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TEMPERATURE AND KINETIC ISOTOPE EFFECT STUDIES ON 5 SUBSTITUTED FUROYL- ALPHA-CHYMOTRYPSINS

The Ohio State University

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STUDIES ON 5 SUBSTITUTED FUROYL-α-CHYMOTRYPSINS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Kim C. Calvo, B.A.

* * * * *

The Ohio State University
1981

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Publications


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Chapter One
Introduction

The understanding of the molecular mechanisms of enzyme catalysis requires a knowledge of the temperature behavior of the reaction rates. In uncatalyzed reactions, i.e. those run in solution, the interpretation of the temperature dependence of the rate is generally straightforward. However, due to the nature of enzyme catalyzed reactions this is not always the case. The temperature dependencies of the enzyme structure, of the ionization of catalytically significant groups, and the individual rates in a multi-step reaction pathway can individually or severally lead to a complex temperature behavior of the observed rate. The ability of the enzyme to bind other molecules in an equilibrium fashion which can affect the overall rate is a further complication. Any attempt to explain the temperature dependence of the reaction kinetics must take these factors into consideration.

The theory relating the general effect of temperature on reaction rate was proposed by Arrhenius (1). His was an empirical relationship based on the observations of van't Hoff (2), concerning the temperature coefficient of the equilibrium constant. Arrhenius reasoned that a similar variation of reaction rate with temperature would be:

\[
\frac{d \ln k}{d t} = \frac{E_a}{RT}
\]  

(1)
For $E_a$ (activation energy) independent of temperature this expression after integration yields

$$k = A \exp(-E_a/RT)$$ (2)

Where $A$ is a constant of integration and represents the rate at infinite temperature. The idea was expanded by Eyring (3) in his derivation of the absolute rate theory. Eyring's model proposes that the conversion of reactants to products passes through an intermediate, metastable structure named the activated complex. It was an unstable structure due to the increase in energy (relative to the ground state) required to reach it (the activation energy). The rate of reaction is simply the number of activated complexes passing to product per unit time. It is further assumed that the activated complex is in equilibrium with the reactants. The theory leads to the following form for the temperature dependence of the rate

$$k = \frac{k'T}{h} \exp(-E_a/RT)$$ (3)

where $k'$ is the Boltzmann constant and $h$ is Planck's constant. Because of the assumption of an equilibrium between reactants and the activated complex, $E_a$ can be considered as a free energy of activation. A plot of $\ln(k/T)$ vs $1/T$ should be linear, providing $\Delta H^\ddag$ (activation enthalpy) and therefore $\Delta S^\ddag$ is constant over the temperature range of interest. This is what is observed for most reactions, including enzyme catalyzed reactions. However, this is not always the case, as there are numerous examples of non-linear Arrhenius plots in enzyme catalyzed reactions (4-23). As will be seen in this work, an unusual
temperature behavior has been observed in a chymotrypsin catalyzed hydrolysis reaction. The analysis of this Arrhenius plot will be developed based on the following discussion.

Han (24) has considered some of the possible reasons for the non-linear temperature behavior of enzyme catalyzed reactions and generally divided them into two groups. The first group (I) relies on the fact that individual steps in a multi-step kinetic pathway can have different temperature dependencies with the result that the overall flux to products can vary non-linearly with temperature. The second group (II) involves various types of secondary equilibria which may kinetically modify the enzyme reaction. The following is a general outline of these conditions for non-linear Arrhenius plots.

I(A). Two parallel pathways may exist between products and reactants. This can be represented as in scheme I.

```
ES \rightarrow^k E + P
E \rightarrow^k' ES
```

scheme I

This model requires that there is a temperature dependent shift in the path by which the product is formed. The reaction with the lower $\Delta H$ will be the predominant path to products in the lower temperature region whereas at high temperatures the larger activation enthalpy will dominate. This will produce concave up curves in a plot of $\ln(\frac{k_{obs}}{T})$ vs $1/T$. Depending on the magnitude of the difference in the activation enthalpies ($\Delta \Delta H^\ddagger$) for the low and high temperature regions, there will be either a gradual upward curvature (small $\Delta \Delta H^\ddagger$) or a very sharp break or discontinuity (large $\Delta \Delta H^\ddagger$). For a true break between the two
limiting linear regions to be observed, the $\Delta H^\#$ must be at least 16 Kcal (29). It should be noted that in the region of transition from high activation enthalpy to low activation enthalpy, the observed rate will be composed of significant contributions from each rate.

I(B). In a two step reaction mechanism (scheme II)

\[
E \rightarrow ES \rightarrow E + P \quad \text{scheme II}
\]

there may be a difference in the temperature dependence of the two steps. The reaction with the higher activation enthalpy will tend to predominate at higher temperatures; however, the overall rate will be limited by the slower reaction. The Arrhenius plot will then be concave down.

The second group provides for various possible secondary equilibria in the reaction scheme.

II(A). If an enzyme mechanism involves a thermal transition of the enzyme leading to inactivation (scheme III)

\[
E + S \rightarrow ES \rightarrow E + P \\
E' + S \rightarrow ES' \quad \text{scheme III}
\]

either of the free enzyme or enzyme substrate complex and the thermally altered enzyme has no catalytic activity then the relative magnitude of the enthalpy for the equilibrium ($\Delta H^O$) determines the shape of the temperature plot. If $\Delta H^O$ is positive or negative, the curve will be concave down. If $|\Delta H^O| >> E_a$, the curve will be very sharply concave down and will resemble an upside down V. In the temperature region before the transition a straight line will be observed which reflects
the true activation energy for the reaction. However, because thermal
denaturation of an enzyme can be very temperature dependent (i.e. the
\[ \Delta C_P^0 \] has a strong temperature dependence (25-27)) the broadness of the
transition can be misleading.

II(B). An equilibrium can exist between the enzyme and a second
species (not the substrate) which is required in the catalysts. This
could be a proton, a metal ion, or a cofactor. This process can then
effect the temperature behavior of the reaction as reflected in the
Arrhenius plot. If A is such a species, two possibilities exist
(scheme IV)

\[
\begin{align*}
E + S & \rightleftharpoons ES \rightleftharpoons E + P \\
EA + S & \rightleftharpoons EAS \\
\end{align*}
\]

or

\[
\begin{align*}
E + S & \rightleftharpoons ES \\
EA + S & \rightleftharpoons EAS \rightarrow E + P \\
\end{align*}
\]

A can act as either an activator or inhibitor. In terms of the Arrhenius plot this is thermodynamically equivalent to the thermal inactivation model (scheme III), except that this is a unimolecular case and therefore the transition is dependent on the concentration of A. Irrespective of whether \( \Delta H^0 \) (the enthalpy of the association of A) is positive or negative, the overall shape of the curve will be concave down. As long as the concentration of A is zero or saturating, a linear region will be observed.

II(C). The third possibility is that a rapid equilibrium can exist between two reactive forms of the enzyme with one form having a different reactivity than the other form (scheme V)
Since there are two rate processes each with a different activation energy, there will be an intersection point for the two independent linear lines in the Arrhenius plot. That point of intersection is $T_b$, and the difference in the $\Delta H^\ddagger$'s for each rate is $\Delta H^\ddagger_b$. If $\Delta H^0$ is defined as the enthalpy for the equilibrium (both equilibria between $E$ and $E'$ and $ES$ and $ES'$ being equal) and $T_o$ the temperature of the midpoint of the equilibrium, then three factors can affect the shape of the Arrhenius curve. If $\Delta H^\ddagger_b \neq \Delta H^0$ and $T_o \neq T_b$ then the temperature plot will show a sigmoidal transition between two limiting linear regions. The linear lines will then give the activation energies for the two rates. If $\Delta H^\ddagger_b \neq \Delta H^0$ and $T_b = T_o$ then two cases are possible. If $\Delta H^0$ is positive, the Arrhenius plot will be a smooth curve, concave up in shape. If $\Delta H^0$ is negative, the curve will be smooth but concave down. The sharpness of the transition is related to the magnitude of $\Delta H^0$ as the larger this value the sharper the transition. So that with a small $\Delta H^0$, the complete transition curve may not be obvious over the small temperature range normally employed in the study of enzyme reactions. Although biphasic, sigmoidal Arrhenius plots are not frequently seen, Sipos and Merkel (20) have reported an example of this type of transition for the $Ca^{+2}$ activated reaction of Trypsin. Also, this case of non-linearity may better explain the results of Kumamoto, Raison and Lyons (28), who reported an actual discontinuity in the Arrhenius plot for succinate oxidation by rat liver mitochondria.
Their explanation for this result was not related to this type of analysis and will be discussed later.

The cases discussed above are the most basic ones. That is, further types of non-linear behavior can arise from combinations of these simple cases. Obviously, the interpretation of a non-linear Arrhenius plot can be quite complex. However, most of the simple cases result in concave down curves. This type of non-linear Arrhenius plot is the most common in the biochemical literature. It is interesting that only one case provides for a sigmoidally shaped curve. As will be seen, this is what has been observed in the first part of the study described in this work.

An alternative interpretation for non-linearity in Arrhenius plots has been advanced by Kumamoto et al. (28) which is based on the concept of a phase change. Kumamoto proposes that this situation can arise in enzymes. That is, at a single temperature two forms of an enzyme coexist, are in equilibrium with each other and are in separate forms. At any temperature away from the equilibrium temperature one form converts completely to the other form. The result, in terms of the Arrhenius plot, is that if the two forms differ in catalytic efficiency (i.e. the ΔG°'s are different but the two forms are still in equilibrium because the transition state for each form is in equilibrium with the same ground state), a discontinuity arises. The sharpness of the transition or break being dependent on the magnitude of the ΔH° (i.e. ΔH°1-ΔH°2). This can explain any break in Arrhenius plots. This is a novel idea indeed, however, considering the error associated with rate measurements as well as the difficulty of proper
temperature control, it would be very difficult to prove. A very large \( \Delta \Delta H^\# \) is required in order to see this type of discontinuity. A more reasonable explanation would be a sharp equilibrium transition (such as scheme V) which would require a large \( \Delta \Delta H^\# \), but would yield a smooth curve for the conversion from one activation enthalpy to another rather than a break or discontinuity.

Chymotrypsin is a particularly well studied enzyme (see for example reference 30), and demonstrates non-linear temperature dependencies in hydrolysis reactions with a number of different substrates; for example N-Benzoyl-D&L-Alanine methyl esters (10), N-Acetyl-L-Phenylalanine methyl ester and N-Acetyl-L-Leucine methyl ester (22). In addition, non-linear breaks are observed in transesterification reactions using 2-(5-n-propyl)-furoyl-Chymotrypsin and several different alcohols (23). The purpose of this study is to investigate the Arrhenius behavior for the hydrolysis of both 2-(5-n-propyl)-furoyl and 2-furoyl-Chymotrypsin esters. Earlier studies in this laboratory have indicated that both substrates show a non-linear temperature dependence in the hydrolysis reaction (23). That non-linearity was arbitrarily represented by two linear lines intersecting at a sharp break point. That study has been extended in this work by a more careful and detailed measurement of the rates as a function of temperature. Although qualitatively similar results have been obtained, an improved method of temperature measurement was used in this work. Also, the low temperature region has been extended. The combination of these two improvements reveal new features of the Arrhenius plot, giving evidence for a smooth transition curve. Due to the complications of the non-linear
temperature dependence, the earlier studies on the mechanism of rate enhancement specificity in Chymotrypsin were somewhat clouded as the actual Arrhenius curves were subject to many interpretations. This study provides somewhat stronger evidence for the earlier interpretation.

A second investigation (31) demonstrated non-linear Arrhenius curves in the alcoholysis reactions using \( p \)-nitro-phenol-2-(5-n-propyl)-furoic acid as substrate. Several alcohols were investigated, all showing similar temperature dependence. Since substitution of alcohol for water as the nucleophile in the reaction did not change the fact that a break in the Arrhenius plot occurs, the possibility of a temperature dependent shift in the rate determining step of the reaction could not be eliminated. In the reaction mechanism of equation 4,

\[
\begin{align*}
R'-\text{OPNP} + \text{ENZ} \rightleftharpoons R'\text{-OENZ} \rightarrow R'-\text{OR} + \text{ENZ}\end{align*}
\]

which represents the reaction of chymotrypsin in hydrolysis or alcoholysis, a temperature dependent shift in the rate determining step may occur at the point alcohol (or water) attacks the acyl enzyme. This is dependent upon the presence of an intermediate of kinetic significance between the acyl enzyme and product. If such a possibility exists, the \((16O/18O)\) kinetic isotope effect (KIE) in the ethanolysis (ethanol-\(18O\)) reaction could show a temperature dependent change. The reaction was studied (32) with the result that the KIE showed unusual behavior and magnitude. The second part of this work describes
a new technique developed for measuring this isotope effect and the application of the technique to studying the $^{16}_O^{18}_O$ KIE associated with the ethyl$^{18}_O$ ester formation of 2-furoic acid and 2-(5-n-propyl)-furoic acid catalyzed by Chymotrypsin.
Chapter Two

Temperature Studies of the Kinetics
of Deacylation of Chymotrypsin

2.1 Background

The mechanism of Chymotrypsin catalysis has been well characterized (30). The enzyme, a serine protease, catalyzes the hydrolysis of peptide bonds (when proteins are substrates), other amide bonds as well as ester linkages. The specificity pocket on the enzyme surface is selective toward large alkyl or aromatic groups. As outlined in equation 5, the reaction proceeds by two half reactions (33).

\[
\begin{align*}
\text{Folinic acid} + \text{EOH} &\rightarrow \text{Folinic acid ester} + \text{HO-NO}_2 \\
\text{Folinic acid} + \text{H}_2\text{O} &\rightarrow \text{Folinic acid ester} + \text{EOH} \\
\text{Folinic acid} + \text{ROH} &\rightarrow \text{Folinic acid ester} + \text{EOH}
\end{align*}
\]

where E-OH is the enzyme and its active site serine, and ROH is any one of several possible nucleophiles (amines or alcohols). With an alcohol present there is competing transesterification and the ratio of product ester to acid increases linearly with increasing alcohol concentration (31, 34). With esters, 5a is the much faster of the two half reactions. Thus by suitable arrangement of conditions \( k_{\text{H}_2\text{O}} \) can be determined directly as a pseudo-first order deacylation reaction (i.e. each active
enzyme producing only one product molecule). This single turnover reaction condition can be obtained by arranging the relative concentrations of reactants according to \([\text{H}_2\text{O}] > [\text{E-OH}] > [\text{substrate}] > K_M\). The experimental conditions for this process has been demonstrated earlier (23) for water.

In those studies (23) the temperature dependence for the deacylation reaction (5b) was studied using a series of 5 substituted n-akly-2-furoic acid derivatives as substrates. These substrates are convenient in that both acylation and deacylation can be monitored. That is acylation can be observed in the release of p-nitrophenol at 347 nm, and deacylation at 245 nm (the point of maximal difference in the spectrum of the free furoic acid and the acylenzyme). In addition, due to the aromatic nature of the furan ring, its binding to the enzyme should be relatively tight. As will be seen, the results of this temperature study qualitatively resemble those obtained earlier but since the temperature region has been extended, new features of the Arrhenius plot have been revealed.

2.2 Methods

2.2.1 Spectrophotometric rate measurements

The deacylation rate for 2-(5-n-propyl)-furoyl-Chymotrypsin and 2-furoyl-Chymotrypsin was measured as a pseudo-first order decay of the acylenzyme. Under the conditions of the assay, \([E_0]\) is greater than or equal to the \([S_0]\), while \([S_0]\) is greater than its \(K_M\). Under these circumstances, there is an initial rapid release of PNP as the enzyme becomes acylated. The acylenzyme then hydrolyzes in a
pseudo-first order process (as the water concentration remains constant during the reaction) releasing the product in its acid form.

### 2.2.1.1 Pseudo-first order kinematics

A stock substrate solution was prepared by dissolving 36 μmoles of p-nitrophenyl-2-(5-n-propyl) furoic acid or 72 μmoles of p-nitrophenyl -2-furoic acid in 10 ml of acetonitrile. A stock enzyme solution was prepared by dissolving 10 mg of alpha-Chymotrypsin (3X crystallized from Worthington) in 10 ml of .001M HCl and was stored at 4°C. The enzyme solution remains stable for one week. The assay procedure described was used for both substrates. To a three ml quartz cuvette (path length 1 cm) in a water thermostatted cell holder was added 2 ml of .1M K$_2$HPO$_4$ buffer (pH = 8.5). The temperature was constantly monitored by use of a thermistor probe immersed to ¼ inch in the top of the buffer. When thermal equilibrium had been reached (about 10 minutes), the stock enzyme solution was added to give a final enzyme concentration of 25-35 μmolar. The absorbance at 245 nm was then monitored until a stable baseline was observed on the recorder output.

The absorbance of the enzyme at 245 nm was approximately .3 O.D. units. Substrate was then added to a final concentration slightly lower than that of the enzyme (20-30 μM) and the absorbance at 347 nm due to the release of p-nitrophenylate was followed until no further increase was observed. The wavelength was then changed to 245 nm so that the first order appearance of the product acid could be recorded. The total absorbance change due to the acid was .03-.1 O.D. units. In all cases, the deacylation process was followed to greater than 3.5 half lives.
The data consisted of voltage readings (taken from the chart recorder) as a function of time. These data were then fit without weighting to the first order equation (6) by a non-linear least squares regression analysis using the subroutine CURFIT (35).

\[ A_t = A_\infty - \Delta A \exp(-kt) \]  

Where \( A_t \) is the voltage reading at any time \( t \) during the course of the reaction, \( A_\infty \) is the voltage at the completion of the reaction, and \( \Delta A \) is the quantity \( A_\infty - A_0 \) (\( A_0 \) is the initial voltage reading). The equation expresses the fact that a first order rate process does not depend on the absolute magnitude of \( A_t \) but only on its percent change with time (i.e. the half time of the reaction is constant throughout the course of product formation).

Thirty to forty pairs of voltage vs time readings were used in the fitting procedure. The three constants of the fit (\( A_\infty \), \( \Delta A \), and \( k \)) were allowed to vary in an unconstrained manner in the search for a best fit. The computer output consisted of the best fit values for \( A_\infty \), \( \Delta A \) and \( k \) plus their associated standard deviations and the residuals for each data set. In all cases, the calculated values of \( A_\infty \) and \( \Delta A \) corresponded closely to those observed on the recorder trace.

2.2.2 Calculations of Arrhenius models

As described in the Introduction, there are many possible models which can account for non-linearity in the temperature dependence of enzymatic catalysis. Some of the models can be eliminated due to their inability to adequately describe the experimental results. Thereby,
each model can be used as a theoretical description of the data. The
temperature dependence given by each model can be derived and tested
for its ability to fit the data.

2.2.2.1 Models with $C_p^\#$ as function of temperature

A). Assuming the temperature dependence of the $C_p^\#$ is given by

$$\Delta C_p^\# = a + bT$$

Where $a$ and $b$ are constants, and essentially the $C_p^\#$ has a linear depen-
dence with temperature. Since

$$\Delta H^\# = \Delta H_0^\# + \int_{T_0}^{T} \Delta C_p^\# \, dT$$

and therefore

$$\Delta H^\# = \Delta H_0^\# + \int_{T_0}^{T} (a + bT) \, dT$$

the temperature dependence of the enthalpy of activation is

$$\Delta H^\# = \Delta H_0^\# + a(T - T_0) + b/2(T^2 - T_0^2)$$

likewise

$$\Delta S^\# = \Delta S_0^\# + a \ln(T/T_0) + b(T - T_0)$$

Substituting into the Arrhenius equation gives:

$$k_{obs} = A \exp\left( \frac{-\Delta H_0^\# - a(T - T_0)}{RT} - \frac{b(T^2 - T_0^2)}{2RT} + \frac{\Delta S_0^\#}{R} + \frac{a}{R} \ln(T/T_0) + \frac{b}{R}(T - T_0) \right)$$

(7)
Where \( T_0 \) is an arbitrary reference temperature, in this case taken to be 310.15 K, \( \Delta H^\circ \) and \( \Delta S^\circ \) are the enthalpy and entropy of activation respectively at \( T_0 \) and \( R \) is the gas constant. \( A \) is the preexponential factor of equation 3. Equation 7 was then used to fit the temperature data for the observed rate. The constants \( \Delta H^\circ \), \( \Delta S^\circ \), \( a \) and \( b \) were allowed to vary in an unconstrained manner in the search for the best fit.

B. Assuming the temperature dependence of the \( C_p^\circ \) is given by

\[
\Delta C_p^\circ = a + bT + cT^2
\]

then

\[
\Delta H^\circ = \Delta H^\circ + a(T-T_0) + b(T^2-T_0^2)/2 + c(T^3-T_0^3)/3
\]

\[
\Delta S^\circ = \Delta S^\circ + a\ln(T/T_0) + b(T-T_0) + c(T^2-T_0^2)/2
\]

and

\[
k_{obs} = A \exp \left( \frac{-\Delta H^\circ - a(T-T_0)}{RT} - \frac{b(T^2-T_0^2)}{2RT} - \frac{c(T^3-T_0^3)}{3RT} + \frac{\Delta S^\circ}{R} \right) + \frac{a\ln(T/T_0)}{R} + \frac{b(T-T_0)}{R} + \frac{c(T^2-T_0^2)}{2R}
\]

The equation 8 with the adjustable parameters \( \Delta H^\circ \), \( \Delta S^\circ \), \( a \), \( b \) and \( c \) was then assumed to fit the data.

C. Assuming the temperature dependence of the \( C_p^\circ \) is given by

\[
\Delta C_p^\circ = a + bT + cT^{-2}
\]

The equation for the temperature dependence is given by
\[ k_{\text{obs}} = A \exp \left( \frac{-\Delta H^0}{RT} - \frac{a(T-T_o)}{RT} + \frac{b(T^2-T_o^2)}{2RT} + \frac{c(1/T-1/T_o)}{RT} + \frac{\Delta S^0}{R} \right) \]

\[ + \frac{a}{R} \ln T/T_o + \frac{b}{R} (T-T_o) - \frac{2c}{R} (1/T^2-1/T_o^2) \]  \quad (9)

Equation 9 with the adjustable parameters \( \Delta H^0, \Delta S^0, a, b \) and \( c \) was then used to fit the data.

2.2.2.2 Models with two first order processes

A). Assume a single acyl enzyme species which can decay by two parallel first order processes which differ in the rate of hydrolysis, and the temperature dependence of this rate,

\[ \begin{align*}
& \xrightarrow{k_1} E + P \\
& \xrightarrow{k_2} EA \\
\end{align*} \]

The expression for the rate of the appearance of \( P \) is

\[ k_{\text{obs}} = (k_1 + k_2) \]

and its temperature dependence is given by

\[ k_{\text{obs}} = A \exp \left( \frac{-\Delta H^1_{1}-\Delta H^2_{2}}{RT} + \frac{\Delta S^1_{1}+\Delta S^2_{2}}{R} \right) \]  \quad (10)

Where \( \Delta H^1_{1} \) and \( \Delta S^1_{1} \) are the activation parameters for the first rate and \( \Delta H^2_{2} \) and \( \Delta S^2_{2} \) are the activation parameters for the second. Equation 10 was then used to fit the data.

B). Assume two different acyl enzyme species at a fixed concentration ratio over the entire temperature range. Each species turns over with a different rate and a different temperature dependence.
The temperature dependence of the observed rate is then

$$k_{obs} = A \exp \left( \frac{-\Delta H_1^f}{RT} + \frac{\Delta S_1^f}{R} \right) + A(1-f)\exp \left( \frac{-\Delta H_2^f}{RT} + \frac{\Delta S_2^f}{R} \right)$$  

(11)

Where $\Delta H_1^f$, $\Delta S_1^f$, $\Delta H_2^f$, $\Delta S_2^f$, and $f$ are the constants which are varied in the search for the best fit.

2.2.2.3 Models involving equilibria

A). Assume two different acyl enzyme species in equilibrium, with the equilibrium rapid relative to the rate of an irreversible step. One form is inactive to turnover. This model can be represented as:

$$\begin{align*}
    & \text{EA} \xrightarrow{k_1} \text{E} + P \\
    & \text{EA'} \xrightarrow{k_2} \text{E'} + P \\
    \end{align*}$$

$$[\text{EA}] + [\text{EA}] = [\text{EA}]_{\text{tot}}$$

$$[\text{EA}] / [\text{EA}]_{\text{tot}} = f$$

$$k_{obs} = (fk_1 + (1-f)k_2)$$

The temperature dependence is given by:
Where $\Delta H^\#$ and $\Delta S^\#$ are the activation parameters associated with the rate $k$ and $\Delta H$ and $\Delta S$ are thermodynamic parameters associated with $K$. These four parameters are varied to obtain the best fit.

B). Assume a mechanism in which two species are in sequential equilibrium with the acyl enzyme. The second species then turns over in a first order process yielding product. This can be represented as:

$$k_{\text{obs}} = \frac{k K_1 K_2}{(1 + K_1 + K_1 K_2)}$$

The temperature dependence of $k_{\text{obs}}$ is given by:

$$k_{\text{obs}} = \frac{A \exp\left(\frac{-\Delta H^\#}{R} + \frac{\Delta S^\#}{R}\right) \exp\left(\frac{-\Delta H_1}{R} + \frac{\Delta S_1}{R}\right) \exp\left(\frac{-\Delta H_2}{R} + \frac{\Delta S_2}{R}\right)}{1 + \exp\left(\frac{-\Delta H_1}{R} + \frac{\Delta S_1}{R}\right) + \exp\left(\frac{-\Delta H_1}{R} - \frac{\Delta H_2}{R} + \frac{\Delta S_1 + \Delta S_2}{R}\right)}$$

Where $\Delta H^\#$ and $\Delta S^\#$ are the activation parameters associated with $k$ and $\Delta H_1$, $\Delta S_1$, and $\Delta H_2$, $\Delta S_2$ are the thermodynamic parameters associated with $K_1$ and $K_2$ respectively. All six parameters were varied in the fitting process.

C). Assume a rapid equilibrium between two acyl enzyme species, both of which turn over, but at different rates.
\[ k_{\text{obs}} = \frac{(k_1 + k_2K)(1+K)}{k_1 + k_2} \]

and the temperature dependence is given by:

\[ k_{\text{obs}} = \frac{A(\exp\left(\frac{-\Delta H_1^d}{RT} + \frac{\Delta S_1^d}{R}\right) + \exp\left(\frac{-\Delta H_2^d}{RT} + \frac{\Delta S_2^d}{R}\right) \exp\left(\frac{-\Delta H}{RT} + \frac{\Delta S}{R}\right)}{1 + \exp\left(\frac{-\Delta H}{RT} + \frac{\Delta S}{R}\right)} \]

(14)

Where \( \Delta H_1^d, \Delta S_1^d \) and \( \Delta H_2^d, \Delta S_2^d \) are the activation parameters for the two rates \( k_1 \) and \( k_2 \) respectively and \( \Delta H, \Delta S \) are the thermodynamic parameters associated with \( K \). All six parameters were varied in the fitting process.

### 2.2.2.4 Fitting process

The equations for the temperature dependence described above were used in the fitting routine GRIDLS (35). The data used was the \( k_{\text{obs}} \) vs T values obtained from the pseudo-first order hydrolysis of 2-(5-n-propyl)-furoyl-Chymotrypsin and 2-furoyl-Chymotrypsin. For each model, several initial estimates for each of the fitting parameters were used. In all cases, the fitting routine converged to give the same best fit values for each fit parameter or at least the same value of the \( x^2 \). The output from the computer program consisted of the best values along with their associated standard deviations, the residuals for each data pair and the chi square value (i.e. the sum of the residuals squared divided by the number of data sets less the number of fitting parameters).
2.3 Materials

2.3.1 Synthesis of the p-nitrophenyl esters of 2-(5-n-propyl)-
furoic acid and 2-furoic acid

The synthesis is that of Baggot (23). Forty millimoles of POCl$_3$ were mixed with forty millimoles of dimethyl formamide for two hours at 0°C. To the deep red solution was added forty millimoles of 5-n-propyl-furan (K & K Labs) over a period of two hours. The solution was allowed to react for two hours then to warm to room temperature. It was then poured over 100 grams of ice and neutralized with Na$_2$CO$_3$. The solution was extracted (3X, 50 ml each) with diethyl ether. The ethereal extract was dried over Na$_2$SO$_4$ and evaporated at reduced pressure yielding a brown oil (the 2-(5-n-propyl)-fural). The aldehyde was oxidized for four days with 120 millimoles of Ag$_2$O in 5% (w/v) NaOH. The solution was filtered and reduced to one half its original volume. The acid precipitated as the solution was acidified with concentrated HCl. The acid was recrystallized from hot ligroine (M.P. 62-64°C, Literature 63-63.5). The yield for both steps was 60%.

Eight millimoles of the acid and nine millimoles of p-nitrophenol (recrystallized from ethanol) were dissolved in 50 ml of anhydrous diethyl ether. Sixteen millimoles of dicyclohexyl carbodiimide were added and the reaction was stirred overnight, filtered and then washed with .05M phosphate buffer. After drying over Na$_2$SO$_4$, the solution was evaporated to dryness and the solid recrystallized (2X) from absolute ethanol (M.P. 65-67°C, Literature 65-67). The yield was 60%.

The 2-furoic acid (Kodak) was recrystallized from toluene. The p-nitrophenol ester was prepared as above and recrystallized (3X) from ethanol (M.P. 162-164, Literature 163.5).
2.3.2 Reagents

Chymotrypsin (lot CDI2LX) three times recrystallized was purchased from Worthington Biochemicals (Freehold, N.J.) and used without further purification. It was dissolved to 10 mg/ml in .001 M HCl. The concentration of the stock was determined using a molar extinction coefficient of $5 \times 10^4$ at 280 nm. The following chemicals were of reagent grade and used without purification; potassium phosphate (mono and dibasic), hydrochloric acid, nitric acid, anhydrous diethyl ether, dimethyl formamide, phosphorous oxychloride, sodium sulfate (anhydrous), magnesium sulfate (anhydrous), ethanol, sodium hydroxide, acetonitrile, sodium carbonate, silver oxide, petroleum ether (B.P. 35-60°C), and methanol. Double distilled deionized water was used throughout.

The buffer used in the kinetic studies was dipotassium hydrogen phosphate (.1 M) in double distilled deionized water. The pH was adjusted to 8.5 with HCl. The pH standard was Fisher brand, pH 7.00 at 25°C.

The p-nitrophenol (Aldrich) was recrystallized from ethanol. 2-Furoic acid (Eastman Kodak) was recrystallized from toluene. 2-n-Propyl furan was purchased from K & K Labs, Inc. Dicyclohexyl carbodiimide was purchased from Eastman Kodak.

2.4 Instruments and Equipment

2.4.1 Spectrophotometer

The kinetic studies were carried out on a Cary 16 UV-VIS spectrophotometer equipped with a Cary model 1626 recorder interface and a NON-LINEAR SYSTEMS digital voltmeter. A linear systems chart recorder
was used. The pen response on the recorder was checked against the voltmeter readings and shown to agree within .5% over the full range of the recorder output. The voltage readings for the kinetic studies could thereby be taken from the voltmeter or the recorder.

The reaction was carried out in a rectangular quartz cell of path length 1.0 cm. The cell holder was of aluminum and water jacketed with water from a Forma constant temperature bath. The flow rate through the cell holder exceeded 200 ml/min.

2.4.2 Thermistor
A Yellow Springs Instruments model 46TUC Telethermometer equipped with a teflon coated model YSI 421 thermistor was used to constantly monitor the temperature in the cell during the reaction. The minimum immersion depth for the thermistor was 1/8 inch and was introduced into the cell through a hole in the cell cap. Temperature readings could easily be made with a .1°C precision. The thermistor was calibrated against a Cenco thermometer over the temperature range 4 to 45°C and found to agree within the limits of the measurement for the thermometer. In all cases, readings for the kinetic runs were taken only after the thermistor indicated the cell temperature was constant.

2.4.3 Miscellaneous Equipment
Melting points were determined on a Thomas Hoover Capillary Melting Point Apparatus and the values uncorrected. The pH was measured with a Heath/Schlumberger model EU-200-50 pH/pION electrometer attached to a digital voltmeter (Digitech). The electrode was a model 4094-L 15
Thomasbrand. Data analysis was performed on either a Nova 1220 Data General minicomputer or an Amdahl 470 computer.

2.5 Results

2.5.1 Validity of the rate constants

The enzyme catalyzed hydrolysis has been shown to be insensitive to small amounts of organic solvents (such as acetonitrile and dimethyl formamide) (36). Investigation into the dependence of the rate on protein concentration indicated no change in rate at 22°C over a ten fold change in enzyme concentration (6-60 μM). In addition, there was no change in the rate in varying the substrate concentration over a ten fold range (6-60 μM), see Table 1. The pH dependence of the rate was shown to have a $pK_a$ of about 7 (23) for both substrates. The use of pH 8.5 then ensures that the observed rates were within 5% of the maximal or pH independent values. The rate also did not vary when the phosphate concentration was changed from .2 to .1 M. Using a simple calculation, it can be shown that the amount of acid formed in the pseudo-first order deacylation reaction was too small to cause a pH change in the reaction greater than .005 pH units. Figure 1 shows the first order curve obtained in the hydrolysis of 2-furoyl-Chymotrypsin.

The conditions for pseudo-order hydrolysis of the acyl enzyme (i.e. $[H_2O] \gg [E_0] \geq [S_0] \gg K_M$) were ensured by measuring the extent of the acylation reaction prior to monitoring the deacylation. This was accomplished by monitoring the burst release of PNP at 347 nm. Only after the O.D. at 347 nm was constant was the spectrophotometer
Table 1

The dependence of 2-(5-n-propyl)-furoxyl-chymotrypsin deacylation on enzyme concentration and substrate concentration

<table>
<thead>
<tr>
<th>Enzyme µM</th>
<th>Substrate µM</th>
<th>$k_{H2O} \text{(min}^{-1})^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>6</td>
<td>0.0436(0.0009)</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>0.0411(0.0011)</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>0.0453(0.0015)</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>0.0465(0.0012)</td>
</tr>
<tr>
<td>60</td>
<td>30</td>
<td>0.0407(0.0009)</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
<td>0.0477(0.0010)</td>
</tr>
<tr>
<td>AVG.</td>
<td></td>
<td>0.0443(0.0029)$^c$</td>
</tr>
</tbody>
</table>

$^a$ All reactions were run in 0.1 M $K_2HPO_4$ buffer pH 8.5 at 22°C.

$^b$ Rate constants determined from non-linear regression analysis.

$^c$ Values in parenthesis are the estimated standard deviations.

$^c$ The average is weighted on the basis of the estimated standard deviations.
Figure 1
Representative first order hydrolysis of 2-furoyl-chymotrypsin at 15°C. The absorbance at 245 nm due to 2-furoic acid is displayed as a function of time as observed on the recorder output.
Absorbance at 245 nm vs. time.

Absorbance values:

- 0.02 at 0.04 units
- 0.04 at 0.06 units
- 0.06 at 0.08 units

Time scale: 3 min
wavelength set to monitor the formation of the acid product. It can also be shown that the amount of acid product formed (ΔOD. 245 nm) was the same as the amount of PNP released (by the ΔOD. 347 nm). Under these conditions then, only the acyl enzyme decay will be observed. The constancy of rate with a ten fold variation of substrate and enzyme concentration indicates that the reaction is indeed first order with respect to the acyl enzyme concentration. In addition, no variation in rate was observed when excess PNP or furoic acid was added to the reaction cell (23).

The observed rates were converted to pH independent rates by using the formula:

\[
k = \frac{K_a k'}{K_a + [H^+]} \quad (15)
\]

Where \( k \) is the observed rate, \( K_a \) is the acid dissociation constant controlling the deacylation reaction (23), \([H^+]\) is the hydrogen ion concentration and \( k' \) is the pH independent rate. At every temperature, the data determining the observed deacylation rate was also fit to an equation for two first order processes. The best fit to the data was consistently obtained using an equation which assumed a single first order increase in the absorbance at 245 nm.

2.5.2 Calculation of Activation Parameters

The pseudo-first order hydrolysis of the acyl enzyme of furoic acid and 2-(5-n-propyl)-furoic acid increased with temperature. The curve, however, showed a distinct non-linearity (Figure 2). The low temperature data (below 10°C) shows a concave down curvature while the
**Figure 2**

The temperature dependence of acyl-chymotrypsin hydrolysis. The rate constant $k$ is in units of $\text{min}^{-1}$ and the temperature is in units of $^\circ\text{K}$. The circles are for the furoyl-chymotrypsin and the squares are for the 2-(5-n-propyl)-furoyl-chymotrypsin.
higher temperature regime displays upward curvature for both substrates. The furoic acid substrate shows a much shallower curvature. The data was analyzed by fitting the curves to models known to display non-linear temperature dependencies in plots of $\ln(k/T)$ vs $1/T$.

The models tested (as outlined in the methods section) are summarized in Table 2. Each model can be considered as a subset of the possibilities outlined in the introduction. Model I shows a concave down curve which upon computer fitting was unable to fit the high temperature regime. Model II gives a concave up curve. Models III, V, Va and VII all show a sigmoidal type of transition. Model IV is concave down as is Model VI. Model VII has the lowest chi squared value and the least amount of systematic variation in the residuals plot. These two criteria for "goodness of fit" (i.e. minimum chi square and lack of systematic variation in a plot of the residuals) were used to evaluate each model. With these two considerations, Model VII was deemed as fitting the data best.

The activation and thermodynamic enthalpies and entropies obtained in the best fit of the data by Model VII (the rapid equilibrium model) are shown in Table 4. As can be seen, the entropy has a major contribution to the equilibrium transition. Also, the activation enthalpies for the high and low temperature forms of the enzyme are equal within one standard deviation, with the entropy of activation contributing to the increased activity of the low temperature form.

The data for the hydrolysis of the furoic acid-Chymotrypsin was not suitable for the fitting procedures to determine the equilibrium parameters. The uncertainties in the activation parameters did not
Table 2
Models tested as explanations for the Cht-PF results of Figure 1

<table>
<thead>
<tr>
<th>MODEL</th>
<th>APPARENT RATE CONSTANT</th>
<th>ADJUSTABLE PARAMETERS</th>
<th>$\chi^2$ a</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. $\text{EA} \xrightarrow{k} \text{EA} \xrightarrow{k} \text{E} + P$</td>
<td>$k_{\text{app}} = kK/(1 + K)$</td>
<td>$\Delta H^\ddagger, \Delta S^\ddagger, \Delta H, \Delta S$</td>
<td>496.7</td>
</tr>
<tr>
<td>II. $\text{EA} \xrightarrow{k_1} \text{E} + P$</td>
<td>$k_{\text{app}} = k_1 + k_2$</td>
<td>$\Delta H^\ddagger, \Delta S^\ddagger, \Delta H_2^\ddagger, \Delta S_2^\ddagger$</td>
<td>1232.0</td>
</tr>
<tr>
<td>III. $\text{E} + A: \Delta G_p^\ddagger = a + bT$</td>
<td>$k_{\text{app}} = k_1 + k_2$</td>
<td>$\Delta H^0, \Delta S^0, a, b$</td>
<td>59.2</td>
</tr>
<tr>
<td>IV. $\text{EA} \xrightarrow{k_1} \text{E} + P$</td>
<td>$k_{\text{app}} = ak_1 + (1 - a)k_2$</td>
<td>$\Delta H^\ddagger, \Delta S^\ddagger, \Delta H_2^\ddagger, \Delta S_2^\ddagger, a$</td>
<td>190.9</td>
</tr>
<tr>
<td>V. $\text{EA} \xrightarrow{k_1} \text{E} + P$</td>
<td>$k_{\text{app}} = ak_1 + (1 - a)k_2$</td>
<td>$\Delta H^0, \Delta S^0, a, b, c$</td>
<td>62.2</td>
</tr>
<tr>
<td>VI. $\text{E} + A: \Delta G_p^\ddagger = a + bT + cT^2$</td>
<td>$k_{\text{app}} = kK/(1 + K + K)$</td>
<td>$\Delta H^\ddagger, \Delta S^\ddagger, \Delta H_2^\ddagger, \Delta S_2^\ddagger, \Delta H^\ddagger, \Delta S^\ddagger$</td>
<td>410.1</td>
</tr>
<tr>
<td>VII. $\text{E} + A: \Delta G_p^\ddagger = a + bT + cT^2$</td>
<td>$k_{\text{app}} = kK/(1 + K)$</td>
<td>$\Delta H, \Delta S, \Delta H_1^\ddagger, \Delta S_1^\ddagger, \Delta H_2^\ddagger, \Delta S_2^\ddagger$</td>
<td>23.2</td>
</tr>
</tbody>
</table>

*a $\chi^2$ is the sum of the residuals squared divided by the number of degrees of freedom, and was obtained from the nonlinear least squares fit of each model to the Cht-PF data of Figure 1.
Table 3
Comparison of the temperature dependence of the rate of hydrolysis of 2-(5-n-propyl)-furoyl-chymotrypsin

<table>
<thead>
<tr>
<th>Temp °C</th>
<th>Method I</th>
<th>Method II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{obs} \times 10^3$ min$^{-1}$</td>
<td>Temp °C</td>
</tr>
<tr>
<td>10.0</td>
<td>17.7 (.27)$^c$</td>
<td>10.5</td>
</tr>
<tr>
<td>12.9</td>
<td>23.7 (.48)</td>
<td>11.9</td>
</tr>
<tr>
<td>15.2</td>
<td>28.4 (.21)</td>
<td>15.5</td>
</tr>
<tr>
<td>18.1</td>
<td>33.1 (.35)</td>
<td>17.8</td>
</tr>
<tr>
<td>19.9</td>
<td>44.0 (1.6)</td>
<td>20.3</td>
</tr>
<tr>
<td>25.0</td>
<td>59.2 (1.7)</td>
<td>25.1</td>
</tr>
<tr>
<td>30.7</td>
<td>98.1 (5.3)</td>
<td>29.2</td>
</tr>
<tr>
<td>34.3</td>
<td>141.0 (1.4)</td>
<td>34.1</td>
</tr>
<tr>
<td>35.8</td>
<td>169.0 (5.9)</td>
<td>35.8</td>
</tr>
<tr>
<td>38.0</td>
<td>207.0 (1.9)</td>
<td>38.1</td>
</tr>
<tr>
<td>40.8</td>
<td>288.0 (1.0)</td>
<td>41.3</td>
</tr>
<tr>
<td>43.4</td>
<td>338.0 (6.0)</td>
<td>42.1</td>
</tr>
</tbody>
</table>

$^a$ Data from reference 23. .2 M phosphate buffer pH 8.5.

$^b$ This work. .1 M phosphate buffer pH 8.5.

$^c$ Numbers in parenthesis represent the estimated standard deviations.
### Table 4

Activation and thermodynamic parameters for the hydrolysis reactions assuming two enzyme forms in rapid temperature dependent equilibrium<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>CHT-PF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CHT-F&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔH</td>
<td>33.6(0.44)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>d</td>
</tr>
<tr>
<td>ΔS</td>
<td>118.0(1.50)</td>
<td></td>
</tr>
<tr>
<td>ΔH&lt;sub&gt;1&lt;/sub&gt;</td>
<td>29.3(1.40)</td>
<td>19.0(0.13)</td>
</tr>
<tr>
<td>ΔS&lt;sub&gt;1&lt;/sub&gt;</td>
<td>-29.6(1.40)</td>
<td>-7.8(0.14)</td>
</tr>
<tr>
<td>ΔH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>28.1(0.21)</td>
<td>19.4(0.23)</td>
</tr>
<tr>
<td>ΔS&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-20.8(0.66)</td>
<td>-5.1(0.66)</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Table 2. Enthalpies are given in units of Kcals mol<sup>-1</sup>, entropies in units of cal mol<sup>-1</sup> deg<sup>-1</sup>

<sup>b</sup> The substrate for the hydrolysis was 2-(5-n-propyl)-furoyl-chymotrypsin.

<sup>c</sup> The substrate for the hydrolysis was 2-furoyl-chymotrypsin.

<sup>d</sup> The results were fit assuming the ΔH and ΔS given under CHT-PF and only allowing the four activation parameters to vary.

<sup>e</sup> Numbers in parenthesis represent the estimated standard deviations.
allow sufficient discrimination between models because the curve was too shallow. The rapid equilibrium model was used to fit the data for the furoic acid substrate based on the equilibrium constant obtained during the fit of the 2-(5-n-propyl)-furoyl-Chymotrypsin data.

2.6 Discussion

2.6.1 Comparison with earlier results

The initial investigation of the temperature dependence of the deacylation of 5 substituted furoyl chymotrypsins indicated non-linear behavior with the points of discontinuity occurring at 20-30°C for the furoyl, 5-ethyl furoyl and 5-n-propyl furoyl substrates (23). This work represents the temperature dependence for the two substrates 2-furoyl and 2-(5-n-propyl)-furoyl chymotrypsins. In examining the correspondence between the individual rates in these two studies, it is seen that near room temperature the actual pH independent rate constants agree quite closely (Table 3). However, in the low temperature region the earlier results tend to be higher than those obtained in this study, and in the high temperature region the earlier rates tend to be lower. The result is that the curve for this data is skewed relative to the earlier data. The reason for this difference may lie in the way the temperatures were measured in these two sets of data. In this work, the temperature was monitored by a thermistor probe inserted into the reaction cuvette. This allowed constant temperature monitoring throughout the reaction. In addition, it allowed the temperature to be measured when the lid to the cell compartment was closed. In the earlier work, temperature was monitored by using a thermometer.
inserted in the cuvette (with the cell compartment lid open) before and after the reaction. It was observed that a temperature difference in the cell occurred when the lid to the cell compartment was open relative to when it was closed. The farther from room temperature the reaction temperature was, the greater the error in the temperature readings. This fact can explain the difference observed between the two sets of rate data.

2.6.2 The temperature dependence of the hydrolysis

The data of Tables 2 and 3 suggest that the simplest explanation for the non-linearity in the Arrhenius plot is the assumption of a temperature dependent equilibrium between two acyl enzyme forms which hydrolyze with different reactivities and at rates slower than their rate of interconversion.

The nature of the two enzyme species can not be deduced from the kinetic results presented here alone. However, a protein dimerization (or higher polymerization) can be excluded from consideration, as seen in Table 1. Chymotrypsin is known to undergo various transitions under different experimental conditions. Of these the alkaline transition is the best characterized. This transition is presumed due to the ionization of isoleucine-16 (the N terminal peptide) alpha amino group. The loss of a proton from this amine removes it from a salt bridge (X-ray data (37)) with aspartate 194. The salt bridge is required for a proper conformation of the active site region of chymotrypsin. In the free enzyme, the $pK_a$ of this salt bridge in the alkaline region appears between 8.5 and 9.0. This pH is near to that used in
Figure 3

(A) Fraction of 2-(5-n-propyl)-furoyl-chymotrypsin in the high temperature form. (B) Fraction of the product acid which proceeds through the high temperature form of the acylated enzyme. Curve A is calculated from the data in Table 4. Curve B is the temperature dependence of the fraction of the product which is formed via the high temperature form and is calculated from \( f k_1 / (f k_1 + (1-f)k_2) \), where \( f \) is the fraction of the high temperature form and \( k_1 \) and \( k_2 \) are the high and low temperature rate constants, respectively.
this study. However, it has been shown (38, 39) that the form at high pH is inactive. Also there is considerable evidence that binding to the active site raises the pK_a of this transition to greater than 10 (40, 41). This transition can not be responsible for the equilibrium postulated here, as both species in the equilibrium transition have activity associated with them.

Wedler et al. (22) have demonstrated transitions in both Arrhenius plots and van't Hoff plots in the hydrolysis of N-acetyl-L-leucine methyl ester, N-acetyl-L-phenyl-alanine methyl ester and N-benzoyl-L-alanine methyl ester at pH 7.8 and 9.6. They have, on the basis of both concave up and concave down Arrhenius and van't Hoff plots interpreted their data as reflecting an equilibrium between two reacting species.

If the scheme of model VII (Table 2) is assumed, by using the best fit equilibrium and activation parameters, the fraction of 2-(5-n-propyl)-furoyl-chymotrypsin in the low and high temperature form can be calculated. In addition, the fraction of the total product released by either form can be computed. The results (Figure 3) are plotted as a function of temperature. It can be seen that the curve for the fraction of the total acyl enzyme which is in the high temperature form (HTF) lies at lower temperatures than the curve representing the fraction of the total product produced by the high temperature form. That is, when the ratio of the fraction in the high temperature form to the fraction in the low temperature form is equal to one, only about 10% of the total product formed comes from the HTF of the acyl enzyme. This indicates that the low temperature form has a greater
intrinsic reactivity. This is reflected in the activation parameters. That is, the greater reactivity of the low temperature form is reflected in its larger entropy of activation (the activation enthalpies of both forms being nearly equal). This may indicate a greater ordering of the transition state in the low temperature form relative to the high temperature form, and may be a consequence of "freezing out" a more conformationally rigid active site at low temperatures. This rigidity may improve specific geometric interactions between the substrate and the active site. Bender (42) has proposed this type of entropic control for the specificity of the hydrolysis reaction catalyzed by chymotrypsin.
Chapter Three

Isotope Effects in Chymotrypsin Catalyzed Transesterification

3.1 Background

3.1.1 Basis of the kinetic isotope effect

The foundations of kinetic isotope effects (KIE) were first outlined by Bigeleisen and Goeppert-Mayer (43) and Bigeleisen (44). Several reviews of specific types and applications of KIE's have appeared. This includes discussions on secondary deuterium isotope effects (45-48), calculations of isotope effects (50, 51), KIE's in solvolysis reactions (52), the use of KIE's in relation to organic reaction mechanisms (53) and the treatment of equilibrium isotope effects (54).

The theoretical basis of the KIE is historically developed within the framework of the absolute rate theory (3). This theory, developed by Eyring, assumes an activated complex or transition state through which the reaction takes place. The activated complex is a metastable "compound" in thermal equilibrium with the reactants. The reaction rate then is the number of activated complexes passing to product per unit time. The equilibrium assumption allows a statistical mechanical calculation of the equilibrium concentration of activated complexes. However, the structure of the activated complex must be known in order for an exact calculation to be made. This requires a detailed knowledge of the potential energy surface for the reaction. Except for the most simple systems, this knowledge of the potential energy surface is
completely lacking. Consequently, certain empirical methods are used to approximate the surface.

The kinetic isotope effect is the ratio of the rates for conversion of reactants to products when the reactant is isotopically substituted with a heavier isotope:

$$\text{KIE} = \frac{k}{k^*}$$

(16)

Where the $k$'s are the rate constants associated with the chemical conversion and the asterisk refers to the isotopically labeled reactant rate. The absolute rate theory provides a framework for the statistical mechanical calculation of the individual rates. According to Bigeleisen (44), the KIE can be divided into three parts:

$$\text{KIE} = \text{MMI} \times \text{EXC} \times \text{ZPE} \times \frac{K \times S \times S^*}{K^* \times S^* \times S^*}$$

(17)

Where MMI refers to the term "mass, momentum of inertia," EXC refers to the term "excitation factor" and ZPE refers to the term "zero point energy." The mathematical expressions for each term are:

$$\text{MMI} = \left[ \left( \frac{M^*}{M} \right) \left( \frac{M^{**}}{M} \right) \right]^{3/2}$$

$$\text{EXC} = \frac{3N-6}{\pi} \int \frac{(1 - e^{-ui})}{(i - e^{-u^*i})} \frac{(1 - e^{-u^*i})}{(i - e^{-ui})}$$

(19)
The M's are the molecular weights, the I's are the moments of inertia for each of the three principal axis, the u's are the vibrational frequencies (\(\frac{\hbar v_i}{kT}\) where \(\hbar\) is Planck's constant, \(k\) is Boltzman's constant, \(v_i\) is the frequency and \(T\) is the temperature), and \(N\) or \(N^\sharp\) is the number of atoms. The double daggers are reserved for the transition state "molecule," It is assumed that there is no isotope effect on the transmission coefficient (K eq 17) or the symmetry number (S).

The MMI factor is generally near unity, as the ratio of the M's are near 1 for reasonably large molecules and the ratio of the I's are near one because they generally depend on the molecular weights. The EXC (represented only as harmonic motion) can contribute to the overall KIE at higher temperatures. However, the isotope effect is generally dominated by the ZPE term, and this fact is the basis for the qualitative interpretations of KIE's. This is illustrated in Figure 4, where the energy vs. reaction coordinate is plotted. The magnitude of the KIE is related to the change in the shape of the potential energy well for the reactants as they proceed through the transition state. When there is a difference in the \(\Delta\)'s (zero point energy vibrations, see Figure 4) between the transition state and the reactants, a KIE will be observed. Two possibilities exist, either the potential energy well for the transition state is broader than the reactant's potential energy well or it is more narrow. Beigleisen has calculated

\[
ZPE = \frac{\exp \sum_i \left( u_i - u_i^\sharp \right)/2}{\exp \sum_i \left( u_i^\sharp - u_i^\sharp \right)/2}
\]

(20)
Figure 4
Energy vs. Reaction Coordinate Diagram. (A) Illustrates the fact that when the potential energy well for the transition state (TS) is broader than that of the ground state (GS) a normal KIE will result due to $\Delta GS > \Delta TS$. (B) When the transition state has a more narrow potential energy well relative to the ground state an inverse KIE will result. $\Delta GS < \Delta TS$. 
the maximum KIE expected for a variety of isotopic substitutions (see Table 5). The maximum KIE values are calculated assuming the transition state has zero bonding of the isotopically labeled atom and the frequency of the "vibration" is large. This means that the stretching vibration along the reaction coordinate is converted completely to translational motion in the transition state. These are primary isotope effects, i.e. those where bond breaking is taking place at the isotopic atom. This can be compared to the KIE's where no bond breaking occurs at the isotopic atom. These are secondary isotope effects and are generally smaller. The calculated maximal values for these secondary isotope effects are presented in Table 6. In Table 5, the trend is obvious. The heavier the isotope pair, the smaller the calculated maximal KIE. The maximum values for the heavier isotopes are relatively small (on the order of 1.15). Measurement of this small a difference in rate is very difficult, consequently not many KIE's have been investigated for C, O, N, or S (the common elements of organic and biochemistry). This work deals with the measurement of an $^{16}\text{O}/^{18}\text{O}$ KIE and therefore in the next section a detailed list of this type of KIE is presented.

3.1.2 Experimental oxygen isotope effects

Kinetic isotope effects can be used in the investigation of reaction mechanisms, especially when isotopic substitution can be made at the point of bond scission. As mentioned in the last section, the heavy isotopes (i.e. C, O, N, and S) generally show very small differences in rates, on the order of 1.02 to 1.08 experimentally. The
Table 5
Estimated maximum ratios in specific rate constants at 25°C^a

<table>
<thead>
<tr>
<th>Isotope pair</th>
<th>KIE^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1\text{H}/^2\text{H}$</td>
<td>18.0</td>
</tr>
<tr>
<td>$^1\text{H}/^3\text{H}$</td>
<td>20.0</td>
</tr>
<tr>
<td>$^6\text{Li}/^7\text{Li}$</td>
<td>1.1</td>
</tr>
<tr>
<td>$^{10}\text{B}/^{11}\text{B}$</td>
<td>1.3</td>
</tr>
<tr>
<td>$^{12}\text{C}/^{13}\text{C}$</td>
<td>1.25</td>
</tr>
<tr>
<td>$^{12}\text{C}/^{14}\text{C}$</td>
<td>1.50</td>
</tr>
<tr>
<td>$^{14}\text{N}/^{15}\text{N}$</td>
<td>1.14</td>
</tr>
<tr>
<td>$^{16}\text{O}/^{18}\text{O}$</td>
<td>1.19</td>
</tr>
<tr>
<td>$^{31}\text{P}/^{32}\text{P}$</td>
<td>1.02</td>
</tr>
<tr>
<td>$^{32}\text{S}/^{35}\text{S}$</td>
<td>1.05</td>
</tr>
</tbody>
</table>

^a From reference 55.

^b Calculated assuming no isotope bonding in the transition state.
Table 6
Estimated maximum ratios of rate constants at 25°C for a secondary isotope effect.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Bonds</th>
<th>Normal\textsuperscript{b}</th>
<th>Inverse\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{CH}}/k_{\text{CD}}$</td>
<td>1.74</td>
<td>0.46</td>
</tr>
<tr>
<td>$k_{\text{CH}'}/k_{\text{CT}}$</td>
<td>2.20</td>
<td>0.33</td>
</tr>
<tr>
<td>$k_{\text{OH}}/k_{\text{OD}}$</td>
<td>2.02</td>
<td>0.37</td>
</tr>
<tr>
<td>$k_{\text{OH}}/k_{\text{OT}}$</td>
<td>2.74</td>
<td>0.24</td>
</tr>
<tr>
<td>$k_{12\text{C}}/k_{13\text{C}}$</td>
<td>1.012</td>
<td>0.983</td>
</tr>
<tr>
<td>$k_{12\text{C}}/k_{14\text{C}}$</td>
<td>1.023</td>
<td>0.968</td>
</tr>
</tbody>
</table>

\textsuperscript{a} From reference 56.

\textsuperscript{b} The lighter isotope molecule reacts faster. Assumes stretching force constant decreases by a factor of 2 in going from reactant to transition state.

\textsuperscript{c} The heavier isotopic molecule reacts faster. Assumes stretching force constant increases by a factor of 2 in going from reactant to transition state.
difficulty of measuring this small a difference in rates has precluded
a large number of examples from appearing in the literature. Also,
the cost of these isotopes has historically been very high, although
they are coming down in price somewhat. This section deals with a
compendium of oxygen isotope effects.

In a study of the oxidation of formic acid by chlorine gas, Ropp,
Danby and Dominy and Ropp and Guillory looked at the $^{13}$C, $^{18}$O and $^2$H
kinetic isotope effects. They observed a carbon KIE of 1.008, no ap-
preciable $^{18}$O KIE and a $^2$H KIE of 2.0. They interpreted these results
in terms of a rate determining abstraction of the C-H hydrogen by a
chlorine atom. The small $^{18}$O KIE indicated a small contribution of
O-H hydrogen abstraction (57, 58).

Taylor (59) has studied the $^{18}$O KIE for the $S_N^2$ displacement
reaction of substituted benzyldimethylsulfonium tosylates:

$$Y^- + \text{CH}_2\text{S(CH}_3\text{)}_2 \rightarrow \text{XCH}_2Y + \text{(CH}_3\text{)}_2\text{S}$$

Using $^{18}$O labeled phenol for $Y^-$, the KIE was measured for $X = \text{p-CH}_3$,
$p\text{-H}$, and $m\text{-Cl}$. The KIE's were 1.0074 (0.0008), 1.0082 (0.0010) and
1.0095 (0.0004) respectively. The KIE's were all very small as expected
for incoming-group isotope effects in displacement reactions. The point
to be made, however, is that the isotopic fractionation at a labeled
incoming atom in a displacement reaction would be expected for both an
$S_N1$ or $S_N2$ mechanism, even though in the $S_N1$ reaction the rate determ-
ining step is carbonium ion (or reactive intermediate) formation which is
not isotopically sensitive. The process of addition of the incoming
group to the reactive intermediate is rate determining as far as the
incoming group is concerned and will result in an isotopic fractiona-
tion providing the process of addition proceeds through an "activated
complex." Small KIE's were also observed by Bartlett (60) in the
reaction of $^{18}O_2$ with free radicals and photoactivated rubrene.

Goldstein (61) measured the $^{13}C$ and $^{18}O$ KIE's in the thermal de-
composition of acetyl peroxide. The $^{18}O$ KIE was 1.023 and the carbonyl
carbon $^{13}C$ KIE was 1.023. On the basis of model calculations, he pro-
posed that carbon-oxygen and carbon-carbon bond cleavage was concerted,
both appearing in the rate determining step.

Workentine (62) found a carbonyl carbon $^{13}C$ KIE of 1.028 and $^{18}O$
KIE of 1.016 in the pyrolysis of dibenzhydryl oxalate and several of
its para substituted derivatives. On the basis of the isotope effect
data (and other data), it was concluded that carbon-carbon and carbon-
oxygen bond cleavage was concerted.

Mitton and Schowen (63) studied the methoxide catalyzed methan-
olysis of aryl benzoates using the carbonyl oxygen labeled with $^{18}O$:

\[
\begin{align*}
\begin{array}{c}
\phi-\text{O}^*-\text{O}^*-\text{X} \\
\times \text{CH}_2\text{O}^- \\
\text{O}\text{-CH}_3
\end{array}
\right\rightleftharpoons
\begin{array}{c}
\phi-\text{O}^*-\text{O}^*-\text{X} \\
\times \text{O}\text{-COCH}_3^- \\
\text{X}\text{C}_6\text{H}_4\text{O}^-
\end{array}
\right\\\
\left\rightleftharpoons
\begin{array}{c}
\phi-\text{O}^*-\text{O}^*-\text{X} \\
\times \text{CH}_2\text{O}^- \\
\text{O}\text{-CH}_3
\end{array}
\right.
\end{align*}
\]

For X-Br and H, the $^{18}O$ KIE was 1.018 and 1.024 respectively. The KIE
is in a sense a secondary $^{18}O$ KIE, as the isotopic bond is not broken
in the reaction. Mitton and Schowen, however, point out that a consi-
derable change in the carbonyl bonding occurs as the transition state
(marked with a $\neq$) is passed through. If the tetrahedral intermediate
formation is rate determining, the KIE is probably better classified as a primary one.

Goering (64) has reported an $^{18}\text{O}$ KIE in the solvolysis of cis-5-methyl-2-cyclohexenyl p-nitrobenzoate in 80% aqueous acetone at 100°C:

For the ether oxygen ($O^*$) labeled with $^{18}\text{O}$, the isotope effect for the solvolysis reaction was 1.08.

Hart and Bourns (65) studied a displacement reaction involving nucleophilic aromatic substitution of piperidine for phenoxide ion (ether $^{18}\text{O}$) in 2, 4-dinitrophenyl phenyl ether:

The last step in the reaction is catalyzed by hydroxide ion so the rate of decomposition of the intermediate should depend on the base concentration. When the concentration of $\text{OH}^-$ is high, the first step is rate limiting, for low base concentration the second step is rate limiting. The $^{18}\text{O}$ KIE as a function of hydroxide ion concentration
was 1.0024 (at .149 M NaOH), 1.007 (.033 M NaOH) and 1.0109 (.005 M NaOH), confirming the mechanism outlined.

Seltzer, Tsolis and Denny (66) have used $^{13}$C and $^{18}$O isotope effects to investigate the acetolysis of three triphenylcarbalkoxyethylphosphonium salts:

$$\begin{align*}
\ddagger & & \mathbf{O} \\
O_3P-\mathbf{CH}_2-C-\mathbf{OR} + \text{HOAC} & \rightarrow & O_3P-\mathbf{CH}_3 + \mathbf{CO}_2 + \text{ROAC} \\
\end{align*}$$

(25)

For $R=\mathbf{\varnothing-CH}_2$, 1-octyl and 2-octyl the $^{13}$C KIE's were 1.045, 1.020, and 1.018; the $^{18}$O KIE's were 1.015, .999, and 1.002 respectively. The large $^{13}$C and $^{18}$O KIE for the benzhydryl case indicates simultaneous bond changes at C and O in the rate determining step, a possible transition state being:

$$\left[ \begin{array}{c}
\ddagger & & \mathbf{O} \\
O_3P-\mathbf{CH}_2-C--O--\mathbf{CH}_2\varnothing
\end{array} \right]$$

(25)

Where the benzhydryl group can stabilize the developing positive charge, favoring O-CH$_2$Ø bond cleavage concerted with C*-CH$_2$ bond cleavage.

Cahill and Taube (67) studied the metal ion catalyzed reduction and oxidation of H$_2$O$_2$, looking at the $^{18}$O KIE for the process. In general, the KIE for oxidation was small, however, for reduction it was considerably larger. In the Fe(II) reduction of H$_2$O$_2$* (only one $^{18}$O) the KIE obtained was 1.068, Sn(II) 1.057, for Ti(III) 1.003, for Cr(II) 1.032 and for Cu(I) 1.055. In the oxidation of H$_2$O$_2$* (one $^{18}$O), the KIE's for a variety of reagents averaged 1.008. Their results indicated a two electron reduction in which rate limiting cleavage of the O-O bond occurred. They calculated, based on the vibrational frequency of the O-O bond, that the expected isotope effect should be 1.061 at most.
Sawyer and Kirsch (68) studied the hydrolysis and hydrazinolysis of methyl formate (ether \(^{18}O\)). In the hydrolysis reaction (using \(H_3O^+\) or \(OH^-\) as nucleophile) the KIE's were relatively small at values of 1.0009 and 1.0091 respectively and 1.0115 using succinate as a general base catalyst. This is what was expected for rate limiting addition of the nucleophile to the carbonyl carbon. Using hydrazine as the nucleophile at pH 7.85 gave an \(^{18}O\) KIE of 1.0621 at 25°C. The addition of hydrazine to the carbonyl carbon is apparently not rate limiting, whereas the breakdown of the tetrahedral intermediate is in this case. The smaller KIE in the hydrolysis is presumably due to the loss of resonance interaction between the ether oxygen and the carbonyl carbon in going from the ester to the transition state.

Gorenstein (69) has determined the \(^{18}O\) KIE associated with the hydrolysis of aryl phosphates labeled with \(^{18}O\) in the ether position. The 2,4-dinitrophenyl dihydrogen phosphate compound hydrolyzed with a KIE of 1.020 at 39°C. This relatively large KIE indicates substantial phosphorous oxygen bond cleavage in the transition state. The KIE for the hydrolysis of dibenzyl 2,4-dintrophenyl phosphate was only 1.007. Gorenstein suggests that the dintrophenyl dihydrogen phosphate hydrolysis may proceed through a dissociative mechanism generating metaphosphate, while the dibenzyl case may involve an addition-elimination mechanism as is postulated for carboxylic acid esters.

O'Leary and Marlier (70, 71) have reported an \(^{18}O\) KIE for the hydrolysis and hydrazinolysis of methyl benzoate (ether \(^{18}O\)). The KIE for hydrolysis was 1.0062, while the KIE for hydrazinolysis was 1.0403. These results are similar to those obtained by Sawyer and Kirsch.
Rosenberg and Kirsch (72) have measured the $^{18}O$ KIE for the hydrolysis of 2,4-dinitrophenyl acetate (ether $^{18}O$) as catalyzed by nicotinamide. Their observation of the KIE = 1.043 is in substantial agreement with the KIE observed by Sawyer (68) and O'Leary (70). They have also measured a V/K KIE for the hydrolysis of 2,4-dinitrophenyl-beta-$d$-galactoside (ether $^{18}O$) catalyzed by Beta-Galactosidase. The observed KIE, expressed as $(V/K)_{16}/(V/K)_{18}$, was 1.039. V/K is defined as the second order rate constant for an enzyme catalyzed reaction extrapolated to zero substrate concentration. The large KIE observed indicates rate determining cleavage of the oxygen bond in the first irreversible step. The KIE was also determined for saturating levels of the substrate. The value obtained was 1.024. This represents the KIE on the $V_{\text{Max}}$ and requires that substantial cleavage of the bond to the isotopically labeled oxygen must occur in the overall rate determining step (72).

Sawyer and Kirsh (73) have also determined the $^{18}O$ KIE associated with the chymotrypsin catalyzed hydrolysis of N-acetyl-1-tryptophan ethyl ester (ethoxyl $^{18}O$) and N-carbomethoxy-1-tryptophan ethyl ester (ethoxyl $^{18}O$). The KIE's reported were for the acylation step (equation 5) which is not rate determining for ester hydrolysis. The observed KIE's were 1.018 and 1.0117 respectively. This was in agreement with the $^{18}O$ KIE's observed for the general base catalyzed hydrolysis.

There was a recent report on the $^{18}O$ KIE associated with the conversion of fumarate to malate (74). The 2S-(2-$^{18}O$)-malate reacted to give a large primary $^{18}O$ KIE which was strongly pH dependent. At pH 8, the KIE was 1.007 increasing in magnitude to 1.073 at pH 5.03. The
data suggest a carbanion intermediate formed by transfer of the 3-R proton of malate to a catalytic group on the enzyme (carboxyl) generating a trigonal acicarboxylic carbanion at C-3 while C-2 remains tetrahedral. Proton transfer to the C-2 hydroxyl generates water and fumarate. The large KIE indicates that at pH 5, C-0 bond cleavage at C-2 is almost completely rate limiting.

Finally, Wang et al. (32) have reported an $^{18}$O KIE for a trans-esterification reaction catalyzed by chymotrypsin. The reaction was that outlined in equation 5. When the ethanol substrate was labeled with $^{18}$O, the KIE for formation of the ester product was found to be .86. This work represents a re-investigation of that KIE by the technique described in the next section.

3.1.3 Methods of KIE measurement

In the broadest sense, KIE measurements are dependent on the rate at which an isotopic species reacts. The KIE then is the ratio of the rates for the reaction of a heavy and a light isotopic compound. In practice, there are two ways by which a KIE can be determined. The first involves the actual measurement of the individual rates, which will be termed a direct measurement. The second involves measuring the isotopic ratios of product or reactant as the reaction proceeds. In this case, actual rates are never measured, only inferred, and this procedure will be termed an indirect measurement.

In direct measurements, some synthesis is required in order to enrich the reactants in the isotope atom desired. This is necessary due to the low natural abundance of the heavy isotopes of the atoms.
normally involved in organic and biological reactions. A direct measurement can be performed in three ways. The first way is to have the reactant completely isotopically labeled in the important reaction center of the molecule. Then, the rate for the conversion of the 100% isotopically labeled compound can be compared to the rate for the natural abundance compound determined in a separate experiment. This is generally not feasible, as most reagents used in the synthesis of the isotopic compound are not pure with respect to that isotope. This fact requires that the isotopic abundance of the labeled reactant be measured very accurately so that the observed rate can be corrected for the presence of the lighter isotope.

The second procedure to directly measure KIE's relies on the ability to simultaneously measure the concentration of both isotopic products (or reactants) as a function of time. Under these circumstances, the rates are measured under exactly the same experimental conditions. This may or may not require physical separation of products from reactants at timed intervals during the reaction. Ideally it would not, and the reaction could be monitored in situ in a continuous fashion. The determination of the isotopic species would probably require a mass spectrometer although perhaps an IR spectrometer could be used.

The third method employs the fact that isotopic substitution can produce a shift in the equilibrium between products and reactants in a chemical reaction. For a bio-chemical reaction in which the products and reactants are at equilibrium, when enzyme is added to the mixture no net change in the concentrations of the products and reactants should be observed (if slight dilution effects are neglected). However, if
100% isotopically labeled reactant is used, when the enzyme is added there is a perturbation in the equilibrium due to the isotope effect for conversion of isotopic reactant to isotopic product and the system moves away from equilibrium. The magnitude of the perturbation can be used to calculate the KIE associated with the interconversion. The method is obviously restricted to systems which can establish equilibrium, that is systems whose equilibrium constant is not too large or too small. In addition, corrections must be applied for reagents other than 100% isotopically labeled. The equilibrium isotope effect must be determined to high precision (109).

There are two ways to indirectly obtain KIE's. The first involves measurement of the change in isotopic composition of the product or reactant as a function of the extent of reaction. If a substrate contains a mixture of labeled and unlabeled species, the relative isotopic composition changes over the course of the reaction because the two species do not react at the same rate. In general, the isotopic composition change in the product is measured after a small extent of reaction \((P^*/P_t)\) and is compared to the isotopic composition after complete reaction \((P^*/P)\). The KIE is calculated from:

\[
KIE = \frac{\log(1-f)}{\log(1-f(P^*/P_t)/(P^*/P))}
\]

where \(f\) is the fraction of reaction. The technique has generally been applied to reactions involving natural isotopic abundance compounds. The products are separated from the reaction mixture and analyzed by an isotope ratio mass spectrometer. The technique is almost exclusively
restricted to reactions where the product is a stable gas (i.e. N₂, NH₃, CO₂, CO, or O₂), or can be converted to a stable gas very easily without too much extra gas being produced (i.e. not too many carbons in a compound which yield extra CO₂ not resulting from the discrimination during the kinetic isotope fractionation). No synthesis is generally required and extremely high precision can be achieved (107). Kirsch has used this technique and modified it somewhat (72).

The second indirect method makes use of doubly labeled substrates. Because some sites of substitution are difficult to measure by the above procedure, comparison is made between unlabeled and doubly labeled substrates. One of the labeled positions (the indicator position, I) is a site that is available for isotopic analysis. The other labeled position (the key position, K) is the position whose isotope effect is desired. The equations are:

\[ \text{IK} \xrightarrow{k} \text{p} \]
\[ I^*K^* \xrightarrow{k^*} p^* \]

If singly labeled substrates are absent, then the product isotope ratio at the indicator position provides an isotope effect which is the product of the isotope effect at the indicator position and the key position.

All of the methods have their advantages and disadvantages. In this work, a direct method of KIE analysis is presented. This method has the advantage that the rates of isotopic product formation are measured in situ and simultaneously. This eliminates errors such as differences in enzyme concentrations, reaction temperature, pH, and
ionic strength, all of which can affect the rate of a chemical reaction. The method does require the synthesis of isotopic substrate, plus it requires a knowledge of the initial isotope ratio of the substrate, but these constraints are relatively minor. The method as it currently stands is only applicable to hydrophobic products (or reactants) which are relatively volatile. However, the wide range of reactions involving these types of compounds allows the method to be generally applicable.

3.1.4 The Permselective Nature of Polymeric Membranes

The method of KIE measurement presented in the current work relies upon the selective permeation of apolar compounds through a synthetic polymeric membrane. The permselectivity of polymeric membranes has been known for several years. Initial studies involved the use of nitrocellulose membranes (76, 77) which are common to this day in the field of ultrafiltration and dialysis. However, the study of the permselectivity of dense polymer membranes (i.e. those characterized by the lack of "pores") was initiated by Graham (75) who regarded the permeation process as solution, diffusion, and re-evaporation. This viewpoint is essentially that held today. Several investigators then began the study of the permeation of gases through these dense membranes (78-80), with Barrer contributing to the mathematical description of the diffusion process (81, 82).

The use of polydimethylsiloxane membranes in gas separations began when Llewellyn (83) incorporated them in his design of an interface between a gas chromatograph and a mass spectrometer. The membrane allowed
efficient permeation of apolar compounds in the vapor phase while excluding to some degree the carrier gas helium. This permitted an increase in the partial pressure of the organic vapor in the ion source of the mass spectrometer and facilitated the production of a mass fragmentograph. These membranes are currently being used as an interface between a liquid chromatograph and a mass spectrometer.

Westover et al. (84) have used dimethylsilicone rubber membranes fabricated as thin fibers in the measurement of apolar compounds dissolved in water. The fibers, with a wall thickness of 25 μm, were coupled to the source of a mass spectrometer, allowing quantitative as well as qualitative measurement of compounds such as alcohols, chlorinated alkanes and aromatics. The technique was extended to measure the rate of hydrolysis of chloromethyl methyl ether and bis (chloromethyl) ether in humid air (85, 86). Response times were generally less than 14 seconds with sensitivity levels ranging to 2 parts per billion. This was accomplished without any lengthy procedural preparations of the solutions prior to analysis. The method has also been used to measure the concentrations of benzene and methyl salicylate in water (87).

The determination of the concentration of polar bio-chemicals in solution has been achieved by the enzymatic conversion of these polar compounds to easily permeable compounds (88). In this way, urea concentrations were determined by measuring the \( \text{CO}_2 \) released during the enzymatic hydrolysis of urea by urease immobilized on a solid support. NADH concentrations were determined by the measurement of ethanol produced in the alcohol dehydrogenase catalyzed reduction of acetaldehyde.
Organ perfusates (89) and individual cells (90) have been used in the measurement of volatile metabolites produced in these systems. In addition, the product of the electrochemical reduction of dibromocyclohexane (i.e. cyclohexene) has been measured mass spectrally as it was produced on a grid electrode mounted on a dimethylsilicone rubber membrane (91).

The selective permeation arises from the fact that the overall permeation of a compound through the membrane is proportional to the product of the compound's solubility in the membrane and its diffusivity through the membrane. With dimethyl silicone rubber, a non-polar medium, the solubility of a compound is related to its hydrophobicity. The membrane acts as a single stage extractor, similar to a single stage liquid extraction, and therefore the partition coefficient between the aqueous solution and the membrane is related to the relative solubility of the compound in the two phases. In general, as the molecule becomes larger and more non-polar, its solubility in the membrane increases. However, this favorable partitioning is offset in part by a decrease in the diffusivity of the compound through the membrane. This can be seen in the expression for the diffusion coefficient:

$$D = \frac{RT}{N \cdot 6\pi\eta} \left( \frac{4N\pi}{3M\nu} \right)^{1/3}$$

Where $D$ is the diffusion coefficient, $T$ the temperature, $N$ is Avagadro's number, $M$ the molecular weight, $\eta$ is the solvent viscosity and $\nu$ the average velocity. It can be seen that the diffusion coefficient is inversely related to the cube root of the molecular weight. It would at first appear that the diffusivity may be favorably altered by increasing
the temperature, however, this is offset somewhat by a decrease in the
solubility of the compound in the membrane (92).

The permselective nature of the dimethylsilicone rubber membrane
has been exploited in the measurement of the solution concentration
of ethyl-2-furoate and ethyl-2-(5-n-propyl)-furoate (93). The esters
are produced by the chymotrypsin catalyzed transesterification using
the p-nitro-phenyl esters of the two acids as substrates. The charac-
terization of the method is discussed in the subsequent sections as is
its use in the measurement of the $^{18}$O KIE associated with the ethanoly-
sis (ethanol -$^{18}$O) of the p-nitrophenyl esters catalyzed by chymotrypsin.

3.2 Methods

3.2.1 Description of the Cell

The cell used in the mass spectrometric studies is shown in Figure
5. The cell consists of 2 stainless steel halves held together by four
brass screws. The upper half of the cell is 1 1/2 inches thick and has a
centrally located conical section in which the reactions are run. A
teflon plug (tapered to fit snugly into the cell region) has holes for
the addition of reagents and one to hold the stirring rod which is
driven by an electric stirrer. There are grooves in the underside of
the upper half which hold the two O-rings in places (the outer supplies
a vacuum seal to the environment, the inner is a liquid seal to hold
the reaction solution in place).

The bottom half of the cell is 1 inch thick stainless steel. The
center region has been bored out and contains a brass plate with several
holes drilled in it which provides mechanical support for the screen
Figure 5
Diagramatic representation of the cell used in the mass spectrometric studies. A) Mechanical stirrer, B) Teflon plug, C) Sample injection ports, D) Dimethyl silicone rubber membrane supported on a polycarbonate filter, E) Stainless steel screen, F) Porous brass support, G) To the mass spectrometer, H) Viton O-rings, J) Reaction compartment with a volume of .5 ml.
upon which the dimethyl silicone rubber membrane, polycarbonate membrane assembly sits. A 1/8 inch hole extends through the bottom of the chamber containing the brass plate through which membrane permeable materials are led to the ion source of the mass spectrometer.

3.2.2 Measurement of cell response

3.2.2.1 Steady-state responses

The diffusion of a permeable compound through a membrane of finite thickness is analogous to the transport of heat through a flat conductor of finite thickness. The equation describing this process has been derived by Carslaw and Jaeger (94).

\[
I(t) = \frac{C_s D A K e}{1 + 2 \sum_{n=1}^{\infty} (-1)^n \exp \left( -\frac{n^2 \pi^2 D t}{l^2} \right)}
\]  

(28)

Where \(C_s\) is the solution concentration of the permeant molecule, \(D\) is the diffusion coefficient in the membrane, \(A\) is the area of the membrane, \(e\) is essentially an extinction coefficient relating intensity to solution concentration, \(K\) is the partition coefficient for the compound between the solution and the membrane, and \(l\) is the membrane thickness. The equation expresses the ion intensity \(I\) as observed at the mass detector of the mass spectrometer as a function of time. The equation holds for a rapid increase in solution concentration at time \(t=0\). (It should be recognized that \(\sum_{n=1}^{\infty} (-1)^n = -\frac{1}{2}\). The validity of equation 28 was checked in the following way. The cell was filled with .4 ml of .1M \(K_2HPO_4\) pH 8.5 buffer. While constantly stirring, 13 \(\mu\)l of ethanol and 20 \(\mu\)l of dimethyl formamide is added. The concentration
of the ethyl ester of furoic acid (or 2-(5-n-propyl)-furoic acid) in the cell is then increased by the rapid addition of the ester stock solution. The stock solution is prepared by diluting 10 mM ester in ethanol 200 fold with the buffer just prior to measurement. The final ester concentration ranged from 1 to 100 µM. The ion intensity is then recorded by repetitively scanning over the parent (m/e=140) or base (m/e=112) peak in the mass spectrum of ethyl-2-furoate. The scanning is accomplished by a circuit of our own design, which linearly varies the acceleration voltage in the mass spectrometer. The output from the ion multiplier of the mass spectrometer is electronically integrated and recorded on a strip chart recorder. The recorded ion current is then used as a measure of the solution concentration of the ester. The data consists of arbitrary intensity units I (which is actually the height of the integrated signal as recorded) versus time readings which are then fit by non-linear regression analysis to equation 28.

Numerous investigations, in which the number of times the computer program had to sum over n (in the series in equation 28) indicated that a simplification of equation 28 could be made. The equation was cast into an empirical form given by equation 29.

\[ I(t) = A_1(1 - A_2 \exp(-A_3 t)) \]  

(29)

Where \( A_1 \) and \( A_2 \) are empirical constants (\( A_2 \) is a correction for terms in the series of equation 29 where \( n>1 \)) and \( A_3 \) is the rate of appearance of the ion signal. This equation was valid for the ethyl-2-furoate, however, a better fit to the data for ethyl-2-(5-n-propyl)-furoate was achieved by using equation 29b.
\[ I(t) = A_1(1 - \exp(-A_3(t+A_2))) \]  
\hspace{1cm} (29b)

In this equation, \( A_1 \) and \( A_3 \) have the same meaning as in equation 29, but \( A_2 \) in equation 29b is a time correction for the initial lag (due to slower diffusion) observed in the time course of the ion signal for ethyl-2-(5-n-propyl)-furoate. Equations 29 and 29b predict that a plateau level in I units will be achieved when \( t=4.16/A_3 \). This plateau (\( A_1 \)) is what is observed in figures 6 and 7.

### 3.2.2.2 Calibration of Response

As seen in equation 28, there are several machine dependent parameters (e, 1, D, etc.). All of these parameters are collected in \( A_1 \) of equation 29. A calibration curve, relating maximal ion intensity (i.e. at \( t=\infty \) in equation 29) to the solution concentration of the ester can then be prepared by multiple additions of standard ester solutions to the cell. The response should be linear as indicated by equation 28. Figures 8 and 9 show the result of such a calibration for ethyl-2-furoate and ethyl-2-(5-n-propyl)-furoate.

### 3.2.2.3 Measurement of the loss of solute from solution by diffusion

Equation 28 describes the flux of the solute through the membrane assuming a jump in concentration at \( t=0 \) from \( C_s=0 \) to \( C_s=C_s \) at all \( t>0 \). It represents the steady state flux of solute through the membrane. In a limited volume reaction chamber, there is a constant loss of solute from solution. The rate of this loss from solution should be a first order process:
**Figure 6**

First order appearance of ethyl-2-furoate after a single rapid addition to the solution in the cell reaction compartment. The solid line is the best fit to the data calculated from equation 29.
Figure 7
First order appearance of ethyl-2-(5-n-propyl)-furoate after a single rapid addition to the solution in the cell. The solid line is the best fit to the data according to equation 29b.
Figure 8

Linear response of the mass spectral ion intensity as a function of the solution concentration of ethyl-2-furoate.
Figure 9

Linear response of the mass spectral ion intensity as a function of the solution concentration of ethyl-2-(5-n-propyl)-furoate.
\[ \frac{P_s}{P_v} \]

\[ P_{s,t} = \Delta P_s \exp(-kt) - P_{s,t = \infty} \] (30)

Where \( P_s \) is the solution concentration of the solute, \( P_v \) is the concentration of the solute in the vapor. Equation 30 shows the integrated form of the rate expression and relates the concentration of the solute in the solution \( (P_{s,t}) \) at time \( t \) to the rate of its loss from solution \( (k) \) with the other constants expressing its percent change with time \( (\Delta P_s = P_{s,t = 0} - P_{s,t = \infty}) \). The data, after a rapid increase in the solution concentration of the ester, appears as an initial first order increase over approximately 1.5 minutes (equation 29) followed by a constant plateau region (lasting about 2-3 minutes) and then by a first order decrease in the ion intensity. The first order decrease was fit to equation 30 yielding best fit values for the three constants. The curve for this first order loss at 25°C is shown in figures 10 and 11.

3.2.3 Rate measurements

3.2.3.1 Spectrophotometric determination of the rate of ethyl ester production

The rate of ethyl ester formation was determined by measuring the steady state formation of p-nitrophenol (31). The reaction was performed by the following procedure. Ethanolic buffer \( (3.0 \text{ ml, } .1M \text{ } \text{K}_2\text{HPO}_4, \text{ } \text{pH } 8.5) \) was added to a 1.0 cm path length cell, and allowed to reach thermal equilibrium. An appropriate amount of substrate stock solution was added so that the final concentration of substrate was 20-40 µM. The absorbance change at 400 nm was then observed. This
Figure 10

First order diffusional loss of ethyl-2-furoate from the cell solution at 25°C.
Figure 11
First order diffusional loss of ethyl-2-(5-n-propyl)-furoate from the cell solution at 25°C.
corresponded to the non-enzymatic hydrolysis of substrate, and was
given by the linear slope of the O.D. vs time as output in the recorder
\( R_0 \). The enzyme stock solution was then added such that the final
enzyme concentration was 2 \( \mu \)M. The absorbance change at 400 nm was
recorded. There was an initial burst of p-nitrophenol release as the
enzyme was acylated, followed by a linear production during the steady
state turnover of the enzyme \( (R) \). The magnitude of the p-nitrophenol
burst in O.D. units was determined by the extrapolation of the two
limiting linear traces back to the point where the enzyme was added.
The magnitude of the burst \( (B) \) is a direct measure of the concentration
of enzyme active sites. The apparent rate is defined by equation 31:

\[
 k_{\text{app}} (\text{min}^{-1}) = \frac{R(\text{OD/min}) - R_0(\text{OD/min})}{B(\text{OD})} \tag{31}
\]

Where \( R \) and \( R_0 \) are the enzymatic and non-enzymatic rates respectively.

The second order rate constant for ester production is determined
by varying the ethanol concentration in the buffer from 0.0 to 1.0 M.
A plot of \( k_{\text{app}} \) vs ethanol concentration then gives as slope the second
order ethanolysis rate (Figure 12).

3.2.3.2 Ethanolysis rate as measured mass spectrometrically

Under the conditions of enzymatic production of ester, there are
two kinetic processes occurring in the mass spectrometer cell. The
first kinetic process is the production of ester, while the second is
the transport of the ester through the membrane and into the ion source
and ion detector of the mass spectrometer. The equation describing
Figure 12
Plot of the variation of the steady state rate of p-nitrophenol release as a function of the ethanol concentration for ethanolsysis of p-nitrophenyl-2-furoate. The slope of the line gives the second order ethanolsysis rate.
this coupled kinetic-diffusion process was derived. The minimal kinetic scheme which can be used to represent the time course for ester production when catalyzed by chymotrypsin is given in scheme VI.

\[
\begin{align*}
E + S & \xrightarrow{k_1^t} EA \xrightarrow{k_2(\text{ETOH})} E + P_2 \\
\text{scheme VI}
\end{align*}
\]

The solution of this scheme can be obtained by the application of the Laplace-Carson transform (95), assuming the concentrations of S and ETOH do not change with time:

\[
P_2(t) = k_o \ [E_o] \left[ t - \frac{1-e^{-k_1 t}}{k_1} \right]
\]

(32)

Where \( P_2 \) is the ethyl ester concentration as a function of time, \([E_o]\) is the total enzyme concentration, \( k_1 \) is the pseudo-first order rate for acylation of the enzyme \( (k_1 = k_1^t [S] + k_2 [\text{ETOH}] ) \) and \( k_o \) is the rate of the steady state appearance of ester \( (k_o = k_2 [\text{ETOH}]/k_1) \). This reaction scheme is far too simple to completely describe the chymotrypsin catalyzed reaction, however, it can be considered as phenomenologically correct. The true construction of the apparent rate constants \( k_o \) and \( k_1 \) from the elementary rate constants will depend on the detailed reaction mechanism. By application of Duhamel's Theorem (94), the coupled enzyme reaction and diffusion process leads to equation 33 (see also appendix B for a derivation of this equation.

\[
I(t) = A_1 k_o [E_o] \left[ t - \frac{1-e^{-k_1 t}}{k_1} - \frac{A_2}{A_3} (1-e^{-A_3 t}) + \frac{A_2}{A_3-k_1} \left( e^{-k_1 t} - e^{-A_3 t} \right) \right]
\]

(33)
Where $A_1$, $A_2$, and $A_3$ are the constants from equation 29 and $k_0$, $E_0$, and $k_1$ come from equation 32. Therefore, by determining $A_1$ (from a plot such as Figure 8) and $[E_0]$ from a burst titration, $k_0$ can be extracted from the linear portion of the I vs time curve, under the conditions of enzymatic catalysis.

The actual procedure is as follows. Buffer (.42 ml of .1M $K_2HPO_4$, pH 8.5), ethanol (13 l of 95%) and enzyme (50 µl of a stock solution containing 20 mg of enzyme/ml of .001M HCl) are added to the reaction chamber. The reaction is initiated by the addition of 20 µl of 12.5 mM p-nitrophenyl-2-furoate (or p-nitrophenyl-2-(5-n-propyl)-furoate) to the rapidly mixed reaction chamber. As before, the base or parent peak of the ester's mass spectrum is scanned and integrated, and the signal output on the chart recorder. The total enzyme concentration was determined by adding an aliquot of the stock enzyme solution to a thermostatted cuvette containing 20 µM p-nitrophenyl-acetate in pH 8.0 phosphate buffer. The absorbance at 400 nm was recorded. The magnitude of the burst of p-nitrophenol release after enzyme addition was determined as outlined in section 3.2.3.1, the burst magnitude was then converted to concentration units using a molar extinction coefficient for p-nitrophenol of 18350 cm$^{-1}$ (96).

3.2.3.3 Stopped flow measurement of the rate constant for the burst

The rate of the initial burst of p-nitrophenol was measured with a Durram stopped flow spectrophotometer. The reaction was performed by mixing equal volumes of the furoic acid substrate (.24 ml of stock
substrate (6.65 mM in dimethyl formamide) dissolved in 20 ml of .2M K₂HPO₄ pH 8.55 buffer which was also .5M in ETOH) and enzyme solution (74.1 mg in 60 ml of .001 M HCl, prepared just prior to use and kept at 4°C until used). The slit width was set at .12 nm, the wavelength at 400 nm. The oscilloscope display was set at .2 volts/division vertically and .5 seconds/division horizontally. The temperature was 25.5°C. The reaction was repeated with the substrate diluted 1:2. Under these conditions, for the first set of reactions the [E₀] = 50 μM and [S₀] = 80 μM. For the second set of reactions the enzyme concentration was the same but the substrate concentration was half (40 μM). The oscilloscope trace was photographed using a 198A Hewlett Packard oscilloscope camera. The picture was digitized using a Bendix Datagrid digitizer and the corresponding voltage vs time data points were fit to the first order curve (equation 6) or the equation 34:

\[ V_t = k_0 t + V_1 (1 - e^{-kt}) \]  

Which was used when [E₀] < [S₀]. V is the voltage at time t, k₀ is the zeroth order (steady state) linear rate, V₁ is the maximal voltage change for the first order burst and k is the rate of the burst. Equation 34 corresponds to equation 32.

3.2.3.4 Kinetic isotope effect measurements

The measurement of the ¹⁶O/¹⁸O ethanolysis KIE was carried out as outlined in section 3.2.3.2. The enzyme concentration in the cell was raised to 80 μM with a substrate concentration of .8 mM. The total ethanol concentration was .5 M, however, it was an approximate equal
molar mixture of $^{16}$O and $^{18}$O ethanol. The intensity versus time data were digitized from the recorder output using a Bendix Datagrid digitizer. In the competitive method, the true KIE associated with $k_o$ (equation 33) can be obtained by correcting for the original $^{18}$O/$^{16}$O ethanol ratio in solution. As seen in equation 32, $k_o$ equals $k_2(ETOH)/k_1$ (scheme VI). The observed zeroth order rate depends on the concentration of ethanol, so that in determining the KIE, the concentrations of the isotopic ethanols must be eliminated from the observed rate. Since only relative rates need be considered, this procedure involves the measurement of the starting ratio of the isotopic ethanols.

$$\text{KIE} = \frac{k^{16}_{obs}}{k^{18}_{obs}} \frac{(ET^{18}_{OH})}{(ET^{16}_{OH})}$$

(35)

Where $k_{obs}^{16}$ and $k_{obs}^{18}$ are the slopes of the linear regions in the I vs time curves obtained from the enzymatic production of the esters when a mixture of isotopic ethanols is used. The I can be measured at either the base (loss of ethylene (97)) or parent peak in the mass spectrum of the ethyl ester product (112, 114 or 140, 142 for the furoic acid substrate or 154, 156 or 182, 184 for the 2-((5-n-propyl)-furoic acid substrate). The ethanol isotope ratio defined in equation 35 was obtained by converting the ethanol to the appropriate ethyl ester and then scanning the appropriate m/e range (as required by the rate measurement) under the same conditions as was used for the particular kinetic determination. This procedure was necessary because some mass discrimination occurs when a magnetic sector mass spectrometer is scanned by varying the acceleration potential (98). The effect of this
procedure is to move the mass ratio of the ethanol to the same mass region as the product of the kinetic run. The ester was prepared by reaction of 3 µl of labeled ethanol (the same ethanol used in the kinetic run) with 300 µl of dry pyridine and 50 µl of the appropriate acid chloride. The reaction mixture was maintained at about 40°C for ten minutes. Aliquots of the reaction mixture were removed and added to the cell in a procedure analogous to that outlined in section 3.2.2.1. The intensity versus time points obtained for each isotopically labeled ethyl ester were fit to equation 29 (furoic acid) or equation 29b (2-(5-n-propyl)-furoate). The $A_1$ values obtained from the fit were then taken as a measure of the concentration of the isotopically labeled ethanol. In a separate experiment, the reaction mixture was separated on a gas chromatograph attached to the mass spectrometer. The ester peak was scanned as above and the area of each isotopic ester was determined from the recorder output, by fitting the intensity versus time data by the subroutine AREA (35) using a tenth degree polynomial. The ratio of the areas then represented the relative concentrations of the isotopically labeled ethanol.

3.2.3.5 Base catalyzed ester hydrolysis

The rate of the base catalyzed hydrolysis of the ethyl-2-furoate was measured in a pseudo-first order reaction. To the cell containing .45 ml of .01 M NaOH was added $^{16}O/^{18}O$ labeled esters in dimethyl formamide (final ester concentration was 20 µM). The first order decay of the parent ions (m/e=140 and 142) were recorded by scanning through the appropriate mass region as described above. The intensity vs time
points were then fit to the equation for a first order decay (equation 30) and the ratio of the first order rates was corrected for the background diffusional loss of ester. Figure 14 is an example of the first order decrease in ethyl ester intensity during base catalyzed hydrolysis.

3.2.4 Gas chromatography analysis

The reaction between ethanol and 2-furoic acid chloride was checked for completion by analysis of the reaction mixture on a GC. A 6 foot stainless steel column 1/8 inch O.D. was packed with 3% OV-225 on Chromosorb A(60/80 mesh). The initial column temperature was 40°C with the detector temperature 300°C and the injector at 250°C. The helium flow rate was 10 ml/min, the hydrogen 30 ml/min, and the air flow rate was 300 ml/min. Under these conditions the retention time for the ethanol was 3.5 minutes. After the ethanol was eluted from the column (five minutes), the temperature of the column was raised to 210°C until the furoic acid eluted. The retention time for the ethyl ester was 12-14 minutes and the acid came off the column in 20-25 minutes. The other major peaks identified as pyridine and the acid chloride.

The response of the GC to ethanol was determined by injection of various volumes of .44% (v/v) ethanol in water. The peak areas due to ethanol were determined by cutting out the peak from the recorder trace and weighing them on an analytical balance. The GC response to the ethanol was linear with respect to the moles of ethanol injected (Figure 15).
First order decrease in the ion intensity of ethyl-2-furoate during the base catalyzed hydrolysis. A) With .01N NaOH added; B) No base squares are for the $^{18}$O-ethyl-ester, circles are for the $^{16}$O-ethyl-ester.
Figure 14

The GC flame ionization detector responses as a function of the moles of ethanol injected into the GC. The FID response is quantitated as the weight of the peak output on the recorder.
3.3 Materials

3.3.1 Synthesis of the acid chlorides

The acid chlorides of both 2-furoic acid and 2-(5-n-propyl) furoic acid were made by reaction of the acids with thionyl chloride. For the 5-n-propyl furoic acid, 6.5 millimoles of the acid was dissolved in 5 ml of SOCl₂. The mixture was refluxed for 1 hour after which time no further HCl gas was evolved. The excess thionyl chloride was distilled off, the remaining liquid cooled then vacuum distilled at 30 mm Hg. The acid chloride was a clear liquid boiling at 140°C and gave a mass spectrum consistent with the proposed structure.

For the furoic acid chloride, 200 millimoles of the acid was dissolved in 20 ml of thionyl chloride and refluxed for 1 hour. The excess SOCl₂ was distilled off and the acid chloride distilled (B.P. 172 Lit. 173-174) was a clear liquid. Both acid chlorides were stored at 4°C in a desiccator.

3.3.2 Synthesis of ¹⁸O-enriched ethanol

The di-n-propyl acetal of acetaldehyde was prepared by mixing 130 gms of propanol and fifty grams of acetaldehyde at 0°C over 30 grams of CaCl₂ (99). The mixture was shaken intermittently over a period of 12 hours. The liquid was decanted and washed 3x with 30 ml of H₂O. The organic layer was dried for 3 hours over Na₂CO₃, filtered and distilled. The acetal fraction was taken at 140-150°C. This fraction was dried over sodium metal and redistilled with the acetal boiling at 145-147°C.
Oxygen-18 labeled acetaldehyde (100) was synthesized by hydrolyzing 17.5 millimoles of the di-n-propanoylthiane with .25 ml of H₂O₁⁸ (approximately 80% atom enriched). HCl gas (3 ml) dried over CaCl₂ was added as a catalyst. The system was under an atmosphere of N₂. Upon warming to 40°C the original two phase system became homogenous. The reaction mixture was stirred for 4 hours after which the ¹⁸O-acetaldehyde was distilled from the mixture (B.P. 21°C) into a receiver cooled to -78°C. Oxygen-18 labeled ethanol was prepared by reduction of the acetaldehyde. Anhydrous diglyme was prepared by distillation of reagent grade diglyme from LiAlH₄ at 20 mm Hg. Four millimoles of LiAlH₄ was added to the anhydrous diglyme, warmed gently and then cooled to 0°C. The ¹⁸O-acetaldehyde was then slowly added as a solution in diglyme to the rapidly stirring LiAlH₄ solution. The remaining LiAlH₄ was removed by the careful addition of 1 ml of water. The ethanol-¹⁸O was distilled (B.P. 76-78°C). The ethanol was 75% ¹⁸O enriched. The yield for both steps was 82%.

3.3.3 Ethyl esters
The ethyl esters of both acids were prepared by reaction of the corresponding acid chlorides with ethanol. The ethyl ester of furoic acid was recrystallized from a water-methanol (70:30, v/v) solution at -78°C (M.P. 32-33 Lit. 34°C). The ethyl ester of the 2-(5-n-propyl)-furoic acid was formed as an oil and was deemed pure by GC/MS analysis.
3.3.4 Reagents

The p-nitrophenyl-2-(5-n-propyl)-furoate and p-nitro-phenyl-2-furoate were prepared as described in section 2.3.1. The following chemicals were purchased and used without further purification: ethanol, dimethyl formamide, pyridine, thionyl chloride, propanol, acetaldehyde, p-nitrophenyl-acetate, CaCl$_2$, Na$_2$CO$_3$, LiAlH$_4$, and H$_2$O-18. The diglyme was dried over CaCl$_2$ and distilled from LiAlH$_4$ at 20 mm Hg.

3.4 Instruments and Equipment

The spectrophotometer system was that outlined in section 2.4. The preliminary GC analysis was performed on a Varian-Aerograph Model 2700. Samples were injected directly on the column and eluted from the OV-225 column with He. The sample was detected by a flame ionization detector and recorded on a Heath chart recorder. A He flow rate of 10 ml per minute was used along with a H$_2$ flow of 20 ml/min and an air flow rate of 300 ml/min. The initial temperature of the column was 40°C which was increased to 210°C after 5 minutes. The injector temperature was 250°C, the detector 300°C.

Isotope analysis was carried out on a Dupont 21-490 single magnetic sector mass spectrometer. The general operating conditions for the mass spectrometer were an ionization voltage of 20 eV at 100 μA. The source temperature was 210°C at a maximum pressure of $10^{-4}$ torr. A Perkin Elmer 990 gas chromatograph attached to the mass spectrometer was used in the ester analysis, using the general conditions outlined above.
The ramp circuit used to scan small regions of the mass spectrum was designed and built by the electronics shop in the department of chemistry. A circuit was included which allowed the output from the mass detector to be electronically integrated. This integrated output was recorded using a Bell and Howell Datagraph model 5-134 (band pass 10 Hz) recorder. The cell used in the mass spectrometric studies was designed and built in the machine shop in the department of chemistry.

3.5 Results

3.5.1 Method characterization

The system response was measured in three ways. The first involved characterization of the response time, the second measured the linearity of the system response to changes in the solution concentration of the ester product, and the third involved measurement of the rate of loss of ester from solution. This allowed determination of the critical parameters required in the kinetic studies.

3.5.1.1 Cell response

The response time was measured by following the ion intensity as a function of time after a rapid addition of ester to the cell solution. The time course for ion intensity increase is given by equation 28. This equation was reduced in form to equation 29 or 29b based on the empirical observation that terms with n>1 in equation 28 were not needed to describe the data. Under the conditions of equation 29 or 29b the response time can be defined as $\frac{0.693}{A_2}$. This gives the time for half maximal signal appearance. Using the ethyl ester of furoic acid
a $t_{1/2}$ of 12 seconds was obtained. With the ethyl ester of 2-(5-n-propyl)-furoic acid, the $t_{1/2}$ obtained was 58 seconds. This represents the difference in the diffusivities of these two compounds through the membrane.

For equation 29 to represent an adequate reduction of equation 28, the value of $A_2$ must approach one. That is, only a small correction for the fractional change of the maximal signal intensity with time can be applied. In five determinations, in which the solution concentration of the ethyl ester of furoic acid was varied, the value of $A_2$ was $.95 \pm .07$. Therefore, since $A_2$ was in fact 1 (within one standard deviation), equation 29 was deemed an adequate representation of the ion signal response. In equation 29b, $A_2$ represents the time lag prior to a strict first order increase in the ion intensity vs time curve. In this case, $A_2$ should approach zero as the response becomes more ideally first order. In the data of Table 7, the value of $A_2$ for the best fit values of equation 29b average out to a time lag of 5.4 seconds. The data for all the fit parameters for the first order increase in the ion intensity for both substrates is presented in Table 7.

The relationship between the ion intensity and the solution concentration of the permeant molecule can be seen in equation 28. The pre-exponential term explicitly contains this concentration expression within it. This is reflected in the $A_1$ term of equation 29 (or 29b). The maximal signal intensity is linearly related to the solution concentration according to this formulation. The data of Table 7 has been plotted to represent this fact. Figure 8 shows the relationship between the ion intensity and the concentration of ethyl-2-furoate, while
Table 7
Values for the parameters of equations 29 and 29b for the first order appearance of the steady state transport of ethyl-2-furoate and ethyl-2-(5-n-propyl)-furoate through the membrane.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ester $\times 10^6$ M</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl-2-furoate$^a$</td>
<td>0.48</td>
<td>55(5)$^b$</td>
<td>.87</td>
<td>3.2(.19)</td>
</tr>
<tr>
<td></td>
<td>1.44</td>
<td>180(3)</td>
<td>1.07</td>
<td>3.1(.22)</td>
</tr>
<tr>
<td></td>
<td>3.35</td>
<td>442(5)</td>
<td>.95</td>
<td>3.2(.16)</td>
</tr>
<tr>
<td></td>
<td>7.13</td>
<td>936(8)</td>
<td>.95</td>
<td>3.3(.13)</td>
</tr>
<tr>
<td></td>
<td>14.57</td>
<td>2010(11)</td>
<td>.92</td>
<td>3.3(.10)</td>
</tr>
<tr>
<td>Ethyl-2-(5-n-propyl)-furoate$^c$</td>
<td>2.27</td>
<td>469(6)</td>
<td>-.16$^d$</td>
<td>.471(.01)</td>
</tr>
<tr>
<td></td>
<td>8.98</td>
<td>1798(11)</td>
<td>-.05</td>
<td>.740(.01)</td>
</tr>
<tr>
<td></td>
<td>17.61</td>
<td>3059(15)</td>
<td>-.09</td>
<td>.840(.01)</td>
</tr>
<tr>
<td></td>
<td>21.49</td>
<td>3857(21)</td>
<td>-.06</td>
<td>.830(.01)</td>
</tr>
</tbody>
</table>

$^a$ Linear regression analysis of the data for $A_1$ vs concentration gives $r = .9998$, $b = -15(13)$, $m = 138.6(1.8) \times 10^6 \text{ M}^{-1}$.

$^b$ Values in parenthesis represent the estimated standard deviations. Fit values from equation 29.

$^c$ Linear regression analysis of the data for $A_1$ vs concentration gives $r = .9998$, $b = 72(79)$, $m = 175(6) \times 10^6 \text{ M}^{-1}$.

$^d$ Fit values from equation 29b where $A_2$ is the time lag in the ester appearance before the data becomes first order.
Figure 9 shows the same plot for the ethyl-2-(5-n-propyl)-furoate. The data can also be analyzed by a linear regression analysis. In this way, the data of Figure 8 gives a linear correlation coefficient of .9998, an intercept of -15(+13) in intensity units and a slope of 138.6(+1.75) M$^{-1}$. The estimated sensitivity level is .1 µM at a signal to noise ratio of 2.

The membrane in acting as a single stage extractor depletes the solution of the permeant molecule. To determine the rate of this diffusional loss, the decrease in the ion intensity after a single rapid increase in the solution of either ester was measured as a function of time. Kinetically, this is a first order process, and the data of Figures 10 and 11 were fit accordingly. The half time for the loss was then calculated and for the ethylfuroate was found to be 13.3(+44) minutes. The rate of loss for the ethyl-2-(5-n-propyl)-furoate was calculated to be 11.6 (+.30) minutes. In both cases, this rate was considerably smaller than the rate to reach the maximal signal intensity during the steady state transport. In the case of ethylfuroate, the ratio of the two rates was greater than 61, while in the case of the other substrate the ratio of the rates was greater than 10. Under these circumstances, if the analysis is not extended over too long a time range, the contribution of the loss due to diffusion to the observed rate will not be significant.

3.5.1.2 Comparison of the ethanolysis rate constants

The rate of ethyl ester formation was determined both spectrophotometrically and mass spectrally. The spectroscopic method was that of
Wang et al. (31). Under the conditions of assay, $[E_0] < [S_0]$ while $[S_0] > K_m$, a steady state release of p-nitrophenol was assured. The pK for the rate process was shown to be around 7 (31). The rate measurements were carried out in phosphate buffer at pH 8.5 and corrected to pH independent values by equation 15. To ensure steady state conditions, $[S_0]$ must be greater than $K_m$. As shown in Figure 24, the $K_m$ for the n-propyl substrate is less than .1μM which is much less than the $[S_0]$ of 20 μM for the spectroscopic rate measurement and the value of $[S_0] = .5$ mM in the mass spectral measurement.

The time dependence of the ion intensity during enzymatic production of ester, is given by equation 33. Figures 16 and 17 show that the theoretical equation fits the observed data for the ethyl-2-furoate and ethyl-2-(5-n-propyl)-furoate production very well. As predicted by equation 33, the curve can be divided into an initial first portion followed by a linear steady state production of ester. The slope of the linear region can be used to obtain a value for the second order transesterification rate constant. The slope of the linear region is given by $A_1 k_o E_0$ as indicated in equation 33. Therefore, the slope can be divided by $E_0$ (the total enzyme concentration) and $A_1$ (which is derived from the slope of a plot such as that in Figure 8) to obtain $k_o$, which is the pseudo-first order rate constant for the ethyl ester production. The true second order rate constant can be obtained by dividing $k_o$ by the ethanol concentration. The data for the comparison of the two methods is given in Table 8. The rate constants are the same within experimental error for both methods and both substrates. The larger standard deviations in the mass spectral measurements arise
**Figure 15**

Mass spectrometer signal response during the enzymatic production of ethyl-2-furoate. The curve on the top is a tracing of the chart recording with the mass spectrometer scanning the base peak (m/e=112). The solid curve was calculated from the best fit of equation 33 to the points.
Figure 16
Mass spectrometer signal response during the enzymatic production of ethyl-2-(5-n-propyl)-furoate. The solid curve is calculated from the best fit of equation 33 to the points.
from the error associated with the measurement of $E_0$ (see section 3.2.3.2).

Equation 33 indicates that the first order rate constants for the pre-steady state should be contained in the first order portion of the curve given in Figure 16. However, stopped flow spectrophotometric measurements of the burst release of p-nitrophenol indicated the pre-steady state portion of the enzymatic reaction had a half life of 2 seconds at 25°C, Table 9. This is too fast to observe in the present system as the response time is on the order of 13 seconds. This is reflected in the best fit value for the $k_1$ rate constant (equation 33) obtained in the fit of the data for Figure 16. The computer calculated a value of $k_1$ equal to $9(\pm20) \times 10^8$ second$^{-1}$. The value of the standard deviation indicates the inability to obtain that rate constant using the present method. It should be pointed out that the initial first order portion of the curve is due solely to the diffusional transport as it approaches a steady state.

The fact that the observed rates for both substrates determined with both methods agreed fairly well confirms the assumption that the diffusional loss of product does not interfere with the rate determinations. The time scale over which an enzyme kinetic run is performed is on the order of 3-5 minutes for both substrates. On this short of a time scale, the diffusional loss would not be expected to interfere. In looking at the data of Figures 16 and 17, it is apparent that the best fit linear line is a true representation of the data and that no systematic deviation below the best fit line is apparent at the longer times. If the assumption that the loss of product by diffusion was
Table 8
Comparison of the steady state rates of ethanolsis determined by the mass spectral method and spectrophotometrically.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Method</th>
<th>$k_{\text{ROH}} \text{M}^{-1}\text{min}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNP-furoate</td>
<td>MS\textsuperscript{a}</td>
<td>.47(.067)</td>
</tr>
<tr>
<td></td>
<td>Spectro.\textsuperscript{b}</td>
<td>.51(.020)</td>
</tr>
<tr>
<td>PNP-2-(5-n-propyl)-furoate</td>
<td>MS</td>
<td>.12(.012)</td>
</tr>
<tr>
<td></td>
<td>Spectro.\textsuperscript{c}</td>
<td>.098(.003)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Determined as outlined in section 3.2.3.2 at 25°C.

\textsuperscript{b} Determined as outlined in section 3.2.3.1 at 25.3°C.

\textsuperscript{c} From reference 103.

\textsuperscript{d} PNP refers to p-nitrophenol.
Table 9

Stopped flow determination of the rate of the burst release of p-nitrophenol during the acylation of chymotrypsin with PNP-2-furoate.\textsuperscript{a}

<table>
<thead>
<tr>
<th>(PNP-2-furoate) x 10^6 M</th>
<th>(k_{\text{burst}} \text{ sec}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>1.297 (.004)</td>
</tr>
<tr>
<td>80</td>
<td>1.279 (.005)</td>
</tr>
<tr>
<td>40</td>
<td>1.272 (.004)</td>
</tr>
<tr>
<td>40</td>
<td>1.342 (.005)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Temperature 25.5°C, \([E_0]\) = 50 \(\mu\)M, [ETOH] = .5 M, the final buffer concentration was .1 M \(K_2\)HPO\(_4\) pH 8.5. The values in parenthesis represent one standard deviation.
incorrect, then that first order process could be included in the derivation of equation 33.

3.5.2 Kinetic isotope effects in transesterifications

The isotope effects to be discussed involve the ethoxyl oxygen of ethanol ($^{18}$O). In equation 5, the reaction to produce the ester is diagrammed. There is a competition between the ethanol and water in the nucleophilic reaction with the acyl enzyme. At 25°C, the percentage of the reaction proceeding to the ester is 75% for the furoyl substrate and 50% for the 5-n-propyl-furoyl substrate at an ethanol concentration of .5 M. Substitution of $^{18}$O labeled ethanol in the reaction will give a corresponding decrease in the amount of each isotopic ester produced. That is, at an ET$^{16}$OH/ET$^{18}$OH ratio of one, only 37% of the product is either $^{16}$O or $^{18}$O ethyl-2-furoate.

3.5.2.1 $^{18}$O Ethanolysis KIE with p-nitrophenyl-2-furoate

As outlined in equation 35, the true KIE depends on the initial ratio of the isotopic ethanols. The determination of this ratio by the formation of the ethyl ester of furoic acid was determined in two ways. The addition of the ester to the cell resulted in a first order increase in the ion intensity with time. The curve was fit to equation 29 for the appearance of both isotopic esters. The ratio of the plateau levels for the ion intensities of the two isotopic ethyl esters was taken as the ratio of the initial isotopic ethanols (I). The second calibration involved injection of the isotopic ethyl esters into the GC attached to the MS. Scanning the effluent from the GC under the
same conditions as (I) above allowed determination of the areas under the two mass peaks (140, $^{16}$O ester, 142, $^{18}$O ester). The ratio of these areas were taken as the ratio of the initial isotopically labeled ethanol. For comparison, the values of the ratios for the two procedures are given in Table 9. The ratio determined by both methods agreed within the respective standard deviations. In subsequent determinations of this ratio the first order procedure was followed.

The value of the $^{16}$O/$^{18}$O KIE was determined by comparing the slopes of the linear region produced during the enzymatic catalyzed formation of the ethyl ester. The ratio of the isotopic ethanol was varied from 0.112 to 2.73. The m/e value which was scanned was also varied. Either the molecular ion (m/e=140 for the $^{16}$O ester) or the base ion (m/e-112 for the $^{16}$O ester) region was scanned. The data presented in Table 11 indicates no significant variation in the apparent KIE when these two variables are changed. The data for a KIE determination is plotted in Figure 18. The weighted average of the apparent KIE determined was 1.013 (+.004), Table 11. In Figure 18 it is apparent that a change over in the ratio of the isotopic esters occurs at about 1 minute. The time region from 0 to 1 minute shows the ratio $^{16}$O/$^{18}$O to be greater than 1, while from 1 minute to the end of the reaction, the $^{16}$O/$^{18}$O ratio is less than 1. This can better be seen in Figure 20 which is a replot of the data from Figure 18. Figure 19 has the ratios of the individual data points plotted as a function of time. The implication is that the apparent KIE is changing in the first minute from a value of about 1.1 to the final apparent KIE of 1.004. The error associated with this representation is rather large, however, the trend does appear real within and between runs.
Table 10
Comparison of the methods for determining the $^{16}_0/^{18}_0$ ratio of the ethanol used in the KIE determination.

<table>
<thead>
<tr>
<th>Method</th>
<th>Ratio($^{18}_0/^{16}_0$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC$^a$</td>
<td>1.182$^c$</td>
</tr>
<tr>
<td></td>
<td>1.164</td>
</tr>
<tr>
<td></td>
<td>1.161</td>
</tr>
<tr>
<td></td>
<td>AVG. 1.169(.009)</td>
</tr>
<tr>
<td>First order$^b$</td>
<td></td>
</tr>
<tr>
<td>plateau levels</td>
<td>1.166(.006)$^d$</td>
</tr>
<tr>
<td></td>
<td>1.159(.007)</td>
</tr>
<tr>
<td></td>
<td>1.160(.007)</td>
</tr>
<tr>
<td></td>
<td>AVG. 1.162(.005)</td>
</tr>
</tbody>
</table>

$^a$ GC separation of the reaction mixture in which the ethyl ester was formed from the isotopically labeled ethanol. The GC peak corresponding to the ethyl ester was scanned by the MS in the selective ion mode.

$^b$ The reaction mixture from $^a$ (see above) was injected into the cell attached to the MS. The MS operated in the selected ion mode was used to follow the first order appearance of the esters parent peak.

$^c$ The ratio of the area of GC peaks for $^{18}_0$-ethyl-2-furoate and $^{16}_0$-ethyl-2-furoate.

$^d$ The ratio of the $A_1$ values from equation 29 for the two isotopic ethyl esters.
Figure 17
Time course for the ion intensity increase during the enzymatic production of ethyl-2-furoate. Circles are for the $^{16}$O-ethanol and the squares are for the $^{18}$O-ethanol.
Figure 18

Plot of the ratios of the ion intensities of the two isotopic ethyl esters as a function of time, demonstrating a change in the apparent KIE.
The graph shows the relationship between time (in minutes) and the ratio $I_{112}/I_{114}$, with apparent KIE values on the vertical axis. The data points indicate a gradual increase in the ratio with time, suggesting a changing KIE over time.
Table 11

$^{16}O^{18}O$ apparent KIE for the ethanolysis of PNP-2-furoate catalyzed by chymotrypsin.\(^a\)

<table>
<thead>
<tr>
<th>m/e Region</th>
<th>$k_{16}^{obs}$/$k_{18}^{obs}$</th>
<th>$\frac{ET_{18}^{OH}}{ET_{16}^{OH}}$</th>
<th>Apparent KIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>140/142(^b)</td>
<td>.863(.004)(^c)</td>
<td>1.163(.004)(^d)</td>
<td>1.004(.005)(^e)</td>
</tr>
<tr>
<td>140/142</td>
<td>.593(.007)</td>
<td>1.683(.030)</td>
<td>.998(.021)</td>
</tr>
<tr>
<td>112/114</td>
<td>.920(.004)</td>
<td>1.106(.010)</td>
<td>1.017(.010)</td>
</tr>
<tr>
<td>112/114</td>
<td>.372(.003)</td>
<td>2.734(.029)</td>
<td>1.016(.013)</td>
</tr>
<tr>
<td>112/114</td>
<td>9.111(.059)</td>
<td>.1117(.001)</td>
<td>1.018(.011)</td>
</tr>
<tr>
<td>AVG.(^f)</td>
<td></td>
<td></td>
<td>1.013(.004)</td>
</tr>
</tbody>
</table>

\(^a\) At 25°C in .1 M PO\textsubscript{4} buffer. [E\textsubscript{0}] = 40-60 M, [ETOH\textsubscript{tot}] = .5M, [S\textsubscript{0}] = .8 mM.

\(^b\) The parent or base peaks for the $^{16}O^{18}O$ labeled esters.

\(^c\) The ratio of the slopes of the linear portion of the I vs time curve for enzymatic formation of the ethyl esters.

\(^d\) The ratio of the ethyl ester formed as indicated in section 3.2.3.4. Expressed as the ratio of the $A_1$ values from equation 29.

\(^e\) Calculated as indicated in equation 35.

\(^f\) The average is weighted based on the standard deviations.
The KIE associated with the hydroxide ion catalyzed hydrolysis of the ethyl-2-furoate (ethoxyl-\(^{18}\)O) was determined as a further check on the method. A mixture of \(^{16}\)O/\(^{18}\)O ethyl esters (total ester concentration of 20 \(\mu\)M) was hydrolyzed in 1 mM NaOH while the ion intensity at m/e 140 and 142 was monitored. The pseudo-first order rates were determined and are presented in Table 12. The observed rates are the sum of the hydrolysis and diffusion rates. The diffusion rates were determined in water in a separate run. The difference between the observed rates and the diffusion rates give the true apparent pseudo-first order hydrolysis rates. As seen in Table 12, the apparent KIE for the hydrolysis is 1.012 (+.0099). It should be noted that there is essentially no isotopic discrimination in the diffusion process as the ratio of the first order diffusion rates are 1.006 (+.006).

3.5.2.2 \(^{18}\)O Ethanolysis KIE with p-nitrophenyl-2-(5-n-propyl)-furoate (PFAPNP)

The apparent KIE associated with the ethanolysis of PFAPNP was determined as outlined in section 3.5.2.1. The isotopic ethanol ratios were varied from 2.141 to .924. The apparent KIE was determined at m/e pairs of 182, 184 (the molecular ion) and 154, 156 (the base peak, from loss of ethylene). The data for the apparent KIE at 25\(^{\circ}\)C is presented in Table 13. At either m/e pair or isotopic ethanol ratios, the apparent KIE was constant at a weighted average value of .90 (+.01). A plot of the KIE determination is shown in Figure 20. The curve shows the change over in the isotope ratio for the \(^{18}\)O labeled ester, in that the curve for the \(^{18}\)O labeled ester initially lies below the curve for
Table 12

$^{16}_0/^{18}_0$ KIE for the hydroxide ion catalyzed hydrolysis of $^{16}_0/^{18}_0$ ethyl-2-furoate(ethoxyl-$^{18}_0$).

<table>
<thead>
<tr>
<th>[OH$^-]\times10^{3}$M</th>
<th>$k_{16}$ min$^{-1}$</th>
<th>$k_{18}$ min$^{-1}$</th>
<th>$\frac{k_{16}}{k_{18}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>.0001$^a$</td>
<td>.05581(.0002)$^c$</td>
<td>.05546(.0002)</td>
<td>1.006(.006)</td>
</tr>
<tr>
<td>1.0</td>
<td>.1247(.0012)$^d$</td>
<td>.1234(.0012)$^d$</td>
<td>1.011(.014)</td>
</tr>
<tr>
<td>1.0</td>
<td>.1241(.0012)$^d$</td>
<td>.1225(.0012)$^d$</td>
<td>1.013(.014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AVG. 1.012(.010)$^e$</td>
</tr>
</tbody>
</table>

$^a$ Reaction was run in H$_2$O.

$^b$ Rate determined from the best fit value obtained from a fit of the data to equation 30.

$^c$ Values in parenthesis are the estimated standard deviations.

$^d$ Rates have been corrected for the contribution of the diffusion process.

$^e$ Average weighted according to the estimated standard deviations.
Table 13

$^{16}O/^{18}O$ apparent KIE for the ethanolysis of PNP-2-(5-n-propyl)-furoate catalyzed by chymotrypsin.$^a$

<table>
<thead>
<tr>
<th>Region</th>
<th>$k_{16}^{obs}/k_{18}^{obs}$</th>
<th>$\frac{ET^{18}OH}{ET^{16}OH}$</th>
<th>Apparent$^e$ KIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>182/184</td>
<td>.85(.003)</td>
<td>1.064(.013)</td>
<td>.90(.011)</td>
</tr>
<tr>
<td>154/156</td>
<td>.81(.004)</td>
<td>1.073(.042)</td>
<td>.87(.030)</td>
</tr>
<tr>
<td>154/156</td>
<td>.81(.003)</td>
<td>1.091(.015)</td>
<td>.88(.013)</td>
</tr>
<tr>
<td>154/156</td>
<td>.413(.002)</td>
<td>2.141(.022)</td>
<td>.88(.011)</td>
</tr>
<tr>
<td>182/184</td>
<td>.833(.005)</td>
<td>1.091(.015)</td>
<td>.91(.014)</td>
</tr>
<tr>
<td>182/184</td>
<td>.843(.004)</td>
<td>1.091(.015)</td>
<td>.92(.013)</td>
</tr>
<tr>
<td>182/184</td>
<td>1.001(.006)</td>
<td>.952(.016)</td>
<td>.93(.016)</td>
</tr>
<tr>
<td>AVG.</td>
<td></td>
<td></td>
<td>.90(.005)$^f$</td>
</tr>
</tbody>
</table>

$^a$ At $25^\circ$C in .1 M PO$_4$ buffer pH 8.5. [E$_o$] = 60-80 µM, [ETOH]$_{tot}$ = .5M, [S$_o$] = .5 µM.

$^b$ The base or parent peak in the mass spectrum of the $^{16}O/^{18}O$ ethyl esters.

$^c$ The ratio of the slopes for the linear portion of the I vs time curve for enzymatic formation of the ethyl esters.

$^d$ The ratio of the ethyl esters formed as indicated in section 3.2.3.4. Expressed as the ratio of the $A_1$ values from equation 29b.

$^e$ Calculated as indicated in equation 35.

$^f$ The average is weighted based on the standard deviations.
the $^{16}O$ ester, while after 2 minutes the curve for the $^{18}O$ ester is above the curve for the $^{16}O$ ester. A replot of the data is shown in Figure 21. The apparent KIE begins with a value of approximately 1.1, decreasing with time to a value of .90. This change in the apparent KIE is relatively constant from run to run.

The variation of the isotope effect with pH at 25°C was measured over the pH range of 6.5 to 8.5. The data presented in Table 14 shows an apparent KIE decrease (i.e. the KIE becomes closer to 1) in going from high to low pH. The value of the apparent KIE at pH 6.5 being .97 with the value increasing to .90 at pH 8.5. The data is plotted in Figure 22. Each data point represents the average of two determinations except the values at pH 6.5 and 8.5. The line is calculated from the best fit values obtained when the KIE vs pH data was fit to equation 15 (with a slight nomenclature change). The pH obtained was 7.34.

The temperature dependence of the apparent KIE was determined over the range 18°C to 38°C. The data in Table 14 indicates the maximal isotope effect occurs at the low temperature end of the data, and is changing relatively slowly with temperature, however, in the region 23°C to 27°C there is a sharp increase in the apparent KIE, followed by a temperature range in which the apparent KIE reaches its minimal value (i.e. near to 1) during which the change with temperature is again relatively small. The data is plotted as the natural logarithm of the apparent KIE vs the inverse of the absolute temperature in Figure 23. The data are for a solution pH of 8.5. Since the diffusion process also has a temperature dependence (101), the variation of the first order rate for diffusion with temperature was studied at
Figure 19

Time course for the ion intensity increase during the enzymatic production of ethyl-2-(5-n-propyl)-furoate. Circles are for the $^{16}$O-ester, squares are for the $^{18}$O-ester.
Figure 20

Plot of the ratios of the two isotopic esters ion intensities as a function of time.
Figure 21

pH dependence of the apparent $^{16}O/^{18}O$ KIE associated with the ethanalysis of PNP-2-(5-n-propyl)-furoate catalyzed by chymotrypsin.
Table 14

pH variation of the $^{16}_0/^{18}_0$ ethanolysis KIE catalyzed by chymotrypsin.

<table>
<thead>
<tr>
<th>pH$^a$</th>
<th>$^{16}_0/^{18}_0$ KIE$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>0.97(.01)</td>
</tr>
<tr>
<td>7.0</td>
<td>0.95(.01)</td>
</tr>
<tr>
<td>7.5</td>
<td>0.93(.01)</td>
</tr>
<tr>
<td></td>
<td>$pK_a=7.34(.04)^c$</td>
</tr>
<tr>
<td>8.0</td>
<td>0.92(.01)</td>
</tr>
<tr>
<td>8.5</td>
<td>0.90(.005)</td>
</tr>
</tbody>
</table>

$^a$ At 25°C. The buffer was .1 M K$_2$PO$_4$ pH adjusted to the indicated value with HCl prior to alcohol addition. [E$_0$] = 60 to 100 μM [S$_0$] = .5 mM, [ETOH] = .5 M.

$^b$ The values in parentheses are the estimated standard deviations.

Each KIE is the average of two determinations, except the value at pH 6.5 (one determination) and pH 8.5.

$^c$ $pK_a$ calculated from a non-linear regression analysis based on equation 15.
Table 15

Temperature dependence of the $^{16}O/^{18}O$ KIE for the ethanolyis of PNP-2-(5-n-propyl)-furoate catalyzed by chymotrypsin.\(^a\)

<table>
<thead>
<tr>
<th>Temperature °C(^b)</th>
<th>Apparent KIE(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.2</td>
<td>0.87(0.010)</td>
</tr>
<tr>
<td>20.5</td>
<td>0.90(0.016)</td>
</tr>
<tr>
<td>23.0</td>
<td>0.87(0.013)</td>
</tr>
<tr>
<td>25.3</td>
<td>0.90(0.005)</td>
</tr>
<tr>
<td>27.2</td>
<td>0.92(0.013)</td>
</tr>
<tr>
<td>29.2</td>
<td>0.94(0.016)</td>
</tr>
<tr>
<td>30.0</td>
<td>0.95(0.014)</td>
</tr>
<tr>
<td>33.0</td>
<td>0.95(0.013)</td>
</tr>
<tr>
<td>38.2</td>
<td>0.96(0.015)</td>
</tr>
</tbody>
</table>

\(^a\) Buffer was 0.1 M $K_2HPO_4$ pH 8.5. $[E_0] = 60-100$ µM, $[S_0] = 0.5$ mM, 
$[ETOH]_{tot} = 0.5$ M.

\(^b\) Temperature measured by a Cenco thermometer immersed in the cell filled with water before and after the reaction.

\(^c\) Each value is from a single determination. The values in parentheses represent the estimated standard deviations.
Figure 22
Arrhenius type plot for the temperature dependence of the apparent KIE. The bars around each point represent the estimated standard deviations.
Table 16

Temperature dependence of the first order rate for the diffusional loss of ethyl-2-(5-n-propyl)-furoate (ethoxyl-16\textsuperscript{0}/18\textsuperscript{0}) from the cell solution.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Temp. °C\textsuperscript{b}</th>
<th>$k^{16}\text{min}^{-1}$</th>
<th>$k^{18}\text{min}^{-1}$</th>
<th>$k^{16}/k^{18}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.2</td>
<td>.240(.006)</td>
<td>.239(.006)</td>
<td>1.004</td>
</tr>
<tr>
<td>25.8</td>
<td>.085(.008)</td>
<td>.086(.008)</td>
<td>.993</td>
</tr>
<tr>
<td>18.2</td>
<td>.058(.0002)</td>
<td>.057(.0002)</td>
<td>1.012</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The cell contained .4 ml of water. The total concentration of the esters was 30\textmu M. The values in parentheses represent the estimated standard deviations.

\textsuperscript{b} Temperature measured by immersion of a Cenco thermometer in the cell before and after measurement of the rates.
Figure 23

Reciprocal plot for the initial rate of hydrolysis of PNP-2(5-n-propyl)-furoate vs the substrate concentration at varying concentrations of ethyl-2(5-n-propyl)-furoate (EF).
A) (EF) = 0.0; B) (EF) = .135 mM; C) (EF) = .230 mM; D) (EF) = .700 mM.
Inset is a replot of the slopes vs the EF concentration.
38°, 25°, and 18°C. The data in Table 16 indicates no isotopic discrimination in the ester diffusion process. The diffusion rate does have a strong temperature dependence, however.

The possibility that the ester product of the reaction was acting as an inhibitor in the enzyme catalyzed steady state transesterification reaction was discounted by measuring the $K_i$ of the ethyl ester. The data is presented in Figure 24 in double reciprocal form. A replot to determine the $K_i$ is also shown in Figure 24. The $K_m$ for the p-nitrophenyl-2-(5-n-propyl)-furoate was less than 1 μM while the $K_i$ of the ethyl ester in the hydrolysis reaction was determined as .40 mM.

3.6 Discussion

The method described in this work allows the use of a mass spectrometer as a detector for a chemical reaction without sample preparation prior to analysis. It is a continuous in situ method. The membrane allows permeant molecules to be concentrated, increasing their partial pressure in the mass spectrometer, relative to their solution concentrations. Since a reaction can be run with heavy and light isotopic substrates, with simultaneous detection of the isotopic products, errors normally associated with direct KIE measurements (i.e. enzyme concentration differences, temperature differences, and pH differences) are eliminated.

The ion intensity response has been shown to be linear with ester concentration from .1 μM to .1 mM. This allows rate measurements over a wide concentration interval without any corrections for non-linearity being required. The sensitivity, measured at a signal to noise ratio
of 2, is .1 µM. This allows analysis of very dilute solutions. This corresponds to the sensitivity normally obtained with a spectrophotometer. The sensitivity is really a function of the time spent in scanning over a peak. In the normal operation of a mass spectrometer this time is on the order of 10-50 milliseconds. By scanning in the manner described in this work, the time spent on one peak can be extended up to 3 seconds. In the experiments reported here, the average time spent in scanning over a peak was 1.2 seconds. The response time for the appearance of the esters mass spectrum was relatively rapid \( t_{1/2} = 12 \text{ seconds} \). This allows the determination of reaction rates as slow as 1.2 min\(^{-1}\) and as fast as 5 min\(^{-1}\).

The response time and sensitivity are dependent on a number of factors among which are the vapor pressure and hydrophobicity of the compound to be measured \((81, 91)\), the chemical nature and thickness of the membrane, the cell and source inlet geometries and the signal to noise characteristics of the mass spectrometer. Of these, the response time can most effectively be decreased by using a thinner membrane. Equation 28 shows that the response time is inversely related to the square of the membrane thickness. With the present cell, a decrease of 25% in response time was observed in going from a membrane thickness of 25 µm to 20 µm. According to equation 28, this should have resulted in a 36% reduction in the response time. The difference is assumed due to some resistance to transport in the inlet system. Further modifications in cell design and inlet system arrangement may allow considerable improvement in the response characteristics of the cell.
Although the mechanism for the enzyme catalyzed reaction (scheme VI) and the empirically reduced equation for diffusion (equations 29 and 29b) are not a complete representation of the process occurring during a measurement, equation 33 does provide a suitable representation of the data (Figures 15 and 16). Several factors were not explicitly considered, including the presence of an unstirred diffusion boundary layer on the solution side of the membrane or the time required to reach solute partitioning equilibrium between the solution and the membrane. Still, the correlation between the second order rate of ethanolysis measured spectrophotometrically and mass spectrally was good. This is a good indication of the accuracy of the method. The error associated with determining the total enzyme concentration has contributed disproportionately to the total error for the mass spectrally measured ethanolysis rate. The ability to collect many data points during a reaction, actually defines the relative rates of formation of the isotopic esters to high precision. In a KIE measurement, only the ratio of the relative rates is required and therefore this ratio can be determined with very high precision (generally on the order of .5% with the current system).

The ability to measure isotope effects with this method was characterized by determining the \( ^{16}O/^{18}O \) KIE associated with the hydroxide ion catalyzed hydrolysis of ethyl-2-furoate (ethoxy-\(^{18}O\)). The observed KIE of 1.012 (.009) is in good with literature values for hydroxide ion catalyzed ester hydrolysis. As reviewed in the background to this chapter, Sawyer obtained an \( ^{16}O/^{18}O \) KIE of 1.0091 (68) for hydroxide ion catalyzed hydrolysis of methyl formate (methoxy-\(^{18}O\)). O'Leary
reported a similar value of 1.0062 (70) for the hydroxide ion catalyzed
hydrolysis of methyl benzoate (methoxy-\textsuperscript{18}O). The interpretation of
the result is that the hydroxide ion attack leading to formation of a
tetrahedral intermediate is rate limiting. The breakdown of the tetra-
hedral intermediate to form the acid and the alcohol occurs rapidly.
The transition state for the reaction involves only limited bond cleav-
age at the site of isotopic substitution and therefore only a small
KIE will be observed.

The KIE's observed in the enzyme catalyzed transesterification
reactions were quite different. The steady state value of 1.013 (.004)
seen with the 2-furoic acid derivative compares with the values obtained
by Sawyer (68) in the hydrolysis of N-acetyl-L-tryptophan-ethyl ester
(KIE = 1.018) and N-carbethoxy-L-tryptophan-ethyl ester (ethoxy-\textsuperscript{18}O)
(KIE = 1.0117). The steady state value for the KIE observed when
using 2-(5-n-propyl)-furoic acid was .90(.005). This value has no
comparable precedent in the literature, except for Wang et al. (34)
who studied the same system. In addition, both substrates demonstrated
a time dependent change in the KIE value, with the early time regime
having a large normal KIE which decreased until the steady state value
for the KIE was reached.

The large steady state KIE can not be due to hydrolysis of the
ethyl ester of 2-(5-n-propyl)-furoate during steady state hydrolysis of
the p-nitrophenyl ester was .40 mM. In as much as the $K_i$ measures the
catalytically competent association of the ethyl ester with chymotryp-
sin, the ethyl ester can not effectively compete with the p-nitrophenyl
ester for the enzyme until greater than 90% reaction has taken
place. This extent of reaction is never achieved under the present conditions.

The value for the KIE depends on the independently measured isotope ratio of the starting ethanol. This ratio is obtained from the ethyl ester of the corresponding acid formed from reaction of the acid chloride and the isotopic ethanol. The reaction was shown to proceed with greater than 99% of the ethanol reacting by GC analysis of the residual ethanol. An isotope effect on this chemical conversion of even 3% (KIE = 1.03) would effect the observed ratio by less than .5% at this extent of reaction (107). This is within the reported error of the KIE.

Both the magnitude and time dependent change in the KIE can be interpreted in terms of scheme VII.

\[
\begin{align*}
E + S & \xrightarrow{k_1} E \cdot S \xrightarrow{k_2} E \cdot A \xrightarrow{k_5 [ETOH]} T \xrightarrow{k_7} E + P_2
\end{align*}
\]

scheme VII

Where \( E \), \( S \), \( E \cdot S \), \( EA \), \( T \), \( ETOH \), \( P_1 \), and \( P_2 \) are the concentrations of the free enzyme, substrate, Michaelis complex, acyl enzyme, a postulated intermediate (the tetrahedral intermediate ), ethanol, p-nitrophenol and ethyl ester respectively. The integrated solution of the kinetic scheme can be accomplished by assuming that the concentrations of \( S \) and \( ETOH \) do not change over the course of the enzymatic reaction. Using the Laplace-Carson transform (95) the solution for the time dependent change in the ethyl ester concentration, as given in Wang et al., is:

\[
P_2 = A_1 [E_0] \left[ \frac{t}{A_2} + \frac{3 - A_3 t - 1}{A_4} + \frac{e^{-A_5 t - 1}}{A_6} + \frac{e^{-A_7 t - 1}}{A_8} \right] \tag{36}
\]
Where the A's represent collections of the individual rate constants of scheme VII, \( t \) is the time, and \( E_o \) is the total enzyme concentration.

By use of a computer, equation 36 can be solved for the concentration of \( P_2 \) as a function of time for both \( ^{16}O \) and \( ^{18}O \) ethanol. A judicious choice of the values for \( k_5, k_6, \) and \( k_7, \) produces a computer calculated change in the isotope effects as a function of time. This is most readily observed when the KIE for \( k_6 \) is large relative to the KIE associated with \( k_5 \) and \( k_7 \) and the absolute magnitude of \( k_6 \) and \( k_7 \) are comparable. This means that the reversal of the intermediate \( T \) to \( EA \) occurs rapidly (relative to \( P_2 \) formation) and with a relatively large KIE (compared to the KIE associated with \( k_5 \)) thereby enriching \( T \) in \( ^{18}O \) label. The \( ^{16}O \) ethanol, being more reactive in the \( k_5 \) step, reacts faster initially, however, the product ratio changes due to the large normal KIE associated with \( k_6 \) until the steady state is reached and the KIE decreases to a constant value. By assuming steady state conditions, the system can be somewhat simplified (103). The steady state treatment for the intermediate \( T \) yields,

\[
T = \frac{k_5[EA][ETOH]}{k_6 + k_7} \tag{37}
\]

and therefore the rate of \( P_2 \) production in the steady state is

\[
P_2 = \frac{k_5k_7[EA][ETOH]}{k_6 + k_7} + P_{2,0} \tag{38}
\]

Where \( P_{2,0} \) is the concentration of \( P_2 \) extrapolated to \( t=0 \) and can be ignored as \( t \) becomes large. The KIE for \( P_2 \) production can be expressed as:
Where the asterisks refer to the $^{18}$O labeled compounds or the rate associated with the heavy atom substitution. The isotope effect on $k_7$ should be small as it is essentially a secondary $^{18}$O KIE and does not involve bond breaking at the site of isotopic substitution. Two limiting cases can arise depending on the relative magnitude of $k_6$ and $k_7$. If (I) $k_6 > k_7$ then equation 39 reduces to

$$
\text{KIE} = \frac{k_{\text{obs}}^*}{k_{\text{obs}}} = \frac{k_{k_6^*}[\text{ETO}]^*}{k_{k_7}[\text{ETO}]^*} = \frac{k_5 k_7 (k_6^* + k_7^*)}{k_5 k_7 (k_6 + k_7)}
$$

(39)

Alternatively, if $k_7 > k_6$ (II), then equation 39 reduces to

$$
\text{KIE} = \frac{k_5 k_7^*}{k_5 k_6}
$$

(40)

If II were the case, then the final steady state KIE would reflect the isotope effect on the formation of T. To obtain an inverse isotope effect for the process would mean that in going through the transition state between EA and T, there is a narrowing of the potential energy well for the transition state relative to that for EA. This narrowing leads to a greater energy difference in the zero point energy (ZPE) levels for $^{16}$O and $^{18}$O vibrations in the transition state relative to the ZPE levels in EA. This produces a greater activation barrier for the light isotope ($^{16}$O), causing the $^{16}$O ethanol to react slower. If case I were valid, then the final KIE would be a reflection of the equilibrium isotope effect for the $^{18}$O ethanol addition to the acyl enzyme to form T. An inverse isotope effect will be observed, even
though a normal isotope effect for both the $k_6$ and $k_5$ steps would occur, if $k_6/k_6^* > k_5/k_5^*$. The final KIE in equation 40 would be less than one.

The mechanism of case I has the effect that in the time required to reach the steady state concentration of $T$, the isotopic composition of $T$ is changing. In the steady state, $T$ is enriched in $^{18}O$ by virtue of the larger KIE on $k_6$ relative to the KIE of $k_5$, implying $T^*$ is more stable than $EA^*$. The $^{18}O$ enrichment of $T$ may be due to the relative isotope effects expected for bond breaking versus bond forming reactions. Bigeleisen (102) indicates that a larger KIE can be expected for the bond breaking step. For equilibrium reactions, the discrimination between bond breaking and bond forming steps leads to an equilibrium KIE. If $T$ is the tetrahedral intermediate, then the stability of $T^*$ relative to $EA$ plus $ETO^*H$ reflects the preference of $^{18}O$ for the stiffer bond in the tetrahedral intermediate. A model which suggests the preference of $^{18}O$ for $T^*$ can be found in the equilibrium isotope effect for transfer of $^{18}O$ in dimethyl ether to methanol in the gas phase. The equilibrium isotope effect for this transfer is 1.022, favoring $^{18}O$ in the dimethyl ether (112).

The magnitude of the final KIE is relatively large for 5-n-propyl derivative. The reason for this large magnitude is problematic, but may be accounted for if two or more kinetic steps of approximately equal rates have relatively large KIE's associated with them and these KIE's are multiplicative. That is, if one step can have a KIE of 1.08 and a second 1.08, the product of these two KIE's would be 1.166. A possibility for the second step may be the general acid assisted hydrogen bond to the ethoxyl oxygen formed during the break down of $T$ to $EA$. 
That there may be a difference between $^{16}\text{O}$ and $^{18}\text{O}$ in the ability to form this hydrogen bond may be reflected in the dimerization constant of $\text{H}_2\text{O}^{16}$ relative to $\text{H}_2\text{O}^{18}$. Pinchas (105, 106) has reported an anomalously low dimerization constant for $\text{H}_2\text{O}^{18}$ in dilute dioxane solutions. At a concentration of .336 M $\text{H}_2\text{O}$ in dioxane the ratio of the dimerization constant for $\text{H}_2\text{O}^{16}$ to $\text{H}_2\text{O}^{18}$ was 1.6. He ascribed this large difference to the decreased ability of $^{18}\text{O}$ to form a hydrogen bond in the dimer formation process. If this is indeed the case, then in the general acid catalyzed decomposition of T to EA, a large isotopic discrimination may result due to the weaker hydrogen bonding ability in T of the $^{18}\text{O}$ labeled ethoxyl oxygen. The KIE for this step would in part be kinetic and in part equilibrium. The largest $^{18}\text{O}$ KIE measured for an enzymatic reaction was in the fumarase catalyzed dehydration of malate. The loss of OH$^-$ from 2S-(2-$^{18}\text{O}$) malate proceeds with a KIE of 1.073 at pH 5 (74). The pK$_a$ for the elimination of the hydroxide ion was reported to be between 5.5 and 6.3. This would indicate the reported KIE at pH 5 is at most only 95% of its maximum value. In the limiting low pH region, the KIE would be 1.10 at most and 1.078 at least. This indicates that relatively large $^{18}\text{O}$ KIE's are possible in enzyme catalyzed reactions. The magnitude of the KIE for the $k_6$ step required to account for the observed change in KIE as well as the steady state KIE is difficult to judge. It depends on the relative magnitude of $k_7$ to $k_6$ as well as the KIE for the $k_5$ step. If $k_7$ is reasonably less than $k_6$ then the equilibrium isotope effect for T formation (i.e. equation 40) could be accounted for by a $k_5$ KIE of 1.042 and a $k_6$ KIE of 1.15. The $k_5$ KIE puts a limit on the initial KIE observed in the
early time regime. The present cell does not allow a very precise
determination of this initial KIE as the low signal to noise ratio in
the region below one minute precludes accurate measurement of the iso-
topic esters mass ratio. The values given above for the KIE's on $k_5$
and $k_6$ could be indicative of the enzyme catalyzed reaction using the
5-n-propyl derivative as substrate as the steady state KIE would there-
fore be .90. The steady state KIE for the furoyl derivative was rela-
tively small. The KIE can be interpreted based on the above discussion,
with the smaller KIE resulting from a larger value of $k_7$ relative to
$k_6$. This results in the forward (to product) breakdown of the interme-
diate $T$ being faster than reversal to $EA$. The large magnitude of the
KIE for the $k_6$ step never gets to exert its full effect. In the limit,
as the ratio of $k_7/k_6$ becomes very large, the final KIE observed would
be that on the $k_5$ step. Because a change in the KIE with time was
observed, $k_7$ can not be too much larger than $k_6$, but the KIE value of
1.013 for the furoyl derivative should place a lower limit on the KIE
for the $k_5$ step.

The $^{16}O/^{18}O$ KIE for transesterification shows a pronounced pH
dependence. Although the errors in the KIE's are large, the data was
fit to a pH titration equation and yielded a $pK_\alpha$ of 7.34 (.04). The pH
variation of $k_{cat}$ for chymotrypsin has been determined for a variety of
substrates (30), and generally the pK falls in the range of 6.9, 7.2.
This pK has been assigned to the ionization of His-57 at the active
site. The pK for the KIE is a little high to correspond to this ion-
ization. Scheme VIII shows the generally accepted mechanism of chymo-
trypsin in the deacylation reaction.
In the kinetic mechanism presented above, as His-57 becomes protonated, the KIE for \( P_2 \) production should become larger (farther from 1). The opposite of what is observed. There are several possible explanations for the observed pH dependence of the KIE. The first explanation involves protonation at a site on the enzyme different from the active site. The KIE could then change as the protein undergoes a conformational change which alters the KIE on one of the elementary steps. Secondly, the intermediate \( T \) could exchange protons to the media, so that the alcoholic oxygen in \( T \) is protonated or the histidine can be deprotonated. The alcoholic oxygen has a solution \( pK_a \) estimated at 11 (108). Protonation of this oxygen may lead to a shift in rate determining step, decreasing the overall KIE for \( P_2 \) production.

The KIE showed a decrease in value as the temperature of the reaction was raised. The change in the KIE was relatively large, due to an abrupt change near 25°C. The KIE transition with temperature is similar to that observed in the hydrolysis reactions in that distinct non-linearity in the Arrhenius plot is seen. If the mechanism by which the non-linearity in the hydrolysis reactions arise is due to a process similar to scheme V, then the \( \Delta H \) for the observed transition in the KIE would have a sign opposite to that for the hydrolysis reaction. The Arrhenius plots for the deacylation reaction using alcohols as nucleophiles show a concave down curvature in the region 20-30°C (31). This
is consistent with the temperature break observed in the KIE analysis. O'Leary (114) has observed a qualitatively similar temperature dependence for the decarboxylation of arginine by *E. coli* arginine decarboxylase. In that study, the KIE changed considerably in magnitude in going from 5°C to 37°C, and an Arrhenius plot for this data showed a distinct non-linearity. As O'Leary points out (111), the temperature dependence of the KIE is negligible based on the absolute rate theory, however, in enzyme catalyzed reactions the temperature can act to effect the partitioning of an intermediate forward or backward, shifting the observed isotope effect.

Finally, the results presented in this work confirm the data of Wang (32). In the transesterification reaction utilizing ethanol, a change in the KIE with time is observed. The KIE is initially normal, decreasing with time to a value less than 1. These results have been confirmed by an independent technique. A further investigation, utilizing a computerized spectrophotometer (72), might be necessary to adequately characterize this unusual isotope effect.
Chapter Four

Conclusions

The temperature dependence of the hydrolysis (this work) and the ethanolysis reactions (31) have been shown to display quantitative differences. The shapes of the Arrhenius plots are opposite in the region of transition between 20 and 30°C. The temperature dependence of the ethanolysis KIE shows a temperature dependence qualitatively similar to the hydrolysis reaction, in that both display a sigmoidal region of transition. If the interpretation of the hydrolysis data is correct, that is, the sigmoidal shape of the Arrhenius plot is due to a rapid equilibrium between acyl enzyme forms which differ in reactivity, then based on the temperature dependence of the KIE, a similar model can account for this observed behavior. The equilibrium between the enzyme forms will, however, have a different sign for the enthalpy. This is reflected in the fact that for the KIE, the Arrhenius curve is concave down around 30°C while the hydrolysis Arrhenius curve is concave up in this region. Numerous reports have indicated a difference in reactivity between different equilibrium forms of chymotrypsin (Eq. 113). The difference in the enthalpies for the proposed equilibrium is presumably due to some effect of the ethanol, even though the alcohol does not exhibit a $K_m$ value. Both water and alcohol attack the same reactive enzyme intermediate, so the effect of the alcohol must be due to some other interaction with the enzyme. This could involve an
effect on the enzyme stability or on its solution structure. Whatever, the nature of the ethanol's effect, it can not be deduced from these kinetic studies alone.

The method presented in this work, although limited to the detection of volatile hydrophobic compounds, should have wide applicability. A wide variety of reactions can be followed, such as the hydrolysis of esters, epoxides, and acetals among others. The ability to convert normally hydrophilic compounds to hydrophobic ones (88) can extend the range of application. The method has been shown to be sensitive and relatively rapid in response. The KIE's obtained with this method have a precision on the order of 1%. The data gives evidence for a change in the KIE with time. This is consistent with the data observed in an earlier study (32). The magnitude of the KIE for the formation of ethyl-2-(5-n-propyl)-furoate was large and inverse, indicating a large KIE in some step of the reaction mechanism, currently interpreted as the step leading from a proposed tetrahedral intermediate to the acyl enzyme.

The substrate used in this study, the 5 substituted 2-furoic acids, have relatively low $K_m$ as far as other chymotrypsin substrates are concerned, however, the rate of the hydrolysis of the enzyme acylated with these substrates is relatively low. This may be an indicator that these substrates do not fit quite right in the enzyme active site and therefore the catalytic activity of the enzyme is reduced. This reduced activity may account for a dramatic increase in the KIE as O'Leary (114) observed in his studies of arginine decarboxylase. Further studies are needed to characterize the KIE reported in this work.
Appendix A

The temperature dependent deacylation rate constants for
the hydrolysis of 2-furoyl-chymotrypsin and 2-(5-n-propyl)
-furoyl-chymotrypsin. a

<table>
<thead>
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<th>$k_{H_2O} \times 10^3$ min$^{-1}$</th>
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<tbody>
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### Appendix A (Continued)

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### Table 1: Enzymatic Reaction Rates

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<th>k&lt;sub&gt;H2O&lt;/sub&gt;x10&lt;sup&gt;3&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;</th>
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<sup>a</sup> Deacylation in .1M K<sub>2</sub>HPO<sub>4</sub> buffer, pH 8.5  [E<sub>0</sub>] ≈ 30 - 35 μM

<sup>b</sup> [S<sub>0</sub>] = 25-30 μM.

<sup>c</sup> Pseudo-first order rate of hydrolysis of the acyl-chymotrypsin.

<sup>c</sup> Temperature determined during reaction by use of a thermistor probe.
Appendix B

Assume a time dependent change in $c_s$ due to any physical or chemical process is given by the function $\phi(\lambda)$ where $\lambda$ is the time over which the change has occurred. Define $J(t)$ by $I(t)/c_s$ for the case when there is an instantaneous step in $c_s$ (see equation 28) with $t$ the time at which the signal is measured. $\phi(\lambda)$ can be approximated by a staircase function as shown in Figure A1, in which $\Delta \lambda$ is a constant small interval, and $\lambda_i = i\Delta \lambda$. The signal intensity measured at time $t$ due to a small step in $c_s$ during the interval $\lambda_i - \lambda_{i-1} = \Delta \lambda$ is given by

$$I_i(t) = \Delta c_{si} j(t - \lambda_i) \quad (A1)$$

provided $\lambda_i < t$. The approximate value for $\Delta c_{si}$ is obtained from the slope of the function $\phi(\lambda)$

$$\Delta c_{si} \approx \phi' (\lambda_i) \Delta \lambda \quad (A2)$$

and by substitution

$$I_i(t) \approx \phi' (\lambda_i) j(t - \lambda_i) \Delta \lambda \quad (A3)$$

The total intensity at time $t$ due to all the small concentration steps is the sum of the individual contributions

$$I(t) = \sum_{i=1}^{n} I_i(t) = \sum_{i=1}^{n} \phi' (\lambda_i) j(t - \lambda_i) \Delta \lambda \quad (A4)$$

Passing to the limit of $\Delta \lambda \to 0$ yields

$$I(t) = \int_{0}^{t} \phi'(\lambda) j(t - \lambda) d\lambda \quad (A5)$$
Taking the derivative of equation 32 and assigning it to $\phi'(\lambda)$ and using equation 29 as the expression $J(t-\lambda)$, equation 33 can be derived by completing the indicated integration.

**Figure A1**
Appendix C

The steady state rate constants used to determine the $K_i$ of ethyl-2-(5-n-propyl)-furoate in the hydrolysis reaction of PNP-2-(5-n-propyl)-furoate catalyzed by chymotrypsin.

<table>
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<tr>
<th>[I]x$10^6$ M$^a$</th>
<th>[S]x$10^6$ M$^b$</th>
<th>$k_{H_2O}x10^3$ min$^{-1}$$^c$</th>
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<tr>
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<tr>
<td>29.3</td>
<td>57.1$^f$</td>
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<tr>
<td>29.8</td>
<td>59.0$^g$</td>
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</table>
Appendix C (Continued)

a The inhibitor was taken to be the ethyl-2-(5-n-propyl)-furoate.
b The substrate was p-nitrophenyl-2-(5-n-propyl)-furoate.
c The rate of hydrolysis in the steady state.
d Linear regression gave the following values for the correlation coefficient (.9954), slope (1.11x10^{-5}), intercept (16.25) in the plot of $k^{-1}$ vs $[S]^{-1}$.
e Linear regression gave the following values for the correlation coefficient (.9999), slope (5.25x10^{-5}), intercept (16.94) in the plot of $k^{-1}$ vs $[S]^{-1}$.
f Linear regression gave the following values for the correlation coefficient (.9957), slope (1.94x10^{-5}), intercept (17.08) in the plot of $k^{-1}$ vs $[S]^{-1}$.
g Linear regression gave the following values for the correlation coefficient (.9936), slope (1.65x10^{-5}), intercept (16.57) in the plot of $k^{-1}$ vs $[S]^{-1}$.
Bibliography

2. van't Hoff, J.H., "Etudes de Dynamique Chimique," 179 (1884).
75. Graham, T., Phil. Mag., 32, 401 (1866).
83. For example see McFadden, W., "Techniques of Combined Gas Chromatography/Mass Spectrometry," Wiley, N.Y., N.Y., 1973, p. 188.


