VARIATION OF THE CONFORMATION OF THE ACTIVE SITE OF α-CHYMOTRYPSIN:

I. HYDROGEN-ION CONCENTRATION STUDIES

II. ELECTROLYTE CONCENTRATION STUDIES

Thesis by

Werner A. Mukatis

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1965

(Submitted December 3, 1964)

To My Parents

ACKNOWLEDGEMENTS

I wish to extend my deepest gratitude and appreciation for the guidance and counsel given me by Professor Carl Niemann.

I thank Dr. George Hein, Dr. J. H. Richards, and Dr. Carole Hamilton for their valuable criticisms and suggestions in the preparation of this thesis.

I am grateful for the financial assistance given me by the California Institute of Technology in the form of scholarships and Teaching and Research Assistantships, to the National Science Foundation for a 1963 Summer Fellowship for Graduate Teaching Assistants, and to E. I. du Pont de Nemours and Company (Inc.) for a Postgraduate Teaching Assistantship.

I cannot express enough thanks to the people who helped make my stay at Cal Tech both stimulating and pleasant.

ABSTRACT

The initial velocities of α-chymotrypsin-catalyzed hydrolyses of acylated amino acid esters follow the rate law

$$v_o = \frac{k_o[E]_o[S]}{K_o + [S]}$$

when the only variables are initial substrate and enzyme concentration. In the above equation, v_o is the initial velocity, $[E]_o$ and $[S]_o$ are initial enzyme and substrate concentration, respectively, and k_o and K_o are a pair of experimentally determined kinetic parameters. Factors such as temperature, ionic strength, hydrogen-ion concentration, structure of substrate, etc., affect the values of k_o and K_o.

Variation in the kinetic parameters k_o, K_o, and k_o/K_o for selected substrates and variations in the ratios of the parameters k_{o1}/k_{o2}, K_{o1}/K_{o2}, and $(k_o/K_o)_1/(k_o/K_o)_2$ for selected pairs of substrates are studied as functions of hydrogen-ion concentration and concentration of added electrolyte.

The results are discussed in terms of possible changes in conformation of the active site of α-chymotrypsin with changing hydrogen-ion concentration and concentration of added electrolyte.

TABLE OF CONTENTS

I. VARIATION OF THE CONFORMATION OF THE ACTIVE SITE OF α-CHYMOTRYPSIN: HYDROGEN-ION CONCENTRATION STUDIES

INTRODUCTION

Fischer (1) in 1894 proposed a theory of enzyme specificity to explain the behavior of yeast toward the stereoisomeric hexoses. He suggested that the combination of the different substrates with one enzyme was analogous to the fitting of a set of different keys to one lock. This idea of a very rigid fit between enzyme and substrate has provided a basis for many studies on the mechanism of enzyme-catalyzed reactions in terms of structural and stereochemical specificity. In some cases, such as the reactions catalyzed by nicotinamide adenine dinucleotide- and nicotinamide adenine dinucleotide phosphate-dependent dehydrogenases, a great deal of progress has been made in understanding the steric course of the reactions by the use of model compounds related to the coenzyme (derivatives of dihydropyridine) and of conformationally determinate substrates (steroids, for example) (2). However, for reactions catalyzed by enzymes requiring no coenzyme, progress generally has not been so great. In the case of the proteinases, nearly universal use of conformationally indeterminate structures has told little about the active site except to confirm its asymmetry (3).

There are a large and ever-increasing number of known proteinases. Similarity of the mechanism of their action has yet to be definitely established although identical peptide fragments associated with the active site of two proteinases, trypsin and α-chymotrypsin, have been isolated (4).

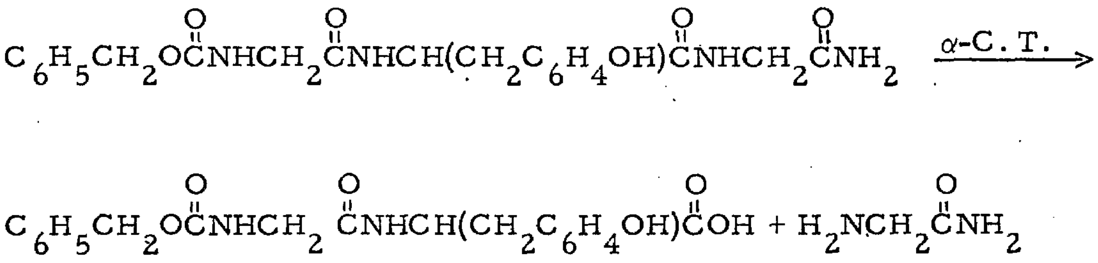
Bovine, pancreatic α-chymotrypsin is a proteinase composed of some 240 amino acid residues. Its three-dimensional structure has not been established, but a primary sequence for its precursor has been proposed (5). The reactions which convert this precursor to α-chymotrypsin are known (6) and involve the splitting of an arginyl-isoleucine bond by trypsin, another pancreatic proteinase, giving rise to π-chymotrypsin. Then, π-chymotrypsin autolyzes, giving rise to δ-chymotrypsin and a dipeptide, serylarginine. The δ-chymotrypsin slowly autolyzes to give α-chymotrypsin and another dipeptide, threonylasparagine.

At present, no mechanism for α-chymotrypsin-catalyzed hydrolyses explains all the known data. Furthermore, a number of important factors such as tertiary structure of the enzyme, presence and function of groups involved in the catalysis (7), relation between model substrates on the one hand and proteins and peptides, the natural substrates of α-chymotrypsin, on the other hand, etc., have yet to be definitely established. These important areas of ignorance have allowed workers in the field to approach the mechanism of α-chymotrypsin-catalyzed hydrolyses from a number of different directions.

Although little work has been done with conformationally determinate substrates, it has proven fruitful to study the steric course of the reaction in terms of structural and stereochemical specificity of the enzyme based on the assumptions of Fischer's lock and key analogy.

One method of approach to the specificity of the enzyme utilizes information from the peptide bonds broken in the partial enzymatic hydrolysis of denatured proteins and polypeptides. Only qualitative information about the structural specificity of the enzyme is gained by this approach. No information concerning the stereochemical specificity can arise from such studies, since proteins generally consist entirely of amino acids of the L-configuration.

The use of model substrates in specificity studies of α-chymotrypsin originated in 1937 when Bergmann and Fruton (8) found that this enzyme catalyzed the hydrolysis of carbobenzyloxyglycyl-L-tyrosylglycinamide at the tyrosylglycyl bond to give carbobenzyloxyglycyl-L-tyrosine and glycinamide.



$$C_6H_5CH_2O\overset{\overset{O}{\|}}{C}NHCH_2\overset{\overset{O}{\|}}{C}NHCH(CH_2C_6H_4OH)\overset{\overset{O}{\|}}{C}NHCH_2\overset{\overset{O}{\|}}{C}NH_2 \xrightarrow{\alpha\text{-C. T.}}$$

$$C_6H_5CH_2O\overset{\overset{O}{\|}}{C}NHCH_2\overset{\overset{O}{\|}}{C}NHCH(CH_2C_6H_4OH)\overset{\overset{O}{\|}}{C}OH + H_2NCH_2\overset{\overset{O}{\|}}{C}NH_2$$

Since then it has been shown that α-chymotrypsin catalyzes the hydrolysis of a large number of low-molecular weight, model substrates, including certain esters, β-keto acids, amides, thiol esters, hydrazides, hydroxamides, small peptides (9), and O^{18} acids (10). Most of these compounds are related to protein structures in that they contain an asymmetric (or potentially asymmetric) center* at the carbon atom alpha to the functional group being hydrolyzed and are of the form $R_1R_2CR_3H$. However, α-chymotrypsin also catalyzes the hydrolysis of substrates such as p-nitrophenyl acetate (11), the dialkylphosphofluoridates (12), derivatives of N,N'-diaryl carbamic acids (13), and esters and amides of trans-cinnamic acid (14).

The kinetic behavior of model substrates has been extensively studied. In general, the initial rates of α-chymotrypsin-catalyzed reactions for model substrates, under conditions where all reaction parameters except enzyme and substrate concentrations are invariant, are described by equation 1.

$$-\frac{d[S]}{dt} = \frac{d[P]}{dt} = \frac{k_o[E]_o[S]}{K_o + [S]} \qquad (1)$$

where [S], [P], and $[E]_o$ are the molar concentrations of substrate, product, and enzyme, respectively; and k_o and K_o are a pair of experimentally determined kinetic parameters. Without regard to mechanism, k_o, K_o, and k_o/K_o may be defined as follows:

---

* except for glycine derivatives

1. $k_o[E]_o$ is the limiting maximum rate at high substrate concentrations.
2. K_o is equal to the substrate concentration at half the maximum velocity.
3. k_o/K_o is the rate constant where substrate concentration is much smaller than K_o.

These definitions, although useful for determining the constants, say nothing about the course of the enzyme-catalyzed reaction. A useful discussion of the variation of the measured parameters must at least start from a proposed mechanism.

From results of kinetic studies on the asymmetric type of model substrates, Huang and Niemann (15) suggested that the substrates might combine with the active site of α-chymotrypsin in several modes and that not all modes lead to hydrolysis. Niemann and coworkers have subsequently developed this idea to explain the structural and stereochemical behavior of a number of model substrates of this enzyme. This has led Hein and Niemann (16a, b) to propose a general theory of the specificity of α-chymotrypsin-catalyzed reactions for model trifunctional substrates of the general formula $R'_1\text{-CONHCHR}_2\text{COR}'_3$ where $R_2 \neq H$.

In its most general form, the theory states that the four groups disposed about the asymmetric center of trifunctional substrates may combine with four complementary loci ρ_1, ρ_2, ρ_3, and ρ_H located at the active site of the enzyme. R_i need not necessarily combine with ρ_i, but, in general, all R_i-ρ_j interactions which do not cause an inversion of configuration in the substrate are possible. The enzyme-substrate complexes formed in this manner are assumed to be fully competitive with one another. Some combinations lead to hydrolysis (productive complexes), while others do not (non-productive complexes).

A representation of the kinetic scheme incorporated in the theory is:

$$E + S \underset{}{\overset{k_{1i}}{\rightleftarrows}} (ES)_i \xrightarrow{k_{2i}} E + P \tag{2}$$

where E, S, and P are again enzyme, substrate, and product, and $(ES)_i$ is one of the fully competitive enzyme-substrate complexes. The rate equation derived from a steady-state treatment of the scheme above is of the same form as equation 1. The interpretation of the kinetic parameters will be given in the Results and Discussion section.

Nine basic postulates are associated with this mechanism. Some are generalizations of large amounts of data while others are necessary for a successful application of the theory. These postulates will be applied in the Results and Discussion section.

The theory in its present form satisfactorily accounts for the structural and stereochemical specificity for both L- and D- trifunctional substrates, where only enzyme and substrate concentration are varied, even in cases where the D-enantiomer is hydrolyzed more rapidly than its optical antipode (18).

Wolf and Niemann (19) have utilized this theory to interpret the behavior of bifunctional substrates ($R'_1-CONHCH_2COR'_3$). Although the theory adequately accounts for the behavior of the bifunctional substrates, more ambiguity exists in the interpretation because of the greater number of degrees of freedom of those compounds. A study by Rapp (20) of a series of acylated glycine and D- and L- alanine methyl esters partially clarifies the relationships between bi- and trifunctional substrates. This theory, modified to incorporate pH effects, will form the basis for discussion of the results of this study.

It is known that reaction parameters other than substrate structure, such as hydrogen-ion concentration, ionic strength, temperature, etc., may have marked effects on the rate of enzyme catalyzed reactions (9). In developing the theory, Hein and Niemann were concerned with the variation of substrate structure (including stereochemistry), while other reaction parameters were held constant. The studies reported here are mainly concerned with the variation of hydrogen-ion concentration while the structures of substrates and selected pairs of substrates remain unchanged. However, the use of several pairs of substrates also yields information for the case of simultaneous variation of substrate structure and pH.

In the many studies made on the effect of hydrogen-ion concentration on α-chymotrypsin-catalyzed reactions, little consideration has been given to the dependence of stereochemical and structural specificity on this parameter. Most studies have been carried out on substrates and inhibitors where variation in structural and stereochemical factors might be masked by other effects. The substrates used in this study consisted of pairs of acylated-D- and L-alanine methyl esters and one pair of L-substrates, α-N-acetyl-O-methyl-L-tyrosine methyl ester and α-N-benzoyl-L-alanine methyl ester. The pairs of enantiomers were chosen to focus attention on changes in stereochemical specificity while the pair of L-substrates focuses attention on changes in structural specificity of the enzyme. Variations in the kinetic parameters k_o and K_o and the ratio k_o/K_o for single substrates, as well as in the ratios k_{o_1}/k_{o_2}, K_{o_1}/K_{o_2}, and $\frac{(k_o/K_o)_1}{(k_o/K_o)_2}$ for the selected pairs were studied as functions of pH.

In principle, a change in pH may affect the substrate, the free enzyme, or the enzyme-substrate complexes (9). Substrates were chosen for which hydrogen-ion effects on the structures of the substrates in solution are negligible over the pH range studied. Thus, attention is brought to bear on possible changes in the free enzyme and enzyme-substrate complexes.

In studying variations in the ratios of the kinetic parameters for selected pairs of substrates, effects caused by different ground states of the substrates are constant for the pair of <u>L</u>-substrates, or zero for the pairs of optical antipodes. By using ratios, effects attributable to changes in the free enzyme cancel exactly; therefore, variations in the magnitude of these ratios with pH for any selected pair of substrates can be attributed entirely to non-identical changes in each of the two sets of enzyme-substrate complexes. This method does not establish whether changes occur before, during, or after combination of the substrate with the enzyme. It can show, however, that the enzyme and/or enzyme-substrate complexes have changed and that this change is kinetically important.

A change in hydrogen-ion concentration may alter the enzyme or enzyme-substrate complexes in three ways:

1. Localized ionization of groups at the active site[*] may

---

[*] The active site is defined as those portions of the enzyme which contribute to the promotion of substrate to products and which include an active center where bond breaking and formation takes place. (See ref. 9, pp. 1288-89.)

take place (21).

2. The active site may undergo a hydrogen-ion induced conformational alteration (22).
3. The two above effects may occur simultaneously.

Theoretical treatments of the subject have concentrated on the first of these possibilities. In an exact steady-state treatment, Laidler (23) has considered the enzyme and enzyme-substrate complex, each in three states of ionization, and has discussed thoroughly the assumptions needed to reduce the resulting kinetic equations to a simpler form. Bender *et al*. (24) have used a slightly modified form of this treatment for two enzyme-substrate complexes in series, and Peller and Alberty (25) have applied a similar treatment to the case where an arbitrary number of intermediates in series is considered. These theoretical treatments and the use of limit-type substrates in experimental studies have focused attention on the effects of ionization of groups at the active site while diverting attention from possible hydrogen-ion induced conformational changes at the active site.

The idea that the conformation of the active site of an enzyme may be altered by various reaction parameters is relatively new. Lumry and Eyring (26), and more recently, Koshland (27), have proposed theories in which the substrate produces a conformational change at the active site. Evidence by Hess and coworkers (28), based on kinetic, chemical, spectroscopic, and optical rotatory dispersion studies, indicates that this may occur in α-chymotrypsin-catalyzed hydrolyses.

Since α-chymotrypsin is a polyampholyte and appears to have charged groups at the active site (29-32), it is reasonable to suppose that protonation or deprotonation of these groups may cause a change in conformation of the active site. Support for this idea was recently obtained by Platt and Niemann (33), who found that the nature of the inhibition process is pH-dependent for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-leucine methyl ester when either formanilide or benzamide is used as an inhibitor. The studies reported here were designed to amplify such information.

RESULTS AND DISCUSSION

I. General

The kinetic scheme used in the Hein-Niemann theory of multiple binding modes may be represented as:

$$E + S \underset{k_{-1i}}{\overset{k_{1i}}{\rightleftharpoons}} (ES)_i \xrightarrow{k_{2i}} E + P \tag{2}$$

where E, S, and P are enzyme, substrate, and product, respectively, and $(ES)_i$ is one of twelve possible complexes an asymmetric substrate ($R'_1CONHCHR_2COR'_3$) may form with four complementary loci (ρ_1, ρ_2, ρ_3, and ρ_H) at the active site of the enzyme. A steady-state treatment of the above scheme yields a rate equation identical in form to equation 1. The expressions for the measured kinetic parameters are:

$$k_o = \frac{\sum_{i=1}^{3} k_{2i}/K_{si}}{\sum_{i=1}^{12} \frac{1}{K_{si}}} \tag{3}$$

$$K_o = \frac{1}{\sum_{i=1}^{12} \frac{1}{K_{si}}} \tag{4}$$

$$\frac{k_o}{K_o} = \sum_{i=1}^{3} k_{2i}/K_{si} \tag{5}$$

$$\text{where } K_{si} = \frac{k_{2i} + k_{-1i}}{k_{1i}} \tag{6}$$

Notice that i = 1, 2, 3 for the terms containing k_{2i} in equations 3 and 5 instead of i =1,2, . . . , 12. This arises from postulate 1 (16b) which states that only complexes which have a COR_3-ρ_3 interaction are potentially productive. A number of studies (34) have suggested that two or more groups are necessary for catalysis of hydrolysis. It is unlikely that these groups would be available in the proper orientation at more than one locus. Consequently, $k_{2i} \cong 0$ for $i > 3$.

Representations of the binding modes for L- and D-substrates of the type $R'_1CONHCHR_2COR'_3$ are presented in Figures 1 and 2, respectively. The potentially productive modes are represented by Figures 1-1, 1-2, and 1-3 for the L-substrates, and Figures 2-1, 2-2, and 2-3, for the D-substrates.

In order to treat effects of pH on enzyme-catalyzed reactions, Laidler (23) has applied an exact steady-state treatment to the case where both the free enzyme and the enzyme-substrate complex exist in three states of protonation, and has discussed fully the assumptions needed to reduce the resulting rate equation to a usable form. This scheme as applied to the case of multiple, competitive complexes, is represented as follows:

$$\begin{tikzcd}
E + S \arrow[r, shift left, "k'_{1i}"] \arrow[d, shift right, "k_{-ae}"'] & (ES)_i \arrow[l, shift left, "k'_{-1i}"] \arrow[r, "k'_{2i}"] \arrow[d, shift right, "k_{-aesi}"'] & E + P & \\
EH + S \arrow[u, shift right, "k_{ae}"'] \arrow[r, shift left, "k_{1i}"] \arrow[d, shift right, "k_{-be}"'] & (EHS)_i \arrow[u, shift right, "k_{aesi}"'] \arrow[l, shift left, "k_{-1i}"] \arrow[r, "k_{2i}"] \arrow[d, shift right, "k_{-besi}"'] & EH + P & (7) \\
EH_2 + S \arrow[u, shift right, "k_{be}"'] \arrow[r, shift left, "k''_{1i}"] & (EH_2S)_i \arrow[u, shift right, "k_{besi}"'] \arrow[l, shift left, "k''_{-1i}"] \arrow[r, "k''_{2i}"] & EH_2 + P &
\end{tikzcd}$$

where E, EH, and EH_2 represent different states of protonation of the free enzyme, and where $(ES)_i$, $(EHS)_i$, and $(EH_2S)_i$ are different states of protonation of the enzyme-substrate complexes. No formal charges are used on any species since the net overall charge at the active site is unknown.

The complete steady-state treatment yields, as expected, a rate equation too complex to interpret. If three assumptions are made, the rate equation can be reduced to the same form as equation 1, where the measured kinetic parameters are:

$$k_o = \left(\sum_{i=1}^{3} k_{2i}/K_{si} \right) / \sum_{i=1}^{12} \frac{1}{K_{si}} \left(1 + \frac{K_{aesi}}{[H]} + \frac{[H]}{K_{besi}} \right) \quad (8)$$

$$K_o = \frac{\left(1 + \frac{K_{ae}}{[H]} + \frac{[H]}{K_{be}}\right)}{\sum_{i=1}^{12} \frac{1}{K_{si}} \left(1 + \frac{K_{aesi}}{[H]} + \frac{[H]}{K_{besi}}\right)} \quad (9)$$

$$k_o/K_o = \left(\sum_{i=1}^{3} \frac{k_{2i}}{K_{si}} \right) / \left(1 + \frac{K_{ae}}{[H]} + \frac{[H]}{K_{be}}\right) \quad (10)$$

The constants in this scheme are defined as:

$$K_{ae} = \frac{k_{ae}}{k_{-ae}} = \frac{[E][H]}{[EH]} \quad (11)$$

$$K_{be} = \frac{k_{be}}{k_{-be}} = \frac{[EH][H]}{[EH]_2} \quad (12)$$

$$K_{aesi} = \frac{k_{aesi}}{k_{-aesi}} = \frac{[ES]_i [H]}{[EHS]_i} \quad (13)$$

$$K_{besi} = \frac{k_{besi}}{k_{-besi}} = \frac{[EHS]_i [H]}{[EH_2S]_i} \quad (14)$$

$$K_{si} = \frac{k_{-1i} + k_{2i}}{k_{1i}} \tag{15}$$

$$K'_{si} = \frac{k'_{-1i} + k'_{2i}}{k'_{1i}} \tag{16}$$

$$K''_{si} = \frac{k''_{-1i} + k''_{2i}}{k''_{1i}} \tag{17}$$

The first assumption needed to obtain a rate equation in the form of equation 1 is that $k_{2_i} >> k'_{2_i}$ and k''_{2_i}. Actually, little is known about the state of ionization of the active site for optimal activity (25). However, this assumption is sufficient to account for the bell-shaped curves obtained for α-chymotrypsin when maximum velocity is plotted against pH (35). A second assumption is that ionizations are rapid with respect to uptake and elimination of substrate and product. If this is true, it can be shown (35) that if either k''_{2_i} or k'_{2_i} are significant, the rate law will lead to a limiting velocity in strong acid or strong base, respectively. No evidence is available on ionization rates of α-chymotrypsin and its complexes with substrates. However, Laidler (35), using methyl hydrocinnamate and Bender _et al_. (24), using α-N-acetyl-_L_-tryptophan ethyl ester, have shown that the bell-shaped curves of rate vs. pH are nearly symmetrical. Since there is no _a priori_ reason to expect the rate involving the "doubly protonated" species to be similar to that of the "non-protonated" species, such symmetry suggests that neither k'_{2_i} nor k''_{2_i} is important.

A third assumption needed to obtain a rate law in the form of equation 1 is that $k_{2_i} << k_{-1_i}$. This implies that K_{s_i} is an equilibrium constant, i.e., that $K_{si} = \frac{k_{-1_i} + k_{2_i}}{k_{1_i}} \simeq \frac{k_{-1_i}}{k_{1_i}}$. Ample support for equilibrium between free enzyme and its complexes with substrates has been given by Neurath and Hartley (36), Laidler (35), and Bender et al. (37).

II. Variation of K_o, k_o, and k_o/K_o for Individual Substrates

A. Variation in K_o

The values of K_o obtained in this study for six pairs of enantiomeric α-N-acyl-alanine methyl esters are listed in Table I and plotted vs pH in Figures 3 through 8. For the one pair of L-substrates, α-N-acetyl-O-methyl-L-tyrosine methyl ester and α-N-benzoyl-L-alanine methyl ester, the values of K_o are listed in Table II and plotted vs pH in Figure 9.

In comparing the data obtained in this study to the limited data available in the literature, certain differences between the two sets of values are evident. Hammond and Gutfreund (38), using acetyl-L-phenylalanine methyl ester as a substrate of α-chymotrypsin, found that K_o was pH-independent from pH 6.5 to 8.0, and that it began to rise above pH 8.0. Laidler and Barnard (39), using methyl hydrocinnamate, found K_o to be constant from pH 7.0 to 8.5, the entire range studied. Cunningham and Brown (40) found K_o values for α-N-acetyl-*L*-tryptophan ethyl ester of 0.093 ± 0.02 mM and 0.084 ± 0.02 mM at pH's of 8.0 and 6.1, respectively. These last two values, although constant within the stated error, do not establish the behavior of K_o between the two pH's given. Gordon (41), using methyl hippurate, concluded that K_o is pH-independent from pH 6.0 to *ca.* pH 8.0, but that it rises rapidly above pH 8.0. The scatter of data in the "pH-independent" range makes this conclusion questionable. More recently, Bender *et al.* (24) have stated that, from pH 5.0 to *ca.* pH 9.0, K_o for α-N-acetyl-*L*-tryptophan ethyl ester is constant and then begins to rise rapidly. The curve of K_o vs pH indicates, however, that there may be a very slight minimum around pH 7. If the slight minimum in K_o for this latter case is ignored, the results from the literature are all compatible with each other. In every case, K_o is independent of hydrogen-ion concentration at low pH. As the pH is increased above 8.0, the values of K_o begin to rise (not all at the same pH) in every case except for methyl hydrocinnamate.* However, for

* Methyl hydrocinnamate was run in 30% methanol solution, whereas all other substrates were run in water. The effects of adding organic solvents to the reaction mixture are not completely

this exception, K_o was not obtained above pH 8.5. An eventual rise in K_o at high pH for methyl hydrocinnamate may occur which would be consistent with the other data from the literature and most of the results obtained in the present study.

In the experiments reported in this thesis, thirteen different substrates were used. One, benzoyl-*L*-alanine methyl ester, was run at an ionic strength of 0.10 as one member of the pair of *L*-substrates and at an ionic strength of 1.00 as one member of a pair of enantiomers, making a total of fourteen cases. In every one of the fourteen cases, a variation in K_o with pH is seen below pH 8.0. In nine of the fourteen cases, K_o exhibits a clear minimum. The five that do not are:

1. α-N-benzoyl-*L*-alanine methyl ester at an ionic strength of 1.00
2. α-N-nicotinoyl-*L*-alanine methyl ester
3. α-N-isonicotinoyl-*L*-alanine methyl ester
4. α-N-nicotinoyl-*D*-alanine methyl ester
5. α-N-furoyl-*D*-alanine methyl ester

For the three *L*-substrates which do not show a clear minimum, K_o *decreases* with *decreasing* pH, and then levels off. For the two

---

understood, and it is possible that the results for methyl hydrocinnamate may not be comparable to the other results.

D-substrates, K_o decreases with increasing pH, and then levels off. It cannot be said for certain that these five cases would each exhibit a minimum if the pH range of investigation were extended, but it is suggested that they would for the following reasons.

Four of the five substrates reported in the literature and all substrates studied here (except the two D-substrates listed above) show an increase in K_o with increasing pH above some pH. Consequently, if the pH range were extended to higher values, a rise in K_o above some pH for these two D-substrates would be expected and would be consistent with the other twelve cases studied here and four of the five cases cited from the literature.

In the present study, all six D-substrates and the five L-substrates which show a clear minimum eventually show an increase in K_o with decreasing pH. Furthermore, in four cases, the minimum occurs around pH 7. The pH range for the three L-substrates not showing a clear minimum was from pH 7.0 to 8.5. If the pH had been lowered below 7.0, a rise in K_o would be expected and would be consistent with the behavior exhibited in the other eleven cases.

It has been suggested (16a, b, 20) for benzoyl-D- and L-alanine methyl esters that only one binding mode is important. These modes are represented in Figures 2-1 and 1-3, respectively. If it is assumed that all the acyl-alanine methyl esters in the present study will have the same predominant binding modes, equation 9 reduces to the following expressions for the D- and L-enantiomers, respectively.

$$K_{oD} = \frac{K_s^{2-1}\left(1 + \frac{K_{ae}}{[H]} + \frac{[H]}{K_{be}}\right)}{\left(1 + \frac{K_{aes}^{2-1}}{[H]} + \frac{[H]}{K_{bes}^{2-1}}\right)} \tag{18}$$

and

$$K_{oL} = \frac{K_s^{1-3}\left(1 + \frac{K_{ae}}{[H]} + \frac{[H]}{K_{be}}\right)}{\left(1 + \frac{K_{aes}^{1-3}}{[H]} + \frac{[H]}{K_{bes}^{1-3}}\right)} \tag{19}$$

The superscripts in equations 18 and 19 replace the "i" subscripts in the general equation and refer to the particular mode under consideration as represented in Figures 1 and 2.

The minima in the K_o-pH profiles may be due to two ionizing groups in the enzyme being involved in binding of the substrate. Dixon (42) has shown that a plot of pK_o^* vs pH may consist of sections of intersecting straight lines. This may be seen from equations 18 and 19 by considering different K/[H] or [H]/K terms important at different pH's. The intersections of these lines

---

* $pK_o \equiv -\log K_o$

correspond to pK values for dissociation of protons from the free enzyme and enzyme-substrate complexes. This treatment, if applied to the data obtained in this study, would yield a spectrum of pK values, since the minima vary by over one-half of a pH unit for the cases where a clear minimum appears, and by as much as one pH unit for the nicotinoyl enantiomers, which do not show a clear minimum.

Furthermore, the idea of two ionizable groups being involved in binding of the substrate is not consistent with the cases from the literature which show a pH-independent range for K_o at low pH and a rise in K_o with increasing pH above pH 8. This latter type of behavior indicates that only one ionizable group is involved in complex formation in these cases. Since there is no a priori reason why some of the substrates should be bound by two ionizable groups and others by one, especially for similar substrates such as N-acetyl-L-phenylalanine ethyl ester and N-acetyl-O-methyl-L-tyrosine methyl ester, it is reasonable to assume that only one ionizable group is involved in complex formation, and that the rise in K_o above pH 8 is associated with deprotonation of this group. The behavior of K_o below pH 8 could be explained by saying that, for the cases where K_o is pH-independent, $K_{be} = K_{bes}$ (see equations 18 and 19), and where K_o shows a minimum, $K_{be} \neq K_{bes}$, This again seems unreasonable, especially for the similar substrates mentioned above which show different behavior below pH 8.

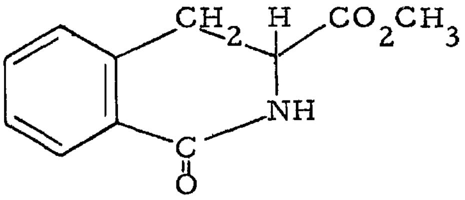
A plausible explanation of the pH behavior of all substrates of α-chymotrypsin discussed is that the variation in K_o with pH is due, at least in part, to a hydrogen-ion-induced conformational change at the active site which alters its structural and stereochemical specificity (43). The minimum would then correspond to the "best" fit of the substrate in one mode or to the optimum distribution of the substrate between two or more modes on an enzyme whose active site is changing conformation with pH.

Three of the five cases from the literature will not be discussed. These cases are methyl hippurate (41), methyl hydrocinnamate (39), and the two values for α-N-acetyl-L̲-tryptophan ethyl ester reported by Cunningham and Brown (40). The first is rejected because of the scatter in the data. The second is rejected because it was run in 30% methanol solution, and the third because only two points are available. The two remaining cases are acetyl-L̲-phenylalanine methyl ester (38) and acetyl-L̲-tryptophan ethyl ester (24).

Acetyl-L̲-phenylalanine methyl esteris a "kinetically good" substrate with high k_o and low K_o values (44, 45, 46). The low K_o value indicates that the substrate is bound well, and the high k_o value indicates that it is bound almost exclusively in a productive mode (16a,b). According to the Hein-Niemann theory, this mode would be represented by Figure 1-1 (16a,b).

For this compound, optimally bound in one mode having no polar or sterically large functionalities on the R_2 group, it is suggested that changes in the conformation of the active site, unless very large, would have a negligible effect either on altering the "best" fit or on changing the distribution of substrate among several modes if one mode predominates to the virtual exclusion of all others. Furthermore, there are no a priori reasons to expect a significant amount of R_i-ρ_j combinations for this substrate. It is only when certain features characteristic of one R group in a "kinetically good" substrate are replaced with or exchanged for features in another R group that significant occurrence of "wrong-way" binding is postulated (16a,b, 20).

The case of α-N-acetyl-L-tryptophan ethyl ester may be an example where a small amount of "wrong-way" binding occurs. Bender et al.(24) concluded that below a pH of about 9.0, K_o is pH-independent; however, from the curve of K_o vs pH, it appears that there is a slight minimum around pH 7. It is possible that the nitrogen on the indolyl group of α-N-acetyl-L-tyrptophan ethyl ester is attracted to a sublocus in ρ_1, possibly to a sublocus at which the acylamino nitrogen of "kinetically good" substrates usually binds. When indole is used as an inhibitor for methyl hippurate (47) and for L-3-carbomethoxydihydroisocarbostyril(I) (16b), the inhibition



I

is mixed (between fully competitive and non-competitive) for the former and non-competitive for the latter substrate. These types of inhibition are normally interpreted in terms of ternary complex formation (33), i.e., complexes composed of substrate, indole, and enzyme. It is expected that methyl hippurate is bound predominantly as represented in Figure 1-3. This places the small hydrogen atom in ρ_1 and may leave open that sublocus in ρ_1 where the nitrogen of indole can combine. It is possible for D- and L- (I), that the L-derivative is also bound in a manner which leaves this same sub-locus in ρ_1 open, whereas for the D-derivative, which is fully competitive with indole, the amido group may be occupying this sublocus. In the case under discussion, α-N-acetyl-L-tryptophan ethyl ester, the indolylmethyl group (R_2) may be distributed between ρ_1 and ρ_2 (Figures 1-1 and 1-2). The lower value of k_o for this substrate as compared to acetyl-L-phenylalanine methyl ester is not inconsistent with this idea since "wrong-way" binding would be expected to lower k_o. A change in conformation of the active site with changing pH might alter the individual K_{si} values with a concomitant change in the distribution of the substrate between the two modes where the indolyl group is in ρ_1 and ρ_2, respectively.

An alternate explanation of the slight minimum in K_o for acetyl-L-tryptophan ethyl ester is that virtually only one mode of binding occurs (all $R_i\rho_i$ interactions), but that the indolyl group, which is large (as compared to the phenyl group of phenylalanine), interacts either sterically or electronically with a sublocus in ρ_2. If a change in pH alters the conformation of ρ_2, or if the sublocus in ρ_2 contains an ionizable group, the K_o value may be altered. Which of these two effects, if either, would be more important is not known and could depend on the steric and electronic demands of the substrate.

In the present study, the plot of K_o vs pH for α-N-acetyl-O-methyl-L-tyrosine methyl ester also exhibits a minimum (Figure 9). This substrate, like acetyl-L-phenylalanine methyl ester, is considered a limit case in which virtually only $R_i\rho_i$ interactions occur (Figure 1-1). In this case, a simple interpretation of the data is that the O-methyl group interacts either sterically or electronically with a sublocus in ρ_2. A change in conformation might be expected to produce a change in K_o. By the use of various N-acetyl-ring-substituted-*L*-phenylalanine methyl esters, Peterson (48) has shown that K_o for these substrates may be quite different from K_o for acetyl-*L*-phenylalanine methyl ester. The variation in K_o as the structure of R_2 is modified has been interpreted in terms of a number of subloci in ρ_2 which are both steric and electronic. Zeller (49) has used a similar idea to explain the variations in K_o for different substrates of monoamino oxidase. The validity of these interpretations will have to await a more complete understanding of the nature of the binding forces associated with the various loci at the active site.

The increase in K_o above some pH has been interpreted by Bender *et al*. (24) in terms of a kinetic scheme represented as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{P_1}{\underset{+}{ES'}} \xrightarrow{k_3} E + P \tag{20}$$

Multiple, competitive binding modes were not considered.

This scheme has been proposed for the case where ES' is an acyl-enzyme intermediate (36) but need not be restricted to this interpretation. A steady-state treatment of equation (20) yields for the kinetic parameters:

$$K_o = k_3K_s/k_2 + k_3 \tag{21}$$

$$k_o = k_3k_2/k_2 + k_3 \tag{22}$$

$$k_o/K_o = k_2/K_3 \tag{23}$$

$$K_s = \frac{k_{-1} + k_2}{k_1} \simeq \frac{k_{-1}}{k_1} \tag{24}$$

For esters, Bender postulated that k_3 (deacylation of the enzyme) is rate-determining at low pH and that as the pH is raised, a point is reached where k_2 (acylation of the enzyme) becomes rate-determining. Consequently, at low pH, K_o reduces to k_3K_s/k_2, and at high pH, K_o begins to rise and to approach K_s. Although the existence of an acyl-enzyme as an intermediate in all α-chymotrypsin-catalyzed reactions has not been definitely established (9, 50), the above scheme could partly explain the rise in K_o for esters at high pH; it does not, however, explain the eventual rise in K_o for substrates used in the present study as the pH is decreased, unless the value of K_o changes with pH. If K_o does change with pH, this is most simply interpreted in terms of an enzyme whose active site is changing conformation.

One final observation is that the variations in K_o over the pH range studied are not large enough to negate conclusions drawn by Hein and Niemann (16a, b) concerning the structural and stereochemical specificity of α-chymotrypsin at the pH optimum. Therefore, if the active site is changing conformation as hydrogen-ion concentration is varied, the changes, though kinetically significant, do not appear to be extremely large.

B. Variation in k_o

The values for k_o are listed in Table I and plotted vs pH in Figures 10 through 15 for the pairs of enantiomers; for the pair of L-substrates, the values are listed in Table II and plotted vs pH in Figure 16.

From these figures, it can be seen that all substrates except two exhibit a sigmoid profile. This sigmoid profile is consistent with earlier work (51, 40). However, Laidler (35), using methyl hydrocinnamate,* and more recently Bender et al. (24), using α-N-acetyl-L-tryptophan ethyl ester, have shown that if the pH range is extended to high enough values, k_o reaches a maximum and then decreases with increasing pH. Whether k_o for the substrates used in this study will decrease at a high pH is not known.

It has been suggested (16, a, b, 20) that for α-N-benzoyl-D-alanine methyl ester, only one mode has a significant k_o, and that

---

* It is reemphasized that this substrate was run in 30% methanol, and comparisons between this substrate and those run in water may not be valid.

this mode is also the predominant binding mode (see Figure 2-1). If this mode is assumed for all the acyl-D-alanine methyl esters used in the present study, equation 8 reduces to equation 25, where

$$k_{oD} = \frac{k_2^{2-1}}{\left(1 + \frac{K_{aes}^{2-1}}{[H]} + \frac{[H]}{K_{bes}^{2-1}}\right)} \qquad (25)$$

superscripts again refer to particular modes represented in Figures 1 and 2 .

For α-N-benzoyl-L-alanine methyl ester it has been suggested (16a, b, 20) that the predominant binding mode is the one represented in Figure 1-3, and the only mode with a significant k_o is the one represented in Figure 1-1. If this is assumed for all the acyl-L-alanine methyl esters in the present study, equation 8 reduces to equation 26.

$$k_{oL} = \frac{k_2^{1-1} \dfrac{K_s^{1-3}}{K_s^{1-1}}}{\left(1 + \frac{K_{aes}^{1-3}}{[H]} + \frac{[H]}{K_{bes}^{1-3}}\right)} \qquad (26)$$

Assuming that k_{2_i} and K_{si} terms are true constants, equations 25 and 26 predict bell-shaped pH-dependencies for k_{oD} and

k_{oL}. This, as was mentioned earlier, has been observed by Laidler (35) using methyl hydrocinnamate and Bender et al. (24) using α-N-acetyl-L-tryptophan ethyl ester as substrates of α-chymotrypsin. The sigmoid shapes of the pH profiles of k_o for substrates used in the present study can be interpreted as the lower half of such bell-shaped curves. Consequently, the curves may be explained by a simple protonation and deprotonation of catalytically active groups in the active site. No information is obtained as to whether conformational changes of the active site are also occurring.

The lack of pH-dependence of k_o for two substrates, α-N-nicotinoyl-D-alanine methyl ester and α-N-isonicotinoyl-D-alanine methyl ester is not easily explained. One possibility is that these two substrates, which are the least reactive trifunctional substrates for which individual kinetic parameters have been obtained, are hydrolyzed by a different mechanism than most trifunctional substrates—a mechanism not involving catalytic groups ionizing in the pH range studied. All substrates could be hydrolyzed partly by this mechanism, for if this rate is small compared to the rate of the normal path, it would not be observed for most substrates. Caplow and Jencks (50), using hydroxylamine as an analog for water in the α-chymotrypsin hydrolysis and hydroxylaminolysis of derivatives of α-N-acetyl-L-tyrosine, have suggested that more than one mechanism may be operating. It is possible, for example, that direct attack by "activated" water competes to a small extent with an acyl-enzyme intermediate in normal reactions.

Whether k_o at low or high pH's approaches zero or some limiting pH-independent rate can be determined in principle by extending the pH range of investigation. However, if this is done, one runs into the problem of enzyme aggregation at low pH (52) and of enzyme denaturation at high pH (24).

Another interpretation of the lack of pH-dependence of k_o for the above two substrates might be that, for very poor substrates, the variation in k_o with pH falls within the large experimental error. However, this does not appear to be a satisfactory explanation. It can be seen in Table V that for each of the six pairs of enantiomers studied, the variation in k_o with pH is greater for the L-compound than for its optical antipode even in the picolinoyl case where the D-isomer has a higher k_o value than its L-enantiomer.

It can also be seen from Table I that for D-enantiomers containing a hetero atom in the α-position of the ring in R'_1, k_o at any pH decreases in the order of decreasing basicity of the hetero atom, i.e., k_o decreases in the order picolinoyl > 2-furoyl > 2-thiophenoyl $\simeq$ benzoyl. Rapp (20) first noticed this behavior in his studies of these compounds at pH 7.90. One possible explanation is that the hetero atom is attracted to a particular sublocus in ρ_2 which orients the substrate for catalysis. If the ring nitrogen of the nicotinoyl and isonicotinoyl derivatives were attracted to the same sublocus, then the order of reactivity: picolinoyl $\gg$ nicotinoyl $>$ isonicotinoyl indicates that as the hetero atom is moved away from the α-position, it disorients the substrate for normal catalysis, and one sees mainly a pH-independent rate due to a competitive pathway, as mentioned earlier. If the hetero atom is attracted to a sublocus in ρ_2, it might also be expected that the value of K_o at any pH for these D-enantiomers would also decrease with respect to K_o for α-N-benzoyl-D-alanine methyl ester since the attraction for the sublocus must be stronger than the normal attraction of ρ_2 for nonpolar side chains, but this is not observed. All values of K_o for D-substrates with hetero atoms in the ring are higher than for the benzoyl derivative under comparable conditions. However, it would also be expected that, due to solvation, the ground state energies of substrates with rings containing hetero atoms would be lower in water than the benzoyl derivative (53), and the higher K_o's for the former substrates may be partly a reflection of this. A similar argument may be made for the fact that the K_o values for the L-enantiomers with a hetero atom in the ring are also larger than K_o for α-N-benzoyl-L-alanine methyl ester.

The fact that one sees a possible correlation of basicity of the hetero atom with k_{oD} but not with K_{oD}, or K_{oL}, may be rationalized from equations 18, 19, and 25. Only in equation 25 (the expression for k_{oD}) do K_{si} terms cancel. Consequently, if factors which stabilize the ground state of substrates in solution also tend to stabilize the enzyme-substrate complex, the effect of the hetero atom would tend to cancel, and other factors could predominate.

Any interpretation of the variation in k_o values at a single pH for the L-enantiomers (except for α-N-acetyl-O-methyl L-tyrosine methyl ester) is complicated because the presumed best binding mode is probably not a productive mode. For the L-enantiomers at one pH

$$k_{oL} = f[H^+] \frac{k_2^{1-1} K_s^{1-3}}{K_s^{1-1}} \qquad (27)$$

If different ground state energies in solution affect K_s^{1-3} and K_s^{1-1} differently for each substrate, any correlation would be difficult to make in terms of the basicity of the hetero atom.

If the hetero atom in R'_1 is attracted to a sublocus in ρ_2 which is important in orientation of the substrate for catalysis, it is predicted that k_o for α-N-(3-furoyl)-D-alanine methyl ester would be much lower than k_o for the 2-furoyl derivative and would also be pH-independent. Furthermore, since the sulfur in the 2-thiophenoyl derivative is probably not basic (54), as indicated by the similarity in k_o and K_o for this substrate and α-N-benzoyl-D-alanine methyl ester, 3-thiophenoyl-D-alanine methyl ester is predicted to have a k_o value similar to the 2-thiophenoyl derivative.

Further tests of the hypothesis that a basic hetero atom in R'_1 is attracted to a sublocus in ρ_2 and results in orientation or disorientation of the substrate for normal catalysis could be made by using various analogs of acetyl-L-phenylalanine methyl ester (a limit case with the predominant binding and productive mode being the same) in which the phenyl ring has been replaced by furyl, pyridinyl, and thiophenyl rings.

C. Variation in k_o/K_o--Inversions in Stereospecificity

The values of k_o/K_o for the pair of L-substrates are listed in Table II and plotted vs pH in Figure 17; for the pairs of enantiomers, these values are listed in Table I and plotted vs pH in Figures 18 through 23. The general shape of the curves agrees with previous work (24,35,38,40).

The ratio k_o/K_o is a measure of the relative reactivity of substrates (16a, b), since this is the specific rate under second order conditions, i.e., the specific rate where saturation of the enzyme is unimportant (see Equation 1, p. 4). The following discussion of reactivity is in terms of k_o/K_o.

Earlier it had been mentioned that for the pairs of enantiomers used in the present study, in only one case was there an inversion of stereospecificity, the reactivity of α-N-picolinoyl-D-alanine methyl ester being greater than that of its optical antipode. Inversions of stereospecificity in α-chymotrypsin-catalyzed reactions have been observed only twice before (55a, b). In the first case, that of D- and L-3-carbomethoxydihydroisocarbostyril (I), the D-compound is hydrolyzed much more rapidly than its L-enantiomer. Although these results were interpreted in terms of the Hein-Niemann theory of structural and stereochemical specificity (16a,b), the results served more to illustrate the pronounced differences between these conformationally-constrained compounds and the more conventional trifunctional substrates. In the second case, Cohen *et al.* showed that D-α-acetoxypropionic acid methyl ester is hydrolyzed faster than its L-antipode, but the degree of stereospecificity is dependent on the concentration of substrate. In the present study, it was thought that the degree of stereospecificity for the enantiomeric pairs of acylalanine methyl esters might be altered sufficiently by a change in pH to cause an inversion since for all these enantiomeric pairs, the reactivities of the D- and L-members of the pairs are similar in magnitude. If a change in pH causes a change in the conformation of the active site with a concomitant change in the relative distribution of the substrates between two binding modes, an inversion in stereospecificity might occur. Although the relative stereospecificity of the substrates varies with pH, no inversion with pH was observed.

As an explanation for the inversion in the case of the picolinoyl derivatives, Rapp (20) postulated that the ring nitrogen in the D-isomer might be directly involved in catalyzing the hydrolysis. Evidence for the presence of histidine at the active site has been obtained (56). The curves of reactivity vs pH for α-chymotrypsin-catalyzed reactions have been interpreted in terms of one ionized form of the imidazole group of this histidine residue being directly involved in the hydrolytic step (56). If, in the case of the picolinoyl-D-alanine methyl ester, the ring nitrogen were oriented so that it, instead of the presumed imidazole, were involved in the catalysis, the curve of k_o/K_o vs pH would be expected to be pH-independent in the range studied since the pK_a of the pyridinium cation is much lower than the imidazolium cation.* This is not observed. All k_o/K_o vs pH profiles resemble titration curves for weak acids. This, however, does not exclude the possibility that,

---

* The imidazolium cation has a pK_a of 7 (56), whereas the pK_a of the protonated nitrogen in picolinic acid has been given as 1.06 (57).

for the D-picolinoyl derivative, catalysis by the ring nitrogen occurs in addition to the normal catalysis.

III. Variation in the Ratios $\frac{K_{o_1}}{K_{o_2}}$, $\frac{k_{o_1}}{k_{o_2}}$, and $(k_o/K_o)_1/(k_o/K_o)_2$ for Selected Pairs of Substrates

One problem involved in correlating kinetic parameters with structural changes in substrates is that it is difficult to determine whether differences in ground state energies of substrates and enzyme in different systems are important. Available evidence indicates that the K_{si} terms in the mechanism under discussion approximate equilibrium constants for the reaction

$$E + S \overset{K_{si}}{\rightleftarrows} (ES)_i \tag{28}$$

where E and S are free enzyme and substrate, respectively, and $(ES)_i$ is one of twelve enzyme-substrate complexes. Each K_{si} term is therefore a measure of the free energy difference in going from free enzyme and substrate to an enzyme-substrate complex. Assume for the moment that the reaction conditions from system to system are such as to keep the ground state energy of the free enzyme identical. Similarly, assume that all substrates in solution have identical free energies. Then the K_{si} terms would be a direct measure of the energy of interaction, both steric and electronic, between substrate and enzyme. These conditions are often tacitly assumed in interpreting the kinetic parameters since the differences in ground state energies of enzyme under various reaction conditions and of substrates have not been determined. Canady et al. (58) have suggested that the variation in K_o for different alkyl esters of methyl hippurate may be due in part to a solubility effect. Further, they (59) have shown that the variation in K_o with ionic strength for methyl hippurate at pH 7.0 can be directly correlated to changes in solubility of the substrate. Inhibition studies (47,32) have shown that the interaction of the active site of α-chymotrypsin with benzamide is considerably more favorable than its interaction with nicotinamide, presumably because of a greater extent of solvation of the latter species (60). In the present study, the possibility that differences in ground states of the substrates account for the lack of correlation of K_o for the D- and k_o and K_o for the L-enantiomers with basicity of the hetero atom, when k_o for the D-enantiomers appeared to be a function of this parameter was discussed in the section on the variation of k_o with pH.

In this section, changes in the ratios of the kinetic parameters for optical antipodes (except for one pair of L-substrates) are discussed. For these pairs, not only are the substrate structures invariant over the pH range investigated, but, for the L-pair, the difference in ground state energies remains constant, and for the D- and L-enantiomers, the difference is zero. Enzyme concentrations were kept as close as possible for the two members of each pair in order to minimize possible differences in ground state energies of enzyme at different concentrations. Consequently, any changes in these ratios with varying hydrogen-ion concentration can be attributed entirely to changes in the enzyme-substrate complexes. Whether the changes take place before, during, or after formation of the complex is not known. The pairs of substrates were chosen to focus attention on conformational changes in the active site as opposed to different states of ionization of catalytically active groups.

A. Variation in K_{o_1}/K_{o_2}

The values for K_{oL}/K_{oD} for the enantiomeric acyl-alanine methyl esters are listed in Table III and plotted vs pH in Figures 24 through 29. K_{oB}/K_{oA} values for the pair of L-substrates are listed in Table IV and plotted vs pH in Figure 30. From the figures, it can be seen that in all cases, the ratio K_{oL}/K_{oD} increases with increasing pH. One curve, that for α-N-benzoyl-L- and D-methyl esters appears to pass through a maximum, but this is uncertain because of the large errors in this ratio. In general, the errors in K_o values are larger than those in k_o or in k_o/K_o because of the form of the equation from which these constants are obtained. The equation is

$$\frac{[S][E]_o}{V} = \frac{1}{k_o}[S] + \frac{K_o}{k_o} \qquad (29)$$

where k_o is the reciprocal of the slope, and k_o/K_o is the reciprocal of the intercept for a plot of $\frac{[S][E]_o}{V}$ vs $[S]$. The errors in each of these are magnified when the ratio is taken to obtain K_o. In general, the error in K_o is ca. 10%, but for very poor substrates, the error is often ca. 20%, and in one case it is 100%. This larger error is increased further when K_{o_1}/K_{o_2} is obtained. Also, the error in K_o is usually larger for D-substrates than for their L-enantiomers, probably because the reactivity of most D-substrates approaches the lower limit of observation by the method used to follow the kinetics. High enzyme concentrations must be used, and at pH 7.90 and pH 8.50, the base-catalyzed reaction and carbon dioxide blank become significant with respect to the enzyme-catalyzed reaction. Although the "blank" reactions are taken into account, any error in them will increase the error in the rate calculated for the enzyme-catalyzed reaction. The optimum pair of enantiomers for determining the kinetic parameters consists of picolinoyl-D- and L-alanine methyl ester, because, for both enantiomers, the blank reactions are relatively small with respect to the enzyme-catalyzed reactions, and high enzyme concentrations are not necessary. For this pair, it can be seen that the curve of K_{oL}/K_{oD} vs pH appears to be sigmoid. The curve for the pair of L-substrates α-N-acetyl-O-methyl-L-tyrosine methyl ester and α-N-benzoyl-L-alanine methyl ester also may be sigmoid, but again the errors are large, and this is uncertain. If all curves of K_{o_1}/K_{o_2} vs pH are assumed to be sigmoid, the results may be discussed as follows.

The predominant binding mode for the L-substrates is represented in Figure 1-3, and for their D-antipodes, in Figure 2-1.

For the ratio of K'_{oL}/K_{oD}, equations 18 and 19 reduce to:

$$\frac{K_{oL}}{K_{oD}} = \frac{K_s^{1-3}}{K_s^{2-1}} \frac{\left(1 + \frac{K_{aes}^{2-1}}{[H]} + \frac{[H]}{K_{bes}^{2-1}}\right)}{\left(1 + \frac{K_{aes}^{1-3}}{[H]} + \frac{[H]}{K_{bes}^{1-3}}\right)} \quad . \tag{30}$$

It can be seen that if $K_{aes}^{2-1} = K_{aes}^{1-3}$, $K_{bes}^{2-1} = K_{bes}^{1-3}$, and if K_s^{2-1} and K_s^{1-3} are true constants, the ratio K_{oL}/K_{oD} is pH-independent. The pH-independence of K_{oL}/K_{oD} is not observed.

Perturbations by the substrate might change the pKa's of ionizing groups at the active site. If this occurs, it is possible that $K_{aes}^{2-1} \neq K_{aes}^{1-3}$ and/or $K_{bes}^{2-1} \neq K_{bes}^{1-3}$. In this case, a sigmoid shape may be explained by a simple deprotonation of an ionizing group at the active site as pH is increased. Whether the deviations from a sigmoid shape are entirely due to experimental error or are partly due to changes in the values of K_s^{2-1} and/or K_s^{1-3} cannot be decided from this ratio.

In the discussion of the individual K_o's, it was concluded that only one ionizing group could be important in combination of the substrate with the enzyme. This conclusion was reached in order to explain that for some substrates, K_o was pH-independent in a certain region. If this is true, equation 30 reduces further to:

$$\frac{K_{oL}}{K_{oD}} = \frac{K_s^{1-3}\left(1 + \frac{[H]}{K_{bes}^{2-1}}\right)}{K_s^{2-1}\left(1 + \frac{[H]}{K_{bes}^{1-3}}\right)} \quad . \tag{31}$$

A sigmoid shape for the plot of K_{oL}/K_{oD} vs pH can still be explained by a simple deprotonation mechanism.

For the pair of L-substrates, the predominant binding mode for α-N-acetyl-O-methyl-L-tyrosine methyl ester (A) is represented in Figure 1-1, whereas for α-N-benzoyl-L-alanine methyl ester (B), again Figure 1-3 represents the predominant binding mode. The simplified expression for K_{oB}/K_{oA} is identical to equation 31. Consequently, the sigmoid curve may again be explained by a simple deprotonation of the complex as pH is increased. The similarity in the pH profiles supports this interpretation.

It may be possible to look for non-sigmoid deviations from a horizontal line by choosing a pair of reactive substrates for which the kinetic constants may be determined more precisely. If two L-substrates "x" and "y" are similar enough in structure and are limit cases, it is possible that differences in K_{besi} for the two would be negligible and equation 31 would reduce further to

$$\frac{K_{ox}}{K_{oy}} = \frac{K_{sx}}{K_{sy}} \tag{32}$$

In this case, deviations from a constant in the ratio as pH is varied must be due to non-identical variations in K_{sx} and K_{sy}. This variation would be most easily explained in terms of an active site of changing conformation. Two major difficulties with this approach are that, in general, errors in kinetics parameters in these systems are not small, and if the substrate structures are similar enough, K_{sx} and K_{sy} may exhibit parallel variation, thus masking changes in the ratio.

B. Variation in k_{o_1}/k_{o_2}

The values for k_{oL}/k_{oD} are listed in Table III and plotted vs pH in Figures 24 through 29 , whereas k_{oB}/k_{oA} values are listed in Table IV and plotted vs pH in Figure 31 . Using the same predominant binding modes and productive modes as for the individual k_o values, the equations for the ratios of the k_o values reduce to equations 33 and 34,

$$\frac{k_{oL}}{k_{oD}} = \frac{k_2^{1-1}\ \dfrac{K_s^{1-3}}{K_s^{1-1}}\left(1 + \dfrac{K_{aes}^{2-1}}{[H]} + \dfrac{[H]}{K_{bes}^{2-1}}\right)}{k_2^{2-1}\left(1 + \dfrac{K_{aes}^{1-3}}{[H]} + \dfrac{[H]}{K_{bes}^{1-3}}\right)} \tag{33}$$

$$\frac{k_{oLA}}{k_{oLB}} = \frac{k_{2A}^{1-1}\left(1 + \dfrac{K_{aes}^{1-3}}{[H]} + \dfrac{[H]}{K_{bes}^{1-3}}\right)_B}{k_{2B}^{1-1}\left(1 + \dfrac{K_{aes}^{1-1}}{[H]} + \dfrac{[H]}{K_{bes}^{1-1}}\right)_A} \tag{34}$$

where the A and B refer to the two members of the pair of L-substrates.

In all cases, even for the pair of L-substrates, it is more certain that the curves are sigmoid. Again, if the k_{si}'s and K_{si}'s are true constants, the sigmoid shape of the curves may be explained by a simple deprotonation of the basic form of a group catalytically involved in the hydrolysis provided $K_{aes}^{2-1} \neq K_{aes}^{1-3}$ and/or $K_{bes}^{2-1} \neq K_{bes}^{1-3}$ in equation 33 and $K_{aes_B}^{1-3} \neq K_{aes_A}^{1-1}$ and/or $K_{bes_A}^{1-1} \neq K_{bes_B}^{1-3}$ in equation 34.

C. Variation in $(k_o/K_o)_1/(k_o/K_o)_2$

The $(k_o/K_o)_L/(k_o/K_o)_D$ values are listed in Table III and plotted in Figures 32 through 36. The $(k_o/K_o)_B/(k_o/K_o)_A$ values are listed in Table IV and plotted vs pH in Figure 37.

In this case, using equation 10, the ratio is exactly:

$$\frac{(k_o/K_o)_{L \text{ or } B}}{(k_o/K_o)_{D \text{ or } A}} = \frac{\left(\sum_{i=1}^{3} \frac{k_{2i}}{K_{si}}\right)_{L \text{ or } B}}{\left(\sum_{i=1}^{3} \frac{k_{2i}}{K_{si}}\right)_{D \text{ or } A}} . \tag{35}$$

Binding modes which are not productive do not appear in the equation. Furthermore, the terms which include ionization constants cancel exactly. These latter terms would always cancel, no matter how many intermediates are postulated, provided that the substrates all interact with the same groups on the enzyme (25). Consequently, any changes in these ratios, except possibly for the nicotinoyl and isonicotinoyl pairs, cannot be due to protonation or deprotonation of groups at the active site. Variations in the ratio with pH must arise from changes in the individual k_{2i} and K_{si} terms. Although some scatter in the data is evident, for five of the seven pairs, a clear variation in the ratio is seen.

These pairs are:

1. nicotinoyl-D- and L-alanine methyl ester
2. isonicotinoyl-D- and L-alanine methyl ester
3. picolinoyl-D- and L-alanine methyl ester
4. benzoyl-D- and L-alanine methyl ester
5. benzoyl-L-alanine methyl ester and α-N-acetyl-O-methyl-L-tyrosine methyl ester

For the D-substrates, in the first two pairs listed, it was postulated that the invariance in k_o with pH might be due to a mechanism which did not involve catalytically active groups ionizing in the pH range studied. In this case, one would expect to see a pH-dependence for $(k_o/K_o)_L/(k_o/K_o)_D$, since the terms containing ionizations of catalytically active groups would not cancel. The expression for the ratio would be:

$$\frac{(k_o/K_o)_L}{(k_o/K_o)_D} = \frac{\left(\sum_{i=1}^{3} k_{2i}/K_{si}\right)_L}{\left(\sum_{i=1}^{3} k_{2i}/K_{si}\right)_D \left(1 + \frac{K_{ae}}{[H]} + \frac{[H]}{K_{be}}\right)} \quad . \tag{36}$$

However, for the last three cases, only a minor dependence (if any) of the ratio is expected, since the rate of the postulated pH-independent mechanism should be small with respect to the rate of the normal path. Consequently, the variation in $(k_o/K_o)_1/(k_o/K_o)_2$ for the last three pairs must be attributed to variations in the k_{2i} and

K_{si} terms. The simplest explanation of these variations is that they are due to hydrogen-ion-induced conformational changes of the active site. This is not inconsistent with the work of Platt and Niemann (33), who found that the nature of the inhibition of α-N-acetyl-L-leucine methyl ester by benzamide or formanilide is pH-dependent. Their results were also interpreted in terms of a hydrogen-ion-induced alteration of the conformation of the active site.

EXPERIMENTAL*

I. Kinetic Runs

Details on the equipment and general kinetic procedure employed in these experiments can be found in the literature (61). The thermostated reaction cell noted in reference 61 has been modified to hold a 20 ml beaker instead of a 50 ml beaker. In all runs, a total reaction volume of 10 ml was used. All runs were made at 25°C.

Kinetic runs were made in the absence of enzyme in order to determine the base-catalyzed hydrolysis rate (substrate blank) and in the absence of substrate and enzyme in order to determine the rate of carbon dioxide absorption by the solution (CO_2 blank) for all runs at and above pH 7.50. At and below pH 7.00, no substrate blank or CO_2 blank could be detected. Although a stream of nitrogen was introduced into the reaction vessel above the solutions, CO_2 was still absorbed slowly by the basic solutions. For fast substrates of α-chymotrypsin, the substrate and CO_2 blank are invariably negligible; however, in the present studies, poor substrates were often used, and both the substrate blank and CO_2 blank had to be taken into account in these cases.

---

* All melting points corrected by a calibration curve prepared by Dr. A. Platt.

Equation 37 describes the rate for all blank reactions under conditions where only the substrate concentration is varied.

$$V = k_6 [S] + k_7 \tag{37}$$

In this equation, $k_6 = k_6'(OH^-)$, $(S)_o$ is the initial substrate concentration, k_7 is the rate of carbon dioxide absorption, and (OH^-) is the hydroxide ion concentration.

The time of the blank runs was varied from 8 to 40 minutes. No variation of the constants k_6 and k_7 could be detected outside of experimental error. Initial substrate concentration was used in calculation of k_6, since the extent of hydrolysis was small (usually less than 2%) over the period of observation. Whenever the velocity of the blank reaction was greater than ca. 2% of the total initial velocity of the enzyme-catalyzed reaction for the most dilute substrate concentration, a correction for the blank was made.

The rates of all reactions were followed by means of a recorder trace. Since the recorder traces for the enzyme-catalyzed reactions consisted of straight or only slightly curved lines, the necessity of correcting the data was easily decided by visual observation of the traces.

II. Treatment of Data

The initial rates of all enzyme-catalyzed reactions in the present study are described by equation 38 under conditions where the only variables are enzyme and substrate concentration.

$$v = \frac{k_o (E)_o (S)}{K_o + (S)} \qquad (38)$$

In this equation, v is the initial velocity, $(E)_o$ is the initial enzyme concentration, (S) is the substrate concentration, and k_o and K_o are a pair of experimentally determined kinetic parameters.

A program has been written for the Datatron 220 by Abrash (62) to obtain the kinetic parameters k_o and K_o. The program employs the method of Booman and Niemann (63) to obtain the initial velocities from the recorder traces. From these initial velocities, a least-squares plot of equation 39 is made.

$$\frac{[S][E]_o}{v} = \frac{1}{k_o}[S] + \frac{K_o}{k_o} \qquad (39)$$

The program as designed by Abrash does not calculate a standard deviation for the intercept, (K_o/k_o). Since values of k_o/K_o were desired, the values for (S) $(E)_o/v$ and (S) which had been accepted by the "T" significance test in the Abrash program were subjected in an identical least-squares treatment which did give a standard deviation for the intercept.

The errors in the ratios k_{o1}/k_{o2}, K_{o1}/K_{o2}, and $(k_o/K_o)_1/(k_o/K_o)_2$ were computed from the standard relationships(64):

$$\frac{A \pm a}{B \pm b} = C \pm \rho \quad \text{and} \quad \rho = \frac{1}{B}\left(b^2 + \frac{a^2 B^2}{A^2}\right)^{\frac{1}{2}} .$$

A subroutine program for calculating the values of ρ was written for the Datatron 220 by Drs. A. Platt and J. R. Rapp.

III. Materials

α-Chymotrypsin. - The enzyme used in these studies was a bovine, salt-free preparation obtained from Worthington Biochemicals Corporation (lot number C.D.I. 6066-67). The protein nitrogen content of the enzyme was determined in this laboratory by Dr. J. R. Rapp and Mr. Gary Neil, using the micro-Kjeldahl method of Redeman (65). A value of 14.91% ± 0.06% was obtained. All values of enzyme concentration were based on a molecular weight of 25,000 and a protein nitrogen content of 16.5%.

D-Alanine methyl ester hydrochloride. - This compound was prepared several times by the method of Brenner and Huber (66). Thionyl chloride was added slowly to a cooled solution of methanol, keeping the temperature below -5°C. The amino acid was then added in portions, and the mixture was stirred overnight at room temperature or for three to four hours at a temperature of 40°C. The methanol was then stripped off in vacuo, and the methyl ester hydrochloride was usually used without further purification. Occasionally the methyl ester hydrochloride was crystallized from methanol-ether mixtures.

L-Alanine methyl ester hydrochloride. - This compound was prepared in the same manner as the D-enantiomer.

Benzoyl-L-alanine methyl ester. - This compound was prepared from L-alanine methyl ester hydrochloride and benzoyl chloride, using Schotten-Bauman conditions. A three-necked, 500 ml, round-bottom flask was fitted with a stirrer and a dropping funnel. The crude amino acid ester hydrochloride obtained from 25 g (0.132 mole) of L-alanine was dissolved in 85 ml of water containing 36.0 g (0.262 mole) of potassium carbonate. To the flask was added 125 ml of chloroform. The mixture was stirred rapidly while 30.8 ml (0.267 mole) of benzoyl chloride was added slowly. The mixture was stirred rapidly for one hour at room temperature. Six ml of pyridine was then added, and the mixture was stirred an additional 20 minutes. After the phases were separated, the organic layer was washed with 50 ml of N HCl and 18 ml of water; it was then dried over anhydrous magnesium sulfate. The crude, oily product obtained after evaporation of the solvent *in vacuo* was crystallized twice from isopropyl ether, yielding fine needles, m.p. 58.5-61.5° (Lit. m.p. 56.5-57.5°) (16b), $[\alpha]_D^{25}$ = 30.2° (c, 5.0% in *sym*-tetrachloroethane) (Lit. $[\alpha]_D^{25}$ = 30.6° (16b).

Benzoyl-D-alanine methyl ester. - This compound was prepared in a manner analogous to that used for the *L*-enantiomer. Two crystallizations from isopropyl ether yielded fine needles, m.p. 58.0-61.0° (Lit. m.p. 56-58°) (16b), $[\alpha]_D^{23}$ = 30.0° (c, 5.0% in sym-tetrachloroethane) (Lit. $[\alpha]_D$ = -30.8°) (16b).

α-N-Acetyl-O-methyl-L-tyrosine methyl ester. - A sample of previously purified material obtained from Mr. R. Peterson in this laboratory was taken up in toluene, treated with Norite, and crystallized from a toluene-ligroin mixture. The product was filtered and dried *in vacuo* over phosphorus pentoxide and paraffin wax yielding fine needles, m.p. 104.5-106.0° (Lit. m.p. 104-105°) (48), $[\alpha]_D^{25}$ = 31.6° (c, 3.6% in methanol) (Lit. $[\alpha]_D^{25}$ = 25.3, c, 0.35% in methanol) (48).

2-Furoyl-L-alanine methyl ester. - To a chloroform solution of *L*-alanine methyl ester hydrochloride (0.057 mole) cooled below 0°C in a 500 ml, three-necked, round-bottom flask fitted with thermometer, dropping funnel, and stirrer, triethylamine (0.114 mole) was added. This was followed by the slow addition of

2-furoyl chloride (0.057 mole) while the temperature was kept below 0°C. The solution was allowed to warm to room temperature and was stirred overnight. After the mixture was washed with N HCl and sodium bicarbonate solution, it was dried over anhydrous sodium sulfate. The solvent was then removed in vacuo, and the slightly colored oil remaining in the flask was distilled at ca. 0.5 mm Hg. The distillate was crystallized once from isopropyl ether, yielding fine, white needles, m.p. 41.5-43.0° (Lit. m.p. 41-42°) (20), $[\alpha]_D^{25}$ = -3.2 (c, 6.0% in water) (Lit. $[\alpha]_D$ = -2.8°, c, 6% in water) (20).

The following substrates had been prepared by J. R. Rapp several months previously (20). These compounds were redried in vacuo, and the melting points were checked. Since all melting points checked well with the values obtained by Rapp (20), these substrates were used without further purification.

Picolinoyl-L-alanine methyl ester. - M.p. 59.0-60.5° (Lit. m.p. 59-60°).

Picolinoyl-D-alanine methyl ester. - M.p. 58.5-60.0° (Lit. m.p. 59-60°).

Nicotinoyl-L-alanine methyl ester. - M.p. 75.0-77.0° (Lit. m.p. 75.5-76.5°).

Nicotinoyl-D-alanine methyl ester. - M.p. 76.0-77.0° (Lit. m.p. 75.5-76.5°).

Isonicotinoyl-L-alanine methyl ester. - M.p. 85.5-87.0° (Lit. m.p. 85.5-86.5°).

Isonicotinoyl-D-alanine methyl ester. - M.p. 85.5-86.5° (Lit. m.p. 85.5-86.5°).

2-Furoyl-L-alanine methyl ester. - M.p. 40.0-41.5° (Lit. m.p. 41-42°).

2-Furoyl-D-alanine methyl ester. - M.p. 40.0-42.0° (Lit. m.p. 41-42°).

2-Thiophenoyl-L-alanine methyl ester.- M.p. 91.0-92.0° (Lit. m.p. 91-92°).

2-Thiophenoyl-D-alanine methyl ester. - M.p. 90.5-92.0° (Lit. m.p. 91-92°).

TABLES

Table I. Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolyses of Enantiomeric Pairs of Acylated Amino Acid Esters

Substrate	No. of Expts.[c]	pH[d]	$[E_o]$ M$\times10^{-4}$	$[S_o]$ mM[e]	$k_o\times10^{2}$ sec^{-1}	K_o mM	$(k_o/K_o)\times10^{2}$ sec^{-1}mM^{-1}
Benzoyl-L-Ala·OMe[b]	12-2	7.00	0.333	0.84-5.87	10.3±0.3	3.3±0.2	3.13±0.08
	10-0	7.50	0.157	1.52-10.62	21.4±0.5	4.1±0.2	5.2 ±0.2
	10-0	7.90	0.206	2.12-14.85	28.0±0.7	5.8±0.4	4.8 ±0.2
	9-1	8.50	0.148	1.52-10.62	33 ±1	7.2±0.4	4.6 ±0.1
Benzoyl-D-Ala·OMe	11-2	7.00	0.703	0.37-2.58	0.8 ±0.1	3.2±0.4	0.26±0.01
	10-0	7.50	0.758	0.55-3.88	1.03±0.05	1.9±0.2	0.54±0.04
	7-1	7.90	0.716	0.55-3.88	1.14±0.01	1.99±0.05	0.55±0.01
	8-0	8.50	0.685	0.51-3.55	1.3 ±0.2	4±1	0.35±0.04
Picolinoyl-L-Ala·OMe	11-1	6.50	0.387	2.88-20.12	1.7 ±0.2	11±2	0.16±0.02
	9-1	7.00	0.374	2.48-17.34	4.0 ±0.2	8.2 ±0.7	0.49±0.02
	10-1	7.50	0.173	2.98-20.86	7.0 ±0.3	7.9 ±0.8	0.88±0.05
	8-1	7.90	0.374	2.48-17.34	12.2 ±0.5	15±1	0.80±0.03
	10-1	8.50	0.298	2.98-20.86	14.9 ±0.8	21±2	0.72±0.02
Picolinoyl-D-Ala·OMe	8-0	6.50	0.402	2.80-19.61	7.2 ±0.2	9.4 ±0.6	0.83±0.03
	11-1	7.00	0.366	2.26-15.85	12.0 ±0.5	7.0 ±0.6	1.7 ±0.1
	10-1	7.50	0.180	1.95-13.64	15.6 ±0.4	5.5 ±0.3	2.8 ±0.1
	10-1	7.90	0.366	2.26-15.85	18.6 ±0.5	6.6 ±0.4	2.8 ±0.1
	10-1	8.50	0.271	1.95-13.64	19.8 ±0.4	8.2 ±0.3	2.41±0.05
Nicotinoyl-L-Ala·OMe	10-0	7.00	0.177	3.80-26.61	36.5 ±0.8	8.0 ±0.8	4.6 ±0.4
	11-0	7.50	0.187	2.49-17.45	50 ±2	12.6 ±0.9	3.9 ±0.1
	8-0	7.90	0.376	2.30-16.12	64 ±6	19±2	3.4 ±0.2
	11-1	8.50	0.187	2.49-17.45	79 ±3	23±1	3.46±0.07

TABLE I (continued)

Substrate	No. of Expts.	pH^d	$[E_o]$M$\times10^{-4}$ ^{e}	$[S_o]$ mM	$k_o\times10^2$ sec^{-1}	K_o mM	$(k_o/K_o)\times10^2$ sec^{-1} mM^{-1}
Nicotinoyl-D-Ala·OMe	10-1	7.00	1.05	1.01-7.11	0.41±0.08	10±3	0.040±0.004
	11-1	7.50	1.26	1.00-6.99	0.40±0.03	5.4±0.7	0.073±0.005
	8-0	7.90	1.05	1.01-7.11	0.37±0.03	3.7±0.5	0.100±0.008
	10-1	8.50	1.24	1.12-7.85	0.39±0.03	4.4±0.6	0.089±0.007
Isonicotinoyl-L-Ala·OMe	10-0	7.00	0.331	3.55-24.87	38±2	18±2	2.2 ±0.1
	10-1	7.50	0.250	2.44-17.09	40±1	14.3±0.6	2.79 ±0.05
	8-0	7.90	0.381	2.32-16.24	53.2±0.4	20±2	2.7 ±0.1
	10-2	8.50	0.257	2.44-17.09	53.9±0.7	21.2±0.4	2.54 ±0.02
Isonicotinoyl-D-Ala·OMe	9-0	7.00	1.50	2.41-16.88	0.19±0.02	8±2	0.025±0.004
	10-0	7.50	1.51	1.05-7.36	0.16±0.02	1.0±0.8	0.2 ±0.1
	8-1	7.90	1.50	2.41-16.88	0.20±0.01	3.0±0.8	0.07 ±0.01
	10-0	8.50	1.62	1.05-7.36	0.18±0.04	1±1	0.1 ±0.1
2-Thiophenoyl-L-Ala·OMe	10-1	7.00	0.231	2.02-14.15	21±2	6±1	3.5 ±0.4
	10-0	7.50	0.247	1.25-8.73	27±2	4.5±0.8	6.1 ±0.7
	10-0	7.90	0.231	2.02-14.15	48±1	7.8±0.4	6.2 ±0.2
	10-1	8.50	0.249	1.25-8.73	54±4	11±1	5.0 ±0.2
2-Thiophenoyl-D-Ala·OMe	10-2	7.00	0.475	1.87-13.13	1.10±0.07	3.2±0.5	0.35 ±0.04
	10-2	7.50	0.507	0.86-6.05	1.19±0.02	1.5±0.1	0.80 ±0.03
	9-0	7.90	0.425	1.87-13.13	1.34±0.09	2.2±0.4	0.60 ±0.08
	9-2	8.50	0.515	0.86-6.05	1.3 ±0.1	2.5±0.6	0.51 ±0.07
2-Furoyl-L-Ala·OMe	10-2	7.00	0.396	4.33-30.31	40±1	25.8±0.8	1.54 ±0.02
	11-2	7.50	0.276	4.33-30.31	49±2	17±1	2.9 ±0.1
	10-0	7.90	0.182	2.51-17.54	54.0±0.4	19±2	2.9 ±0.1
	10-0	8.50	0.404	4.91-34.37	71±5	30±3	2.4 ±0.1

TABLE I (continued)

Substrate	No. of Expts.	pH[d]	$[E_o]$Mx10^{-4}[e]	$[S_o]$ mM	k_ox10^2sec^{-1}	K_o mM	(k_o/K_o)sec^{-1}mM^{-1} x10^2
2-Furoyl- _D_-Ala·OMe	9-1	7.00	0.302	1.70-11.90	3.2 ± 0.3	13 ± 2	0.25 ± 0.01
	10-1	7.50	0.365	1.63-11.41	3.6 ± 0.1	7.2 ± 0.5	0.50 ± 0.02
	9-1	7.90	0.302	1.70-11.90	4.0 ± 0.3	8 ± 1	0.51 ± 0.04
	8-0	8.50	0.363	1.63-11.41	3.7 ± 0.5	8 ± 2	0.45 ± 0.06

a. In aqueous solutions at 25.0° and 1.00 M in sodium chloride except where noted

b. α-N-Benzoyl-_L_-alanine methyl ester

c. First number refers to number of kinetic runs performed; second number refers to number of points rejected by a statistical reiterative procedure

d. All pH values are within ±0.05 pH units

e. Based upon a molecular weight of 25,000 and a nitrogen content of 16.5%. The enzyme is a Worthington preparation, Lot No. CDI-6066-67.

Table II. Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolyses of Two Acylated-L-Amino Acid Esters

Substrate	No. of Expts.[c]	pH[d]	$[E_o]$ μM[e]	$[S_o]$ mM	$k_o \times 10^2$ sec^{-1}	K_o mM	$k_o/K_o \times 10^2$ sec^{-1} mM^{-1}
A[b]	10-0	6.00	12.0	0.64-5.13	15 ± 1	4.1 ± 0.4	3.7 ± 0.2
	12-0	6.50	12.2	0.64-5.14	22 ± 2	3.9 ± 0.6	5.8 ± 0.5
	20-1	7.00	8.9	0.47-4.27	35 ± 2	2.9 ± 0.2	11.8 ± 0.4
	9-1	7.50	6.5	0.49-3.94	49 ± 1	3.2 ± 0.1	15.4 ± 0.3
	19-1	7.90	6.2	0.52-4.23	50 ± 3	2.7 ± 0.2	18.3 ± 0.8
	10-1	8.50	6.5	0.49-3.94	54 ± 2	4.6 ± 0.2	11.7 ± 0.2
	10-0	9.00	7.9	1.13-9.07	55 ± 5	8 ± 1	7.4 ± 0.4
B[b]	9-0	6.00	50.4	3.77-29.65	1.7 ± 0.1	8.0 ± 0.8	0.21 ± 0.02
	11-0	6.50	11.5	1.37-10.94	3.2 ± 0.4	6 ± 2	0.58 ± 0.09
	10-0	7.00	32.7	1.72-13.73	9.3 ± 0.6	6.3 ± 0.9	1.5 ± 0.1
	10-0	7.50	11.5	1.37-10.94	16 ± 1	6 ± 1	2.6 ± 0.3
	18-0	7.90	13.0	1.35-14.28	26 ± 2	10 ± 1	2.7 ± 0.1
	8-0	8.50	12.7	1.65-13.21	22 ± 2	13 ± 2	1.6 ± 0.1
	10-0	9.00	6.2	3.95-31.63	30 ± 3	28 ± 4	1.1 ± 0.1

a. In aqueous solutions at 25.0° and 0.100 M NaCl

b. A = α-N-acetyl-O-methyl-L-tyrosine methyl ester and B = α-N-benzoyl-L-alanine methyl ester

c. First number refers to number of kinetic runs performed; second number refers to number of points rejected by a statistical reiterative procedure

d. All pH values are within ±0.05 pH units

e. Based on a molecular weight of 25,000 and a nitrogen content of 16.5%. The enzyme is a Worthington preparation, Lot No. CDI 6066-67.

Table III. Ratios of the Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolyses of Enantiomeric Pairs of Acylated Amino Acid Esters

Substrate	pH	k_{oL}/k_{oD} [a]	K_{oL}/K_{oD} [a]	$\dfrac{(k_o/K_o)_L}{(k_o/K_o)_D}$ [a]
Benzoyl-Ala·OMe[b]	7.00	13±2	1.0±0.1	12.3±0.6
	7.50	21±1	2.2±0.3	9.6±0.8
	7.90	24.6±0.7	2.9±0.2	8.8±0.4
	8.50	26±5	2.0±0.6	13 ±2
Picolinoyl-Ala·OMe	6.50	0.24±0.02	1.1±0.3	0.19±0.02
	7.00	0.33±0.02	1.1±0.1	0.28±0.02
	7.50	0.45±0.02	1.4±0.2	0.31±0.02
	7.90	0.66±0.03	2.3±0.2	0.28±0.01
	8.50	0.76±0.04	2.5±0.2	0.30±0.01
Nicotinoyl-Ala·OMe	7.00	90±20	0.8±0.3	110±20
	7.50	120±10	2.3±0.3	54±4
	7.90	170±20	5 ±1	34±3
	8.50	200±20	5.1±0.8	39±3
Isonicotinoyl-Ala·OMe	7.00	200±20	2.4±0.7	90±20
	7.50	250±30	10±10	20±10
	7.90	270±10	6±2	41±9
	8.50	290±60	20±20	20±20
2-Thiophenoyl-Ala·OMe	7.00	19±2	2.0±0.5	10±2
	7.50	23±2	3.0±0.5	7.6±0.8
	7.90	36±3	3.5±0.7	10±1
	8.50	43±6	4±1	10±1
2-Furoyl-Ala·OMe	7.00	12±1	2.0±0.3	6.1±0.3
	7.50	13.6±0.7	2.4±0.2	5.7±0.3
	7.90	13.4±0.9	2.4±0.4	5.6±0.4
	8.50	19±3	4±1	5.4±0.7

a. The errors in these ratios were obtained from the relationships:

$$\frac{A \pm a}{B \pm b} = C \pm \rho \quad \text{and} \quad \rho = \frac{1}{B}\left(b^2 + \frac{a^2B^2}{A^2}\right)^{\frac{1}{2}}$$

b. α-N-Benzoyl-alanine methyl ester

Table IV. Ratios of the Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolyses of Two Acylated-L-Amino Acid Esters

pH	$k_{oB}/k_{oA} \times 10^2$	K_{oB}/K_{oA}	$\frac{\left(k_o/K_o\right)_B}{\left(k_o/K_o\right)_A} \times 10^2$
6.00	11±1	1.9±0.3	5.7±0.5
6.50	15±2	1.5±0.4	10±2
7.00	24±1	2.4±0.4	12±1
7.50	32±3	1.9±0.4	17±2
7.90	51±4	3.5±0.5	15±1
8.50	40±3	2.9±0.4	14±1
9.00	55±6	3.8±0.7	14±1

a. The substrates used were B = α-N-benzoyl-L-alanine methyl ester and A = α-N-acetyl-O-methyl-L-tyrosine methyl ester

b. The errors in these ratios were obtained from the relationships:

$$\frac{A \pm a}{B \pm b} = C \pm \rho \quad \text{and} \quad \rho = \frac{1}{B}\left(b^2 + \frac{a^2B^2}{A^2}\right)^{\frac{1}{2}}$$

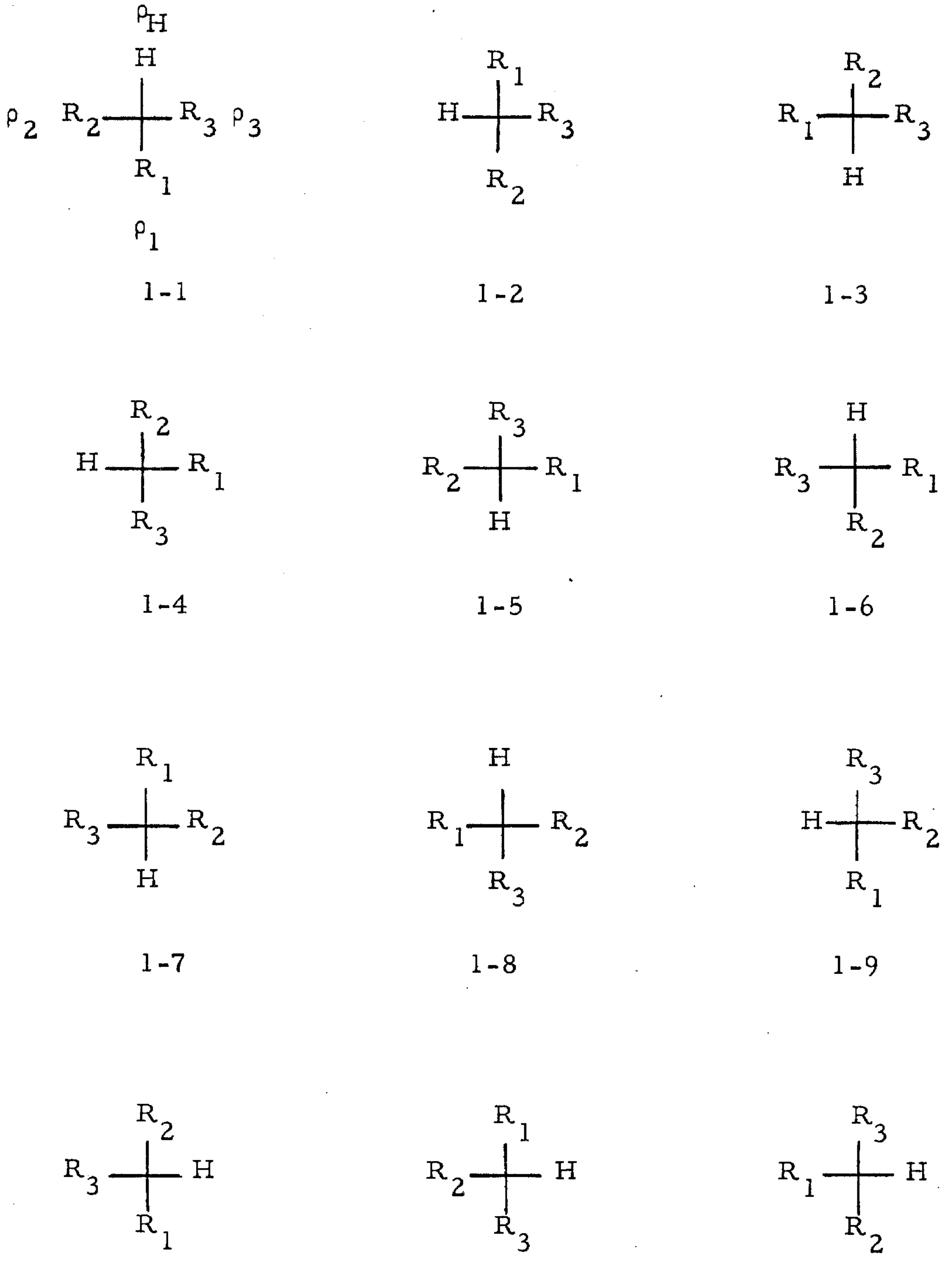
Table V. The Relative Sensitivity of k_o to Changes in pH for Enantiomeric Substrates of α-Chymotrypsin.

α-N-acyl-L-alanine methyl ester[a]	pH	% max. of k_{oL}	Change in % max. of k_{oL}
Nicotinoyl	7.00	46	54
	8.50	100	
Furoyl	7.00	56	44
	8.50	100	
Isonicotinoyl	7.00	70	30
	8.50	100	
Thiophenoyl	7.00	39	61
	8.50	100	
Benzoyl	7.00	31	69
	8.50	100	
Picolinoyl	7.00	11	89
	8.50	100	
α-N-acyl-D-alanine methyl ester[a]			
Picolinoyl	7.00	36	64
	8.50	100	
Furoyl	7.00	76	24
	8.50	100	
Thiophenoyl	7.00	78	22
	8.50	100	
Benzoyl	7.00	62	38
	8.50	100	
Nicotinoyl	7.00	105	-5
	8.50	100	
Isonicotinoyl	7.00	105	-5
	8.50	100	

[a] arranged in order of decreasing k_o

FIGURES

Figure 1.[*]--Possible Orientations of an $\underline{L}$-Substrate at the Active Site of α-Chymotrypsin



1-1 1-2 1-3

1-4 1-5 1-6

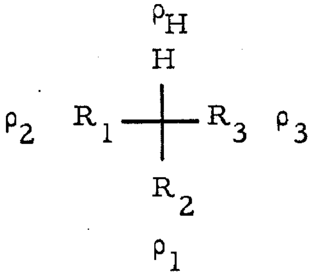
1-7 1-8 1-9

1-10 1-11 1-12

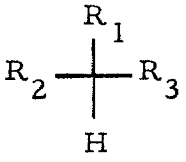
---

[*] Figure 1-1 shows the four loci ρ_1, ρ_2, ρ_3, ρ_H at the active site as well as the four complementary groups on the substrate where R_1- = R'_1 CONH-, R_3- = COR$'_3$, H = H, and R_2 is the side chain of the amino acid derivative.

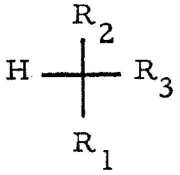
Figure 2.* --Possible Orientations of a <u>D</u>-Substrate at the Active Site of α-Chymotrypsin



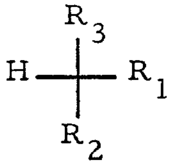
2-1



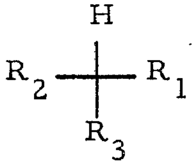
2-2



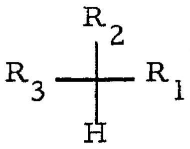
2-3



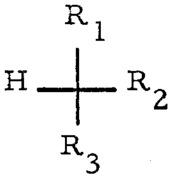
2-4



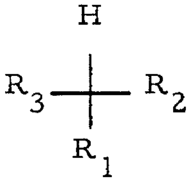
2-5



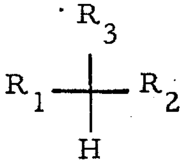
2-6



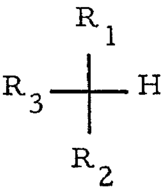
2-7



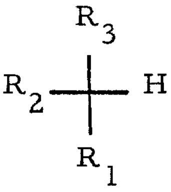
2-8



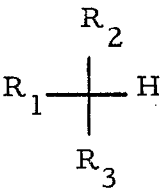
2-9



2-10



2-11



2-12

---

* Figure 2-1 shows the four loci ρ_1, ρ_2, ρ_3, and ρ_H at the active site as well as the four complementary groups on the substrate where $R_1 = R'_1CONH-$, $R_3- = COR_3-$, H = H, and R_2 is the side chain of the amino acid derivative.

Figure 3.--Dependence of K_o on hydrogen-ion concentration

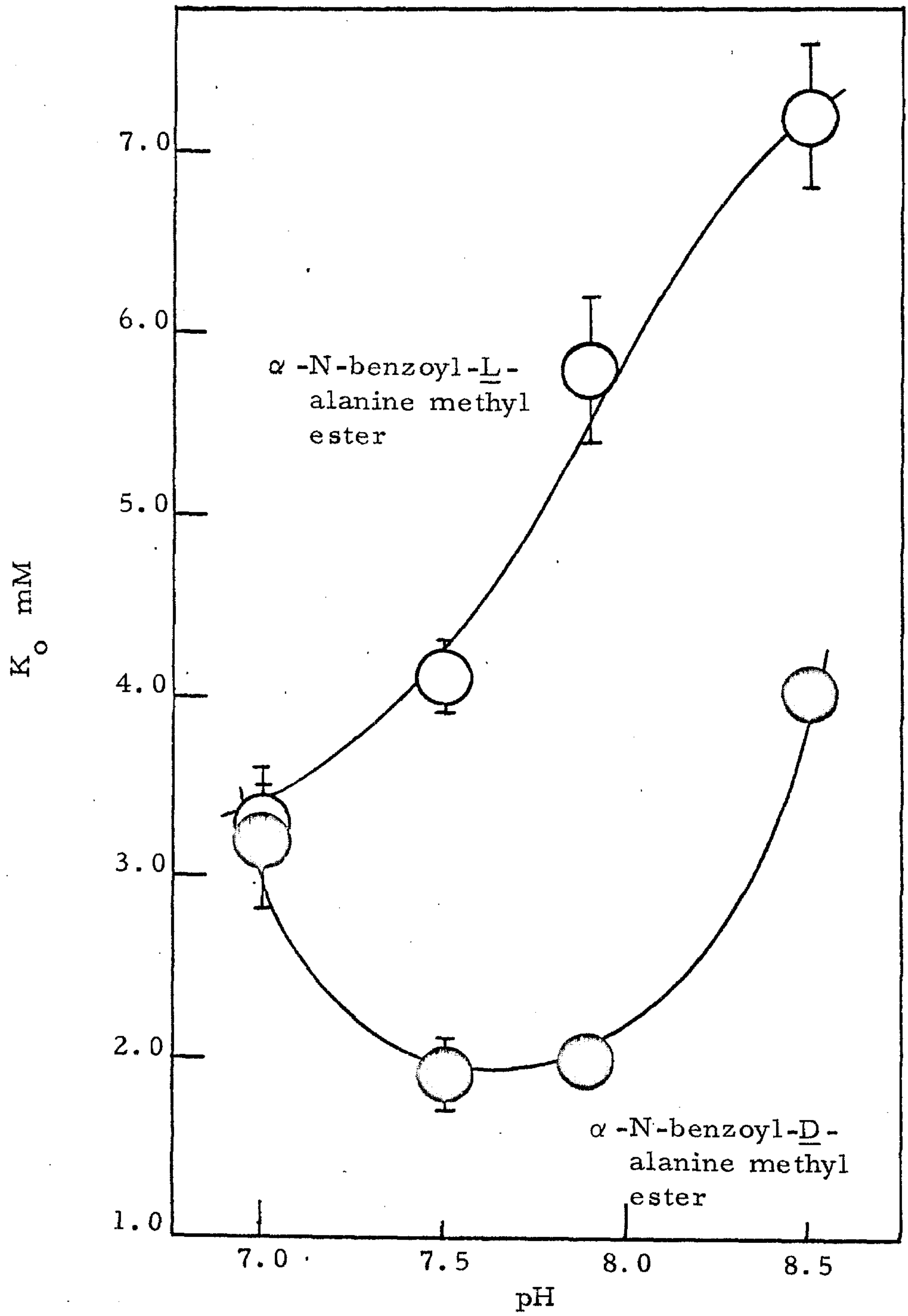


Figure 4.--Dependence of K_o on hydrogen-ion concentration

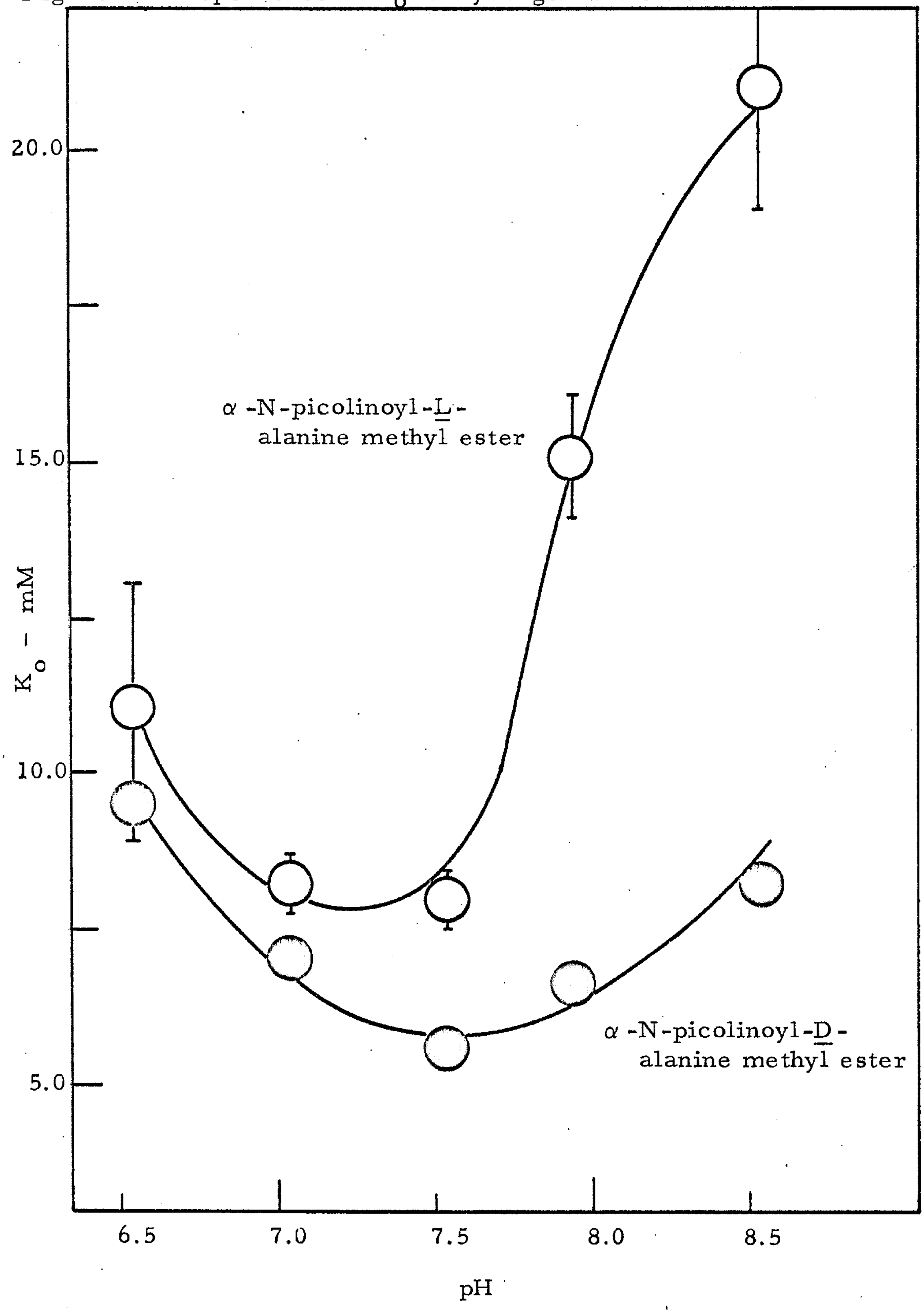


Figure 5.--Dependence of K_o on hydrogen-ion concentration

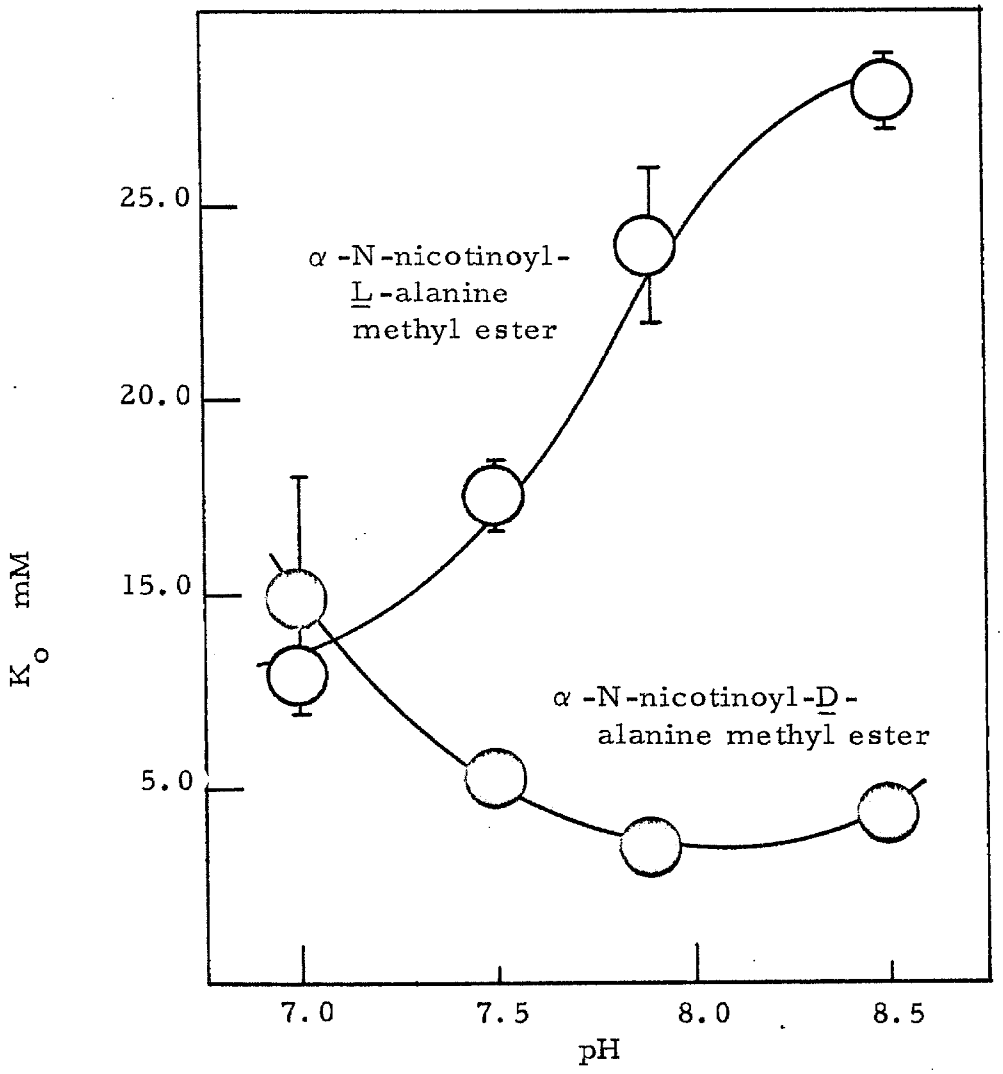


Figure 6.--Dependence of K_o on hydrogen-ion concentration

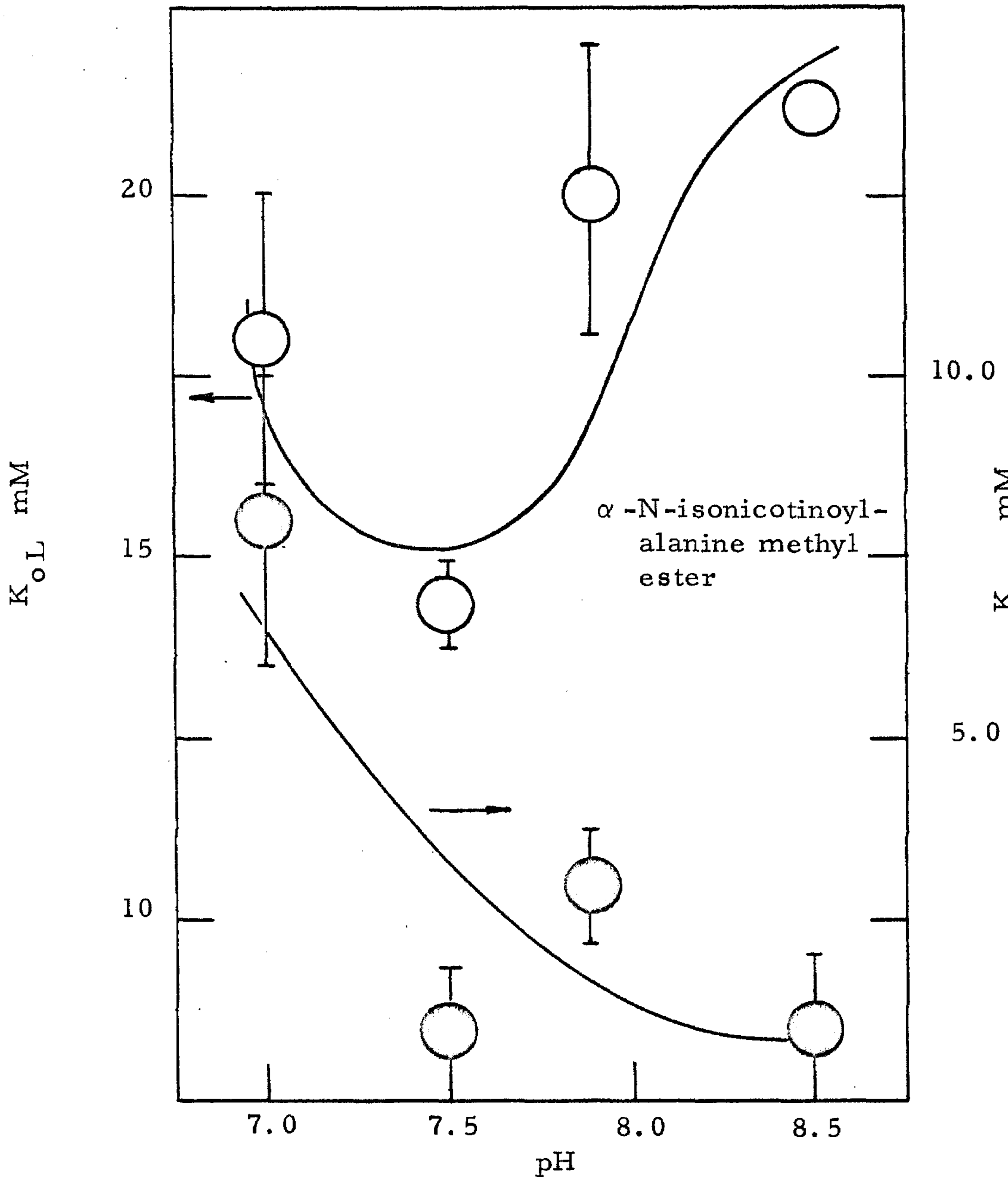


Figure 7.--Dependence of K_o on hydrogen-ion concentration

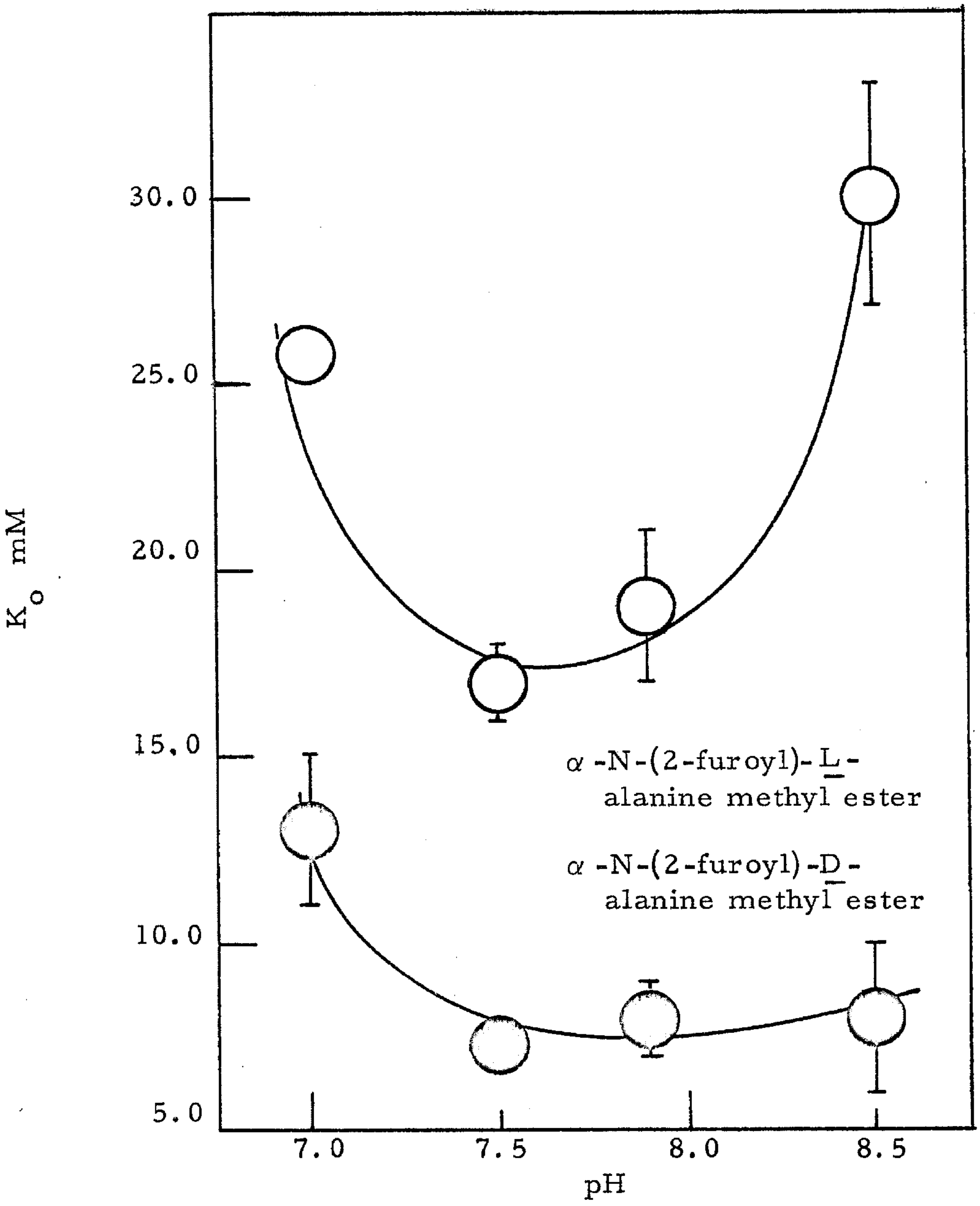


Figure 8.--Dependence of K_o on hydrogen-ion concentration

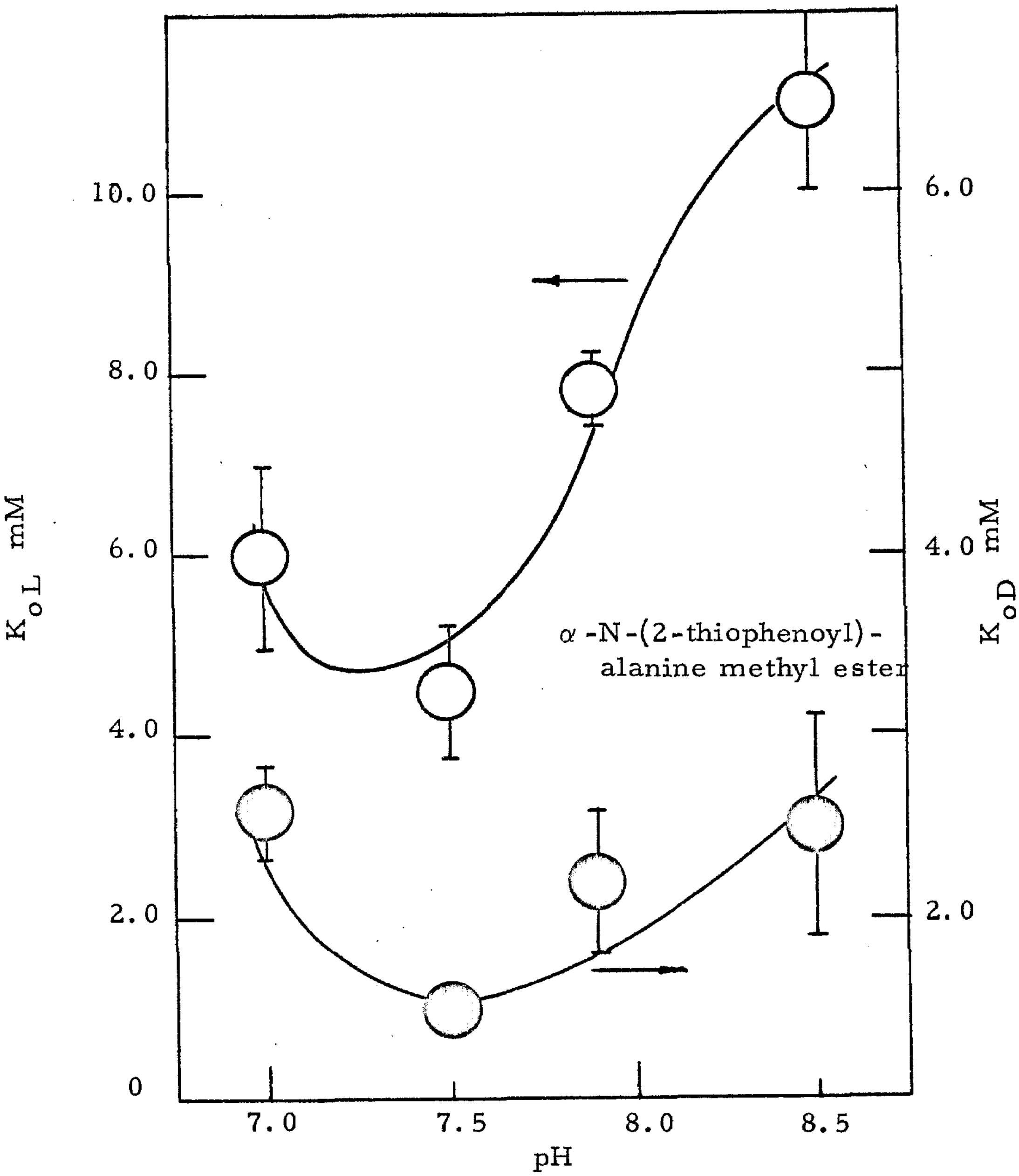


Figure 9.--Dependence of K_o on hydrogen-ion concentration

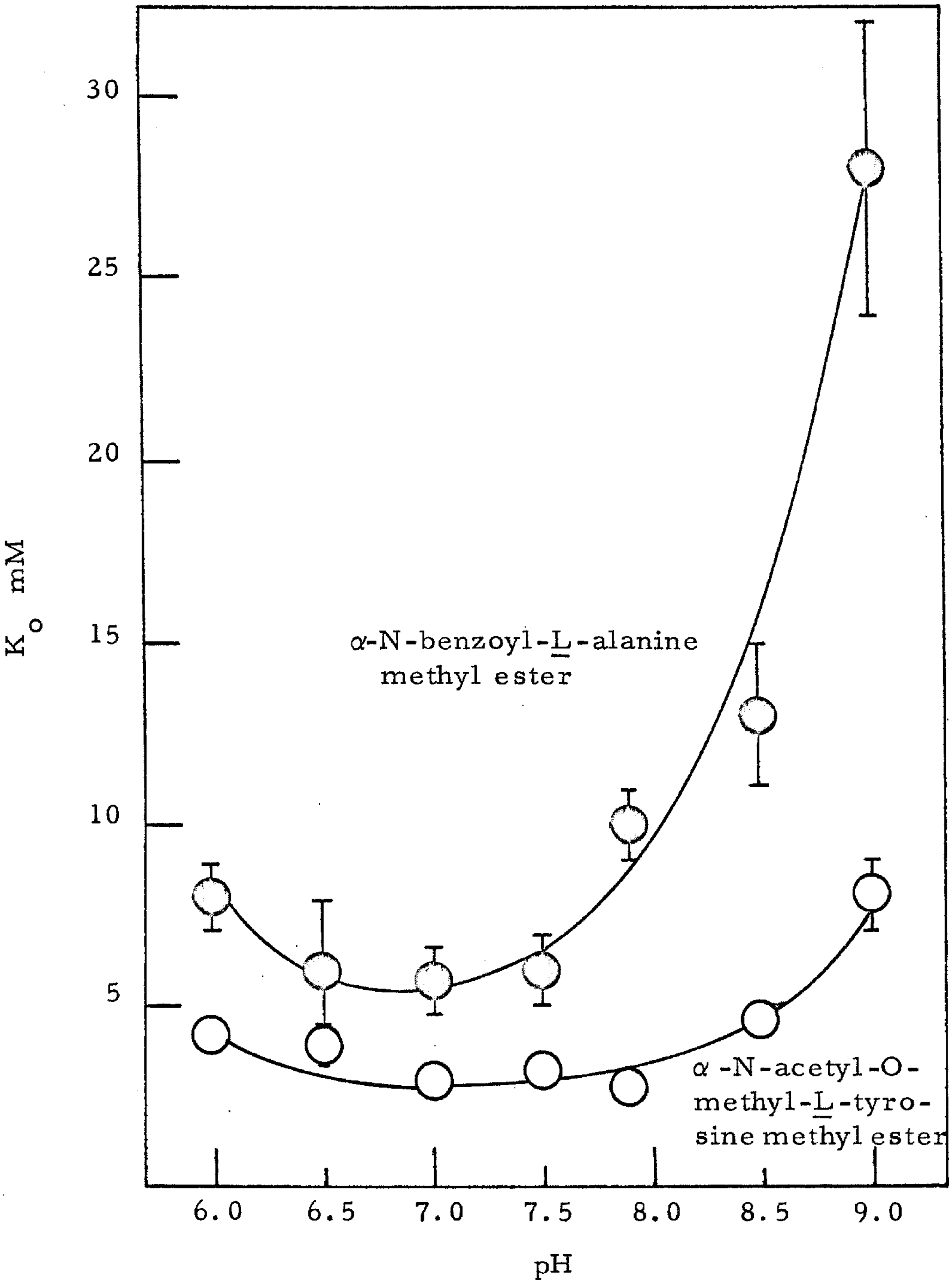


Figure 10.--Dependence of k_o on hydrogen-ion concentration

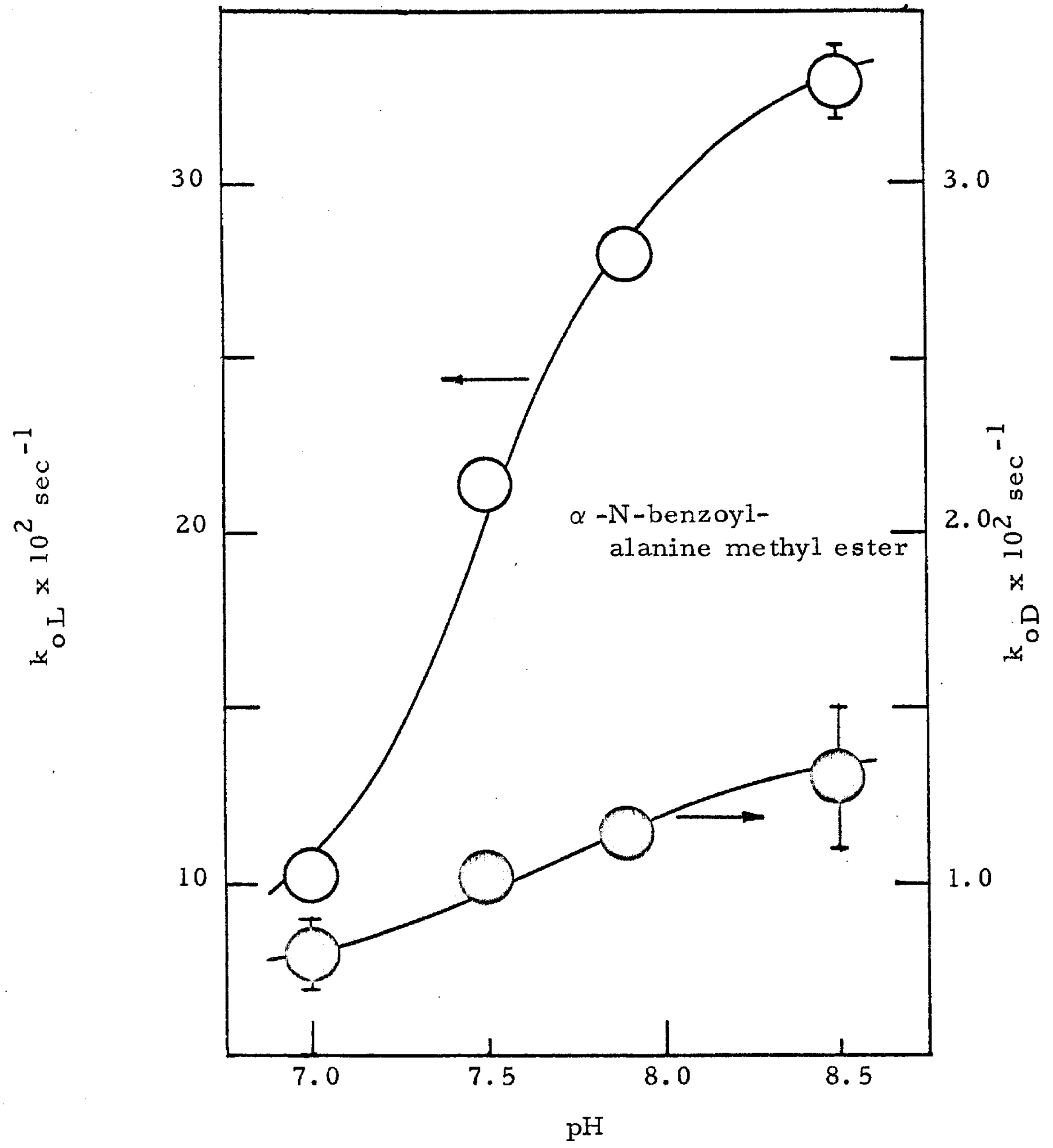


Figure 11.--Dependence of k_o on hydrogen-ion concentration

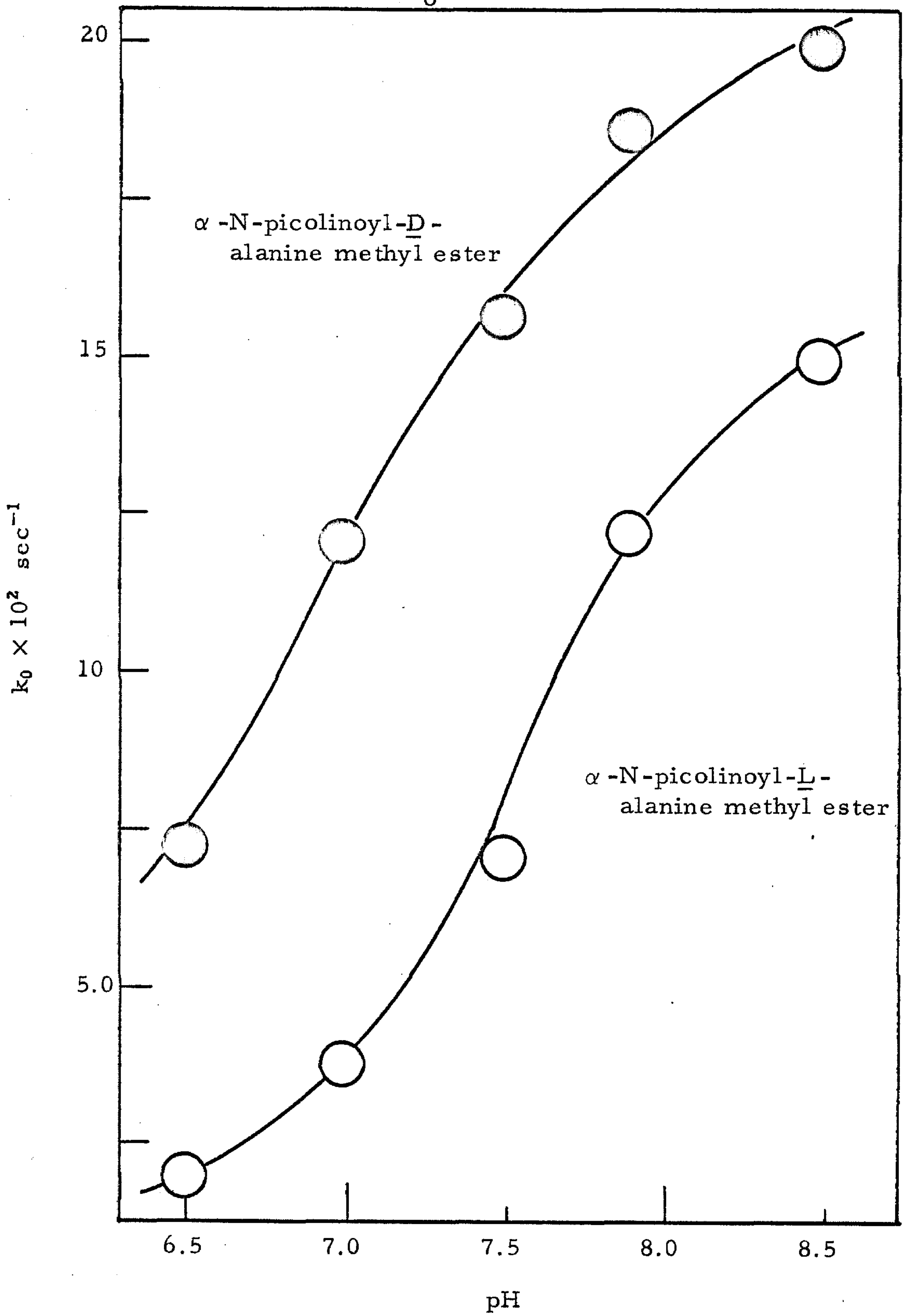


Figure 12.--Dependence of k_o on hydrogen-ion concentration

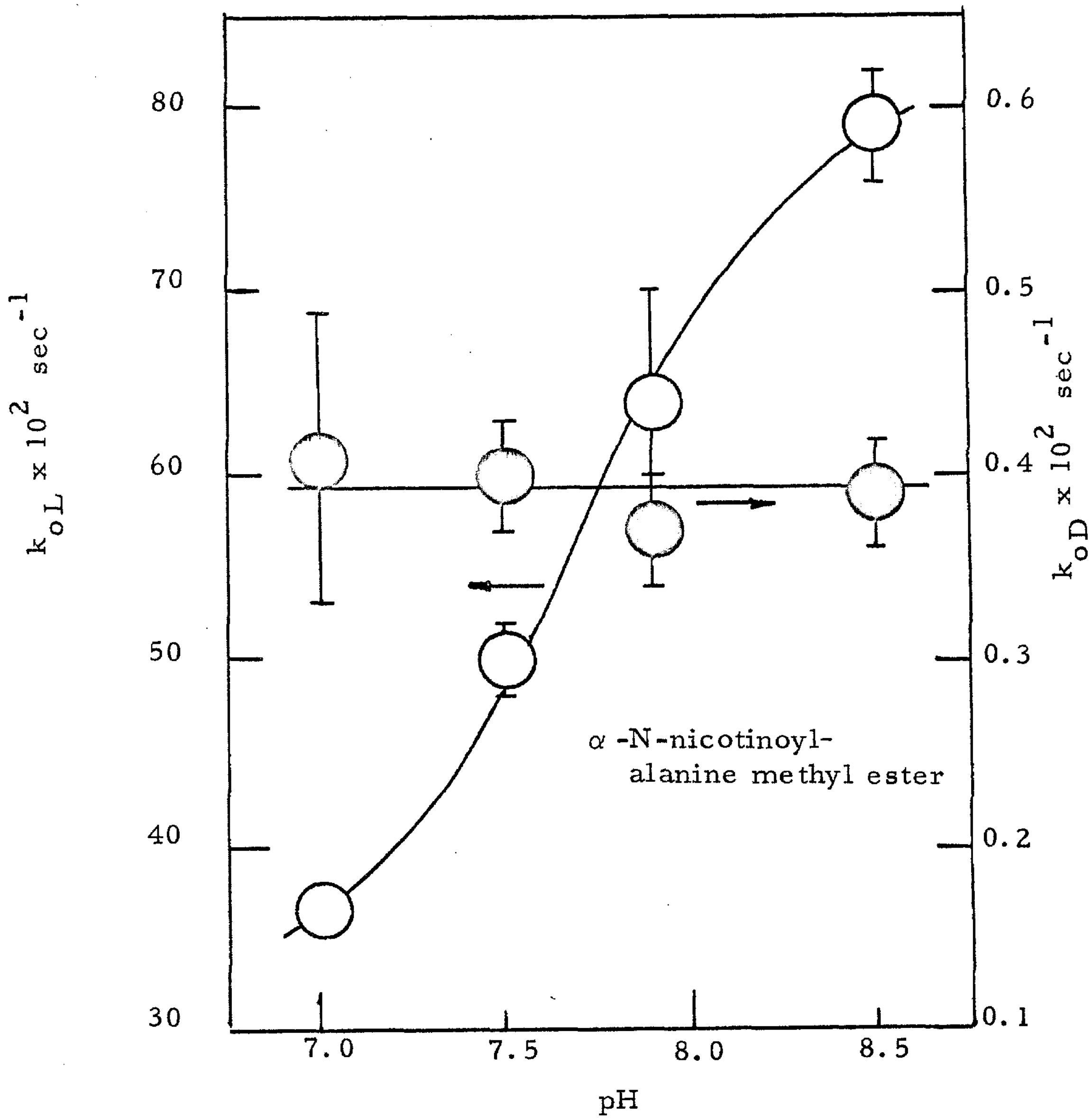


Figure 13.--Dependence of k_o on hydrogen-ion concentration

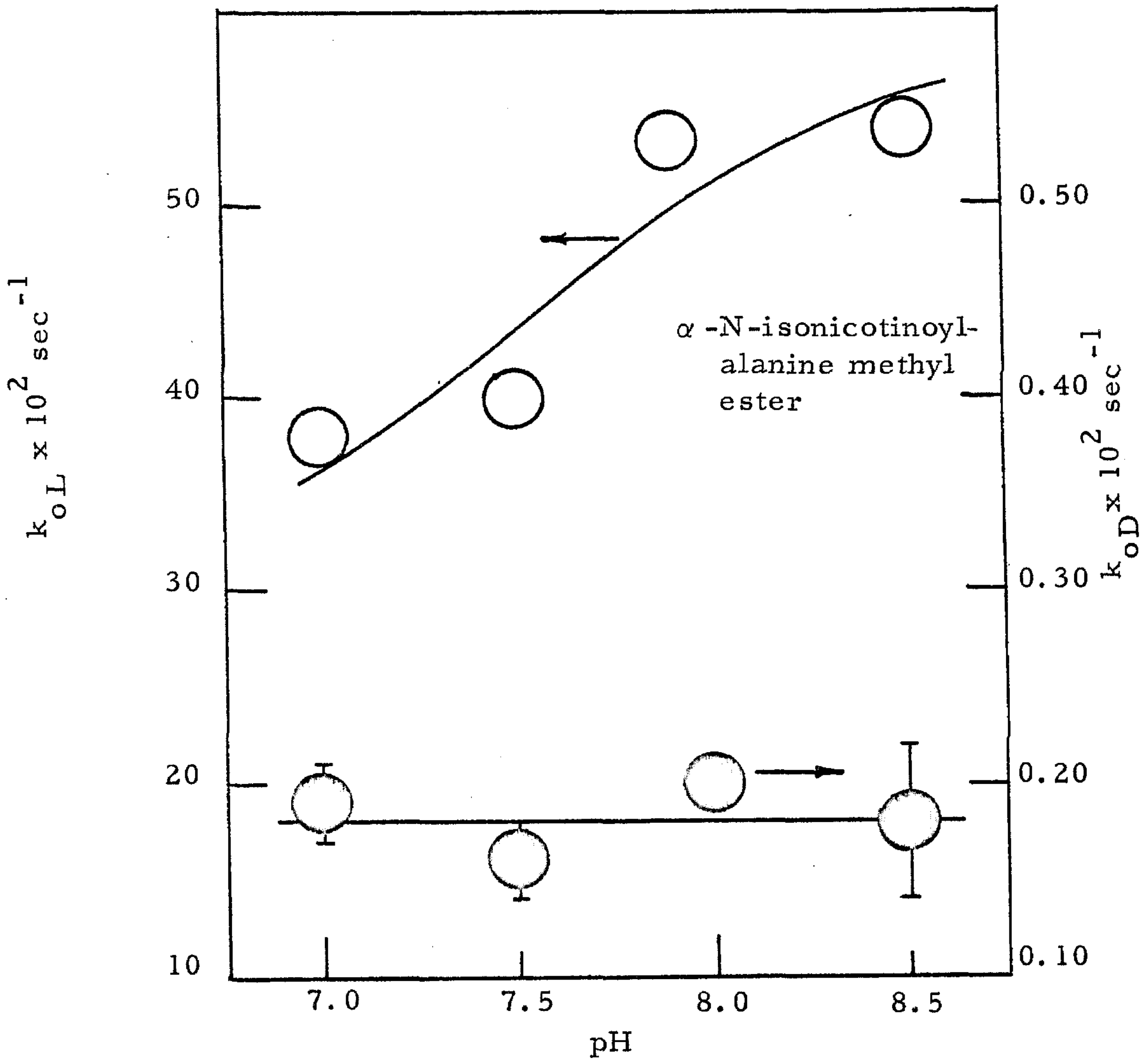


Figure 14.--Dependence of k_o on hydrogen-ion concentration

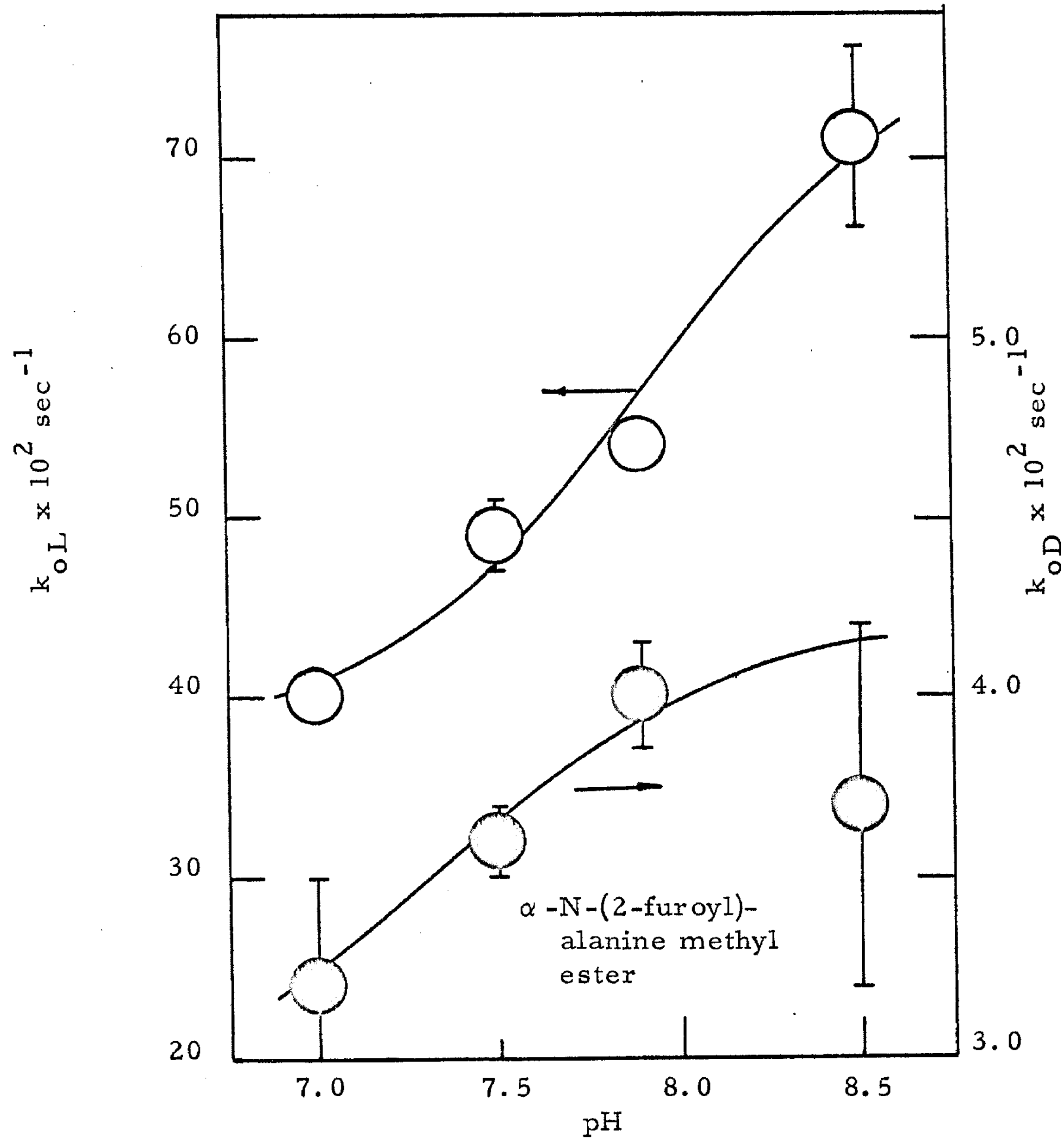


Figure 15.--Dependence of k_o on hydrogen-ion concentration

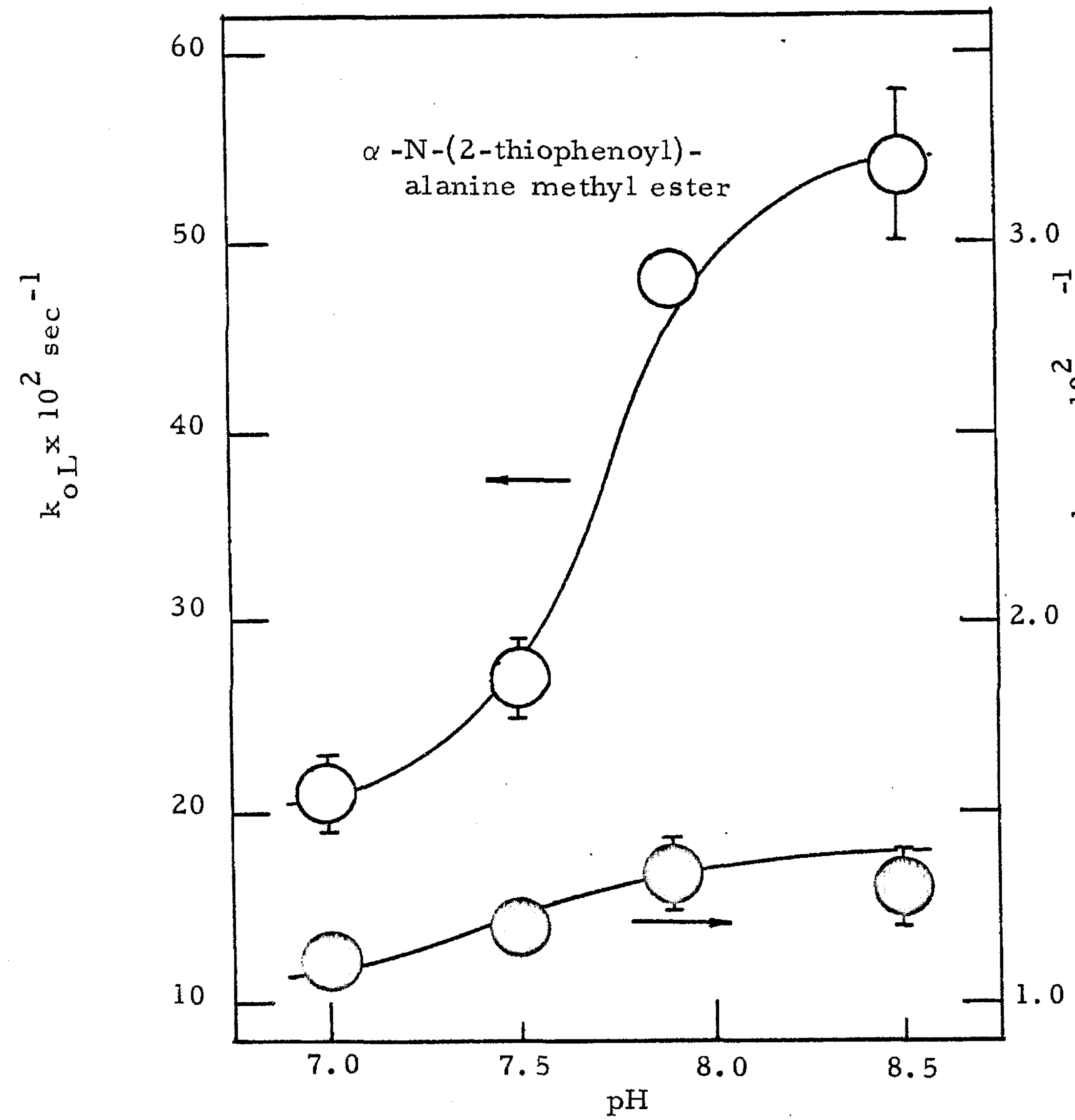


Figure 16.--Dependence of k_o on hydrogen ion concentration.

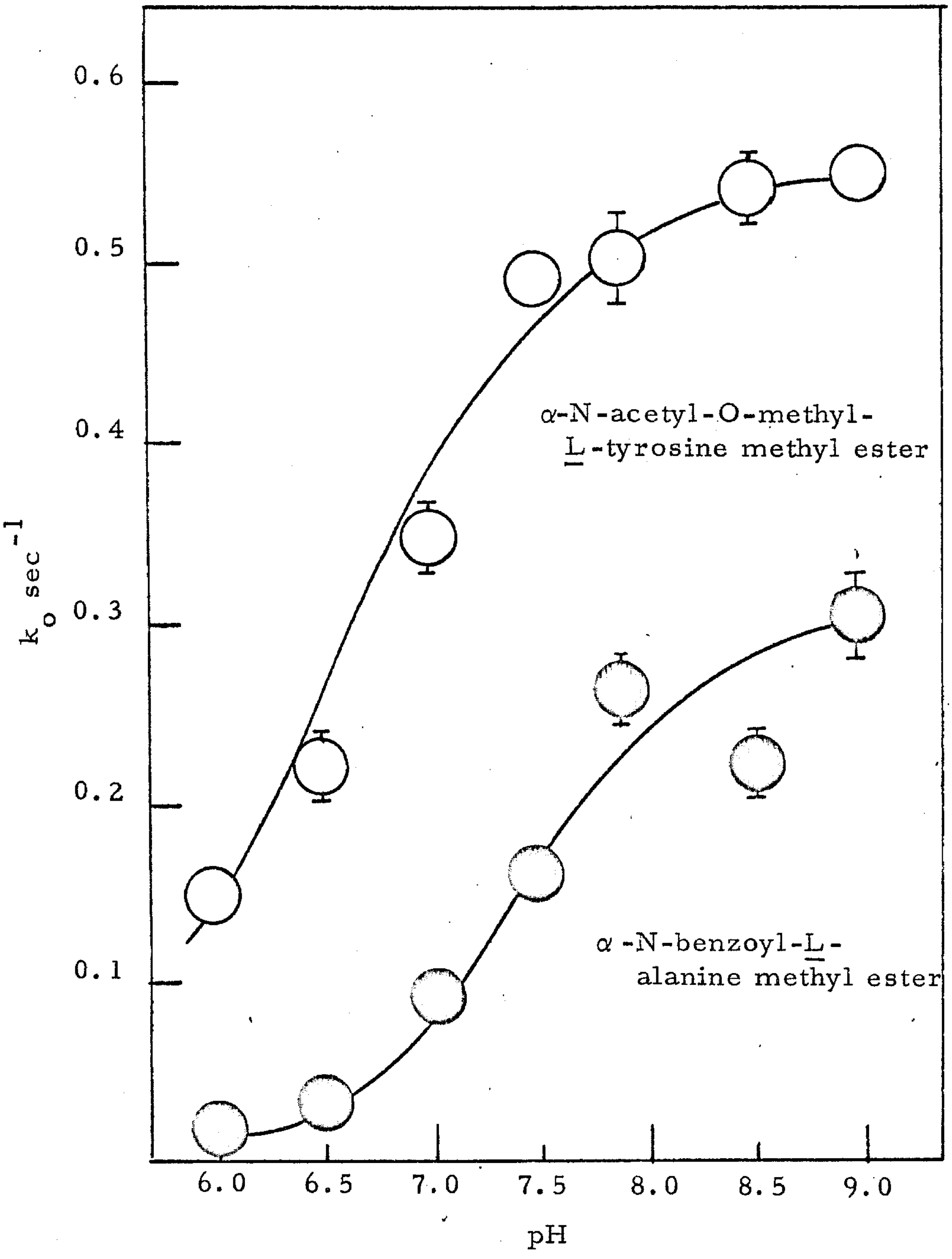


Figure 17.--Dependence of the ratio k_o/K_o on hydrogen-ion concentration

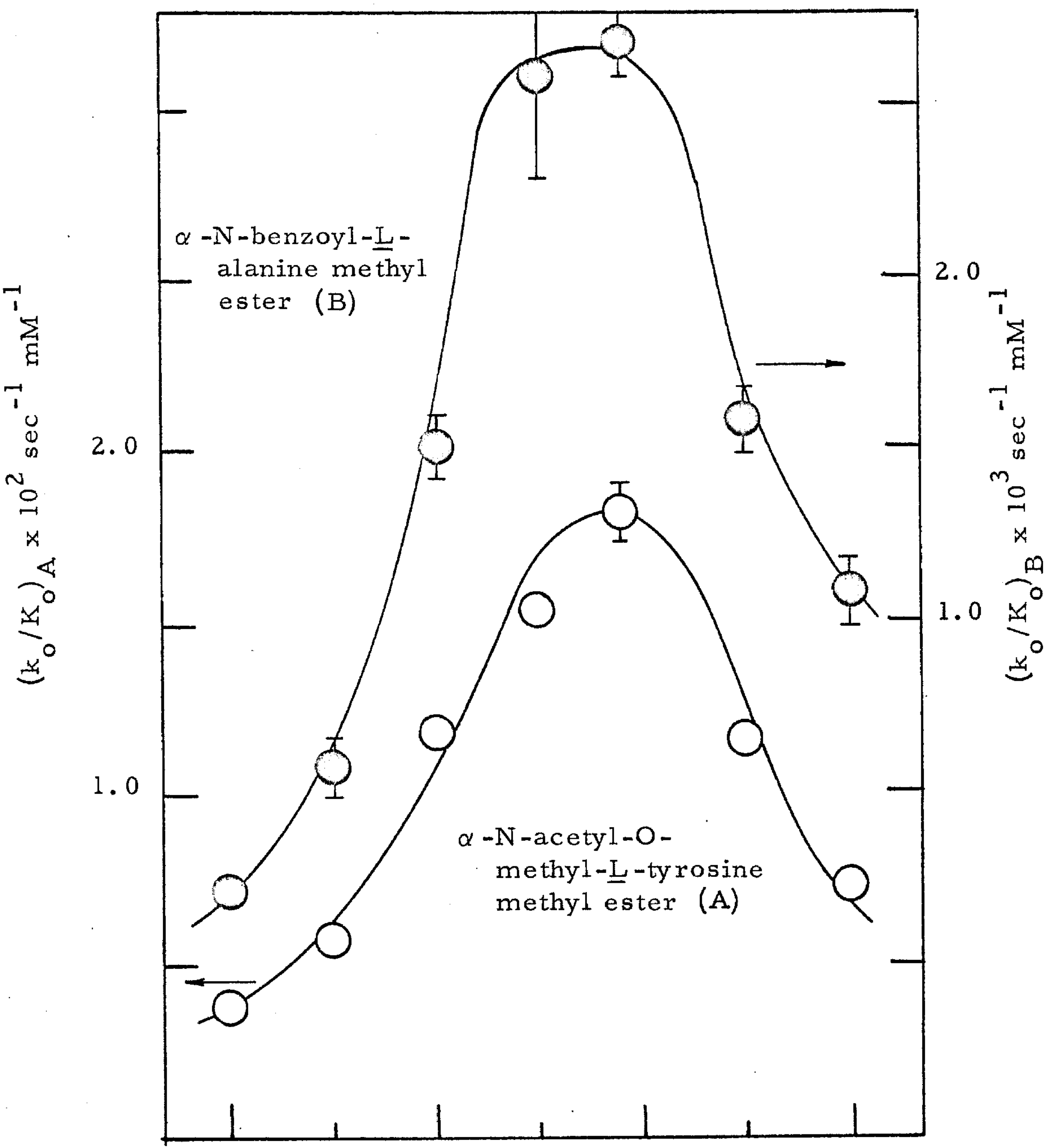


Figure 18.--Dependence of the ratio k_o/K_o on hydrogen-ion concentration

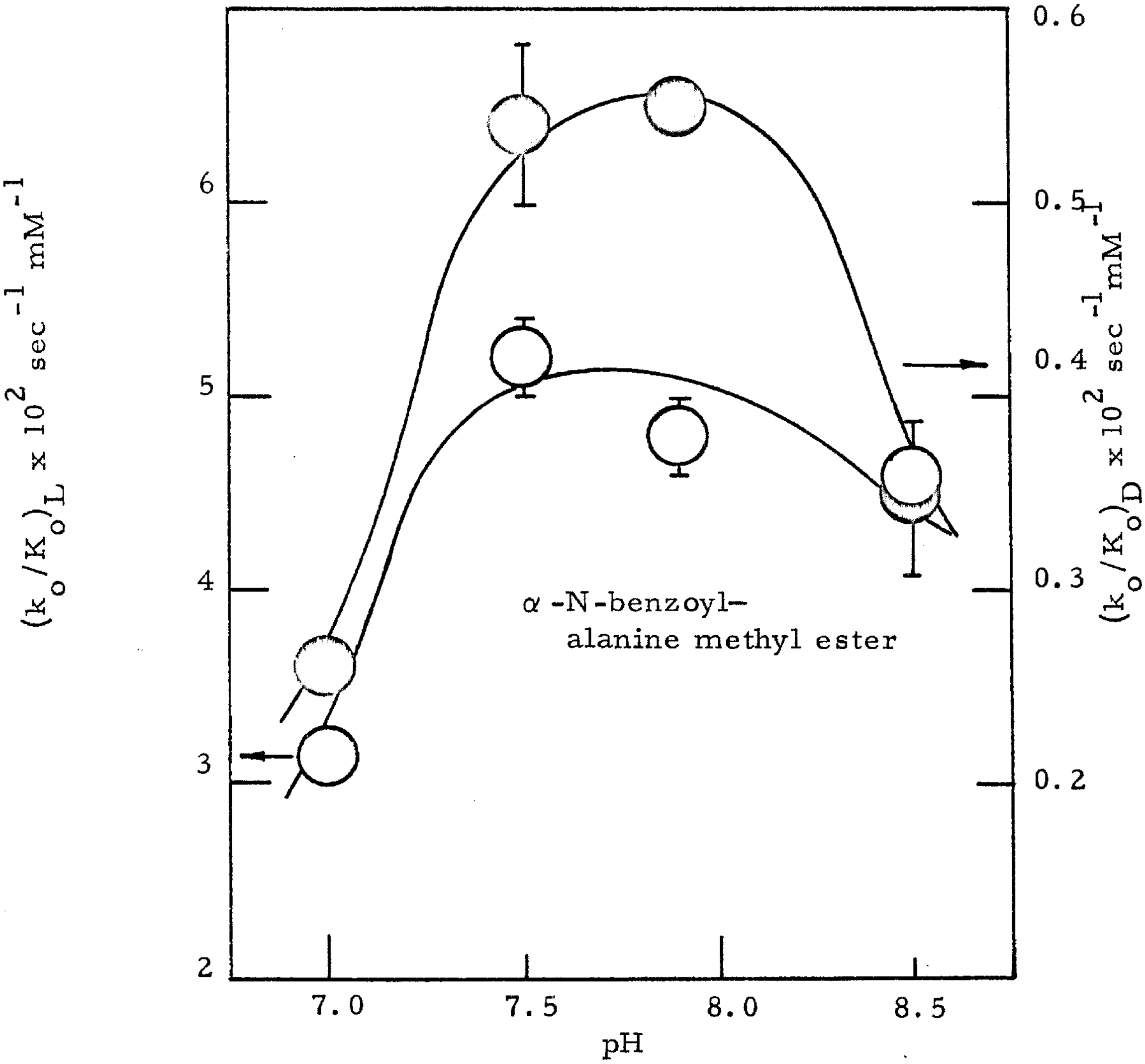


Figure 19.--Dependence of the ratio k_o/K_o on hydrogen-ion concentration

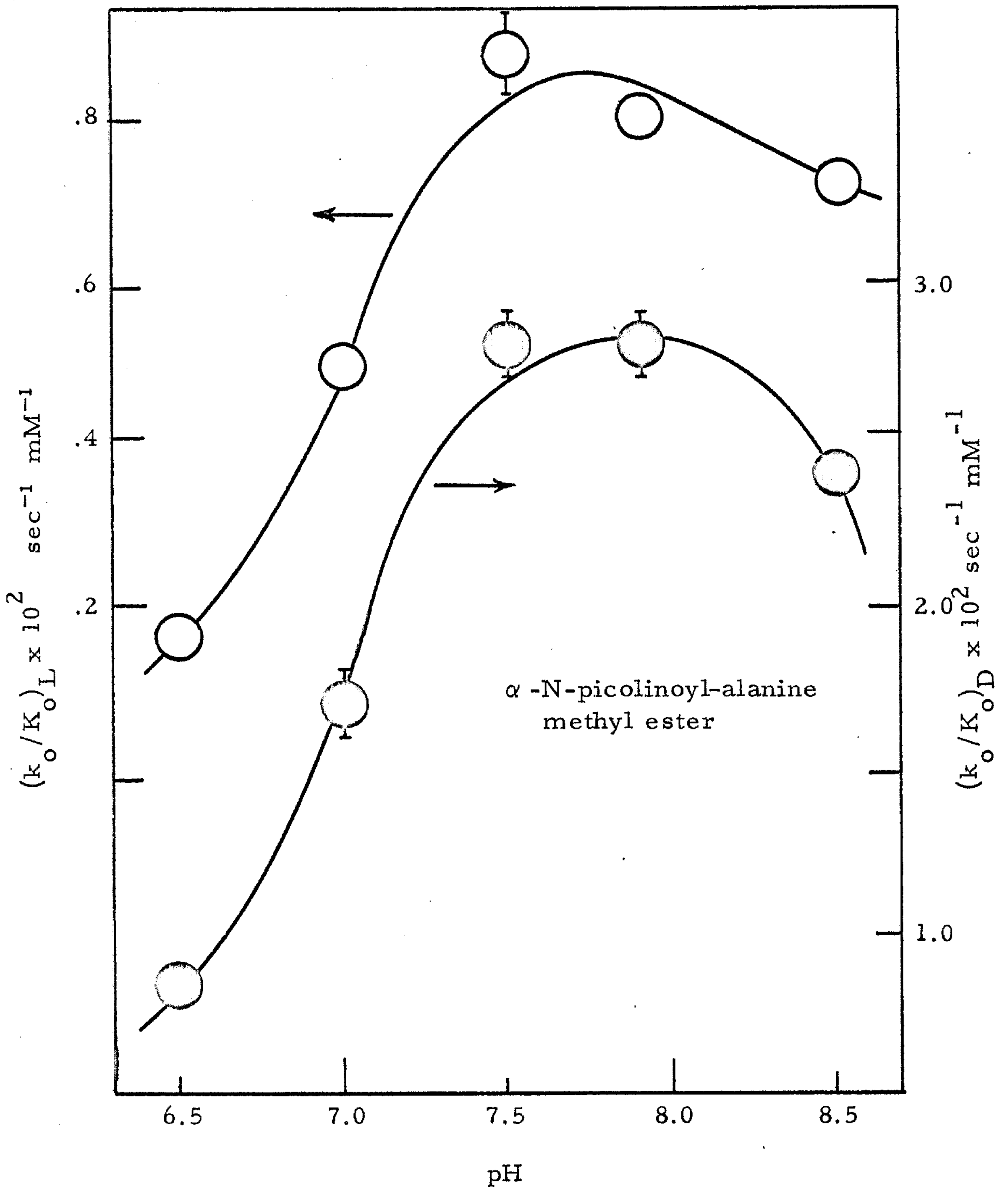


Figure 20.--Dependence of the ratio k_o/K_o on hydrogen-ion concentration

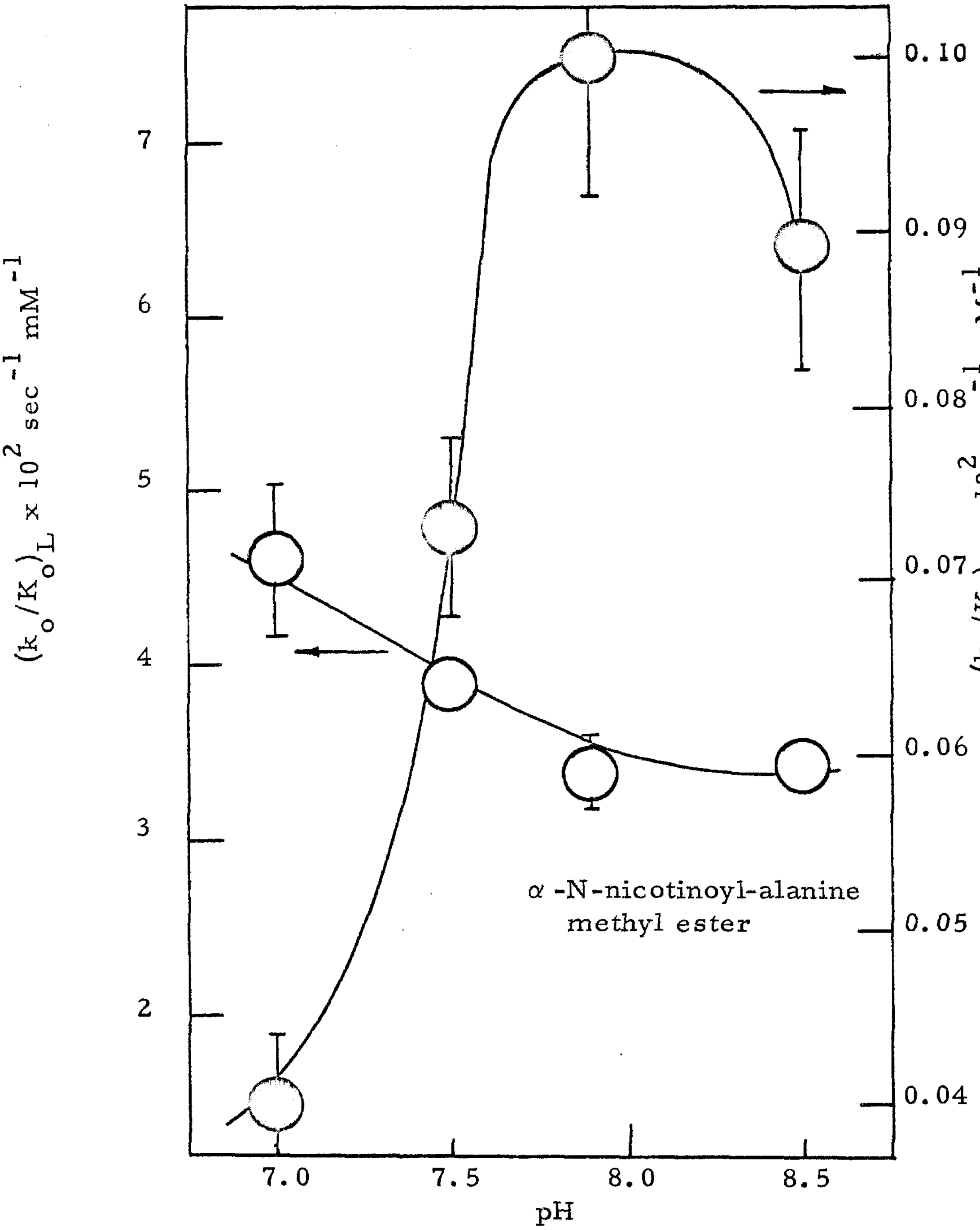


Figure 21.--Dependence of the ratio k_o/K_o on hydrogen-ion concentration

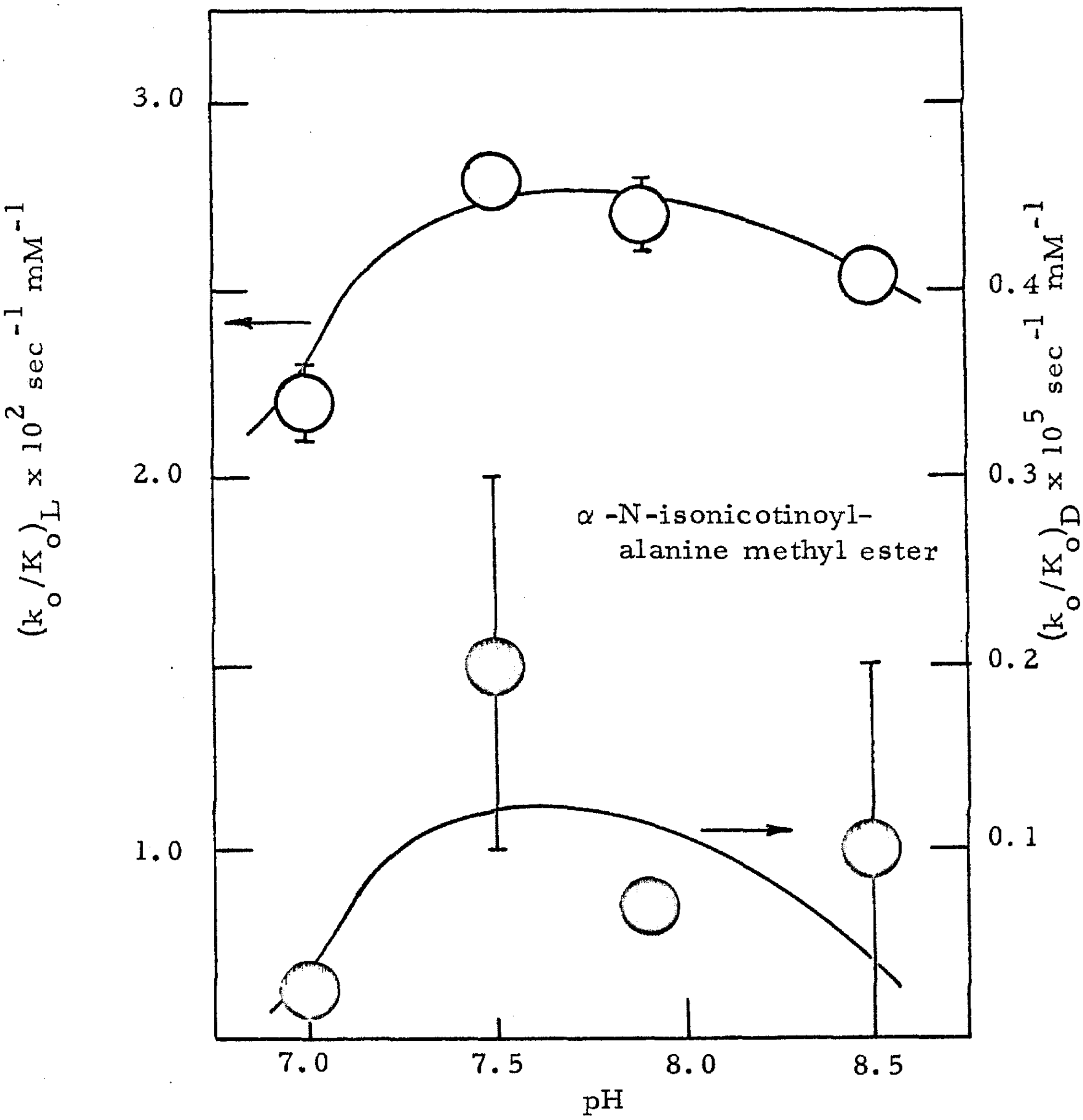


Figure 22.--Dependence of the ratio k_o/K_o on hydrogen-ion concentration

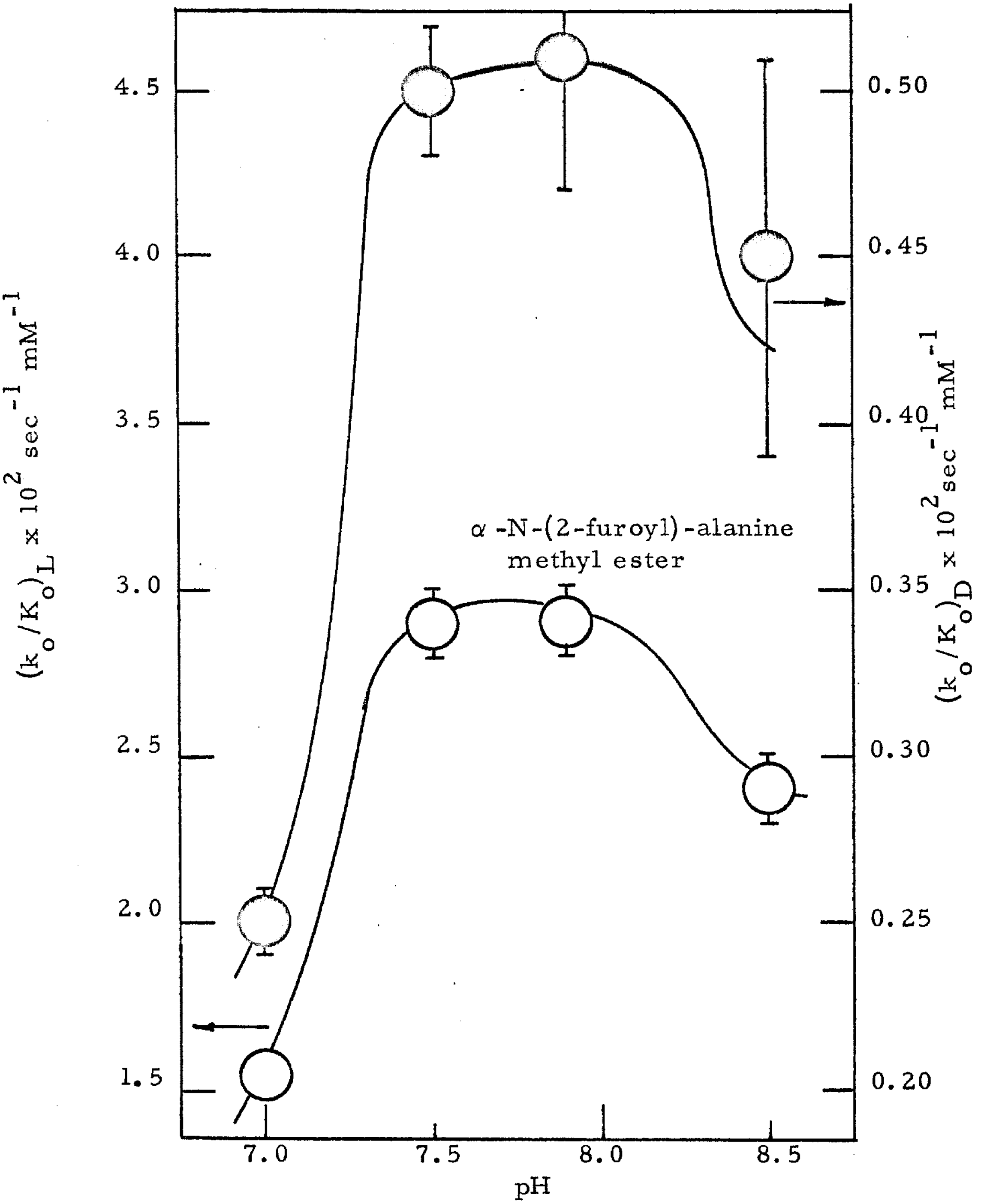


Figure 23.--Dependence of the ratio k_o/K_o on hydrogen-ion concentration

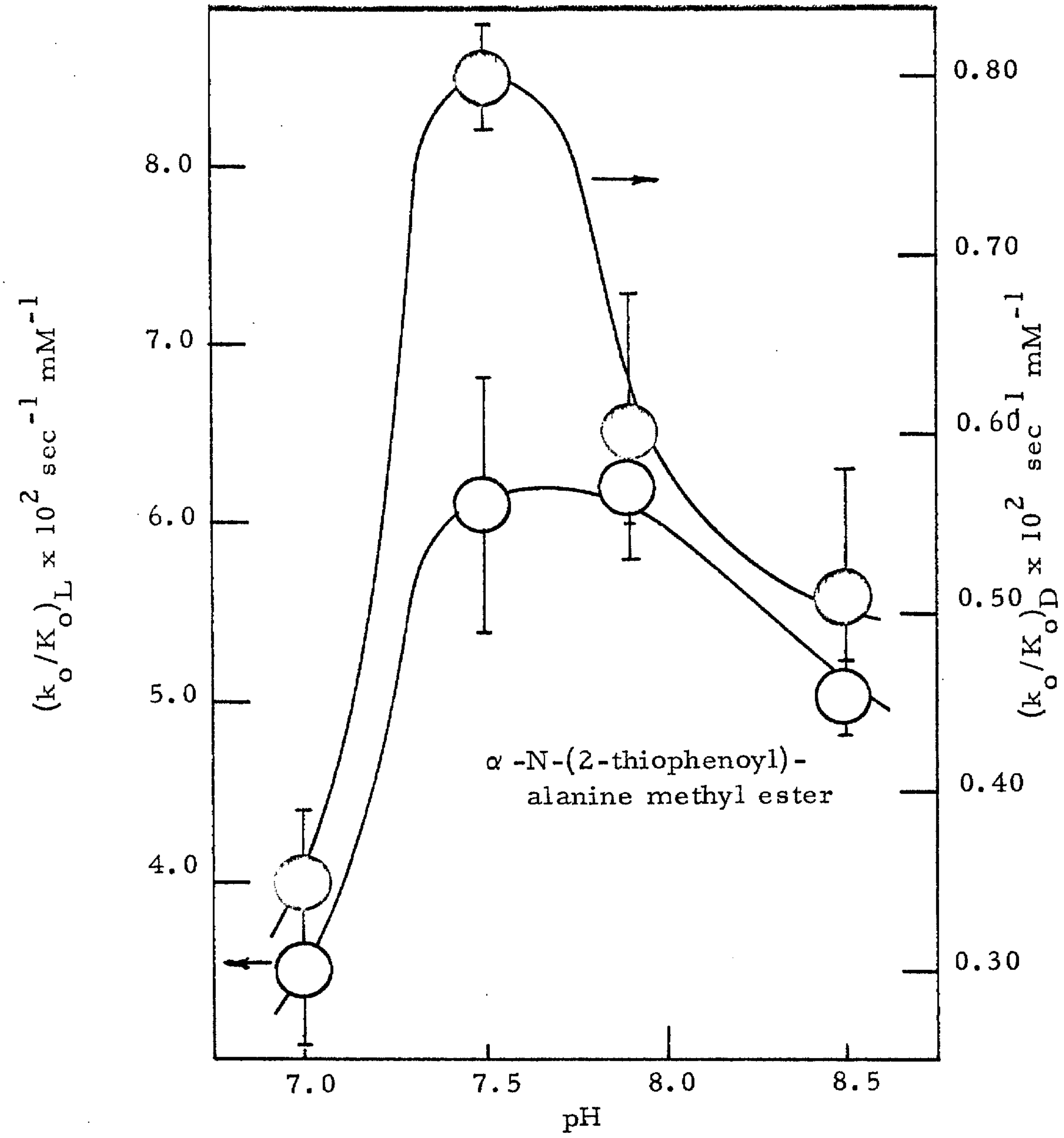


Figure 24.--Dependence of the ratios k_{oL}/k_{oD} and K_{oL}/K_{oD} on hydrogen-ion concentration

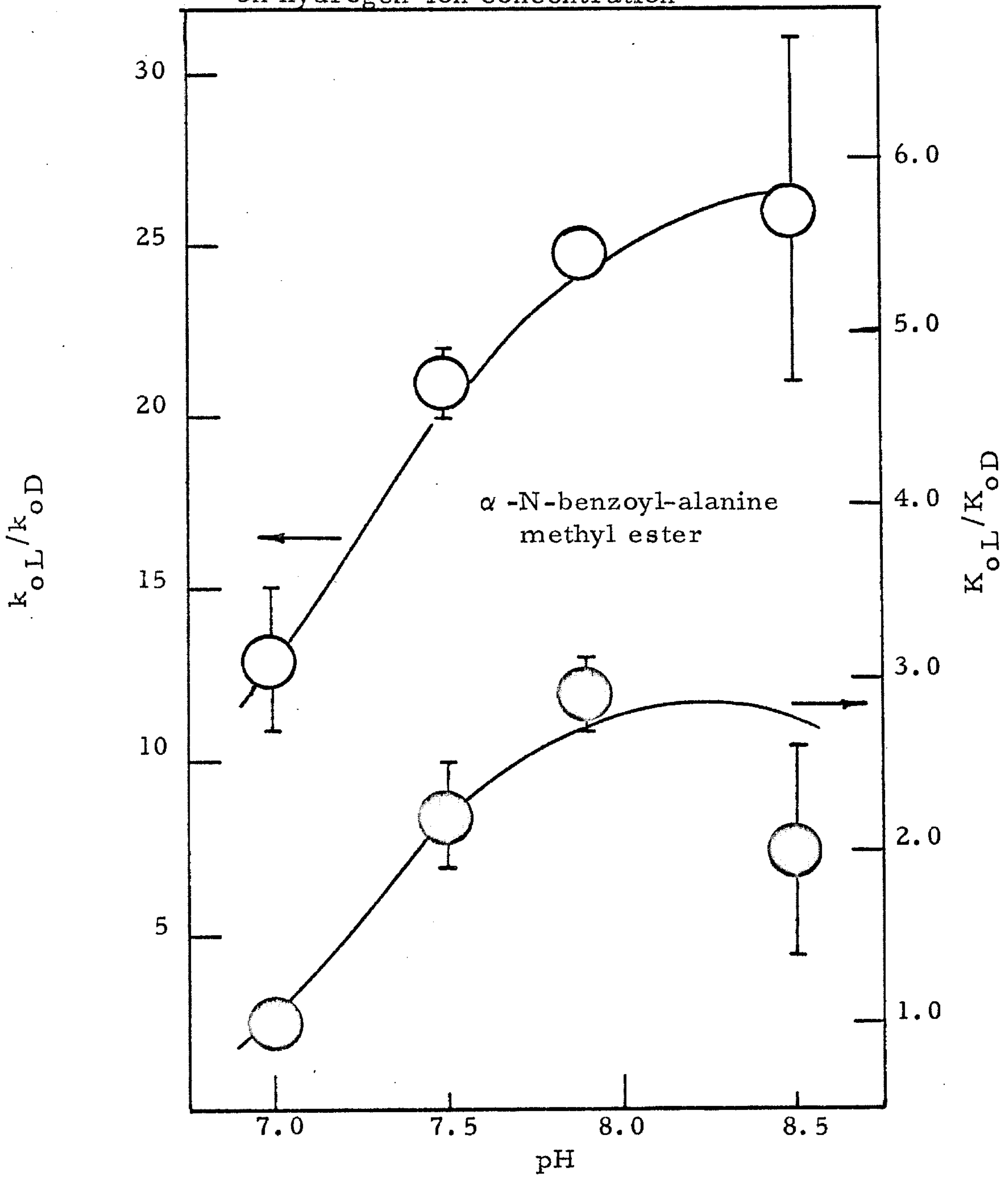


Figure 25.--Dependence of the ratios k_{oL}/k_{oD} and K_{oL}/K_{oD} on hydrogen-ion concentration

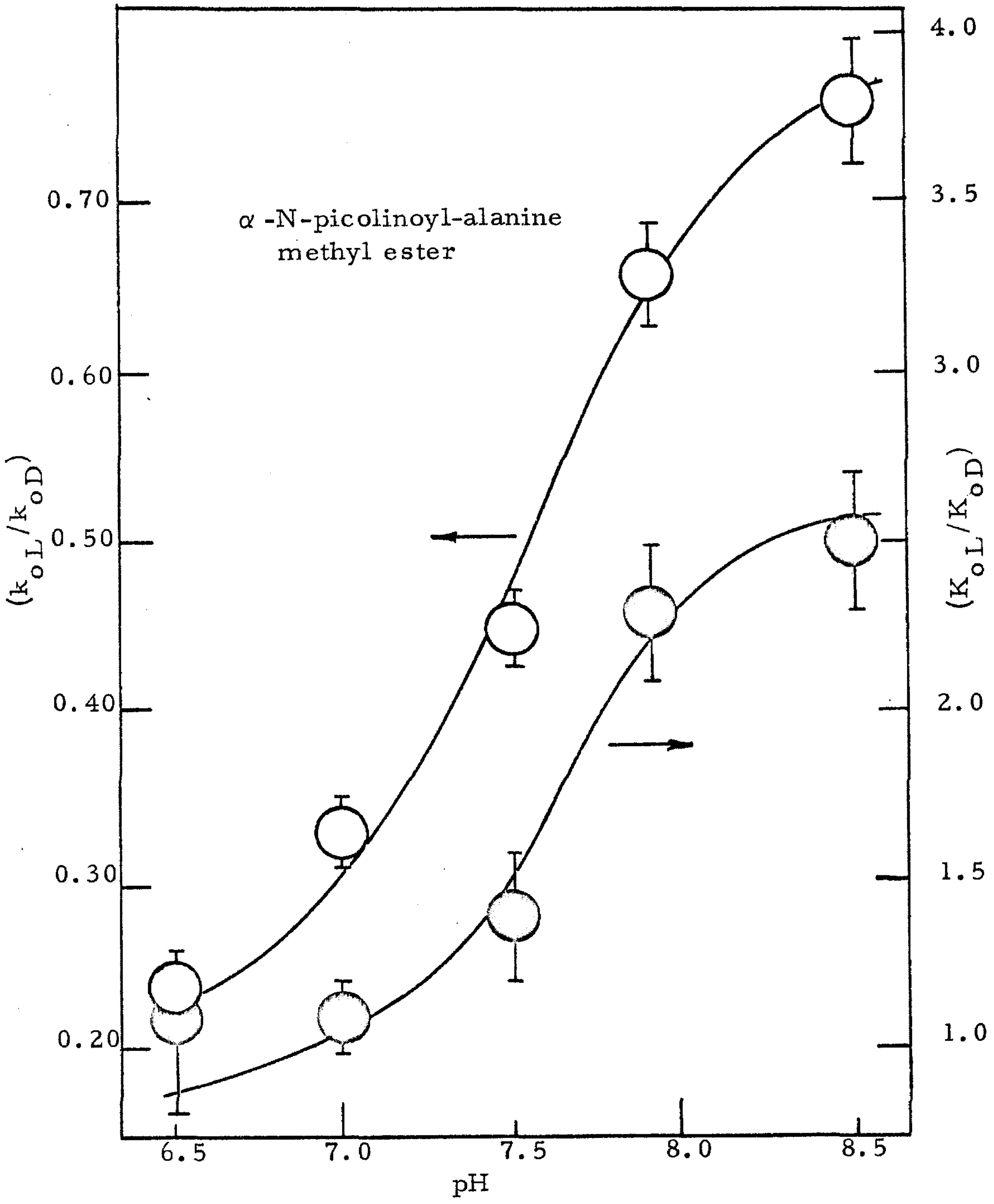


Figure 26.--Dependence of the ratios k_{oL}/k_{oD} and K_{oL}/K_{oD} on hydrogen-ion concentration

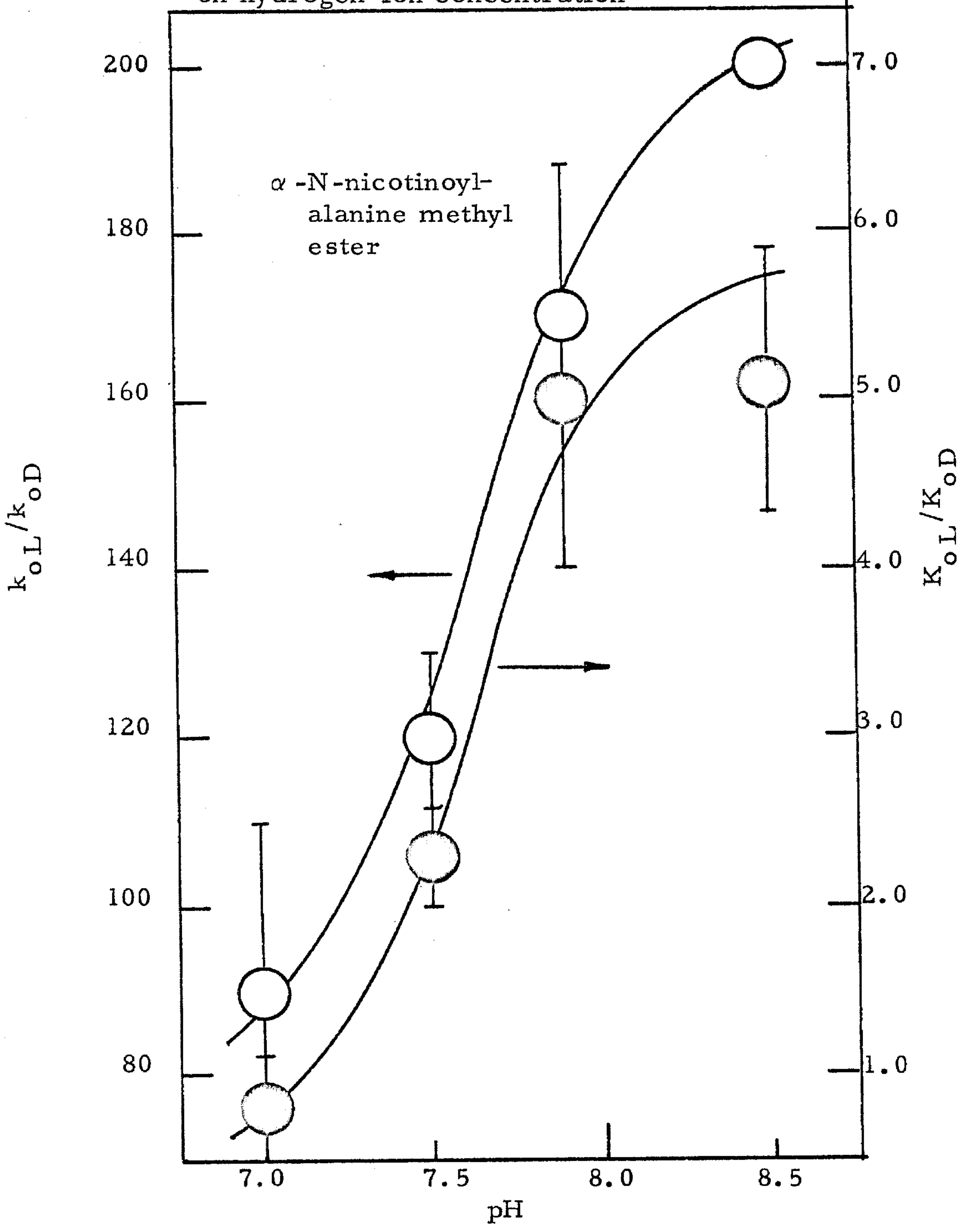


Figure 27.--Dependence of the ratios k_{oL}/k_{oD} and K_{oL}/K_{oD} on hydrogen-ion concentration

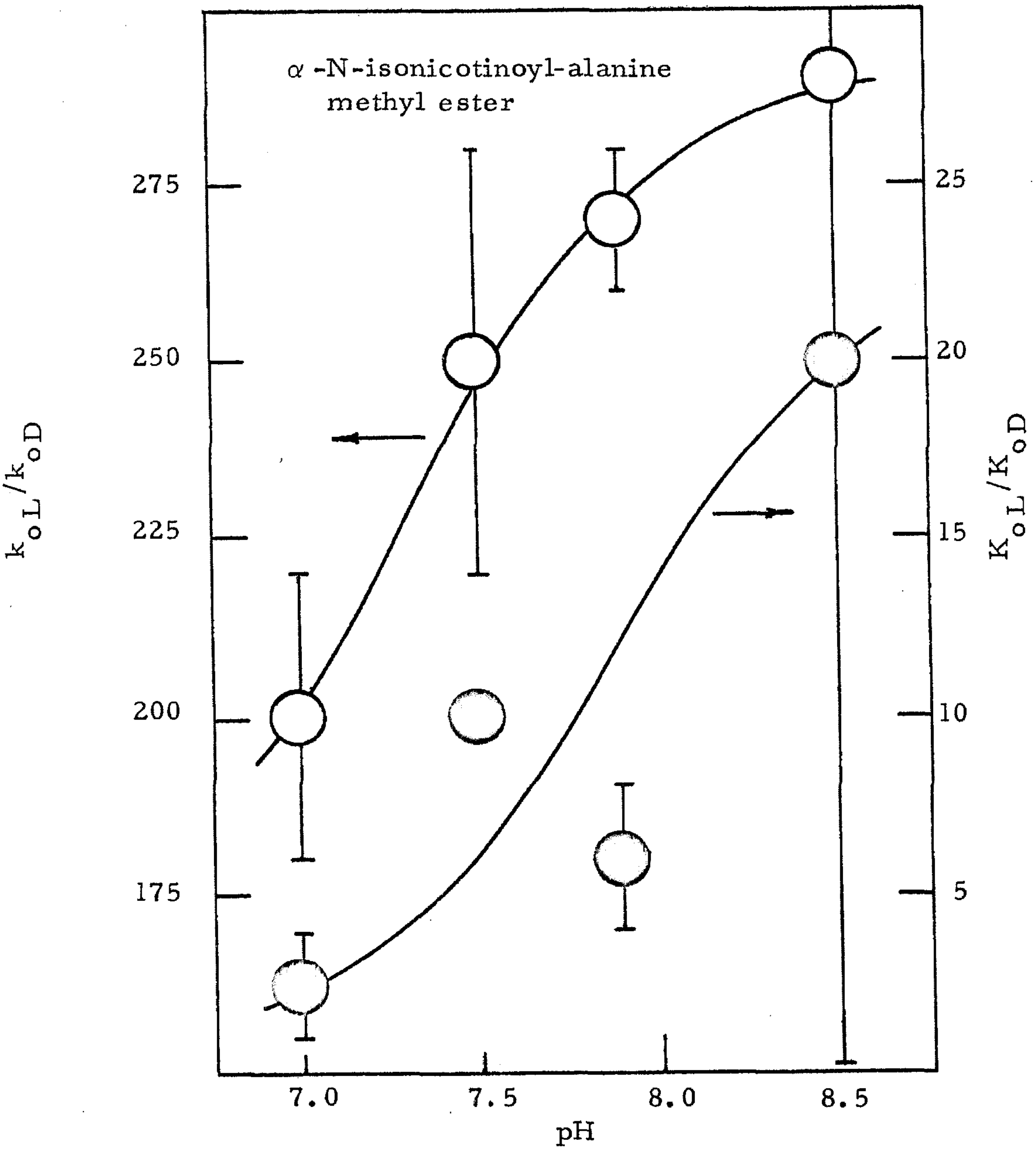


Figure 28.--Dependence of the ratios k_{oL}/k_{oD} and K_{oL}/K_{oD} on hydrogen-ion concentration

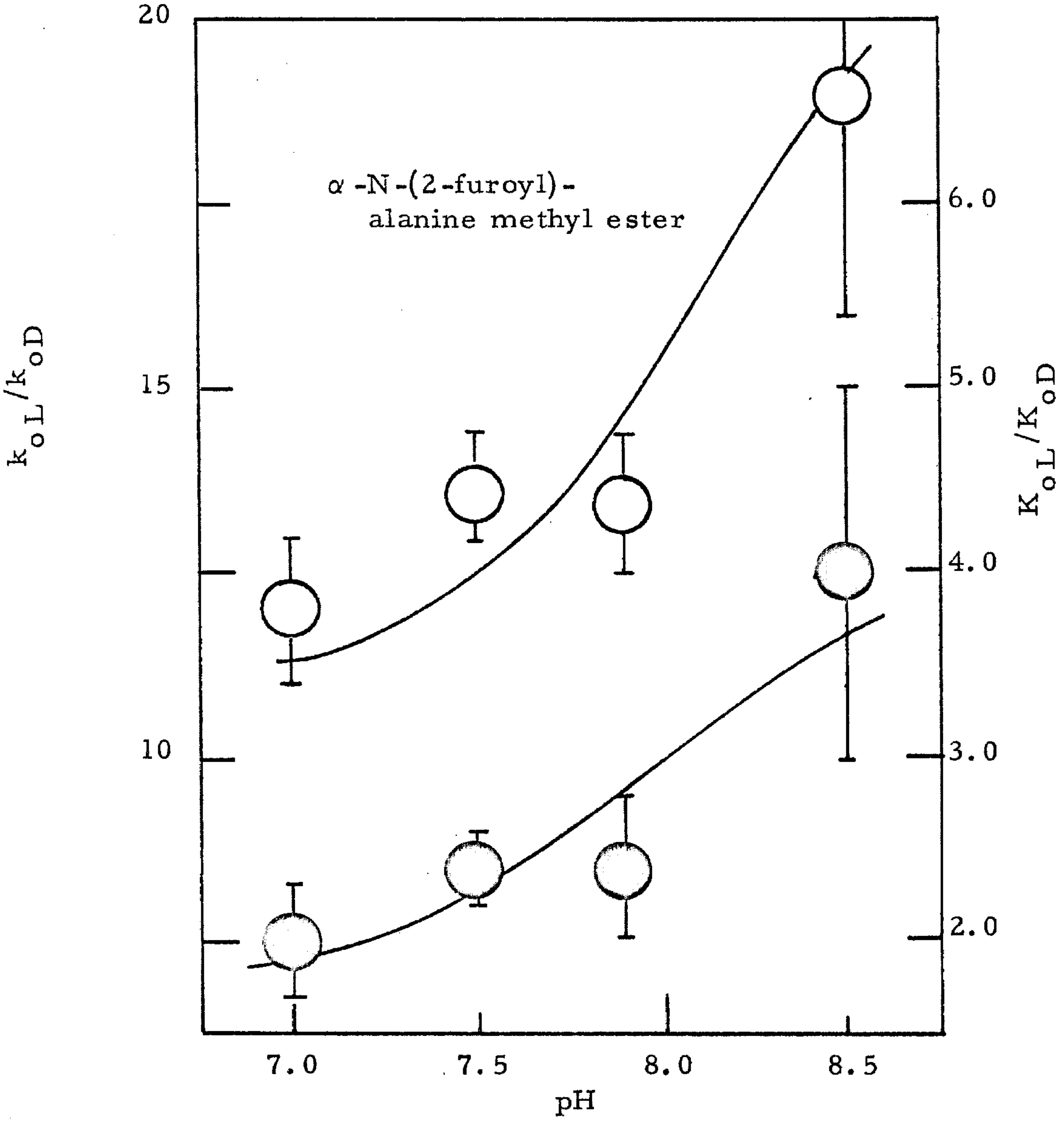


Figure 29.--Dependence of the ratios k_{oL}/k_{oD} and K_{oL}/K_{oD} on hydrogen-ion concentration

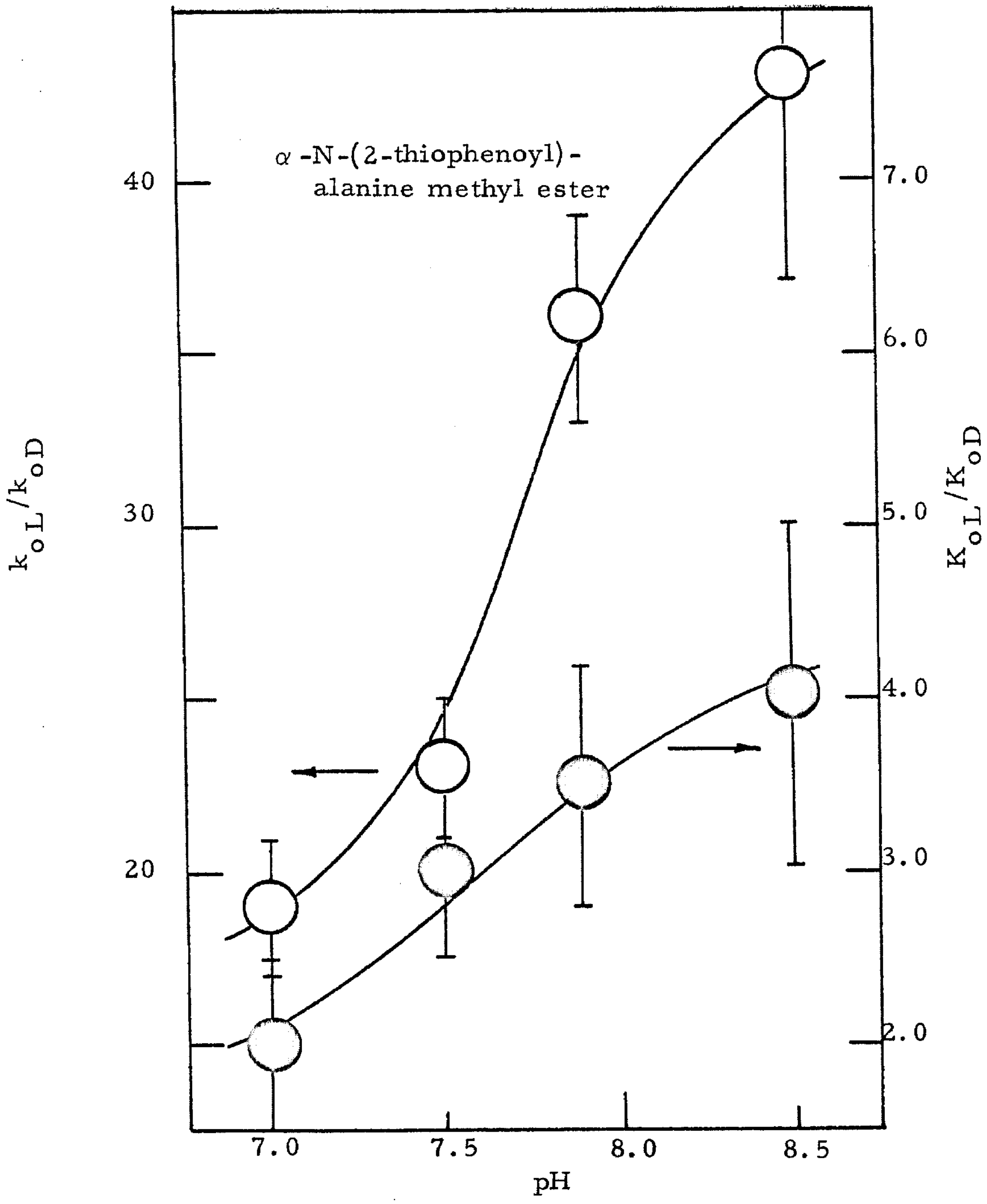


Figure 30. --Dependence of the ratio K_{oB}/K_{oA} on hydrogen-ion concentration

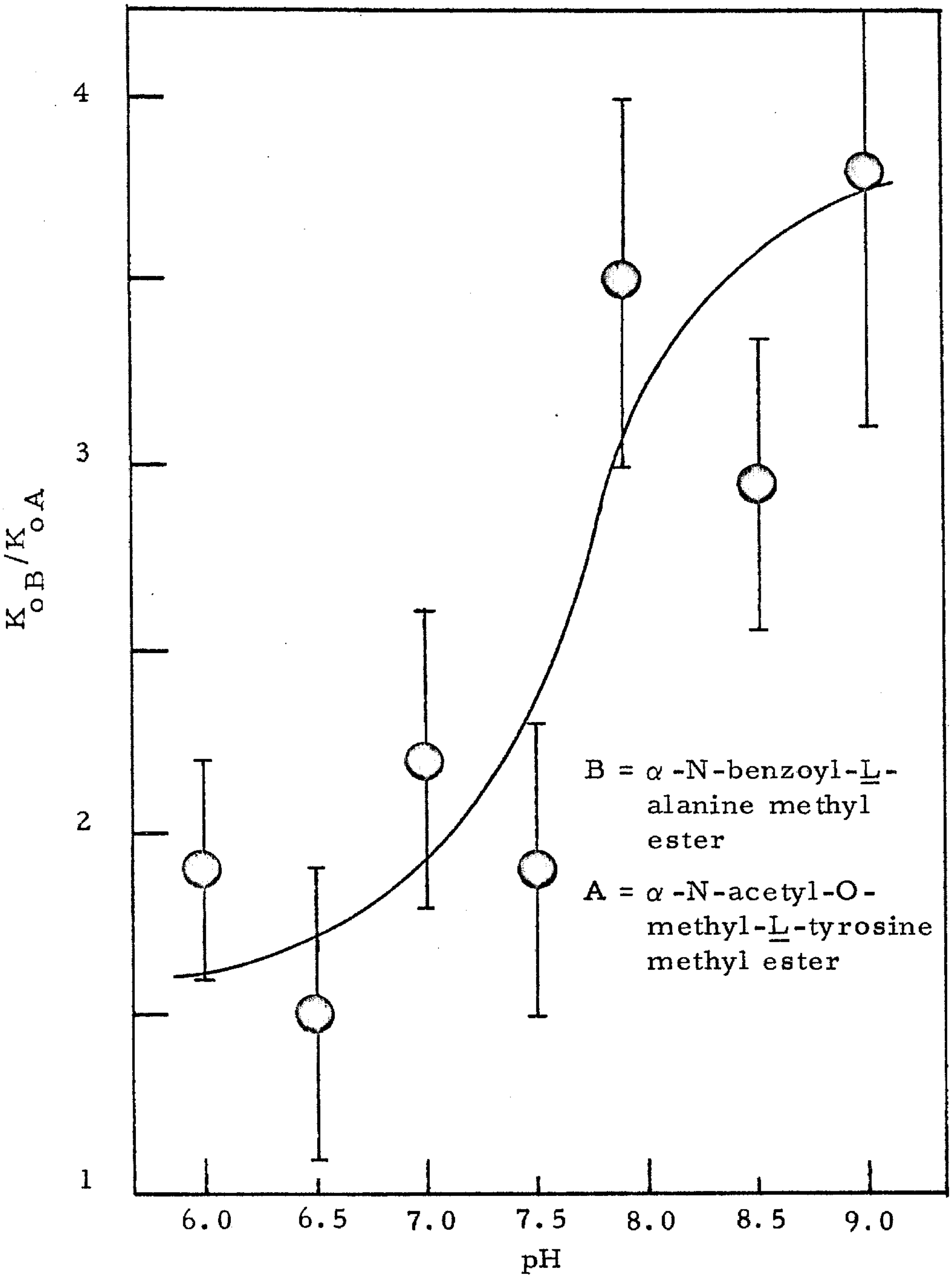


Figure 31.--Dependence of the ratio k_{oB}/k_{oA} on hydrogen-ion concentration

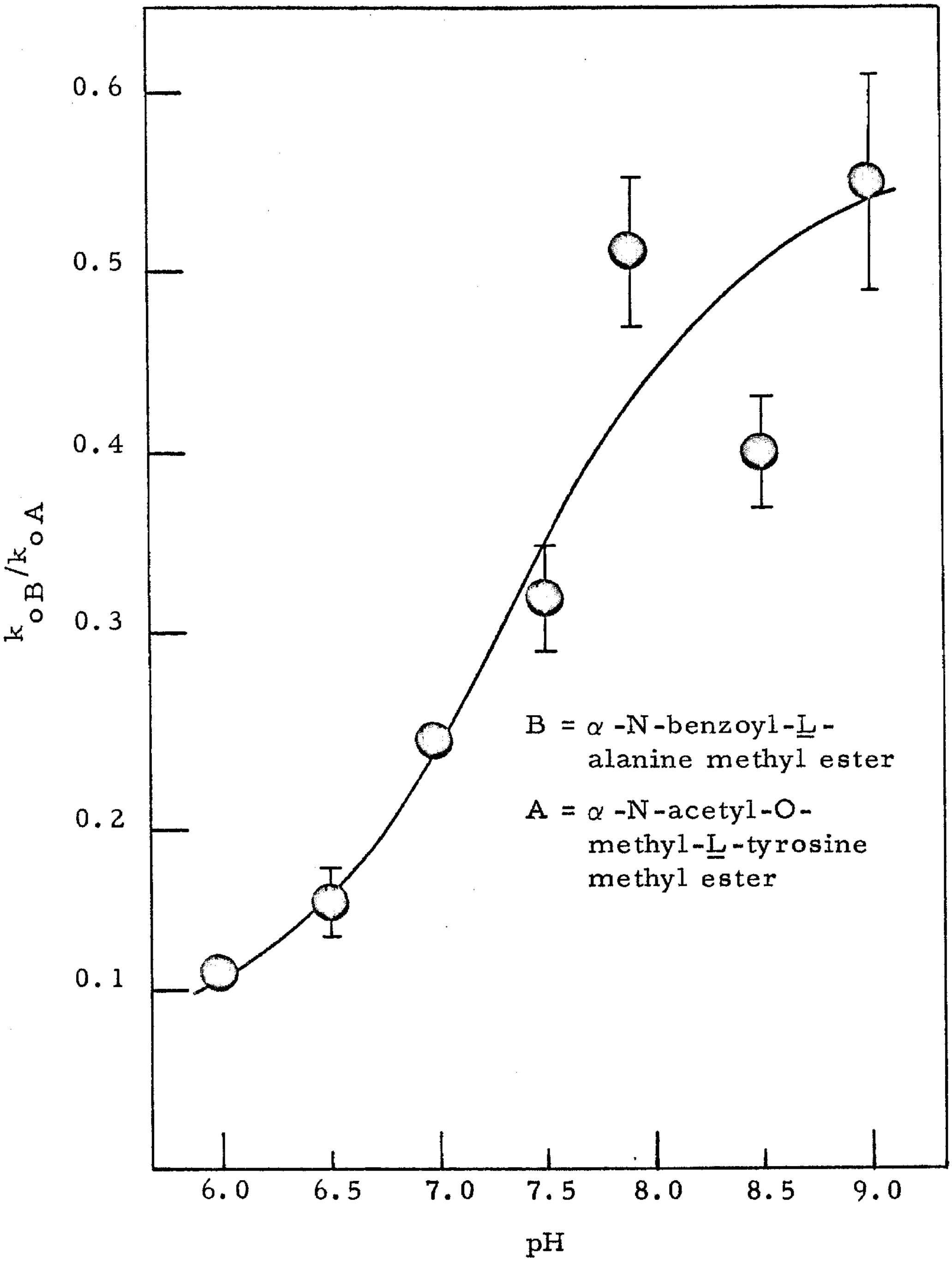


Figure 32.--Dependence of the ratio $(k_o/K_o)_L/(k_o/K_o)_D$ on hydrogen-ion concentration

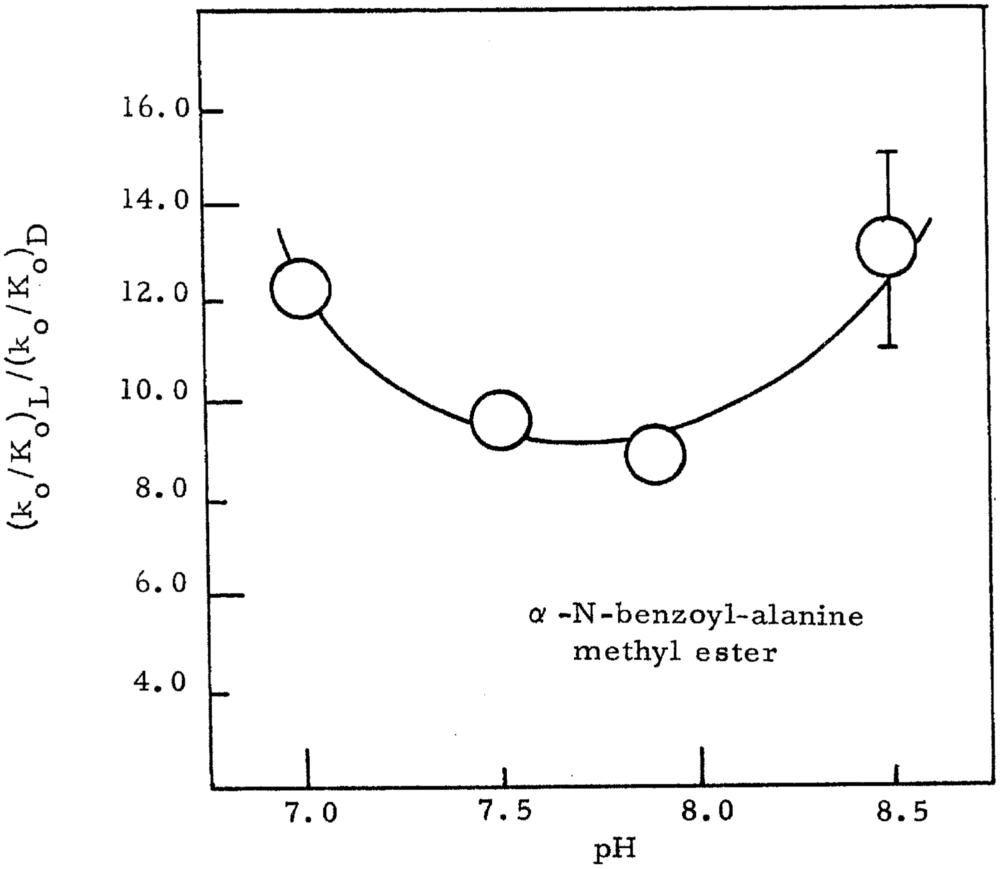


Figure 33.--Dependence of the ratio $(k_o/K_o)_L/(k_o/K_o)_D$ on hydrogen-ion concentration

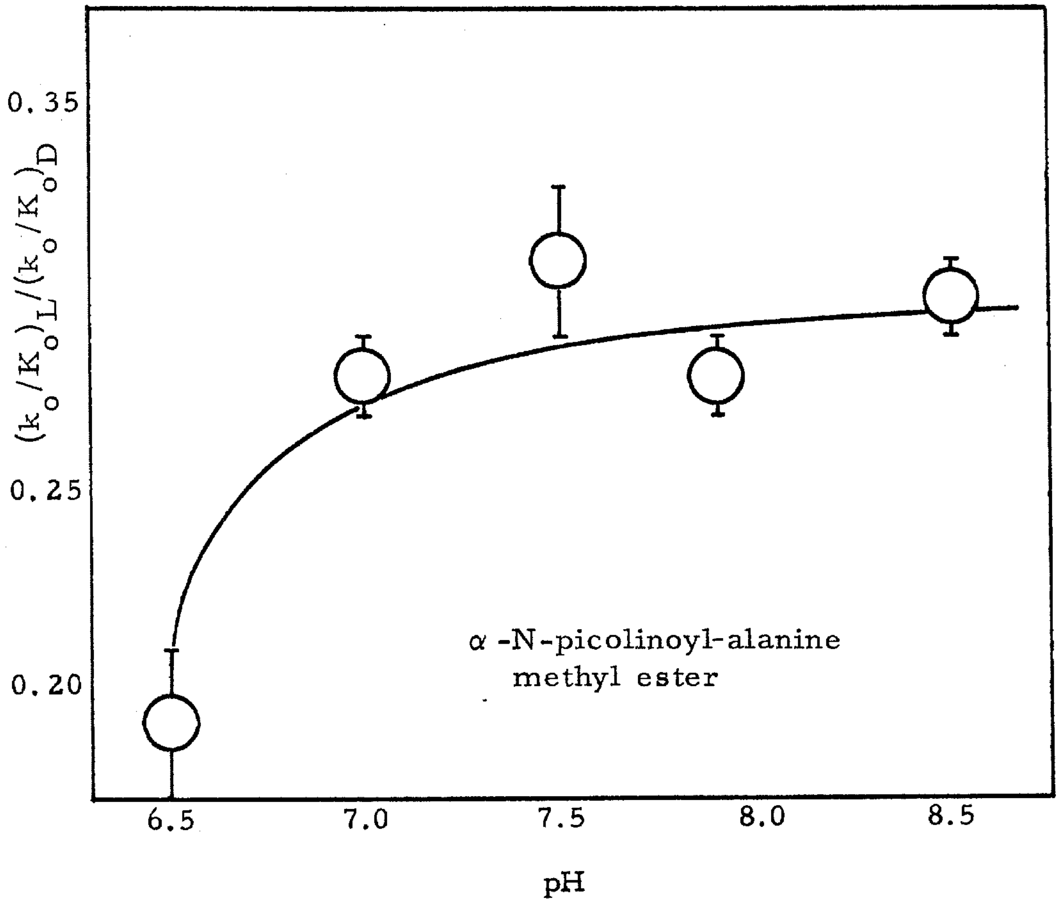


Figure 34.--Dependence of the ratio $(k_o/K_o)_L/(k_o/K_o)_D$ on hydrogen-ion concentration

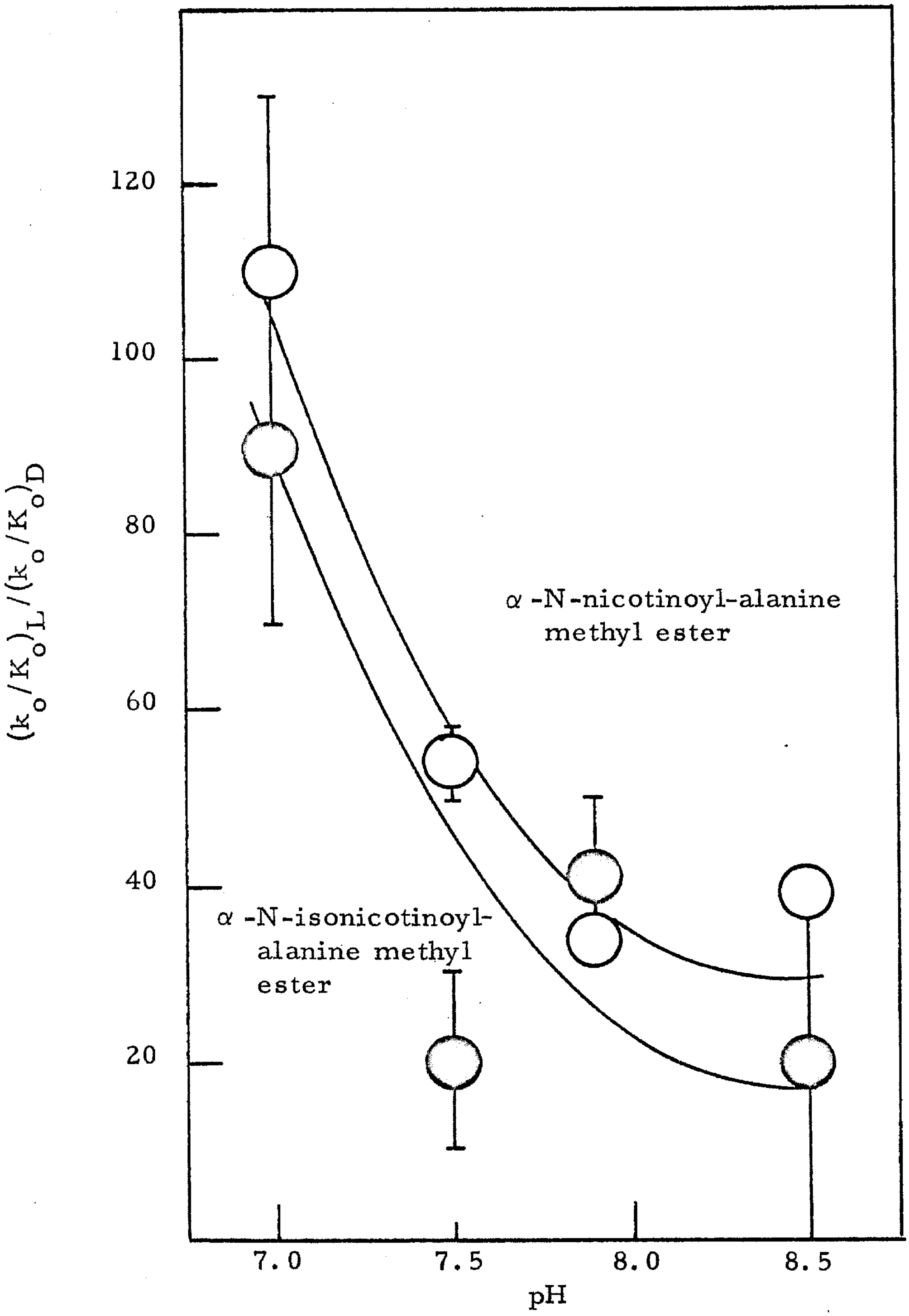


Figure 35.--Dependence of the ratio $(k_o/K_o)_L/(k_o/K_o)_D$ on hydrogen-ion concentration

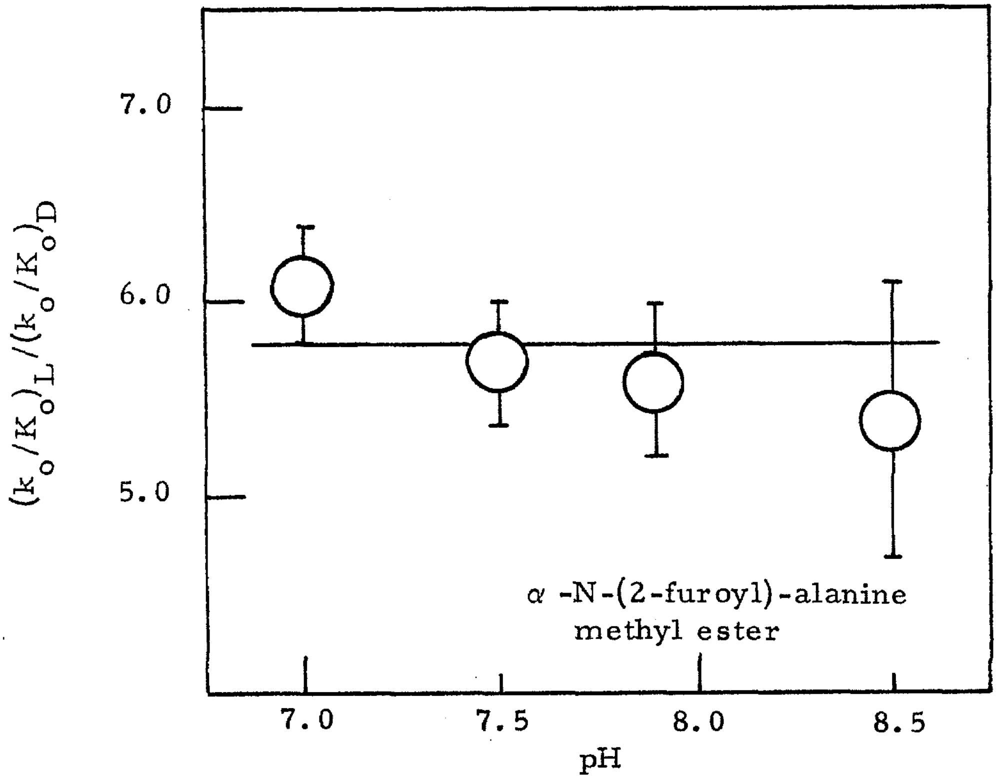


Figure 36.--Dependence of the ratio $(k_o/K_o)_L/(k_o/K_o)_D$ on hydrogen-ion concentration

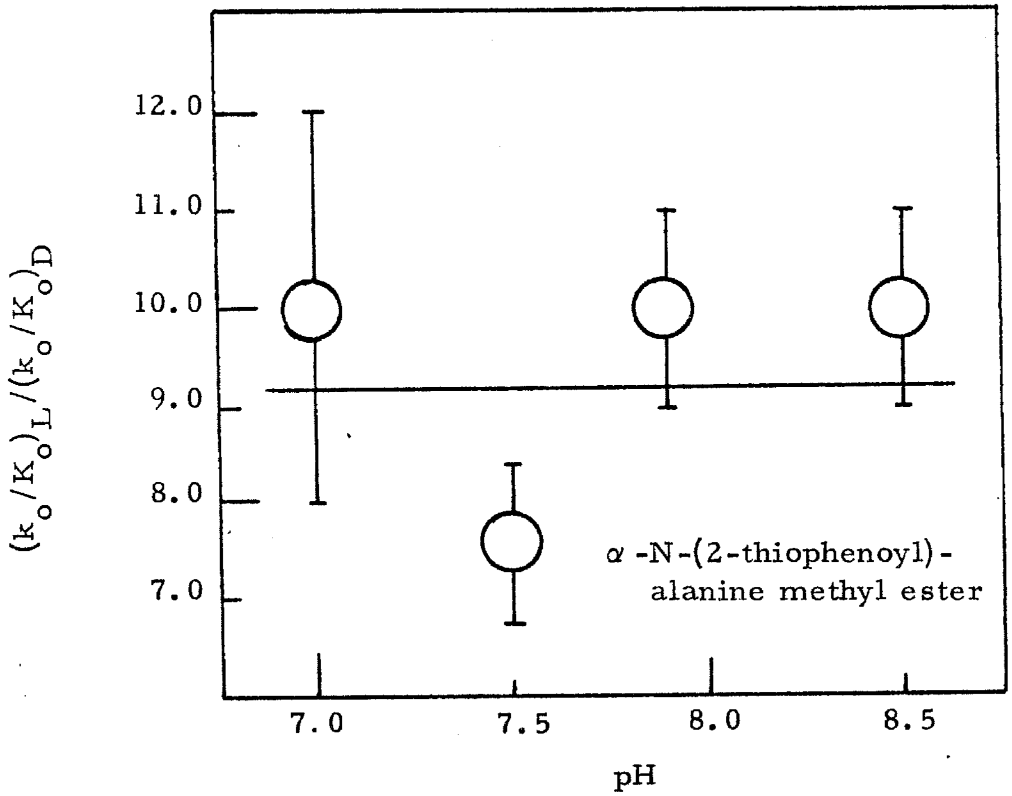
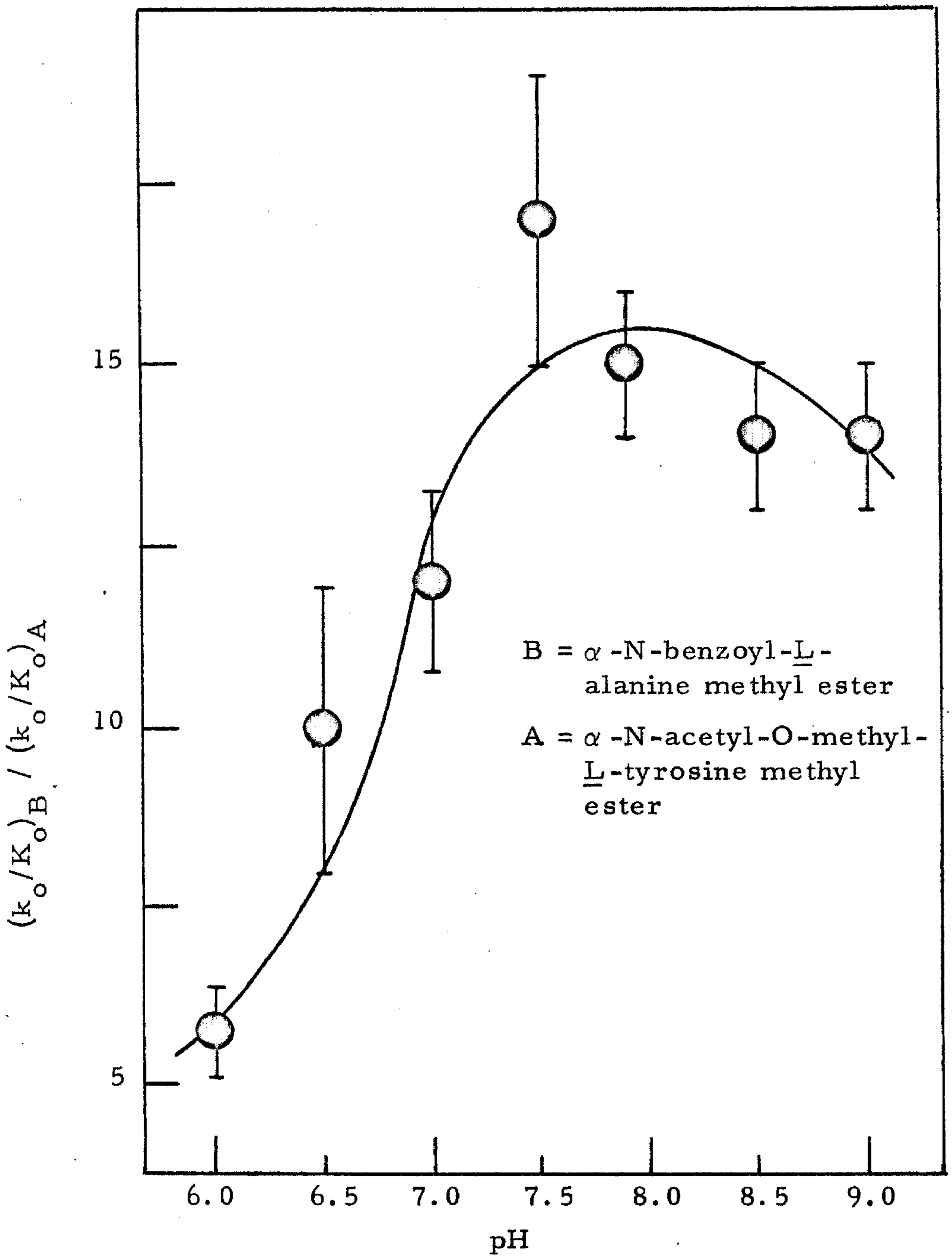


Figure 37. --Dependence of the ratio $(k_o/K_o)_B/(k_o/K_o)_A$ on hydrogen-ion concentration



References

1. E. Fischer, Chem. Ber., 27, 2985 (1894).
2. For a review, see G. W. Wolstenholme and C. M. O'Connor, in Steric Course in Microbiological Reactions, CIBA Foundation Study Group No. 2, Little, Brown, and Co., Boston, Mass. (1959).
3. L. Pasteur, Compt. rend., 46, 615 (1858).
4. K. A. Walsh, D. L. Kauffman, K. S. V. Sampath Kumar, and H. Neurath, Proc. Natl. Acad. Sci., U.S., 51, 301 (1964).
5. B. S Hartley, Nature, 201, 1284 (1964).
6. P. Desuelle, in "The Enzymes," P. D. Boyer, H. Lardy, and K. Myrback, eds. (Academic Press, New York, ed. 2, 1960), vol. 4, pp. 104-108.
7. For a review, see D. E. Koshland, Jr., D. H. Strumeyer, and W. J. Ray, Jr., in Enzyme Models and Enzyme Structures, Brookhaven Symposium in Biology, No. 15 (1962), pp. 101-133.
8. M. Bergmann and J. S. Fruton, J. Biol. Chem., 118, 405 (1937).
9. C. Niemann, Science, 143, 1287 (1964).

10. M. L. Bender and K. C. Kemp, J. Am. Chem. Soc., 79, 116 (1957).

11. B. S. Hartley and B. A. Kilbey, Biochem. J., 50, 672 (1952).

12. A. K. Balls and E. F. Jansen, Advan. Enzymol., 13, 321 (1952)

13. B. F. Erlanger and W. Cohen, J. Am. Chem. Soc., 85, 348 (1963).

14. M. L. Bender, G. R. Schonbaum, and B. Zerner, ibid., 84, 2540, 2562 (1962).

15. H. T. Huang and C. Niemann, ibid., 73, 3223 (1951).

16. a) G. E. Hein and C. Niemann, Proc. Natl. Acad. Sci., U. S., 47, 1341 (1961); b) J. Am. Chem. Soc., 84, 4487, 4495 (1962).

17. S. G. Cohen, J. Crossley, E. Khedouri, and R. Zand, J. Am. Chem. Soc., 84, 4163 (1962).

18. J. R. Rapp and C. Niemann, ibid., 85, 1896 (1963).

19. J. P. Wolf III and C. Niemann, Biochemistry, 2, 82, 493 (1963).

20. J. R. Rapp, Ph. D. Thesis, California Institute of Technology, 1964, pp. 1-44.

21. J. S. Friedenwald and G. D. Maengwyn-Davies, in Symposium on the Mechanism of Enzyme Action, W. D. McElroy and H. B. Glass, eds., Johns Hopkins Press, Baltimore, 1954, pp. 141-202.

22. W. A. Mukatis and C. Niemann, Proc. Natl. Acad. Sci., U. S., 51, 397 (1964).

23. K. J. Laidler, Trans. Faraday Soc., 51, 528, 540, 550 (1955).

24. M. L. Bender, G. E. Clement, F. J. Kezdy, and H. d'A. Heck, J. Am. Chem. Soc., 86, 3680 (1964).

25. L. Peller and R. A. Alberty, ibid., 81, 5907 (1959).

26. R. Lumry and H. Eyring, J. Phys. Chem., 58, 110 (1954).

27. D. E. Koshland, Jr., Proc. Natl. Acad. Sci., U. S., 44, 98 (1958).

28. For a list of references, see B. H. Havsteen and G. P. Hess, J. Am. Chem. Soc., 85, 791 (1963).

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

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

31. H. T. Huang and C. Niemann, J. Am. Chem. Soc., 74, 5963 (1952).

32. R. J. Foster and C. Niemann, ibid., 77, 3365, 3370 (1955).

33. A. Platt and C. Niemann, Proc. Natl. Acad. Sci., U. S., 50, 817 (1963).

34. M. L. Bender, Chem. Revs., 60, 53 (1960).

35. K. J. Laidler, The Chemical Kinetics of Enzyme Action, Oxford University Press, London (1958), pp. 117-162.

36. H. Neurath and D. S. Hartley, J. Cellular Comp. Physiol., 54, Suppl. 1, 179 (1959).

37. B. Zerner, R. P. M. Bond, and M. L. Bender, J. Am. Chem. Soc., 86, 3674 (1964).

38. B. R. Hammond and H. Gutfreund, Biochem. J., 61, 187 (1955).

39. K. J. Laidler and M. L. Barnard, Trans. Faraday Soc., 52, 497 (1956).

40. L. W. Cunningham and C. S. Brown, J. Biol. Chem., 221 287 (1956).

41. T. P. Gordon, Ph. D. Thesis, California Institute of Technology, 1959.

42. M. Dixon, Biochem. J., 55, 161 (1953).

43. J. Monod, J. P. Changeau, and F. Jacob, J. Molecular Biol., 6, 306 (1963).

44. R. L. Peterson, unpublished results in this laboratory.

45. J. B. Jones and C. Niemann, Biochemistry, 2, 498 (1963).

46. M. L. Bender and W. A. Glasson, J. Am. Chem. Soc., 82, 3336 (1960).

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

48. R. L. Peterson, Ph. D. Thesis, California Institute of Technology, 1965.

49. E. A. Zeller, Ann. N. Y. Acad. Sci., 107, 811 (1963).

50. M. Caplow and W. P. Jencks, J. Biol. Chem., 239, 1640 (1964).

51. B. R. Hammond and H. Gutfreund, Biochem. J., 61, 187 (1955).

52. R. B. Martin and C. Niemann, J. Am. Chem. Soc., 80, 1473 (1958).

53. R. L. Bixler and C. Niemann, ibid., 80, 2716 (1958).

54. A. Albert, Heterocyclic Chemistry, The Athlone Press, London, 1959, p. 208.

55. a) G. E. Hein, R. B. McGriff, and C. Niemann, J. Am. Chem. Soc., 70, 2610 (1960).

b) S. G. Cohen, J. Crossley, E. Khedouri, and R. Zand, ibid., 84, 4163 (1962).

56. D. E. Koshland, Jr. and W. J. Ray Strumeyer, Jr., in Symposium on Enzyme Models and Enzyme Systems, Brookhaven National Laboratory, Upton, New York (1962), pp. 101-119.

57. P. O. Lumme, Suomene Kem, 30B, 173 (1957).

58. G. H. Nelson, J. L. Miles, and W. J. Canady, Arch. Biochem. Biophys., 96, 545 (1962).

59. J. L. Miles, G. A. Robinson, and W. J. Canady, J. Biol. Chem., 238, 2932 (1963).

60. H. T. Huang and C. Niemann, J. Am. Chem. Soc., 74, 101 (1952).

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

62. H. I. Abrash, Ph. D. Thesis, California Institute of Technology, 1961, pp. 34-36.

63. K. A. Booman and C. Niemann, J. Am. Chem. Soc., 78, 3642 (1956).

64. F. Daniels, J. H. Mathews, J. W. Williams, P. Bender, and R. A. Alberty, Experimental Physical Chemistry, McGraw-Hill Book Company, Inc., 5th ed., (1956), p. 328.

65. C. E. Redeman, Ind. Eng. Chem., 11, 635 (1939).

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

II. VARIATION OF THE CONFORMATION OF THE ACTIVE SITE OF α-CHYMOTRYPSIN: ELECTROLYTE CONCENTRATION STUDIES

INTRODUCTION

In Section I of this thesis, the effect of hydrogen-ion concentration on the measured kinetic parameters for individual substrates and on the ratios of the kinetic parameters for selected pairs of these substrates was studied. It was shown that variations in these parameters and ratios of the parameters could not be completely explained in terms of ionization of groups at the active site involved in the catalysis or in terms of a change in rate-determining step in the hydrolysis if two steps occur. The simplest explanation of the observed behavior is that variations in the kinetic parameters and ratios of parameters with pH are at least in part due to hydrogen-ion induced conformational changes at the active site of the enzyme which alters its structural and stereochemical specificity.

In this section the effects of sodium chloride concentration on the measured kinetic parameters for individual substrates and ratios of these parameters for selected pairs of substrates are investigated. The same substrates used in the hydrogen-ion studies are used here; however, these latter studies are not as extensive.

For two pairs of substrates, the concentration of added sodium chloride was varied from 0 to 2.0 M at pH 7.90 and 25.0°C.

These pairs are:

(a) α-N-benzoyl-D-and-L-alanine methyl esters.

(b) α-N-acetyl-O-methyl-L-tyrosine methyl ester and α-N-benzoyl-L-alanine methyl ester.

Benzoyl-L-alanine methyl ester was used as one member of a pair of L-substrates and as one member of a pair of enantiomeric substrates in order to study variations both in structural and stereochemical specificity. Comparison in the ratios of kinetic parameters for five other pairs of enantiomeric acyl-alanine methyl esters were made at pH 7.90 and two concentrations of sodium chloride (0.10 M and 1.00 M). The kinetic parameters at a sodium chloride concentration of 0.10 M had been measured earlier by Rapp (1). The six pairs of enantiomeric acyl-alanine methyl esters are:

1. benzoyl
2. picolinoyl
3. nicotinoyl
4. isonicotinoyl
5. 2-thiophenoyl
6. 2-furoyl

For the first three pairs, comparisons of the relative stereochemical specificity were also measured at pH's other than 7.9.

One purpose of this study was to determine whether significant conformational changes occur at the active site of α-chymotrypsin with variation in electrolyte concentration. According to the Hein-Niemann theory (2a,b), each asymmetric substrate may form twelve different complexes with the active site of the enzyme (see Figures 1 and 2, Section I of this thesis, pp. 68 and 69). Many substrates will be bound predominantly in one mode. Individual members of each pair of substrates which would exhibit different principal binding modes were chosen. If changes in the active site of the enzyme occur with varying electrolyte concentration, these changes might then be reflected by a different variation of the kinetic parameters for each member of the pair. If the two members of the pair were similar in structure and thus had the same principal binding mode, changes in the conformation might be masked by parallel behavior of the two members of the pair.

Another purpose of this investigation was to determine whether a change in electrolyte concentration could effect an inversion of stereochemical specificity. This would also be indicative of a change in conformation of the active site of the enzyme with varying electrolyte concentration.

In the course of the present investigations, it was necessary to use α-chymotrypsin from two sources. Ratios of the measured kinetic parameters were always obtained using the same enzyme preparation. However, in order to investigate the possibility that the two samples of α-chymotrypsin might have different kinetic activities, both samples were used to obtain the kinetic constants for one substrate, α-N-benzoyl-L-alanine methyl ester at several concentrations of added electrolyte.

RESULTS AND DISCUSSION

General

The effects of electrolyte addition on α-chymotrypsin-catalyzed reactions are not fully understood. Possible effects may be discussed under the broad classifications of general and specific effects. As in the case of hydrogen-ion concentration, electrolyte addition may modify parts of the enzyme which have no influence on the active site. Effects due to such modifications cannot be considered since the kinetic technique employed is sensitive only to changes at the active site.

I. General Effects of Electrolyte Addition

A. Changes in the properties of water

Addition of electrolytes alters the bulk dielectric constant of water (3). The effect is linear with salt concentration and has been expressed as

$$D = D_o + 2\delta c \tag{1}$$

(3), where D_o and D are the dielectric constants of pure water at 25°C and of the salt solution, respectively, c is the salt concentration, and δ is the molar depression of the dielectric constant. For sodium chloride, $\delta = -5.5$ ℓ./mole, and the dielectric constant decreases from 78.54 for pure water (4) to 56.54 for a 2.0 M sodium chloride solution. This represents a 28% decrease in bulk dielectric constant, which can be important (3); however, the change in microscopic dielectric constant may be an even more important consideration (3). Little is known about the latter (3). In either case, however, a change in the dielectric constant could result in changes of the pKa's of catalytically active groups at the active site (3,5), changes in the state of aggregation of the enzyme involving the active site (6), and/or changes in the conformation of the active site (3). Furthermore, a change in dielectric constant could alter the affinity of substrate for enzyme due to a salting out of the substrate (7). Any of these phenomena could change the rate of the reaction.

Another effect of salt addition might be a decrease in the concentration of free water due to hydration of the ions of added electrolyte. Since water is involved in the hydrolysis, a decrease in its concentration might decrease the reaction rate. For sodium chloride, the hydration number of the sodium cation has been given as 4 (8). In 2.0 M sodium chloride solutions, this amounts to a decrease in free water concentration of ca. 15%, which could be significant.

B. Change in the activity coefficients of the reaction components

When an electrolyte is added to a water solution of molecules with charged groups, the electric potential of the charged groups is decreased. This reduction in electric potential changes the rate of reactions between charged groups and has been referred to as the primary salt effect (3, 5).

At low ionic strengths, the Debye-Huckel limiting law for activity coefficients as applied to Bronsted's general velocity formula for reactions in non-ideal solutions (5, 9) predicts no primary salt effect between a neutral molecule and an ion or between two neutral molecules. Since the substrate is neutral, no primary salt effect is expected at low ionic strengths. However, at the ionic strengths used in the present study, the Debye-Huckel limiting law does not hold, and a primary salt effect can occur. This is due to the fact that activity coefficients of neutral molecules vary at high ionic strengths. Unfortunately, it is difficult to predict even the sign of this effect on the rate (9).

Another possible result of changing the activity coefficients is the alteration of the pKa's of ionizable groups on the enzyme. If one form of an ionizing group or groups at the active site is necessary for catalysis, a change in ionic strength could alter the concentration of the catalytically active form of the group(s). This has been called the secondary salt effect (3, 5).

II. Specific Effects of Salt Addition

Any association of an ion with a group that is more pronounced than expected from simple electrostatic attraction is called a specific effect (3). This phenomenon is well-known for enzyme reactions, particularly for certain multivalent ions (e.g. Ca^{++}). Besides the possibilities already mentioned, specific ions could act as competitive inhibitors and, if not easily displaced by substrate, could alter the shape of the active site. Although it is often assumed that the common monovalent ions such as Na^+, K^+, and Cl^- are not specifically bound, Lewis and Saroff(10) have demonstrated differential binding of Na^+ and K^+ to myosin. More pertinent, Castañeda-Agullo _et al._ (5) have shown that the rate of α-chymotrypsin-catalyzed hydrolyses is altered in the presence of different monovalent cations including sodium. Although they observed that different anions had no specific effect in the range of ionic strength studied (_ca._ 0.3 to 0.6), Martin and Niemann (11) have shown that at 1.00 M salt concentrations, monovalent anions including chloride also have specific effects on the rate of α-chymotrypsin-catalyzed reactions. Consequently, the use of sodium chloride to vary the ionic strength does not preclude the possibility that the conformation of the active site is altered by the space-filling properties of the ions. Specific ions need not necessarily combine at the active site. Combinations at other points on the enzyme may modify the conformation of the entire molecule including the active site.

III. Variation in K_o, k_o, and k_o/K_o for Individual Substrates

The values of K_o, k_o, and k_o/K_o for all substrates are tabulated in Tables I, II, and III. For the three substrates listed at the top of page 113, log K_o is plotted vs ionic strength (sodium chloride concentration) in Figure 1.

The decrease in K_o with increasing ionic strength is qualitatively in agreement with previous studies on the α-chymotrypsin-catalyzed hydrolysis of acetylated amino acid esters (5, 7, 11).

In an elegant study by Canady *et al.* (7), it was shown that at pH 7.0, plots of -log K_o vs ionic strength for four alkyl esters of hippuric acid were linear. It was also shown that the slopes of log solubility of these substrates vs ionic strength were linear (12). For each substrate, the slope of the plot of -log K_o vs ionic strength was approximately equal in magnitude but opposite in direction to the slope of the corresponding plot of log solubility vs ionic strength. In the above studies, k_o did not vary significantly.

Since Foster (13) has shown, using acetyl-chymotrypsin, that deacylation of the enzyme is not sensitive to variations in ionic strength, Canady et al. (7) concluded that the change in K_o with ionic strength at pH 7.0 is almost entirely due to changes in solubility of the substrate whether or not an acyl-enzyme exists.

In the present studies at pH 7.90, two considerations indicate that the change in K_o cannot be due solely to changes in the solubility of the substrates. The first is that there are deviations from linearity in the plots of log K_o vs ionic strength. If the only variation produced by addition of salt were in the solubility of the substrate, such plots should be straight lines as observed by Canady et al. (7). Figure 1 shows that this is not the case for benzoyl-D-alanine methyl ester or N-acetyl-O-methyl-L-tyrosine methyl ester. Secondly, if it is assumed that K_o is an equilibrium constant or combination of equilibrium constants (see equation 9, p. 15), the fractional increase or decrease in K_o between any two ionic strengths should be identical for enantiomers if K_o is due solely to a "salting out" of substrate. Within experimental error, this is usually but not always the case. For example, K_o for picolinoyl-D-alanine methyl ester changes from 17±1 to 6.6±0.4, whereas K_o for the L-enantiomer varies from 18±1 to 15±1, as the ionic strength is changed from 0.10 to 1.0. Therefore, at pH 7.90, factors besides solubility of the substrate must be important in determining the magnitude of K_o.

For the same three substrates listed at the top of page 113, log k_o is plotted vs ionic strength (sodium chloride concentration) in Figure 2. The increase in k_o with increasing ionic strength (sodium chloride concentration) is also qualitatively in agreement with previous results of α-chymotrypsin-catalyzed hydrolyses of esters at pH 7.90 (5, 11) but not at pH 7.0 (7), since, as was noted, Canady et al. (7) showed that at the latter pH, k_o for a series of alkyl esters of hippuric acid was independent of ionic strength. The positive salt effect on k_o which appears at pH 7.9 but not at pH 7.0 is difficult to interpret, although it may be related to the appearance of a negative charge or charges at the active site at pH 7.9 (14a, b), which largely disappear by pH 6.9 (14b).

Log k_o/K_o is plotted vs ionic strength (sodium chloride concentration) in Figures 3, 4, and 5. The plots of log k_o/K_o vs ionic strength can be considered to be reasonably straight lines above an ionic strength of ca. 0.10.

Castañeda-Agullo et al. (5) have suggested that α-chymotrypsin may be treated as a simple negative ion, and that at low ionic strengths the secondary salt effect predominates. This would give rise to a positive salt effect as observed. However, at higher ionic strengths, other factors such as salting out of the substrate must also become important as indicated by Canady et al. (7). Separation of the contributions of individual factors to the overall salt effect on the individual kinetic parameters K_o, k_o, and k_o/K_o is difficult.

One point is worth discussing. **If, for illustrative purposes,** it is assumed that plots of log k_o/K_o vs electrolyte concentration for all substrates in the present study will be approximately linear* between concentrations of 0.10 M and 1.00 M, then the values of log k_o/K_o for substrates where only two points are available may be plotted vs ionic strength and the slopes of the lines obtained. This has been done in Figure 6. One curve, that for α-N-isonicotinoyl-D-alanine methyl ester, is not included because the large error in the K_o value at an ionic strength of 0.10 makes the value of k_o/K_o questionable.

The relative slopes of all the lines except one vary between 0.22 and 0.33. The line for the one exception, α-N-nicotinoyl-D-alanine methyl ester, has a slope of 0.07, i.e., the variation in k_o/K_o with ionic strength for this particular substrate is much smaller than that for the other substrates. If, as the results in Section I indicate, this substrate is being hydrolyzed mainly via a pH-independent mechanism, the secondary salt effect would be

---

* This relationship need not be linear, but the magnitudes of changes in log k_o/K_o with ionic strength are more easily discussed in terms of the slopes of straight lines.

unimportant. On the other hand, if the major reaction path for the other substrates involves catalysis by one ionized form of a weak acid, it would be expected that the secondary salt effect might be significant for these substrates. Consequently, the smaller variation in log k_o/K_o vs ionic strength for α-N-nicotinoyl-D-alanine methyl ester as compared to other substrates is consistent with the above interpretation, but the different slopes for each substrate indicate that other factors may also be important.

IV. Variation in the Ratios K_{o1}/K_{o2}, k_{o1}/k_{o2}, and $(k_o/K_o)_1/(k_o/K_o)_2$ for Selected Pairs of Substrates

A. Possible changes in conformation of the active site.

The values of K_{o1}/K_{o2}, k_{o1}/k_{o2}, and $(k_o/K_o)_1/(k_o/K_o)_2$ for all substrates are tabulated in Tables IV, V, VI, and VII. In Figures 7 and 8, K_{oL}/K_{oD}, k_{oL}/k_{oD}, and $(k_o/K_o)_L/(k_o/K_o)_D$ are plotted vs ionic strength (sodium chloride concentration) for the first pair of substrates listed at the top of page 113. The scatter in the ratios for this pair is large, and no significant trend in these ratios is seen outside of experimental error. Whether the apparent independence of the ratios for this pair of substrates is due to changes being too small or to the occurrence of compensating changes is not known.

For the other enantiomeric acyl-alanine methyl esters, which were studied at only two ionic strengths, changes in k_{oL}/k_{oD}, K_{oL}/K_{oD}, and $(k_o/K_o)_L/(k_o/K_o)_D$ with electrolyte concentration are evident (see Table VI). A more complete study of the effect of added electrolyte on the enantiomers which show the largest changes would have to be done to determine whether the changes are significant.

For the second pair of substrates listed at the top of page 113, although it cannot be stated conclusively, there appears to be a variation in both k_{o1}/k_{o2} and K_{o1}/K_{o2} with ionic strength (see Figure 9). It appears that as the ionic strength is increased, both ratios increase, reach a maximum around an ionic strength of 0.10, then both ratios decrease until, in each case, the curves pass through a broad minimum and then increase again.

Figure 10 is a plot of $(k_o/K_o)_1/(k_o/K_o)_2$ vs ionic strength (sodium chloride concentration) for this second pair. Here the ratio appears to be independent of ionic strength until above an ionic strength of 1.0 ; the ratio then begins to decrease.

The variations in these three ratios must be due to nonidentical changes in the two sets of enzyme-substrate complexes. Some of the factors discussed under the headings of General and Specific Salt Effects which may affect the rate of individual reactions as the concentration of added electrolyte is varied will cancel out when ratios of the kinetic parameters are taken. The remaining factors which might bring about the variation in the ratios illustrated in Figures 9 and 10 are now listed and discussed.

1. A different variation in solubility of each substrate with ionic strength.

This is unlikely because of the similarity in structure of the two substrates. Canady et al. (12) have shown that as the ester group of four esters of hippuric acid is changed from methyl to ethyl to n-propyl to isopropyl, the slopes of the curves of log solubility of these esters vs ionic strength are 0.21, 0.23, 0.22, and 0.20, respectively, representing a negligible variation. In the present case, both substrates are methyl esters, both contain an amido group and a phenyl group, and the molecular formulas for the two substrates are $C_{13}H_{17}O_4N$ and $C_{11}H_{13}O_3N$, respectively. It is expected that, since there are only minor differences in the structures of the two substrates in the present study, the variation in solubility of the two substrates with ionic strength would be approximately parallel, and therefore these variations would cancel in the ratios of the kinetic parameters.

2. Aggregation of the enzyme involving the active site

Dimerization of α-chymotrypsin is known to occur under certain conditions and to increase with increasing ionic strength (6, 15). This dimerization is more important at low pH. At pH 8.0, 25°C, and a sodium chloride concentration of 0.30 M, it has been shown (6) that association of α-chymotrypsin is kinetically unimportant for initial enzyme concentrations as high as 8×10^{-4}M, and that under these conditions the association is probably negligible even up to concentrations as high as 10^{-3}M (6). Since the highest enzyme concentration used in the present study was below 10^{-4}M, all enzyme concentrations are below the upper limit. It is still possible that enzyme dimerization becomes important at electrolyte concentrations higher than 0.30 M, but this is very unlikely, since the association is known to decrease the activity of the enzyme (6), and from sodium chloride concentrations of 0.30 M to 2.0 M, log k_o/K_o increases linearly (see Figures 3, 4, and 5).

3. Dissimilar variations in the concentrations of catalytically active groups in the enzyme substrate complexes as electrolyte concentration is changed.

The presence of different substrates at the active site may perturb the pKa's of catalytically active groups in a non-identical manner. This could result in non-identical variations in the concentration of catalytically active groups as the salt concentration is changed, the variations being due to a secondary salt effect or a dielectric effect.

4. Changes in the conformation of the active site of the free enzyme.

This effect could be caused by changes in the dielectric constant, a secondary salt effect, or the binding of electrolyte ions to the enzyme. These phenomena need not necessarily occur at the active site since changes in the effective concentration of charged groups away from the active site could change the conformation of the entire enzyme.

5. Modification of the shape of active site by the space-filling properties of ions.

As a simple illustration of this, consider the case where electrolyte ions bind specifically at the active site. If one substrate, because of its particular structure (shape), were able to form ternary complexes (enzyme-ion-substrate) and binary complexes (enzyme-substrate) with equal facility, then as the concentration of electrolyte and concomitantly the concentration of enzyme-ion complexes were increased, no salt effect due to this factor would be observed. If the other substrate, due to its structure (shape), were only able to form binary complexes (enzyme-substrate), then as the enzyme-ion complexes were increased, a salt effect due to this factor would be observed. Consequently, the space-filling properties of electrolyte ions could cause a variation in any or all of the ratios of kinetic parameters as the electrolyte concentration is increased, especially at high electrolyte concentrations.

Factors 3, 4, and 5 may all cause variations in the ratios of the kinetic parameters. None of these factors by themselves can easily explain the complex variation in the plots of k_{oB}/k_{oA} and K_{oB}/K_{oA} vs ionic strength (sodium chloride concentration), but it cannot be concluded that a change in conformation of the active site occurs as ionic strength is increased. However, the changes in these ratios with electrolyte concentration up to an electrolyte concentration of <u>ca</u>. 1.5 M are not very large—not nearly as large as the changes exhibited when pH is varied. Consequently, if variations in electrolyte concentration alter the conformation of the active site, the magnitude of this alteration is small, unless conformational changes are being compensated for fortuitously.

B. Attempts to cause an inversion in stereospecificity with changes in ionic strength.

In Tables V and VII are tabulated the values of $(k_o/K_o)_D/(k_o/K_o)_L$ for the pair of benzoyl-alanine methyl esters at various ionic strengths. These values for the other five enantiomer pairs of acyl-alanine methyl esters at two ionic strengths are tabulated in Table VI. From the tables it can be seen that although the relative stereospecificity varies with ionic strength, the changes are not large and in no case is an inversion in stereospecificity observed.

V. Comparison of the Kinetic Activities of Two Preparations of α-Chymotrypsin

Figure 4 contains plots of log k_o/K_o vs ionic strength (sodium chloride concentration) for α-N-benzoyl-L-alanine methyl ester using two enzyme preparations from different sources. It can be seen that at comparable ionic strengths, the values of k_o/K_o for the Armour preparation are always below those for the Worthington preparation. Above an ionic strength of ca. 0.1, the relationship between log k_o/K_o and ionic strength is linear. In this range, two separate lines of approximately equal slope are obtained.

The difference in reactivity for the two preparations appears to be concentrated more in K_o (central curve in Figure 1) than in k_o (central curve in Figure 2), although the scatter in the values of k_o and K_o is somewhat greater than the scatter in k_o/K_o.

Since the kinetic constants are obtained from the slope and intercept of a least squares plot of equation 2, any error in the

$$\frac{[S][E]_o}{v} = \frac{K_o}{k_o} + \frac{1}{k_o}[S] \qquad (2)$$

determination of the amount of active enzyme in a preparation would be seen in k_o but not in K_o, and therefore this factor cannot explain the different activity of the two preparations. This leaves an alternate possibility that one preparation(or both)contains a natural inhibitor, which, if fully competitive with the model substrate, would affect K_o but not k_o. The kinetic properties of different preparations of α-chymotrypsin have not been investigated extensively. However, in one study, Huang and Niemann (16a) redetermined the kinetic constants of α-N-acetyl-L-tryptophanamide using a Worthington preparation of α-chymotrypsin. Previously (16b), these constants had been determined using an Armour preparation of the same enzyme. No differences in the kinetic constants were observed.

Further investigations are needed to determine the cause of the difference in activity of the two enzyme preparations used in the present study.

EXPERIMENTAL

I. Equipment and Procedure Used for Kinetic Studies

The equipment and procedures used in these studies are described in the Experimental section in Section I. A reference to the literature is also given.

II. Treatment of Data to Obtain Kinetic Constants

This is described in the Experimental section in Section I.

III. Materials

α-Chymotrypsin. - Two different preparations were used in the present studies. A Worthington preparation (Lot no. C.D.I. 6066-67) and an Armour preparation (Lot no. T-97207) were employed. The nitrogen contents of these preparations are:

	% Protein Nitrogen
Worthington Lot. no. C.D.I. 6066-67	14.91±0.06
Armour Lot. no. T-97207	13.97±0.11

The substrates used in this study are:

1. α-N-acetyl-O-methyl-L-tyrosine methyl ester
2. α-N-benzoyl-D-and-L-alanine methyl esters
3. α-N-picolinoyl-D-and-L-alanine methyl esters
4. α-N-nicotinoyl-D-and-L-alanine methyl esters

5. α-N-isonicotinoyl-D-and-L-alanine methyl esters
6. α-N-(2-furoyl)-D-and-L-alanine methyl esters
7. α-N-(2-thiophenoyl)-D-and-L-alanine methyl esters

Methods of synthesis, physical constants, etc. for these compounds are given in the Experimental part of Section I.

TABLES

TABLE I. Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolysis of Two L-Substrates[a]

Substrate	No. of Expts.[c]	Ionic Strength μ	$[E_o]$μM[d] μ	$[S_o]$mM	$k_o \times 10^2 sec^{-1}$	K_o mM	$k_o/K_o \times 10^2 sec^{-1} mM^{-1}$
A[b]	23-3	0.00	4.83-6.97	0.52-4.84	40±3	3.3±0.4	12.0±0.8
	9-1	0.01	7.26	0.57-4.53	49±4	4.0±0.5	12.3±0.6
	9-1	0.05	5.00	0.55-4.40	55±3	3.0±0.3	18.4±0.8
	10-0	0.10	6.25	0.53-3.70	46±3	2.5±0.3	18.5±0.9
	9-0	0.10	1.44[e]	0.60-4.82	47±1	3.1±0.2	15.0±0.4
	8-1	0.15	6.25	0.53-3.70	52±1	2.8±0.1	18.9±0.3
	8-0	0.20	7.26	0.57-4.53	64±3	3.3±0.3	19.2±0.7
	9-1	0.50	4.83	0.52-3.66	79±5	2.7±0.3	30 ±2
	11-0	1.00	4.34	0.55-3.84	80±5	1.8±0.3	44 ±4
	8-0	1.50	4.34	0.55-3.29	79±5	1.0±0.2	80 ±10
	6-0	2.00	4.34	0.55-2.74	69±1	0.45±0.04	130 ±10
B[b]	9-1	0.00	13.0	1.78-14.28	17±1	8.4±0.7	2.05±0.09
	10-1	0.01	17.8	1.74-13.90	22±1	11 ±1	2.00±0.08
	10-0	0.05	14.3	1.41-11.28	21±1	8.7±0.8	2.37±0.09
	9-0	0.10	12.8	1.35-9.48	26±3	9.7±0.6	2.6 ±0.2
	10-0	0.10	33.6[e]	1.92-15.36	23±1	9.6±0.6	2.41±0.08
	9-1	0.15	12.8	1.35-9.48	25±1	8.8±0.6	2.81±0.07
	9-1	0.20	14.3	1.41-11.28	20±1	6.3±0.3	3.11±0.06
	10-0	0.50	9.72	1.37-11.00	28±2	6.7±0.8	4.2 ±0.3
	9-1	1.00	9.72	1.37-11.00	30±1	4.9±0.3	6.1 ±0.2
	8-1	1.50	7.36	1.37-8.19	30±1	3.4±0.2	9.0 ±0.4
	8-0	2.00	7.36	1.37-6.83	33±2	3.1±0.5	11 ±1

TABLE I (continued)

a. In aqueous solutions at 25.0° and pH 7.90±0.05

b. A = α-N-acetyl-O-methyl-L-tyrosine methyl ester and
B = α-N-benzoyl-L-alanine methyl ester

c. First number refers to number of kinetic runs performed; second number refers to number of points rejected by a statistical reiterative procedure

d. Based on a molecular weight of 25,000 and a nitrogen content of 16.5%. The enzyme is a Worthington preparation, Lot No. CDI 6066-67, except where noted (see "e")

e. The enzyme is an Armour preparation, Lot No. T-97207

TABLE II. Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolyses of Benzoyl-D-and L-Alanine Methyl Esters[a]

Substrate	No. of Expts.[b]	Ionic Strength[c]	$[E_o]$ μM[d]	$[S_o]$ mM	$k_o \times 10^2$ sec^{-1}	K_o mM	$k_o/K_o \times 10^2$ sec^{-1} mM^{-1}
Benzoyl-	13-0	0.00	30.7	2.37-21.34	19±1	18±2	1.09±0.04
L-Ala·	9-0	0.01	32.5	1.23-9.82	18±2	12±2	1.51±0.07
OMe	10-0	0.10	33.6	1.92-15.36	23±1	9.6±0.6	2.41±0.08
	8-1	0.50	33.9	2.19-17.54	27±1	8.7±0.8	3.2 ±0.2
	10-0	1.05	20.6	2.12-14.85	28±1	5.8±0.4	4.8±0.2
	8-1	1.50	73.6	1.37-8.19	30±1	3.4±0.2	9.0±0.4
	8-0	2.00	73.6	1.37-6.83	33±2	3.1±0.5	11±1
Benzoyl-	10-1	0.00	51.2	2.84-22.73	0.60±0.03	6.0±0.9	0.10±0.01
D-Ala·	9-1	0.01	55.6	1.54-15.50	0.68±0.02	3.8±0.4	0.18±0.01
OMe	10-1	0.10	53.9	1.10-8.78	0.71±0.02	2.2±0.2	0.31±0.02
	9-0	0.50	56.7	2.51-20.10	0.96±0.05	2.8±0.8	0.34±0.08
	10-0	1.05	62.4	0.52-3.66	1.0 ±0.1	1.4±0.3	0.7 ±0.1
	9-0	1.50	76.7	0.34-2.03	1.3 ±0.1	1.3±0.2	0.88±0.08
	9-0	2.00	72.8	0.15-1.87	1.2 ±0.1	1.1±0.3	1.1 ±0.1

a. In aqueous solutions at 25.0° and pH = 7.90 ± 0.05

b. First number refers to number of kinetic runs performed; second number refers to number of points rejected by a statistical reiterative procedure

c. The ionic strength reported here is due to added sodium chloride and does not take into account any ionic strength effects due to charged groups on the enzyme

d. Based on a molecular weight of 25,000 and a nitrogen content of 16.5%. The enzyme for both D and L substrates from $\mu = 0$ to $\mu = 1.05$, inclusive, is an Armour preparation, Lot No. T 97207. The enzyme for both D and L substrates for $\mu = 1.50$ and $\mu = 2.00$ is a Worthington preparation, Lot No. CDI-6066-67.

TABLE III. Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolysis of Several Substrates[a]

Substrate	No. of Expts.	pH	Ionic Strength μ	$[E_o]$[d] μM	$[S_o]$ mM	$k_o \times 10^2$ sec^{-1}	K_o mM	$(k_o/K_o) \times 10^2$ sec^{-1} mM^{-1}
Benzoyl-L-Ala·OMe[b]	10-0	8.50	2.00	25.3	0.25-6.27	31±2	3.2±0.4	9.4 ±0.6
D-[b]	10-0	8.50	2.00	56.5	0.10-2.37	1.5±0.2	2.5±0.6	0.60±0.06
Nicotinyl-L-Ala·OMe[b]	8-1	8.50	2.00	19.5	2.24-11.20	46.5±0.8	9.1±0.3	5.11±0.06
D-[b]	9-0	8.50	2.00	99.0	0.33-4.09	0.27±0.05	1.2±0.7	0.22±0.09
Picolinoyl-L-Ala·OMe	10-0	7.00	0.100	37.8	2.13-17.05	2.85±0.2	13±2	0.22±0.02
D-	10-1	7.00	0.100	26.5	2.25-18.01	12.4±0.5	15.0±0.9	0.83±0.02

a. In aqueous solution at 25.0°

b. Acyl-L-Ala·OMe = α-N-acyl-L-alanine methyl ester

c. The first number refers to the number of kinetic runs performed; the second number refers to the number of points rejected by a statistical reiterative procedure

d. Based on a molecular weight of 25,000 and a nitrogen content of 16.5%. The enzyme is a Worthington preparation, Lot No. CDI-6066-67.

TABLE IV. Ratios of the Kinetic Parameters for a Pair of L-Substrates of α-Chymotrypsin[a]

Ionic Strength μ	k_{oB}/k_{oA}[b]	K_{oB}/K_{oA}[b]	$\frac{(k_o/K_o)_B}{(k_o/K_o)_A}$[b]
0.00	0.43±0.03	2.5±0.4	0.17±0.01
0.01	0.44±0.04	2.8±0.4	0.16±0.01
0.05	0.38±0.03	2.9±0.4	0.13±0.01
0.10	0.55±0.07	3.9±0.5	0.14±0.01
0.10	0.49±0.02	3.1±0.3	0.16±0.01
0.15	0.47±0.02	3.2±0.2	0.148±0.004
0.20	0.31±0.02	1.9±0.2	0.16±0.01
0.50	0.35±0.03	2.5±0.4	0.14±0.01
1.00	0.37±0.03	2.7±0.4	0.14±0.01
1.50	0.38±0.03	3.2±0.6	0.12±0.02
2.00	0.48±0.03	7±1	0.07±0.01

a. The substrates used were B = α-N-benzoyl-L-alanine methyl ester and A = α-N-acetyl-O-methyl-L-tyrosine methyl ester

b. The errors in these ratios were obtained from the relationships:

$$\frac{A \pm a}{B \pm b} = C \pm \rho \quad \text{and} \quad \rho = \frac{1}{B}\left(b^2 + \frac{a^2B^2}{A^2}\right)^{\frac{1}{2}}$$

TABLE V. Ratios of the Kinetic Parameters for Benzoyl-D-and-L-Alanine Methyl Esters[a]

Ionic Strength μ	$k_{oD}/k_{oL} \times 10^{2}$ [b]	$K_{oD}/K_{oL} \times 10^{1}$ [b]	$\frac{(k_o/K_o)_D}{(k_o/K_o)_L} \times 10^{2}$ [b]
0.00	3.1 ± 0.2	3.5 ± 0.6	9 ± 1
0.01	3.7 ± 0.4	3.2 ± 0.6	12 ± 1
0.10	3.0 ± 0.1	2.3 ± 0.3	13 ± 2
0.50	3.5 ± 0.2	3 ± 1	11 ± 3
1.05	3.7 ± 0.4	2.4 ± 0.6	15 ± 2
1.50	3.8 ± 0.4	3.9 ± 0.9	10 ± 1
2.00	3.7 ± 0.5	4 ± 2	10 ± 2

a. The substrates chosen were α-N-benzoyl-L-alanine methyl ester and its D-enantiomer

b. The errors in these ratios were obtained from the following relationships:

$$\frac{A \pm a}{B \pm b} = C \pm \rho \quad \text{and} \quad \rho = \frac{1}{B}\left(b^2 + \frac{a^2 B^2}{A^2}\right)^{\frac{1}{2}}$$

TABLE VI. Ratios of the Kinetic Parameters for Several Enantiomeric Pairs of Substrates of α-Chymotrypsin

Substrate	Ionic Strength μ	k_{oL}/k_{oD} [c]	K_{oL}/K_{oD} [c]	$\frac{(k_o/K_o)_L}{(k_o/K_o)_D}$ [c]
Benzoyl-Ala·OMe[a]	0.10	31±2	4.0±0.7	7.7±0.6
	1.00	28±3	4±1	6.8±0.6
Picolinoyl-Ala·OMe[a]	0.10[b]	0.42±0.02	1.1±0.9	0.40±0.01
	1.00	0.66±0.03	2.3±0.2	0.28±0.01
Nicotinoyl-Ala·OMe[a]	0.10[b]	126±7	6±1	20±3
	1.00	170±20	5±1	34±3
2-Thiophenoyl-Ala·OMe[a]	0.10[b]	29±4	2.0±0.6	14±2
	1.00	36±3	3.5±0.7	10±1
2-Furoyl-Ala-OMe[a]	0.10[b]	13±1	2.6±0.4	5.1±0.4
	1.00	13±1	1.5±0.2	9±1

a. Acyl-Ala·OMe means α-N-acyl-alanine methyl ester

b. The values for the ratios were obtained from values obtained by J. Rapp, Ph.D. Thesis, Calif. Inst. of Technology (1963) pp. 4, 5.

c. The errors in these ratios were obtained from the following relationships:

$$\frac{A \pm a}{B \pm b} = C \pm \rho \quad \text{and} \quad \rho = \frac{1}{B}\left(b^2 + \frac{a^2 B^2}{A^2}\right)^{\frac{1}{2}}$$

TABLE VII. Ratios of the Kinetic Parameters for Several Enantiomeric Pairs of Substrates of α-Chymotrypsin

Substrate	pH	Ionic strength μ	k_{oL}/k_{oD}[b]	K_{oL}/K_{oD}[b]	$\frac{(k_o/K_o)_L}{(k_o/K_o)_D}$[b]
Benzoyl-Ala·OMe[a]	8.50	2.00	20±3	1.3±0.3	15±2
Nicotinoyl-Ala·OMe[a]	8.50	2.00	170±30	7±4	20±10
Picolinoyl-Ala·OMe[a]	7.00	0.100	3.45±0.09	0.8±0.1	0.27±0.02

a. Acyl-Ala·OMe = α-N-acyl-alanine methyl ester

b. The errors in these ratios were obtained from the following relationships:

$$\frac{A \pm a}{B \pm b} = C \pm \rho \quad \text{and} \quad \rho = \frac{1}{B}\left(b^2 + \frac{a^2B^2}{A^2}\right)^{\frac{1}{2}}$$

FIGURES

Figure 1.--Log K_o as a function of ionic strength.

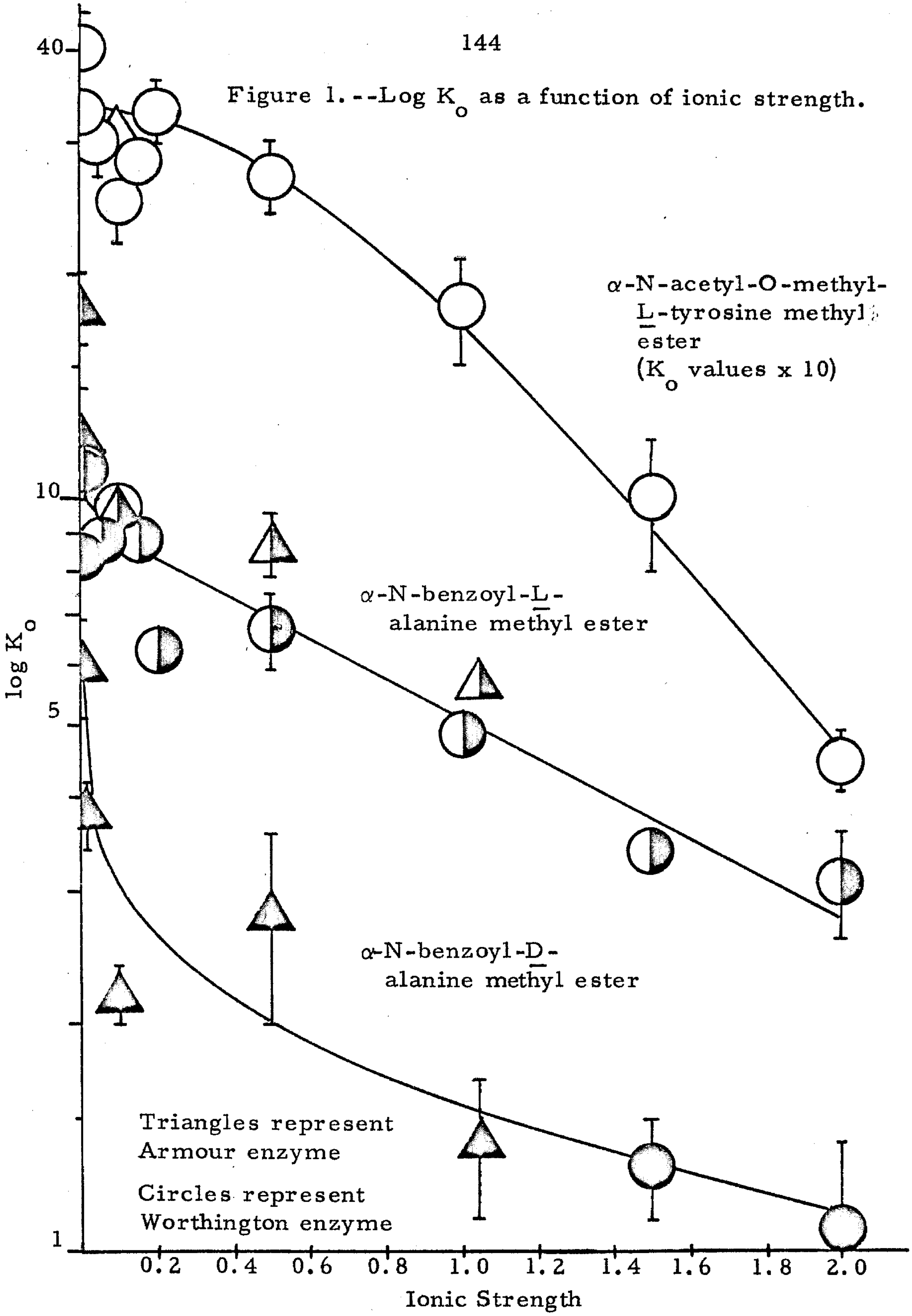


Figure 2.--Log k_o as a function of ionic strength.

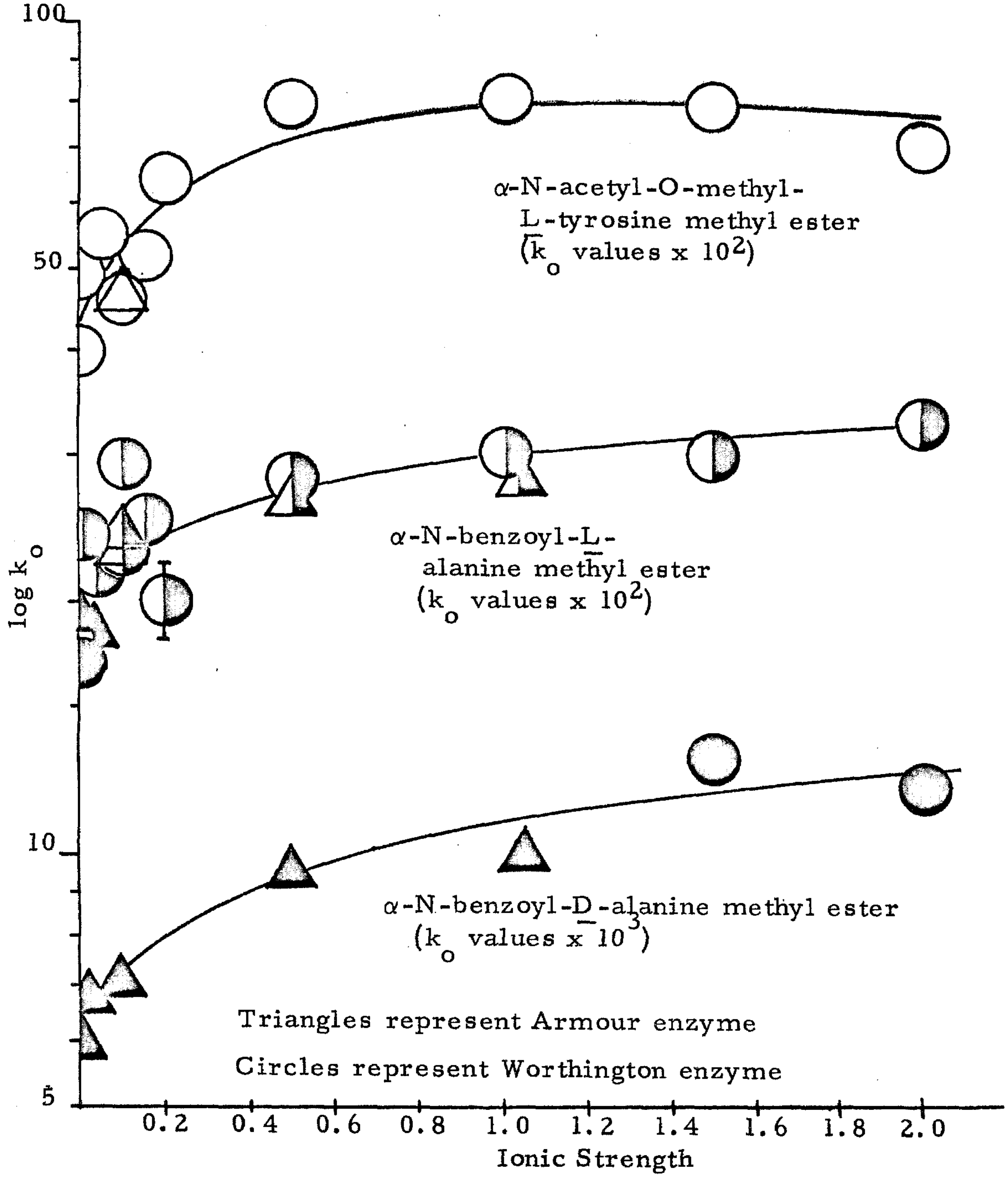


Figure 3.--Log k_o/K_o as a function of ionic strength.

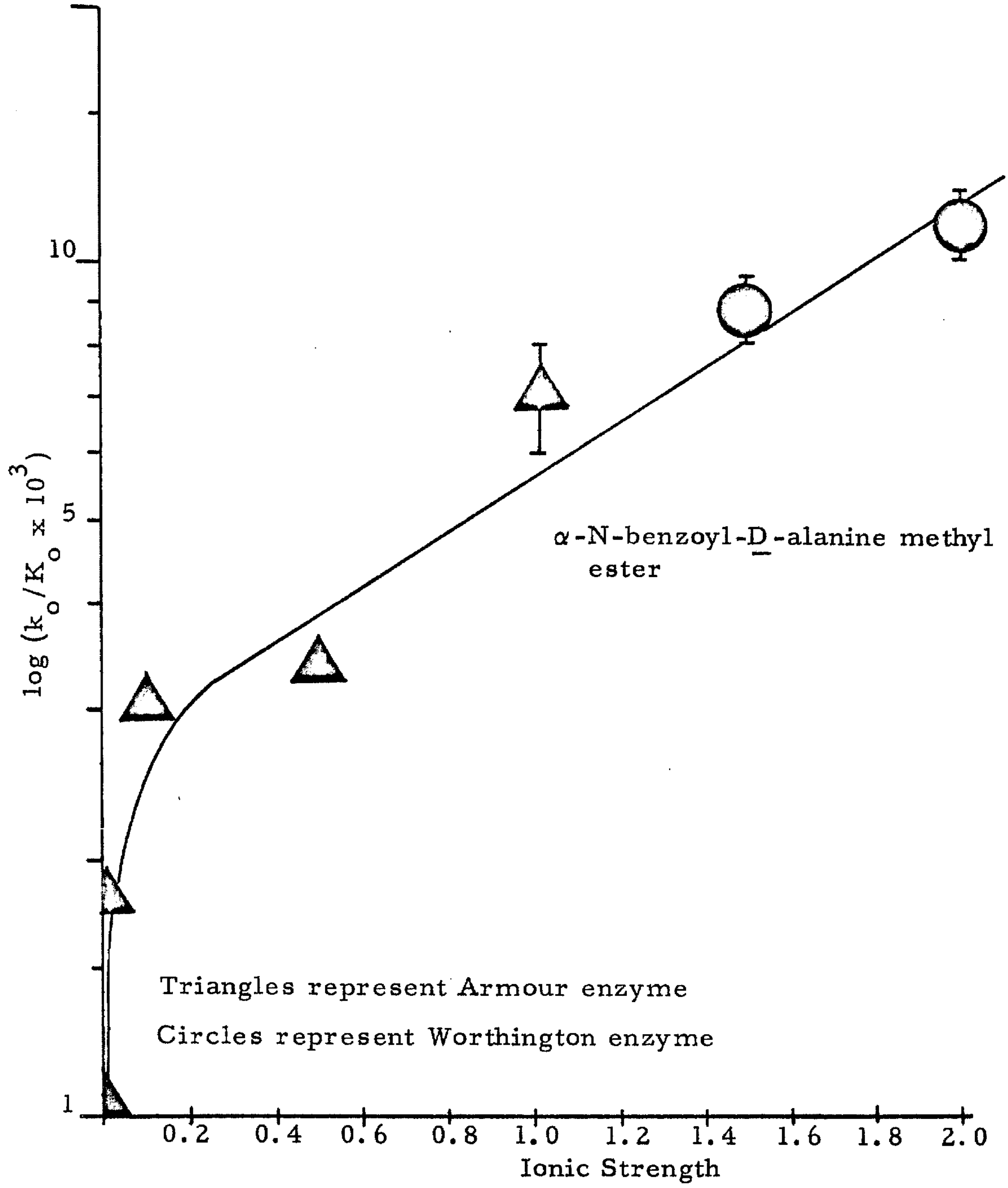


Figure 4.--Log k_o/K_o as a function of ionic strength.

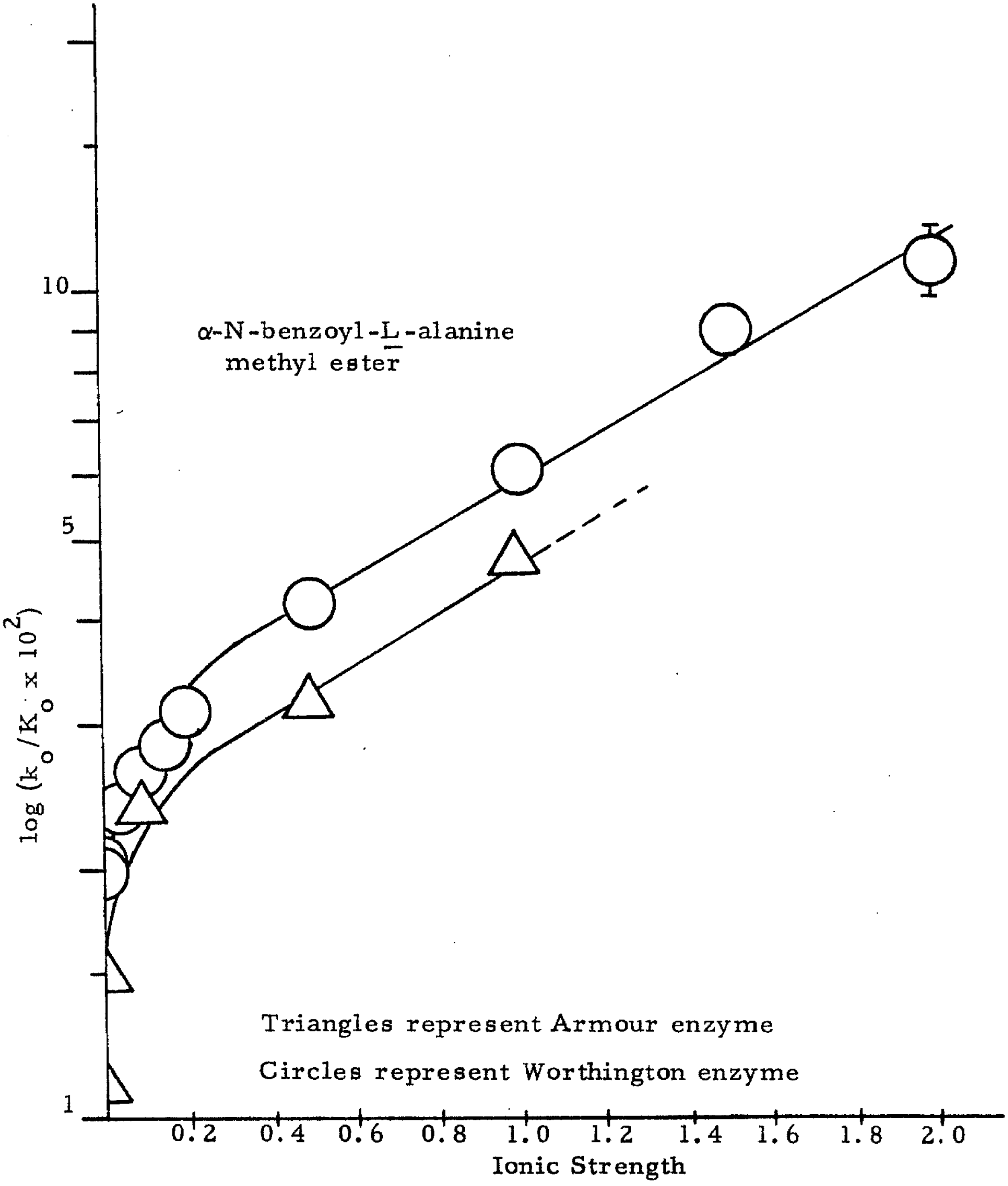


Figure 5.--Log k_o/K_o as a function of ionic strength.

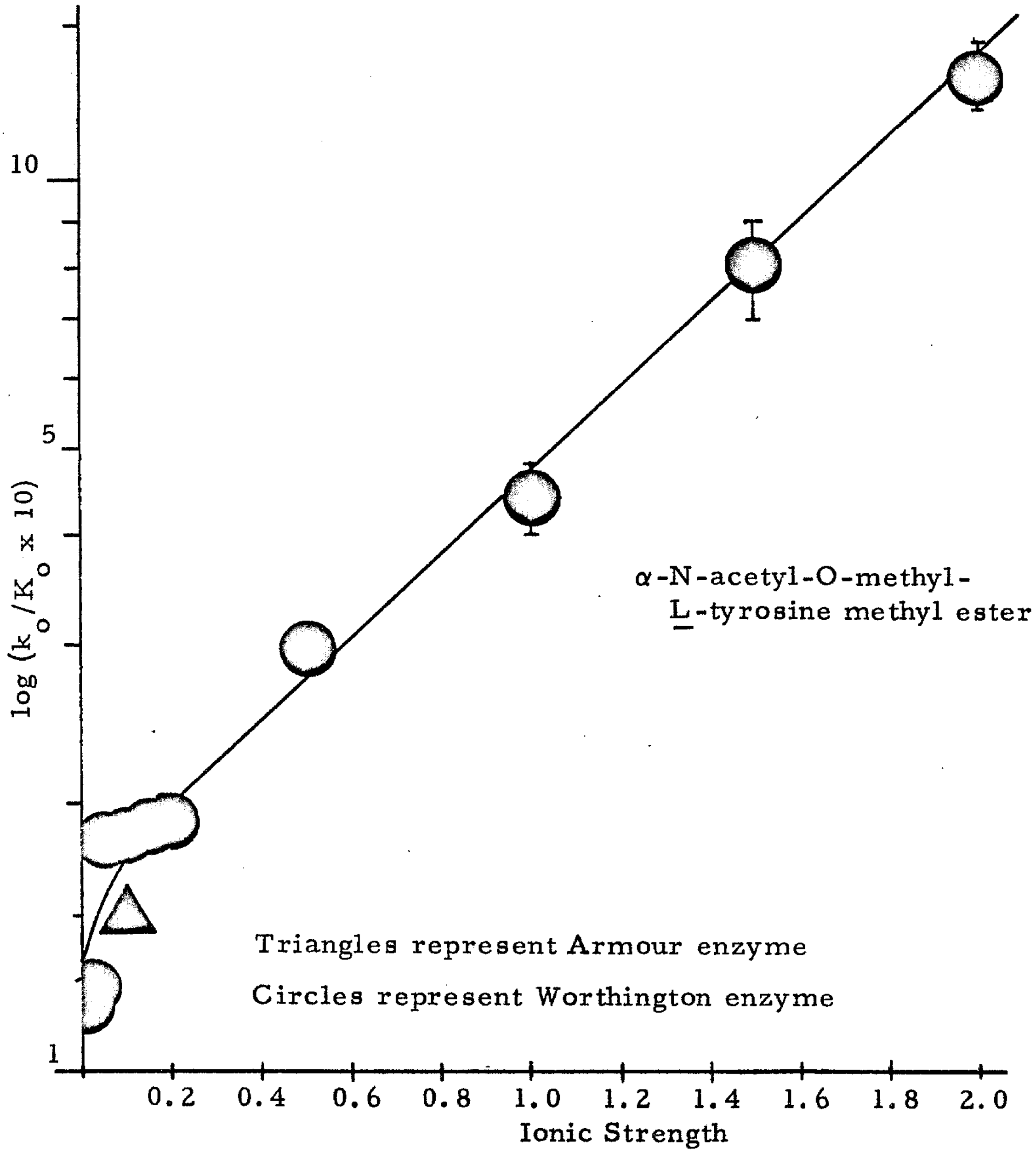
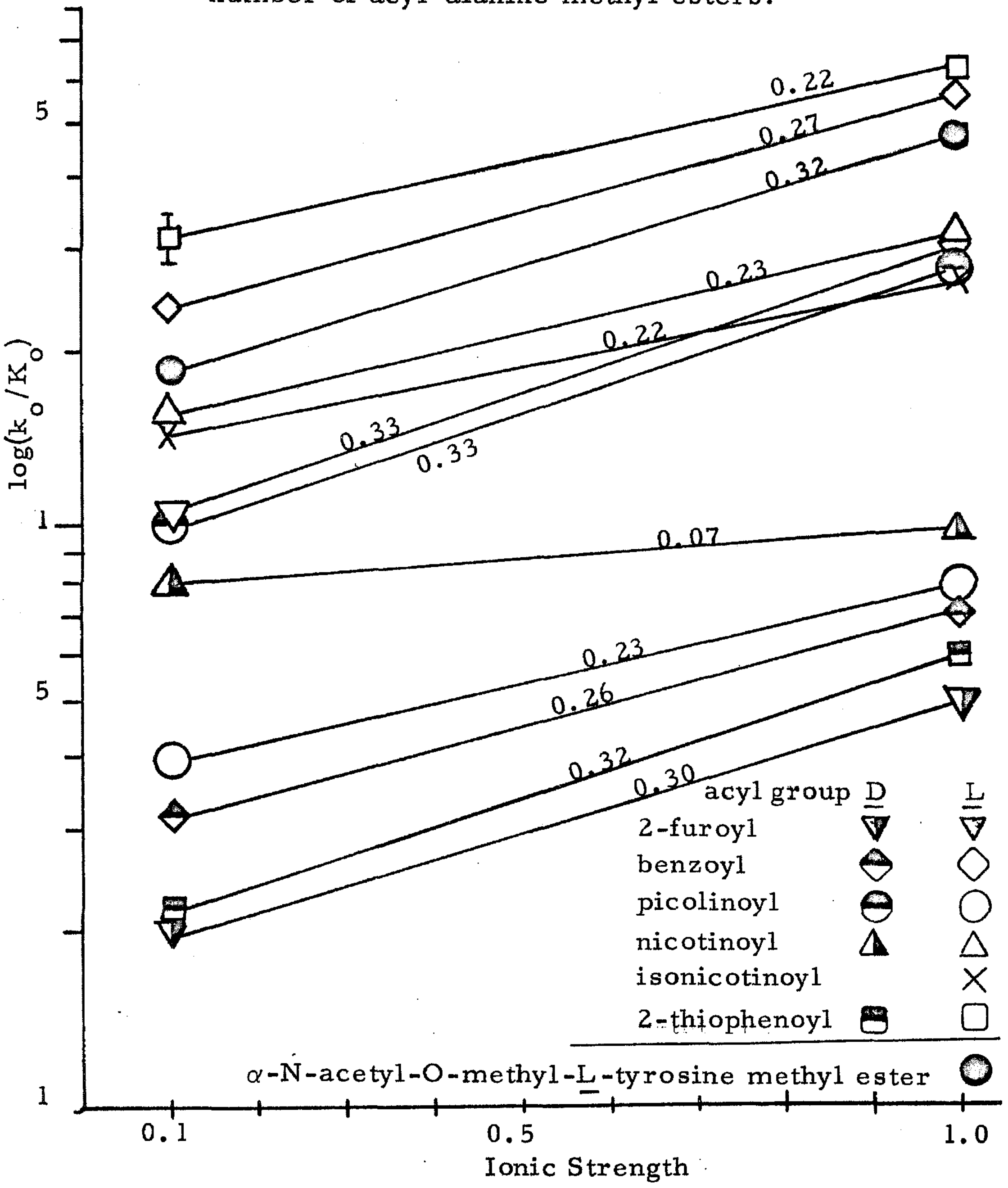


Figure 6.--Log k_o/K_o as a function of ionic strength for a number of acyl-alanine methyl esters.[a]



[a] One derivative of tyrosine is also shown.

Figure 7.--Dependence of the ratios K_{oD}/K_{oL} and k_{oD}/k_{oL} for α-N-benzoyl-D-and-L-alanine methyl esters upon ionic strength.

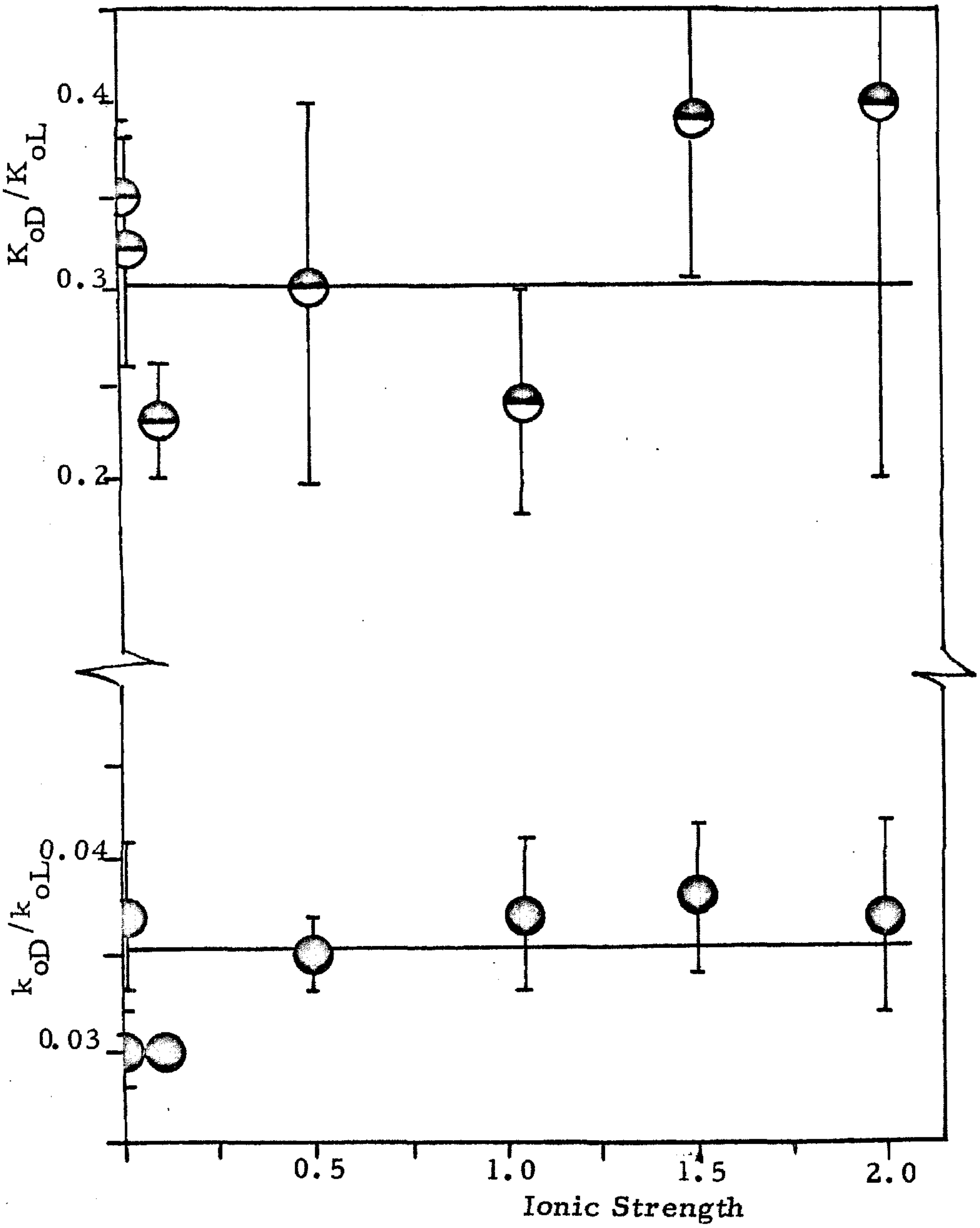


Figure 8.--Dependence of the ratio $(k_o/K_o)_D/(k_o/K_o)_L$ for α-N-benzoyl-D- and L-alanine methyl esters upon ionic strength.

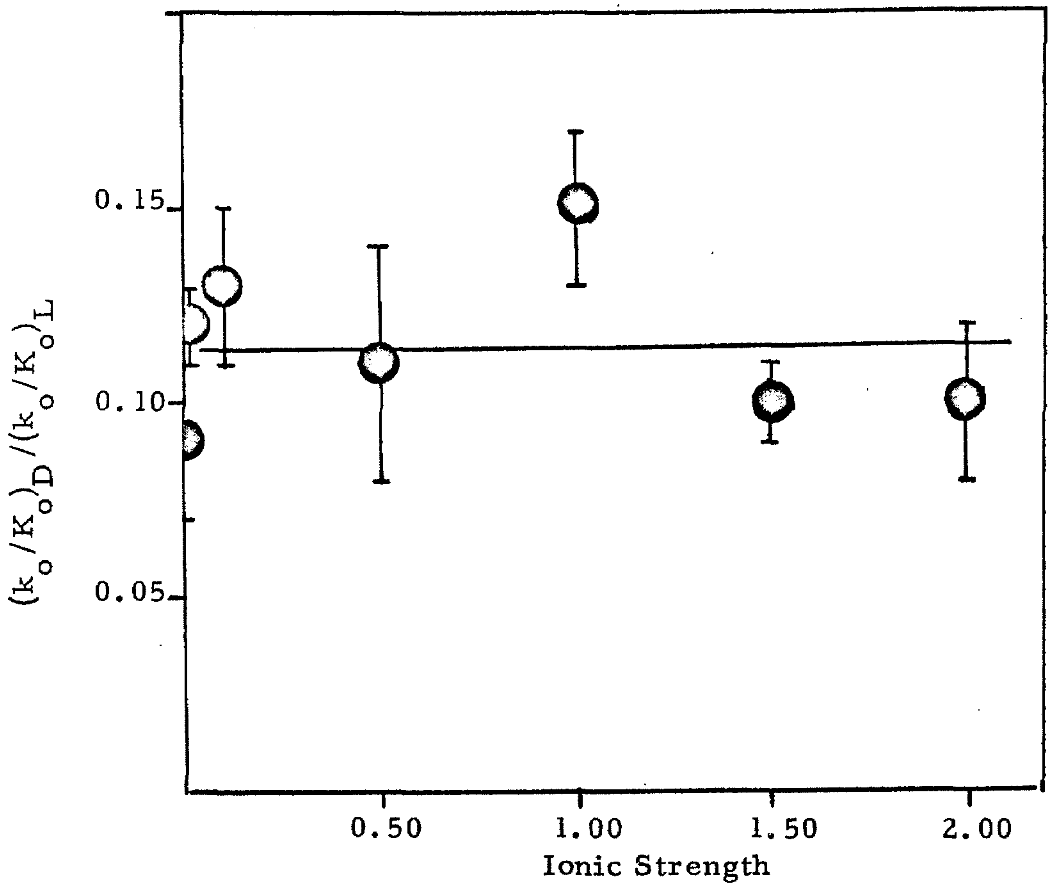


Figure 9.--Dependence of the ratios k_{oB}/k_{oA} and K_{oB}/K_{oA} for α-N-benzoyl-L-alanine methyl ester (B) and α-N-acetyl-O-methyl-L-tyrosine methyl ester (A) upon ionic strength.

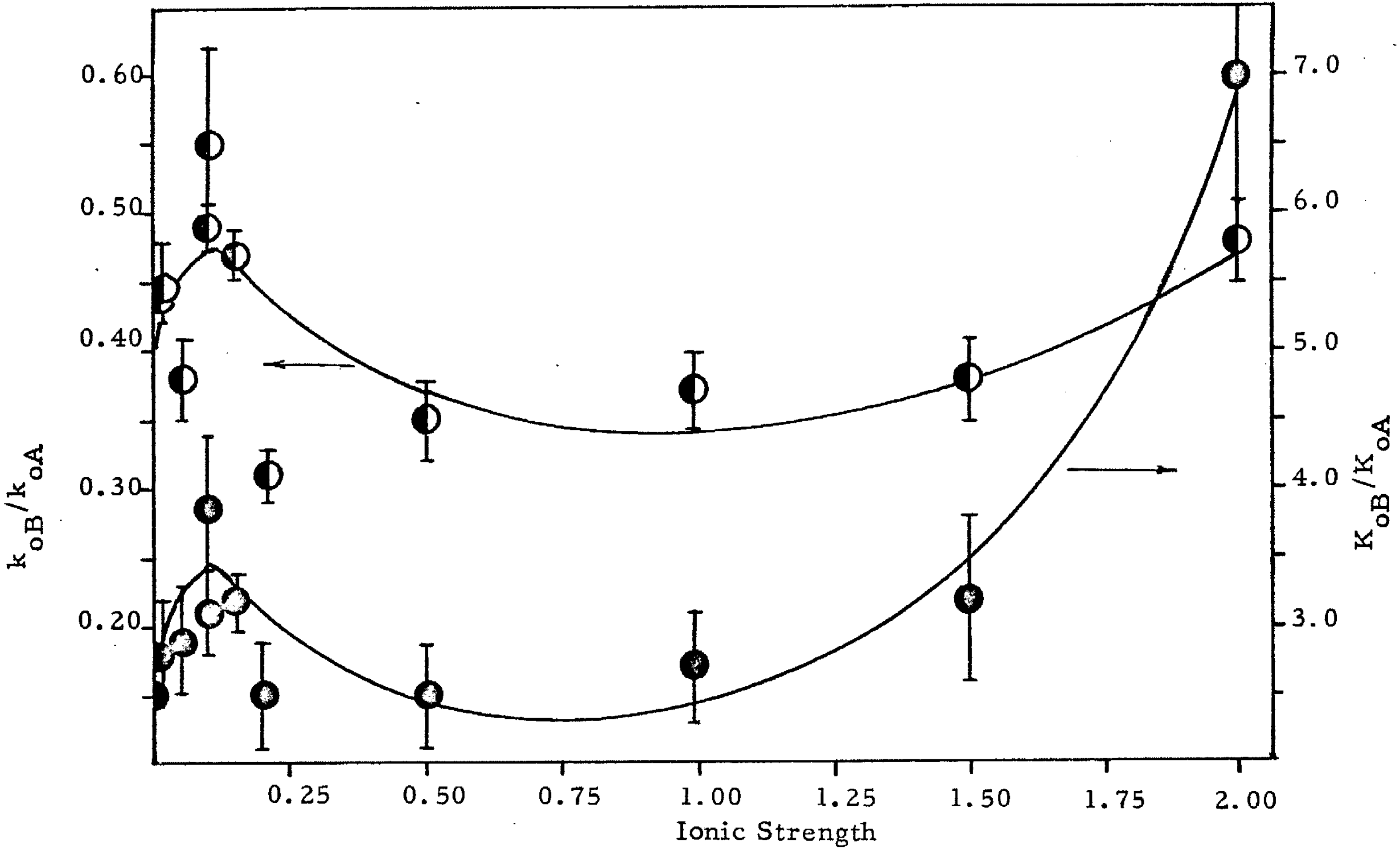
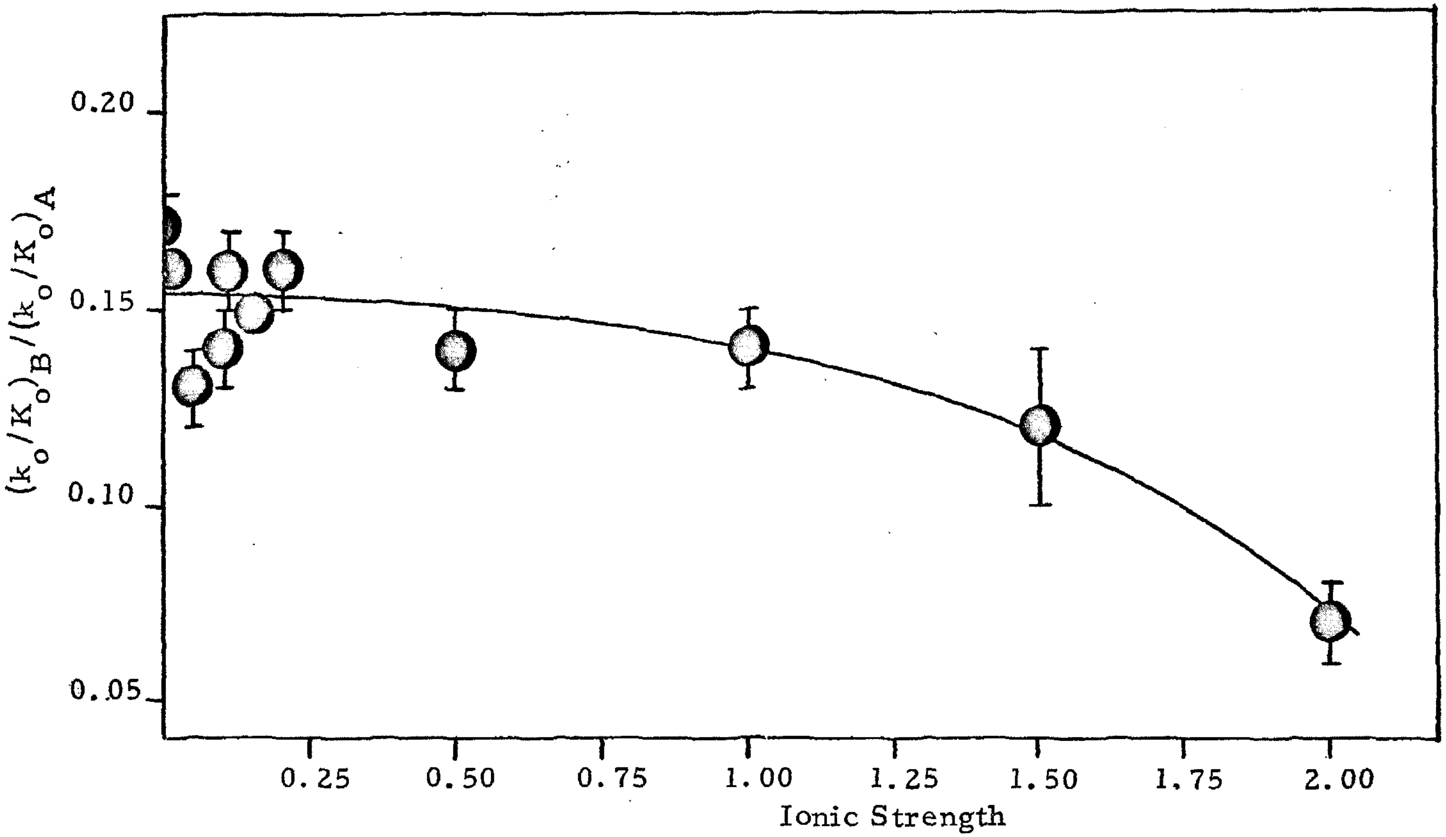


Figure 10.--Dependence of the ratio $(k_o/K_o)_B/(k_o/K_o)_A$ for α-N-benzoyl-L-alanine methyl ester (B) and α-N-acetyl-O-methyl-L-tyrosine methyl ester (A) upon ionic strength.



References

1. J. R. Rapp, Ph. D. Thesis, California Institute of Technology, 1964.

2. a) G. E. Hein and C. Niemann, Proc. Natl. Acad. Sci., U. S., 47, 1341 (1961);

   b) J. Am. Chem. Soc., 84, 4487, 4495 (1962).

3. J. L. Webb, Enzyme and Metabolic Inhibitors, Vol. 1, Academic Press, New York (1963), Chapter 15, pp. 799-839.

4. Handbook of Chemistry and Physics, 38th ed., C. D. Hodgman, R. C. Weast, and S. M. Selby, eds., Chemical Rubber Publishing Co., Cleveland (1956), p. 2332.

5. M. Castañeda-Agullo, L. M. Del Castillo, J. R. Whitaker, and A. L. Tapped, J. Genl. Physiol., 44, 1103 (1961).

6. K. A. Booman and C. Niemann, Biochim. Biophys. Acta, 26, 439 (1957).

7. J. L. Miles, D. A. Robinson, and W. J. Canady, J. Biol. Chem., 238, 2932 (1963).

8. H. S. Harned and B. B. Owen, The Physical Chemistry of Electrolytic Solutions, Reinhold Publishing Corp., New York, 3rd ed. (1958), p. 546.

9. A. A. Frost and R. G. Pearson, Kinetics and Mechanism, John Wiley and Sons, Inc., 2nd ed. (1961), p. 152.

10. M. S. Lewis and H. A. Saroff, J. Am. Chem. Soc., 79, 2112 (1957).

11. R. B. Martin and C. Niemann, ibid., 80, 1481 (1958).

12. R. J. Larese, D. A. Robinson, W. F. Brassine, and W. J. Canady, J. Phys. Chem., 66, 897 (1962).

13. R. J. Foster, J. Biol. Chem., 236, 2461 (1961).

14. a) H. Neurath and G. W. Schwert, Chem. Revs., 46, 69 (1950);

b) R. J. Foster and C. Niemann, J. Am. Chem. Soc., 77, 3365 (1955).

15. R. F. Steiner, Arch. Biochem. Biophys., 53, 457 (1954).

16. a) H. T. Huang and C. Niemann, J. Am. Chem. Soc., 74, 4713 (1952);

b) ibid., 73, 1555, 3223, 3228, 4039 (1951); 74, 101 (1952).

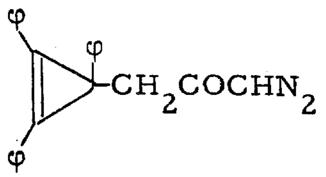
PROPOSITIONS

PROPOSITION I

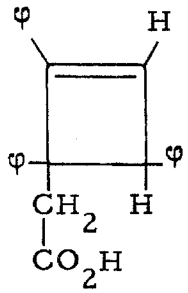
A mechanism is proposed to account for the unexpected product obtained in the Wolff rearrangement of 1-(1, 2, 3-triphenylcyclopropenyl)-3-diazopropan-2-one. The available evidence on the product is compatible with two structures; one structure is compatible with the proposed mechanism; the other structure is preferred by the author. A method is proposed for determining which product is correct.

- - - - - - - - - - - - - - -

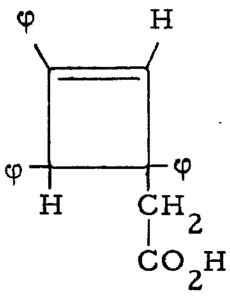
Small (1) has carried out a Wolff rearrangement on 1-(1, 2, 3-triphenylcyclopropenyl)-3-diazopropan-2-one (I) using silver oxide in a dioxane-water mixture. The unexpected product was tentatively identified as (1, 2, 4-triphenylcyclobut-2-enyl) acetic acid (II) on the basis of the infrared, ultraviolet, and NMR spectra and the carbon-



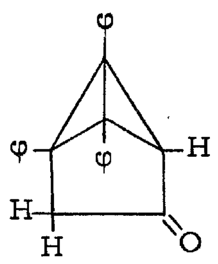
I



II



III

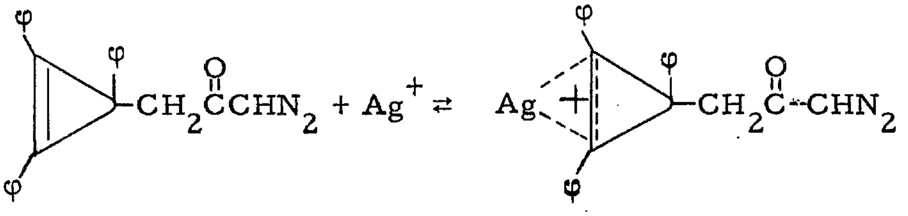


IV

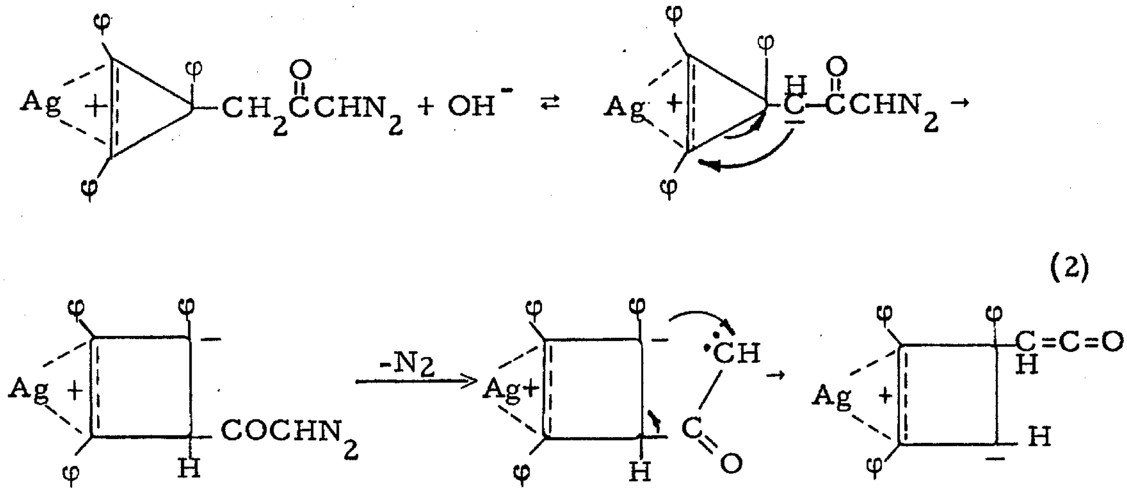
hydrogen analysis. A referee has pointed out that the evidence is also compatible with structure III. Small, however, prefers structure II. On the basis of knowledge about the decomposition of α-diazoketones, it is felt that Small's preference for II is not justified.

The silver ion-catalyzed decomposition of α-diazoketones is believed to go through an α-keto carbene (2). One piece of evidence which indicates that the reaction may in some cases be a free-radical chain process is not very convincing (3). The intermediate carbene rearranges to a ketene via an alkyl (aryl) shift. The ketene has been isolated in several cases (4); however, in the presence of water it is hydrolyzed to an acid (2). An optically active alkyl group migrates mainly with retention of configuration (5).

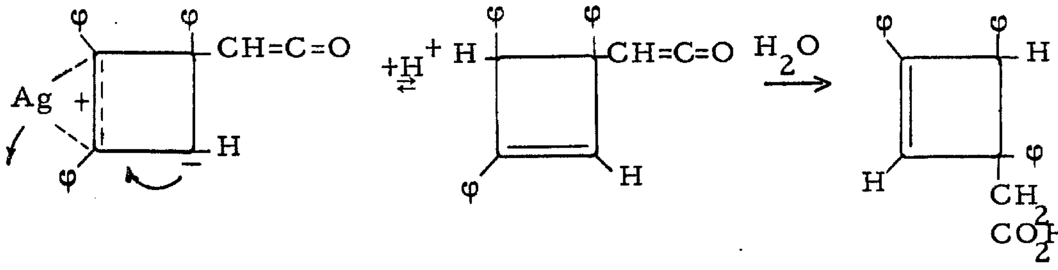
The following reaction scheme is proposed for the decomposition of the α-diazoketone.



(1)



(2)



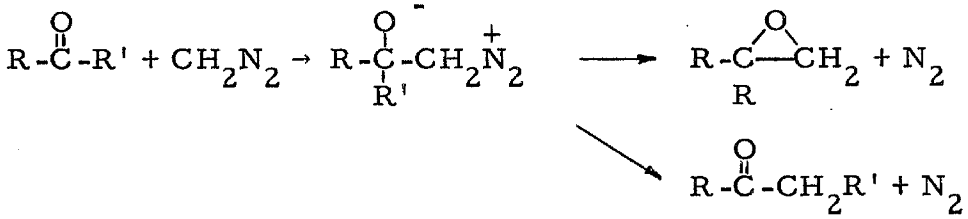
(3)

III

The proposed reaction scheme is straightforward except for two steps. In equation 2, the carbanion α- to the keto group attacks the cyclopropenyl double bond.

This double bond should be prone to nucleophilic attack for one or both of two possible reasons. The silver-ion complex and/or the phenyl groups would decrease the density of negative charge at the double bond. Silver-ion complexes with double bonds are well known (6).

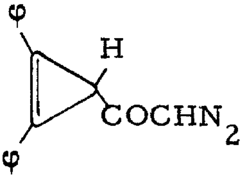
There seems to be no precedence in the Wolff rearrangement for the migration of the cyclobutenyl ring as shown in equation 2; however, it has been shown for keto carbenes decomposed in the presence of copper and copper salts, that attack by nucleophiles competes with rearrangement (7a, b). Also, in the reaction of diazomethane with ketones, attack by the oxyanion on the intermediate competes with rearrangement (8). (See equation 4)



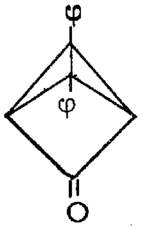
(4)

Decomposition of the α-ketodiazo compound (I) in boiling benzene with copper as a catalyst yields 4, 5, 6-triphenyltricyclo-[2. 1. 1. 0]hexane-2-one (IV). Small's preference for II over III as the product of the silver-ion catalyzed decomposition of (I) is summed up in a footnote in reference 1. Small (1) states, "A mechanism involving base cleavage of a silver complex of IV (or a similar species) leads directly to II whereas mechanisms leading to III involve several intermediates which we feel would collapse just as readily to 3, 4, 5-triphenylphenol. But in fact no phenol can be detected by infrared analysis of the crude product from the attempted Wolff rearrangement of I."

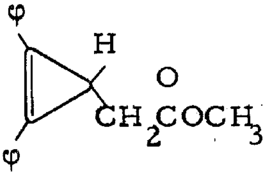
There is no reason to expect that the decomposition of I with silver oxide will go through a silver complex of IV (or a similar species) simply because the reaction of I with copper leads to IV. In fact, there is evidence which indicates that the two reactions are different mechanistically. The decomposition of α-diazoketones using Cu as a catalyst in the presence of various nucleophiles results predominantly in unrearranged material in which the nucleophile attacks the carbene directly (7a, b), whereas when silver salts are used, rearrangement to the ketene usually occurs (5). Also, Masamune (9) has shown that irradiation of 1, 2-diphenylcyclopropenyldiazomethyl ketone (V) results in tricyclo[1. 1. 1. 0]-pentan-2-one (VI). This product is analogous to IV and indicates that the mechanism here may be similar to the copper-catalyzed thermal decomposition (10). Treatment of V with silver benzoate (and presumably methanol) led to (VII), the normal rearrangement product (9). Once again, if a silver complex of VI were an intermediate in the silver ion-catalyzed reaction, the simplest mechanism



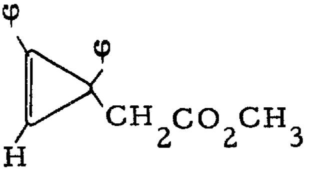
V



VI



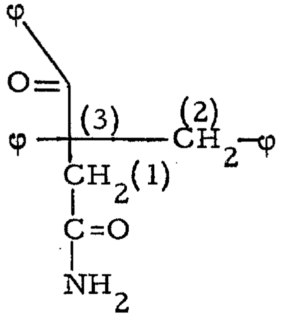
VII



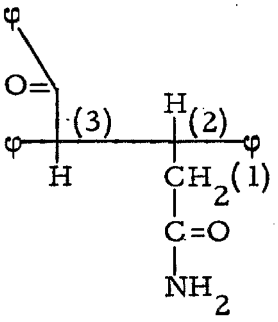
VIII

for base-catalyzed decomposition of VI would yield VIII instead of VII, the rearrangement product obtained (9). Consequently, it seems that structure III is more likely than II as the product obtained when I is decomposed using silver-ion catalysis.

In order to determine whether II or III is the actual product, it is suggested that the product (II or III) be converted to an amide, the double bond ozonized under oxidative conditions, and the resulting acid decarboxylated. This would produce IX or X, which could be distinguished by NMR spectroscopy.



IX



X

All protons except those on C_1, C_2, and C_3 should be far downfield and give similar spectra. The protons on C_1 in either IX or X should be furthest upfield and should absorb around $\tau = 7.9$ (11). In X, but not in IX, the CH_2 group would be split into a doublet by the adjacent hydrogen.

References

1. A. Small, J. Am. Chem. Soc., 86, 2091 (1964).
2. E. S. Gould, Mechanism and Structure in Organic Chemistry, Holt, Reinhart, and Winston, New York, 1959, pp. 627-8.
3. M. S. Newman and P. F. Beal, J. Am. Chem. Soc., 72, 5161 (1950).
4. H. Staudinger and H. Hirzel, Chem. Ber., 49, 2523 (1916).
5. K. B. Wiberg and T. W. Hutton, J. Am. Chem. Soc., 78, 1640 (1956).
6. M. A. Muhs and F. T. Weiss, J. Am. Chem. Soc., 84, 4697 (1962).
7. a) P. Yates and J. Fugger, Chem. Ind., 1511 (1957);
   b) P. Yates, J. Am. Chem. Soc., 74, 5376 (1952).
8. D. J. Cram and G. S. Hammond, Organic Chemistry, McGraw-Hill Book Company, Inc., New York (1959), p. 466.
9. S. Masamune, J. Am. Chem. Soc., 86, 735 (1964).
10. G. Stork and J. Ficini, ibid., 83, 4678 (1961).
11. L. M. Jackman, Applications of Nuclear Magnetic Resonance Spectroscopy, Pergamon Press, Ltd., New York (1959), p. 57.

PROPOSITION II

Mixed carboxylic-carbonic anhydrides of acylamino acids or peptides are useful intermediates in peptide synthesis. However, the peptides obtained from the mixed anhydrides are often racemized to varying degrees. Whether the racemization of the mixed anhydride or of an oxazolone derived from the mixed anhydride is more important has not been established.

The following two experiments are proposed:

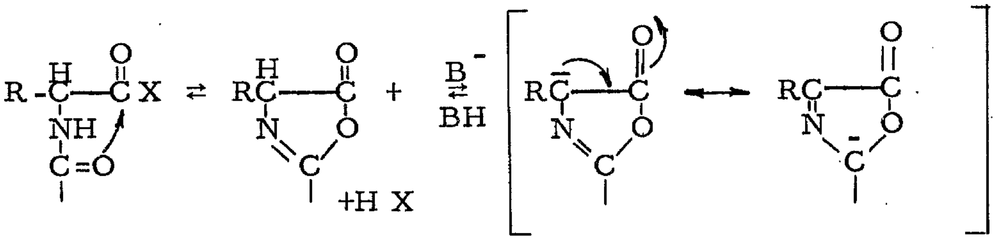
(1) Measurement of the rate of loss of optical activity and measurement of the rate of oxazolone formation from the solution of mixed anhydride in the presence of various tertiary amines (bases).

(2) Independent measurement of the rate of racemization of the oxazolone derived from the mixed anhydride under similar conditions.

These experiments should establish which of the two possible intermediates is more important in racemization.

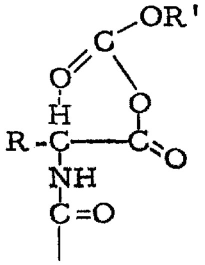
- - - - - - - - - -

Racemization in peptide synthesis still poses an important problem. Except for acid azides (1), all other carboxyl-activated derivatives of amino acids and peptides have been reported to give varying amounts of racemic product in peptide-forming reactions (2a, b, c). Although much evidence (2a, b, c) strongly suggests that racemization of carboxyl-activated acylamino acids and peptides usually occurs via formation of an intermediate oxazolone (see equation 1), racemization may be brought about with derivatives in which oxazolone formation is precluded (2d, 3) (e.g. if the amido hydrogen is replaced by a methyl group,an oxazolone cannot form).


(1)

Also, no peptide oxazolone has been isolated (4).

With mixed carboxylic-carbonic anhydrides, racemization may occur by direct abstraction of the α-hydrogen of the anhydride. In the mixed anhydride, ionization may be facilitated by structures like I (2d).



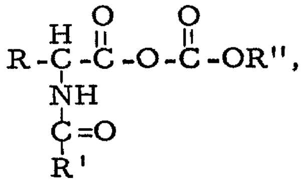
I

Recently, Applewhite and Nelson (5), using ethyl chloroformate as a condensing agent, have demonstrated that racemization in peptide synthesis may be suppressed by inverse addition of reagents in the anhydride-forming step. Their results were interpreted in terms of proton abstraction and racemization of the mixed anhydride although the data did not permit them to exclude oxazolone formation. Normally, peptide synthesis through mixed anhydrides is carried out in two steps. In the first (anhydride-forming) step, a chloroformate ester is added to a solution containing the substituted ammonium salt of an acylamino acid or peptide. In the second (peptide-forming) step, a carboxyl-protected amino acid or similarly protected peptide is added to the solution. Goodman and Levine (4) have shown that for oxazolones, racemization is much faster than ring opening by a nucleophile. Consequently, any oxazolone formed in the first step should result in racemic peptide. Carter and Stevens (3) have shown that the formation of oxazolone is base-catalyzed. The base causing the racemization and/or oxazolone formation in the first step is presumably the carboxylate anion. Additional racemization can occur in the second step either by abstraction of the α-hydrogen of the mixed anhydride or by more oxazolone formation catalyzed by the added nucleophile (base).

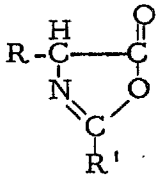
When inverse addition is used in the anhydride-forming step, the acylamino acid and tertiary amine are added slowly to an excess of chloroformate ester (5). The carboxylate anion is thus reacted immediately. The latter procedure results in much higher optical purity of the peptide product than the former procedure (5). The small amount of racemization observed in the product after inverse addition may result either from abstraction of the α-hydrogen of a small amount of oxazolone formed in the second step or from abstraction of the α-hydrogen from the mixed anhydride in the second step.

If one assumes that formation of the mixed anhydride by inverse addition to an excess of chloroformate ester is virtually complete and causes little or no racemization of the anhydride, the specific rotation of the mixed anhydride may be obtained directly from this solution. If a tertiary amine is then added, the kinetics of oxazolone formation and of change in rotation of the solution may be analyzed according to the following scheme:

$$\begin{tikzcd}
I^{*} \arrow[r, "k_2"] \arrow[d, "k_1"'] & II^{*} \arrow[d, "k_3"'] \\
I \arrow[r, "k_2"] & II
\end{tikzcd}$$

where I^{*}, I ≡ mixed anhydride ≡ 

$R\text{-}\underset{\underset{\underset{R'}{|}}{\underset{|}{\underset{C=O}{\underset{|}{NH}}}}}{\overset{H}{C}}\text{-}\overset{O}{\overset{\|}{C}}\text{-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-}OR''$,

II, II^{*} ≡ oxazolone ≡  and the asterisk denotes an optically active substance.

The rate of formation of total oxazolone may be measured either by infrared or ultraviolet spectroscopy (4). The oxazolone may be prepared independently (4), and its specific rotation and rate of racemization may be obtained. Thus, k_2 and k_3 may be obtained, and only k_1 is needed to complete the analysis.

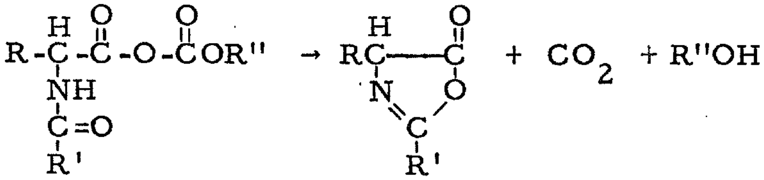
More rapid loss of optical activity than of oxazolone formation warrants the conclusion that the oxazolone is not the more important racemizing intermediate. In all other cases, a detailed kinetic analysis may be used to obtain k_1. The polarimetric rate is given by:

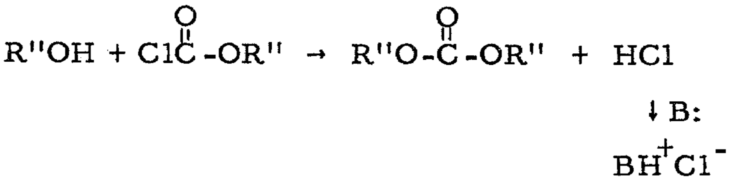
$$\frac{-d\alpha}{dt} = [I_o]\, e^{-(k_1+k_2)t}\left(k_1[\alpha]_{DI}^* + k_2[\alpha]_{DI}^* - k_2\,[\alpha]_{D\,III}^*\right)$$

$$+\frac{k_2\,[I_o]}{(k_3-k_1-k_2)}\left(e^{-(k_1+k_2)t} + e^{-k_3 t}\right)$$

where $[I_o]$ = initial concentration of mixed anhydride, $\frac{d\alpha}{dt}$ and t are measurable, and k_1 is the only unknown.

The measurement of k_2 may have to be made for small conversions of mixed anhydride to oxazolone since the formation of oxazolone produces an alcohol. The alcohol should react with the excess chloroformate ester producing a carbonate diester and HCl. The HCl would then react with tertiary amine, giving a salt which may significantly affect the kinetics of the reactions.





$$R''OH + Cl\overset{O}{\overset{\|}{C}}\text{-}OR'' \rightarrow R''O\text{-}\overset{O}{\overset{\|}{C}}\text{-}OR'' + \underset{BH^+Cl^-}{\underset{\downarrow B:}{HCl}}$$

Since no peptide oxazolone has ever been isolated, it is also suggested that this method employing excess chloroformate ester could be used to prepare these oxazolones. The loss of carbon dioxide and the capture of the alcohol by excess choroformate ester would drive the reaction to completion.

References

1. T. Wieland and H. Determann, Angew. Chem. (International edition), 2, 358 (1963).

2. For reviews of the extensive literature, see:

   a) Ref. 1;

   b) F. Wegand, A. Prox, and L. Schmidhammer, Angew. Chem. (International edition), 2, 183 (1963);

   c) N. Albertson, Org. Reactions, 12, 157 (1962);

   d) A. Neuberger, Adv. Protein Chem., IV, 333-362 (1948).

3. H. E. Carter and C. M. Stevens, J. Biol. Chem., 133, 117 (1940).

4. M. Goodman and L. Levine, J. Am. Chem. Soc., 86, 2918 (1964).

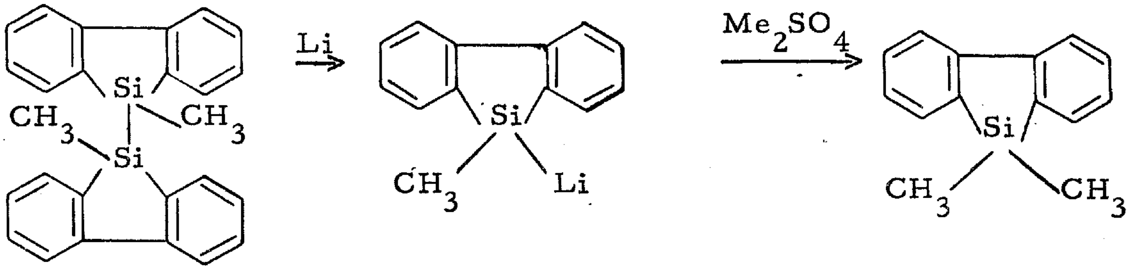
5. T. H. Applewhite and J. S. Nelson, Tet. Let., 15, 819 (1964).

PROPOSITION III

A synthesis of potassium silacyclopentadienyl is proposed. The anion may possess aromatic properties similar to cyclopentadienyl anion.

- - - - - - - - - - - - - -

No compound containing a silicon atom in an aromatic ring has ever been isolated. Gilman and Gorsich (1) reported that treatment of I with lithium, a reagent known to cleave silicon-silicon bonds, yielded a green mixture (presumably containing II) which, when reacted with dimethyl sulfate, yielded III. More recently, Benkeser and coworkers (2) have attempted to prepare potassium silacyclopentadienyl in order to test the anion for aromatic stability. To date they have not reported success.

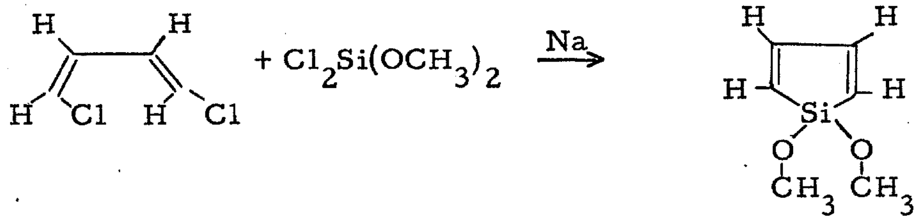


I

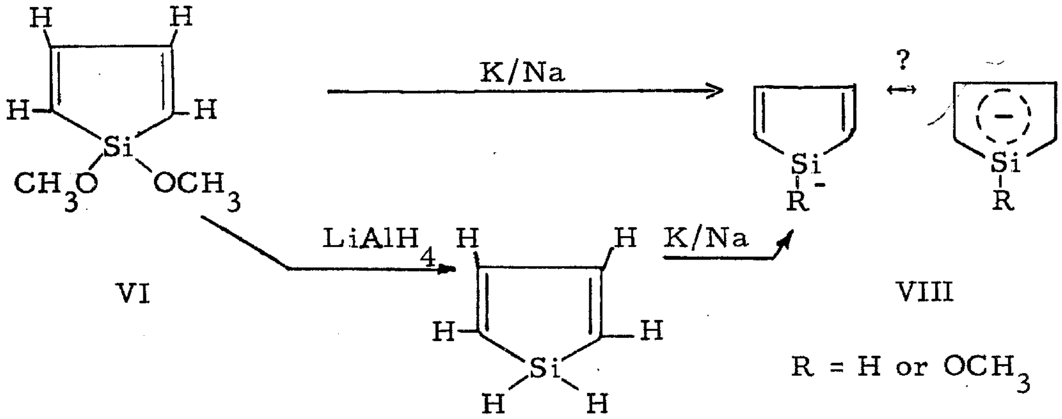
II

III

The following synthesis of potassium silacyclopentadienyl is proposed:



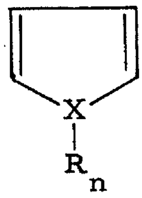
IV V VI



VI VII VIII

R = H or OCH$_3$

Gilman <u>et al.</u> (4) reacted 1,4-dilithio-1,2,3,4-tetraphenyl-1,3-butadiene with dichlorodimethylsilane to obtain 1,1-dimethyl-2,3,4,5-tetraphenyl-1-silacyclopentadiene, a reaction analogous to that proposed in equation 1. Similar reactions using 1,4-dilithio-1,3-butadienes have been used (5) to prepare various tetraphenylsubstituted heterocycles of the general formula:



where R_n = $(\text{alkyl})_n$ or $(\text{aryl})_n$.

1,4-Dichloro-1,3-butadiene (IV) is known (3). Use of dichlorodimethoxysilane and sodium is suggested, since Hiratsuka (6) has shown that halogens on silicon can be replaced in preference to alkoxy groups using sodium in a Wurtz-Fittig reaction.

The formation of the anion may be attempted in two ways. Sodium-potassium alloy in ether has been used to prepare triarylsilyl potassium compounds from the chlorides, hydrides, or alkoxides (7a). Consequently, VI may be reacted with sodium-potassium alloy or may first be reduced to the hydride. Lithium aluminum hydride reduction of alkoxysilanes is well known (7b, 8). The possibility exists that ring opening may occur by cleavage of the silicon-carbon bond; however, Benkeser et al. (2) have used lithium aluminum hydride to reduce dichlorodivinylsilane to divinylsilane and 1,1-dichloro-1-sila-2-cyclopentene to sila-2-cyclopentene. The pure products were obtained by vapor phase chromatography.

Whether the silacyclopentadienyl anion will exhibit resonance stabilization is open to question. Benkeser et al. (9) have carried out an LCAO-MO calculation on silacyclopentadienyl anion which incorporates the silicon $3p_z$, $3d_{xz}$, and $3d_{yz}$ orbitals. A resonance energy of approximately 36 kcal/mol was obtained. However, the ultimate test of stability of the anion would have to await its synthesis and measurement of its physical properties.

References

1. H. Gilman and R. D. Gorsich, J. Am. Chem. Soc., 80, 3243 (1958).
2. R. A. Benkeser, N. Yoichiro, J. L. Noe, R. F. Cunico, and P. H. Gund, ibid., 86, 2446 (1964).
3. H. G. Viehe, Angew. Chem., 75, 793 (1963).
4. H. Gilman, S. G. Cottis, and W. H. Atwell, J. Am. Chem. Soc., 86, 1596 (1964).
5. Braye, E. H., W. Hubel, and I. Caplier, ibid., 83, 4406 (1961).
6. K. Hiratsuka, Jap. Pat. No. 5330 (1951); Chem. Abstr., 47, 9345 (1953).
7. a) C. Eaborn, Organosilicon Compounds, Academic Press, Inc., London (1960), p. 103;

   b) ibid., p. 196.
8. L. H. Sommer, C. L. Frye, G. A. Parker, and K. W. Michael, J. Am. Chem. Soc., 86, 3271 (1964).
9. R. A. Benkeser, R. F. Grossman, and G. M. Stanton, J. Am. Chem. Soc., 84, 4727 (1962).

PROPOSITION IV

Recently, Cruickshank and Sheehan (1) synthesized a pentapeptide which catalyzed the hydrolysis of p-nitrophenyl acetate at a rate six times greater than that reported previously for any other synthetic peptide. The mechanism of the catalysis is unknown. Two experiments are proposed to determine whether more than one catalytic group is involved in the reaction and whether this peptide may prove useful as a model for the active site of α-chymotrypsin and other hydrolytic enzymes.

- - - - - - - - - - -

The pentapeptide, L-threonyl-L-alanyl-L-seryl-L-histidyl-L-aspartic acid has been synthesized and investigated as a catalyst in the hydrolysis of p-nitrophenyl acetate (1). Three of the amino acids, L-histidine, L-serine, and L-aspartic acid, have been found at or near the active site of a number of hydrolytic enzymes, including α-chymotrypsin (2). Although it has been fairly clearly established that hisitidine and serine are both directly involved in α-chymotrypsin-catalyzed hydrolysis (3a, b, c), aspartic acid has been implicated only by its being located adjacent to the reactive seryl group and from one pH study (4) in which the authors concluded that a group of pKa of *ca.* 4 is involved in the acylation reaction. Bender and Kezdy (5) have suggested that the active conformation of α-chymotrypsin may be due to an electrostatic interaction between an ammonium group (from terminal isoleucine) and a carboxylate anion. Aspartic acid may furnish the carboxylate anion for the electrostatic interaction.

Sheehan and McGregor (6) have previously shown that cyclo-glycyl-*L*-histidyl-*L*-serylglycyl-*L*-histidyl-*L*-seryl catalyzes the hydrolysis of p-nitrophenyl acetate at a rate no greater than that of any compound containing an imidazole group.

Since α-chymotrypsin-catalyzed reactions are believed to involve both serine and imidazole, it would be important to know whether the catalytic activity of the pentapeptide is due to more than one group, and also which group(s) are involved.

It is first proposed that the pH-rate profile of this reaction be studied between pH 3 and 8. The shape of this curve would determine whether imidazole and/or aspartic acid are involved in the catalysis. If the only function of aspartic acid in α-chymotrypsin is to maintain an active conformation, it is expected that the reaction catalyzed by the pentapeptide would show no pH-dependence below *ca.* pH 5.

In order to determine whether serine is involved in the catalysis, it is suggested that the method of Koshland *et al.* (3b) be used to dehydrate the serine (and threonine) residues. The modified peptide should show no more activity than any imidazole-containing compound if the serine (or threonine) is involved in the catalysis. This simple experiment would not be able to distinguish between serine or threonine being involved. However, the two experiments would tell whether more than one group was involved in the catalysis and, consequently, whether this peptide could prove useful as a model for the active site of a number of hydrolytic enzymes.

References

1. P. Cruickshank and J. C. Sheehan, J. Am. Chem. Soc., 86, 2070 (1964).

2. M. L. Bender, G. R. Schonbaum, and G. A. Hamilton, J. Polymer Sci., 49, 75 (1961).

3. a) For a recent review, see D. E. Koshland, Jr., D. H. Strumeyer, and W. J. Ray, Jr., in Enzyme Models and Enzyme Structures, Brookhaven Symposia in Biology, No. 15, (1962), pp. 101-133.

   b) D. H. Strumeyer, W. N. White, and D. E. Koshland, Jr., Proc. Natl. Acad. Sci., U.S., 50, 931 (1963).

   c) J. Kallos and D. Rizok, J. Molec. Biol., 9, 255 (1964).

4. J. A. Stewart, H. S. Lee, and J. E. Dobson, J. Am. Chem. Soc., 86, 1537 (1964).

5. M. L. Bender and F. J. Kezdy, J. Am. Chem. Soc., 86, 3704 (1964).

6. J. C. Sheehan and D. N. McGregor, J. Am. Chem. Soc., 84, 3000 (1964).

PROPOSITION V

It is proposed that anhydro-chymotrypsin[*] be used as a substitute for α-chymotrypsin in the study of the enzyme-catalyzed hydroxylaminolysis of acetyl-L-tyrosine and the hydrolysis of acetyl-L-tyrosinehydroxamate. This may shed some light on whether all α-chymotrypsin-catalyzed reactions proceed via an acyl-enzyme intermediate.

\- - - - - - - - - - - -

Whether all α-chymotrypsin-catalyzed reactions proceed via an acyl-enzyme intermediate is an open question (3,4,5). The use of hydroxylamine as a water analog has not clarified the situation (3,4,5). For example, Epand and Wilson (3) have reported that the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate is slower than that for other hippurate esters. If these reactions proceed through an acyl-enzyme, then the slow step in the methyl hippurate hydrolysis must be formation of the acyl-enzyme. However, Bernhard *et al.* (4) have reported that addition of hydroxylamine increases the rate of disappearance of methyl hippurate several fold. This result requires that decomposition of the acyl-enzyme be rate-determining. Thus, the two results appear to be

---

[*] Anhydro-chymotrypsin is α-chymotrypsin from which the active serine residue has been converted to a dehydroalanine residue, i.e., a water molecule has been removed from serine(1,2).

inconsistent with each other.

Caplow and Jencks (5) have recently studied the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine ethyl ester (ATE) and acetyl-L-tyrosinehydroxamate (ATH) in the presence of varying concentrations of hydroxylamine. They have also studied the α-chymotrypsin-catalyzed synthesis of acetyl-L-tyrosinehydroxamate (ATH) from acetyl-L-tyrosine(5). In the enzyme-catalyzed hydrolysis of ATH in the presence of varying amounts of hydroxylamine, Caplow and Jencks (5) found that the observed ratios of $k_{\text{hydroxylaminolysis}}/k_{\text{hydrolysis}}$ are higher than the same ratios calculated from the hydrolysis and hydroxylaminolysis of ATE. If both substrates, ATE and ATH, react via the same acyl-enzyme, the observed and calculated ratios $k_{\text{hydroxylaminolysis}}/k_{\text{hydrolysis}}$ should be identical. Caplow and Jencks concluded that these results offer no support for the acyl-enzyme hypothesis, but that the differences are not sufficiently large to be used as conclusive evidence against the acyl-enzyme hypothesis.

Clearer results were obtained by Caplow and Jencks (5) in the α-chymotrypsin-catalyzed synthesis of ATH from AT. They analyzed the reaction terms of equation 1.

$$AT + E \overset{K_s}{\rightleftarrows} \underset{I}{[AT\cdots E]} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \underset{II}{[AT\text{-}E]} \underset{\underset{\pm NH_2OH}{k_{-2}}}{\overset{k_2}{\rightleftarrows}} \underset{III}{[AT\cdots H]} \rightleftarrows ATH + E \tag{1}$$

In equation 1, I and III are enzyme-substrate and enzyme-product complexes, respectively, II is an acyl-enzyme, and E is free enzyme. Using a steady-state treatment of equation 1 in which k_{-2} is considered unimportant initially, they obtained an expression for the initial rate of the forward reaction. This expression is given in equation 2.

$$\text{rate} = \frac{k_1 k_2\,[NH_2OH]\,[I]}{k_{-1} + k_2\,[NH_2OH]} \tag{2}$$

Equation 2 predicts that the rate will be linear with increasing hydroxylamine concentration as long as $k_2[NH_2OH] \ll k_{-1}$, but when $k_2[NH_2OH] \simeq k_{-1}$, the rate should deviate from linearity. Since Caplow and Jencks knew the ratio $k_2[NH_2OH]/k_{-1}$ to be 1.3 in 3.0 M hydroxylamine from the reaction involving ATE, it was expected that a significant deviation from linearity in the rate should occur at this hydroxylamine concentration. No deviation was observed, although the calculated and observed rates are identical at low hydroxylamine concentrations.

An alternative hypothesis suggested by Caplow and Jencks (5) is that not all α-chymotrypsin-catalyzed reactions proceed via an acyl-enzyme. For example, hydroxylamine may react directly with complex I (see equation 1). The principle of microscopic reversibility predicts that the hydrolysis of ATH should follow the same path as the hydroxylaminolysis of AT.

Since Bender and coworkers (6) have obtained evidence that acetyl-*L*tyrptophan forms an acyl-enzyme with α-chymotrypsin, it is possible that hydroxylamine reacts with both I and II (see equation 1). If methyl hippurate also reacted by these two paths, the results of Epand and Wilson (3) would be compatible with those of Bernhard *et al.* (4).

In order to determine whether hydroxylamine (or water) can react with an enzyme-substrate complex or by any mechanism other than an acyl-enzyme, it is proposed that the synthesis of ATH from AT and the hydrolysis of ATH be studied using anhydro-chymotrypsin as a substitute for α-chymotrypsin. It has been reported (7) that anhydro-chymotrypsin binds substrates of α-chymotrypsin and that these substrates are not hydrolyzed (7, 12). Since the serine hydroxyl is absent from anhydro-chymotrypsin, an acyl-enzyme cannot form. If the hydroxylaminolysis of AT and the hydrolysis of ATH are catalyzed by anhydro-chymotrypsin, this result would strongly suggest that the same reactions catalyzed by α-chymotrypsin may proceed, at least in part, via a pathway not involving an acyl-enzyme. If these reactions are not catalyzed by anhydro-chymotrypsin, this result would, however, not establish conclusively that an acyl-enzyme is a necessary intermediate. Removal of the serine hydroxyl group from α-chymotrypsin may modify the enzyme to an extent where it cannot be used as a valid model.

References

1. D. H. Strumeyer, W. N. White, and D. E. Koshland, Jr., Proc. Natl. Acad. Sci., 50, 931 (1963).
2. J. Kallos and D. Rizok, J. Molec. Biol., 9, 255 (1964).
3. R. M. Epand and I. B. Wilson, J. Biol. Chem., 238, 1718 (1963).
4. S. A. Bernhard, W. C. Coles, and J. F. Nowell, J. Am. Chem. Soc., 82, 3043 (1960).
5. M. Caplow and W. P. Jencks, J. Biol. Chem., 239, 1640 (1964).
6. F. J. Kezdy, G. E. Clement, and M. L. Bender, J. Am. Chem. Soc., 86, 3690 (1964).
7. Information obtained in a seminar delivered by D. E. Koshland at the California Institute of Technology, November 9, 1964.