A STUDY OF THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF p-NITROPHENYL 5-N-ALKYL-2-FUROATES

A Thesis

Presented in Partial Fulfillment of the Requirements for

the Degree Master of Science

By

Joseph E. Baggott, B.A.

The Ohio State University

1975

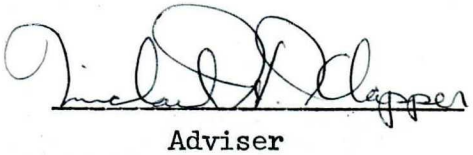
Reading Committee:

Dr. L.J. Berliner

Dr. M.H. Klapper

Dr. R.M. Mayer

Approved By



Adviser

Department of Chemistry

ACKNOWLEDGMENTS

The author wishes to sincerely thank Dr. Michael H. Klapper for his advice and suggestions during the course of this study.

Also, the author wishes to thank the many fellow graduate students for their encouragement and counsel.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Attempts to describe and define α-chymotrypsin specificity have been numerous (1-3). Steric, polar and hydrophobic considerations among members of a homologous series of substrates are generally similar. Therefore, studies of α-chymotrypsin specificity for members of a homologous series of substrates provide useful information concerning the mechanism of specificity (3-7).

In this study the hydrolysis of p-nitrophenyl esters of 5-n-alkyl-2 furoic acids was catalyzed by α-chymotrypsin. These substrates were chosen for several reasons. The furan moiety is a relatively small aromatic ring and should bind strongly to the active site of α-chymotrypsin. Secondly, alkyl substitutions were made at the 5 position in order to minimize steric considerations and maintain the position of the carboxyl group relative to the aromatic group constant. Thirdly, acylation and deacylation could be monitored spectrophotometrically because both p-nitrophenol and the furoic acids are chromophores. The furoic acids have low molar extinction coefficients above 300 mμ while p-nitrophenol absorbs strongly in the region 300 to 400 mμ. Thus acylation and deacylation can be monitored separately without interference. Direct spectrophotometric observation of the deacylation reaction facilitates calculation of precise and accurate rate constants without the

difficulties and approximations inherent in steady state Michaelis Menten kinetics (2). Finally, the acyl portion of the substrates contains no other functional groups except for the carboxyl group. This eliminates polar and charged group interactions with the enzyme.

Enzymatic catalysis and specificity was measured by comparing deacylation rate constants and activation parameters with alkaline saponification rate constants and activation parameters. Alkaline saponification reactions were chosen as a reference since there are similarities to the enzymatic reaction (2,8).

The results of this investigation reveal similarities to other investigations which are discussed with respect to theories of enzymatic specificity. Klapper's development and elucidation of the "lock and key" model appears to be especially applicable to relative specificity of α-chymotrypsin for homologous substrates (9).

MATERIALS AND METHODS

A. Reagents

α-Chymotrypsin was purchased from Worthington Biochemical Company (Lot CDS 2CA) and was used without further purification. Stock solutions were prepared by dissolving 100 mg of enzyme in 10 ml of water adjusted to pH 3.0 with HCl. The concentration was estimated using a molar extinction coefficient of 5.0×10^4 at 280 mμ (10). Stock enzyme solutions were stored at 5°C.

2-Furoic acid was purchased from Eastman and recrystallized from benzene. 5-Methylfuran-2-aldehyde (Lot 95999), 2-ethylfuran (Lot 8360-A), 2-n-propylfuran (Lot 3359 AF), 2-n-butylfuran and 2-amylfuran were purchased from K and K Laboratories, Inc. 1-(3-Dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (Lot 100537) was purchased from Aldrich Chemical Company. Acetonitrile was distilled once and stored over molecular sieves. p-Nitrophenol was recrystallized from 95% ethanol before use. Water was double distilled before using.

The following commercially available reagent grade chemicals were used: benzene, ethyl ether, anhydrous ethyl ether, 95% ethanol, concentrated hydrochloric acid, carbon tetrachloride, sodium carbonate, N,N-dimethylformamide, sodium hydroxide, anhydrous magnesium sulfate, petroleum ether (B.P. 65-110), sodium phosphate

(dibasic), phosphorous oxychloride ($POCl_2$), silver oxide (Ag_2O), tetramethylsilane, and sodium borate.

B. Synthesis of Substrates

2-n-Alkylfurans were formylated to yield 5-n-alkylfuran-2-aldehydes by the general procedure of Traynelis (11). Forty millimoles of phosphorous oxychloride and N,N-dimethylformamide were mixed at 5°C and stirred for twenty minutes. Twenty millimoles of the 2-n-alkylfuran was then added over a twenty minute period. The reaction mixture was stirred at 5°C for two hours and then for two hours at room temperature. The reaction mixture was then poured onto 70 ml of ice and neutralized with sodium carbonate. A brown oil separated from the aqueous layer upon standing overnight. The oil was separated from the aqueous layer with the aid of ether, and the aqueous layer was extracted with five 30 ml portions of ether. The extracts were combined and the ether was evaporated under reduced pressure leaving a brown oil. The oil was used without further purification.

5-n-Alkylfuran-2-aldehydes were oxidized to their corresponding acids by the following procedure. Twenty millimoles of 5-methylfuran-2-aldehyde or the oil from the formylation reaction was added to a mixture of sixty millimoles of silver oxide in 250 ml of a 5% aqueous sodium hydroxide solution. The reaction mixture was stirred vigorously for 60 to 70 hours at room temperature. The reaction mixture was filtered and the filtrate evaporated under reduced pressure to 60% of the original volume. The acids were

precipitated by the slow addition of concentrated hydrochloric acid to the rapidly stirred filtrate at 5°C. The precipitate was filtered off, dried and recrystallized from petroleum ether (B.P. 65-110). Yields were 50-60% overall for the two steps. The acids were identified and characterized by their melting points, U.V. and NMR spectra given in Table 1.

p-Nitrophenyl esters of the 5-n-alkyl-2-furoic acids were synthesized by the following procedure. Eight millimoles of the acid and nine millimoles of p-nitrophenol were dissolved in 50 ml of anhydrous ether. Sixteen millimoles of 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride was added to the solution and the mixture was stirred overnight. The ether solution was filtered, washed repeatedly with 0.05M phosphate buffer pH 7.0, dried over anhydrous magnesium sulfate and evaporated under reduced pressure. The solid residue was recrystallized from 95% ethanol. Emphasis was placed on purity and yields were only 20-30%.

The p-nitrophenyl esters were identified and characterized by their elemental analyses, melting points and U.V. spectra. The data is listed in Table 2. Microanalyses were performed by Galbraith Lab., Inc., Knoxville, Tenn. In addition, the purity of each ester was calculated from the change in optical density at 347 mμ upon base hydrolysis ($\epsilon = 5.7 \times 10^3$ $cm^{-1}mole^{-1}$ liter, 25°C for p-nitrophenol). The esters were found to be 95-100% pure. Stock substrate solutions were made by dissolving 20 to 50 micromoles of the ester in 10 ml of acetonitrile.

C. Equipment and Buffers

Melting points are uncorrected and were performed on a Thomas Hoover Capillary Melting Point Apparatus. U.V. spectra of the furoic acids, their p-nitrophenyl esters and the difference spectra were performed on a Unicam SP800A spectrophotometer equipped with a model 2095 Forma constant temperature bath and thermostated cell block. The U.V. spectra of the furoic acids and their p-nitrophenyl esters were measured at 27°C in 0.1M phosphate buffer at pH 7.0 containing 0.5% v/v or less acetonitrile. N.M.R. spectra were measured at room temperature in carbon tetrachloride containing a small amount of tetramethylsilane as the reference. N.M.R. spectra were performed on a Varian A-60 spectrometer.

Phosphate, carbonate and borate buffers were titrated with sodium hydroxide or hydrochloric acid solutions to the desired pH. The pH at each temperature was determined on a Model 12 Corning pH meter with Model 4094-L15 Thomas electrode. Coleman (pH 7.00, 25°C) and Sargent-Welch (pH 10.00, 25°C) standard buffers were used as references.

All kinetic measurements were made on a Cary 16 spectrophotometer equipped with a model 1626 Cary recorder interface, a Non-linear Systems series 5000 digital voltmeter and a Model EU-205-11 Heath strip chart recorder. Voltages could be read simultaneously from the digital voltmeter and chart recorder. The accuracy of the chart recorder was determined by calculating rate constants from data taken from the voltmeter and chart recorder. The accuracy of

the chart recorder was ± 0.5%.

Enzymatic and nonenzymatic reactions were carried out in rectangular quartz cells with 1.0 or 5.0 cm path length. Cells were thermostatted by means of an aluminum cell block and a Model 2095 Forma constant temperature bath. The flow rate through the cell block was 400 ml/min. The temperature was measured using Cenco -10°C to 60°C thermometer having divisions of 0.2°C.

Rate constants, Vm's, Km's, pKa's, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ were calculated using a Nova 1220 Data General computer.

D. Enzymatic Kinetics

The accepted sequence for α-chymotrypsin catalyzed hydrolysis of esters is as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

where E, S, ES, ES', P_1 and P_2 correspond to the free enzyme, the substrate, the Michaelis complex, the acyl-enzyme, the alcohol and the acid respectively (2). If P_2 is a chromophoric acid and $k_2 \gg k_3$, then it should be possible to measure the rate of deacylation spectrophotometrically. This is the case with p-nitrophenyl esters of 5-n-alkyl-2-furoic acids.

Acylation of α-chymotrypsin can be monitored as the release of p-nitrophenol (P_1) and the increase in optical density at 347 mμ or 400 mμ. Under conditions where (E) > (S) acylation is complete upon

mixing enzyme with substrate. The deacylation rate constant (k_3) can be calculated from the rate of appearance of P_2, disappearance of ES' or from the concerted process. The extent of deacylation was measured spectrophotometrically at 245 mμ where the molar extinction coefficient of the enzyme is relatively small and the molar extinction coefficient of the furoic acid is relatively large. This method is similar to that of Bender et al. (16,17) who measured the rate of deacylation of trans-cinnamoyl-chymotrypsin at 250 mμ and Inward and Jencks (18) who measured the rate of deacylation of 2-furoyl-chymotrypsin at 265 mμ.

The deacylation of 5-n-alkyl-2-furoyl-chymotrypsin was monitored by the following procedure. Enzyme solutions were prepared by the addition of the appropriate amount of enzyme stock solution to 17.0 ml of buffer in the thermostatted 5.0 cm path length cell, such that the final concentration of enzyme was 5.0 μM. The solution was stirred thoroughly using a glass rod and was allowed to reach thermal equilibrium. This required ten minutes or longer. Substrate stock solution was then added by means of a Hamilton syringe, and the solution was stirred. Substrate concentrations were 0.87 to 6.1 μM depending on the experiment. The acylation reaction was monitored at 347 mμ until no further change in optical density was observed. The deacylation reaction was then monitored at 245 mμ. Optical density changes measured at 245 were 0.01 to 0.1 absorbance units depending on the substrate used, its concentration and the extent of deacylation before measurements

were made. The optical density of the enzyme solution was 0.5 absorbance units under these conditions. Air was used as a reference since no significant change in the optical density at 245 mμ of the enzyme solution was seen over the time interval required to measure the deacylation reaction. The temperature of the reaction solution was measured before and after each kinetic run and the average was taken as the true value. The addition of enzyme stock solution or substrate stock solution did not affect the pH of the buffers used.

The data consisted of unweighted voltage and time sets which were computer fit by non-linear regression to the first order rate equation (1), using the method described by Bevington (19):

$$A_t = A_\infty - \Delta A e^{-k_3 t} \qquad (1)$$

where A_t is the voltage at any time during the course of the reaction, A_∞ is the voltage at infinite time and ΔA is the quantity $A_\infty - A_{t=0}$. First order processes do not depend on the absolute values of A_t, but only on the percent change of A_t with time. Twenty to sixty data sets were used for each kinetic run and reactions were monitored well into the third half-life or longer. The computer output consisted of A_∞, ΔA, k_3, their respective standard deviations and the residual for each data set so that any trends in the data could easily be seen.

Steady state reactions were performed by the following procedure. Enzyme solutions were prepared by the addition of the appropriate amount of enzyme stock solution to 17.0 ml of buffer in a thermostatted 5.0 cm path length cell, such that the final concentration of enzyme was 0.50 to 1.0 μM. The solution was stirred thoroughly using a glass stirring rod and allowed to reach thermal equilibrium. This required ten minutes or longer. Substrate stock solution was then added by means of a Hamilton syringe, such that the final concentration was 1.0 to 30 μM. The reaction solution was stirred. The steady state rate was monitored at 347 mμ where the molar extinction coefficient of p-nitrophenol is 5.7×10^3 $cm^{-1}mole^{-1}$ liter at 25°C. Spontaneous hydrolysis rates were subtracted from the enzymatic rates.

The data consisted of unweighted substrate concentrations and initial velocities in moles $l^{-1}min^{-1}$. The Michaelis-Menten equation was computer fit by non-linear regression, using the method described by Bevington yielding V_m and $K_{m(app)}$ and their standard deviations (19). The deacylation rate constant was obtained by dividing V_m by the enzyme concentration.

The titration of the free enzyme was performed by the general method outlined by Caplow and Jencks (8). The concentration of free enzyme can be determined by measurement of the burst of p-nitrophenol or the steady state rate of release of p-nitrophenol following the burst as long as the concentration of the titrant is greater than that of the enzyme and much greater than that of its $K_{m(app)}$. The amount

of free enzyme present at any time will be proportional to the magnitude of the burst and the steady state rate of release of p-nitrophenol. The amount of free enzyme present at any time can be used as a measure of the extent of deacylation.

The acyl-enzyme was prepared in a thermostatted beaker by the addition of substrate stock solution, such that the final concentration was 3.0 μM, to a 5.0 μM enzyme solution. Aliquots of the acyl-enzyme solution were titrated with 20 μM p-nitrophenyl 2-furoate during the course of the reaction. The burst was monitored at 400 mμ twenty seconds after mixing of the titrant and the steady state rate was then measured.

Rate constants for the deacylation of 5-methyl and 5-ethyl-2-furoyl-chymotrypsin were calculated from burst optical density and time data or steady state rate and time data. The data was computer fit to the first order rate equation (1).

The apparent pK_a's of the deacylation reaction were determined by calculating k_3's over a range of 1.0 to 2.3 pH units. Data sets consisted of pH's and weighted k_3's. Data sets were computer fit by non-linear regression to equation (2), using the method described by Bevington (19).

$$k_3 = \frac{K_a}{K_a + (H^+)} \; k'_3 \qquad (2)$$

where k_3 is the deacylation rate constant at any pH, K_a is the apparent acid dissociation constant governing the deacylation rate

and k'_3 is the pH independent deacylation rate constant. The output consisted of k'_3, the apparent pK_a and their standard deviations.

The apparent pK_a at any temperature was estimated from hand drawn plots of pK_a vs $\frac{1}{T}$ and k_3's were corrected to k'_3's at each temperature using equation 2, when phosphate buffers pH 8.4 - 8.6 were employed. Rate constants were considered as pH independent when carbonate buffers at pH 10.0 were employed.

The standard deviations of the pH independent rate constants were determined by equation (3):

$$\sigma' = \left[\sigma^2 \left(1 + \frac{(H^+)}{K_a}\right)^2\right]^{\frac{1}{2}} \qquad (3)$$

where σ' is the standard deviation of the pH independent rate constant and σ is the standard deviation of the rate constant measured at a given pH and temperature.

E. Saponification Kinetics

Alkaline saponification rates were measured in 10 millimolar borate buffer pH 10.50, measured at 25°C. The reaction was monitored by recording the increase in optical density at 400 nm. Since alkaline saponification is also a pseudo first order reaction the rate constants were calculated using equation (1). The validity of this method was checked by measuring the alkaline saponification rate constants of p-nitrophenyl 2-furoate in 10 and 30 mM borate buffer and in unbuffered sodium hydroxide solutions as a function of the pH of the buffers and solutions.

F. Difference Spectra

Difference spectra were measured in 1.0 cm path length cells. Substrate stock solutions were added to obtain a final concentration of 30 μM to an equal concentration of enzyme solution. The U.V. spectra were scanned from 230 mμ to 315 mμ periodically throughout the course of the reaction. The difference spectra were calculated by subtracting the intermediate spectrum or the final spectrum (taken after 6 half lives) from the initial spectrum.

G. Calculation of Activation Parameters

The enthalpy and entropy of activation for enzymatic and non-enzymatic reactions were calculated from a least squares computer fit of equation 4, using a method described by Deming (20).

$$k'_3 = \frac{KT}{h} e^{-\Delta H^{\neq}/RT + \Delta S^{\neq}/R} \qquad (4)$$

where h is Planck's constant, K is the Boltzmann constant, k'_3 is the pH independent enzymatic rate constant or the saponification rate constant, R is the gas constant and T is the absolute temperature. The data consisted of weighted temperatures and weighted k'_3's. Errors in the temperature were determined by measuring the temperature fluctuations of the solution in the reaction cell. The computer output consisted of $\Delta H^{\neq}$, $\Delta S^{\neq}$, their standard deviations and residuals for both the rate constants and temperatures. Since $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for the alkaline saponification results were compared on a relative basis, no correction was made for the heat of ionization of borate buffer.

RESULTS AND DISCUSSION

A. Validity of the Rate Constants

In order to test the validity of the rate constants calculated from measurements at 245 mμ, deacylation rate constants were calculated by three additional methods: Michaelis Menten kinetics, titration of the free enzyme with p-nitrophenyl 2-furoate by the measurement of the magnitude of the burst of p-nitrophenol (burst plot) and by measurement of the steady state rate of release of p-nitrophenol following the burst (steady state plot). Titrations of the free enzyme were performed with titrant concentrations of 20 μM, well above that of the enzyme (5.0 μM) and the $K_{m(app)}$ of the titrant (0.69 μM).

The rate constants calculated by the four methods are in good agreement as shown in Table 3. The deacylation rate constant furoyl-chymotrypsin at 25°C calculated from measurements at 245 mμ is in good agreement with Inward and Jencks rate constant calculated from measurements at 265 mμ (18). This would be expected if optical density measurements truly represent the extent of deacylation. It is assumed that no change in the apparent pK_a for the deacylation rate constant occurred under the varied conditions.

The pseudo first order deacylation rate constant should be independent of moderate substrate and product concentrations provided that p-nitrophenol is not competing with water as the acyl group

acceptor (18). In this respect moderate concentrations of products would not be expected to alter the observed deacylation rate constant, if pseudo first order dissappearance of ES' and pseudo first order appearance of P_2 is the only process measured at 245 mμ. In order to test these assumptions the deacylation rate constant of 2-furoyl-chymotrypsin was determined at various initial concentrations of substrate and products. The reactions were performed at low pH to minimize the absorbance of p-nitrophenol at 245 mμ. Slight upward trends in the rate constants are seen with increasing substrate and p-nitrophenol concentration in Table 4A. It seems unlikely that this effect is due to p-nitrophenol activation. The concentration of p-nitrophenol where the trend in the rate constants is observed is about an order of magnitude greater than the concentration of substrate where the trend is observed. Therefore, the upward trend in the rate constants is not dependent upon the concentration of p-nitrophenol. Differences in the rate constants are most likely caused by the small optical density changes when low substrate concentrations were used and the large background optical density at 245 mμ when high product concentrations were used. Averages of the rate constants are very close, indicating that there is no real change.

Under the conditions (E) > (S), the excess or unacylated enzyme could attack the acyl-chymotrypsin producing a nicked or partially digested acyl-chymotrypsin which has a markedly different activity with respect to the native acyl-chymotrypsin. It is reasonable to expect that this process would occur most rapidly at high temperature

and pH. Therefore, the effect of the enzyme concentration on the rate constant of the deacylation of 5-ethyl-2-furoyl chymotrypsin at 43.8°C and pH 8.60 was studied. No significant effect on the rate constant was observed as shown in Table 4-B. Since the highest enzyme concentration used was three times that of the substrate concentration, it is reasonable to argue that this pseudo-autolytic process is insignificant over the period of time required to measure the deacylation reaction.

It is well known that deacylation rate constants are independent or nearly independent of small concentrations of acetonitrile and changes in the ionic strength (22,23). Deacylation rate constants of 2-furoyl-chymotrypsin were calculated from measurements at 245 mμ using varied acetonitrile concentrations and ionic strengths. Rate constants were determined at pH's close to the apparent pK_a of the deacylation reaction in order to detect any sensitivity to acetonitrile concentration or ionic strength. No effect was seen over a ten fold range of ionic strengths or up to 0.70% (v/v) acetonitrile as seen in Table 5.

The difference spectra of 2-furoyl-chymotrypsin vs. chymotrypsin plus 2-furoic acid, 5-ethyl-2-furoyl chymotrypsin vs. chymotrypsin plus 5-ethyl-2-furoic acid and the intermediate difference spectra taken during the deacylation of 5-ethyl-2-furoyl-chymotrypsin are shown in Figures 1 and 2. Since the pK_a of these furoic acids is in the range of 3.0 to 4.0, the difference spectra, measured at pH 7.00 or pH 8.20, are of the completely ionized acids. The difference spectra of

5-ethyl-2-furoyl-chymotrypsin taken during the deacylation show a true isosbestic point (Figure 2-B) which indicates that only two interconvertible species are present, namely the acylated and free enzyme. The difference spectrum of 2-furoyl-chymotrypsin versus chymotrypsin and 2-furoic acid is therefore consistent with the fact that the same pseudo first order rate constant is obtained when calculated from measurements at 245 and 265 mμ as shown in Table 3.

B. The Temperature Dependence of the Apparent pKa of the Deacylation Reaction

The calculation of precise and accurate pH independent rate constants requires that the pK_a of the deacylation reaction be known at each temperature. From the results given in Table 6, substrates of this homologous series do not have the same pK_a's. Dupaix et al. observed the same behavior with a variety of homologous series (3). The apparent pK_a of the deacylation reaction of 2-furoyl-chymotrypsin and the pH independent rate constant at 25°C are 6.95 and 0.101 min^{-1} respectively which is in good agreement with 7.1 and 0.11 min^{-1} obtained by Inward and Jencks (18). The heat of ionization of the catalytically important group is also dependent on the nature of the substrate as shown in Figure 3. The ΔH of ionization for the methyl, ethyl and n-propyl substituted substrates is 11 kcal/mole about twice that of the H and n-butyl substituted substrates which are 5 and 6 kcal/mole respectively. The ΔH of ionization of acetyl-chymotrypsin was determined by Fife and Milstien to be 7 kcal/mole and that for the chymotryptic hydrolysis of N-acetyl-tryptophan ethyl ester was determined by Cunningham and Brown to be 11 kcal/mole (7,24). The

values presented here are within that range. The heat of ionization of imidazole and the imidazole nitrogen of L-histidine are 8.8 and 7.1 kcal/mole respectively in aqueous solution (25).

Some non-linearity is indicated in the plots shown in Figure 3 with respect to the methyl and ethyl substituted substrates. A straight line was drawn in the plots as an approximation that will introduce only very minimal error in the calculation of pH independent deacylation rate constants.

C. Activation Parameters for the Deacylation of 5-N-Alkyl-2-Furoyl-Chymotrypsin

Deacylation rate constants of 5-n-alkyl-2-furoyl-chymotrypsins were measured over a temperature range of 10 to 49°C, as shown in Table 7. The rate constants were measured at pH 8.4 to 8.6 in order to minimize corrections to pH independent values. The plots of ln $\frac{k_3'}{T}$ vs $\frac{1}{T}$ for the n-propyl, ethyl and unsubstituted substrates were not linear, but showed a break in continuity occurring between 20 and 30°C. The most striking example is the n-propyl substituted substrate, which is shown in Figure 4. The plots of the other substrates appeared linear, however, this may be due to the limited low temperature range studied. The n-butyl substituted substrate is shown as an example in Figure 5. In order to separate the two temperature regions of the plots and minimize any subtle deviations from linearity, rate constants which were measured at temperatures greater than 28 to 30°C were used to calculate activation parameters for the high temperature region and those rate constants measured at lower temperature were used to calculate activation parameters in the low

Discontinuity in Arrhenius plots of chymotrypsin catalyzed reactions have been observed before. Breaks in the Arrhenius plots of the chymotrypsin catalyzed hydrolysis of N-benzoyl-d- and -l-alanine methyl esters were observed by Kaplan and Laidler (26). The breaks occurred at 25°C. A break in the Arrhenius plot for the chymotrypsin catalyzed hydrolysis of N-acetyl-l-tryrosine ethyl ester was observed by Glick (27). In pure water the break occurred at 25°C with an activation enthalpy of 8 and 14 kcal/mole in the low and high temperature regions respectively. A close examination of the data presented by Bender et al. in the Arrhenius plot of the deacylation rate constant of trans-cinnamoyl-chymotrypsin reveals a break occurring at approximately 25°C (28). It is also worthwhile noting that the activation enthalpy for the deacylation of trans-cinnamoyl-chymotrypsin at temperatures equal to or less than 25°C is lower than the activation enthalpy at higher temperatures. These results are consistent with our calculated activation parameters as shown in Table 8 and the nonlinearity of the plots of the apparent pK_a of the deacylation reaction versus $\frac{1}{T}$ for the methyl, ethyl and unsubstituted substrates.

Breaks in linearity in Arrhenius plots could be interpreted as a change in the rate limiting step or the existence of two catalytically active forms of the acyl-enzyme which are in rapid equilibrium. Changes in the acyl-enzyme structure which would alter the activity might be accompanied by changes in the U.V. spectrum

of the acyl-enzyme. During the deacylation the two forms of the acyl-enzyme would not show a true isosbestic point in the U.V. spectrum. However a true isosbestic point was seen during the deacylation of 5-ethyl-2-furoyl-chymotrypsin at 27°C where concentrations of both forms of the acyl-enzyme should be significant. This suggests that the discontinuity that is observed is due to a change in the rate limiting step, but does not rule out the possibility of two catalytically active forms with very similar U.V. spectra.

Activation enthalpies in the high temperature region trend upwards as the deacylation rates constants increase. The notable exception is the amyl substituted substrate which shows an increase in activation enthalpy compensated for by an increase in the activation entropy.

The deacylation rate constants of 2-furoyl-chymotrypsin were measured at several different temperatures at pH 10.0. The rate constants at this high pH were treated as pH independent values. Binding of inhibitors and substrates to α-chymotrypsin cause proton uptake at high pH, with an apparent pK_a for this process of 8.8 (29,30). In addition large changes in the lattice parameters of crystalline α-chymotrypsin at or above pH 9.0 have been observed (31). This would suggest that there is another conformer of α-chymotrypsin at or above pH 9. The effect of the process of proton uptake and the apparent change in conformation on the deacylation rate constant of 2-furoyl-chymotrypsin was tested. Rate constants

measured at pH 10.0 are compared to those calculated using equations 2 and 4. The results given in Table 9 show that there is no effect on the rate constants or the activation parameters. This is consistent with Bender et al., who showed that the deacylation rate constant of trans-cinnamoyl-chymotrypsin was virtually unaffected over a liberal range of alkaline pH's (17).

D. The Intrinsic Reactivity of the Substrates

The intrinsic reactivity of the substrates was measured by the rate of alkaline saponification and the activation parameters for the saponification reaction. Linear free energy relationship for the deacylation of para and meta substituted benzoyl-chymotrypsins ($\rho = 2.1$) were found to be very similar to the linear free energy relationship for the alkaline saponification ($\rho = 2.04$) of meta and para substituted p-nitrophenyl benzoates (8). This would indicate a similarity in mechanism. Since hydroxide ion is small, steric considerations should be minimized, and differences in rates should reflect primarily inductive effects.

The alkaline saponification reactions were performed in 10 mM borate buffer at pH 10.50 (25°C). The rate constants for the alkaline saponification of p-nitrophenyl 2-furoate were found to be dependent upon the pH of the solution. The plot of log k_{OH} vs pH was found to be linear with buffered and unbuffered solutions as shown in Figure 6. The average second order rate constant for the saponification of p-nitrophenyl 2-furoate was $4.74 \times 10^{3} m^{-1} min^{-1}$ at 25°C

which compares favorably with $4.9 \times 10^{2} m^{-1} min^{-1}$ determined by Marshall et al. (21). The alkaline saponification rates were measured at seven temperatures for each substrate, as shown in Table 10. The pH was not corrected for temperature, since activation parameters would be compared on a relative basis. Initial ester concentrations were 1.0 μM which is well below the buffer concentration and the concentration of hydroxide ions.

The activation parameters for the alkaline saponification reaction are given in Table 10. The enthalpy of activation for H substituted ester appears to be somewhat lower than the others in the series. Since this ester does not have an electron donating group at the 5 position its saponification rate constants are approximately three times higher than the others in the series.

E. Corrected Activation Parameters

Activation parameters for the alkaline saponification reaction were subtracted from the activation parameters for the enzymatic hydrolysis for each substrate to yield corrected activation enthalpies and entropies, shown in Table 11. This is equivalent to dividing the enzymatic rate constant by the saponification rate constant at each temperature studied and thus normalizing the reactivity of each substrate.

Using arguments presented by Klapper we have good justification for using corrected activation parameters as a measure of enzymatic activity or specificity (9). Any chemical reaction may be arbitrarily

divided into three steps: vaporization of the reference state molecule from the reference phase, transformation of the reference state molecule to the transition state molecule in the gas phase and condensation of the transition state molecule into the reference phase. Using this path it is therefore possible to divide the total free energy of activation into two parts according to equation (5):

$$\Delta G^{\neq} = \Delta G^{\neq}_{int} + \Delta G^{\neq}_{med} \qquad (5)$$

where $\Delta G^{\neq}$ is the total free energy of activation, $\Delta G^{\neq}_{int}$ is the intrinsic free energy of activation which is required to transform the reference state molecule to the transition state molecule in the gas phase and $\Delta G^{\neq}_{med}$ is the medium dependent free energy of activation which is dependent upon the free energies of vaporization of the reference state and transition state molecules. $\Delta G^{\neq}_{int}$ depends only on the nature of the reaction and the reacting molecule. For a reaction occurring in bulk water and at an enzyme's active site the quantity $\Delta\Delta G^{\neq}$ represents the difference in the medium dependent free energies of activation according to equation (6):

$$\Delta\Delta G^{\neq} = \Delta G^{\neq}_{(enz)} - \Delta G^{\neq}_{(H_2O)} = \Delta G^{\neq}_{med}(enz) - \Delta G^{\neq}_{med}(H_2O) \qquad (6)$$

$\Delta\Delta G^{\neq}$ is therefore a measure of catalytic activity and enzymatic specificity when different reacting molecules are compared, provided that the same or equivalent mechanisms function in both bulk water and at an enzyme's active site.

The corrected activation enthalpies at high temperature for the H, methyl, ethyl and n-propyl substituted substrates trend upwards, while the corrected entropies vary in concert with the values of the deacylation rate constants. The n-butyl substituted substrate shows a decrease in the corrected activation enthalpy and an increase in the absolute value of the corrected activation entropy, which is consistent with its lower rate constant. However, the amyl substituted substrate shows an increase in the corrected activation enthalpy compensated by a decrease in the absolute value of the corrected activation entropies. In this respect, the amyl substituted substrate seems to be inconsistent with the rest. One explanation for this result would be that this substrate is non-productively bound, requiring that the acyl group proceed an enthalpically unfavorable process before deacylating. The fact that this is the largest substrate lends credence to this explanation.

The dramatic lowering of the corrected activation enthalpy for the n-propyl substituted substrate at low temperature remains an enigma. In the low temperature region enthalpic control of the deacylation reaction appears to be dominant, in contrast to entropic control in the high temperature region.

CONCLUSIONS

Following the model proposed by Klapper in which the reacting molecule is treated as a hard sphere, the enzyme's active site is treated as a rigid spherical cavity, and water is treated as a hard sphere liquid, it can be shown that enzymatic specificity can be controled by the volume of the reacting molecule. Klapper derived the corrected entropy of activation as a function of both the volume of the spherical cavity and the volume of the reacting molecule, for the case where the reacting molecule would fit into the cavity. In theory the volumes of reacting molecules would determine the specificity for a particular enzyme, provided no change in reaction mechanism occurred. The specificity for different substrates would be reflected in changes in the corrected entropies of activation for molecules which have volumes smaller than the volume of the active site. Molecules with volumes greater than that of the active site would either be non-productively bound or excluded from the active site and thus have low specificities (9).

This model closely resembles the "lock and key" model proposed by Fisher, with two exceptions (32). First, Klapper's model allows for large changes in enzymatic specificity for substrates that do fit into the active site. Secondly, these changes in enzymatic specificity will depend upon the volume of the substrate and will be

reflected in changes in the corrected entropies of activation. It must be emphasized that the above concepts serve only as a model for enzymatic specificity and are not meant to mimic reality. It is obvious that an enzyme's active site and a substrate may not be spherical in shape. Therefore, it is reasonable that the shape of a substrate could exclude it from the active site even if its volume was less than that of the active site's. However shape considerations can be minimized by using only structurally similar or homologous substrates with unbranched alkyl chains. In this respect relative specificities among members of a homologous series of substrates provide more useful and unencumbered data.

In general the corrected entropies of activation, in the high temperature region shown in Table 11, correlate better with the corrected free energies of activation than do the corrected enthalpies of activation. The exception is the 5-amyl substituted substrate.

Implicit in Klapper's model is the conclusion that the relative specificity of an enzyme for structurally similar substrates should be a function of the volume of the substrate, as already mentioned. Maximum or minimum specificities should correspond to a particular substrate volume when comparing the enzymatic rates for a homologous series of substrates. In Figure 7 the corrected acyl-chymotrypsin deacylation rate constants are plotted versus the volume of the acyl portion of four homologous series of p-nitrophenyl esters. In all four series a maximum corrected deacylation rate constant occurs at

an acyl volume of 130 to 160 $Å^3$. This observation indicates that α-chymotrypsin is sensitive to the volume of the substrate. The apparent differences in volume required for maximum specificity may only reflect inherent difficulties in calculating volumes of small molecules and comparing volumes of aliphatic, aromatic and heterocyclic aromatic substrates (33).

Invoking a rigid active site is in sharp contrast to the induced fit theory proposed by Koshland to explain differences in specificity of chymotrypsin for n-alkyl esters (34). In the induced fit theory, the shape of the substrate as well as the relative positions of hydrophobic, polar and charged groups are critical to enhanced or depressed specificity (34,35). In the four homologous series shown in Figure 7, the shapes of the substrates are not similar nor is the position of the carboxyl moiety relative to the aromatic moiety. This suggests that Klapper's model is better equipped to explain these relative specificities than is the induced fit model.

The sensitivity of chymotrypsin to the volume of the substrate is obviously not limited to volume changes in the acyl group. Inward and Jencks demonstrated that when water was replaced with amines or alcohols, aminolysis and alcoholysis of 2-furoyl-chymotrypsin was observed (18). Using a variety of amines they showed that the rate of aminolysis was nearly independent of the pK_a of the amine except for small primary amines. Marked differences in rates

of aminolysis were seen with such structurally similar amines as ammonia and methylamine, hydroxyl- and methoxyamine, hydrazine and methylhydrazine. This suggests that the rate of aminolysis of 2-furoyl-chymotrypsin is sensitive to the volume of the acyl acceptor. This conclusion is consistent with the acyl group acceptor occupying volume in the active site and thus influencing the reactivity.

Bender et al. has proposed some of the essential features of Klapper's model (28). Bender demonstrated that the freezing of rotation of bonds caused by a rigid active site could cause the observed differences in the rates of deacylation of acetyl-, trans-cinnamoyl, N-acetyl-L-tryptophanyl- and N-acetyl-L-tyrosyl-chymotrypsins. The entropy of activation was shown to be the controling factor in deacylation. This is consistent with Klapper's model. In general as the substrate volume increases, more bonds can be frozen. The possibility of freezing or restricting bond rotation increases as the volume of the substrate approaches the volume of the active site.

The results and conclusions presented here should serve to stimulate a reinvestigation of the "lock and key" model of enzymatic specificity especially with regard to Klapper's recent work.

APPENDIX

TABLE 1

PHYSICAL PROPERTIES OF 5-N-ALKYL-2-FUROIC ACIDS[a]

5-n-alkyl Substitution	M.P.	λ max.	N.M.R. splitting, δ(ppm), Number of protons
CH_3-	107 - 109 (Lit. 107-108.5)[b]	255	Singlet, 2.4, 3.1 Doublet, 6.1, 1.0 Doublet, 7.1, 1.0
CH_3CH_2-	92 - 94 (Lit. 93 - 94)[c]	256	Triplet, 1.3, 3.0 Quadruplet, 2.7, 1.9 Doublet, 6.1, 1.0 Doublet, 7.1, 1.0
CH_3-$(CH_2)_2$-	62 - 64 (Lit. 63 - 63.5)[d]	257	Triplet, 1.0, 2.8 Multiplet, 1.1-1.9, 2.1 Triplet, 2.7, 1.9 Doublet, 6.1, 1.0 Doublet, 7.2, 1.0
CH_3-$(CH_2)_3$-	71 - 73	257	Triplet, 0.9, 3.0 Multiplet, 1.1-1.9, 4.4 Triplet, 2.7, 2.0 Doublet, 6.1, 0.9 Doublet, 7.2, 1.1
CH_3-$(CH_2)_4$-	59 - 61	257	Triplet, 0.9, 3.0 Multiplet, 1.1-1.9, 6.2 Triplet, 2.7, 2.1 Doublet, 6.1, 1.0 Doublet, 7.1, 1.0

[a] Measurements were performed as described in the text.

[b] Obtained from Runde et al. (12).

[c] Obtained from Reichstein et al. (13).

[d] Obtained from Novitskii et al. (14).

TABLE 2

PHYSICAL PROPERTIES OF p-NITROPHENYL 5-N-ALKYL-2-FUROATES[a]

5-n-alkyl substitution	M.P.	λ max.		%C	%H	%N
				$C_{11}H_7O_5N$		
H-	162 - 164 (Lit. 164-165)[b]	270	Calc.	56.66	3.03	6.01
			Found	56.73	3.03	5.86
				$C_{12}H_9O_5N$		
CH_3-	113 - 115	282	Calc.	58.30	3.67	5.67
			Found	58.35	3.70	5.49
				$C_{13}H_{11}O_5N$		
CH_3CH_2-	74 - 76	283	Calc.	59.77	4.24	5.36
			Found	59.36	4.45	5.33
				$C_{14}H_{13}O_5N$		
CH_3-$(CH_2)_2$-	65 - 67	283	Calc.	61.09	4.76	5.09
			Found	61.08	4.80	5.49
				$C_{15}H_{15}O_5N$		
$CH_3(CH_2)_3$-	62 - 64	287	Calc.	62.28	5.23	4.84
			Found	61.97	5.23	5.26
				$C_{16}H_{17}O_5N$		
CH_3-$(CH_2)_4$-	79 - 81	287	Calc.	63.36	5.65	4.62
			Found	63.11	5.94	5.12

[a]Measurements were performed as described in the text.

[b]Obtained from Bender et al. (15).

TABLE 3

THE COMPARISON OF THE DEACYLATION RATE CONSTANTS OF 5-N-ALKYL-2-FUROYL-CHYMOTRYPSINS CALCULATED FROM MICHAELIS-MENTEN KINETICS, TITRATION METHODS AND MEASUREMENTS AT 245 mμ[a]

5-n-alkyl substitution	pH	$\frac{V_M}{(E_o)}$ x $10^3 min^{-1}$	$K_{M(app)}$ μM	Burst plot	k_3 x $10^3 min^{-1}$ S.S. plot[b]	245 mμ
H	7.00	52 ± 3	0.69 ± 0.33			56.1 ± 1.4
H	7.8	66[c]				
H	7.80					81.7[d]
H	8.2					104 ± 5.6[e]
H	8.20					99.4 ± 1.8
CH_3-	7.11	6.4 ± 0.5	< 0.5			6.25 ± 0.06
CH_3-	7.34			9.63 ± 0.65	10.5 ± 1.3	8.35 ± 0.15
CH_3CH_2-	7.11	12 ± 0.7	< 0.5			12.1 ± 0.1
CH_3CH_2-	7.34			21.6 ± 0.62	17.8 ± 4.3	17.2 ± 0.74

[a]The reactions were performed in 0.2 molar phosphate buffer, u = 0.5 and 1.0% (v/v) or less acetonitrile. Michaelis Menten kinetics and kinetics measured by titration methods were performed as described in the text. Kinetics calculated from measurements at 245 mμ contained 5.0 and 4.0μM initial enzyme and substrate concentrations respectively, and were performed as described in the text. Errors are expressed as ± one standard deviation. Rate constants were measured at 25°C.

[b]Steady state titration plot.

[c]Obtained from Marshall et al. (21).

[d]This value was calculated from k'_3 and its apparent pKa at 25°C given in Table 6, using equation (2).

[e]Calculated from values obtained from Inward and Jencks (18). The rate constants were calculated from measurements at 265 mμ.

TABLE 4A

THE EFFECT OF SUBSTRATE AND PRODUCT CONCENTRATION ON THE DEACYLATION RATE CONSTANT OF 2-FUROYL-CHYMOTRYPSIN AT pH 6.20 AND 25°C

$(S)_o$ μM	p-Nitrophenol μM	2-Furoic acid μM	$k_3 \times 10^3$ min^{-1}
0.87	0.0	0.0	11.6 ± 0.58
2.6	0.0	0.0	12.3 ± 0.79
3.5	0.0	0.0	13.8 ± 0.38
6.1	0.0	0.0	13.6 ± 0.13
			Average 12.8 ± 1
2.0	9.2	0.0	11.4 ± 0.3
2.0	18	0.0	12.2 ± 0.33
2.0	43	0.0	13.7 ± 0.33
2.0	65	0.0	13.5 ± 0.43
2.0	87	0.0	13.6 ± 0.45
			Average 12.9 ± 1
2.0	0.0	3.9	12.4 ± 0.19
2.0	0.0	7.8	13.5 ± 0.28
2.0	0.0	16	13.6 ± 0.17
2.0	0.0	27	12.6 ± 0.18
			Average 13.0 ± 0.6

Reactions were performed in 0.2 molar phosphate, u = 0.4, 0.1% (v/v) or less acetonitrile. Deacylation rate constants were calculated from measurements at 245 mμ as described in the text; $(E)_o$ = 5.0 micromolar. Errors in the rate constants and averages represent ± one standard deviation.

TABLE 4B

THE EFFECT OF CHYMOTRYPSIN CONCENTRATION ON THE DEACYLATION RATE CONSTANT OF 5-ETHYL-2-FUROYL-CHYMOTRYPSIN

$(E)_o$ μM	$k_3 \times 10^3$
4.2	206 ± 2.9
7.4	210 ± 3.8
12	217 ± 9.8

Deacylation rate constants were calculated from measurements at 245 mμ as described in the text; $(S)_o$ = 4.0 micromolar, 43.8°C, 0.2 molar phosphate buffer pH 8.60, u = 0.6 and 0.08% (v/v) acetonitrile. Errors represent ± one standard deviation.

TABLE 5

THE EFFECT OF IONIC STRENGTH AND ACETONITRILE CONCENTRATION ON THE DEACYLATION RATE CONSTANT OF 2-FUROYL-CHYMOTRYPSIN AT 25°C

pH	Phosphate Concentration M	Ionic Strength μ	%(v/v) CH_3CN	$k_3 \times 10^3$ min^{-1}
7.00	0.05	0.09	0.10	55.3 ± 1.3
7.00	0.5	0.9	0.10	57.0 ± 1.4
7.53	0.02	0.06	0.10	78.3 ± 0.44
7.53	0.2	0.6	0.10	78.1 ± 0.7
6.90	0.2	0.5	0.0	47.5 ± 1.4
6.90	0.2	0.5	0.10	47.8 ± 1.8
6.90	0.2	0.5	0.70	45.9 ± 0.9

Deacylation rate constants were calculated from measurements at 245 mμ as described in the text; $(E)_o$ = 5.0 micromolar; $(S)_o$ = 4.0 micromolar. Errors in the rate constants represent ± one standard deviation.

TABLE 6

THE EFFECT OF TEMPERATURE ON THE APPARENT pKa OF THE DEACYLATION REACTION OF 5-N-ALKYL-2-FUROYL-CHYMOTRYPSINS

5-n-alkyl substitution	Temp. °C	pH	$k_3 \times 10^3 min^{-1}$	$k_3' \times 10^3 min^{-1}$	pK_a
H	25.0	8.41	93.6 ± 2.8	101 ± 2.4	6.95
		8.20	99.4 ± 1.8		± .07
		7.90	92.5 ± 1.4		
		7.59	79.6 ± 1.3		
		7.35	69.9 ± 2.1		
		7.00	56.1 ± 1.4		
		6.20	12.8 ± 0.9		
	31.4	8.44	164 ± 3.5	174 ± 3	6.87
		8.04	172 ± 4.7		± .06
		7.45	156 ± 7.4		
		7.08	110 ± 2.4		
		6.82	76.6 ± 1.9		
		6.38	42.9 ± 0.42		
	39.2	8.45	359 ± 10	366 ± 7	6.80
		7.95	357 ± 12		± .08
		7.49	301 ± 15		
		7.12	242 ± 5		
		6.87	196 ± 3		
		6.33	93.6 ± 2.1		
CH_3-	25.0	8.41	14.9 ± 0.23	15.4 ± 0.28	7.27
		8.20	13.4 ± 0.14		± .05
		7.91	12.5 ± 0.11		
		7.60	11.1 ± 0.2		
		7.34	8.35 ± 0.15		
		7.11	6.25 ± 0.06		
		6.77	3.76 ± 0.40		

Table 6 (continued)

5-n-alkyl substitution	Temp. °C	pH	$k_3 \times 10^3 min^{-1}$	$k_3' \times 10^3 min^{-1}$	pK_a
CH_3-	31.4	8.43	24.8 ± 0.7	26.2 ± 0.47	6.97
		8.03	24.4 ± 0.33		± .05
		7.45	19.0 ± 0.4		
		7.08	14.9 ± 0.34		
		6.82	11.4 ± 0.29		
		6.42	5.67 ± 0.13		
	39.2	8.45	55.1 ± 0.75	58.6 ± 1.9	6.88
		7.95	55.1 ± 0.8		± .07
		7.49	50.2 ± 0.66		
		7.11	34.9 ± 0.55		
		6.87	28.4 ± 0.38		
		6.61	21.1 ± 0.38		
CH_3CH_2-	25.0	8.41	31.0 ± 0.42	32.6 ± 0.86	7.33
		8.20	29.2 ± 0.22		± .06
		7.91	24.4 ± 0.24		
		7.60	21.6 ± 0.16		
		7.35	17.2 ± 0.74		
		7.11	12.1 ± 0.1		
		6.44	4.25 ± 0.13		
	31.4	8.44	56.1 ± 0.6	59.5 ± 0.98	7.11
		8.03	54.3 ± 0.8		± .05
		7.45	43.7 ± 1.3		
		7.08	28.5 ± 0.48		
		6.82	19.7 ± 0.24		
		6.42	10.2 ± 0.19		
	39.2	8.45	124 ± 5	131 ± 4.8	7.02
		7.96	123 ± 1.8		± .07
		7.49	95.8 ± 1.3		
		7.11	68.3 ± 1		

Table 6 (continued)

5-n-alkyl substitution	Temp °C	pH	$k_3 \times 10^3 min^{-1}$	$k_3' \times 10^3 min^{-1}$	pK_a
CH_3CH_2	39.2	6.87	54.7 ± 0.7		
		6.60	37.3 ± 0.7		
$CH_3(CH_2)_2$-	25.0	8.48	62.0 ± 4.8	59.2 ± 1.7	7.09
		8.11	55.9 ± 2.1		± .06
		7.82	50.2 ± 1.1		
		7.64	45.9 ± 0.48		
		7.35	35.4 ± 0.91		
		6.94	24.6 ± 0.17		
		6.71	17.8 ± 0.14		
		6.54	11.2 ± 0.26		
	35.8	8.36	164 ± 3.4	169 ± 5.9	6.82
		8.02	158 ± 2		± .08
		7.71	155 ± 1.8		
		7.35	132 ± 1.2		
		6.99	94.4 ± 0.9		
		6.67	73.0 ± 0.7		
$CH_3(CH_2)_3$-	25.0	8.40	20.1 ± 1	21.1 ± 0.72	6.96
		8.17	21.0 ± 0.86		± .09
		7.95	18.9 ± 1.2		
		7.44	14.6 ± 0.74		
		7.03	12.5 ± 0.81		
	37.8	8.36	70.7 ± 1.5		
		3.01	68.5 ± 0.53		
		7.71	62.4 ± 0.64		
		7.36	56.8 ± 0.5		
		6.99	45.8 ± 0.39		
		6.67	30.8 ± 0.33		
$CH_3(CH_2)_4$-	42.7	8.41	40.7 ± 1.4	42.2 ± 0.75	6.80
		8.04	41.5 ± 0.59		± .08

Table 6 (continued)

5-n-alkyl substitution	Temp. °C	pH	$k_3 \times 10^3 min^{-1}$	$k_3' \times 10^3 min^{-1}$	pK_a
$CH_3(CH_2)_4$-	42.7	7.78	38.0 ± 1.3		
		7.38	33.8 ± 0.82		
		7.11	29.5 ± 0.9		
	48.8	8.55	75.5 ± 3.1	79.2 ± 4	6.75
		7.82	76.9 ± 2.4		± .10
		7.45	60.5 ± 3.1		
		7.02	51.7 ± 1.6		

Rate constants were calculated from measurements at 245 mμ as described in the text; $(E)_o$ = 5.0 micromolar, $(S)_o$ = 4.0 micromolar, 0.2 molar phosphate, μ = 0.4-0.6, 0.1% (v/v) or less acetonitrile. Errors in the rate constants and pKa's represent ± one standard deviation.

TABLE 7

THE TEMPERATURE DEPENDENCE OF THE DEACYLATION RATE CONSTANT OF 5-N-ALKYL-2-FUROYL-CHYMOTRYPSINS

5-n-alkyl substitution	T°C	pH	$k_3 \times 10^3 min^{-1}$	$k_3' \times 10^3 min^{-1}$
		Low temperature region		
H	12.4 ± 0.4	8.60	28.3 ± 0.47	29.3 ± 0.49
	15.8 ± 0.4	8.60	40.7 ± 0.64	42 ± 0.66
	20.0 ± 0.3	8.41	64.9 ± 1.9	68.4 ± 2
	25.0 ± 0.2	--	---	101 ± 2.4
	28.2 ± 0.2	8.44	132 ± 2.6	136 ± 2.7
		High temperature region		
	31.4 ± 0.3	--	---	174 ± 3
	34.6 ± 0.3	8.47	231 ± 6	236 ± 6.1
	39.2 ± 0.4	--	---	366 ± 7
	40.7 ± 0.4	8.44	409 ± 24	418 ± 26
	43.3 ± 0.4	8.45	495 ± 17	504 ± 17
	46.7 ± 0.4	8.47	691 ± 20	703 ± 20
		Low temperature region		
CH_3-	20.0 ± 0.3	8.42	8.90 ± 0.11	9.60 ± 0.12
	25.0 ± 0.2	--	---	15.4 ± 0.28
		High temperature region		
	28.2 ± 0.2	8.47	19.2 ± 0.1	20.0 ± 0.11
	31.4 ± 0.3	--	---	26.2 ± 0.47
	34.6 ± 0.3	8.47	36.5 ± 0.34	37.5 ± 0.35
	39.2 ± 0.4	--	---	58.6 ± 1.9
	46.7 ± 0.4	8.46	107 ± 1.7	109 ± 1.7
	48.4 ± 0.4	8.60	130 ± 1.7	131 ± 1.7

Table 7 (continued)

5-n-alkyl substitution	T°C	pH	$k_3 \times 10^3$	$k'_3 \times 10^3$
		Low temperature region		
CH_3CH_2-	15.8 ± 0,4	8.43	12.1 ± 0.27	13.9 ± 0.3
	20.0 ± 0.3	8.42	18.7 ± 0.22	20.7 ± 0.24
	25.0 ± 0.2	--	---	32.6 ± 0.86
	28.2 ± 0.2	8.47	42.5 ± 0.6	44.8 ± 0.63
		High temperature region		
	28.2 ± 0.2	8.47	42.5 ± 0.6	44.8 ± 0.63
	31.4 ± 0.3	--	---	59.5 ± 0.98
	34.6 ± 0.3	8.47	80.0 ± 1	83.0 ± 1
	39.2 ± 0.4	--	---	131 ± 4.8
	40.7 ± 0.4	8.44	150 ± 1.7	154 ± 1.8
	43.3 ± 0.4	8.46	198 ± 2.1	202 ± 2.1
		Low temperature region		
$CH_3(CH_2)_2$-	10.0 ± 0.4	8.60	16.6 ± 0.25	17.7 ± 0.27
	12.9 ± 0.4	8.60	22.4 ± 0.45	23.7 ± 0.48
	15.2 ± 0.4	8.60	27. ± 0.2	28.4 ± 0.21
	18.1 ± 0.3	8.60	31.7 ± 0.34	33.1 ± 0.35
	19.9 ± 0.3	8.60	37.9 ± 0.41	39.4 ± 0.43
	22.5 ± 0.3	8.48	42.1 ± 1.5	44 ± 1.6
	25.0 ± 0.2	--	---	59.2 ± 1.7
		High temperature region		
	30.7 ± 0.2	8.54	95.8 ± 5.2	98.1 ± 5.3
	34.3 ± 0.3	8.42	137 ± 1.4	141 ± 1.4
	35.8 ± 0.3	--	---	169 ± 5.9
	38.0 ± 0.4	8.50	203 ± 1.9	207 ± 1.9
	40.8 ± 0.4	8.48	283 ± 1	288 ± 1
	43.4 ± 0.4	8.49	334 ± 6	338 ± 6.1
	48.1 ± 0.4	8.60	621 ± 9.1	627 ± 9.2

Table 7 (continued)

5-n-alkyl substitution	T°C	pH	$k_3 \times 10^3$	$k'_3 \times 10^3$
		Low temperature region		
$CH_3(CH_2)_3$-	20.4 ± 0.3	8.48	12.3 ± 0.39	12.8 ± 0.4
	25.0 ± 0.2	--	---	21.1 ± 0.72
	29.7 ± 0.2	8.50	27.0 ± 1.1	27.7 ± 1.1
		High temperature region		
	32.7 ± 0.3	8.49	44.4 ± 1.5	45.4 ± 1.5
	34.3 ± 0.3	8.43	55.3 ± 1.6	56.7 ± 1.6
	37.8 ± 0.4	--	---	71.9 ± 1.2
	39.5 ± 0.4	8.48	81.7 ± 2.4	83.1 ± 2.5
	43.8 ± 0.4	8.48	129 ± 1.5	131 ± 1.5
	46.9 ± 0.4	8.49	173 ± 4.8	175 ± 4.9
$CH_3(CH_2)_4$	30.0 ± 0.2	8.52	9.71 ± 0.32	9.93 ± 0.33
	32.8 ± 0.3	8.50	14 ± 0.37	14.3 ± 0.38
	35.6 ± 0.3	8.49	16.8 ± 0.51	17.2 ± 0.52
	38.0 ± 0.4	8.52	23.2 ± 0.9	23.7 ± 0.91
	40.5 ± 0.4	8.55	30.9 ± 0.52	31.5 ± 0.53
	42.7 ± 0.4	--	---	43.2 ± 0.75
	43.5 ± 0.4	8.60	45.3 ± 0.45	45.9 ± 0.46
	44.7 ± 0.4	8.60	47.0 ± 0.57	47.6 ± 0.58
	48.6 ± 0.4	8.60	78.4 ± 1	79.5 ± 1
	48.8 ± 0.4	--	---	79.2 ± 4

The rate constants were calculated as described in the text and Table 6. Errors represent ± one standard deviation unit.

TABLE 8

THE ACTIVATION PARAMETERS FOR THE DEACYLATION OF

5-N-ALKYL-2-FUROYL-CHYMOTRYPSINS

5-n-alkyl substitution	ΔH kcal/mole	-ΔS e.u.
	High temperature region	
H-	16.92 ± 0.24	14.61 ± 0.77
CH_3-	17.24 ± 0.24	17.27 ± 0.81
CH_3CH_2-	18.21 ± 0.34	12.47 ± 1.11
$CH_3(CH_2)_2$-	19.84 ± 0.68	6.10 ± 2.18
$CH_3(CH_2)_3$-	17.42 ± 0.71	15.89 ± 2.28
$CH_3(CH_2)_4$-	20.89 ± 0.51	7.00 ± 1.64
	Low temperature region	
H-	15.79 ± 0.52	18.23 ± 1.75
CH_3CH_2-	15.78 ± 0.28	20.52 ± 0.94
$CH_3(CH_2)_2$-	12.19 ± 0.61	31.47 ± 2.09

Error represents ± one standard deviation.

TABLE 9

THE TEMPERATURE DEPENDENCE OF THE DEACYLATION RATE CONSTANT OF 2-FUROYL-CHYMOTRYPSIN AT pH 10.0, AND THE ACTIVATION PARAMETERS[a]

$T^{\circ}C$	$k_3 \times 10^3$ min^{-1}	$k'_3 \times 10^3$ [b] min^{-1}
	Low temperature region	
16.3 ± 0.4	42.2 ± 2.2	44.9
21.1 ± 0	81.6 ± 1.7	71.5
24.8 ± 0.2	105 ± 4.2	101
29.0 ± 0.2	159 ± 6.2	149
	High temperature region	
35.0 ± 0.3	280 ± 6.4	247
39.2 ± 0.4	387 ± 13	363

Activation parameters in the low temperature region

$\Delta H^{\neq}$	$\Delta S^{\neq}$
16.3 ± 1.8	16.5 ± 6.1

[a] The rate constants were calculated from measurements at 245 mμ as described in the text; 0.2 M carbonate buffer, μ = 0.4, 0.1% (v/v) acetonitrile, $(E)_o$ = 5.0 μM, $(S)_o$ = 4.0 μM. Errors represent ± one standard deviation unit.

[b] The rate constants were calculated from values measured at low pH's using equations 2 and 4.

TABLE 10

THE TEMPERATURE DEPENDENCE OF THE ALKALINE SAPONIFICATION RATE CONSTANTS OF p-NITROPHENYL 5-N-ALKYL-2-FUROATES

5-n-alkyl substitution	T°C	$k_{OH} \times 10^3 min^{-1}$	ΔH kcal/mole	-ΔS e.u.
H	28.4 ± 0.2	240 ± 1.2	19.06	6.33
	31.8 ± 0.3	338 ± 2.9	± 0.28	±0.92
	36.0 ± 0.3	549 ± 4.2		
	39.4 ± 0.4	746 ± 7.2		
	42.0 ± 0.4	943 ± 8.2		
	43.1 ± 0.4	1130 ± 8.1		
	46.1 ± 0.4	1490 ± 16		
CH_3-	25.0 ± 0.2	54.2 ± 1	19.52	7.05
	28.4 ± 0.2	74.1 ± 0.3	± 0.31	±1.03
	36.0 ± 0.3	179 ± 1.4		
	39.4 ± 0.4	251 ± 1.3		
	42.0 ± 0.4	325 ± 2.4		
	43.1 ± 0.4	375 ± 2.7		
	46.1 ± 0.4	484 ± 3.7		
CH_3CH_2-	21.4 ± 0.3	35.5 ± 0.3	19.35	7.63
	25.0 ± 0.2	50.4 ± 0.4	± 0.29	±0.94
	28.4 ± 0.2	77.5 ± 0.44		
	36.0 ± 0.3	175 ± 1		
	39.4 ± 0.4	247 ± 2.4		
	42.0 ± 0.4	308 ± 2.8		
	43.1 ± 0.4	365 ± 4.1		
$CH_3(CH_2)_2$-	25.0 ± 0.2	48.7 ± 0.25	19.59	7.01
	28.4 ± 0.2	68.8 ± 0.39	± 0.22	± 0.73
	31.8 ± 0.3	104 ± 0.57		
	36.0 ± 0.3	156 ± 0.9		

Table 10 (continued)

5-n-alkyl substitution	T°C	$k_{OH} \times 10^3$	ΔH kcal/mole	-ΔS e.u.
$CH_3(CH_2)_2$-	39.4 ± 0.4	237 ± 2.3		
	42.0 ± 0.4	300 ± 3.3		
	43.1 ± 0.4	333 ± 2		
	46.1 ± 0.4	461 ± 3.4		
$CH_3(CH_2)_3$-	25.0 ± 0.2	46.4 ± 0.32	19.23	8.24
	28.4 ± 0.2	69.6 ± 0.39	± 0.31	±1.01
	31.8 ± 0.3	101. ± 0.6		
	36.0 ± 0.3	160 ± 1.6		
	39.4 ± 0.4	211 ± 1.3		
	42.0 ± 0.4	280 ± 2		
	43.1 ± 0.4	342 ± 4.4		
	46.1 ± 0.4	416 ± 4.3		
$CH_3(CH_2)_4$-	25.0 ± 0.2	44.3 ± 0.43	19.33	8.04
	28.4 ± 0.2	64.8 ± 0.2	± 0.22	±0.74
	31.8 ± 0.3	92.8 ± 0.53		
	36.0 ± 0.3	151 ± 1.1		
	39.4 ± 0.4	210 ± 2.1		
	43.1 ± 0.4	290 ± 3		
	46.1 ± 0.4	418 ± 5		

The reactions were performed in 10 millimolar borate buffer at pH 10.50 as described in the text. Errors represent ± one standard deviation.

TABLE 11

THE CORRECTED ACTIVATION PARAMETERS FOR THE DEACYLATION OF 5-N-ALKYL-2-FUROYL-CHYMOTRYPSINS

5-n-alkyl substitution	ΔΔH kcal/mole	-ΔΔS e.u.
	High temperature region	
H-	-2.14 ± 0.37	8.28 ± 1.20
CH_3-	-2.26 ± 0.39	10.22 ± 1.32
CH_3CH_2-	-1.14 ± 0.45	4.84 ± 1.46
$CH_3(CH_2)_2$-	0.25 ± 0.72	-0.91 ± 2.28
$CH_3(CH_2)_3$-	-1.81 ± 0.78	7.65 ± 2.50
$CH_3(CH_2)_4$-	1.56 ± 0.56	-1.04 ± 1.81
	Low temperature region	
H-	-3.27 ± 0.59	11.90 ± 1.99
CH_3CH_2-	-3.57 ± 0.40	12.89 ± 1.34
$CH_3(CH_2)_2$-	-7.40 ± 0.65	24.46 ± 2.22

Errors represent ± one standard deviation.

Figure 1. The Difference Spectrum of 2-Furoyl-Chymotrypsin Versus Chymotrypsin Plus 2-Furoic Acid.

The difference spectrum was measured as described in the text; $(E)_o$ = 30 micromolar, $(S)_o$ = 30 micromolar, 0.2 molar phosphate buffer, pH = 7.00, μ = 0.5, 0.8% (v/v) acetonitrile and 25°C.

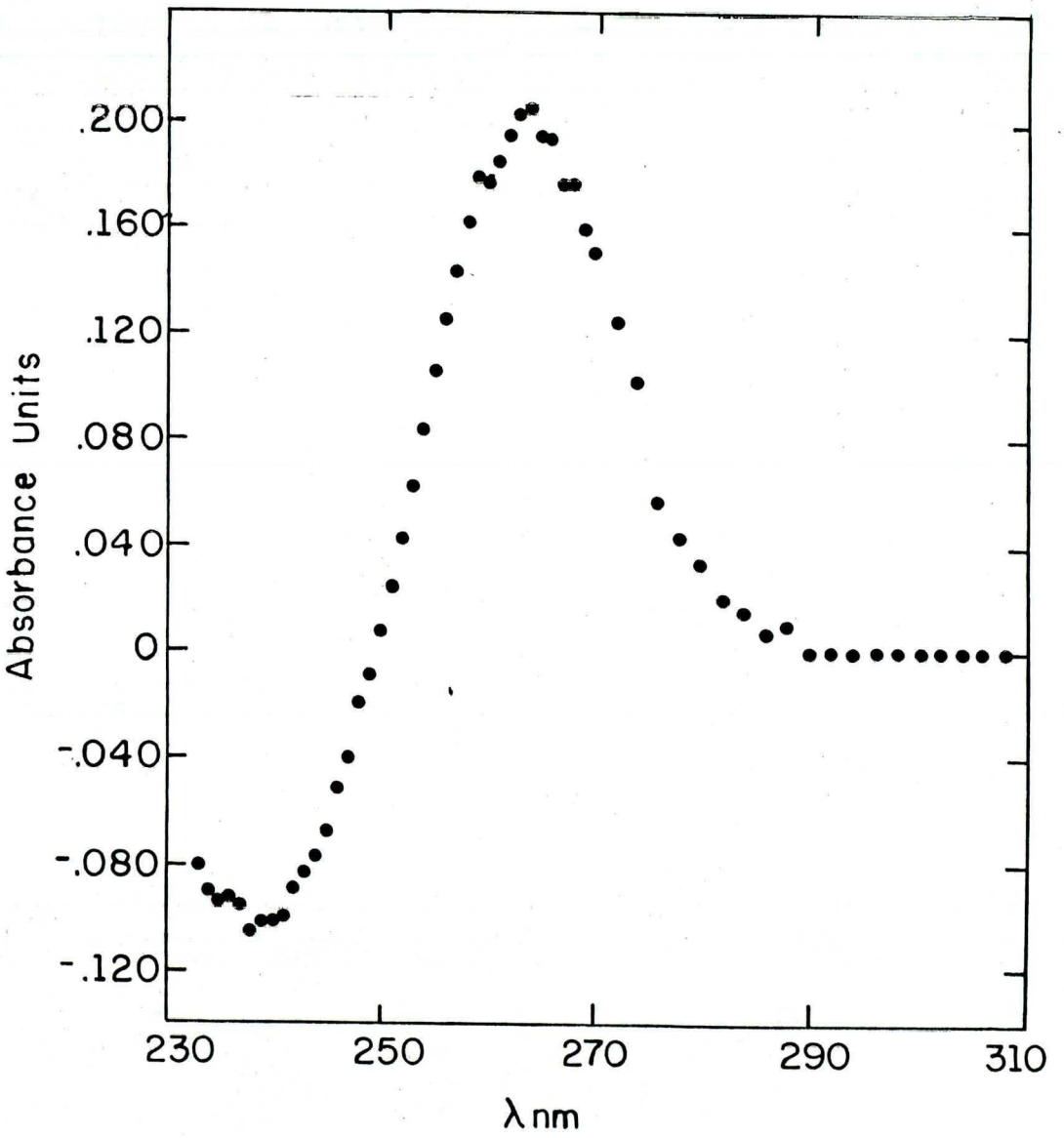

.200
.160
.120
.080
.040
0
-.040
-.080
-.120
Absorbance Units
230
250
270
290
310
λnm

Figure 1

Figure 2-A. The Difference Spectrum of 5-Ethyl-2-Furoyl-Chymotrypsin Versus Chymotrypsin Plus 5-Ethyl-2-Furoic Acid

The difference spectrum was measured as described in the text; $(E)_o$=30 micromolar, $(S)_o$ = 30 micromolar, 0.2 molar phosphate buffer, pH = 8.20, μ = 0.6, 0.5% (v/v) acetonitrile and 27°C.

Figure 2-B. The Difference Spectra of 5-Ethyl-2-Furoyl-Chymotrypsin Versus Chymotrypsin Plus 5-Ethyl-2-Furoic Acid During the Deacylation Reaction

The difference spectra were measured at various times during the deacylation reaction. Conditions are the same as in Figure 2-A.

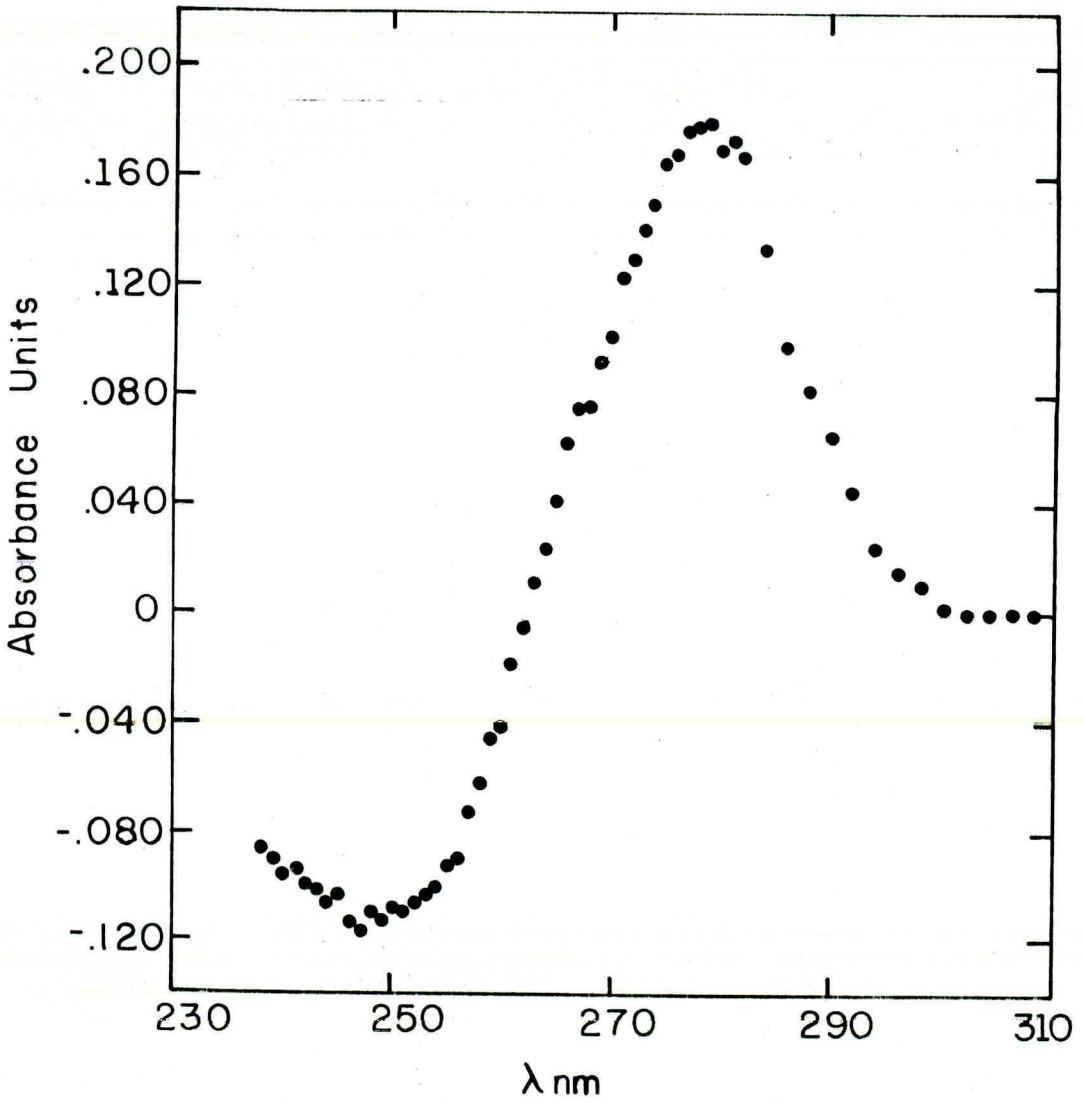

.200
.160
.120
.080
.040
0
-.040
-.080
-.120
Absorbance Units
230
250
270
290
310
λ nm

Figure 2-A

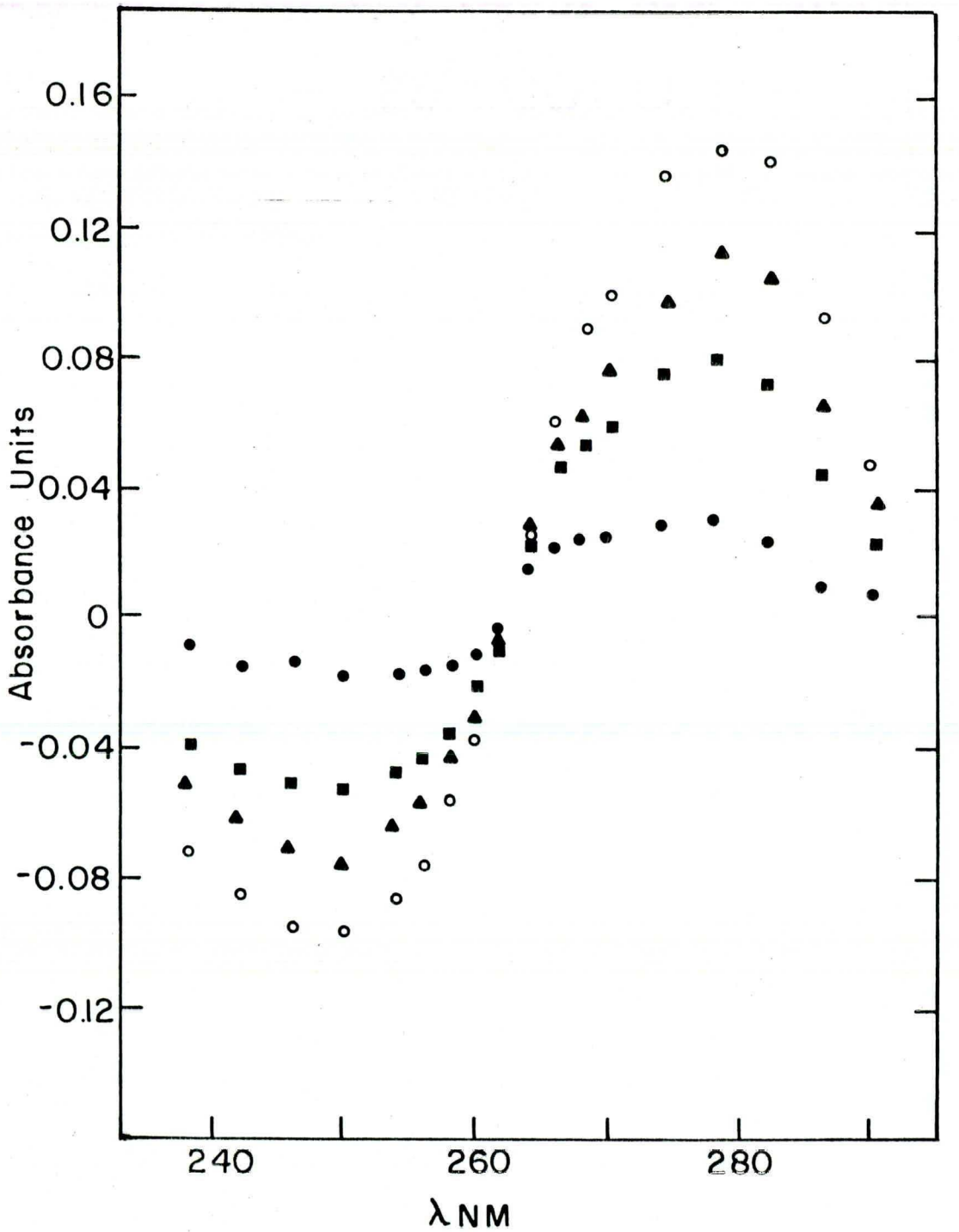


Figure 2-B

Figure 3. The Temperature Dependence of the Apparent pKa of the Deacylation Reaction of 5-N-Alkyl-2-Furoyl Chymotrypsins

The apparent pKa is plotted vs. $\frac{1}{T}$. CH_3, CH_3CH_2, etc. indicate the 5-n-alkyl substitution. Conditions are described in Table 6.

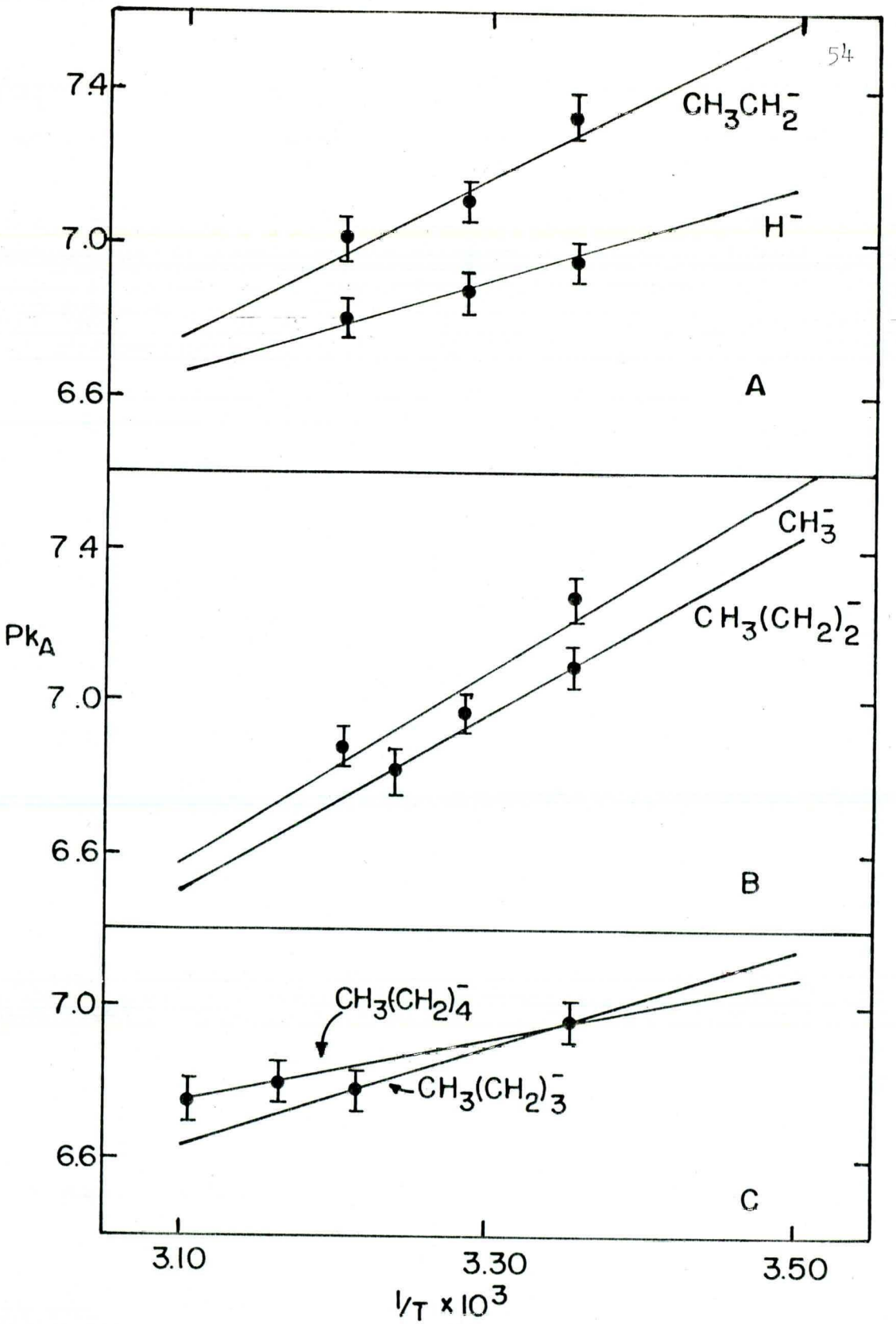

7.4
7.0
6.6
$CH_3CH_2^-$
H^-
A
7.4
7.0
6.6
CH_3^-
$CH_3(CH_2)_2^-$
B
pK_A
7.0
6.6
$CH_3(CH_2)_4^-$
$CH_3(CH_2)_3^-$
C
3.10
3.30
3.50
$1/T \times 10^3$

Figure 3

Figure 4. The Temperature Dependence of the pH Independent Rate Constants for the Deacylation of 5-n-Propyl-2-Furoyl-Chymotrypsin

The ln of $\frac{k'_3}{T}$ is plotted versus $\frac{1}{T}$ according to equation 4. Conditions are the same as those in Table 7.

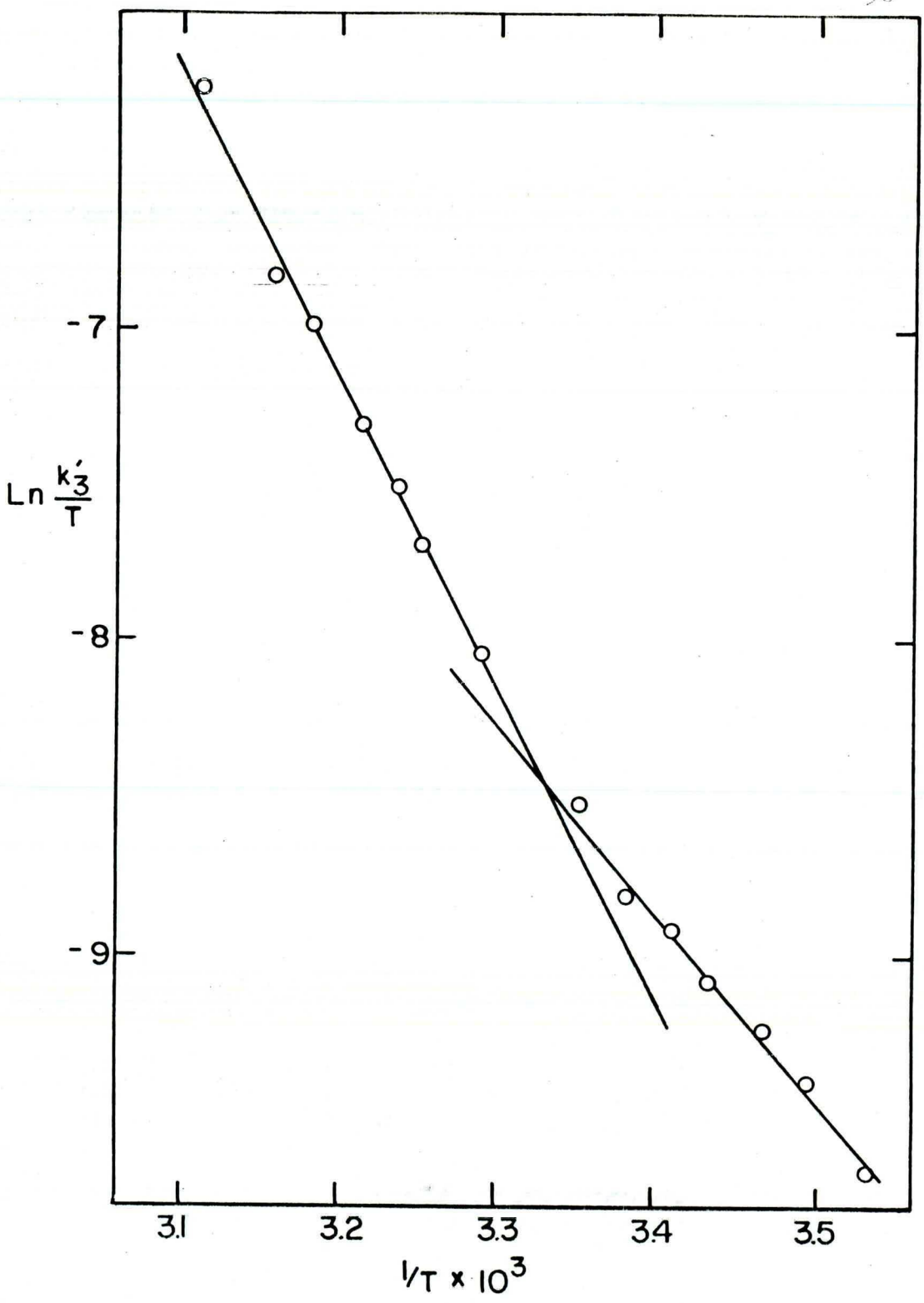


Figure 4

Figure 5. The Temperature Dependence of the pH Independent Rate Constants for the Deacylation of 5-N-Butyl-2-Furoyl-Chymotrypsin

The ln of $\frac{k'_3}{T}$ is plotted versus $\frac{1}{T}$ according to equation 4. Conditions are the same as those in Table 7.

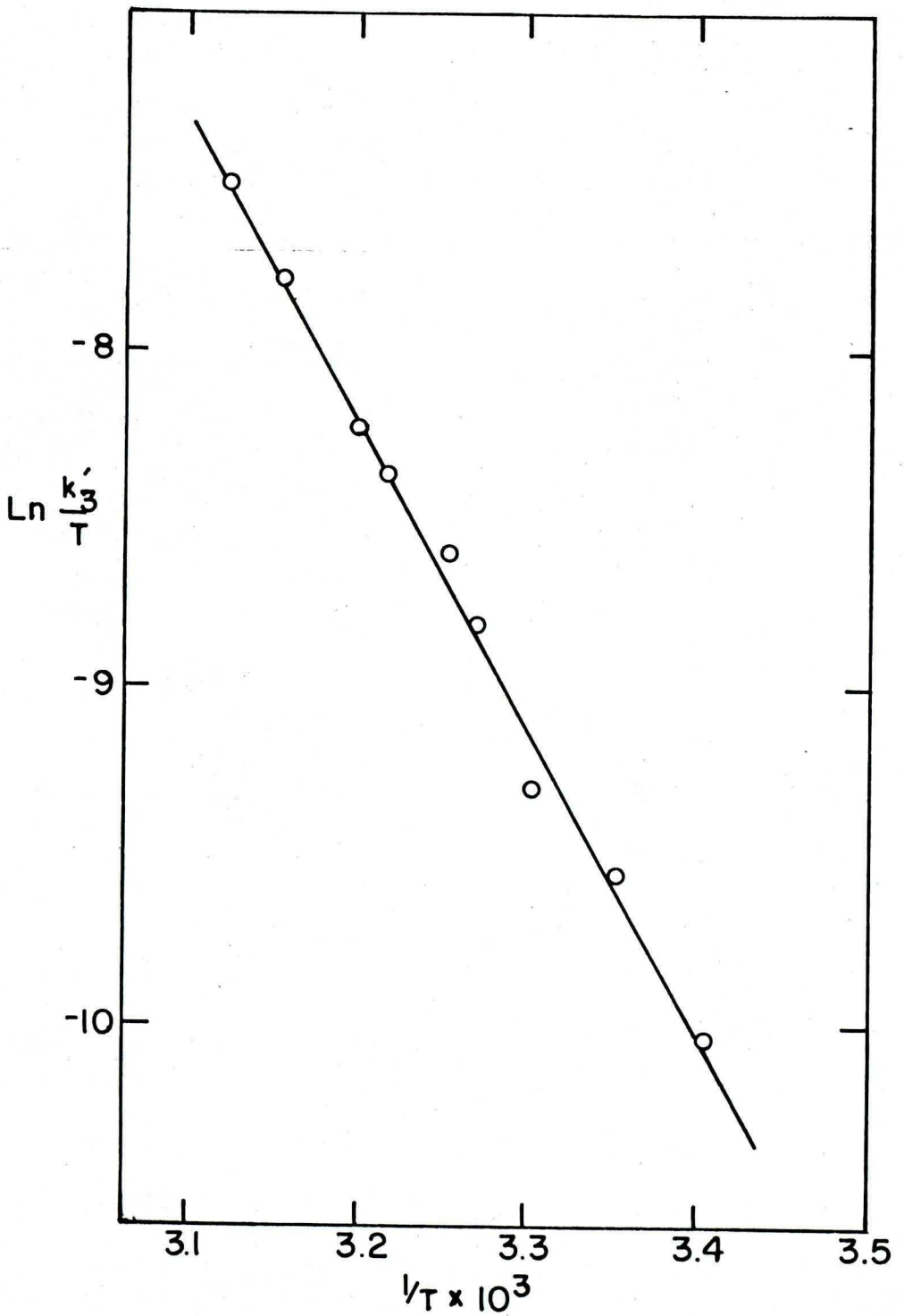


Figure 5

Figure 6. The pH Dependence of the Alkaline Saponification Rate Constant of p-Nitrophenyl 2-Furoate

The log of the alkaline saponification rate constant of p-nitrophenyl 2-furoate plotted versus the pH. Rate constants were measured at 25°C. Open circles represent rate constants measured in 10 millimolar borate buffer. Open squares represent rate constants measured in unbuffered sodium hydroxide solution. The open triangle represents the rate constant measured in 30 millimolar borate buffer. The initial concentration of p-nitrophenyl 2-furoate was 1 μM.

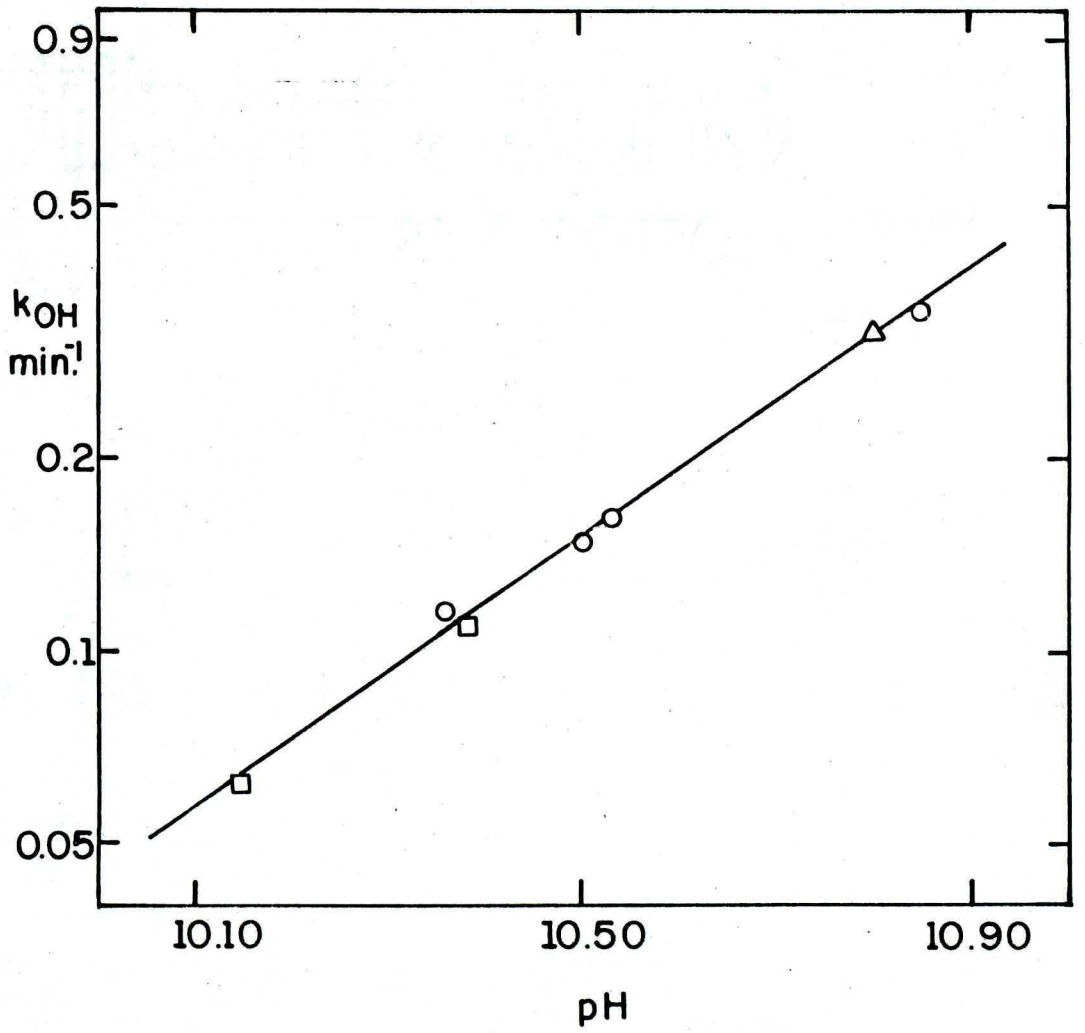
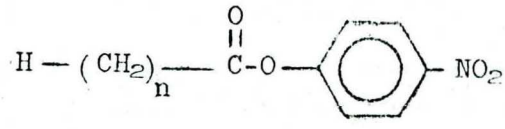


Figure 6

Figure 7. The Dependence of the Corrected Deacylation Rate Constant and the Relative Corrected Deacylation Rate Constant on the Volume of the Substrate.

Volumes are in $Å^3$ and represent the volume of the acyl moiety ($R\text{-}CO_2\text{-}$) of the substrate $R\text{-}CO_2\text{-}\emptyset\text{-}NO_2$. Volumes were calculated from data obtained from Edward (33). Rate constants were measured at 25°C.

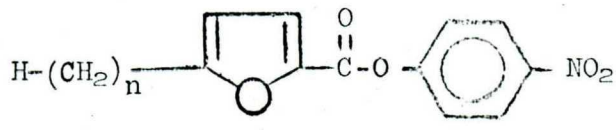
A: Closed circles and solid lines represent the series



$n = 1,2,3,4,5,6,7,8$

where k_3/k_{OH} for p-nitrophenyl acetate is taken as the reference; pH 7.8. Values were obtained from Marshall and Akgün (6).

Open circles and dashed lines represent the series:

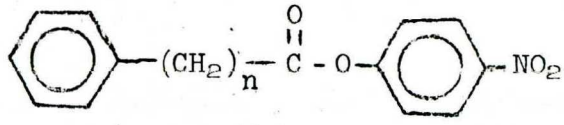


$n = 1,2,3,4,5$

where k'_3/k_{OH} for p-nitrophenyl furoate is taken as a reference. The rate constant for the deacylation of 5-amyl-2-furoyl-chymotrypsin

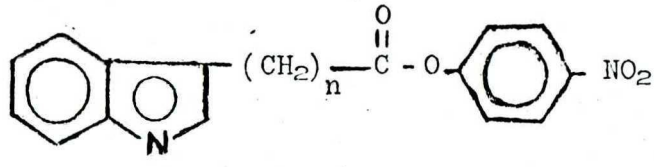
at 25°C was obtained by extrapolation using equation 4. Conditions are described in Table 6.

B: Closed circles and solid lines represent the series:



n = 1,2,3,4

and open circles and dashed lines represent the series:



n = 1,2,3

In both series the pH independent rate constants are corrected for inductive and steric effects using the Taft equation; the values were obtained from Dupaix et al. (3).

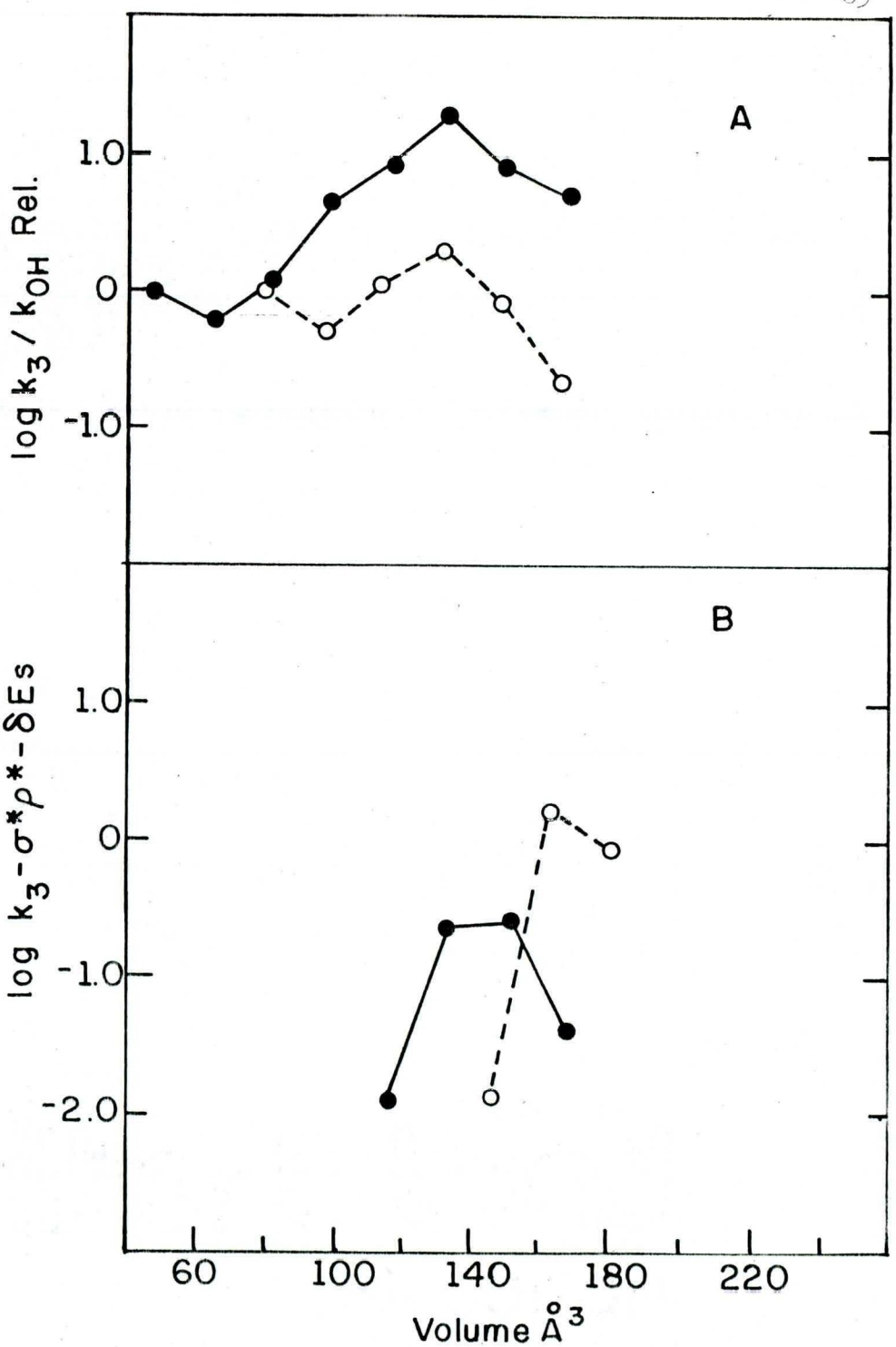

A
B
1.0
0
-1.0
log k_3 / k_{OH} Rel.
log $k_3 - \sigma^* \rho^* - \delta E_s$
1.0
0
-1.0
-2.0
60
100
140
180
220
Volume Å^3

Figure 7

BIBLIOGRAPHY

1. Hein, G.E., and Niemann, C., J. Am. Chem. Soc., 84, 4495 (1962).

2. Bender, M.L., and Kezdy, F.S., Ann. Rev. Biochem., 34, 49 (1965).

3. Dupaix, A., Bechet, J.J., and Roucous, C., Biochemistry, 12, 2559 (1973).

4. Hofstee, B.H.J., Biochim. Biophys. Acta, 24, 211 (1957).

5. Hofstee, B.H.J., Biochim. Biophys. Acta, 32, 182 (1959).

6. Marshall, T.H. and Akgün, A., J. Biol. Chem. 246, 6019 (1971).

7. Fife, T.H. and Milstien, Biochemistry 6, 2901 (1967).

8. Caplow, M., and Jencks, W.P., Biochemistry 1, 883 (1962).

9. Klapper, M.H., Prog. Biog. Chem. 2, 55 (1973).

10. Laskowski, M. in Biochemist's Handbook (Long, C., ed), p. 304, Van Nostran-Reinhold (1961).

11. Traynelis, V.J., Miskel, J.J., and Sowa, J.R., J. Org. Chem., 22, 1269 (1957).

12. Runde, M.M., Scott, E.W., and Johnson, J.R., J. Am. Chem. Soc., 52, 1284 (1930).

13. Reichstein, T., and Zschokke, H., Helv. Chim. Acta 15, 1124 (1932).

14. Novitskii, K. Yu., Gresl, Kh., and Yur'ev, Yu.K., Khim. Geterotsikl Soedin, 2, 832 (1966).

15. Bender, M.L., Begue-Canton, M.L., Balkeley, R.L., Brubacher, L.J., Feder, J., Gunter, C.R., Kezdy, F.J., Killhefer, J.V.,Jr., Marshall, T.H., Miller, C.G., Roeske, R.W., and Stoops, J.K. J. Am. Chem. Soc., 88, 5890 (1966).

16. Bender, M.L., Schonbaum, G.R., and Zerner, B., J. Am. Chem. Soc., 84, 2540 (1962).

17. Bender, M.L., Schonbaum, G.R., and Zerner, B., J. Am. Chem. Soc., 84, 2562 (1962).

18. Inward, P.W. and Jencks, W.P., J. Biol. Chem. 240, 1986 (1965).

19. Bevington, P.R., Data Reduction and Error Analysis for the Physical Sciences, p. 237, McGraw-Hill (1969).

20. Deming, W.E., Statistical Adjustment of Data, p. 218, Dover Publications (1964).

21. Marshall, T.H., Whitaker, J.R., and Bender, M.L., Biochemistry 8, 4671 (1969).

22. Clement, G.E., and Bender, M.L., Biochemistry 2, 836 (1963).

23. Martinek, K., Yatsimirskii, A.K., and Berezin, I.V., Mol. Biol. 5, 96 (1971).

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

25. Christensen, J.J., Izatt, R.M., Wrathall, D.P., and Hansen, L.D., J. Chem. Soc. A, 8, 1212 (1969).

26. Kaplan, H., and Laidler, K.J., Can. J. Chem. 45, 547 (1966).

27. Glick, D.M., Biochim. Biophys. Acta, 250, 390 (1971).

28. Bender, M.L., Kedzy, F.G., and Gunter, C.R., J. Am. Chem. Soc. 86, 3714 (1964).

29. Keizer, J., and Bernhard, S.A., Biochemistry 5, 4172 (1966).

30. Wedler, F.C., and Bender, M.L., J. Am. Chem. Soc. 91, 3894 (1969).

31. Mavridis, A., Tulinsky, A., and Liebmann, M.N., Biochemistry 13, 3661 (1974).

32. Fisher, E., Chem. Ber., 27, 2985 (1894).

33. Edward, J.T., J. Chem. Educ. 47, 261 (1970).

34. Koshland, D.E., Jr., and Neet, K.E., Ann. Rev. Biochem., 37, 359 (1968).

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