

Collected Kinetic Constants*

Compound	K_s [a]	k_3 [b]	$\frac{k_3}{K_s}$ [c]	k_B [d]
Methyl N-acetyl sarcosinate	not a substrate			67.1±2.0
Methyl N-phenyl glycinate	0.0296±0.0036	1.11±0.03	375	76.2±2.7
Methyl N-acetyl-l-alaninate	61.1±1.0	188±3	30.8	88.4±1.7
Methyl N-acetyl-d-alaninate	not a substrate			88.4±1.7
Ethyl aceturate	9.57±0.50	1.95±0.05	2.04	67.5±1.2

a) In units of 10^{-2}M.
b) In units of 10^{-4}M min^{-1} (mg. protein-nitrogen per ml.)$^{-1}$.
c) In units of 10^{-3} M min^{-1} (mg. protein-nitrogen per ml.)$^{-1}$.
d) In units of M^{-1}min^{-1}.
e) The α-chymotrypsin-catalyzed hydrolysis of methyl picolinurate was too fast to follow in the pH stat at the enzyme concentration used.
f) The base-catalyzed hydrolysis of methyl α-acetamidoisobutyrate was too slow to follow in the pH stat under the conditions used to evaluate k_B for the other compounds given.

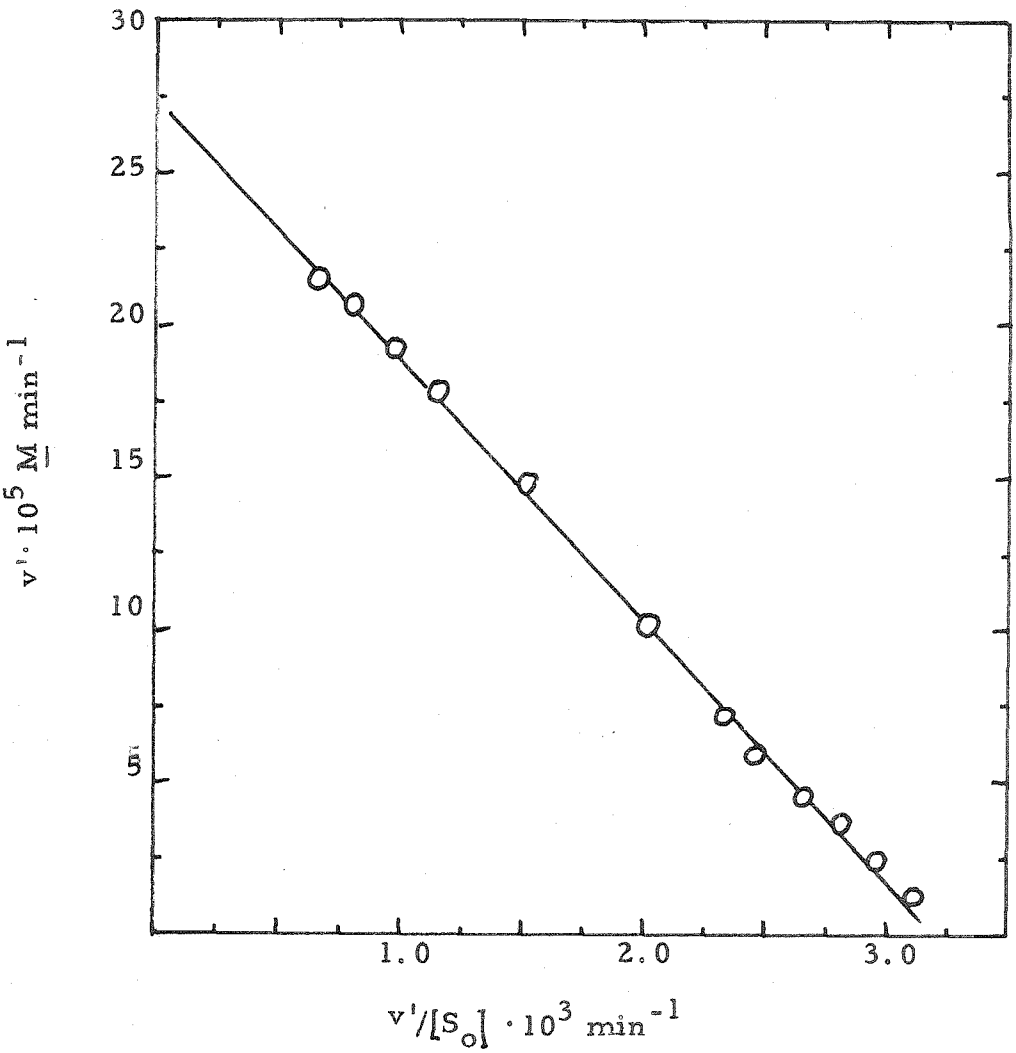
*These constants were determined from experiments conducted under conditions identical to those described in table II. The specific data are given in the appendices A and B.

Figure 3

Methyl mesylurate

v' versus $v'/[S_o]$

over 60 fold concentration range



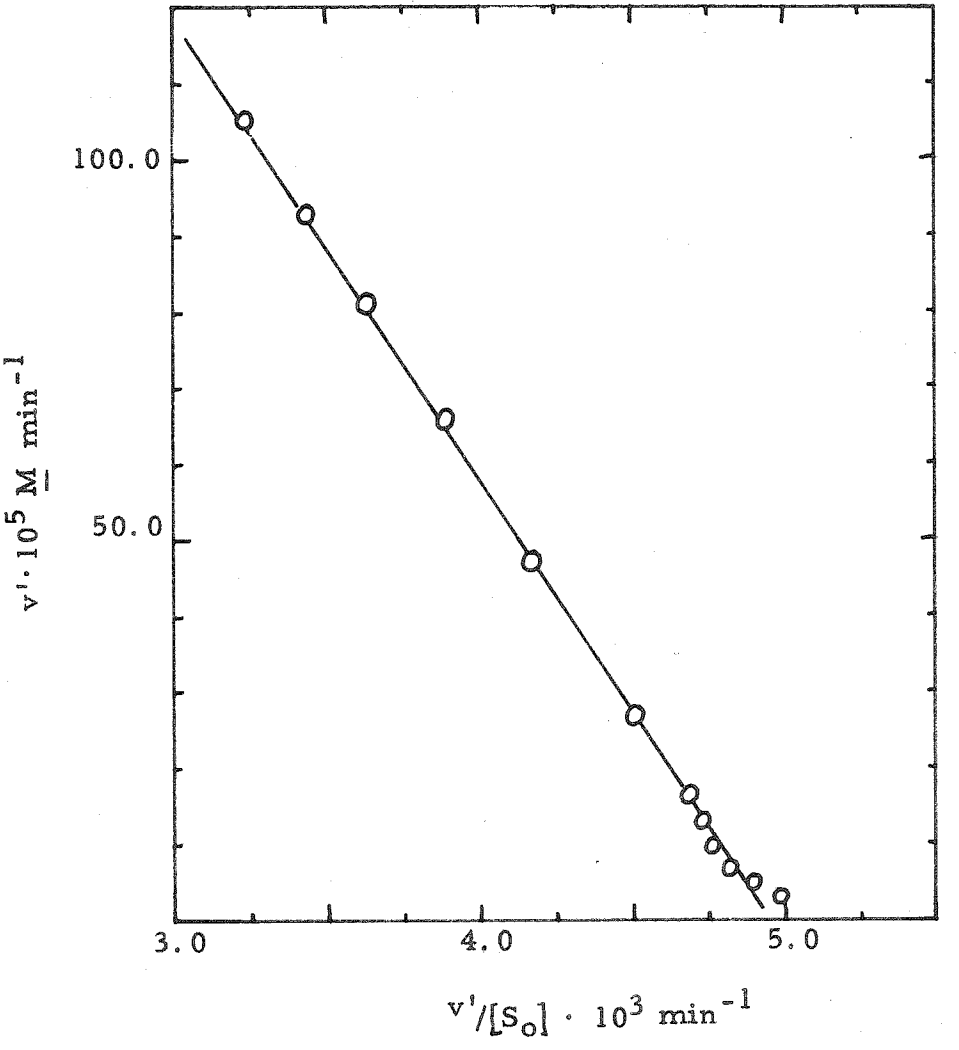
$$0.06 \lesssim \frac{[S_o]}{K_s} \lesssim 3.69$$

Figure 4

N-Acetyl- l-alanine Methyl Ester

v' versus $v'/[S_o]$

over 60 fold concentration range



$$0.01 \lesssim \frac{[S_o]}{K_s} \lesssim 0.5$$

plots for ethyl aceturate and methyl ethoxyaceturate are shown in figure 5. It is obvious, in view of equation 3, that from such data we can only determine the ratio of k_3 to K_s and not the individual constants. Also, it has been considered adequate that when, in a v' versus $v'/[S_o]$ plot, points deviate from a straight line at low v' values and if these points afford a linear v' versus $[S_o]$ plot, they may be ignored when determining k_3 and K_s from the former plot. An instance where this criterion has been applied is in the case of ethyl aceturate given in another section of this thesis.

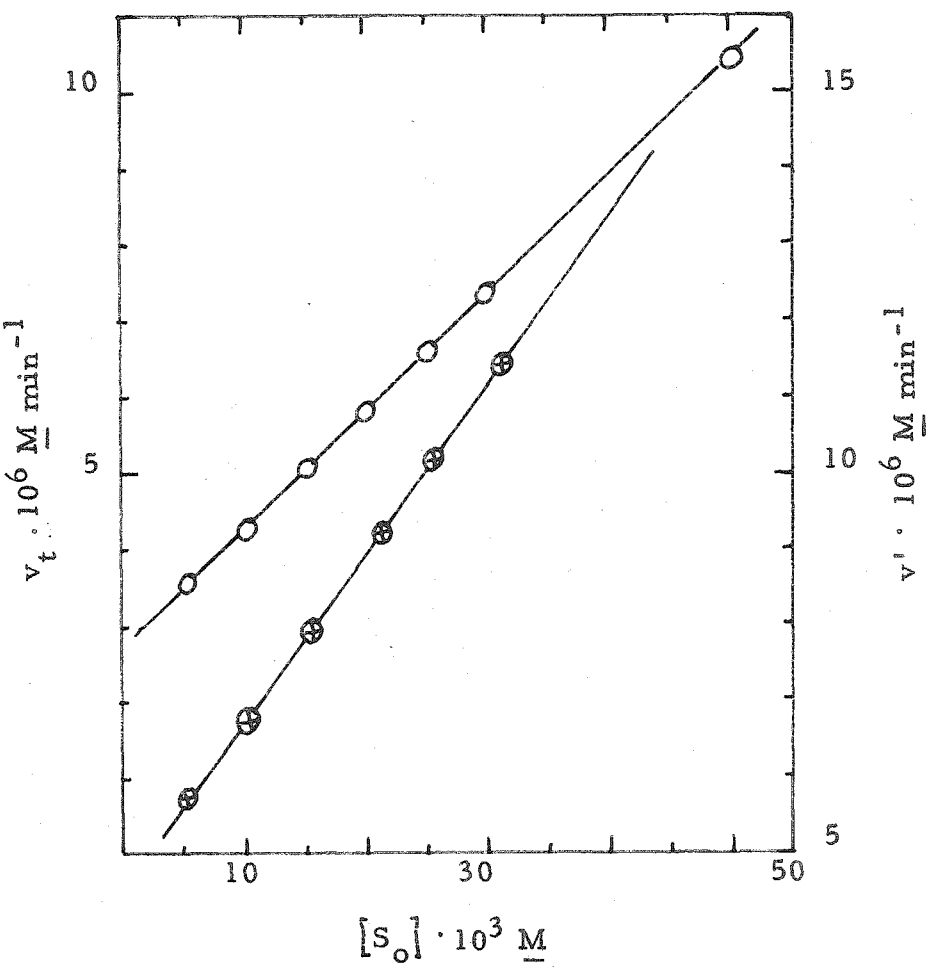
The third kind of v' versus $[S_o]$ behavior predicted by equation 3 is demonstrated by methyl isonicotinurate and methyl N-phenyl glycinate. It is seen in figure 6 that the velocity of the α-chymotrypsin-catalyzed hydrolysis of these compounds reaches a maximum value. This situation wherein v' is independent of $[S_o]$ is referred to as V_{max}; that is, that velocity observed when all of the catalytically active sites on the enzyme in the system are combining with substrate. The values obtained for V_{max} (the ordinate intercept on an Eadie plot, see equation 6) got by extrapolation are in satisfactory agreement with those determined graphically by drawing a smooth curve through the data to the ordinate. The values found for methyl N-phenyl glycinate are 17.6 and $16.0 \cdot 10^{-6}$ M min^{-1} (mg. protein-nitrogen per ml.)$^{-1}$ by the extrapolation and graphical methods, respectively. For methyl isonicotinurate one finds the

Figure 5

Ethyl Aceturate
and
Methyl Ethoxyaceturate

$v' \cdot 10^6$ versus $[S_o] \cdot 10^3$

for Low $[S_o]$ Concentrations



○ Ethyl aceturate, left ordinate

⊕ Methyl ethoxyaceturate, right ordinate

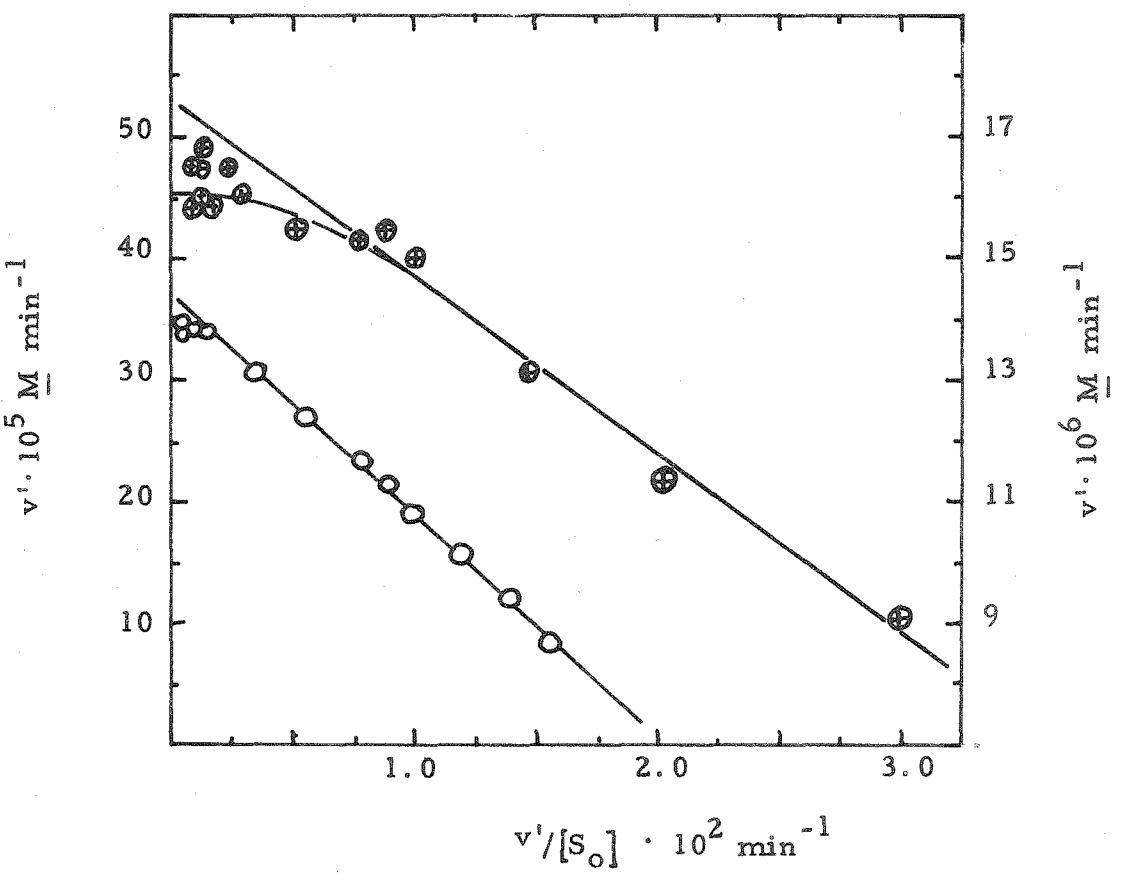
Figure 6

Methyl isonicotinurate

Methyl N-phenyl glycinate

Exhibition of V_{max} in plot of

v' versus $v'/[S_o]$



○ Methyl isonicotinurate, left ordinate

⊛ Methyl N-phenyl glycinate, right ordinate

values 36.7 and $34.5 \cdot 10^{-6}$ M min^{-1} (mg. protein-nitrogen per ml.)$^{-1}$ by the extrapolation and graphical methods, respectively, for V_{max}.

This study has experimentally demonstrated all those cases predicted on the basis of equation 3 and shown that the α-chymotrypsin-catalyzed hydrolysis of the esters considered in this work may be adequately described by this equation. Also, one may see by inspection of table III that there is no apparent correlation between k_B and any of the constants, k_3, K_s or k_3/K_s. The constant k_3/K_s is that which one would determine if the reaction obeyed pseudo first-order kinetics, i.e., when $[S_o] \ll K_s$. Thus one may conclude that, in the acylated glycine methyl ester series, the pK_a of the acid corresponding to the acyl moiety has little to do with determining the velocity of the enzyme-catalyzed ester hydrolysis.

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Part II

THE NATURE AND PROPERTIES OF THE ENZYME-SUBSTRATE COMPLEX PRODUCED UNDER CONDITIONS OF VERY HIGH SUBSTRATE CONCENTRATIONS

Activation

When the data from the α-chymotrypsin-catalyzed hydrolysis of methyl aceturate wherein the concentration of substrate was varied over a 120 fold range are presented as in an Eadie plot (see figure 7) one finds that there are two linear segments connected by a curve. This behavior is without explanation if one only considers the Michaelis-Menten formulation of enzymic catalysis unless one admits of more than one active center or site capable of catalyzing the formation of products per molecule of enzyme. This alternative contradicts much experimental evidence that seems to indicate that α-chymotrypsin has but one such site per protein molecule (1, 2).

Such behavior is predictable if one uses an alternative formulation wherein the enzyme-substrate complex may combine with another molecule of substrate at high substrate concentrations and thus have its rate of breakdown to product changed. The derivation of the kinetic equation applicable in this case is as follows.

Consider the system represented by equations 1 and 2.

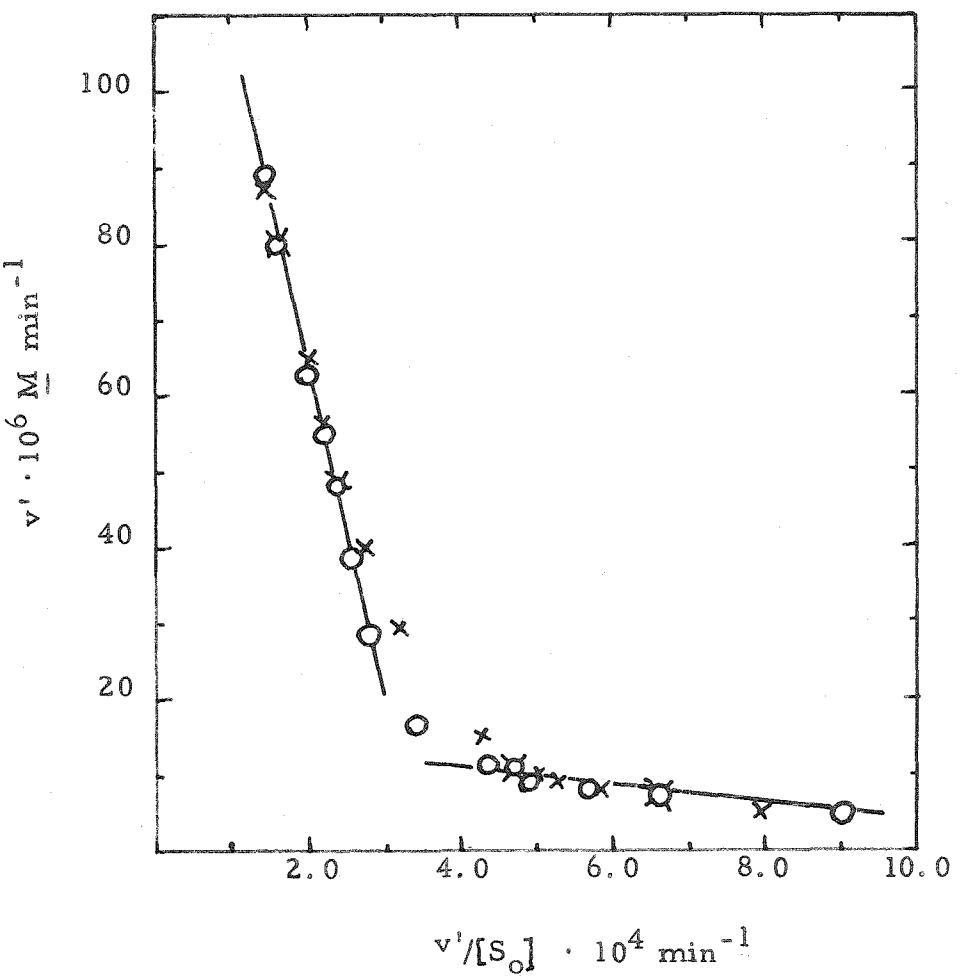
$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P \qquad (1)$$

$$ES + S \underset{k_5}{\overset{k_4}{\rightleftarrows}} ES_2 \xrightarrow{k_6} ES + P \qquad (2)$$

Figure 7

Methyl Aceturate

Activation by Excess Substrate



○ Experimental data (see table IV).

× Points calculated from equation 16 using constants given in text.

The velocity of the sum of reactions 1 and 2 is

$$v = \frac{dP}{dt} = k_3[ES] + k_6[ES_2] \tag{3}$$

and from a material balance one obtains

$$[E_o] = [E] + [ES] + [ES_2] . \tag{4}$$

In order to derive a simple equation for v in terms of experimentally measurable quantities one must satisfy the following condition that

$$[S_o] \doteq [S] \gg [ES] + [ES_2] . \tag{5}$$

Then, imposing steady-state conditions, one may write

$$O = \frac{d[ES]}{dt} = k_1[E][S] - (k_2 + k_3)[ES] - k_4[ES][S] + (k_5 + k_6)[ES_2] \tag{6}$$

$$O = \frac{d[ES_2]}{dt} = k_4[ES][S] - (k_5 + k_6)[ES_2] . \tag{7}$$

Defining the quantities

$$K_{s1} \equiv \frac{k_2 + k_3}{k_1} \tag{8a}$$

$$K_{s2} \equiv \frac{k_5 + k_6}{k_4} \tag{8b}$$

allows one to write with equations 7, 6, 8a and 8b

$$O = [E][S] - K_{s1}[ES] \tag{9}$$

$$O = [ES][S] - K_{s2}[ES_2] . \tag{10}$$

Substitution of 4 in 9 gives

$$O = [E_o][S] - [ES][S] - [ES_2][S] - K_{s1}[ES] , \tag{11}$$

and 10 in 11

$$O = [E_o][S] - [ES][S] - \frac{[ES][S]^2}{K_{s2}} - K_{s1}[ES] . \tag{12}$$

Solving 12 for $[ES]$ affords

$$[ES] = \frac{[E_o][S]}{[S] + \frac{[S]^2}{K_{s2}} + K_{s1}} . \tag{13}$$

Combining 3 with 10 and rearranging one obtains

$$v = \left(k_3 + \frac{[S] k_6}{K_{s2}} \right) [ES] \tag{14}$$

which, when substituted in with 13 and rearranged yields

$$v = \frac{(k_3 K_{s2} + [S] k_6)[E_o][S]}{[S]^2 + K_{s2}[S] + K_{s1}K_{s2}} . \tag{15}$$

If one conducts the kinetic experiments such that the measurement of v occurs very soon after the beginning of the reaction we may write 15, in view of 5, as

$$v_o = \frac{(k_3 K_{s2} + [S_o] k_6)[E_o][S_o]}{[S_o]^2 + K_{s2}[S_o] + K_{s1}K_{s2}} . \tag{16}$$

If one assigns values for the constants in equation 16 such that $K_{s1} \doteq 50\ K_{s2}$ and $k_6 \doteq 10\ k_3$ and calculates v_i as a function of S_i therefrom with $K_{s1} \lesssim S_i \lesssim K_{s2}$ and presents the results in an Eadie plot one obtains a curve of the form shown in figure 7. In particular, if one uses $K_{s1} = 9.2 \cdot 10^{-3}$ M, $K_{s2} = 4.2 \cdot 10^{-1}$ M, k_3 and k_6 equal to $5.8 \cdot 10^{-5}$ and $8.8 \cdot 1.0^{-3}$ M min^{-1} (mg. protein N per ml.)$^{-1}$, respectively, and values for S_{oi} equal to experimental S_{oi} one will obtain those points in figure indicated by "x". These calculations are presented in table IV. From this agreement between theory and experiment it is seen that equation 16 adequately describes the experimental results. Therefore, one may conclude that equations 1 and 2 are admissible as representations of the system examined.

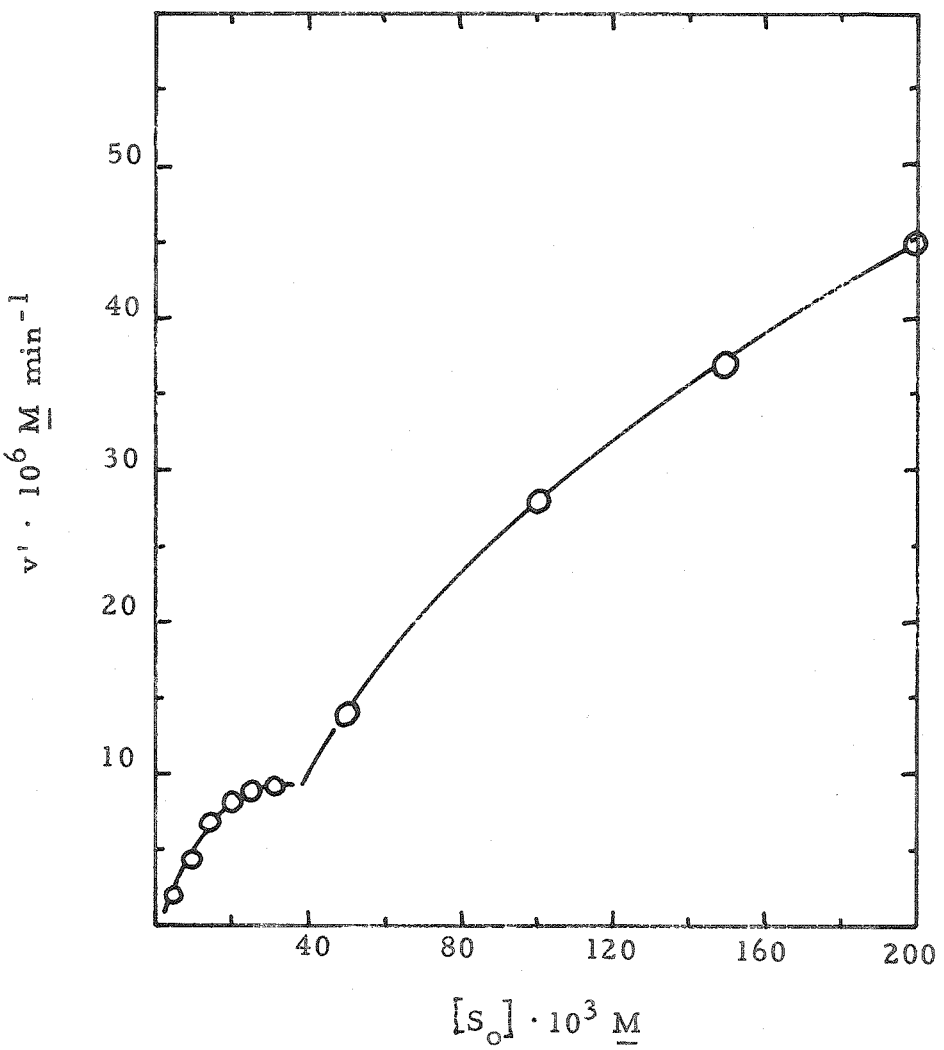
Figure 8 is a plot of v' versus $[S_o]$ for the same data. It can be readily seen that the relation is not linear. Therefore, one may conclude that the case is not one where $[S_o] \ll K_s$ and that the slopes and intercepts of the linear segments of figure 7 do have meaning with respect to defining a K_{s_x} and k_{n_x} as from equation 3 of the preceding section.

The simplicity of the explanation offered by equations 1 and 2 for the phenomenon observed with methyl aceturate and that it, at the same time, does not conflict with other experimental data makes it rather attractive. In fact, the "anomalous" results of Castaneda-Agullo and Del Castillo (3) are immediately explainable in the same manner.

Figure 8

Methyl Aceturate

Plot of v' versus $[S_o]$



One may become quite sophisticated and write equations 1 and 2 in much more detail as to the nature of the various ES(s). In the first instance it was assumed that all of the ES(s) were identical but this need not be the case. The other possible cases are, with equation 1 understood to be included as the first reaction:

$$\begin{aligned} &ES + S \rightleftharpoons ES_2 \rightharpoonup ES^{\ddagger} + P \\ &ES^{\ddagger} \rightleftharpoons E + S \end{aligned} \tag{17}$$

$$\begin{aligned} &ES^{\ddagger} + S \rightleftharpoons ES_2 \rightharpoonup ES^{\ddagger} + P \\ &ES^{\ddagger} \rightleftharpoons E + S \end{aligned} \tag{18}$$

$$\begin{aligned} &ES^{\ddagger} + S \rightleftharpoons ES_2 \rightharpoonup ES + P \\ &ES^{\ddagger} \rightleftharpoons E + S \end{aligned} \tag{19}$$

$$\begin{aligned} &ES^{*} + S \rightleftharpoons ES_2 \rightharpoonup ES^{\ddagger} + P \\ &ES^{*} \rightleftharpoons E + S \\ &ES^{\ddagger} \rightleftharpoons E + S \end{aligned} \tag{20}$$

where $ES \neq ES^{*} \neq ES^{\ddagger}$.

Thus, it can be seen that one may have many formally different representations of the system but all will yield equations for v of exactly the same form as equation 16 and therefore are all equally admissible. Since there is no experimental criterion available at the present for deciding between these possibilities, equations 1 and 2 will be used as they are the least complicated and most readily interpreted in terms of the Michaelis-Menten formulation of enzyme-catalyzed reactions.

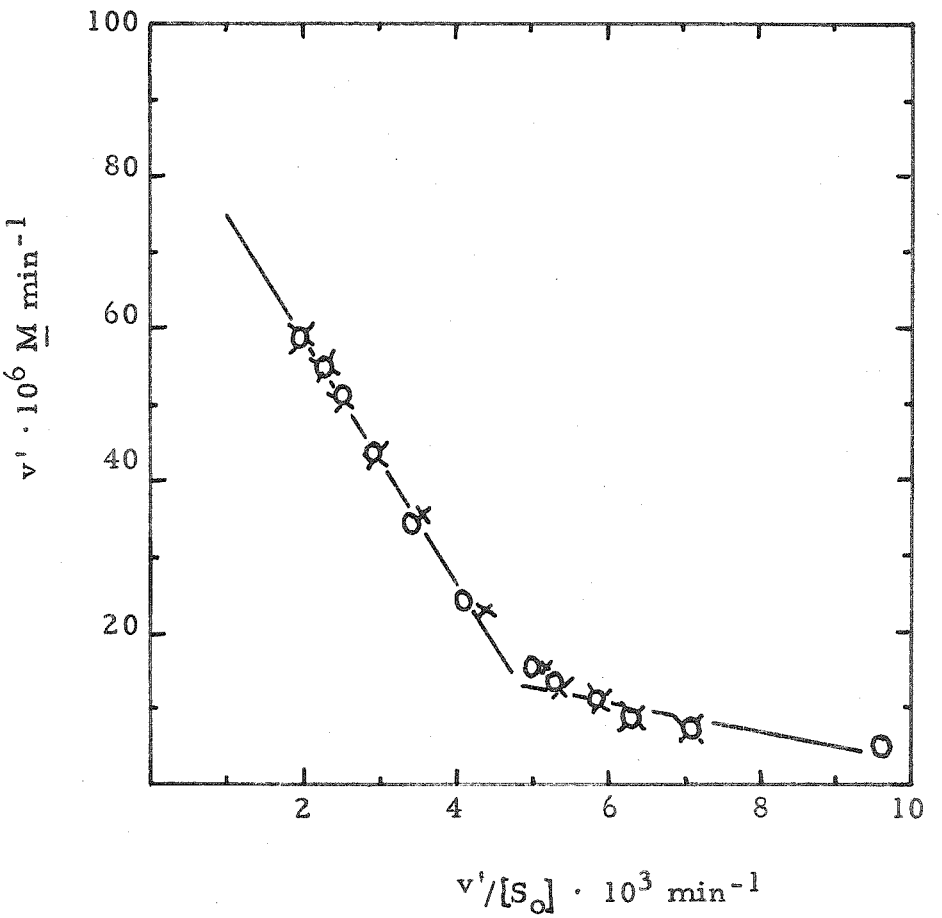
This kind of interaction, that is, of the enzyme-substrate complex with excess substrate to produce a complex containing two molecules of substrate which decomposes to give products at a faster rate than does the complex containing only one substrate molecule is without precedence in the literature. The nearest approach is that found in the system fumarase-fumarate wherein the enzyme rather than ES is activated, possibly by excess substrate. This system has not been fully investigated as yet and the decision as to the nature of the activator is still equivocal (4).

Methyl aceturate is not the only case wherein the dependence of v versus $\frac{v}{[S_o]}$ requires the use of equation 16 for its description. Methyl propionurate, when examined over a 60 fold range of $[S_o]$, affords data which exhibit the same behavior in an Eadie plot as methyl aceturate. These data are shown in figure 9 and summarized in table IV. Those points marked "x" are the results of calculations with equation 16 using $K_{s1} = 9.0 \cdot 10^{-3}$ M, $K_{s2} = 1.8 \cdot 10^{-1}$ M, $k_3 = 5.0 \cdot 10^{-5}$ and $k_6 = 5.6 \cdot 10^{-4}$ M min^{-1} (mg. protein N per ml.)$^{-1}$, respectively, and $[S_o]_i$ equal to those of the experimental points.

Figure 9

Methyl Propionurate

Activation by Excess Substrate



O Experimental data (see table IV)

X Points calculated from equation 16 using constants given in text.

Table IV

Activation of the Enzyme-Substrate Complex

by Excess Substrate[a]

Methyl aceturate				Methyl propionurate			
$v' \cdot 10^6$ [b]		$v'/[S_o] \cdot 10^4$ [c]		$v' \cdot 10^6$ [b]		$v'/[S_o] \cdot 10^4$ [c]	
found	calc.	found	calc.	found	calc.	found	calc.
4.52	3.95	9.04	7.90	4.81	5.49	9.62	11.0
6.65	6.65	6.65	6.65	7.04	7.00	7.04	7.00
8.65	8.85	5.77	5.90	9.44	9.59	6.29	6.39
9.85	10.8	4.92	5.39	11.73	11.91	5.86	5.96
12.23	12.5	4.89	5.02	13.18	13.9	5.27	5.56
13.28	14.2	4.43	4.74	15.28	16.0	5.09	5.25
17.36	20.2	3.47	4.03	21.30	22.1	4.26	4.30
28.39	32.2	2.84	3.22	33.94	35.5	3.39	3.55
38.59	40.3	2.57	2.69	43.8	44.0	2.92	2.93
47.62	50.0	2.38	2.50	51.2	50.1	2.56	2.50
55.68	56.9	2.23	2.27	55.0	55.0	2.20	2.20
62.72	62.8	2.09	2.09	58.9	58.7	1.96	1.96
79.58	79.8	1.59	1.60				
88.47	85.8	1.47	1.43				

$k_3[E_o] = 9.6 \cdot 10^{-6}$ M min^{-1}
$K_{s1} = 9.2 \cdot 10^{-3}$ M
$k_6[E_o] = 1.4 \cdot 10^{-4}$ M min^{-1}
$K_{s2} = 4.2 \cdot 10^{-1}$ M

$k_3[E_o] = 8.0 \cdot 10^{-6}$ M min^{-1}
$K_{s1} = 9.0 \cdot 10^{-3}$ M
$K_{s2} = 1.8 \cdot 10^{-1}$ M
$k_6[E_o] = 9.0 \cdot 10^{-5}$ M min^{-1}

a) Experimental conditions described in footnotes to table II and in appendix A.
b) In units of M min^{-1}.
c) In units of min^{-1}.

Inhibition

When one displays the data from the α-chymotrypsin-catalyzed hydrolysis of methyl nicotinurate in an Eadie plot as in figure 10, it is obvious that the simple Michaelis-Menten formulation is not adequate to accommodate all of the data. At high $[S_o]$ it is seen that v' decreases at a rapid rate as $[S_o]$ increases. This suggests that at high concentrations the substrate is inhibiting the enzyme-catalyzed reaction. This interpretation may be formulated in the following manner.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \tag{21}$$

$$ES + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES_2 \tag{22}$$

For the system represented by equations 21 and 22 it is true that

$$v = \frac{dP}{dt} = k_3[ES] \tag{23}$$

$$[E_o] = [E] + [ES] + [ES_2] \tag{24}$$

and if

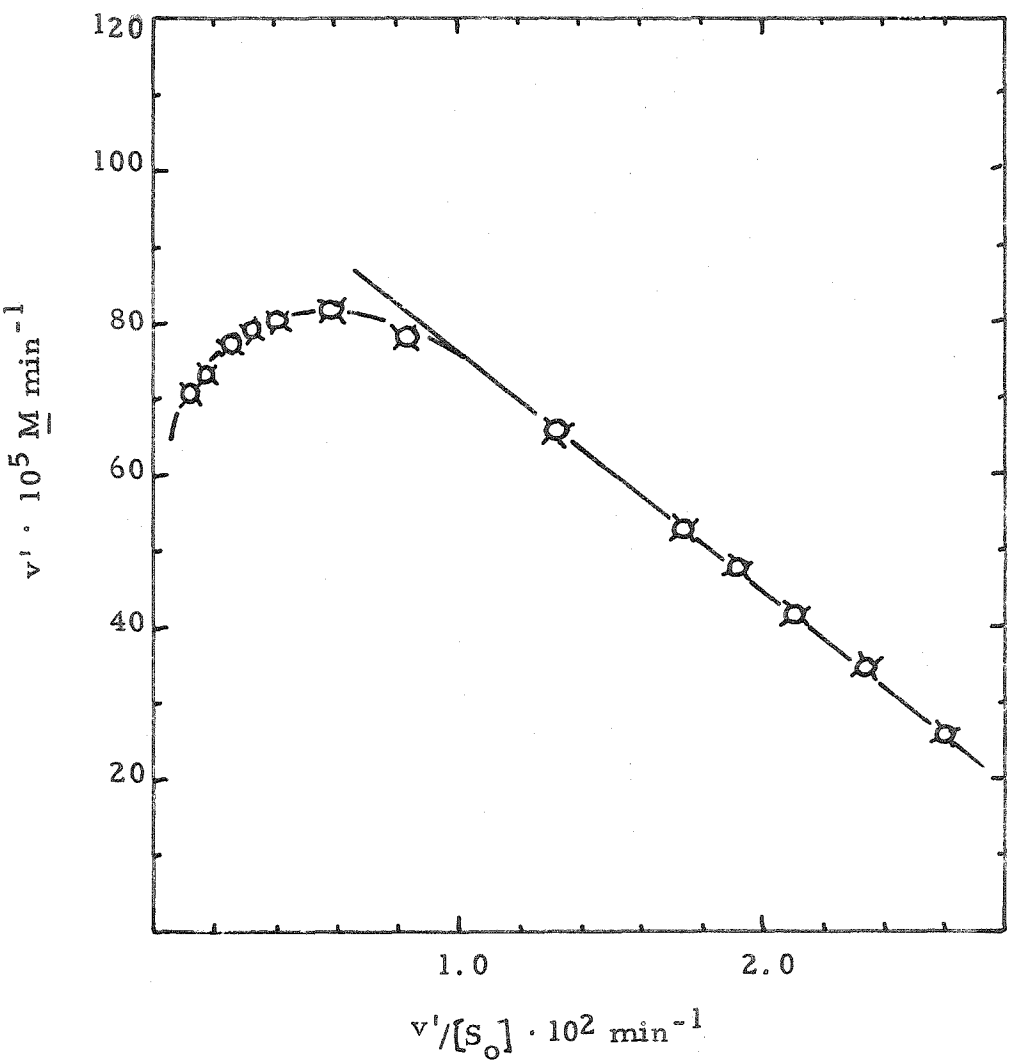
$$[S_o] \doteq [S] \gg [ES] + [ES_2] \tag{25}$$

one may write when steady-state are applied

Figure 10

Methyl Nicotinurate

Inactivation by Excess Substrate



O Experimental data (see table V).

× Points calculated from equation 34 using constants given in text.

$$0 = \frac{d[ES]}{dt} = k_1[E][S] - (k_2 + k_3)[ES] - k_4[ES][S] + k_5[ES_2] \quad (26)$$

$$0 = \frac{d[ES_2]}{dt} = k_4[ES][S] - k_5[ES_2] \quad . \quad (27)$$

Solving 27 for $[ES_2]$

$$[ES_2] = \frac{k_4}{k_5}[ES][S] \quad (28)$$

and substituting 28 in 24 yields

$$[E] = [E_o] - [ES] - \frac{k_4}{k_5}[ES][S] \quad . \quad (29)$$

Defining the quantities

$$K_s \equiv \frac{k_2 + k_3}{k_1} \quad (30)$$

$$K_I \equiv \frac{k_5}{k_4} \quad (31)$$

one may now combine equations 30, 31, 29, 26 and 27 to obtain

$$0 = [S][E_o] - [S][ES] - \frac{[ES][S]^2}{K_I} - K_s[ES] \quad (32)$$

which may be solved for $[ES]$ and combined with 23 to yield the desired equation for v:

$$v = \frac{K_I k_3 [S][E_o]}{[S]K_I + [S]^2 + K_s K_I} \quad . \quad (33)$$

If one considers only those v(s) measured very soon after the reaction begins equation 33 may be written as

$$v_o = \frac{K_I k_3 [S_o][E_o]}{[S_o]K_I + [S_o]^2 + K_s K_I} \quad . \tag{34}$$

It is seen from equation 34 that if $K_s K_I \ll [S_o] K_I + [S_o]^2$ then a plot of $\frac{1}{v_o}$ versus $[S_o]$ will be linear; viz.,

$$\frac{1}{v_o} = \frac{[S_o]}{K_I k_3 [E_o]} + \frac{1}{k_3 [E_o]} \quad . \tag{35}$$

Also, it is apparent that when $[S_o]^2 \ll [S_o]K_I + K_s K_I$ equation 34 reduces to the familiar Michaelis-Menten form. Therefore, at the lower limit of the range of $[S_o]$ one may determine a K_s in the usual manner and at the upper limit equation 35 may be used to find a K_I.

Fortunately, it is possible to determine K_I directly from the data without having to resort to curve-fitting. The solubility of methyl nicotinurate permits working with an aqueous system as concentrated as 1.0 M and one may vary $[S_o]$ over a 100 fold range. Figure 11 shows the data obtained at high $[S_o]$ plotted according to equation 35.

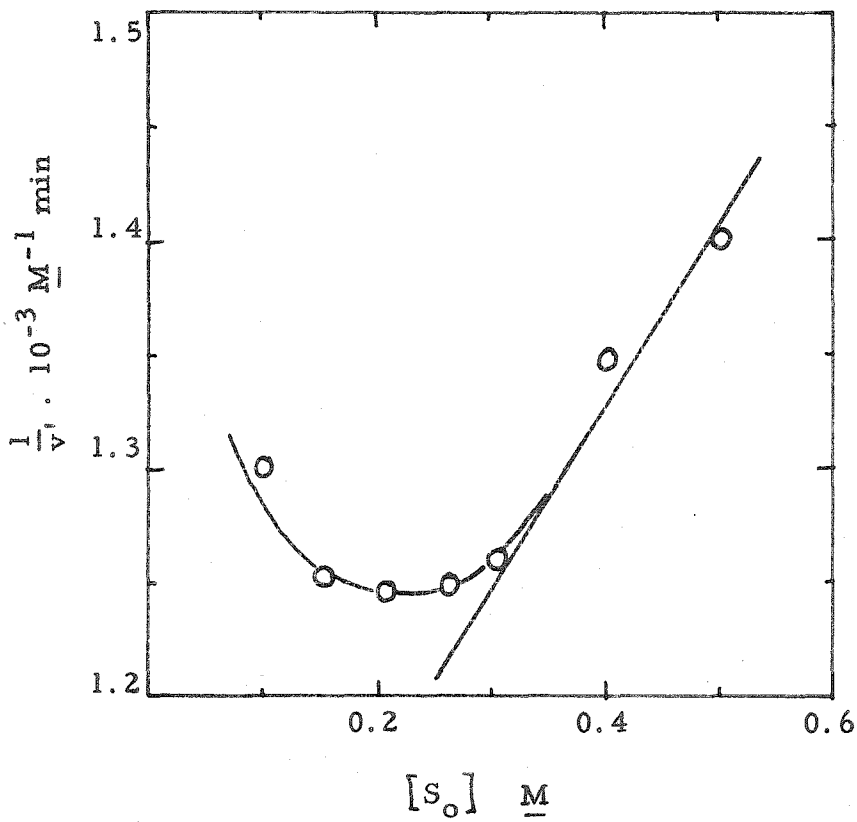
Using the constants $K_s = 3.25 \cdot 10^{-2}$ M, $k_3[E_o] = 1.10 \cdot 10^{-3}$ M min^{-1} and $K_I = 1.0$ M it is seen in figure 10 and table V that equation 34 describes the experimental data almost quantitatively. One may conclude therefrom that equations 21 and 22 are admissible as representations of this system.

Figure 11

$\frac{1}{v'}$ versus $[S_o]$

for Methyl Nicotinurate at

High $[S_o]$



tangent slope = $8.3 \cdot 10^2$ M^{-2} min

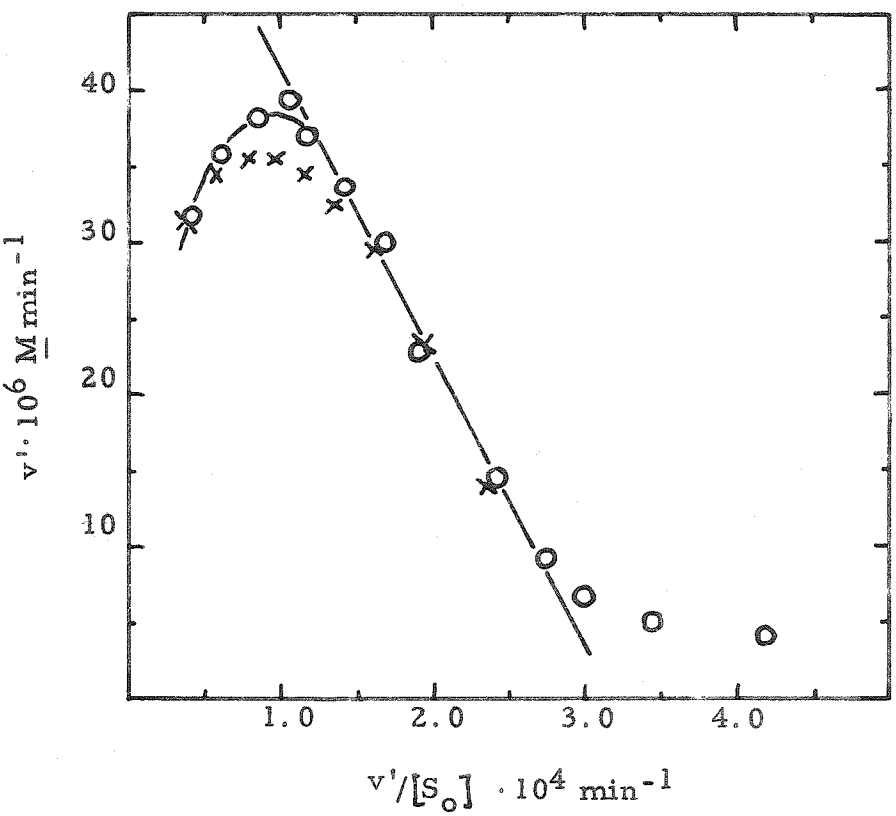
K_I = 1.1 M (see equation 35)

Equations 34 and 35 also describe the experimental data obtained for the system α-chymotrypsin-ethyl aceturate as is shown in figure 12 and table V. At first sight it seems incongruous that the methyl and ethyl aceturic esters should behave in such drastically different manners at high concentrations in the presence of the enzyme. A hypothesis which rationalizes these data is presented in the last section of this thesis.

Figure 12

Ethyl Aceturate

Inactivation by Excess Substrate



O Experimental data (see table V).

× Points calculated from equation 34 using constants given in text.

Table V

Inactivation of the Enzyme-Substrate Complex

by Excess Substrate[a]

Methyl nicotinurate				Ethyl aceturate			
$v' \cdot 10^5$ [b]		$v'/[S_o] \cdot 10^2$ [c]		$v' \cdot 10^6$ [b]		$v'/[S_o] \cdot 10^4$ [c]	
found	calc.	found	calc.	found	calc.	found	calc.
13.9	14.7	2.79	2.94	14.4	14.0	2.40	2.33
25.9	25.8	2.59	2.58	22.6	23.2	1.89	1.93
34.5	34.6	2.31	2.31	30.0	29.0	1.67	1.61
41.6	42.0	2.08	2.10	33.9	32.3	1.41	1.34
47.7	47.3	1.91	1.89	37.0	34.5	1.23	1.15
52.7	52.1	1.76	1.74	39.6	35.2	1.10	0.98
65.3	64.7	1.31	1.29	38.2	35.4	0.85	0.79
77.1	77.2	0.77	0.77	35.8	34.1	0.60	0.57
80.3	80.5	0.54	0.54	31.6	31.9	0.42	0.42
79.8	80.7	0.40	0.40				
79.4	79.7	0.32	0.32				
78.1	78.1	0.26	0.26				
72.9	74.3	0.18	0.19				
70.9	70.9	0.14	0.14				

$K_s = 3.25 \cdot 10^{-2}$ M
$k_3[E_o] = 1.1 \cdot 10^{-3}$ M min^{-1}
$K_I = 1.0$ M

$K_s = 3.2 \cdot 10^{-1}$ M
$k_3[E_o] = 9.0 \cdot 10^{-5}$ M min^{-1}
$K_I = 5.4 \cdot 10^{-1}$ M

a) Experimental conditions given in footnotes to table II and in appendix A.
b) In units of M min^{-1}.
c) In units of min^{-1}.

The Inhibition of Methyl Aceturate with Aceturamide

In view of the interpretation of the data of the system α-chymotrypsin-methyl aceturate it was anticipated that if aceturamide could simulate the ester and bind to the enzyme with a molecule of ester it might show an apparent activation effect. That is, one might anticipate that one of the ternary enzyme-substrate-inhibitor complexes could go to products faster than ES.

To test this hypothesis the enzyme-catalyzed hydrolysis of methyl aceturate was followed at constant $[S_o]$ with $[I_o]$ being varied over a 12 fold range. The velocities observed and the corresponding $[I_o]$ are given in table VI. It can be seen that there is no increase in v_o and, in fact, there is a slight, but significant decrease. This decrease in v_o with increasing $[I_o]$ implied that aceturamide was having some effect in the system.

A second experiment was carried out wherein $[I_o]$ was held constant and $[S_o]$ varied over a 60 fold range. These data are shown in figure 13. It is apparent that, at an $[I_o]$ of $1.00 \cdot 10^{-1}$ M aceturamide has no discernible effect if $[S_o] > 3.0 \cdot 10^{-2}$ M. It would appear that when $[S_o] < 3.0 \cdot 10^{-2}$ M one has a case where, in the presence of inhibitor the apparent K_s and k_3 values both decrease. At present, no explanation can be offered for this phenomenon observed at low $[S_o]$.

Table VI

Velocities observed at constant $[S_o]$ as $[I_o]$ was varied 12 fold for the system α-chymotrypsin-methyl aceturate and aceturamide.*

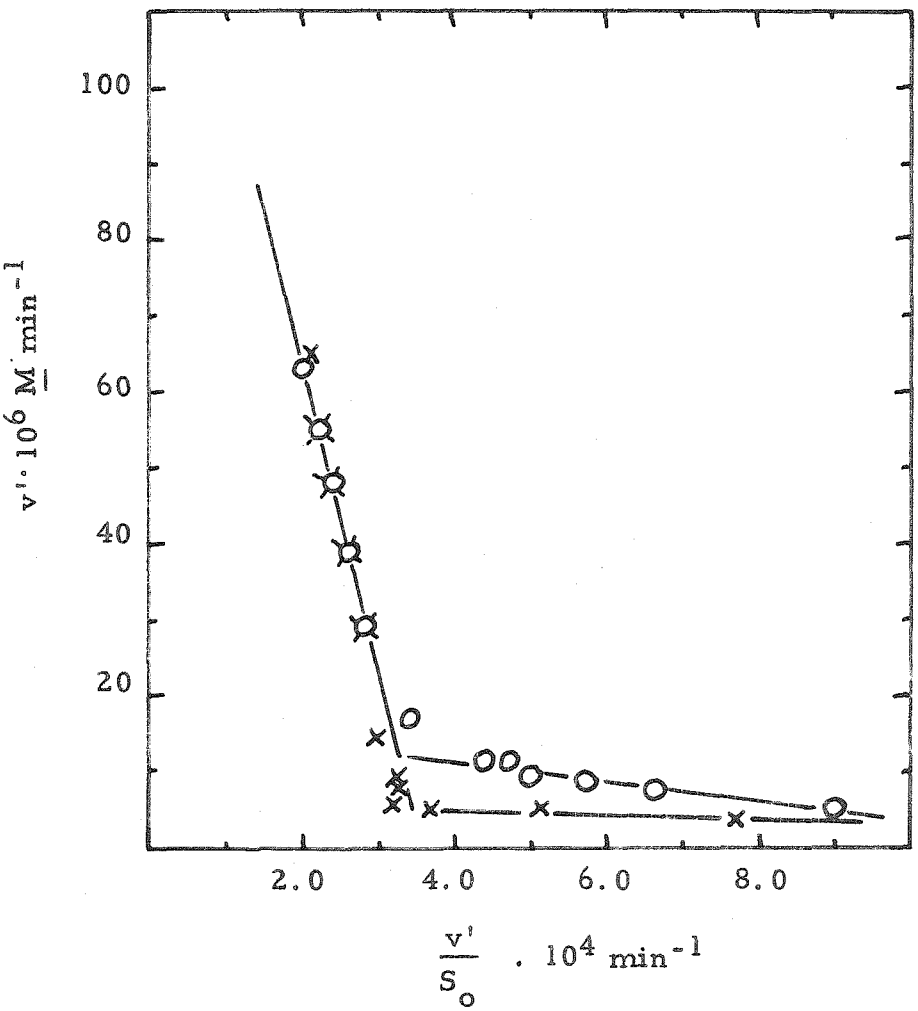
$v_t\ 10^6$ M min^{-1}	$I_o \cdot 10^2$ M	$S_o \cdot 10^2$ M
11.24	0	2.5
11.38	5.0	2.5
11.38	10.0	2.5
11.58	15.0	2.5
11.42	20.0	2.5
10.58	25.0	2.5
10.71	30.0	2.5
10.20	50.0	2.5
9.75	60.0	2.5

*Each reaction carried out at pH 7.90 ± 0.03 and containing 1.00 ± 0.03 mg. crystalline bovine α-chymotrypsin (Armour & Co., lot no. 283) in CO_2 free water and under an atmosphere of nitrogen.

Figure 13

Inhibition of Methyl Aceturate by Aceturamide

$v' \cdot 10^6$ versus $v'/[S_o] \cdot 10^4$



O Methyl aceturate without inhibitor (see table VII).

X Methyl aceturate - $1.00 \cdot 10^{-1}$ M aceturamide.

Table VII

Methyl Aceturate Inhibited by Aceturamide[a]

$v_t \cdot 10^6$[b]	$v_b \cdot 10^6$[b]	$v' \cdot 10^6$[b]	$S_o \cdot 10^3$[c]	$v'/S_o \cdot 10^4$[d]
4.52	0.67	3.85	5.0	7.70
6.48	1.34	5.14	10.0	5.14
7.54	2.01	5.53	15.0	3.69
9.09	2.68	6.41	20.0	3.20
11.58	3.35	8.23	25.0	3.29
13.90	4.02	9.88	30.0	3.29
21.65	6.70	14.95	50.0	2.99
42.56	13.40	29.16	100	2.92
57.80	20.10	37.70	150	2.51
75.54	26.80	48.74	200	2.44
88.50	33.50	55.00	250	2.20
105.8	40.20	65.60	300	2.19

a) Experimental conditions given in footnotes to table II and appendix A.
b) In units of $\bar{M}$ min^{-1}.
c) In units of $\bar{M}$.
d) In units of min^{-1}.

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Part III

A HYPOTHESIS CONCERNING

α-CHYMOTRYPSIN-SUBSTRATE COMPLEXES

When one determines a "constant" by the method of chemical kinetics the only significance that can be attached to it is that it, when used in an appropriate equation, will reproduce the experimental data with satisfactory accuracy. The identification of this "constant" with a quantity having physical reality rests entirely upon the ingenuity of the theorist. His ability to formulate an abstraction immediately relatable to physical reality which by logical means affords an equation of appropriate form having identifiable parameters and is amenable to experimental examination determines whether or not a set of data is meaningful. One may go so far as to say that experiments have only two purposes, that is to decide whether a given theory is or is not acceptable and to provide a basis for formulating new theories. The data given in the first two sections of this thesis will now be utilized in both ways.

In this work the kinetics of the hydrolyses of 19 compounds have been shown to be adequately described by the Michaelis-Menten equation given as equation 3 in part I. Therefore, it will be assumed that the formulation given in equation 2 of part I does, in fact, describe reality. The interpretation of equation 2 is quite straightforward as it says that substrate and enzyme combine to form a complex which then breaks down to give products. Since syntheses are also observed in the presence of enzymes one may

conclude that the action of an enzyme is that of a catalyst. That is to say that it is able to affect the rate at which an equilibrium is attained but not its position.

In view of this last statement it is obvious that equation 3 of part I contains a tacit assumption, i.e., the rate of the reaction of product plus enzyme to give enzyme-substrate complex is so slow as to be negligible with respect to the other rates. It has been shown (1) that this assumption is unnecessary and that one will obtain an equation of the same form as equation 3 of part I without this restriction. Thus, even though equation 3 adequately describes the dependence of the kinetics of the hydrolysis of a substrate in the presence of α-chymotrypsin as a function of the concentration of substrate, one may not use this fact with more force than to imply that equation 2 is an acceptable abstraction. It is obvious that kinetic studies cannot decide the question of whether or not a given representation is unique or true; they can only form a basis for deciding on the acceptability of a given representation. This question has been decided in the affirmative for the work contained in the previous parts of this thesis.

In order to progress further toward an understanding of the mechanism of enzyme catalysis it is necessary to examine equation 2 of part I in the light of other experimental data. It has been shown by chemical methods (2, 3) that there must be more than one

intermediate and therefore the symbol ES must stand for a cycle or sequence of enzyme-substrate complexes. Assuming that the determined k_3 is the rate constant of the slowest step of the reaction sequence going from enzyme-substrate complex to products and free enzyme and that K_s is the dissociation constant associated with that complex one may now interpret the given data in terms of a specific mechanism.

One may assume that the enzyme and substrate combine in solution to form a loosely bound complex, the amount of which is determined by the mass action law and whose formation and dissociation are very facile. In addition, it is necessary that some of these complexes can proceed along a mechanistically unknown reaction path to give products and free enzyme. It is now possible to postulate a physical picture of the events taking place.

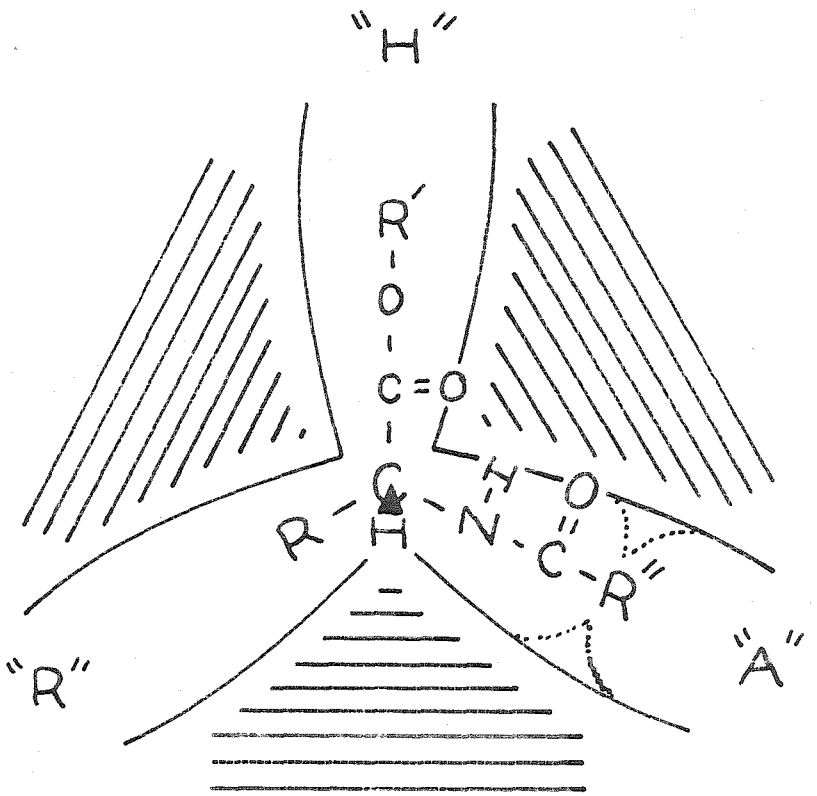
When one considers the fact that α-chymotrypsin is capable of catalyzing the hydrolysis of peptide bonds in a large molecule such as casein (3) it is possible to deduce that the place on the enzyme where catalysis is occurring must be schematically representable as in figure 14. It will be assumed that there is but one such site per molecule of enzyme and, further, all of the enzyme molecules in the system contain such a site. The manner in which the substrate molecule is bound to the enzyme has been suggested as being due to van der Waals' forces but, for now, this point will be neglected.

Figure 14

Schematic Representation of a

Substrate "on" the Active

Site of α-Chymotrypsin



"H" - hydrolytic site

"R" - amino acid side-chain binding site

"A" - acylamido binding site

Figure 14 says that, in the enzyme-substrate complex, there must be a space to put each of the four groups appended to that carbon atom which is alpha to the bond which will be cleaved. An assumption which must be made concerning these "slots" is that one, and only one, contains those structures necessary to catalyze the reaction which occurs. This "slot" will be designated as the H (for hydrolysis) leg. Figure 14 shows the substrate as being an α-acylamido ester but this is not meant to be restrictive. In addition, it is necessary that that structure of the enzyme which initiates the reaction to products should be relatively immobile within the H leg.

It has been suggested, "...the enzyme-substrate complex, defined by the Michaelis-Menten constant, consists of absorbed substrate on the enzyme in which the ester linkage (or that linkage which will be ruptured)* has not been altered by the enzyme; that is, binding involves those parts of the (substrate)* molecule other than the ester group." (2) This concept has immediate confirmation in this work if one considers those constants of methyl hippurate, methyl nicotinurate, methyl isonicotinurate and methyl-2-furourate given in table III. It is seen that K_s of the substrates changes only slightly while k_3 varies over two orders of magnitude. If the labile ester group were involved in forming that ES complex given by K_s then one should expect much larger changes in K_s because of the large

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*Parentheses contain this author's comment.

variations in k_3 which implies very large changes in the nature of the intermediate which goes to products.

If, however, the changes in k_3 were due to the position of the labile bond relative to the hydrolysis-initiating structure in the H leg, then the large changes in k_3 are understandable, not in terms of difference of binding to the enzyme but in placement of the substrate at the active site. Thus, k_3 may be said to contain a large entropy factor. In fact, if one considers the above-mentioned series of substrates in detail, it is seen that, as the hetero-atom approaches that position which would be occupied by the nitrogen atom of the second acylamido moiety away from the labile bond in a peptide, k_3 approaches a maximum value. The velocity of the hydrolysis of methyl picolinurate was too fast to measure, a fact which lends credence to the above hypothesis.

This ability of the acylamido group to orient the substrate is almost completely lost when the amino acid substrate bears a side-chain on the α-carbon atom. As can be seen from the data in table VIII, very large changes in either of the amide or α-acylamido groups produce very modest changes in K_s and k_3 when the substrate amino acid is tyrosine.

Table VIII[a]

Substrate	K_s [b]	k_3 [c]
Acetyl-l-tyrosylglycinamide	23±3	7.5±1.0
Acetyl-l-tyrosinamide	32±4	2.4±0.3
Nicotinyl-l-tyrosinamide	12±3	5.0±1.0

a) Taken from reference (5).
b) In units of 10^{-3} M.
c) In units of 10^{-3} $\bar{M}$ min^{-1} (mg. protein-nitrogen per ml.)$^{-1}$

It is interesting to consider the effect of removing all the hydrogen atoms and substituting bulkier groups on those carbon atoms alpha to the labile linkage and those positions which would be alpha to peptide links in a protein. Such substitutions have been made in the compounds, methyl α-acetamidoisobutyrate, methyl pivalurate and methyl trichloroaceturate. None of these compounds are substrates of α-chymotrypsin. Therefore one may conclude that these alpha hydrogen atoms are involved in binding to the enzyme or fit in a place which is inaccessible to a larger atom or group.

It is also interesting to note that substitution of the hydrogen atom on the nitrogen atom of the first alpha acetamido group also prevents a compound from being a substrate for the enzyme. As shown in table III, methyl N-acetylsarcosinate and methyl succinimidoacetate are not substrates for α-chymotrypsin.

The very large k_3 found for methyl N-acetyl-l-alaninate

strongly suggests that when one of the two alpha hydrogens of glycine is substituted by a methyl group the resulting bulk about the alpha carbon atom orients the substrate optimally with respect to the position of the labile linkage within the H leg. Two such substitutions destroy the ability of the compound to act as a substrate, possibly because the α-methyl group cannot "fit" in the place of hydrogen. When acylated glycine methyl esters are considered it has been shown above that the labile linkage can be considered as being moved to and fro in the H leg. Therefore, one may conclude that position on the active site is reflected in changes in k_3 while the total amount of enzyme-substrate complex present is shown by K_s.

The work contained in part II of this thesis is now interpretable in terms of the picture given in figure 14. With an acylated glycine ester as the substrate it is obvious that the R leg is unoccupied in the ES complex.

It seems intuitively obvious to identify the ES_2 complex with that situation where a second molecule of substrate combines with the ES complex by binding at the empty R leg. Then one would anticipate a change in the rate of breakdown to products if the second molecule of substrate were to "nudge" the first so as to obtain a more favorable position of the labile group within the H leg of the active site.

This interpretation immediately afforded the proposition that the behavior of v with respect to [S] shown by methyl aceturate should be different than that shown by methyl propionurate or ethyl aceturate. It was in fact shown that methyl propionurate behaved exactly as methyl aceturate at very high substrate concentrations but a completely different effect was seen with ethyl aceturate. This latter compound was shown to obey the kinetic expression derived from the instance wherein ES_2 could not go to products. The physical picture is simply that the second molecule of substrate "nudges" the first one too far, in or out, of the H leg for the catalysis to take place.

One further detail of figure 14 may be filled in when one considers the fact that methyl l-pyroglutamate is a substrate, albeit a poor one, for the enzyme. This fact implies that on the active site as shown as the shaded area between legs R and A in figure 14 there is no protuberance on the enzyme which prevents the substrate from "laying down" on the active site. In other words, there is no "hump" of enzyme coming up out of the plane of figure 14 in this area.

The problem of how the enzyme is able to show stereospecificity may now be considered. It has been shown that one hydrogen atom on the carbon atom alpha to the labile linkage is necessary for a compound to function as a substrate. Therefore, in view of the original assumption that the H leg is unique, it follows that if that hydrogen is binding as in a "go-no go" situation such that "no go"

precludes further reaction then the enzyme can distinguish between a methyl or an acetamido group in legs R and A of the active site. It is necessary that only one of the two legs can be discriminatory as only one definite attachment is necessary to effect the desired result.

Thus, it has been shown that the concept contained in figure 14 is of great utility, in that, with it, and the Michaelis-Menten formulation of enzymic catalysis one can correlate a great amount of experimental data.

References

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APPENDIX A

Data from the α-chymotrypsin-catalyzed and base-catalyzed hydrolyses of some substituted glycine esters and related compounds.

All reactions were conducted under the following conditions. The system was made up to a volume of 10.0 ml., 0.5 M in sodium chloride, held at pH 7.90±0.01 (except where noted) and titrated under an atmosphere of nitrogen in a pH stat.

The enzyme concentration used in each set of experiments is given with that entry in the units mg. enzyme per ml. as that was the number determined by weighing. To convert mg. enzyme per ml. to mg. protein-nitrogen per ml. multiply the former figure by 0.160. The enzyme used was Armour & Co. crystalline bovine chymotrypsin, lot number 283.

Velocities are given in the units M min^{-1} and concentrations in M. In all cases the velocity of the reaction was measured before 10% hydrolysis had occurred.

The order of listing is the same as in table III.

	enzymic catalysis *			base catalysis *		
	$v_t \cdot 10^6$	$v' \cdot 10^6$	$S_o \cdot 10^3$	$v_t \cdot 10^6$	$S_o \cdot 10^3$	pH
Methyl aceturate	5.19	4.52	5.0	1.55	10.0	7.90
	7.93	6.65	10.0	5.41	10.0	8.50
E_o = 1.073	10.57	8.65	15.0	13.27	25.0	8.50
mg/ml	12.40	9.85	20.0	23.42	15.0	9.00
	15.42	12.23	25.0	32.81	20.0	9.00
	17.11	13.28	30.0			

*v_t is that velocity obtained directly from pH stat trace; i.e., the rate of appearance of acidic products from all reactions occurring in system. v' is v_t less the velocity of the base-catalyzed reaction and is assumed to be due only to the enzyme-catalyzed reaction.

	enzymic catalysis			base catalysis		
	$v_t \cdot 10^6$	$v' \cdot 10^6$	$S_o \cdot 10^3$	$v_t \cdot 10^6$	$S_o \cdot 10^3$	pH
Methyl propionurate	5.59	4.81	5.0	1.47	10.0	7.90
	8.60	7.04	10.0	4.77	10.0	8.50
	11.78	9.44	15.0	12.00	25.0	8.50
E_o = 1.041	14.85	11.73	20.0	21.21	15.0	9.00
mg/ml	17.08	13.18	25.0	28.17	20.0	9.00
	19.96	15.28	30.0			
Methyl isobutyrate	5.58	4.89	5.0	1.39	10.0	7.90
	11.12	9.73	10.0	4.58	10.0	8.50
	13.63	11.56	15.0	10.23	25.0	8.50
E_o = 1.037	17.36	14.58	20.0	18.60	15.0	9.00
mg/ml	21.24	17.78	25.0	23.94	20.0	9.00
	24.35	19.28	30.0			
Methyl isovalurate	11.95	9.89	5.103	1.55	10.206	7.90
	21.16	17.04	10.206	4.43	10.206	8.50
	28.71	22.53	15.309	10.61	25.515	8.50
E_o = 1.022	33.16	24.92	20.412	17.89	15.309	9.00
mg/ml	39.50	29.20	25.515	23.51	20.412	9.00
	44.45	32.09	30.618			
Methyl pivalurate	2.57*		5.151	1.61	10.302	7.90
	5.70*		25.755	3.65	10.302	8.50
				9.02	25.755	8.50
E_o = 1.021				14.87	15.453	9.00
mg/ml				19.27	20.604	9.00
Methyl phenaceturate	5.35	4.98	2.50	0.73	5.0	7.90
	6.57	5.84	5.00	2.62	5.0	8.50
	8.56	7.45	7.50	7.20	15.0	8.50
E_o = 1.060	9.69	8.21	10.00	7.86	5.0	9.00
mg/ml	11.02	9.17	12.50	14.67	10.0	9.00
	12.14	9.92	15.00			

*velocity at 8 minutes.

	enzymic catalysis			base catalysis		
	$v_t \cdot 10^6$	$v' \cdot 10^6$	$S_o \cdot 10^3$	$v_t \cdot 10^6$	$S_o \cdot 10^3$	pH
Methyl chloroaceturate	10.91	10.02	5.0	2.69	10.0	7.90
	18.72	16.94	10.0	8.45	10.0	8.50
	25.80	23.13	15.0	19.53	25.0	8.50
E_o = 1.003	32.56	29.00	20.0	35.05	15.0	9.00
mg/ml	38.85	34.40	25.0	45.78	20.0	9.00
	44.53	39.19	30.0			
Methyl dichloroaceturate	12.12	10.46	5.0	2.72	10.0	7.90
	19.62	16.30	10.0	8.59	10.0	8.50
	26.70	21.72	15.0	20.11	25.0	8.50
E_o = 1.001	32.09	25.45	20.0	35.82	15.0	9.00
mg/ml	36.56	28.26	25.0	48.6	20.0	9.00
	41.55	31.59	30.0			
Methyl trichloroaceturate	3.88*		5.091	3.27	10.182	7.90
	8.16*		25.455	8.87	10.182	8.50
				21.21	25.455	8.50
E_o = 1.022				38.70	15.273	9.00
mg/ml				47.51	20.364	9.00
Methyl ethoxyaceturate	7.39	5.29	5.126	4.20	10.252	7.90
	12.09	7.89	10.252	9.84	10.252	8.50
	16.05	9.75	15.378	27.40	25.630	8.50
E_o = 1.021	20.28	11.88	20.504	49.08	15.378	9.00
mg/ml	24.21	13.71	25.630	62.52	20.504	9.00
	28.64	15.66	30.756			
Methyl thio-ethoxyaceturate	5.53	4.11	5.0	2.63	10.0	7.90
	9.04	6.20	10.0	5.58	10.0	8.50
	11.33	7.07	15.0	11.96	25.0	8.50
E_o = 0.998	13.85	8.17	20.0	20.70	15.0	9.00
mg/ml	15.64	8.54	25.0	27.36	20.0	9.00
	17.94	9.42	30.0			

*velocity at 8 minutes

	enzymic catalysis			base catalysis		
	$v_t \cdot 10^6$	$v' \cdot 10^6$	$S_o \cdot 10^3$	$v_t \cdot 10^6$	$S_o \cdot 10^3$	pH
Methyl nicotinurate	26.16	25.94	10.0	2.20	10.0	7.90
	34.92	34.59	15.0	5.57	10.0	8.50
	42.08	41.64	20.0	11.85	25.0	8.50
E_o = 1.008 mg/ml	48.30	47.75	25.0	20.94	15.0	9.00
	53.38	52.72	30.0	26.37	20.0	9.00
	66.40	65.30	50.0			
Methyl iso-nicotinurate	78.7	77.6	5.0	2.23	10.0	7.90
	134.9	132.7	10.0	6.43	10.0	8.50
	175.3	171.9	15.0	15.48	25.0	8.50
E_o = 1.031 mg/ml	205.3	199.8	20.0	27.28	15.0	9.00
	227.3	221.7	25.0	36.79	20.0	9.00
	244.9	238.2	30.0			
	283.7	272.5	50.0			
	337.6	315.2	100.0			
	366.6	333.0	150.0			
Methyl picolinurate	1456		5.061	2.09	10.304	7.90
	too fast to follow			7.21	10.304	8.50
				17.11	25.760	8.50
E_o = 1.005 mg/ml				31.49	15.456	9.00
				39.24	20.608	9.00
Methyl 2-furourate	451	451	5.0	1.81	10.0	7.90
	768	768	10.0	4.75	10.0	8.50
	993	993	15.0	11.02	25.0	8.50
E_o = 1.005 mg/ml	1168	1168	20.0	23.17	15.0	9.00
	1261	1261	25.0	33.10	20.0	9.00
	1361	1361	30.0			

	enzymic catalysis			base catalysis		
	$v_t \cdot 10^6$	$v' \cdot 10^6$	$S_o \cdot 10^3$	$v_t \cdot 10^6$	$S_o \cdot 10^3$	pH
Methyl mesylurate	18.24	16.56	5.194	3.35	10.35	7.90
	34.38	31.02	10.39	10.83	10.35	8.50
	48.28	43.24	15.58	27.70	25.88	8.50
E_o = 1.005	61.35	54.63	20.78	49.06	15.53	9.00
mg/ml	72.51	64.11	25.97	66.50	20.71	9.00
	83.40	73.32	31.16			
	123.2	106.4	51.94			
	189.4	155.8	103.9			
	233.1	182.7	155.8			
	265.3	198.1	207.8			
	292.4	208.4	259.7			
	315.7	214.9	311.6			
	17.75	16.07	5.18			
	32.45	29.10	10.35			
	46.65	41.62	15.53			
	59.55	52.85	20.71			
	73.10	64.72	25.88			
	83.10	73.05	31.06			
Methyl-3-indolylurate	43.2	42.6	0.689	1.10	2.58	7.90
	69.3	68.1	1.378	2.12	2.58	8.50
	84.1	82.3	2.067	3.48	6.45	8.50
E_o = 1.021	88.2	85.8	2.756	5.96	3.87	9.00
mg/ml	94.6	91.6	3.445	7.39	5.16	9.00
	104.6	101.0	4.134			
Methyl p-amino-hippurate	22.91	22.32	2.50	1.19	5.00	7.90
	37.59	36.40	5.00	2.71	5.00	8.50
	47.26	45.48	7.50	5.43	12.50	8.50
E_o = 1.021	55.21	52.94	10.00	9.98	7.50	9.00
mg/ml	61.10	58.14	12.50	12.79	10.00	9.00
	66.50	61.95	15.00			
Methyl succin-imidoacetate	4.36*		7.92	5.83	15.8	7.90
	16.81*		47.52	20.48	15.8	8.50
				47.56	39.6	8.50
E_o = 1.030				86.8	23.8	9.00
mg/ml				120.3	31.7	9.00

*velocity at 8 minutes.

	enzymic catalysis			base catalysis		
	$v_t \cdot 10^6$	$v' \cdot 10^6$	$S_o \cdot 10^3$	$v_t \cdot 10^6$	$S_o \cdot 10^3$	pH
Methyl carbo-ethoxyamido-acetate	26.90	25.74	5.258	2.32	10.52	7.90
	43.89	41.57	10.52	6.07	10.52	8.50
	65.98	62.50	15.77	12.68	26.29	8.50
	83.30	78.66	21.03	21.98	15.77	9.00
E_o = 1.025 mg/ml	99.20	93.40	26.29	28.11	21.03	9.00
	112.6	105.6	31.55			
Methyl l-pyro-glutamate	28.6	21.6	5.608	13.05	10.88	7.90
	51.5	37.6	11.22	131.5	32.64	8.50
	73.7	52.9	16.82	234.5	21.76	9.00
E_o = 1.068 mg/ml	96.9	69.1	22.43	326.3	27.20	9.00
	121.0	86.2	28.04			
	140.5	98.8	33.65			
Methyl α-acet-amidoisobutyrate	too slow to follow			too slow to follow		
E_o = 1.030 mg/ml	$v < 10^{-6}$M min^{-1} @ $[S_o] > 5 \cdot 10^{-3}$M			$v < 10^{-6}$M min^{-1} @ $[S_o] > 5 \cdot 10^{-3}$M		
Methyl β-acet-amidopropionate	3.61*		5.65	0.92	11.30	7.90
	7.94*		33.90	1.86	11.30	8.50
				3.67	28.25	8.50
E_o = 1.020 mg/ml				5.29	16.95	9.00
				6.64	22.60	9.00
Methyl N-acetyl-sarcosinate	3.82*		10.54	1.47	21.08	7.90
	8.35*		52.70	5.21	21.08	8.50
				12.44	52.70	8.50
E_o = 1.007 mg/ml				22.69	31.62	9.00
				28.40	42.16	9.00
Methyl N-phenyl-glycinate	9.32	9.27	0.303	1.96	7.27	7.90
	11.58	11.48	0.606	3.68	7.27	8.50
	13.14	12.98	0.909	6.64	21.82	8.50
E_o = 0.996 mg/ml	14.70	14.49	1.212	10.30	10.91	9.00
	15.33	15.07	1.515	12.60	14.54	9.00
	15.28	14.97	1.818			

*velocity at 8 minutes.

	enzymic catalysis			base catalysis		
	$v_t \cdot 10^6$	$v' \cdot 10^6$	$S_o \cdot 10^3$	$v_t \cdot 10^6$	$S_o \cdot 10^3$	pH
Methyl N-acetyl-l-alaninate	29.5	28.7	5.736	1.34	11.38	7.90
	57.0	55.4	11.47	4.13	11.38	8.50
	84.1	81.7	17.21	9.00	28.45	8.50
E_o = 1.003	111.1	107.9	22.94	16.74	17.07	9.00
mg/ml	138.5	134.5	28.68	21.87	22.76	9.00
	164.9	160.1	34.42			
	256.8	248.8	55.14			
	476.7	460.7	110.3			
	667.0	643.0	165.4			
	834.9	802.9	220.6			
	973.2	933.2	275.7			
	1105	105.7	330.8			
Methyl N-acetyl-d-alaninate	5.16*		5.41	1.59	10.82	7.90
	9.42*		27.06	4.17	10.82	8.50
				8.84	27.06	8.50
E_o = 1.003				15.73	16.24	9.00
mg/ml				19.92	21.65	9.00
Ethyl aceturate	6.96	5.04	15.0	1.28	10.0	7.90
	8.12	5.56	20.0	3.16	10.0	8.50
	9.72	6.52	25.0	6.36	25.0	8.50
E_o = 1.015	11.07	7.23	30.0	11.25	15.0	9.00
mg/ml	16.87	10.47	50.0	14.25	20.0	9.00
	29.22	16.42	100			
	37.55	18.35	150			
	46.64	21.04	200			
	55.32	23.32	250			

*velocity at 8 minutes.

The author would like to acknowledge his grateful appreciation to Mr. A. N. Kurtz for providing the program tapes with which the above calculations were made on a Datatron No. 205 computer.

APPENDIX B

Synthesis of compounds considered in text.* ** The order of listing is the same as in table III.

(A) Glycine methyl ester hydrochloride

This compound was synthesized from glycine and methanol by the method of Brenner and Huber (1) in ca. 95% yield and was used without further purification.

(I) Methyl aceturate

Aceturic acid, prepared by the method of Herbst and Shemin (2), was esterified with methanol as per Brenner and Huber (1). The crude product was distilled at 150 μHg and 88°C. The distillate crystallized on standing. It was then recrystallized twice from di-isopropyl ether. The final yield was ca. 80%. The melting point was 57.0-58.7°.

Analysis:	calculated	C: 45.8	H: 6.9	N: 10.7
$C_5H_9NO_3$ (131)	found	C: 45.7	H: 7.0	N: 10.6

(II) Methyl propionurate

Glycine methyl ester hydrochloride (40 gm.) was dissolved

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*All melting points corrected.
**Analyses by Dr. A. Elek.

in 75 ml. of distilled water saturated with NaCl and Na_2CO_3. One hundred fifty ml. of toluene were added and the system was homogenized with a Vibro-stirrer and maintained at 0° with an ice bath. Propionyl chloride (18.7 gm.) was made up to 100 ml. of solution in toluene. This solution was added, 10 ml. at a time, to the homogenized system. Solid Na_2CO_3 was added as needed to maintain the pH above 7. After the last of the solution of the chloride had been added the mixture was stirred an additional 15 min., then transferred to a 500 ml. separatory funnel and allowed to separate into two layers. The toluene layer was removed and reserved. The aqueous layer was extracted three times with 100 ml. portions of ethyl acetate. The aqueous solution was discarded and the ethyl acetate extracts combined with the toluene phase. The solvents were removed in vacuo pressure on a steam cone. The residue, 13 gm. of a light oil, was distilled at 110° and 1.5 mm. Hg. The distillate crystallized on standing and was recrystallized to give long needles from diisopropyl ether. The yield was ca. 55% and the product had a m.p. of 37.0-38.0°.

Analysis:	calculated	C: 49.6	H: 7.6	N: 9.6
$C_6H_{11}NO_3$ (145)	found	C: 49.4	H: 7.4	N: 9.5

(III) Methyl isobutyrurate

Isobutyryl chloride was prepared by refluxing isobutyric acid with thionyl chloride in toluene for one hr. The solution was

cooled, made up to 100 ml. with toluene and used to acylate glycine methyl ester as in II. The product was a light oil which crystallized after distillation at 90° and 300 Hg. Recrystallization from diiso-propyl ether afforded the product in ca. 51% yield as long needles, m.p. 50.8-51.6°.

Analysis:	calculated	C: 52.8	H: 8.2	N: 8.8
$C_7H_{13}NO_3$ (159)	found	C: 52.9	H: 8.3	N: 8.7

(IV) Methyl isovalurate

This compound was prepared as in III using isovaleric acid as the starting material. The product was distilled twice at 96.5° and 300 μHg. The distillate, n^{25} = 1.4481, resisted crystallization.

Analysis:	calculated	C: 55.4	H: 8.7	N: 8.1
$C_8H_{15}NO_3$ (173)	found	C: 55.4	H: 8.7	N: 8.0

(V) Methyl pivalurate

Pivaloyl chloride was obtained by slowly distilling a solution of one mole of pivalic acid dissolved in three moles of benzoyl chloride as according to Brown (3). The acid chloride was used to acylate glycine methyl ester as in II. The product was distilled at 111-112° and 4 mm. Hg. The distillate crystallized after standing at 4° for 5 months but could not be recrystallized except from the melt. The product, n_D^{25} = 1.4492, was obtained in ca. 80% yield.

Analysis:	calculated	C: 55.4	H: 8.7	N: 8.1
$C_8H_{15}NO_3$ (173)	found	C: 55.3	H: 8.6	N: 8.2

(VI) Methyl phenaceturate

Phenacetyl chloride (Eastman) was used to acylate glycine methyl ester as in II. The crude product, obtained in ca. 60% yield, crystallized readily from diisopropyl ether in long needles, m.p. 89.3-90.8°.

Analysis:	calculated	C: 63.7	H: 6.3	N: 6.8
$C_{11}H_{13}NO_3$ (207)	found	C: 63.6	H: 6.2	N: 6.9

(VII) Methyl chloroaceturate

Using chloroacetic acid and the procedure as in V, a crude product was obtained in 90.0% yield. This material was distilled at 105° and 500 μHg to give a distillate which crystallized on standing. Recrystallization from diisopropyl ether gave a product, m.p. 43.6-44.6°.

Analysis:	calculated	C: 36.3	H: 4.8	N: 8.5
$C_5H_8NO_3Cl$ (166)	found	C: 36.4	H: 4.9	N: 8.5

(VIII) Methyl dichloroaceturate

Using dichloroacetic acid and the procedure in III, a crude product was obtained in ca. 50% yield. This material, after three recrystallizations from diisopropyl ether had m.p. 63.1-64.5°.

Analysis:	calculated	C: 30.0	H: 3.5	N: 7.0
$C_5H_7NO_3Cl_2$ (200)	found	C: 30.2	H: 3.6	N: 6.9

(IX) Methyl trichloroaceturate

Using trichloroacetic acid and the procedure as in V, a crude product was obtained in ca. 80% yield. This material was

distilled at 95.6° and 200 μ Hg to give a distillate of n_D^{25} = 1.4953.

Analysis:	calculated	C: 25.6	H: 2.6	N: 6.0
$C_5H_6NO_3Cl_3$ (234)	found	C: 25.5	H: 2.5	N: 5.9

(X) Methyl ethoxyaceturate

Ethoxyacetic acid was synthesized from chloroacetic acid and sodium ethoxide according to the procedure of Rothstein (4). The acid chloride was used to acylate glycine methyl ester as in III. The product was distilled at 97-100° and 300 μ Hg and was obtained in ca. 80% yield. n_D^{25} = 1.4481.

Analysis:	calculated	C: 48.0	H: 7.5	N: 8.0
$C_7H_{13}NO_4$ (175)	found	C: 48.2	H: 7.3	N: 7.9

(XI) Methyl thioethoxyaceturate ($EtSCH_2CONHCH_2CO_2CH_3$)

Thioethoxyacetic acid was prepared from chloroacetic acid and sodium mercaptide according to the procedure of Ramberg (5). The acid chloride was made and used to acylate glycine methyl ester as in III. The product was recrystallized twice from diiso-propyl ether to give the ester, m.p. 60.7-62.7°, in ca. 80% yield.

Analysis:	calculated	C: 44.0	H: 6.8	N: 7.3
$C_7H_{13}NO_3S$ (191)	found	C: 44.0	H: 6.8	N: 7.4

(XII) Methyl nicotinurate

Nicotinyl chloride was synthesized from sodium nicotinate and thionyl chloride in toluene according to the procedure of Meyer and Graff (6). The toluene solution of the acid chloride was filtered, diluted to 100 ml. with toluene and used to acylate glycine methyl ester as follows.

Glycine methyl ester hydrochloride was suspended in anhydrous chloroform, 2.2 equivalents of triethylamine added to the suspension, cooled to 0° in an ice bath, and the chloroform solution now treated with small portions of the acid chloride solution with shaking and cooling between each addition. After all the acid chloride had been added the reaction mixture was allowed to stand at room temperature for one hr., transferred to a 500 ml. separatory funnel and washed three times with dilute aqueous sodium bicarbonate solution saturated with salt. The aqueous washings were discarded and the solvent removed from the chloroform solution in vacuo. The residue was a light oil which crystallized on standing. It was recrystallized from diisopropyl ether to give long needles, m.p. 69.9-71.7°. The yield was ca. 80%.

Analysis:		calculated	C: 55.7	H: 5.2	N: 14.4
$C_9H_{10}O_3N_2$	(194)	found	C: 55.6	H: 5.2	N: 14.3

(XIII) Methyl isonicotinurate

The acid chloride of isonicotinic acid was obtained by refluxing the acid in excess thionyl chloride for 18 hr. The solution was cooled and 200 ml. of dry hexane added to precipitate a brown, microcrystalline substance. When an attempt was made to distill this material in vacuo thionyl chloride distilled leaving a black tar. It was thought that the product obtained might be a molecular addition compound of thionyl chloride and the nicotinyl chloride. Further attempts to purify the material were abandoned and this material was

used to acylate glycine methyl ester as in XII except that 5.2 equivalents of triethylamine were used. The product, after recrystallization from diisopropyl ether, was obtained in ca. 60% yield and had m.p. 112.0-113.5°.

Analysis:	calculated	C: 55.7	H: 5.2	N: 14.4
$C_9H_{10}N_2O_3$ (194)	found	C: 55.7	H: 5.2	N: 14.5

(XIV) Methyl picolinurate

Picolinyl chloride was obtained by refluxing picolinic acid and thionyl chloride in toluene for 24 hr. The solution was diluted to 100 ml. with toluene and used to acylate glycine methyl ester as in XII. The product was an oil which was distilled twice at 123° and 200 μHg. The final product was a colorless liquid. The yield was 20%.

Analysis:	calculated	C: 55.7	H: 5.2	N: 14.4
$C_9H_{10}N_2O_3$ (194)	found	C: 55.5	H: 5.2	N: 14.3

(XV) Methyl 2-furourate

Furoyl chloride (Eastman) was used to acylate glycine methyl ester as in II to give a crude product in 75% yield. Two recrystallizations from diisopropyl ether gave the product in long, slender needles having m.p. 70.1-70.9°.

Analysis:	calculated	C: 52.5	H: 4.9	N: 7.7
$C_8H_9NO_4$ (183)	found	C: 52.6	H: 4.9	N: 7.7

(XVI) Methyl mesylurate

Methane sulfonyl chloride (Eastman) was used to sulfonate glycine methyl ester as in II to a crude product in 90%

yield. This material was distilled at 125° and 350 μHg. The distillate crystallized on standing and had m.p. of 32.0°.

Analysis:	calculated	C: 28.7	H: 5.4	N: 8.4
$C_4H_9NO_4S$ (167)	found	C: 28.7	H: 5.3	N: 8.2

(XVII) Methyl 3-indolylurate (Methyl 3-indolecarboxyglycinate)

Crude 3-indolyl chloride (3-indolecarbonyl chloride) obtained as per Peterson, Wolf and Niemann (7) was used to acylate glycine methyl ester as in XII. The product was obtained in a crude yield of 30% based on indole. Recrystallization from toluene-ethyl acetate gave the product as blunt needles having m.p. 169-171°.

Analysis:	calculated	C: 62.1	H: 5.2	N: 12.1
$C_{12}H_{12}N_2O_2$ (232)	found	C: 61.9	H: 5.3	N: 12.1

(XVIII) Methyl p-aminohippurate

p-Aminohippuric acid (Matheson)was esterified with methanol according to Brenner and Huber (1) in 75% yield. The product was recrystallized from ethyl acetate to give short needles, m.p. 131.9-133.1°.

Analysis:	calculated	C: 57.7	H: 5.8	N: 13.5
$C_{10}H_{12}O_3N_2$ (208)	found	C: 57.6	H: 5.8	N: 13.4

(XIX) Methyl succinimidoacetate

Succinimidoacetic acid was obtained by heating an equimolar mixture of glycine and succinic anhydride on an oil bath at 190° for three hours. The reaction product was cooled, ground and esterified with methanol according to Brenner and Huber (1).

The final product was obtained in ca. 60% yield and after recrystallization from ethyl acetate-diisopropyl ether had m.p. 93.6-95.4°.

Analysis:	calculated	C: 49.1	H: 5.3	N: 8.2
$C_7H_9NO_4$ (171)	found	C: 49.1	H: 5.2	N: 8.3

(XX) Methyl carboethoxyamidoacetate

Ethyl chloroformate (Matheson) was used as in II to treat glycine methyl ester. The product was obtained as an oil which was distilled at 73.1° and 250 μHg. The distillate was a colorless liquid having $n_D^{25} = 1.4367$. The yield was 60%.

Analysis:	calculated	C: 44.7	H: 6.9	N: 8.7
$C_6H_{11}NO_4$ (161)	found	C: 44.8	H: 6.8	N: 8.6

(XXI) Methyl l-pyroglutamate

l-glutamic acid was heated at 170-175° for 1 hr. The product was cooled, ground and esterified with methanol as per Brenner and Huber (1). The ester was distilled at 113° and 200 μHg. $\alpha_D^{23} = +0.91°$ (C = 2% in water).

Analysis:	calculated	C: 50.3	H: 6.3	N: 9.8
$C_6H_9NO_3$ (143)	found	C: 50.4	H: 6.3	N: 9.8

(XXII) Methyl α-acetamidoisobutyrate

α-Aminoisobutyric acid was esterified by the Brenner and Huber method (1) with methanol to give a quantitative yield of the ester hydrochloride. This was acylated with acetic anhydride in chloroform containing two equivalents of triethylamine to give the product in 90% yield. After two recrystallizations from diisopropyl ether the product had m.p. 100.0-100.5°.

Analysis:	calculated	C: 52.8	H: 8.2	N: 8.8
$C_7H_{13}NO_3$ (159)	found	C: 52.7	H: 8.2	N: 8.8

(XXIII) Methyl β-acetamidopropionate

The methyl ester of β-alanine was synthesized by the Brenner and Huber method (1). The methyl ester hydrochloride was treated with acetyl chloride in toluene as in II. The product was obtained in 85% yield as a light oil. This oil was distilled at 93.6° and 300 μHg to give a colorless distillate having n_D^{25} = 1.4537.

Analysis:	calculated	C: 49.6	H: 7.6	N: 9.6
$C_6H_{11}NO_3$ (145)	found	C: 49.5	H: 7.6	N: 9.6

(XXIV) Methyl N-acetyl sarcosinate

Sarcosine methyl ester hydrochloride was prepared and treated with acetyl chloride as in XXIII to give a light oil. This material was distilled at 61° and 110 μHg to give the final product, n_D^{25} = 1.4490, in ca. 50% yield.

Analysis:	calculated	C: 49.6	H: 7.6	N: 9.6
$C_6H_{11}NO_3$ (145)	found	C: 49.5	H: 7.6	N: 9.6

(XXV) Methyl N-phenylglycinate

N-phenylglycine (Matheson) was esterified with methanol by Brenner and Huber's method (1) to give a 60% yield of the desired product. The ester, recrystallized twice from water-ethanol, had a final melting point of 48.1-49.0°. Literature value, m.p. 48° (8).

(XXVI) Methyl N-acetyl-l-alaninate

l-Alanine was esterified with methanol according to the procedure of Brenner and Huber (1). The product was acetylated with acetic anhydride in dilute, aqueous sodium hydroxide. This product was extracted with ethyl acetate and the solvent removed in vacuo. The residue, a light oil, was distilled at 76.0° and 250 μ Hg to give the final product, α_D^{23} = -91.72° (C = 2% in water), in 55% yield.

Analysis:	calculated	C: 49.6	H: 7.6	N: 9.6
$C_6H_{11}NO_3$ (145)	found	C: 49.8	H: 7.7	N: 9.6

(XXVII) Methyl N-acetyl-d-alaninate

N-acetyl-dl-alanine was obtained from dl-alanine and acetic anhydride as in I. The product was esterified with methanol according to Brenner and Huber (1). Thirty gms. of the N-acetyl-dl-alanine methyl ester was added to a solution of 100 mgm. α-chymotrypsin, 2.9 gm. sodium chloride and 6.8 gm. sodium bicarbonate in 200 ml. of distilled water in a loosely stoppered flask. The reaction mixture was held at room temperature for 48 hrs. and then extracted 3 times with 150 ml. portions of ethyl acetate. The ethyl acetate solutions were combined and the solvent removed in vacuo. The product, obtained in 60% yield, was crystallized from diisopropyl ether and found to melt too close to room temperature to permit taking an accurate melting point with the apparatus at hand. The

crystallization solvent was removed by filtering through chilled ware and the crystals washed twice with cold hexane. The product, $\alpha_D^{25} = +85.44^\circ$ (C = 2% in water), melted on standing at room temperature.

(XXVIII) Ethyl aceturate

Glycine ethyl ester hydrochloride (Matheson) was acetylated with acetic anhydride in dilute aqueous sodium hydroxide. The product was extracted with ethyl acetate and the solvent removed in vacuo leaving an oily residue. The residue was distilled at 90° and 300 μHg to give, in 70% yield, a product which crystallized on standing. After recrystallization from diisopropyl ether it had m.p. 47.0-48.5°. Literature value, m.p. 48° (9).

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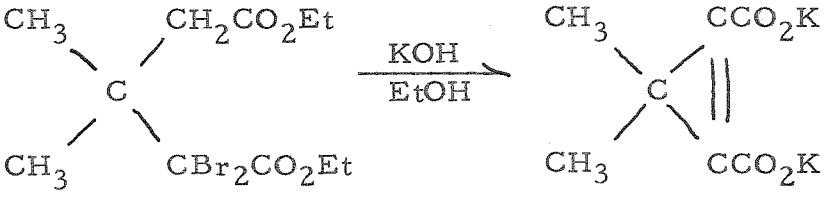
Propositions

I. It is proposed that the postulated structure for the active site of acetylcholinesterase containing separate anionic and cationic sites is untenable because it does not admit of a release mechanism for the choline after the hydrolysis of the ester (1).

II. The compound N_2F_2 is known but its higher homologue, N_2F_4, is not. It is proposed that the higher fluoride can be synthesized by electrolyzing $N_2H_6F_2$ in liquid HF at 0° C. as per the method of Simons (2).

III. Perkin and Thorpe (3) attempted the synthesis of 3,3-dimethyl 1,2-cyclopropenedicarboxylic acid by the following reaction:

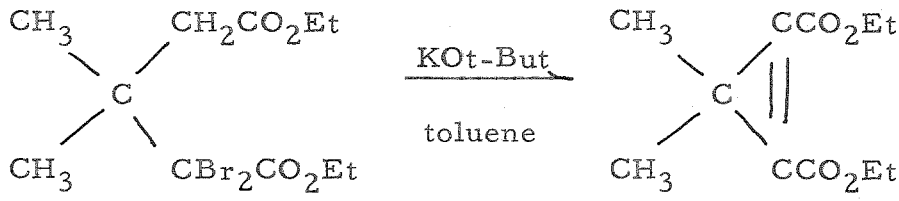


The product they obtained, instead of the cyclopropene, was 3,3-dimethyl-2-ethoxy-1,2-cyclopropanedicarboxylic acid. They suggested that they indeed had formed the cyclopropene first but it had reacted to add solvent across the double bond.

In a similar manner Wiberg, et al. (4) tried to dehydrohalogenate ethyl 2-bromo cyclopropanecarboxylate with potassium

t-butoxide in t-butanol. They obtained, instead, ethyl 2-t-butoxy cyclopropanecarboxylate. The fact that cyclopropane derivatives are relatively inert to S_n1 and S_n2 reactions lends support to the hypothesis that the cyclopropene was an intermediate. Its existence is further substantiated by the results of reactions run in solvent t-butanol-d. Deuterium was found in the 1 and 2 positions of the product.

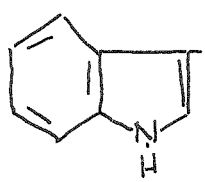
It is proposed that the synthesis of ethyl 3,3-dimethyl-1,2-cyclopropenedicarboxylate can be effected by the following scheme:



By running the reaction in refluxing toluene with t-butoxide one gains the advantages of retarding the addition of alkoxide by steric hindrance and also of azeotroping off the t-butanol as soon as it forms.

IV. It is proposed that in the Brenner and Huber method of esterification (5) the chlorosulfonyl ester is not the alkylating agent but rather it is the dialkylsulfite.

V. It is proposed that α-chymotrypsin be reacted with



where R is $-OC(NH)NH_2$ or $-NHC(NH)OCH_3$

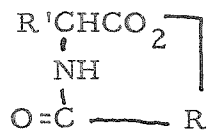
since it has been shown that O-methyl isourea can guanylate chymotrypsinogen (6). The object of this experiment would be to label the active site such that one of its components could be identified.

VI. It is proposed that the enzyme blank reaction observed with α-chymotrypsin is due to the hydrolysis of a small amount of protein artifact present in the enzyme preparation. The large amount of base taken up by this reaction might be due to the hydrolysis product ionizing after the reaction as a result of undergoing a major conformational change.

VII. Schwimmer, et al. (7) have identified melezitose as a trace constituent of White Rose potatoes. It is proposed that their identification is not unambiguous as there is another non-reducing sugar, gluco-sucrose, not considered by them which could easily be confused with melezitose on a chromatogram (8, 9).

VIII. It is proposed that the work of Weil, et al. (10) be repeated to determine whether the 50% photo-inactivated enzyme will react with diisopropylphosphorofluoridate. This experiment will answer the question of whether histidine is involved in this reaction or not when compared with the work of Wood and Balls (11).

IX. It is proposed that a compound of the following type be investigated to determine whether or not it could function as a substrate for α-chymotrypsin.



By varying the size and nature of R one could assess the requirements of the enzyme as to orientation of the ester carbonyl group.

X. C. S. Hudson has suggested, "... It seems improbable that the soft-scale insects or aphids make the sugar (melezitose)." (12) It is proposed that this view is untenable in view of the work of Wolf and Ewart (13).

XI. It is proposed that the Florey factor I (14) from mammalian central nervous tissue is a mixture containing gamma-aminobutyric acid and, in addition, at least two more compounds which can inhibit the crayfish stretch receptor preparation.

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