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PART I THE MECHANISMS OF ACYLATION OF CHYMOTRYPSIN BY PHENYL ESTERS OF BENZOIC ACID AND ACETIC ACID PART II THE RAPID HETEROLYSIS OF INDOPHENYL ACETATE BY ALPHA(1)-ACID GLYCOPROTEIN IN THE COMMERCIAL PREPARATION OF HORSE SERUM CHOLINESTERASE

THOMAS SCOTT SHOUPE

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PART II.

THE RAPID HETEROLYSIS OF INDOPHENYL ACETATE BY α_1-ACID GLYCOPROTEIN IN THE COMMERCIAL PREPARATION OF HORSE SERUM CHOLINESTERASE

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This thesis is dedicated to

Suzanne, Jeanne, Harry and Max.

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PART I

The kinetics of the acylation of α-chymotrypsin by a series of substituted phenyl p-nitrobenzoates have been studied by stopped-flow and conventional spectrophotometry. Electron withdrawal in the leaving group accelerates the rate of acylation, and the rho value obtained for eight esters is +1.96. There is also good correlation of the rate constants with the pK_a of the leaving group ($\beta = -0.78$). An analysis of these parameters compared with those for similar enzymatic reactions and the analogous non-enzymatic reactions provides insight into the nucleophilic character of the group in the enzyme which attacks the acyl carbon of these substrates. Previous studies of the reactivity of chymo-

trypsin toward active non-specific esters have indicated that either acylation is general-base catalyzed or that a transient acylimidazole chymotrypsin (His 57) is formed en route to the acyl enzyme.

Kinetic solvent deuterium isotope effects of 1.14 and 1.30 for the acylation by p-nitrophenyl p-nitrobenzoate and p-nitrophenyl benzoate, respectively, taken together with the structure-reactivity results, tend to favor a mechanism for acylation by phenyl benzoates in which the initial reaction is a nucleophilic attack by an imidazole of the enzyme. Further isotope effect data, the results of studies of dioxane inhibition of acylation by the homologous series of esters, p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, and p-nitrophenyl hydrocinnamate, and the effect of dioxane inhibition on the structure-reactivity correlations for the acylation by phenyl benzoates, were also found to be consistent with the intermediacy of an acylimidazole upon acylation by phenyl benzoate substrates.

While it was previously felt that phenyl acetates proceeded to the acyl enzyme by the same path, a kinetic isotope effect of 2.10 for the acylation by p-nitrophenyl thiolacetate and a leaving group rho of 2.05 for acylation by phenyl acetates renders their mechanism of acylation less clear.

Part II

Indophenyl acetate (IPA) undergoes rapid hydrolysis in the presence of commercial preparations of horse serum cholinesterase. The kinetics have been monitored in a stopped-flow spectrophotometer at pH 7.9. A biphasic reaction was observed consisting of a very rapid first-order liberation of indophenolate anion followed by a slower steady-state release. The first-order rate constant for the burst process was found to be independent of initial substrate concentration over a large range of concentrations (7.0×10^{-6} - 2.0×10^{-4} M), and dependent upon the initial enzyme concentration (0.5 - 4.0 mg/ml). Subsequent studies with the inhibitors eserine, tetrabutylammonium iodide, and o-nitrophenyl dimethyl carbamate, indicated that the rapid burst of indophenolate was caused not by the cholinesterase enzyme but by another component of the preparation. The commercial preparation was fractionated and the burst active component was isolated. The first-order rate constant for the rapid cleavage of IPA by the isolated component exhibited the same substrate and enzyme concentration dependence as the commercial preparation. Studies with the isolated component and subsequent analyses have led to the tentative identification of the burst active component as serum α_1-acid

glycoprotein.

PART I.

THE MECHANISMS OF ACYLATION OF CHYMOTRYPSIN BY PHENYL ESTERS OF BENZOIC ACID AND ACETIC ACID

INTRODUCTION

A Brief History of the Mechanism of Chymotrypsin Catalysis.

α-Chymotrypsin is an enzyme which is synthesized in the pancreas. The biological function of this enzyme is to catalyze the hydrolysis of peptide bonds during the digestion of protein material. It is one of a class of proteolytic enzymes called "serine proteases" (1), which are so classified because of their reactions with organophosphates to yield an inactive enzyme phosphorylated on the hydroxyl of a serine moiety of the enzyme. While the biological specificity of chymotrypsin is for peptide bonds formed by the carboxyl group of amino acids with aromatic side chains, it will catalyze the hydrolysis of a wide range of amides as well as esters of specific and non-specific substrates. Much of the work attempting to elucidate the catalytic mechanism of chymotrypsin has involved the study of its reaction with these non-physiological substrates. Recent reviews have examined aspects of the catalytic mechanism of chymotrypsin (2,3,4,5), but an abbreviated history of the subject will aid in placing the following study in perspective.

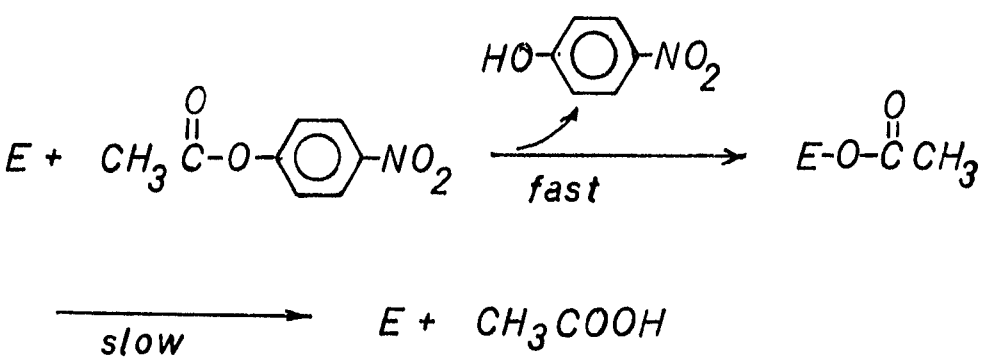
Early workers found that chymotrypsin reacts stoichiometrically with diisopropylfluorophosphate

(DFP) to form diisopropylphosphorylchymotrypsin (6), and subsequently the presence of serine phosphoric acid was found in the hydrolysate of the DFP inhibited enzyme (7), thus implicating a serine residue in the catalytic mechanism. The effect of pH upon the catalysis of neutral esters indicated that the catalytic rate constant increased as a residue with a pK_a of approximately 7 ionized (8). This piece of evidence implicated the imidazole of a histidine residue in the catalysis because an ionizing group in a protein with a pK_a of about 7 is usually thought to be a histidine residue. Reagents which alkylate histidine specifically were later shown to inactivate the enzyme, confirming the importance of this residue (9,10). When the amino acid sequence of chymotrypsin was finally determined these catalytically important residues were identified as Ser 195 and His 57 (11,12). Much investigation into the catalytic mechanism of chymotrypsin has centered on the determination of the functions of these two residues.

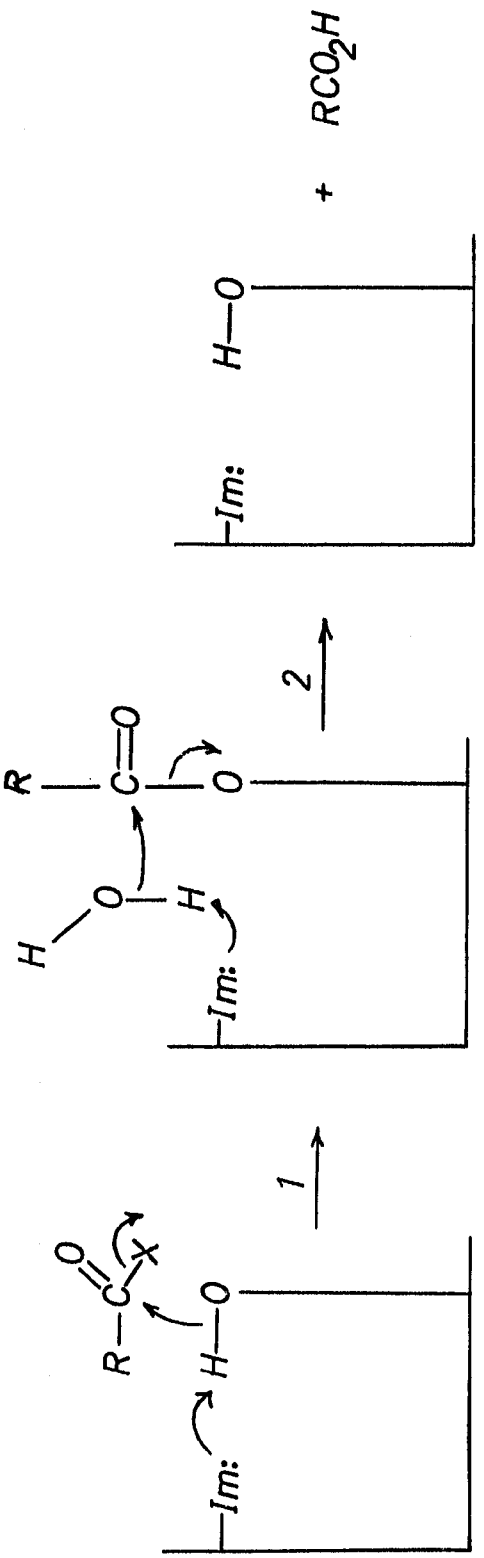
A typical pH-rate profile for the reaction of chymotrypsin with neutral substrates is bell shaped indicating the mechanistic importance of two ionizing groups in the catalytic reaction. The rate of the reaction increases as a group with an apparent pK_a of approximately 7 ionizes. This group has been identified as the imidazole of His 57 (4). The basic limb of the

profile indicates that the rate decreases with the ionization of a group with an apparent pK_a of about 8.5. Studies have shown that deacylation, represented by k_{cat}, is independent of pH at alkaline pH while K_m is pH dependent (13). Therefore, the ionizing group with a pK_a of 8.5 affects the substrate binding. Subsequent studies have shown that the ionizing group with a pK_a of 8.5 is the α-amino group of Ile 16, which is responsible for a conformational change of the enzyme triggered by the ionization of this residue (14). Binding of substrates is favored for the enzyme conformation in which this group is protonated.

Hartley and Kilby first observed a biphasic hydrolysis of p-nitrophenyl acetate by chymotrypsin and proposed a mechanism by which the enzyme is rapidly acylated by the ester accompanied by a stoichiometric release of p-nitrophenol followed by a slower deacylation (Scheme I) (15). With the aid of rapid reaction



Scheme I



Scheme II

detection techniques Gutfreund and Sturtevant (16) were able to examine the kinetics of each step in the enzyme catalyzed hydrolysis of p-nitrophenyl acetate and put the mechanism into the general form of Equation (1-1).

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E\tilde{}S \xrightarrow{k_2} E\text{-}P_2 \xrightarrow{k_3} E + P_2 \qquad (1\text{-}1)$$

$$E\tilde{}S \xrightarrow{k_2} P_1$$

They also suggested that the chymotrypsin catalyzed hydrolysis of specific ester substrates proceeded by the same mechanism (17,18). It was Cunningham (18) who first discussed the functions of the serine and histidine moieties in the catalytic mechanism. It was suggested that acylation proceeded by general-base activation of the serine hydroxyl by the imidazole of the histidine resulting in a serine acylated enzyme, and subsequent deacylation proceeds by analogous imidazole general-base catalyzed attack of water on the acyl enzyme resulting in regeneration of the enzyme. This general mechanism is shown in Scheme II.

The existence of an acyl enzyme intermediate in reactions of non-specific substrates was substantiated by the isolation of crystalline trimethylacetylchymotrypsin from acylation by p-nitrophenyl trimethylacetate (19) and the observation that the deacylation of cinnamoylchymotrypsin was independent of the nature of the

ester leaving group as would be predicted by the existence of a common intermediate (20). Titration of the active sites of chymotrypsin allowing accurate determinations of enzyme concentration was a direct consequence of the existence of stable acyl enzyme intermediates (21). Examination of the Michaelis constants for the chymotrypsin catalyzed hydrolysis of esters and their corresponding amides indicated that for esters deacylation of the enzyme is rate determining for turnover while for amide substrates acylation is rate determining (22,23). Evidence for the generality of the acyl enzyme intermediate has been compiled elsewhere (24,25).

Acylation rate constants for a series of substituted anilides of acetyl-L-tyrosine decrease with increasing electron withdrawal (26). This trend is opposite to that observed for ester substrates. A mechanism in which a proton is transferred to the anilide in the transition state was proposed. This hypothesis has been questioned because the pH dependence of acylation and deacylation is consistent with the imidazole of His 57 being in the basic form for activity (27). Alternate explanations for this electronic effect involving rate determining breakdown of the tetrahedral intermediate to the acyl enzyme (27) and competitive nonproductive binding (28) have been proposed.

The successful determination of the three dimensional structure of chymotrypsin by x-ray crystallo-

graphy demonstrated the proximity of the His 57 and Ser 195 residues giving credence to the postulate that the imidazole of His 57 can act as a general-base in the activation of the serine hydroxyl (29,30).

The tosyl group of tosylchymotrypsin was bound in a hydrophobic pocket adjacent to the active site. This hydrophobic pocket has become known as the "tosyl hole". The competitive inhibitors dioxane and indole also bind in the "tosyl hole". It has been proposed that hydrophobic side chains of substrates, such as the phenyl ring of tyrosine, are bound in the "tosyl hole" decreasing K_s and orienting the reactive carbonyl of the substrate at the active site, therefore influencing the rate of catalysis. Results of subsequent x-ray work led to the propostion that Asp 102 is hydrogen bonded to His 57 which in turn is hydrogen bonded to Ser 195 resulting in a "charge-relay" activation of the hydroxyl group of the serine enhancing its ability to act as a nucleophile during acylation of the enzyme. (31). Since the time scale involved for the determination of an x-ray structure is so large reactive intermediates are not accessible by this method, therefore, at present it remains for other techniques to examine the dynamic processes of catalysis.

Of the two steps in the chymotrypsin catalyzed hydrolysis of ester substrates, acylation and deacyla-

tion, the latter has been the object of the most intensive investigation. It has been concluded from structure-reactivity correlations (32,33,34,35) and solvent deuterium isotope effects (32,36) that the deacylation reaction for non-specific ester substrates proceeds by imidazole catalyzed attack of water on the acyl enzyme. Model building has shown that in the acyl enzyme a water molecule can be placed so that it is hydrogen bonded to the imidazole of His 57 with its oxygen oriented toward the nearby carbonyl carbon of the acyl group which is consistent with a general-base catalyzed deacylation mechanism (37).

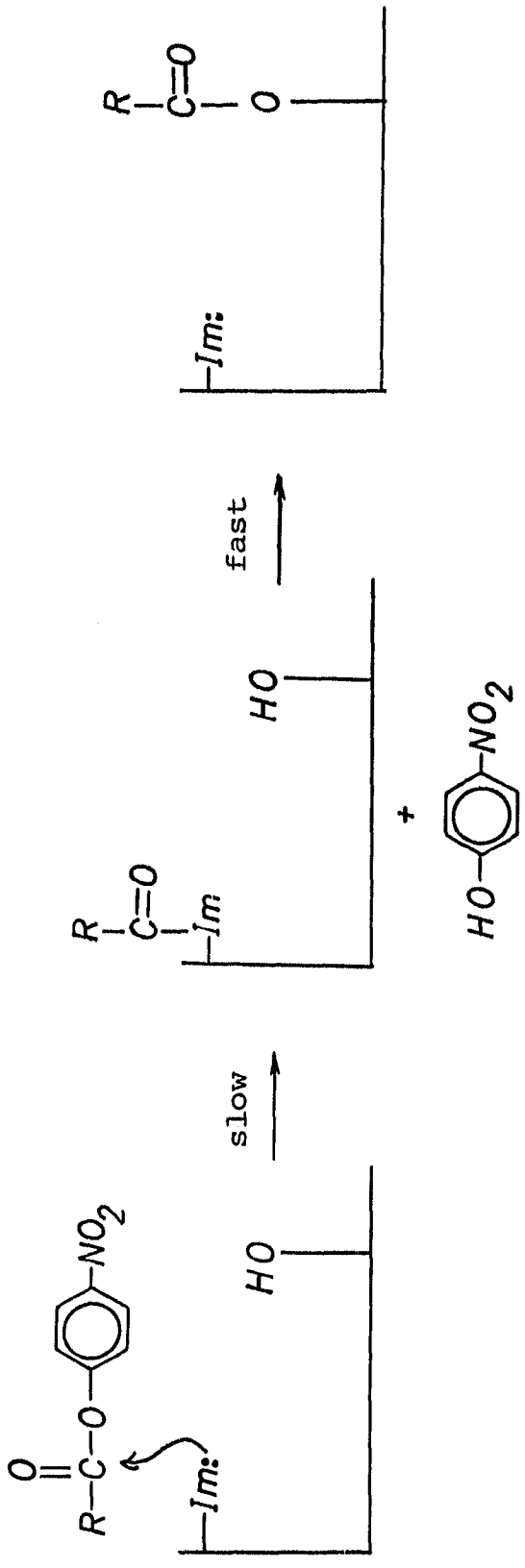
The mechanism of the acylation step is less clear (5,38,39). It has been implied (25) and generally accepted (40,41) that acylation is the symmetrical reverse of deacylation and proceeds by general-base (His 57) activation of Ser 195, which in turn acts as a nucleophile to generate the serine acylated enzyme (Scheme II)(18). In the past there has been speculation about acylimidazole intermediates being formed during acylation (42,43), and during deacylation (44,45), and during inhibition of the enzyme (46). The one piece of experimental evidence that led to these hypotheses was the observation of a rapid increase in absorbance at 245 nm followed by a slow decay when the pH of a solution of acetylchymotrypsin was raised from 3.5 to 8.9 (45). The

absorbance at 245 nm was consistent with an acylimidazole species, but Hess subsequently showed these spectral phenomena to be due to pH difference spectra and light scattering due to different degrees of molecular aggregation (47).

An experiment designed to distinguish between acylation at serine or histidine involved the measurement of the number of protons released upon acylation by p-nitrophenyl acetate (16,17). Acylation at serine at neutral pH would result in the release of 1 mole of p-nitrophenoxide and 1 mole of protons, while acylation at protonated imidazole would result in the liberation of 1 mole of p-nitrophenoxide, 1 mole of protons, and a fraction of a mole of protons depending upon the extent of protonation of the imidazole at the pH used. At pH 6.6 a net loss of protons from the buffer was observed. These results were seen to be in accord with serine acylation accompanied by a change in pK_a of a basic group in the enzyme from approximately 6.6 to 7.3. The values agree with the pK_a values found for acylation and deacylation reactions of chymotrypsin with p-nitrophenyl acetate of 6.59 and 7.3 respectively (48). These results would also be consistent with acylation at imidazole followed by a rapid N→O acyl transfer to serine.

The question of the existence of an acylimidazole intermediate then lay dormant until recently, when Hubbard and Kirsch postulated such an intermediate for the acylation of chymotrypsin by non-specific p-nitrophenyl and 2,4-dinitrophenyl benzoate esters (38,49). It was concluded from a comparison of the magnitude of rho for reactions of various nucleophiles and of chymotrypsin with substituted p-nitrophenyl and 2,4-dinitrophenyl benzoates that chymotrypsin acts as a neutral rather than anionic nucleophile toward these substrates. Kinetic solvent isotope ratios and other data together with the rho analysis led to the suggestion that in the case of phenyl benzoates acylation may occur by direct nucleophilic attack by the imidazole of His 57, yielding a transient acylimidazole which rapidly collapses to the Ser 195 acylated enzyme (38) (Scheme III). Since the substrates studied were non-specific, it did not seem unreasonable to expect that the geometry of their interaction with the active site of the enzyme might be sufficiently different from that for physiological substrates to provide for an alternative mechanism of acylation.

Because Scheme III seems to apply only to phenyl benzoates (although earlier it was thought that in addition phenyl acetates would react in an analogous manner) and because of the reports (5,39,50) that acylation can



Scheme III

only proceed through general-base activation of serine, it appeared appropriate to conduct further experiments which would shed light upon the pathway for the acylation of chymotrypsin by these phenyl esters.

A detailed examination of the structure-reactivity correlations for the acylation of chymotrypsin by phenyl benzoates was undertaken. These analyses along with information developed from the study of solvent deuterium isotope effects on the acylation by several non-specific and one specific ester substrates were examined in the context of the hypothesis of an acylimidazole intermediate on the path to acylation by phenyl benzoate substrates. Because of the reliance of subsequent analyses on structure-reactivity relationships the following introductory section on their applicability to enzyme systems is included.

An Introduction to the Use of Structure-Reactivity Correlations in the Study of Enzyme Mechanisms. The relationship between the free-energy difference between products and reactants of a chemical system, and the equilibrium constant for a reaction is shown in Equation 1-2.

$$\Delta G^{\circ} = -RT\ln K \qquad (1\text{-}2)$$

The rate of reaction is proportional to the concentration of activated complex, and transition state theory maintains that the activated complex is in continuous equilibrium with the reactive species. It is therefore possible to relate rate constants, k_R, and equilibrium constants to free-energy differences between species as shown in the following equations.

$$\Delta G^{\neq} = -RT\ln K^{\neq} \qquad (1\text{-}3)$$

$$k_R = \frac{kT}{h} K^{\neq} \qquad (1\text{-}4)$$

Thus the rate constant is a function of the free-energy difference between the ground state and the activated complex (Equation 1-5).

$$k_R = \frac{kT}{h} e^{-\Delta G^{\neq}/RT} \qquad (1\text{-}5)$$

or,

$$\log k_R = \log \frac{kT}{h} - \frac{\Delta G^{\neq}}{2.3RT} \qquad (1\text{-}6)$$

The Hammett equation (1-7) is an empirical relationship between the rate constants of a standard reaction and a similar reaction in which a substituent on one of the reactants distant from the reaction center has been changed.

$$\log k_R = \log k_o + \sigma\rho \qquad (1\text{-}7)$$

Substitution of Equation 1-6 into Equation 1-7 yields

$$\log \frac{kT}{h} - \frac{\Delta G^{\neq}}{2.3RT} = \log \frac{kT}{h} - \frac{\Delta G_o^{\neq}}{2.3RT} + \sigma\rho \qquad (1\text{-}8)$$

therefore,

$$\frac{(\Delta G^{\neq} - \Delta G_o^{\neq})}{2.3RT} = -\sigma\rho \qquad (1\text{-}9)$$

For a second series of reactions

$$\frac{(\Delta G'^{\neq} - \Delta G_o'^{\neq})}{2.3RT} = -\sigma\rho' \qquad (1\text{-}10)$$

From Equations 1-9 and 1-10 it can be seen that a linear relationship exists between the free-energy of activation for the two series of reactions (Equation 1-11).

$$\Delta G^{\ddagger} - {}^{\rho}/_{\rho'}\Delta G'^{\ddagger} = \text{constant} \qquad (1\text{-}11)$$

For similar types of compounds undergoing similar reactions, the perturbation in energy caused by a given substituent in one of the compounds is proportional to the perturbation in energy caused by the same substituent in one of the other compounds. Therefore, it is valid to compare rho values obtained from different series of similar reactions.

The rho constant is a function of the particular reaction being studied and can be related to the development of charge at the reaction center in the transition state. For example, a developing negative charge would be stabilized by electron withdrawing substituents and would yield a positive rho, while a developing positive charge would be favored by electron donating substituents and result in a negative rho. The magnitude of rho can give an indication of the extent of charge development in forming an activated complex.

Since its inception the Hammett correlation has been refined with various substituent scales reflecting

various properties of select substituents, such as resonance interaction (51). Also the linear free energy concept has been extended to include reactions of aliphatic compounds and nonconjugated ring systems (51,52). A wide body of literature exists analyzing linear and nonlinear structure-reactivity correlations (53,54,55,56) and has shown their value and validity in the investigation of molecular transformations.

The success of structure-reactivity correlations in interpretation of conventional organic reaction mechanisms has led to the hope that they might be of use in the analysis of more complex interactions, such as reactions in biological systems. Quantitative correlations between the structure of drug analogues and their efficacy has been demonstrated (57,58). The application of structure-reactivity correlations to the binding of small molecules to proteins has also proven useful (35). Recent reviews have outlined the scope of the utility of these correlations in the investigation of enzyme mechanisms (35,59,60). In the following study conclusions about the mechanism of the acylation of the enzyme chymotrypsin will be made based in part upon such analyses.

The very nature of enzymatic catalysis puts severe restraints upon the use of structure-reactivity correlations. An enzymatic reaction consists minimally of a rapid adsorption step resulting in an equilibrium

between the enzyme, the substrate, and the Michaelis complex, followed by a rearrangement of covalent bonds to finally yield product and free enzyme. This scheme is illustrated in Equation (1-12). Many enzyme mechanisms

$$E + S \overset{K_s}{\rightleftharpoons} (E\sim S) \xrightarrow{k_2} E + P \qquad (1\text{-}12)$$

are more complex in that they have stable intermediates along the path to enzyme turnover.

Therefore, to make use of structure-reactivity correlations in the study of enzyme reactions one must first distinguish which step of the reaction the variation in structure is affecting. Since the adsorption step involves direct steric interactions it is likely that substantial changes in structure of the substrate will be reflected in this step. Fortunately chymotrypsin lacks the elegant specificity of many other enzymes, and it is possible to choose series of substrates or reaction conditions which allow the experimentor to discount structure variation on the equilibrium constant K_s. Thus it is possible to determine directly the effect of the electronic interaction of substituents on well chosen substrates with the reaction center of the enzyme.

To what extent is it valid to compare enzymatic with non-enzymatic model structure-reactivity correlations in postulating a mechanism? The very fact that such correlations can be obtained experimentally indicates a regular dependence of structural variation upon covalent bond reordering at the reactive center. Can the resultant parameter be compared with similar parameters for non-enzymatic reactions to draw conclusions about the nature of the mechanism?

If a series of substrates can be chosen for which there is independent evidence favoring a certain mechanism and the results of a comparison of enzymatic with non-enzymatic structure-reactivity correlations are consistent with this mechanism, such a comparison is seen to be valid. For example, the deacylation of a series of benzoylchymotrypsins yields solvent deuterium isotope effects which support a general-base catalysis mechanism (36,61). A rho value of 2.0 obtained for the deacylation of a series of meta and para substituted benzoylchymotrypsins (32) when compared with values for non-enzymatic reactions of the substituted benzoates is found to be consistent with the participation of basic or nucleophilic groups rather than acid catalysis. Such consistency between independent mechanistic probes speaks for the

validity of the use of structure-reactivity correlations in this case. Each case must be examined independently, but with the use of caution and independent corroboration where possible structure-reactivity correlations can be a powerful tool for elucidating enzymatic mechanisms.

A quote from Leffler and Grunwald will help to put the use of structure-reactivity correlations as a mechanistic probe into perspective.

> "Our discussion would therefore be incomplete if we did not occasionally attempt to identify a particular mechanism. In doing so, we run the risk that our identification might be in error. But it should be noted that even when we are wrong, the mistake in the identification will in no way affect the validity of the extrathermodynamic relationship that suggested the identification." (62).

EXPERIMENTAL

Materials: α-Chymotrypsin was purchased from Worthington Biochemical (3 x crytallized activation product of 3 x crytallized zymogen, 45 units/mg) and used without further purification. p-Bromophenol, m-bromophenol, m-chlorophenol, m-acetylphenol, p-nitrophenol, p-nitrobenzoyl chloride, hippuric acid, and 99.8% D_2O were purchased from Aldrich. N-trans-Cinnamoylimidazole was obtained from Eastman and was recrystallized repeatedly from cyclohexane prior to use (mp 132-132.7°; lit. mp 133-133.5°) (21). Deuterium chloride (38% in 99% D_2O) was purchased from Stohler Isotope. Dioxane (Spectrograde) was obtained from Eastman and used without further purification. All buffer reagents were reagent grade and buffers were prepared immediately prior to use with glass distilled water. The pH meter was standardized using Fisher standard buffers.

The esters p-nitrophenyl p-nitrobenzoate and p-nitrophenyl benzoate were those used in a previous study (38).

The pH values of buffer and reaction solutions were determined with an Orion Research Model 701 digital pH meter. Kinetic runs were carried out in either a

Gibson-Durrum stopped-flow spectrophotometer with a Kel-F flow path, a 2 cm path length cuvette, and thermostated temperature control (± 0.1° C), or in a Cary 14 recording spectrophotometer with a thermostated cell holder. All spectrophotometric determinations for analytical wave lengths were carried out in the Cary 14. Kinetic results were analyzed with the aid of computer programs written for the University of New Hampshire's IBM 360 Model 50 computer.

Carbon, hydrogen and nitrogen analyses were carried out in the Chemistry Department at the University of New Hampshire by Deanna Cardin.

The Synthesis of Substituted Phenyl p-Nitrobenzoates. The method previously described by Kirsch (63) was employed for the synthesis of a series of substituted phenyl p-nitrobenzoates. A typical procedure consisted of the addition of an equivalent amount of p-nitrobenzoyl chloride in sodium dried toluene to the appropriate phenol dissolved in pyridine. The reaction mixture was stirred for 15 minutes after completion of the addition of the p-nitrobenzoyl chloride. The mixture was then evaporated to dryness in a rotary evaporator, and the resulting solid was dissolved in chloroform. The chloroform solution was washed with sodium bicarbonate followed by H_2O and was dried over sodium sulfate. The chlo-

roform was evaporated and the solid residue was recrystallized from 95% ethanol until a sharp reproducible melting point was obtained.

The following esters were synthesized in this manner: p-cyanophenyl p-nitrobenzoate (64), p-chlorophenyl p-nitrobenzoate (64), p-bromophenyl p-nitrobenzoate, m-nitrophenyl p-nitrobenzoate (64), m-chlorophenyl p-nitrobenzoate, p-bromophenyl p-nitrobenzoate, and m-acetylphenyl p-nitrobenzoate. The melting points and carbon, hydrogen, nitrogen analyses are collected in Table 2-1.

Synthesis of Substituted Phenyl Acetates. p-Cyanophenyl acetate and p-chlorophenyl acetate were synthesized by the method of Bender and Nakamura (67) from acetic anhydride and the appropriate phenol. A representative synthesis of p-cyanophenyl acetate follows.

To 75 ml of sodium dried benzene in a 250 ml round bottomed flask 11.9 gm (0.12 mol) acetic anhydride, 11.2 gm (0.094 mol) p-cyanophenol, and 3 ml of dry pyridine freshly distilled from barium oxide were added. The mixture was stirred and refluxed for one hour; subsequently the reaction mixture was cooled and washed two times each with saturated sodium bicarbonate, H_2O, 1.5% sodium hydroxide, 0.1 M hydrochloric acid and H_2O. The organic phase was dried over sodium sulfate. The

Table 2-1. Analytical Data for Substituted Phenyl p-Nitrobenzoates.

Phenyl Substituent	Calculated C	H	N	Found C	H	N	MP °C	Lit MP °C
p-CN[c]	62.69	3.01	10.44	62.28	2.83	10.26	187.5-189	
p-Cl							170-171	171[a]
p-Br[c]	48.47	2.50	4.35	48.22	2.57	4.30	181.9-183	
m-Br[c]	48.47	2.50	4.35	48.36	2.63	4.37	106-107	
m-Cl[c]	56.22	2.91	5.05	56.00	2.75	4.98	95.5-97.0	
m-NO_2							172.5-174	174-175[b]
m-$COCH_3$[c]	63.16	3.89	4.91	63.32	3.96	4.89	131.9-133	

(a) Reference 65.

(b) Reference 66.

(c) New Compounds.

solvent was removed using a rotary evaporator and an oil which crystallized on cooling was obtained. The solid was dissolved in petroleum ether, filtered, and cooled, yielding white crystals which were collected in a Buchner funnel. The crystals melted at 56.5-58.0° C. Anal: Calcd. for $C_9H_7NO_2$: C, 67.08, H, 4.38; N, 8.69. Found: C, 66.92; H, 4.43; N, 8.67.

p-Chlorophenyl acetate was isolated as a liquid, bp 112° C at 16.5 mm. Anal: Calcd for $C_8H_7O_2Cl$: C, 56.33; H, 4.14. Found: C, 56.45; H, 4.12.

p-Nitrophenyl acetate and phenyl acetate were obtained from Aldrich and were purified by recrystallization and distillation respectively.

Synthesis of p-Nitrophenyl Phenylacetate and p-Nitrophenyl Hydrocinnamate. The method previously described by Kirsch (63) was utilized for the synthesis of these esters. An equivalent amount of the appropriate acid chloride dissolved in toluene was added to p-nitrophenol (recrystallized several times from benzene) dissolved in a 2:1 mixture of diethyl ether and pyridine. The mixture was stirred for one hour and the solvent was removed under reduced pressure. The solid residue was dissolved in chloroform and extracted five times with 5% sodium bicarbonate, two times with 0.01 M HCl, and two times with water. The organic layer was dried over magnesium sulfate and the solvent was evaporated. The resi-

due was recrystallized repeatedly from 95% ethanol until a sharp melting point was obtained.

Analytical data for the synthesized esters are as follows:

p-Nitrophenyl phenylacetate, mp 63-64.5° (lit. mp 62-63°)(68). Anal: Calcd. for $C_{14}H_{11}NO_4$: C, 65.37; H, 4.31; N, 5.44. Found: C, 65.32; H, 4.29; N, 5.40.

p-Nitrophenyl Hydrocinnamate, mp 97.4-98.2° (lit. mp 97-98°)(69). Anal: Calcd. for $C_{15}H_{13}NO_4$; C, 66.42; H, 4.83; N, 5.16. Found: C, 66.21; H, 4.70; N, 5.11.

Synthesis of p-Nitrophenyl Thiolacetate. To 5.0 gm (0.032 mol) of p-nitrothiophenol (twice crystallized from cyclohexane) in pyridine 5.6 gm (0.072 mol) of acetyl chloride in toluene was added. The reaction mixture was stirred for 10 minutes while cooled in an ice bath. The solvent was then evaporated and the pale yellow solid residue was dissolved in chloroform. The resulting solution was washed several times with 5% sodium bicarbonate followed by H_2O, dried over sodium sulfate, and the chloroform was evaporated. The solid product was recrystallized several times from cyclohexane and twice from 95% ethanol. Off-white needles were obtained; mp 80.5-81.0° C. (lit. mp 82.3-82.6° C. (70). Anal: Calcd. for $C_8H_7NO_3S$: C, 48.74; H, 3.58; N, 7.11. Found: C, 48.83; H, 3.49; N, 7.17.

The Sythesis of p-Nitrophenyl Hippurate. p-Nitrophenyl hippurate was prepared by the method of Williams (40) from hippuroyl chloride and p-nitrophenol.

A. Preparation of hippuroyl chloride: 5.37 gm (0.03 mol) of finely ground hippuric acid was suspended in 60 ml of dry chloroform and cooled in an ice bath. 9.32 gm (0.045 mol) of finely ground phosphorous pentachloride was added over 5 minutes with stirring. 60 ml of dry petroleum ether was added, the mixture stirred, and then rapidly filtered in a Buchner funnel. The resulting white solid was washed with petroleum ether and stored in vacuo in a desiccator.

B. Preparation of p-nitrophenyl hippurate: 2.78 gm (0.02 mol) of p-nitrophenol (recrystallized from benzene) was dissolved in 60 ml of dry CH_2Cl_2 and 50 ml of pyridine, and was cooled in an ice bath with stirring. 4.27 gm (0.02 mol) of hippuroyl chloride was added as a solid with continued stirring in the ice bath. After 15 minutes the ice bath was removed and the mixture was allowed to stir at room temperature for 3 hours. The solvent was removed leaving a red-brown oil, which was dissolved in chloroform, washed with dilute hydrochloric acid, 5% sodium bicarbonate, and H_2O, and then dried over sodium sulfate. The chloroform was removed yielding a yellow solid which was recrystallized twice from

95% ethanol yielding yellow crystals, mp 169.1-169.8° C (lit. mp 170-171° C(40); 167-168° C (71)). Anal: Calcd. for $C_{15}H_{12}N_2O_5$: C, 60.00; H, 4.03; N, 9.33. Found: C, 59.81; H, 4.19; N, 9.49.

Determination of the Operational Normality of Commercial Batches of Chymotrypsin. A modification of the method of Schonbaum, Zerner, and Bender (21) was employed to determine the operational normality of the chymotrypsin solutions. The active site of chymotrypsin is titrated with N-trans-cinnamoylimidazole (NTCI) yielding trans-cinnamoylchymotrypsin. It is assumed there is one active site per chymotrypsin molecule and one NTCI molecule reacts with each active site. From the known molar absorptivities of NTCI and trans-cinnamoylchymotrypsin (9.37×10^3 M^{-1} and 0.42×10^3 M^{-1} respectively) at 335 nm the operational normality can be determined. (61).

A typical determination was made in the following manner. A stock solution of NTCI (recrystallized from sodium dried cyclohexane) is prepared in acetonitrile (0.475 mg/ml). 36.84 mg of enzyme (Worthington CDI 3AF) is dissolved in pH 5.0 buffer (0.1 M acetate). 100 µl of the stock solution of NTCI was added to 3.0 ml buffer in a 1 cm path length cuvette and the absorbance at 335 nm versus time was recorded. Extrapolating

to zero time gave the absorbance of NTCI at 335 nm and was designated A_2.

100 ml of stock NTCI was added to 3.0 ml of the enzyme solution in a 1 cm path length cuvette using as a blank an equal volume of unreacted enzyme solution. The absorbance at 335 nm extrapolated to zero time was recorded as A_1.

From the known molar absorptivities of NTCI and trans-cinnamoylchymotrypsin at 335 nm the normality of the chymotrypsin solution was determined from the following equation.

$$N = \frac{A_2 - A_1}{8.66 \times 10^3 \ M^{-1}}$$

where,

$$8.66 \times 10^3 \ M^{-1} = \frac{3.0 \text{ ml}}{3.1 \text{ ml}} (\varepsilon_{335}\text{NTCI} - \varepsilon_{335} \text{ trans-cinnamoylchymotrypsin})$$

From the absorbance of the chymotrypsin solution at 280 nm an empirical equation can be derived relating the A_{280} of the enzyme solution to its operational normality. The following are the results of the determination for Worthington batch CDI 3AF.

	A_1	A_2	$A_{280}/10$
trial 1	0.252	0.731	0.280
trial 2	0.254	0.724	0.280
trial 3	0.251	0.726	0.280
avg.	0.252	0.727	0.280

$$N = \frac{(0.727) - (0.252)}{8.66 \times 10^{3}\ M^{-1}} = 5.48 \times 10^{-5}\ M$$

$$N = 19.57 \times A_{280}/10 \times 10^{-5}\ M$$

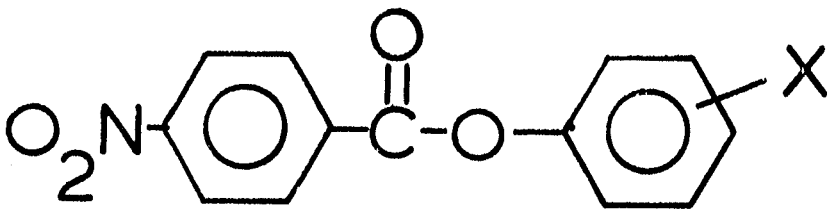
The operational normalities of Worthington batches of chymotrypsin used in this study are collected in Table 2-2.

Determination of the Kinetics of Acylation of Chymotrypsin by Substituted Phenyl p-Nitrobenzoates. The acylation of chymotrypsin by substituted phenyl p-nitrobenzoates was carried out under the conditions of initial enzyme concentration in excess of initial substrate concentration. Under these conditions a pseudo-first-order reaction is expected from which the second-

Table 2-2. Operational Normality as a Function of $A_{280}/10$

Batch No.	Normality as a Function of $A_{280}/10$
CDI 1IB	$19.25 \times A_{280}/10 \times 10^{-5}$
CDI 34J847	$19.87 \times A_{280}/10 \times 10^{-5}$
CDI 34M608	$20.18 \times A_{280}/10 \times 10^{-5}$
CDI 3AF	$19.57 \times A_{280}/10 \times 10^{-5}$

order acylation rate constant, k_2/K_s, can be determined by dividing the resulting first-order k_{obs} by the initial enzyme concentration (38). The reactions of the following esters were monitored in a Cary 14 recording spectrophotometer fitted with a 0.0 - 0.2 absorbance unit slide wire and a thermostatted cuvette holder.



X = p-NO_2, p-CN, p-Cl, p-Br, m-Cl, m-NO_2 m-Br, m-$COCH_3$

Stock solutions of substrate were prepared in acetonitrile such that an addition of approximately 10 µl of stock solution to a 3.0 ml volume of enzyme solution gave an initial substrate concentration of 5×10^{-6} M. Enzyme solutions were prepared by addition of weighed samples of enzyme to the appropriate buffer (5% v/v CH_3CN) to give an approximate concentration of 5×10^{-5} M. Precise enzyme concentrations were deter-

mined from their absorbance at 280 nm and the appropriate conversion factor determined from NTCI titration.

3.0 ml of enzyme solution were pipetted into each of two cuvettes, one being used as a blank and the other being placed in the thermostated (25.0 $\pm$ 0.1° C) cuvette holder. After sufficient time for the enzyme solution to become equilibrated at 25.0° C the cuvette was removed and the substrate stock solution was rapidly introduced with a microliter syringe. The cuvette was shaken, returned to the cuvette holder, and the instrument recorder was started.

The reactions were monitored by following the disappearance of substrate at wavelengths which were determined from spectra of the ester starting materials and their reaction products with chymotrypsin. The analytical wavelengths used in the kinetic determinations are given in Table 2-3.

The absorbance readings at given time intervals (A_t) and the absorbance at infinite time (A_∞) were determined from a time versus absorbance graph of the reaction. Plots of $\ln(A_t - A_\infty)$ versus time were analyzed for anticipated first-order kinetics using the program library STATPACK of the University of New Hampshire Computer Center. This program performs linear regression analyses and provides correlation coefficients for the straight lines generated. The slopes of these

Table 2-3. Analytical Wavelengths Used for the Determination of the Rate of Acylation of Chymotrypsin by Substituted Phenyl p-Nitrobenzoates.

Ester	λ Analytical (nm)
p-Cyanophenyl p-nitrobenzoate	300
p-Chlorophenyl p-nitrobenzoate	264
p-Bromophenyl p-nitrobenzoate	264
m-Nitrophenyl p-nitrobenzoate	250
m-Chlorophenyl p-nitrobenzoate	263
m-Bromophenyl p-nitrobenzoate	263
m-Acetylphenyl p-nitrobenzoate	270

plots (which were linear for at least 3 half lives) represented k_{obs}. The second-order rate constants for acylation, k_2/K_s, were obtained by dividing k_{obs} by the initial enzyme concentrations.

The acylation reaction of m-acetylphenyl p-nitrobenzoate was too slow to obtain a reliable A reading. Therefore, a modified Guggenheim method was used to obtain k_{obs} (72).

The acylation by p-nitrophenyl p-nitrobenzoate was monitored in a Gibson-Durrum stopped-flow spectrophotometer by following the liberation of p-nitrophenyl at 330 nm below pH 6.5, and the p-nitrophenolate anion at 400 nm above pH 6.5. The first-order rate constants, k_{obs}, were determined using computer program TRDATA as outlined in a separate section.

Determination of the Kinetics of Acylation of Chymotrypsin by Substituted Phenyl Acetates. The acylation of chymotrypsin by p-nitrophenyl acetate, p-cyanophenyl acetate, p-chlorophenyl acetate, and phenyl acetate was examined. All reactions were studied under second-order conditions and were monitored by following the appearance of the phenolic products. The analytical wavelengths are presented in Table 2-4.

Enzyme solutions were prepared in 0.1 M phosphate buffer (pH 7.47, 5% v/v CH_3CN) and concentrations

Table 2-4. Analytical Wavelengths Used in Monitoring the Phenolic Products of the Acylation of Chymotrypsin by Substituted Phenyl Acetates.

Ester	λ Analytical (nm)
p-Nitrophenyl acetate	400
p-Cyanophenyl acetate	247
p-Chlorophenyl acetate	270[a]
Phenyl acetate	270

(a) 0.0 - 0.2 slide wire.

were determined spectrophotometrically. Reactions were initiated by addition, at t = 0, of substrate stock solution in CH_3CN with a microliter syringe to the thermostated (25.0 ± 0.1° C) reaction cuvette. Enzyme solutions were used as blanks. The reactions were monitored by measuring absorbance as a function of time. Initial enzyme concentrations were in the range of $5 - 6 \times 10^{-5}$ M and initial substrate concentrations were in the range of $3 - 5 \times 10^{-5}$ M.

The second-order rate constants for acylation by p-nitrophenyl acetate and p-cyanophenyl acetate were analyzed using Equation 2-1 (73).

$$\frac{kt}{\delta} - C = \log (1 + r/A_\infty - A_t) \qquad (2\text{-}1)$$

$$\delta = 2.303/b\ (n - 1)$$

$$a = [E_o]$$

$$b = [S_o]$$

$$a = nb$$

$$r = (n - 1)(A_\infty - A_o)$$

The resultant rate constants were obtained by linear regression analysis of Equation 2-1 using the STATPACK library. The second-order rate constant for acylation by p-nitrophenyl acetate obtained by this

method, 1425 M^{-1} sec^{-1}, compared favorably with the second-order rate constant obtained for the same reaction under pseudo-first-order conditions, 1580 M^{-1} sec^{-1}. The pseudo-first-order rate constant from which this constant was derived was determined in the stopped-flow spectrophotometer.

Because of complications arising from competing deacylation of the enzyme the second-order acylation rate constants for p-chlorophenyl acetate and phenyl acetate were determined from initial rates of reaction. The molar absorptivities of the p-chlorophenol and phenol leaving groups were determined from the initial concentration of substrate and the absorbance at infinite time. Once the molar absorptivity of the product is known the initial rate is determined from the tangent to the first 10% of the total change of the absorbance versus time graph. The second-order rate constant is obtained by the solution of Equation 2-2. Rate constants obtained by this method for p-cyanophenyl acetate compared favorably with those obtained using Equation 2-1.

$$\text{Initial Rate} = k[E_o][S_o] \qquad (2\text{-}2)$$

The Rates of the Acylation of Chymotrypsin by Ester Substrates in H_2O and D_2O. The rates of the acylation of chymotrypsin by p-nitrophenyl p-nitrobenzoate, p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, p-nitrophenyl hydrocinnamate, p-nitrophenyl thiolacetate, and p-nitrophenyl hippurate, were determined as a function of pH (pD) in H_2O and D_2O.

For determinations in H_2O all buffers were prepared using glass distilled water. Below pH 8.25 buffers were prepared by mixing quantities of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4, both 5% v/v CH_3CN. Above pH 8.25 buffers were made from 0.1 M $Na_4P_2O_7$ (5% v/v CH_3CN) by adjusting the pH with concentrated HCl. For reactions in D_2O all buffers were prepared in 99.8% deuterium oxide. The phosphate salts were deuterated by repeated dissolution in D_2O followed by drying. It was assumed that by this procedure all exchangeable protons were replaced with deuterium atoms. Buffers were prepared in a manner analogous to those in H_2O. Those below pD 8.65 were made by mixing 0.1 M NaD_2PO_4 and 0.1 M Na_2DPO_4 both 5% v/v CH_3CH, and those above pD 8.65 were prepared by adjusting 0.1 M $Na_4P_2O_7$ with 38% DCl in 99% D_2O. The pH values read from the pH meter were converted to pD values by the formula pD = pH + 0.40 (74). All buffer solutions were 5% v/v CH_3CN before the pH was determined.

Enzyme was weighed in a 10 ml beaker and dissolved in 5.0 ml of the appropriate buffer. Enzyme concentration was determined spectrophotometrically. At pH values below 8.25 (pD 8.65) substrate solutions were prepared by the addition of approximately 5 µl of a stock solution of substrate in CH_3CN to 5.0 ml of buffer. In order to prevent buffer mediated hydrolysis of the substrate, at pH values above 8.25 (pD 8.65), stock solution was added to 0.1 M $NaNO_3$, 5% CH_3CN.

The acylation reactions were monitored in the stopped-flow spectrophotometer by following the liberation of p-nitrophenol below pH 6.5 at 330 nm, p-nitrophenolate anion above pH 6.5 at 400 nm, or p-nitrothiolphenolate anion at 409 nm. Enzyme and substrate were thermostated at 25.0° C before mixing. Rate constants were determined from Polaroid photographs of oscilloscope tracings representing a graph of optical transmission versus time in a manner described below.

Analysis of Kinetic Results from the Stopped-Flow Spectrophotometer. The Durrum-Gibson stopped-flow spectrophotometer is fitted with a monochromator which focuses ultraviolet or visible light through a 2 cm thermostated reaction cuvette. The transmitted light is detected by a photomultiplier tube and a signal proportional to the per cent of light transmitted

is displayed on an oscilloscope screen. The reactant solutions are driven pneumatically from each of two thermostated drive syringes through a Kel-F mixing chamber into the reaction cuvette, and ultimately into the stop syringe which triggers the oscilloscope display.

A reaction which exhibits a change in absorbance due to a change in concentration of the reactants and products can therefore be monitored by triggering the oscilloscope upon rapid mixing of the reactants. The resulting trace will be a time versus percent transmission profile of the reaction. By converting per cent transmission to absorbance, which is proportional to the concentration of the absorbing species, and plotting the logarithm of absorbance at infinite time (A_∞) minus absorbance at time $\underline{t}$ (A_t) (for a reaction in which absorbance increases) versus time, the first-order kinetics can be tested and a rate constant obtained.

Reaction profiles are recorded on Polaroid photographs of the oscilloscope tracings. Figure 2-1 represents a typical photographic recording of a reaction profile.

The 100% transmittance line is determined by adjusting the photomultiplier voltage with the cuvette filled with water. The optical density at infinite

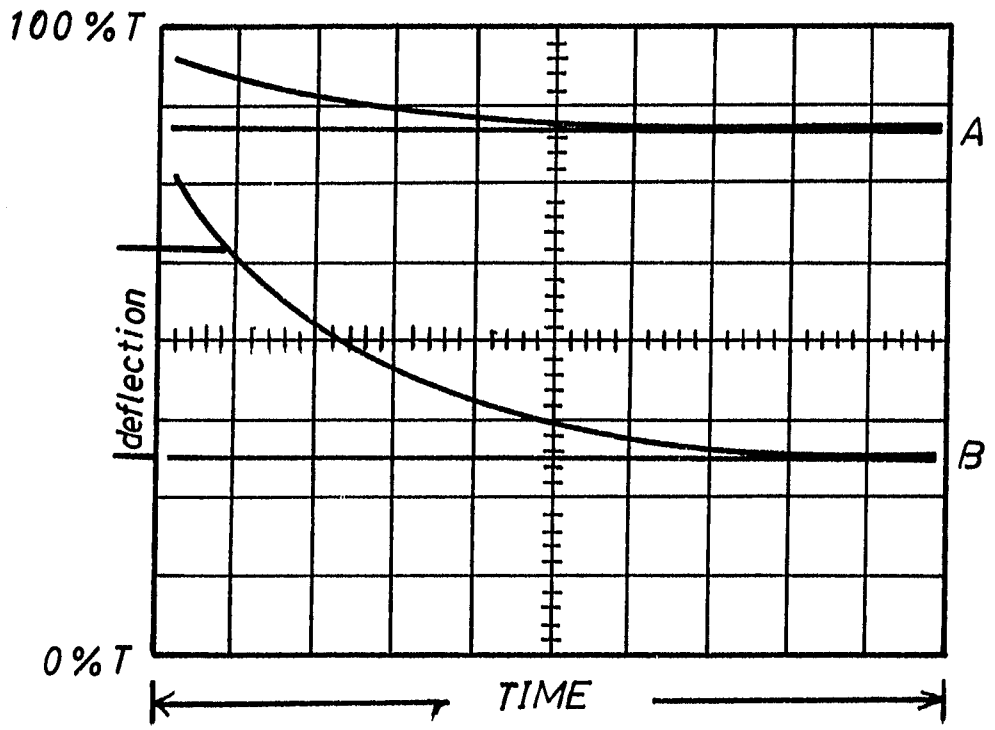


Figure 2-1. Typical Photographic Recording of a Reaction Profile.

time for the reaction is determined on the 100% transmittance scale (Trace A in Figure 2-1). By adjusting the voltage on the differential amplifier the transmittance scale can be expanded, which will result in a magnified transmittance versus time graph (Trace B in Figure 2-1). From the magnified graph the deflection between transmittance at a given time and transmittance at infinite time can be measured. With the aid of computer program TRDATA these deflections can be related to transmittance differences on the 0 - 100% transmittance scale, which are then converted to differences in absorbance versus time. The computer program gives a plot of the logarithm of the difference between absorbance at time t and absorbance at infinite time versus time. From this plot the first-order rate constant, k_{obs}, can be calculated.

The Acylation of Chymotrypsin by p-Nitrophenyl p-Nitrobenzoate in the Presence of 20% v/v Methanol. An attempt was made to monitor spectrophotometrically in the Cary 14 the burst of p-nitrophenol upon the acylation of chymotrypsin by p-nitrophenyl p-nitrobenzoate in pH 8.00 (0.1 M phosphate) buffer which was either 20% v/v CH_3CN or 20 v/v CH_3OH. A stock solution of ester in CH_3CN (1.08 mg/ml) was prepared. The addition of 10 µl of stock solution to 3.0 ml of enzyme solution

gave an ester concentration of 1.25×10^{-5} M. Limited solubility of the ester dictated a low concentration. Enzyme solutions were prepared in each buffer.

1) pH 8.00, 20% v/v CH_3CN $[E_o] = 5.42 \times 10^{-6}$ M
2) pH 8.00, 20% v/v CH_3OH $[E_o] = 4.34 \times 10^{-6}$ M

3.0 ml of enzyme solution was pipetted into the reaction cuvette and 3.0 ml of the appropriate buffer was added to the blank cuvette. 10 µl of stock solution of the substrate was added rapidly to each cuvette, the recorder of the instrument was switched on, and the liberation of p-nitrophenolate was monitored as a function of time at 400 nm using the 0.0 - 0.2 slide wire. This procedure was followed with both buffer systems 1 and 2.

The Rates of Acylation of Chymotrypsin by Ester Substrates in Dioxane and Acetonitrile Cosolvents. The rates of acylation of chymotrypsin by p-nitrophenyl acetate, p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, and p-nitrophenyl hydrocinnmate were examined at pH 8.00 (0.1 M phosphate) in 5% v/v dioxane and 5% v/v acetonitrile respectively. The reactions were monitored in the stopped-flow spectrophotometer under the conditions of initial enzyme concentration in at

least ten fold excess over initial substrate concentrations by monitoring the release of p-nitrophenolate ion at 400 nm. All reactions were run at 25.0 ± 0.1° C and rate constants were determined in a manner outlined elsewhere using program TRDATA.

The Rates of Acylation of Chymotrypsin by Ester Substrates in H_2O and D_2O using Dioxane as the Cosolvent.

The rates of acylation of chymotrypsin by p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, and p-nitrophenyl hydrocinnamate were determined in H_2O and D_2O in the pH independent region of the pH-rate profile at pH 8.00 and pD 8.40 (0.1 M phosphate, 5% v/v dioxane) respectively. For all reactions the initial enzyme concentrations were in at least ten fold excess over initial substrate concentrations. For determinations in H_2O the buffers were prepared using glass distilled water and for reactions in D_2O all buffers were prepared in 99.8% deuterium oxide using deuterated phosphate salts. The pH values read from the pH meter were converted to pD values by the formula pD = pH + 0.40 (74).

Enzyme concentrations were determined spectrophotometrically and substrate solutions were prepared by adding microliter quantities of dioxane stock solutions of the ester to the appropriate buffer immediate-

ly prior to each run. The reactions were monitored in the stopped-flow spectrophotometer by following the appearance of p-nitrophenolate anion at 400 nm. All reactions were run at 25.0 ± 0.1° C. Rate constants were determined in the manner outlined elsewhere using program TRDATA.

Determination of the Kinetics of Acylation of Chymotrypsin by p-Nitrophenyl Esters of Substituted Benzoic Acids in 5% v/v Dioxane. The acylation of chymotrypsin by a series of four substituted p-nitrophenyl benzoates was studied in the stopped-flow spectrophotometer under conditions of initial enzyme concentration greater than initial substrate concentration. Dioxane (Eastman spectrograde), an inhibitor of chymotrypsin (75), was used as the cosolvent to effect the solution of the organic ester substrates. The four esters examined in this study, p-nitrophenyl p-nitrobenzoate, p-nitrophenyl p-cyanobenzoate, p-nitrophenyl m-fluorobenzoate, and p-nitrophenyl benzoate, were those used in a previous study (38).

The reactions were run in 0.1 M phosphate buffer (pH 8.0; 5% v.v dioxane) at 25.0 ± 0.1° C. Enzyme concentrations were determined spectrophotometrically and substrate solutions were prepared immediately prior to each run by adding microliter quantities of

a dioxane stock solution to the buffer in order to give an initial substrate concentration of 3.0×10^{-6} M. The reactions were monitored by following the liberation of p-nitrophenolate anion at 400 nm. Rate constants were determined using program TRDATA.

RESULTS

The Acylation of Chymotrypsin by Substituted Phenyl p-Nitrobenzoates. The acylation of chymotrypsin by substituted phenyl p-nitrobenzoates under the conditions of $[E_o] >> [S_o]$ exhibited first-order kinetics. Therefore by the equation

$$\text{rate} = k_{obs}[S],$$

the reaction was first-order in substrate. Previously, the acylation by a series of substituted p-nitrophenyl benzoates was studied under identical conditions of $[E_o] >> [S_o]$ and the reactions were all found to be first-order in enzyme, i.e. $k_{obs} = k/[E]$ (38,76). Other workers have demonstrated first-order kinetics for substrate and for enzyme under conditions of $[E_o] >> [S_o]$ for the acylation of chymotrypsin by p-nitrophenyl acetate (77,78). Bender has also demonstrated true second-order kinetics for the acylation of chymotrypsin by phenyl acetates where $[E_o] \tilde{=} [S_o]$ (67). Therefore, since the esters studied here are either structurally very similar or the same as those previously studied there is ample precedent to assume first-order kinetics in each species.

The composite second-order rate constants for

the acylation of chymotrypsin, k_2/K_s (see Discussion) were determined for eight meta and para substituted phenyl p-nitrobenzoates at pH 5.05 (0.1 M acetate, 5% v/v CH_3CN), and are collected in Table 3-1. A low pH was chosen to reduce the rates of reaction in order that those reactions which must be monitored in the ultra violet range could be followed in a conventional recording spectrophotometer. A double beam spectrophotometer was necessary because of the substantial absorbance of the enzyme at these wavelengths. In order to have confidence that the acylation by different substrates has the same pH dependence, the acylation reactions of three esters with substituents of widely differing polarities, p-NO_2, p-CN, and p-Cl, were examined at four different pH values between 5 and 7. The resulting Hammett plots constructed for each pH by plotting the logarithm of the second-order rate constants versus the sigma minus substituent parameter yielded the same rho within experimental uncertainty. The results are summarized in Figure 3-1 and Tables 3-2 through 3-5. Previous studies have demonstrated the efficacy of studying the catalytic mechanism of chymotrypsin at low pH (79). It is safe to compare values of k_2/K_s for similar substrates at a constant pH because pK_a differences for the acidic limb of the pH rate profile are very small (38,48,80,81).

Table 3-1. Kinetic Parameters for the Acylation of Chymotrypsin by X-Phenyl p-Nitrobenzoates at pH 5.05 (0.1 M acetate, 5% v/v CH_3CN) and 25.0° C.

Ester X-	$[S_o]$ x 10^6 M	$[E_o]$ x 10^5 M	k_{obs} x 10^3 sec^{-1}	(Std Dev)	k_2/K_s M^{-1} sec^{-1}	pK_a [a] l.g.	σ^- [b]
p-NO_2	4.9	5.84	74.1	(4.5)	1269	7.15	1.00
p-CN	5.0	4.86	33.5	(3.4)	688	7.53	0.89
p-Cl	4.8	5.25	2.66	(0.28)	50.7	9.10	0.228
p-Br	4.9	4.95	2.80	(0.09)	56.5	9.06	0.232
m-NO_2	4.9	4.94	8.51	(0.35)	172	8.04	0.71
m-$COCH_3$	4.9	5.02	1.31	(0.17)	26.2	9.25	0.376
m-Cl	4.8	5.47	1.98	(0.04)	40.9	9.76	0.373
m-Br	4.9	5.59	1.93	(0.06)	39.0	8.75	0.391

(a) Reference 84.

(b) Substituent parameters from Reference 51.

Table 3-2. Kinetic Parameters for the Acylation of Chymotrypsin by X-Phenyl p-Nitrobenzoates at pH 5.5 (0.1 M acetate, 5% v/v CH_3CN) and 25.0° C.

Ester X-	$[S_o]$ x 10^6 M	$[E_o]$ x 10^5 M	k_{obs} x 10^3	(Std Dev) sec^{-1}	k_2/K_s M^{-1} sec^{-1}	σ^-
p-NO_2	4.9	5.77	89.7	(5.6)	1555	1.0
p-CN	5.0	5.05	41.2	(3.6)	816	0.89
p-Cl	4.8	5.13	3.72	(0.2)	72.5	0.23

Table 3-3. Kinetic Parameters for the Acylation of Chymotrypsin by X-Phenyl p-Nitrobenzoates at pH 6.0 (0.1 M phosphate, 5% v/v CH_3CN) and 25.0° C.

Ester X-	$[S_o]$ x 10^6 M	$[E_o]$ x 10^5 M	k_{obs} x 10^3 sec^{-1} (Std Dev)	k_2/K_s M^{-1} sec^{-1}	σ^-
p-NO_2	4.9	5.79	140.4 (12.2)	2425	1.0
p-CN	5.0	5.07	52.0 (5.5)	1026	0.89
p-Cl	4.8	5.12	7.90 (0.61)	154.3	0.23

Table 3-4. Kinetic Parameters for the Acylation of Chymotrypsin by X-Phenyl p-Nitrobenzoates at pH 7.0 (0.1 M phosphate, 5% v/v CH_3CN) and 25.0° C.

Ester -X	$[S_o]$ x 10^6 M	$[E_o]$ x 10^5 M	k_{obs} (Std Dev) x 10^3 sec^{-1}	k_2/K_s M^{-1} sec^{-1}	σ^-
p-NO_2	4.9	5.53	449 (15)	8136	1.0
p-CN	5.0	4.89	79.3 (14)	1622	0.89
p-Cl	4.8	5.08	25.9 (2.3)	510	0.23

Table 3-5. Rho Values for the Acylation of Chymotrypsin by Substituted Phenyl p-Nitrobenzoates at Various pH Values and 25.0° C.

pH	Rho
5.0	1.8
5.5	1.8
6.0	1.8
7.0	1.7

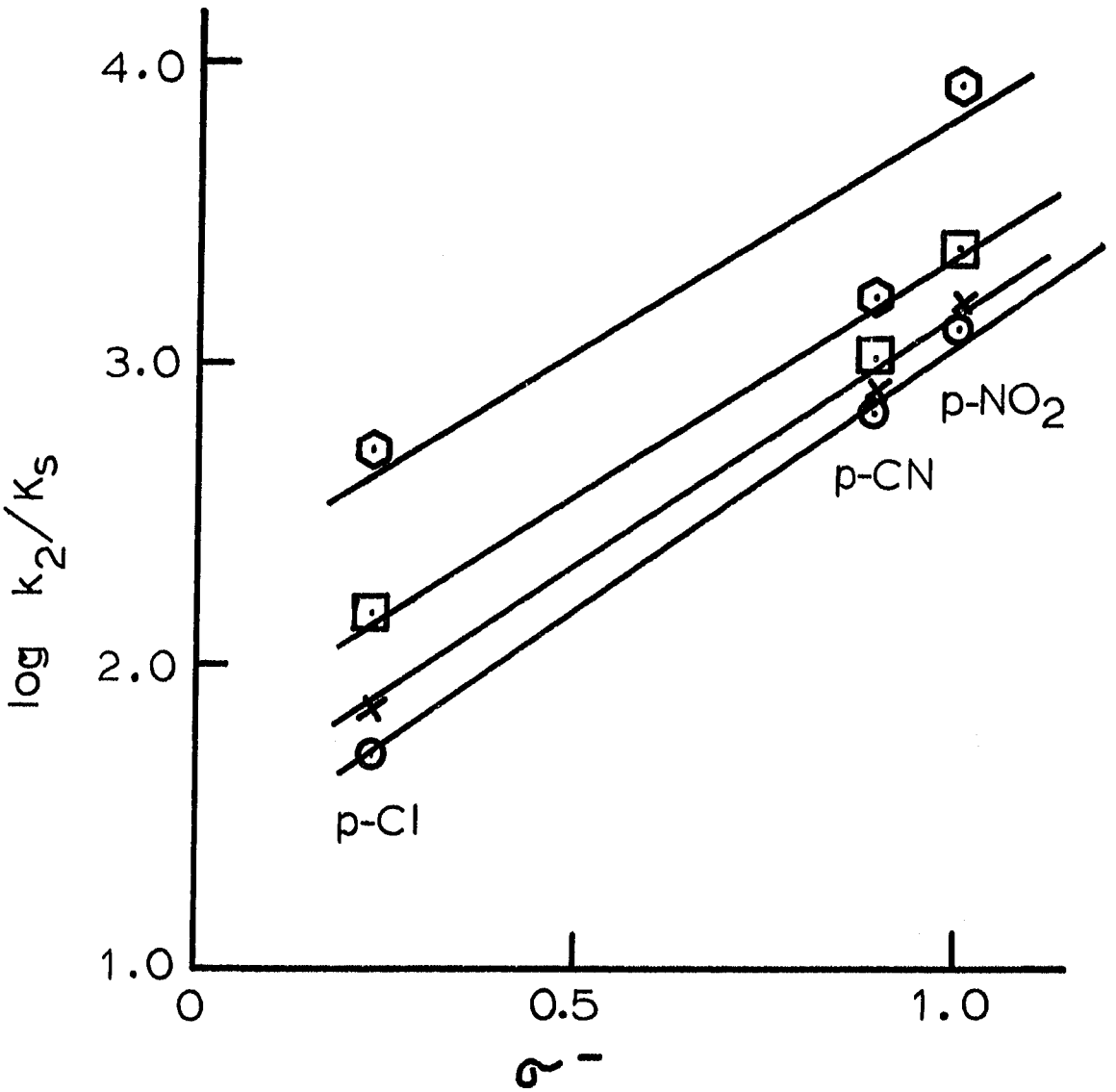


Figure 3-1. Hammett Plots for the Acylation of Chymotrypsin by Substituted Phenyl p-Nitrobenzoates at pH Values between 5 and 7. ⊙, pH 5.05, ρ = 1.8; X, pH = 5.5, ρ = 1.8; ⊡, pH=6.0, ρ = 1.8, ⊙, pH = 7.0, ρ = 1.7.

To monitor the acylation reaction the disappearance of substrate was followed, whereas in previous studies (38,67) the appearance of phenolic product was monitored. In order to assure that both procedures yield the same kinetic data the acylation of chymotrypsin by p-nitrophenyl benzoate was monitored by both methods at pH 5.05 (0.1 M acetate, 5% v/v CH_3CN). The resulting second-order rate constants for acylation derived from the disappearance of substrate and appearance of product are 346 $\pm$ 10 M^{-1} sec^{-1} and 363 $\pm$ 11 M^{-1} sec^{-1} respectively. These are statistically equivalent, and therefore, it is justifiable to compare kinetic parameters obtained by both methods.

Plots of the logarithm of k_2/K_s versus sigma minus and the pK_a of the phenolic leaving group are presented in Figures 3-2 and 3-3 respectively. A rho of 1.96 $\pm$ 0.30 and a β_{lg} of -0.78 were obtained.

The Acylation of Chymotrypsin by Substituted Phenyl Acetates. Second-order acylation rate constants were determined for a series of four para substituted phenyl acetates at pH 7.47 (0.1 M phosphate, 5% v/v CH_3CN). The rate constant obtained for acylation by p-nitrophenyl acetate, 1425 M^{-1} sec^{-1}, is comparable to the rate constant obtained previously at pH 7.58 in 4.68% CH_3CN, 1370 M^{-1} sec^{-1} (82). These kinetic para-

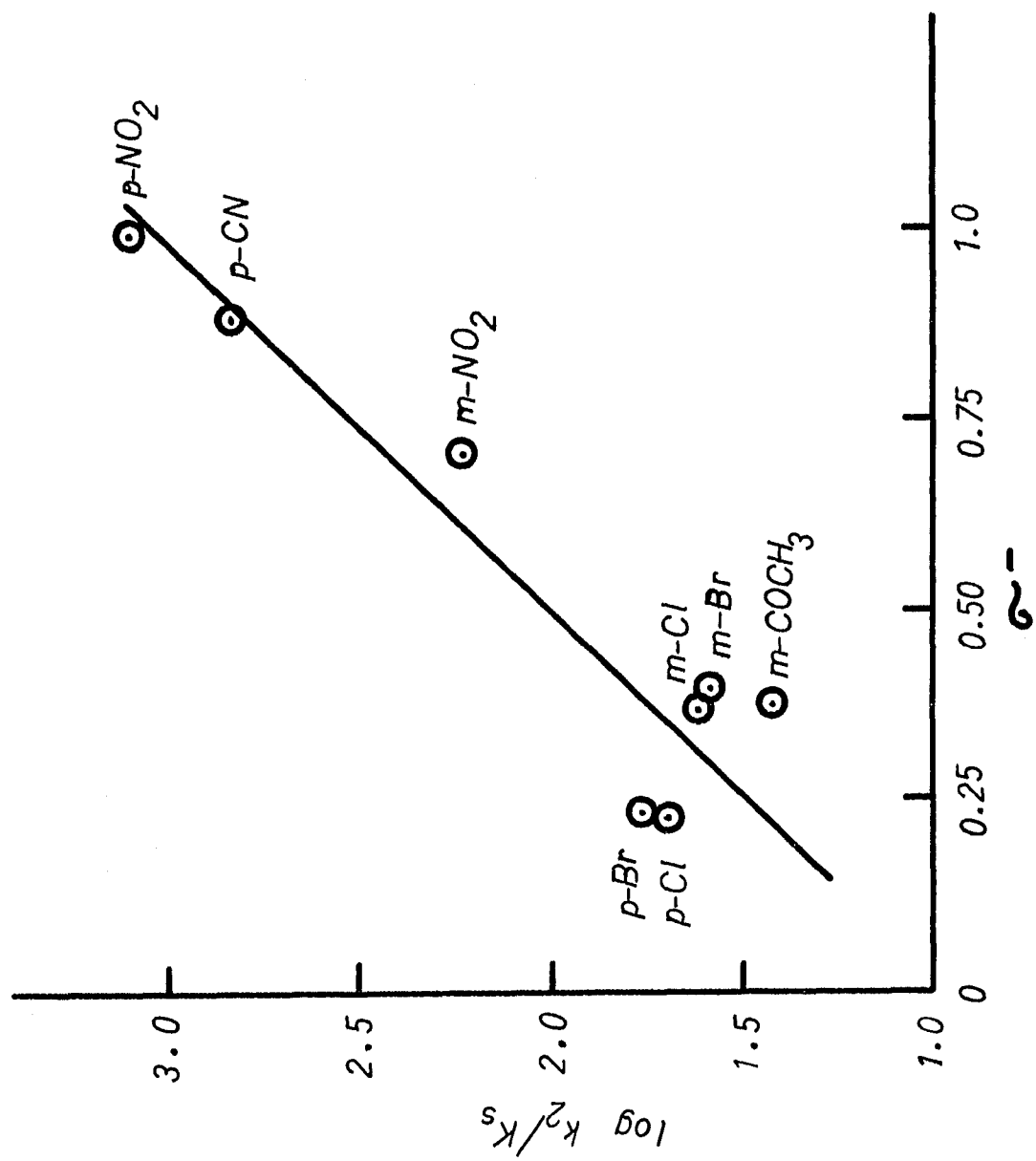


Figure 3.2. Hammett Plot for the Acylation of Chymotrypsin by Substituted Phenyl p-Nitrobenzoates at pH 5.05. $\rho = 1.96 \pm 0.30$.

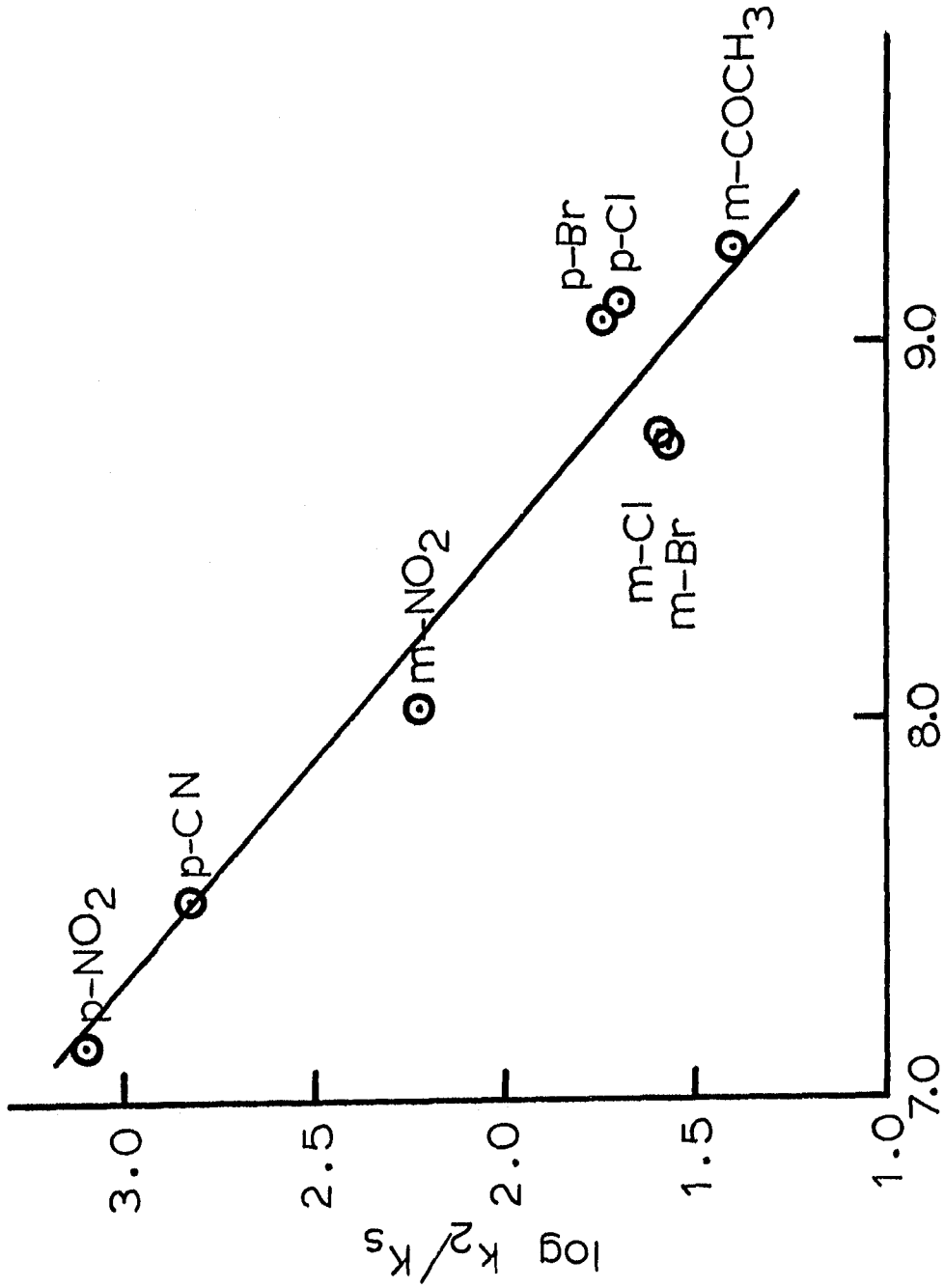


Figure 3-3. Brønsted Plot for the Acylation of Chymotrypsin by Substituted Phenyl p-Nitrobenzoates at pH 5.05. $\beta_{lg} = -0.78$.

meters along with values for a different series of phenyl acetates presented in a previous study (67) are compiled in Tables 3-6 and 3-7 respectively. Since the experiments in this previous study were carried out at pH 7.92 in 10% CH_3CN, their values, for the purpose of comparison, were normalized to give the same rate constant for p-nitrophenyl acetate as was found in this study. Plots of the logarithms of these rate constants versus sigma minus and the leaving group pK_a are presented in Figures 3-4 and 3-5 respectively. A rho of 2.05 $\pm$ 0.32 and a β_{lg} of -0.92 were obtained.

Acylation Rates as a Function of pH and pD. The rates of the acylation of chymotrypsin by p-nitrophenyl p-nitrobenzoate, p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, p-nitrophenyl hydrocinnamate, p-nitrophenyl thiolacetate, and p-nitrophenyl hippurate, were determined as a function of pH and pD. In the case of the acylation by p-nitrophenyl p-nitrobenzoate it became necessary to use two different batches of enzyme. Inexplicably the two batches gave second-order rate constants at the same pH which differed by an average of 12.7%. Multiple determinations of the normality of enzyme batches CDI 34M608 and CDI 34J847 showed a variation of 1% and 2% respectively, therefore, this deviation cannot be attributed to errors in the determination of enzyme normality. In order to overcome

Table 3-6. Kinetic Parameters for the Acylation of Chymotrypsin by X-Phenyl Acetates at pH 7.47 (0.1 M phosphate, 5% v/v CH_3CN) and 25.0° C.

Ester -X	$[S_o]$ x 10^5 M	$[E_o]$ x 10^5 M	k_2/K_s M^{-1}	(Std Dev) sec^{-1}	pK_a l.g.	σ^-
p-NO_2	2.93;2.94	5.85;2.94	1425	(40)	7.15	1.0
p-CN	2.59	5.18	430	(11.2)	7.53	0.89
p-Cl	4.60	5.28	29.6	(0.7)	9.10	0.23
p-H	5.33	5.33	3.93	(0.03)	9.78	0.0

Table 3-7. Kinetic Parameters for the Acylation of Cmymotrypsin by X-Phenyl Acetates Obtained from the Study of Bender and Nakamura[a] and Normalized to the Value Obtained for p-Nitrophenyl Acetate in the Current Study.

Ester -X	k_2/K_s M^{-1} sec^{-1}	Normalized k_2/K_s M^{-1} sec^{-1}	pK_a l.g.	σ^-
p-NO_2	563	1425	7.15	1.0
p-CHO	227	575	7.66	1.13
m-NO_2	21.3	53.9	8.04	0.71
m-CHO	15.8	40.0	9.02	0.36
m-$COCH_3$	4.6	11.6	9.19	0.38

(a) Reference 67.

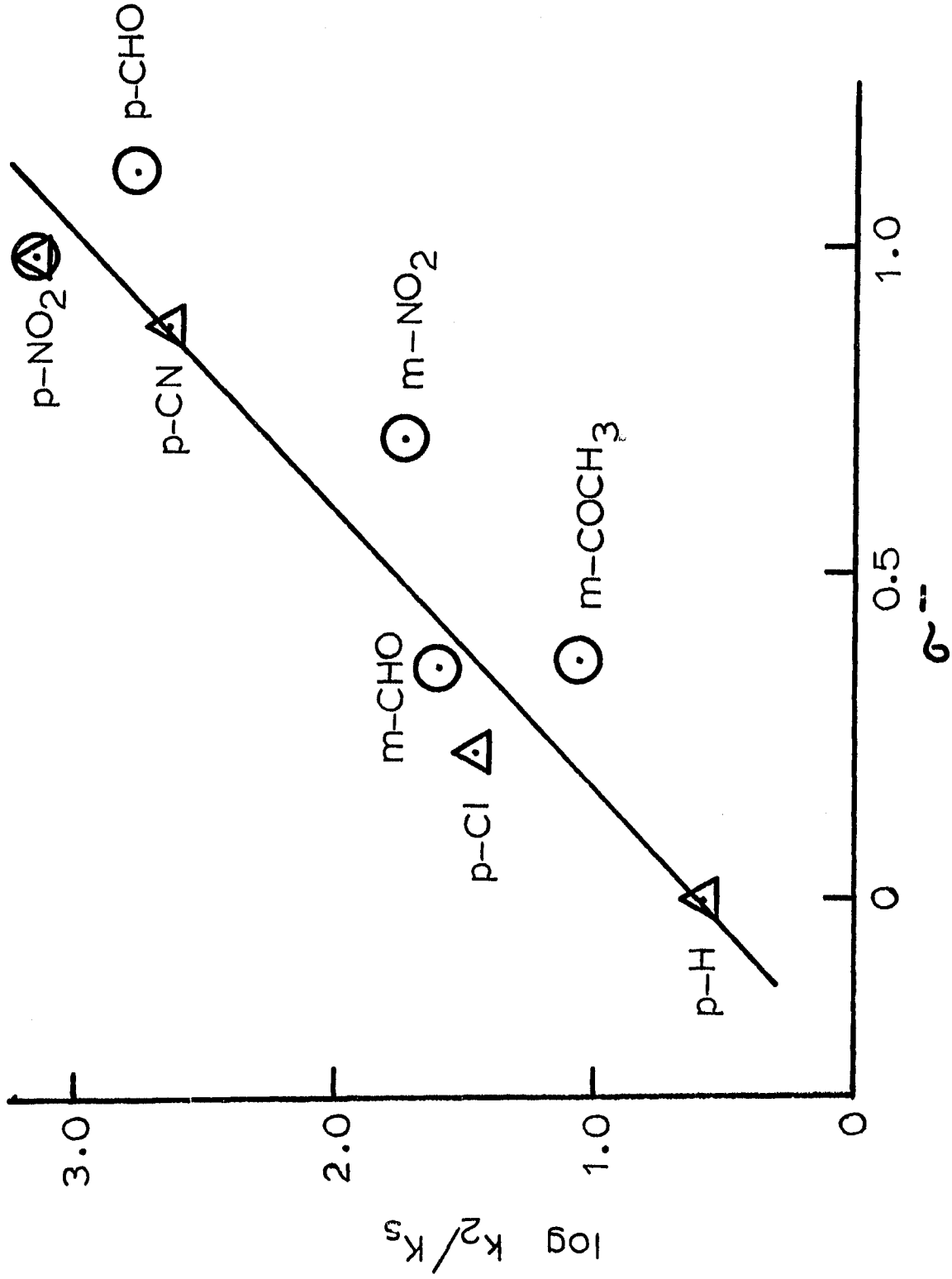


Figure 3-4. Hammett Plot for the Acylation of Chymotrypsin by Substituted Phenyl Acetates at pH 7.47. △, Values from this Study; ⊙, Normalized Values of Bender and Nakamura (67). $\rho = 2.05 \pm 0.32$.

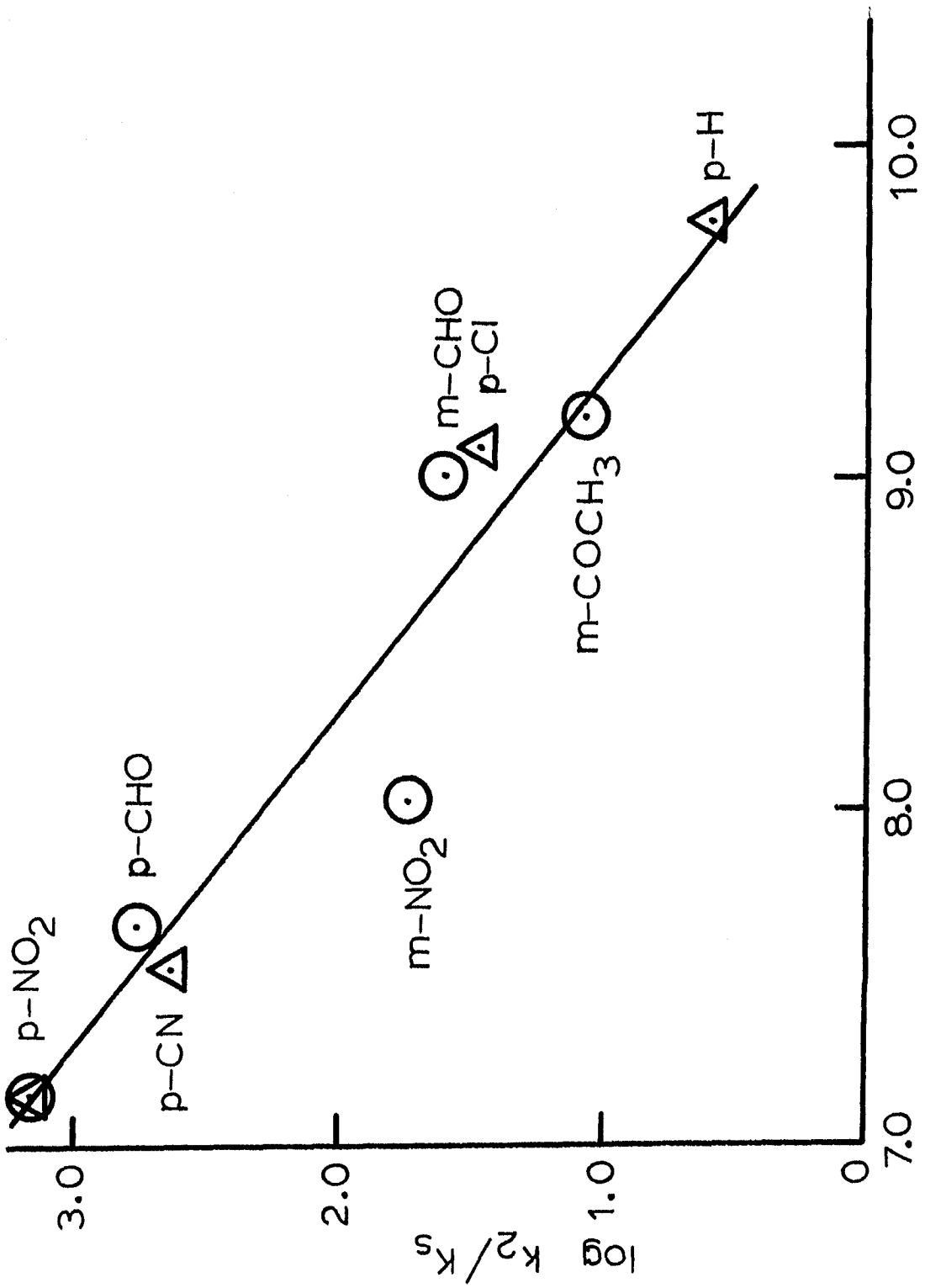


Figure 3-5. Brønsted Plot for the Acylation of Chymotrypsin by Substituted Phenyl Acetates at pH 7.47. Δ, Values from this Study; ⊙, Normalized Values of Bender and Nakamura (67). $\beta_{lg} = -0.92$.

this problem and allow results from both batches to be compared the rate constants obtained with the Worthington batch CDI 34M608 were normalized to those obtained with batch CDI 34J847. The normalization factor is given in Equation 3-1. The supporting data for this

$$k_2/K_s \ (43J) = 1.127 \times k_2/K_s \ (34M) \qquad (3\text{-}1)$$

normalization factor is given in Table 3-8. All other pH-rate profiles were determined with the same batch of enzyme, therefore, normalization in those cases was not necessary.

Compilations of the second-order rate constants of acylation as a function of pH and pD are presented in Tables 3-9 through 3-20. Plots of k_2/K_s versus pH and pD are exhibited in Figures 3-6 through 3-11. The values of pK_1, pK_2, and the pH (pD) independent rate constant, k^{lim}, for acylation by p-nitrophenyl p-nitrobenzoate, p-nitrophenyl benzoate, and p-nitrophenyl thiolacetate, were determined from a non-linear regression analysis (83) of Equation 3-2, and are reported in Table 3-21.

$$k = \frac{k^{lim}}{1 + (H^+)/K_1 + K_2/(H^+)} \qquad (3\text{-}2)$$

Table 3-8. Determination of the Normalization Factor for Worthington Batches CDI 34J847 and CDI 34M608 from the Acylation of Chymotrypsin by p-Nitrophenyl p-Nitro-Benzoate at 25.0° C.

pH	k_2/K_s (34J) $M^{-1}sec^{-1}$	k_2/K_s (34M) $M^{-1}sec^{-1}$	% Difference
8.00	12780	11250	13.6
7.50	11640	10450	11.4
9.39	6670	5890	13.2

(a) The average difference is 12.7%, therefore to yield comparable rate constants k_2/K_s for Batch 34M must be multiplied by 1.127.

Table 3-9. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl p-Nitrobenzoate as a Function of pH in H_2O at 25.0° C.

pH	$[S_o]$ x 10^6 M	$[E_o]$ x 10^5 M	Enzyme Batch	k_{obs} x 10 sec^{-1}	(Std Dev)	k_2/K_s M^{-1} sec^{-1}	Norm k_2/K_s M^{-1} sec^{-1}
6.10	5.0	5.61	34J	1.70	(0.06)	3030	3030
6.35	5.0	5.46	34J	2.07	(0.10)	3790	3790
6.85	5.0	5.23	34J	3.78	(0.18)	7230	7230
7.10	5.0	5.43	34J	4.77	(0.14)	8780	8780
7.50	5.0	5.29	34J	6.16	(0.11)	11640	11640
7.50	5.17	5.38	34M	5.62	(0.10)	10450	11780
7.69	2.99	6.51	34M	7.73	(0.17)	11870	13380
7.90	5.0	5.37	34J	6.63	(0.08)	12350	12350
8.00	5.17	5.80	34J	7.41	(0.13)	12780	12780
8.00	5.17	5.75	34M	6.47	(0.12)	11250	12680
8.29	2.99	6.60	34M	8.42	(0.16)	12760	14380
8.76	2.99	5.65	34M	5.29	(0.07)	9360	10550
9.17	5.17	5.86	34M	4.49	(0.11)	7660	8630
9.39	5.17	5.43	34J	3.62	(0.10)	6670	6670
9.39	5.17	6.06	34M	3.57	(0.06)	5890	6640
9.67	5.17	5.93	34M	2.85	(0.11)	4810	5420

Table 3-10. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl p-Nitrobenzoate as a Function of pD in D_2O at 25.0° C.

pD	$[S_o]$ x 10^6 M	$[E_o]$ x 10^5 M	k_{obs} x 10	(Std Dev) sec^{-1}	k_2/K_s M^{-1} sec^{-1}	Norm k_2/K_s M^{-1} sec^{-1}
6.40	5.02	6.14	1.60	(0.08)	2610	2940
6.71	5.06	6.33	2.01	(0.09)	3180	3580
7.02	5.06	6.81	2.87	(0.19)	4210	4740
7.51	2.99	6.31	4.94	(0.37)	7830	8820
7.69	5.06	6.81	4.34	(0.15)	6370	7180
7.92	5.28	6.34	5.45	(0.05)	8600	9690
8.17	5.28	6.23	6.61	(0.15)	10610	11960
8.29	2.99	5.95	6.29	(0.21)	10570	11910
8.41	5.28	6.73	7.79	(0.24)	11580	13050
8.60	2.99	5.63	6.42	(0.11)	11400	12850
9.45	3.05	6.35	4.66	(0.22)	7340	8270
9.75	3.05	5.85	3.75	(0.22)	6410	7220
9.99	3.05	5.83	2.83	(0.17)	4850	5460

(a) All runs made with enzyme batch 34 M.

Table 3-11. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Benzoate as a Function of pH in H_2O at 25.0° C.

pH	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs} (Std Dev) $\times 10^2$ sec^{-1}	k_2/K_s $M^{-1}sec^{-1}$
6.88	4.0	5.79	5.24 (0.52)	905
7.47	4.0	5.51	8.75 (0.69)	1585
7.89	4.0	5.27	9.93 (0.36)	1885
8.02	4.0	5.95	10.8 (0.48)	1815
8.13	4.0	5.42	9.98 (0.59)	1840
8.58	4.0	5.35	9.21 (0.11)	1720
9.05	4.0	5.64	6.67 (0.13)	1185

Table 3-12. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Benzoate as a Function of pD in D_2O at 25.0° C.

pD	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs}	(Std Dev) $\times 10^2$ sec^{-1}	k_2/K_s $M^{-1}sec^{-1}$
7.95	3.32	6.17	8.21	(0.56)	1330
8.31	3.32	5.77	8.80	(0.17)	1530
8.48	3.32	5.57	8.67	(0.30)	1560
8.58	3.32	6.28	9.31	(0.41)	1480
8.83	3.32	6.15	8.34	(0.27)	1360
9.25	3.32	5.76	7.38	(0.26)	1280

Table 3-13. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Thiolacetate as a Function of pH in H_2O at 25.0° C.

pH	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs} (Std Dev) $\times 10^2$ sec^{-1}	k_2/K_s $M^{-1}sec^{-1}$
6.47	3.30	6.15	4.18 (0.22)	680
6.99	3.30	5.76	6.74 (0.25)	1170
7.40	3.30	5.88	8.94 (0.24)	1520
7.80	3.30	5.81	9.76 (0.36)	1680
8.00	3.30	6.19	13.20 (0.54)	2130
8.10	3.30	5.96	10.53 (0.31)	1770
8.21	3.30	5.95	10.44 (0.36)	1750
8.55	3.30	5.39	8.88 (0.28)	1650
9.30	3.30	6.30	5.35 (0.18)	840
9.45	3.30	5.15	4.45 (0.17)	864

Table 3-14. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Thiolacetate as a Function of pD in D_2O at 25.0° C.

pD	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs}	(Std Dev) $\times 10^2$ sec^{-1}	k_2/K_s $M^{-1}sec^{-1}$
6.92	3.30	5.59	1.78	(0.05)	318
7.39	3.30	5.63	3.08	(0.08)	547
7.77	3.30	5.82	4.46	(0.17)	766
8.19	3.30	5.75	4.88	(0.14)	849
8.40	3.30	5.80	5.14	(0.20)	886
8.51	3.30	6.25	5.18	(0.24)	829
8.64	3.30	6.00	5.29	(0.23)	882
8.90	3.30	5.48	5.15	(0.11)	939
8.32	3.30	5.70	4.97	(0.11)	872
9.78	3.30	5.36	3.83	(0.13)	715
10.07	3.30	5.59	2.92	(0.18)	522

Table 3-15. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Hippurate as a Function of pH in H_2O at 25.0° C.

pH	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs} (Std Dev) sec^{-1}	k_2/K_s $M^{-1}sec^{-1} \times 10^{-4}$
7.87	3.24	5.39	1.28 (0.02)	2.37
7.90	3.24	5.10	1.23 (0.08)	2.41
8.06	3.24	5.95	1.25 (0.02)	2.11
8.21	3.24	5.40	1.20 (0.03)	2.22

Table 3-16. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Hippurate as a Function of pD in D_2O at 25.0° C.

pD	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs} (Std Dev) sec^{-1}	$k_2/K_s \times 10^{-4}$ $M^{-1}sec^{-1}$
8.33	3.17	5.48	0.794 (0.017)	1.45
8.61	3.17	5.27	0.778 (0.029)	1.48
8.52	3.17	6.24	0.816 (0.025)	1.31

Table 3-17. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Phenylacetate as a Function of pH in H_2O at 25.0° C.

pH	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs} (Std Dec) sec^{-1}	$k_2/K_s \times 10^{-4}$ $M^{-1}sec^{-1}$
7.50	3.0	5.24	2.73 (0.06)	5.21
7.80	3.0	5.34	2.95 (0.06)	5.52
8.00	3.0	5.19	2.94 (0.13)	5.66
8.10	3.0	5.09	2.74 (0.08)	5.38
8.20	3.0	5.20	2.98 (0.10)	5.73

Table 3-18. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Phenylacetate as a Function of pD in D_2O at 25.0° C.

pD	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs} (Std Dev) sec^{-1}	$k_2/K_s \times 10^{-4}$ $M^{-1}sec^{-1}$
7.93	3.0	5.26	1.78 (0.13)	3.38
8.20	3.0	5.31	2.08 (0.03)	3.92
8.37	3.0	5.30	2.28 (0.03)	4.30
8.50	3.0	5.35	2.32 (0.15)	4.34
8.66	3.0	5.24	2.22 (0.05)	4.24

Table 3-19. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Hydrocinnamate as a Function of pH in H_2O at 25.0° C.

pH	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs} (Std Dev) sec^{-1}	$k_2/K_s \times 10^{-5}$ $M^{-1}sec^{-1}$
7.50	3.0	5.24	23.2 (0.3)	4.43
7.80	3.0	5.34	24.3 (0.7)	4.55
8.00	3.0	5.19	25.9 (0.6)	5.00
8.10	3.0	5.09	25.8 (0.6)	5.07
8.20	3.0	5.20	25.8 (0.9)	4.96

Table 3-20. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl Hydrocinnamate as a Function of pD in D_2O at 25.0° C.

pD	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_{obs} (Std Dev) sec^{-1}	$k_2/K_s \times 10^{-5}$ $M^{-1}sec^{-1}$
7.93	3.0	5.26	11.1 (0.8)	2.11
8.20	3.0	5.31	12.7 (0.2)	2.39
8.37	3.0	5.30	14.2 (0.1)	2.68
8.50	3.0	5.35	14.0 (0.7)	2.62
8.66	3.0	5.24	13.2 (0.2)	2.52

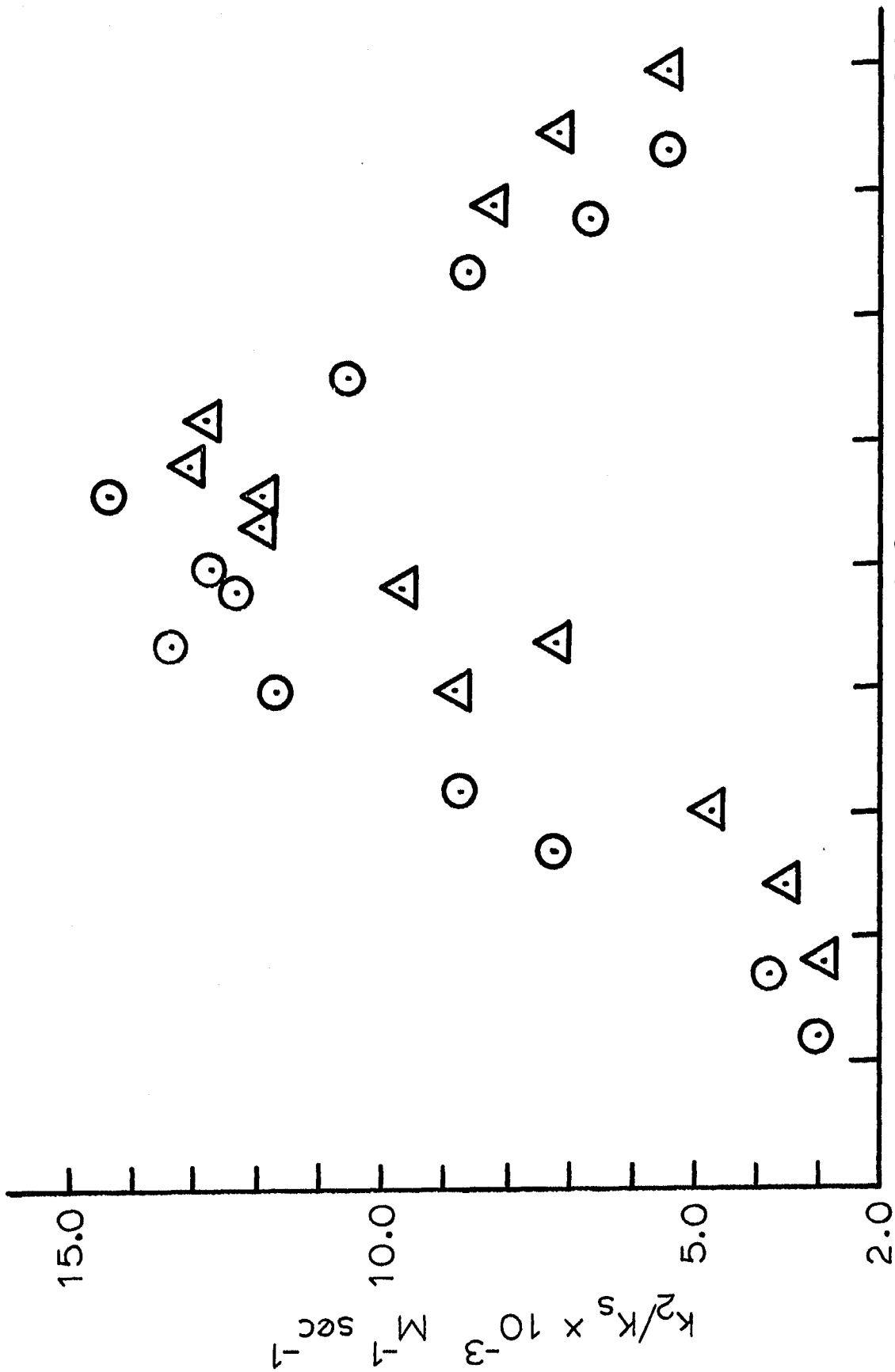


Figure 3-6. pH(pD) - Rate Profile of the Acylation of Chymotrypsin by p-Nitrophenyl p-Nitrobenzoate. ⊙, H_2O; Δ, D_2O

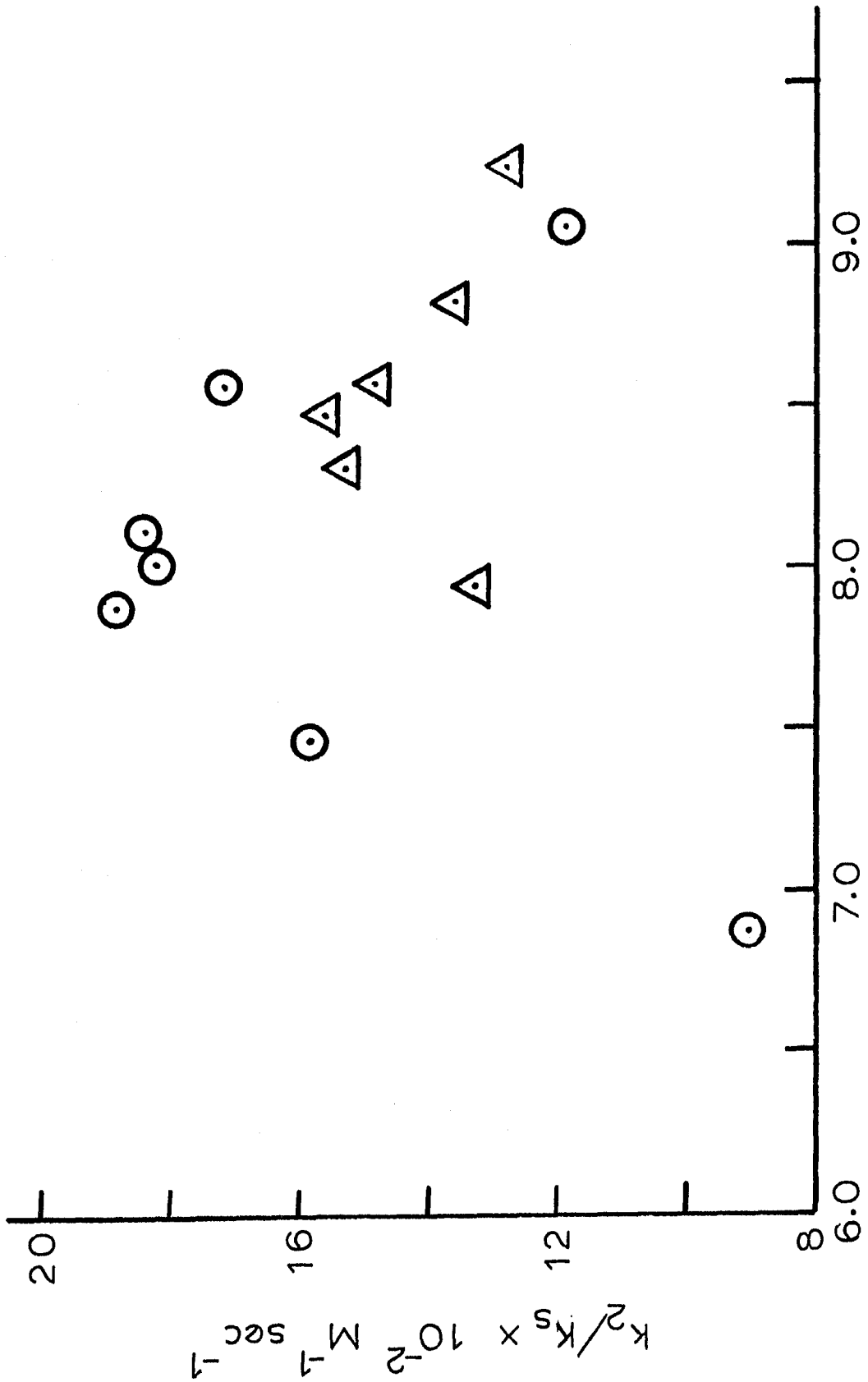


Figure 3-7. pH(pD) - Rate Profile for the Acylation of Chymotrypsin by p-Nitrophenyl Benzoate. ⊙, H_2O; △, D_2O.

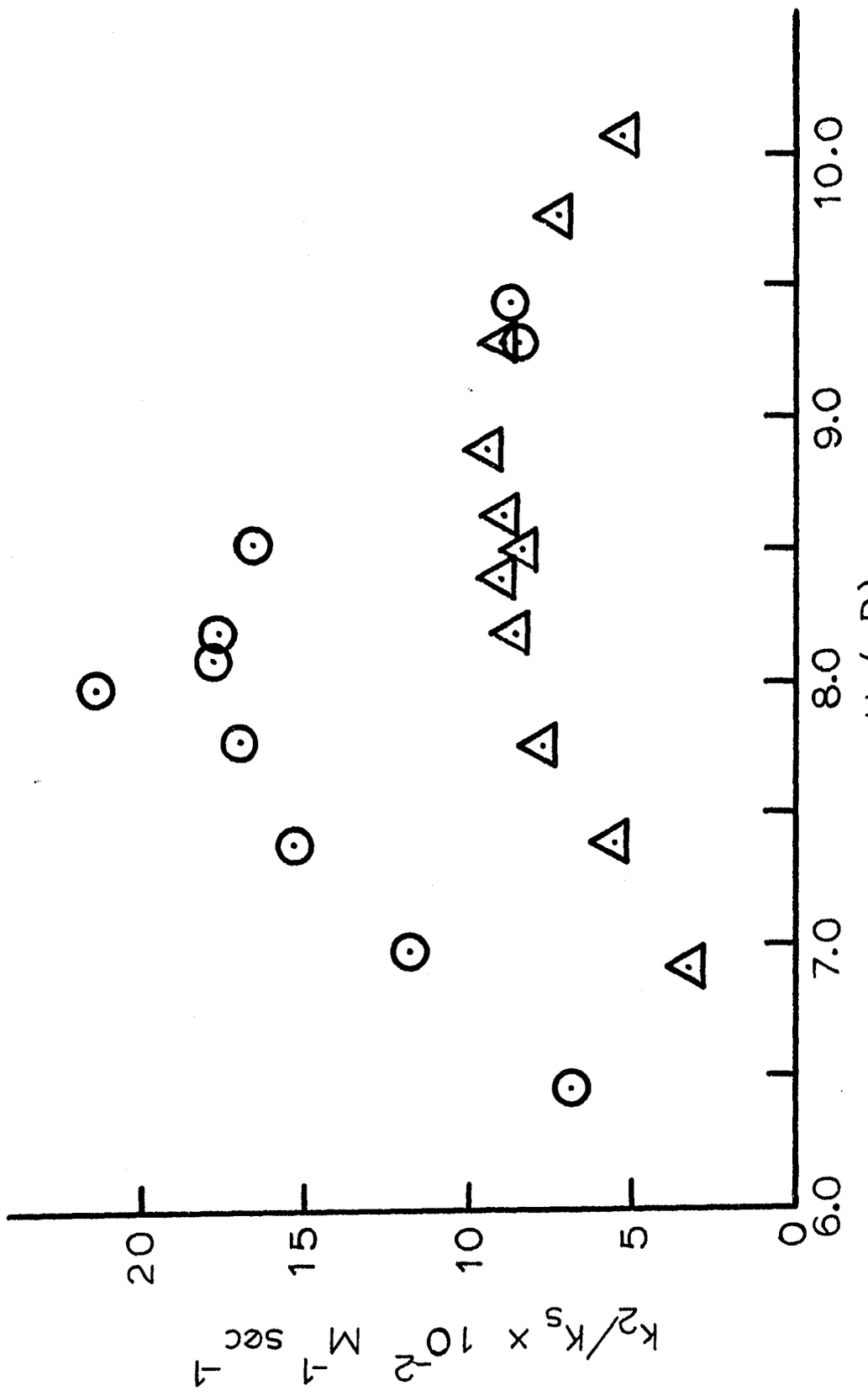


Figure 3-8. pH(pD) - Rate Profile for the Acylation of Chymotrypsin by p-Nitrophenyl Thiolacetate. ⊙, H_2O; △, D_2O.

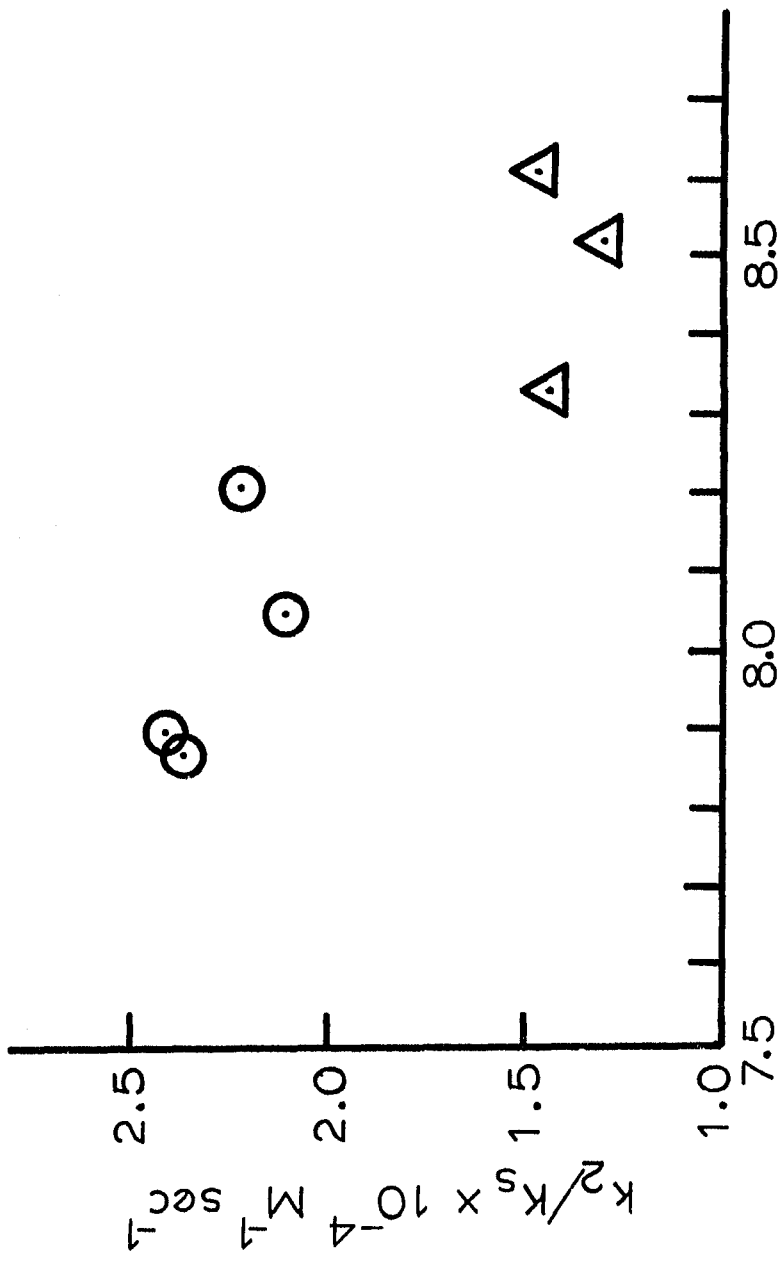


Figure 3-9. pH(pD) - Rate Profile for the Acylation of Chymotrypsin by p-Nitrophenyl Hippurate. ⊙, H_2O; △, D_2O.

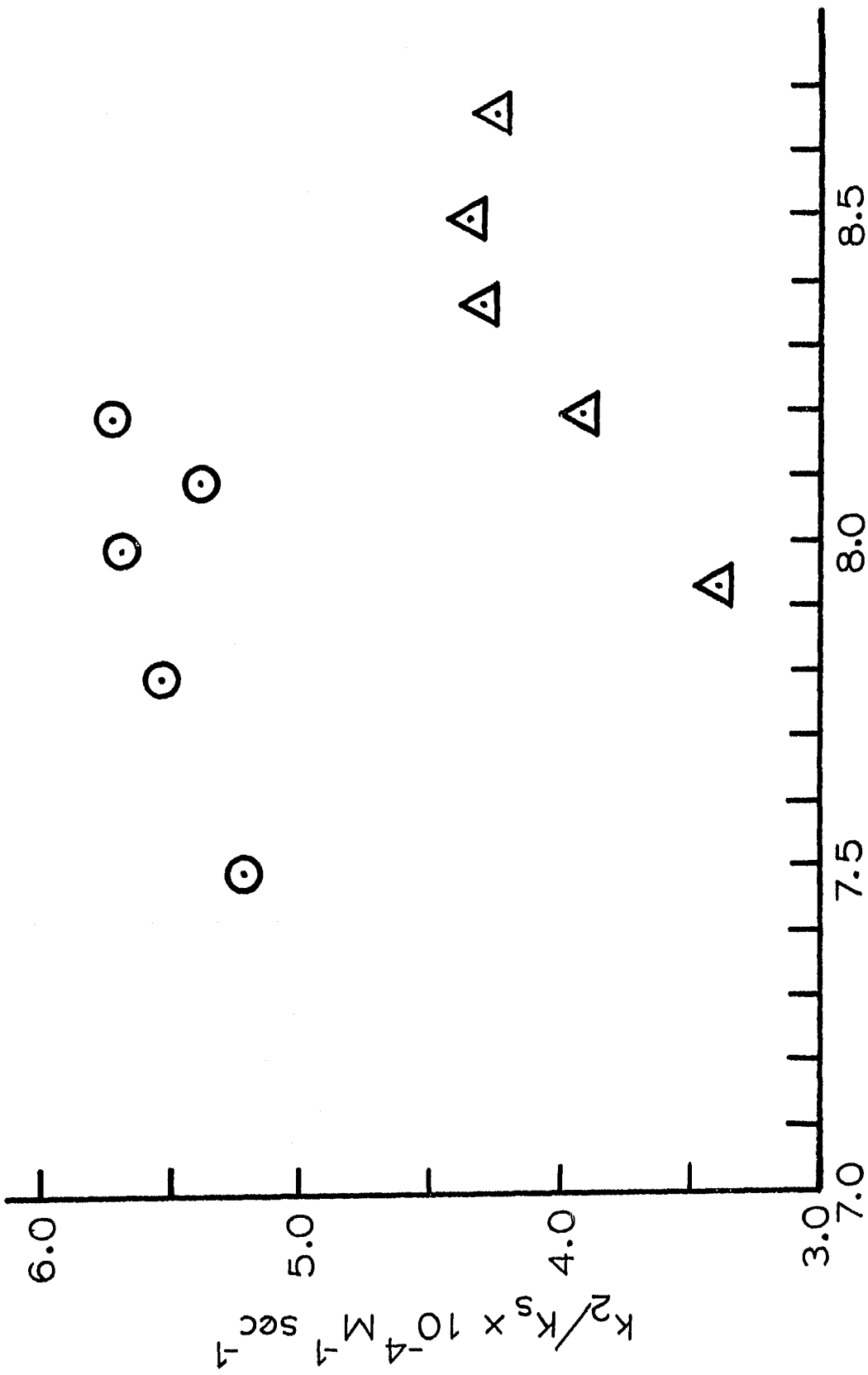


Figure 3-10. pH(pD) - Rate Profile for the Acylation of Chymotrypsin by p-Nitrophenyl Phenylacetate. ⊙, H_2O; △, D_2O.

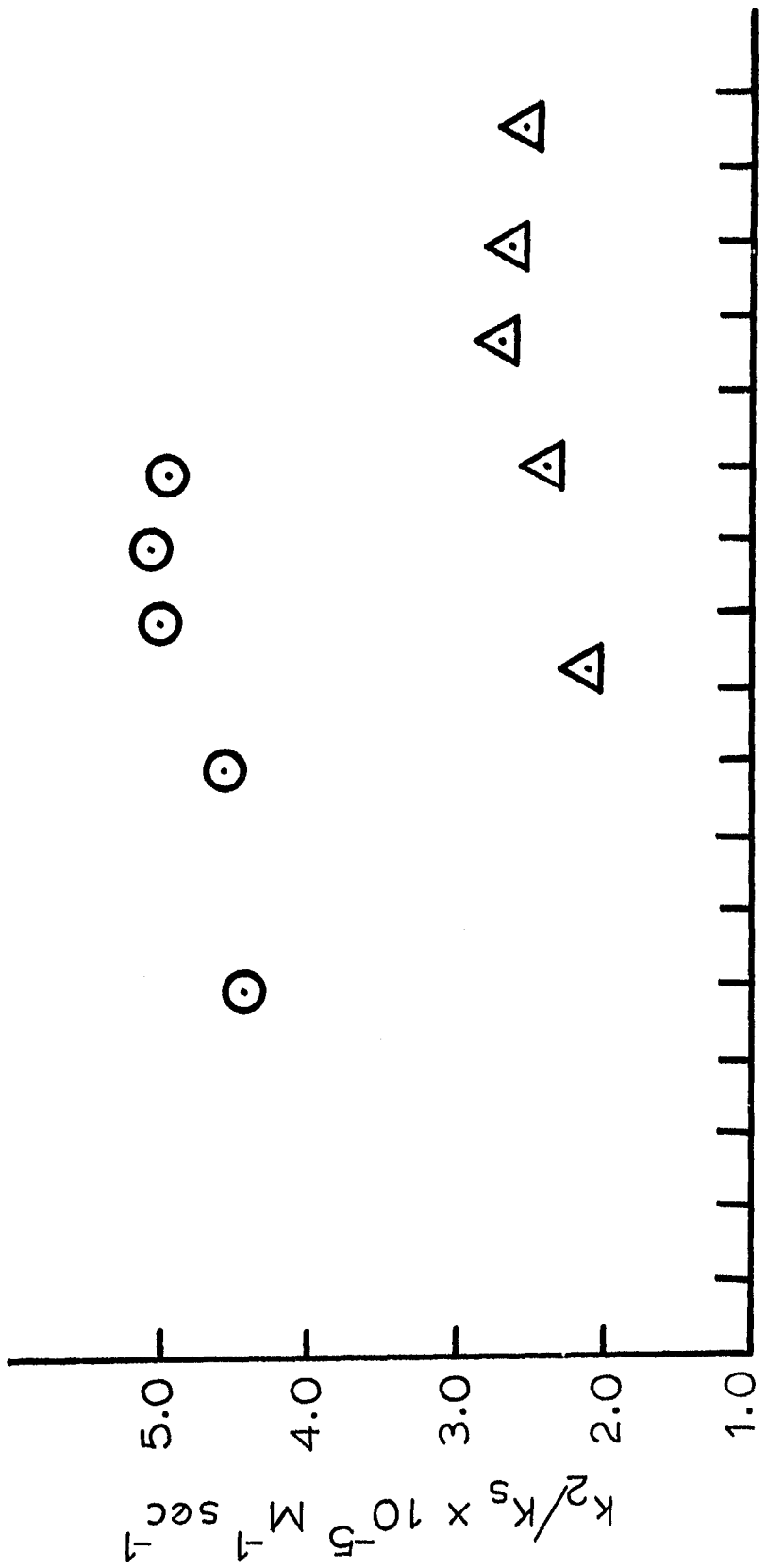


Figure 3-11. pH(pD) - Rate Profile for the Acylation of Chymotrypsin by p-Nitrophenyl Hydrocinnamate. ⊙, H_2O; Δ, D_2O.

Table 3-21. Acylation of Chymotrypsin by Ester Substrates in H_2O and D_2O.

Ester	pK_1	pK_2	k^{lim} M^{-1} sec^{-1} x 10^{-3}	Solvent	k_{H_2O}/k_{D_2O}
p-Nitrophenyl	6.80	9.39	14.0	H_2O	
p-Nitrobenzoate	7.31	9.79	12.3	D_2O	1.14
p-Nitrophenyl	7.03	9.14	2.19	H_2O	
Benzoate	7.30	9.72	1.68	D_2O	1.30
p-Nitrophenyl	6.87	9.20	2.02	H_2O	
Thiolacetate	7.24	10.20	0.96	D_2O	2.10
p-Nitrophenyl			(24)	H_2O	
Hippurate			(15)	D_2O	(1.6)
p-Nitrophenyl			(57)	H_2O	
Phenylacetate			(43)	D_2O	(1.3)
p-Nitrophenyl			(510)	H_2O	
Hydrocinnamate			(260)	D_2O	(2.0)

The pH (pD) independent acylation rate constants for p-nitrophenyl phenylacetate, p-nitrophenyl hydrocinnamate and p-nitrophenyl hippurate were estimated from values obtained in the pH independent region of the acylation pH-rate profile (pH 7.50 - 8.20 and pD 7.93 - 8.66). These estimated values are also presented in Table 3-21. The resulting solvent deuterium isotope effects (k_{H_2O}/k_{D_2O}) on acylation are 1.04, 1.30, 1.3, 2.0, 2.10, and 1.6 for p-nitrophenyl p-nitrobenzoate, p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, p-nitrophenyl hydrocinnamate, p-nitrophenyl thiolacetate, and p-nitrophenyl hippurate respectively (Table 3-21).

The Acylation of Chymotrypsin by p-Nitrophenyl p-Nitrobenzoate in the Presence of Added Methanol. The acylation of chymotrypsin by p-nitrophenyl p-nitrobenzoate at pH 8.00 (0.1 M phosphate) with the initial concentration of substrate in at least two-fold excess over enzyme was monitored by following the appearance of the p-nitrophenolate anion at 400 nm. The reaction was carried out in two different pH 8.00 phosphate buffers, one being 20% v/v CH_3CN and the other being 20% v/v CH_3OH.

When the reaction was carried out in 20% v/v CH_3CN an initial rapid release of p-nitrophenolate followed by a much slower steady-state liberation was

observed. The magnitude of the burst was obtained by extrapolating the steady-state absorbance versus time curve to zero time. In the case of the 20% v/v CH_3OH buffer an apparent continuous release of p-nitrophenolate until all of the ester was hydrolyzed was observed. The total change of absorbance was taken as the absorbance value at which the curve leveled off. The magnitudes of the observed bursts and the theoretical magnitudes of the bursts assuming one to one stoichiometry of the enzyme to p-nitrophenol liberated are collected in Table 3-22. The theoretical values were determined from the molar absorptivity of the p-nitrophenolate anion at 400 nm, 18,320 (77), and the pK_a of p-nitrophenol, 7.14 (67). Taking into account the blank rate in 20% v/v CH_3OH of 0.016 absorbance units per minute the total absorbance change for conversion of all of the ester to products was found to be 0.212 absorbance units, while the calculated theoretical value was 0.201. Considering that the blank rate will actually be hyperbolic in nature, these two value confirm the validity of the extinction coefficient of pK_a value used above.

The significance of these results will be discussed below.

Table 3-22. The Analysis of the p-Nitrophenolate Liberation Upon the Acylation of Chymotrypsin by p-Nitrophenyl p-Nitrobenzoate[a].

Cosolvent	$[E_o]$ x 10^6 M	$[S_o]$ x 10^5 M	Obs. Burst ΔA	Theoret. Burst ΔA
CH_3CN	5.42	1.25	0.049	0.087
CH_3OH	4.34	1.25	0.132	0.070

(a) Reactions were run in pH 8.00 (0.1 M phosphate) buffer which was either 20% CH_3CN or 20% v/v CH_3OH.

The Acylation of Chymotrypsin by p-Nitrophenyl Esters in 5% v/v Dioxane and 5% v/v Acetonitrile. The second-order rate constants for the acylation of chymotrypsin, k_2/K_s, were determined for p-nitrophenyl acetate, p-nitrophenyl benzoate, p-nitrophenyl phenyl acetate, and p-nitrophenyl hydrocinnamate at pH 8.00 (0.1 M phosphate) in 5% v/v dioxane and 5% v/v acetonitrile. The rate constants for the reactions in dioxane and acetonitrile are collected in Table 3-23. Ratios of the rate constants for each ester in the two cosolvents are presented in Table 3-23.

The Acylation of Chymotrypsin by Some p-Nitrophenyl Esters in H_2O and D_2O using Dioxane as the Cosolvent. The acylation of chymotrypsin by p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, and p-nitrophenyl hydrocinnamate was studied in H_2O and D_2O with dioxane cosolvent. A pH of 8.00 or pD of 8.40 was chosen for these reactions because these pH values fall in the pH-independent region of the pH-rate profile for acylation (38,48,81). From the second-order rate constants obtained at pH 8.00 and pD 8.40 an estimate of the solvent deuterium isotope effects upon the acylation by these esters in 5% v/v dioxane can be made. The rate constants and approximate isotope effects are given in Table 3-24.

Table 3-23. The Acylation of Chymotrypsin by p-Nitrophenyl Esters in 5% v/v Dioxane and 5% v/v Acetonitrile at pH 8.00 and 25.0° C.[a]

Ester	Cosolvent	k_2/K_s (Std Dev) M^{-1} sec^{-1}	k_A/k_D
p-Nitrophenyl	Acetonitrile	1520 (40)	
Acetate	Dioxane	708 (26)	2.15
p-Nitrophenyl	Acetronitrile	1870 (30)	
Benzoate	Dioxane	1350 (50)	1.39
p-Nitrophenyl	Acetonitrile	54600 (500)	
Phenylacetate	Dioxane	18900 (300)	2.89
p-Nitrophenyl	Acetonitrile	498000 (13000)	
Hydrocinnamate	Dioxane	198000 (6000)	2.52

(a) Substrate concentrations were uniformly 3.0×10^{-6} M; Enzyme concentrations were 5.18×10^{-5} M in acetonitrile and 5.04×10^{-5} M in dioxane.

Table 3-24. Acylation of Chymotrypsin by Ester Substrates in H_2O and D_2O with 5% v/v Dioxane Cosolvent at pH 8.00 (pD 8.40) and 25.0° C.

Ester	Solvent	$k_2/K_s \times 10^{-3}$	(Std Dev) M^{-1} sec^{-1}	k_{H_2O}/k_{D_2O}
p-Nitrophenyl Benzoate	H_2O	1.35	(0.05)	
	D_2O	0.82	(0.03)	1.7
p-Nitrophenyl Phenylacetate	H_2O	18.9	(0.3)	
	D_2O	10.9	(0.7)	1.7
p-Nitrophenyl Hydrocinnamate	H_2O	198	(6)	
	D_2O	101	(5)	2.0

The Acylation of Chymotrypsin by Substituted p-Nitrophenyl Benzoates in 5% v/v Dioxane. The second-order rate constants for the acylation of chymotrypsin by p-nitrophenyl p-nitrobenzoate, p-nitrophenyl p-cyanobenzoate, and p-nitrophenyl m-fluorobenzoate, and p-nitrophenyl benzoate were determined at pH 8.00 (0.1 M phosphate, 5% v/v dioxane). A previous study examined the acylation by a much larger selection of substituted benzoates (38) in 5% v/v acetonitrile. The four esters used in this study were chosen based on two criteria: 1) a large difference in the σ substituent parameters on the benzoate moiety, and 2) the logarithms of their acylation rate constants when determined in acetonitrile fell on or very near the slope of the Hammett plot in the previous study. By following these two criteria a Hammett plot generated from the kinetic parameters for these four esters should give a good correlation. The rate constants determined in this study are presented in Table 3-25 and a plot of the logarithm of k_2/K_s versus the polar substituent constant, sigma, is presented in Figure 3-12. A rho value of 0.60 was obtained.

Table 3-25. Kinetic Parameters for the Acylation of Chymotrypsin by p-Nitrophenyl X-Benzoates at pH 8.00 (0.1 M phosphate, 5% v/v dioxane) and 25.0° C.

X	$[S_o] \times 10^6$ M	$[E_o] \times 10^5$ M	k_2/K_s (Std Dev) M^{-1} sec^{-1}	σ
p-NO_2	3.0	5.32	4490 (190)	0.778
p-CN	3.0	5.32	3740 (390)	0.66
m-F	3.0	5.32	2880 (90)	0.337
p-H	3.0	5.32	1490 (50)	0.0

(a) σ Values obtained from reference 51.

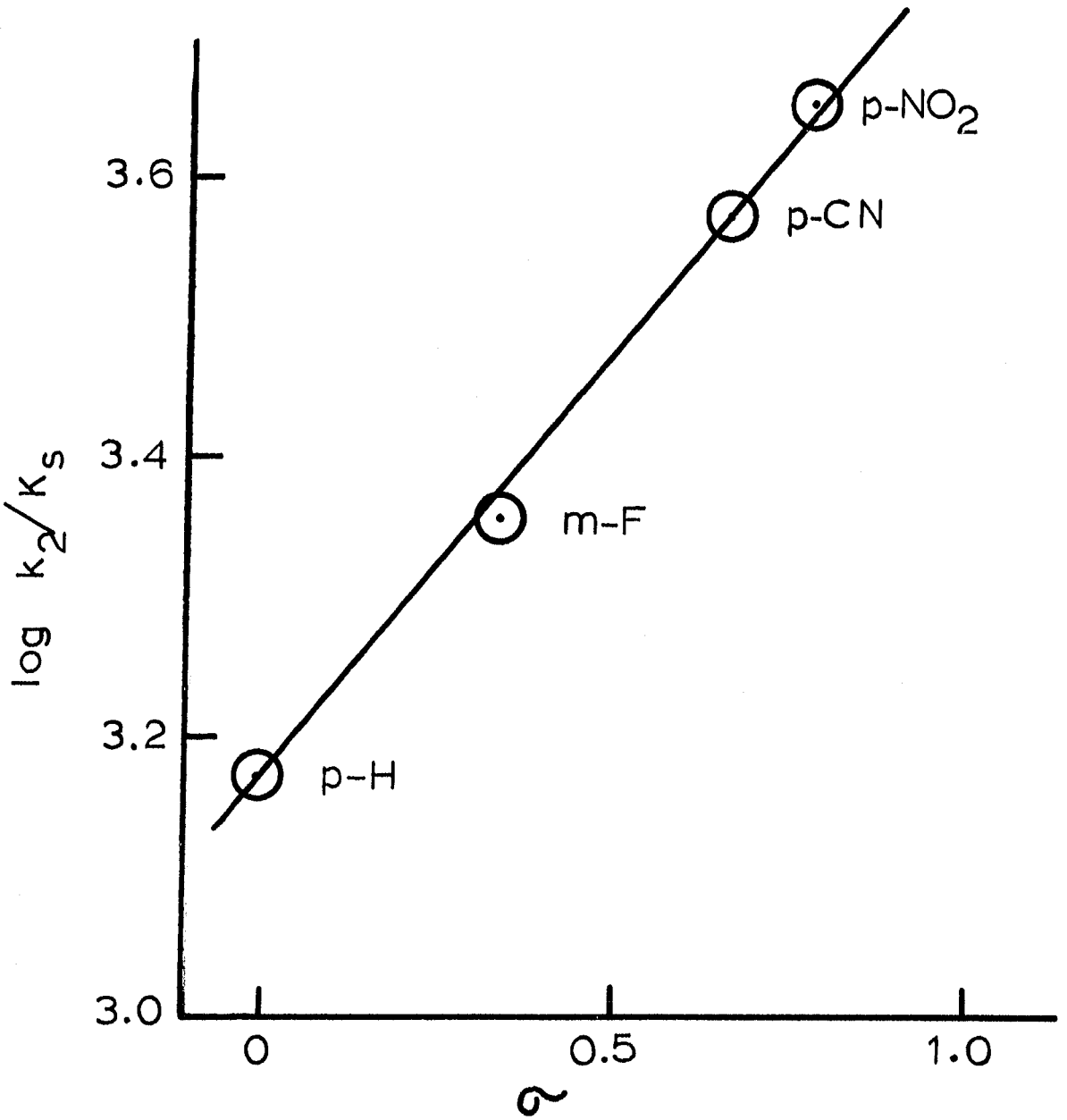


Figure 3-12. Hammett Plot for the Acylation of Chymotrypsin by Substituted p-Nitrophenyl Benzoates at pH 8.00. $\rho = 0.60$.

DISCUSSION

The Mechanistic Significance of the Components of the Second-Order Rate Constant Obtained for the Acylation of Chymotrypsin Under Pseudo-First-Order Conditions.

The minimal mechanism for the catalytic hydrolysis of substrates by chymotrypsin can be described by Equation 4-1.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \sim S \xrightarrow{k_2} E\text{-}P_2 \xrightarrow{k_3} E + P_2 \qquad (4\text{-}1)$$

$$E \sim S \xrightarrow{k_2} P_1$$

A general steady-state solution of Equation 4-1 yields the following kinetic parameters:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$$

$$K_m = \left(\frac{k_3}{k_2 + k_3} \right) \left(\frac{k_{-1} + k_2}{k_1} \right)$$

For the case of chymotrypsin catalyzed hydrolysis of ester substrates the following assumptions can

be made:

$$k_{-1} >> k_2$$

$$k_2 >> k_3$$

Thus the expressions for k_{cat} and K_m can be simplified becoming,

$$k_{cat} = k_3$$

$$K_m = \left(\frac{k_{-1}}{k_1}\right) \frac{k_3}{k_2} = K_s \frac{k_3}{k_2}$$

If the hydrolysis of the substrate is catalyzed according to Equation 4-1 the ratio k_{cat}/K_m becomes,

$$\frac{k_{cat}}{K_m} = \frac{k_3}{K_s(k_3/k_2)} = \frac{k_2}{K_s}$$

Equation 4-1 can also be examined by standard kinetic analysis.

$$\frac{d[P_1]}{dt} = k_2[E\sim S]$$

The dissociation constant of the Michaelis complex, K_s, is expressed as follows:

$$K_s = \frac{[E][S]}{[E\sim S]}$$

Solving the above expression for [E~S] and substituting that value in the rate expression we have,

$$\frac{d[P_1]}{dt} = \frac{k_2[E][S]}{K_s}$$

Under pseudo-first-order conditions of $[E_o] >> [S_o]$, and assuming saturation of the enzyme by substrate is of negligible importance, the following relationship obtains.

$$\frac{d[P_1]}{dt} = k_{obs}[S]$$

with,

$$k_{obs} = \frac{k_2[E]}{K_s}$$

therefore,

$$\frac{k_{obs}}{[E]} = \frac{k_2}{K_s}$$

One additional assumption becomes apparent at this point. The operational second-order rate constant is determined by dividing k_{obs} by the initial enzyme concentration, $[E_o]$. To be precise, the k_{obs} should be divided by the instantaneous concentration of enzyme [E], which equals $[E_o] - [E\sim S]$. But since initial enzyme concentration is in great excess of initial substrate concentration $[E_o]$ will be much greater than $[E\sim S]$, and therefore, $[E_o] \tilde{=} [E]$. Thus the assumption is sound.

It is now seen that the value of k_{cat}/K_m determined by steady-state analysis of Equation 4-1 can be related to k_{obs} through the term k_2/K_s. Other studies of the acylation of chymotrypsin by ester substrates have used the acylation rate constant k_{cat}/K_m determined from steady state parameters rather than direct observation of the acylation reaction (40,85). In the present study acylation was usually studied under pseudo-first-order conditions and the second-order acylation rate constant, k_2/K_s, was determined from the observed

first-order rate constant and the initial enzyme concentration, $[E_o]$. The rate constants derived by both methods are shown above to be equal.

It can be seen from the above analysis that a substituent effect observed upon the acylation of chymotrypsin by ester substrates can manifest itself in two processes, the enzyme-substrate equilibrium to form the Michaelis complex, [E~S], and the actual acylation process. The former would be observed in K_s and the latter in k_2. For the acylation of chymotrypsin by esters in which the polarity of the leaving groups is altered it can be assumed that K_s is independent of the substituent because chymotrypsin does not appear to have a distinct binding site for the leaving group of the ester (85,86 87). Nevertheless, it is believed that the binding specificity of chymotrypsin is at least partially hydrophobic in nature; therefore bulky alkyl substituents may affect K_s, and should be used only with caution in polarity correlations. The fact that the structure-reactivity correlations presented herein generate good straight lines for the dependence of rate on the polarity of the substituents is good empirical evidence that steric or hydrophobic effects are constant except for alkyl substituents.

Analysis of Structure-Reactivity Correlations for the Acylation of Chymotrypsin by Substituted Phenyl Esters of Benzoic and Acetic Acids. A study of the effect of structural variation of the phenolic leaving groups of phenyl esters of benzoic acid and acetic acid upon the acylation of chymotrypsin, as expressed in the complex rate constant, k_2/K_s, was carried out. Two such relationships were studied. Hammett and Brønsted correlations were analyzed in an attempt to ascertain the identity of the nucleophilic moiety in the active site of the enzyme which directly participates in the acylation reaction. It was concluded that for phenyl benzoate substrates acylation proceeds by direct nucleophilic attack by the imidazole of the His 57 residue resulting in a transient acylimidazole intermediate along the path to the Ser 195 acylated enzyme. For the case of acylation by phenyl acetates there is conflicting evidence but it is felt now that the evidence is more in favor of these esters acylating by the conventional general-base catalyzed activation of the Ser 195.

Analysis of the Hammett Correlation. In a previous study the effect of substitution upon the benzoic acid moiety of a series of p-nitrophenyl and 2,4-dinitrophenyl benzoates was examined. Correlation of the sigma

substituent parameter with the logarithms of the acylation rate constants were constructed and rho values of 0.97 ± 0.11 and 1.6 ± 0.3 respectively were found (38). Rho values in this range indicated little stabilization of the transition state by electron withdrawing substituents. Therefore one would expect little if any charge to be developed in the transition state at the reaction center relative to the ground state. From knowledge of the potentially reactive moieties in the active site of chymotrypsin, the imidazole of His 57, the hydroxyl of Ser 195, and the carboxylate of Asp 102, one can postulate three transition states (I-III) which meet the no net change in charge criterion. (Figure 4-1).

Transition state I would require nucleophilic attack by an unactivated alcohol and is deemed unlikely because unactivated alcohols are poor nucleophiles and would not be expected to generate enzymatic rates. Also, no function is provided for the catalytically important group of pK_a 6-7 seen in the pH-rate profile for acylation. Transition state II would allow for significant activation of the serine, and would meet the no net change in charge criterion by participation of an unidentified functional group in general-acid catalysis. This alternative is rendered unlikely by subsequent solvent deuterium isotope effect studies, and the neutralization of charge in the transition state may take place at too

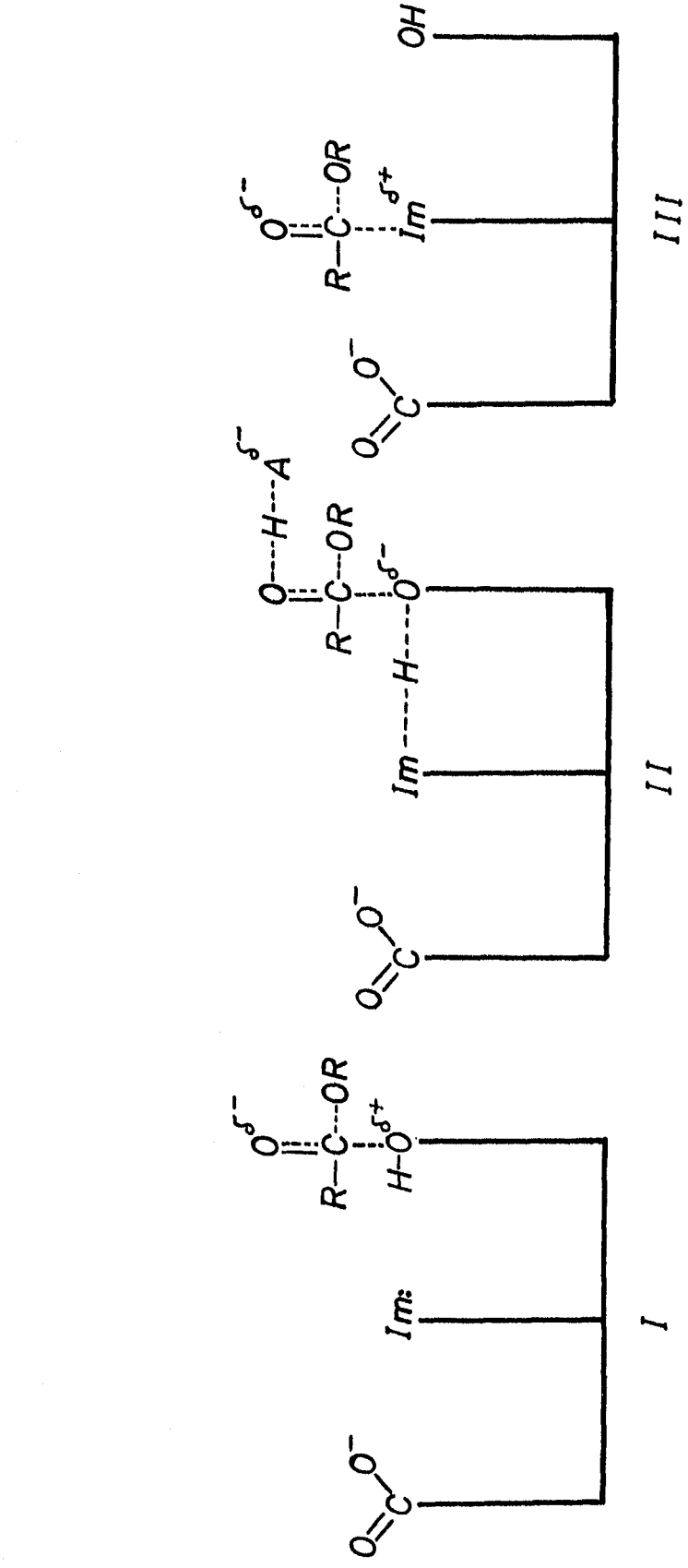


Figure 4-1. The Postulated Transition States for which the No Net Change in Charge Criterion is Met.

great a distance from the reaction center to affect rho. Transition state III represents direct nucleophilic attack by imidazole to generate a transient acylimidazole species which rapidly collapses to the serine acylated enzyme.

A comparison of the rho values obtained for the acylation of chymotrypsin by the substituted p-nitrophenyl and 2,4-dinitrophenyl benzoates with rho values for the reactions of various nucleophiles with the same esters is presented in Table 4-1. A correspondence with rho values obtained with neutral nitrogen nucleophiles such as imidazole rather than strong anionic nucleophiles such as HO^- and N_3^- is noted. This is also consistent with a transition state with little or no net change in charge from the ground state.

In order to test the internal consistency of this analysis, the acylation by a series of substituted phenyl p-nitrobenzoates was examined. A rho of 1.96 $\pm$ 0.30 was obtained. The sigma minus substituent parameter was chosen for this correlation because any negative charge developed on the phenolic oxygen in the transition state will be stabilized by resonance. A better correlation is obtained for the p-CN and p-NO_2 substituents using this procedure in line with the proposition that sigma minus gives better linearity than sigma for many reactions of phenols and phenolic esters

Table 4-1. Rho Values for the Reactions of Nucleophiles with Substituted p-Nitrophenyl and 2,4-Dinitrophenyl Benzoates.

Nucleophile	Rho (Std Dev)
p-Nitrophenyl Benzoates	
Chymotrypsin	0.97 (0.11)[a]
Imidazole	1.21 (0.07)[b]
NH_3	1.43 (0.02)[c]
$(CH_3)_2NOH$	1.28[d]
OH^-	2.01 (0.01)[e]
N_3^-	1.8[d]
2,4-Dinitrophenyl Benzoates	
Chymotrypsin	1.6 (0.30)[a]
Imidazole	1.73 (0.07)[a]
OH^-	2.20 (0.09)[a]

(a) Reference 38.
(b) Reference 32.
(c) Reference 88.
(d) Reference 89.
(e) Reference 63.

(51). The sigma minus for p-NO_2 is used as 1.0 as suggested by Bruice (90).

The rho values obtained for the acylation of chymotrypsin by substituted phenyl p-nitrobenzoates and phenyl acetates are shown in Table 4-2 along with the rho values for the reactions of various nucleophiles with these esters. It can be seen that the rho value for the phenyl p-nitrobenzoates from this study more closely resembles those obtained for neutral nitrogen nucleophiles rather than strong anionic oxygen species. The extent to which the strength of an anionic oxygen nucleophile, as measured by its basicity, will affect the resulting rho obtained by variation of the leaving group of phenolic esters will be discussed during the analysis of the Brønstead relationship.

A composite Hammett plot for the acylation by substituted phenyl acetates, including rate constants from this study and normalized rate constants from the study of Bender and Nakamura (67), yield a rho value of 2.05 ± 0.32. Again, this value appears to be consistent with neutral nitrogen being the nucleophile, but in light of solvent deuterium isotope effects of approximately two for acylation by p-nitrophenyl acetate (92) and p-nitrophenyl thiolacetate, the mechanism of acylation by phenyl acetates is unclear and will be discussed below.

Table 4-2. Rho Values for Reactions of Various Nucleophiles with Substituted Phenyl p-Nitrobenzoates and Substituted Phenyl Acetates.

Nucleophile	Rho (Std Dev)
Phenyl p-Nitrobenzoates	
Chymotrypsin	1.96 (0.30)[a]
OH^-	1.14 (0.06)[b]
NH_3	2.6[c]
Phenyl Acetates	
Chymotrypsin	2.05 (0.32)[a,d]
OH^-	0.8[e]
$(HOCH_2)_3CH_2O^-$	0.98[f]
Imidazole	1.8[g]
NH_3	2.1[g]

(a) This study.

(b) Reference 63.

(c) Reference 88. Based upon only two esters, p-nitrophenyl p-nitrobenzoate and p-chlorophenyl p-nitrobenzoate. Therefore, represents only an approximate values.

Table 4-2. References continued.

(d) Reference 67.

(e) Reference 98.

(f) Reference 91.

(g) Reference 90.

In order to provide insight into the significance of the rho obtained for non-specific phenyl p-nitrobenzoates it is of interest to compare this value with those obtained for substituted phenyl esters of specific substrates. It is generally accepted that specific ester substrates acylate the enzyme by general-base catalyzed activation of the Ser 195 hydroxyl group, which in turn acts as the primary nucleophile. Such leaving group studies have been carried out for the acylation by substituted phenyl hippurates (40,85), phenyl N-methanesulfonyl-L-phenylalaninates (40), and phenyl N-benzoyloxycarbonyl-L-tryptophanates (85). The results of these studies are summarized in Table 4-3. The rho values for these series are significantly less than that obtained for the non-specific phenyl p-nitrobenzoates. This difference could signal a change in mechanism of acylation by the two types of esters in which the specific esters acylate by general-base catalysis while the phenyl p-nitrobenzoates are subject to nucleophilic attack by the imidazole of His 57.

A summary of the Hammett relationships for the acylation of chymotrypsin by a variety of ester substrates is also of interest. In this study and a previous one (38) Hammett rho values for the variation of substituents on the acyl and phenolic portions of substi-

Table 4-3. Rho Values for the Acylation Chymotrypsin by Substituted Phenyl Esters of Specific Ester Substrates.

Ester Series	Rho
Hippurates	0.63[a]
Hippurates	0.5[b]
N-Mesyl-L-Phenylalaninates	0.45[b]
N-Cbz-L-Tryptophanates	0.46[a]

(a) Reference 85.

(b) Reference 40.

tuted phenyl benzoates were determined to be 0.97 and 1.96 respectively. These rho values are internally consistent in approximating model reactions of neutral nitrogen nucleophiles rather than strong anionic nucleophiles. Leaving group substituent effects for three series of specific phenyl esters yield rhos of less than one, which are similar to values obtained for oxygen nucleophiles such as hydroxide ion. Recently the acylation by a series of acyl substituted aliphatic p-nitrophenyl esters was examined (41), and substituent effects upon the second-order acylation rate constants were analyzed using the Taft-Ingold relationship. Contributions of polar, steric, and specific effects were separated and a rho*, representing the polar contribution of the substituents, was found to be 2.18 $\pm$ 0.17, a value consistent with nucleophilic attack by the activated serine. Assuming that aliphatic p-nitrophenyl esters acylate by the conventional general-base mechanism, an assumption at least partially borne out by solvent deuterium isotope effect studies and the rho* for acyl substituents, a consistent picture of two different mechanisms for acylation becomes apparent. The rho values for the acyl and leaving group portions of the two types of esters, phenyl benzoates and those which evidence indicates undergo general-base catalyzed acylation (specific phenyl and aliphatic p-nitrophenyl

esters), are complementary. The supporting data for this conclusion are presented in Table 4-4.

Brønsted Analysis of Acylation. It has been determined that for a series of structurally similar nucleophiles the strength or efficacy of the nucleophile can be correlated with its base strength as manifested in its pK_a by conventional Brønsted analysis (93,94,95). Such a Brønsted analysis can also be applied to structurally similar leaving groups in nucleophilic displacement reactions (95,96). A detailed analysis of the reactivity of nucleophilic reagents toward a series of acetate esters with structurally similar leaving groups with pK_a values in the range 2 to 10 has been carried out (96). It is possible to divide the nucleophiles into two classes, anionic and neutral nitrogen, and to make mechanistic distinctions between the two by relating the pK_a of the nucleophile to the Brønsted slope (β_{lg}) derived by plotting the logarithm of the second-order nucleophilic rate constant versus the pK_a of the leaving group.

Let us begin by examining the mechanism of nucleophilic ester heterolysis by anionic nucleophiles. A general scheme for this reaction is provided in Equation 4-2.

Table 4-4. A Comparison of the Rho Values for the Acylation of Chymotrypsin by Phenyl Benzoates, Specific Phenyl Esters, and Aliphatic p-Nitrophenyl Esters.

Ester Series	Rho Acyl	Rho Leaving Group
Phenyl Benzoates	0.97[a]	1.96[c]
Specific Phenyl Esters	----	0.45 - 0.63[d,e]
Aliphatic p-Nitrophenyl Esters	2.18[b]	----

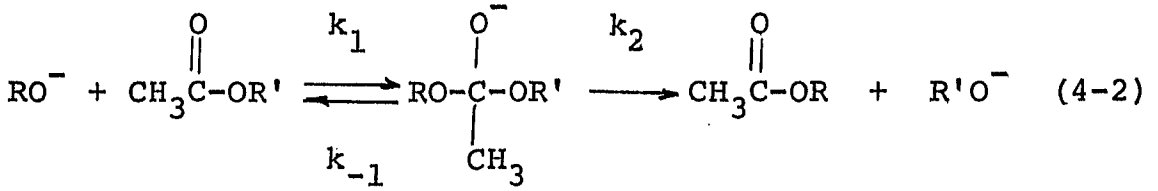
(a) Reference 38.

(b) Reference 41.

(c) This study.

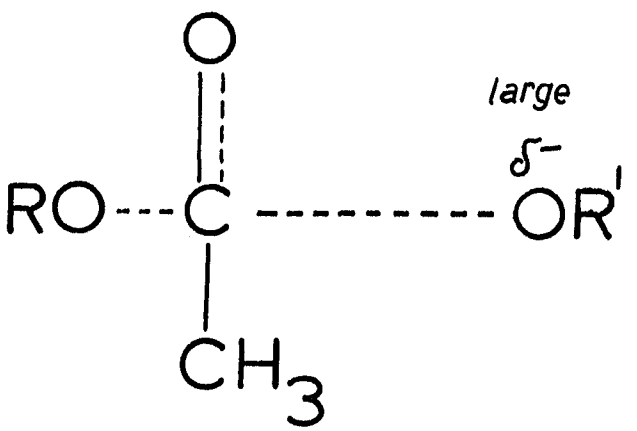
(d) Reference 85

(e) Reference 40.



(4-2)

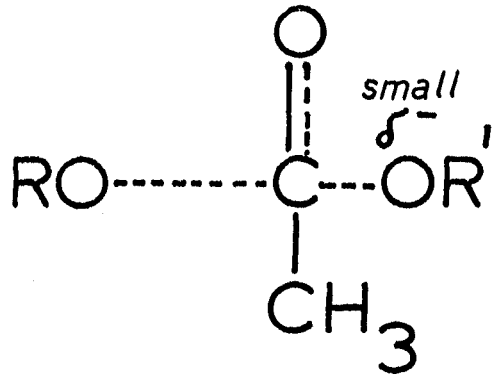
If it is assumed that the pK_a of RO^- is less than the pK_a of $R'O^-$, or RO^- is less basic than $R'O^-$, the RO^- moiety, by virtue of being better able to bear a negative charge, is the better leaving group. The breakdown of the tetrahedral intermediate will be rate determining, and by the Hammond postulate (97), the transition state will resemble IV. There will be a large degree of bond formation by the nucleophile, and consequently, significant bond breaking by the leaving group. The rate of the reaction would be very sensitive



IV

to the basicity of the nucleophile (large β) and it would be expected that the rate would also be very sensitive to electron withdrawing substituents on the leaving group (large $-\beta_{lg}$ or rho).

On the other hand, if the attacking nucleophile, RO^-, is more basic than the leaving group, $R'O^-$, k_1 would become rate determining, and the transition state (V) would resemble attack on the ester. Since little charge is lost from the nucleophile or gained by the leaving group in the transition state, the rate would be expected to exhibit little sensitivity to the basicity of either group.



V

Such a difference in transition states should be reflected in the Brønsted plots of the logarithms of the rate constants versus the pK_a of the leaving group. Using the data of Jencks and Gilchrist (96) the difference can

be graphically displayed. By constructing Brønsted plots for the reactions of phenyl acetate, p-nitrophenyl acetate, and 2,4-dinitrophenyl acetate with anionic nucleophiles of greater and lesser basicity than the leaving groups (pK_4 4 - 10), numerical values of β_{lg} can be obtained (Figures 4-2 and 4-3). The results are summarized in Table 4-5.

The larger absolute values of β_{lg} for nucleophiles with pK_a values lower than those of the displaced leaving groups (β_{lg} ~ -0.7) are consistent with significant charge development on the phenolic oxygen and rate determining expulsion of the leaving group, as predicted from transition state IV. The low absolute values of β_{lg} (~ 0.2 - 0.3) are consistent with transition state V. Since the magnitude of β_{lg} will parallel rho for leaving group variation, the difference between the rho values obtained for the alkaline hydrolysis of phenyl acetates, 0.8 (98), and the intramolecular nucleophilic heterolysis of phenyl glutarates, 2.9 (90), can rationally be explained. In the case of the alkaline hydrolysis the nucleophile is more basic than the phenolic leaving groups; therefore the rate will not be sensitive to electron withdrawing substituents; hence a small rho. For the phenyl glutarates the nucleophilic moiety, the glutarate anion (pK_a ~ 5), will be less basic than the

Table 4-5. β_{lg} Values for the Reactions of Anionic Nucleophiles with Phenyl Acetate Esters.[a]

Nucleophile	pK_a	Nucleophile	β_{lg}
CH_3O^-	15.5		-0.28
$HC\ CCH_2O^-$	13.55		-0.30
$CF_3CH_2O^-$	12.37		-0.31
AcO^-	4.61		-0.55
N_3^-	4.0		-0.63
F^-	3.1		-0.69

(a) Data abstracted from reference 96.

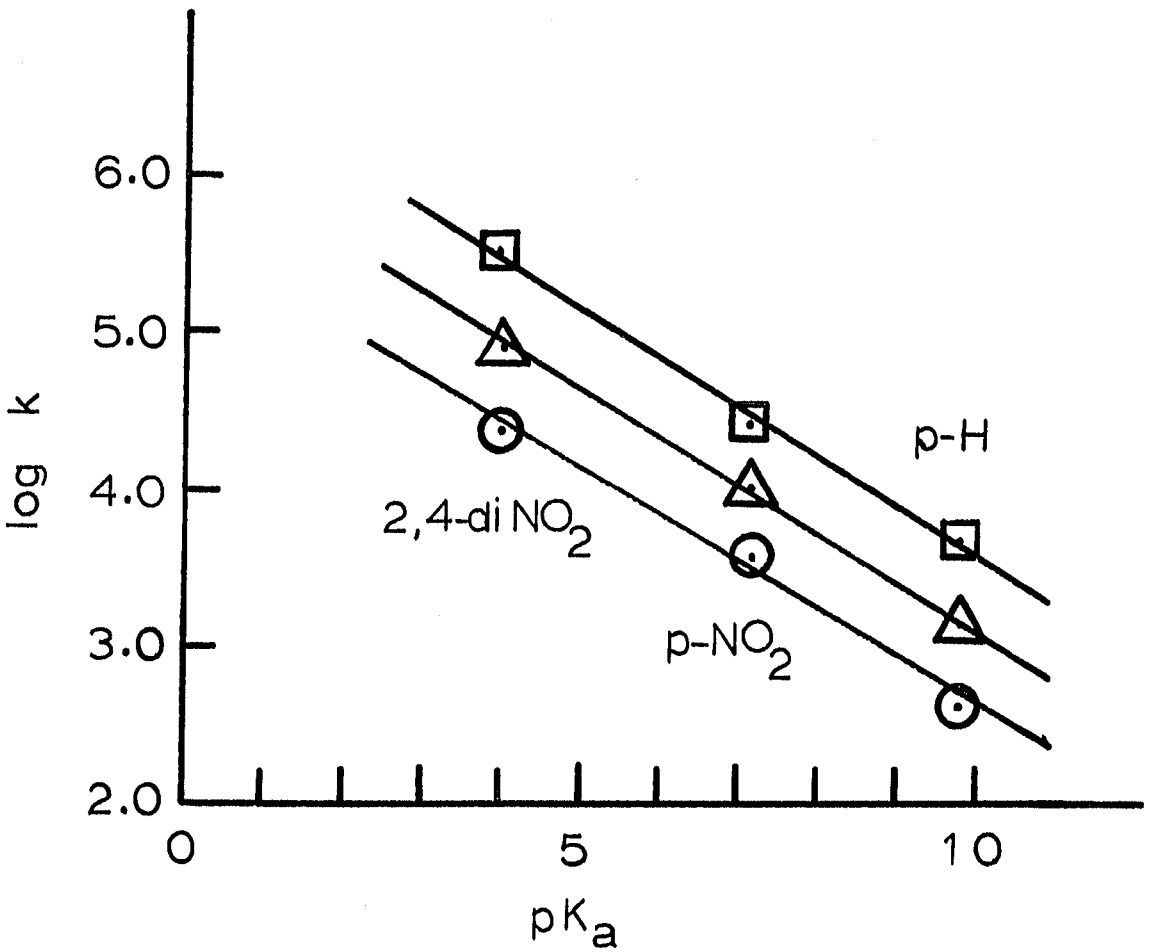


Figure 4-2. Brønsted Plots for Nucleophilic Heterolysis of Substituted Phenyl Acetates by Nucleophiles of Greater Basicity than the Phenolic Leaving Groups. Data Abstracted from Reference 96. Nucleophiles: ⊙, $CF_3CH_2O^-$; △, HC C - CH_2O^-; ⊡, CH_3O^-.

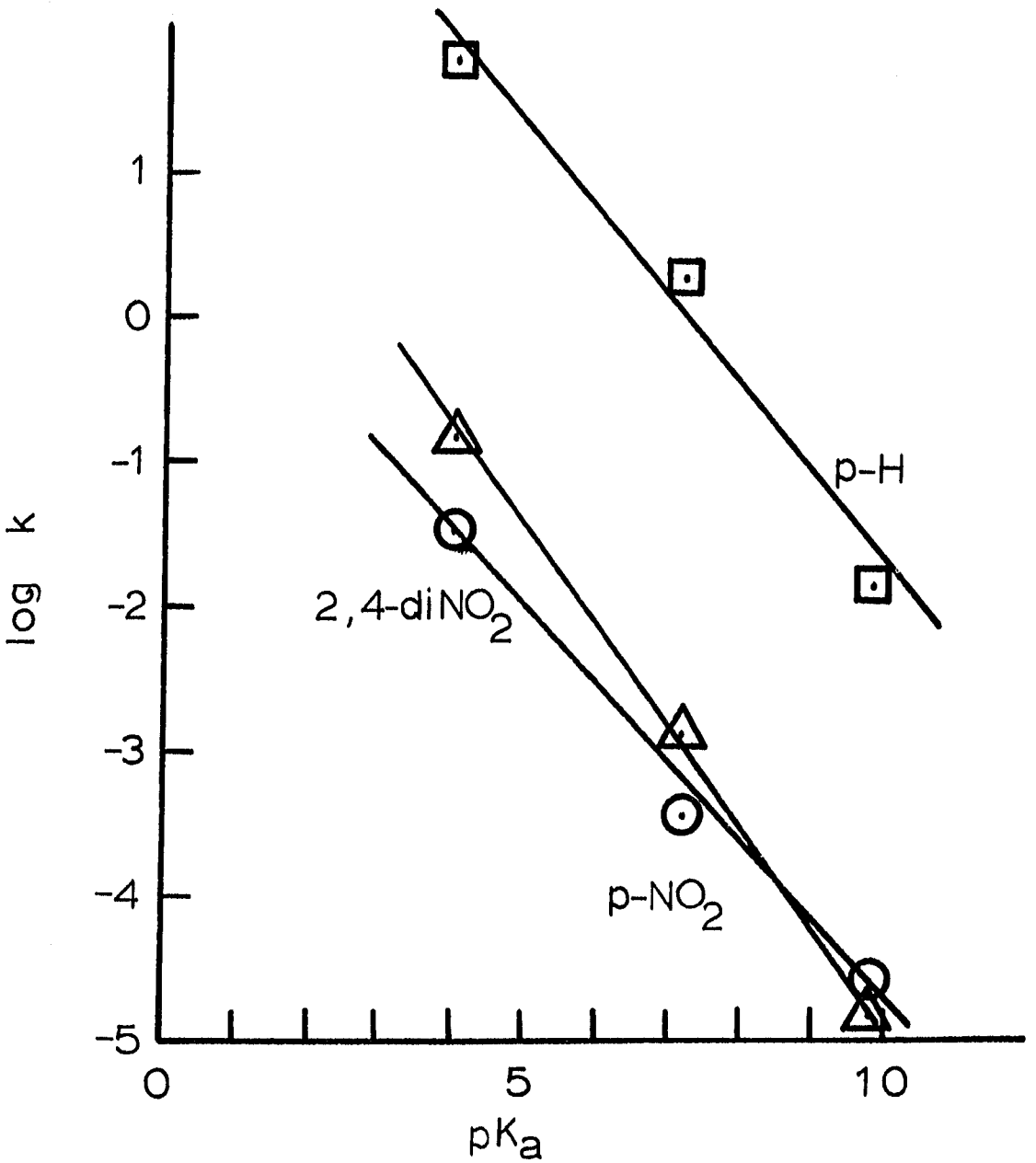


Figure 4-3. Brønsted Plots for the Nucleophilic Heterolysis of Substituted Phenyl Acetates by Nucleophiles More Acidic than the Phenolic Leaving Groups. Data Abstracted from Reference 96. Nucleophiles: ⊙, AcO^-; △, F^-; ⊡, N_3^-.

monosubstituted phenolic leaving groups, resulting in great sensitivity to electron withdrawing substituents and a large rho. Thus it is not necessary to invoke intramolecularity of the reaction to rationalize the large rho obtained as was done by Bender and Nakamura (67).

Jencks found that neutral nitrogen nucleophiles of similar structure gave linear plots of log k versus the pK_a of the nucleophile over a large range of pK_a values (96). The resulting β was approximately 0.8, indicating significant bond formation in the transition state. An implication of this is a build up of negative charge on the oxygen of the leaving group, and hence a large negative β_{lg} for leaving group variation. Values for β_{lg} of approximately -0.7 to -1.0 are observed for neutral nitrogen nucleophiles over a large range of basicity. Of interest is a β_{lg} of -0.6 for the reaction of imidazole with three substituted phenyl acetates. Again, this observation is consistant with the observed rho for the nucleophilic reaction of imidazole with phenyl acetates of 1.8 (90).

An analysis of the relationship of the rate of acylation of chymotrypsin by a series of phenyl p-nitrobenzoates with the pK_a values of the phenolic leaving groups can now give an indication of the character

of the nucleophilic group in the active site. Imidazole and serine would be predicted to yield two different β_{lg} values for a series of phenyl esters. From estimates based on model compounds, the pK_a of the serine hydroxyl would be expected to be approximately 13, significantly more basic than the phenolic leaving groups, and would be expected to yield a β_{lg} in the range of -0.2 to -0.3. The imidazole of His 57, being a neutral nitrogen nucleophile, would be expected to yield a β_{lg} in the range of -0.7 to -1.0. The observed β_{lg} for acylation by substituted phenyl p-nitrobenzoates, -0.78, is approximately what would be expected for nucleophilic attack by imidazole.

Deuterium Solvent Isotope Effects. Supporting evidence for a mechanism involving a transient acylimidazole species can be found in the examination of deuterium solvent isotope effects upon the acylation by phenyl benzoates. Previously isotope effects determined from the pH(pD) independent acylation rate constants of 1.07 and 1.64 were found for the acylation by p-nitrophenyl p-trifluoromethylbenzoate and 2,4-dinitrophenyl benzoate respectively (38). Present work extended this study to examine the isotope effects upon the acylation by p-nitrophenyl p-nitrobenzoate and p-nitrophenyl benzoate, with the resulting values being 1.04 and 1.30

respectively (Table 3-21). A transition state with rate determining proton transfer, such as general-base activation of the serine hydroxyl, would be expected to show isotope effects of greater than two (61). Isotope effects of this magnitude are observed for the deacylation of acylchymotrypsins derived from specific (48) and non-specific (32,99) ester substrates. It is generally agreed that deacylation proceeds by a general-base mechanism. Nucleophilic attack by imidazole would require no such proton transfer in the transition state and deuterium solvent isotope effects of approximately one would be predicted.

It is also noteworthy that two point Hammett rhos determined for p-nitrophenyl p-nitrobenzoate and p-nitrophenyl benzoate in H_2O and D_2O are 1.04 and 1.00 respectively. Both of these values are comparable to the rho value for p-nitrophenyl benzoates of 0.97 found previously (38), and are consistent with a mechanism involving nucleophilic attack by imidazole.

An approximate solvent deuterium isotope effect of 1.6 was found for the acylation by the specific ester substrate p-nitrophenyl hippurate. It is expected that this ester acylates by general-base catalyzed activation of serine, therefore an isotope effect of approximately 2 would be predicted. The value of 1.6 is approximate,

based upon an abbreviated pH-rate profile, and could easily indicate general-base catalysis. A more thorough study was carried out with p-nitrophenyl thiolacetate which, according to Frankfater and Kézdy (70), acylates by the same mechanism as p-nitrophenyl acetate. A deuterium isotope effect of 2.10 was found which is consistent with general-base activation of Ser 195.

The pK_1 values obtained from the pH-rate profiles for the acylation by p-nitrophenyl p-nitrobenzoate and p-nitrophenyl thiolacetate of 6.80 and 6.87 respectively (see Table 3-21) were found to be in close agreement with pK_1 values found previously for specific (80) and nonspecific ester substrates (38,48). Since the acylation pK_1 is virtually substrate independent it is valid to compare the acylation rate constants for a series of structurally similar substrates at a constant pH to generate a structure-reactivity correlation.

Unequivocal analysis of solvent isotope effects for enzyme reactions is difficult at best (60,87), because of solvent effects on conformational structure, pre-equilibrium proton transfers, and medium effects. Therefore, solvent isotope effects of approximately one are consistent with, but not unequivocal proof of, the intermediacy of a transient acylimidazole.

The Acylation of Chymotrypsin in the Presence of Added Methanol. If the acylation of the enzyme is rapid compared to deacylation, which is the case for ester substrates (16), acylation by a p-nitrophenyl ester should exhibit an initial liberation of one mole of p-nitrophenol for each mole of enzyme. Assuming that the mechanism of acylation proceeds through an acylimidazole intermediate an added nucleophile such as methanol might compete with the Ser 195 for the acyl group bonded to the imidazole. A consequence of such a competition would be an increased concentration of liberated p-nitrophenol upon acylation. The acylation of chymotrypsin by p-nitrophenyl p-nitrobenzoate was studied in pH 8.00 buffers, one 20% v/v CH_3CN, which would not act as a competing nucleophile, and the other 20% v/v CH_3OH, which could act as a competing nucleophile. From the results outlined in Table 3-22 it is clear that the added methanol caused an increase in burst magnitude. The less than theoretical burst obtained for 20% v/v CH_3CN can be explained by lack of saturation of the enzyme due to the low concentration of substrate used. Constraints were placed upon the substrate and enzyme concentrations because of the limited solubility of the substrate and the detection

limits of the spectrophotometer. Nevertheless a rapid liberation of p-nitrophenolate followed by a slow steady-state was observed in 20% v/v CH_3CN.

The continuous burst observed with the 20% v/v added CH_3OH could signal competition between the methanol and Ser 195 for the acylimidazole, but an examination of previous studies indicates the most likely explanation to be an enhanced rate of deacylation of the serine acylated enzyme caused by the added methanol. Bender found that the rate of deacylation of trans-cinnamoylchymotrypsin in 6.18 M CH_3OH was enhanced approximately 10 fold over the absence of methanol (100). Since 20% v/v CH_3OH corresponds to approximately 6.25 M CH_3OH, it can be assumed that the deacylation of p-nitrobenzoylchymotrypsin will be enhanced approximately 10 fold. Caplow and Jencks (32) determined k_3 for deacylation of p-nitrobenzoylchymotrypsin at pH 7.07 to be 2.17×10^{-2} min^{-1}. Assuming this value to be approximately 60% of the value of k_3 at pH 8.0 (38) and a 10 fold rate enhancement in 20% v/v CH_3OH, a half-life of approximately 2 minutes would result. Such rapid deacylation of p-nitrobenzoylchymotrypsin would account for the observation of an apparent continuous burst of p-nitrophenolate.

In retrospect it is not surprising that no

competition was found. The proximity of the serine hydroxyl to the acylimidazole could give an effective concentration of 55 M (101) for serine which is much greater than the actual concentration of CH_3OH. Also any additional catalytic effects at the active site would increase the effective concentration disparity. In addition, it has been suggested, based on model building of the imidazole acylated active site of another serine protease, subtilisin, that the reactive moieties are effectively shielded from the solvent (39). If this observation can be extrapolated to the active site of chymotrypsin, the intervention of a methanol molecule to compete with serine becomes unlikely. Therefore, the apparent increased magnitude of the burst of p-nitrophenolate in 20% CH_3OH is most readily explained by enhanced rate of deacylation of the serine acylated enzyme caused by the participation of the added methanol.

Imidazole to Serine Acyl Transfer. A basic implication of an acylation mechanism involving rate determining nucleophilic attack by imidazole is a subsequent rapid acyl transfer from the imidazole of His 57 to the Ser 195. It has been suggested such a facile transfer is very unlikely because the substitution on the imidazole destroys its ability to activate the

serine hydroxyl and there are no solvent molecules in the vicinity to accept the serine proton in the event of a transfer (39).

Chymotrypsin containing 3-methyl His 57 has been studied by x-ray crystallography (102), and it was demonstrated that the methylated nitrogen remained hydrogen bonded to the Ser 195. It is possible to speculate that an acylated imidazole would also remain hydrogen bonded to Ser 195, and therefore, provide the driving force for a rapid intramolecular transacylation. It is not necessary to invoke proton transfer to a solvent molecule or an unidentified enzyme residue. A concerted transfer of the proton to the N-3 of the imidazole will provide a receptor for the proton while at the same time facilitating the acyl transfer by providing a partial positive charge on N-3 stabilizing the transition state for acyl transfer. The postulated transition state is shown in Figure 4-4.

Such a direct four-center switching mechanism will also be favored if spatial juxtaposition brought the serine oxygen within bonding distance of the carbonyl carbon of the acyl group, which seems a reasonable assumption considering the compact nature of the active site.

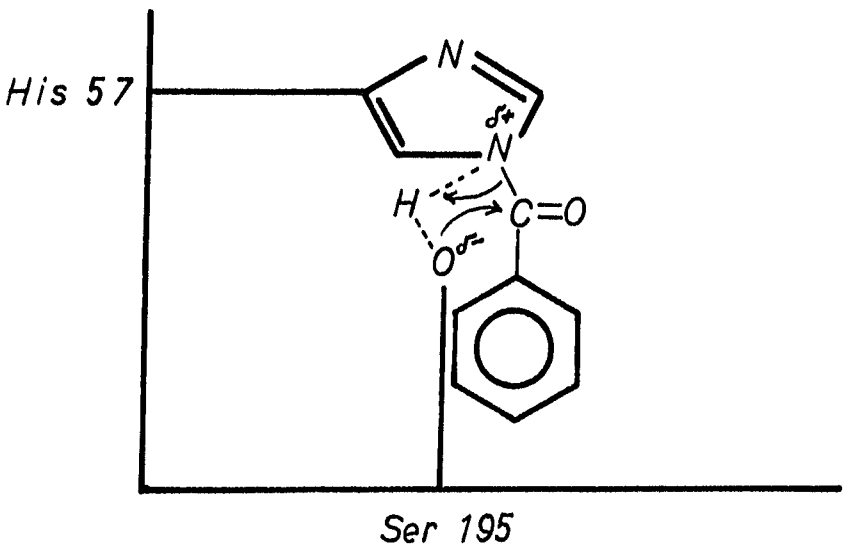


Figure 4-4. Postulated Transition State for the Imidazole to Serine Acyl Transfer.

Significance of a Perturbed pK_a of Ser 195. The proposed "charge-relay" system at the active site of chymotrypsin, consisting of Asp 102, His 57, and Ser 195, calls for significant negative charge to be developed on the serine oxygen near neutral pH (31). Studies with competitive inhibitors have shown that an ionizing group results in the active site of the enzyme developing a net negative charge above pH 7.3 (103). One of the implications of these two pieces of information is the possibility that the apparent pK_a of Ser 195 is perturbed from near 13 for the free amino acid to approximately 7. Calculations on the active site of chymotrypsin have predicted a serine pK_a of approximately 8 (104), and there is precedent for large perturbations of the pK_a of amino acid residues in enzymes (105,106). It is informative to examine the consequences such a perturbation would have on the analysis of the structure-reactivity and other correlations.

An operational pK_a of approximately 7 would render the Ser 195 moiety a better leaving group than the substituted phenoxide ions in the transition state on the conventional general-base catalyzed path to the serine acylated enzyme for phenyl p-nitrobenzoate and phenyl acetate substrates. The transition state would then have significant charge developed on the phenolic

oxygen resulting in a great degree of sensitivity to the basicity of the leaving group or a large β_{lg} (see the discussion of Brønsted correlations). This situation would also be reflected in a large positive rho since sigma minus substituent constants for phenols parallel their relative basicities.

A relatively low pK_a for serine can provide an attractive explanation for the observed leaving group rhos and β_{lg} values for the phenyl p-nitrobenzoates and phenyl acetates without having to invoke an acylimidazole intermediate, but it leaves unexplained the rho of 0.97 found for variation in the acyl portion of a series of p-nitrophenyl benzoates. Nucleophilic attack by ionized serine would lead to a transition state in which a negative charge is developed adjacent to the reaction center and a rho of approximately 2 would be expected (38). Also, the solvent deuterium isotope effects of approximately one observed for p-nitrophenyl benzoates could conceivably be rationalized by a pre-equilibrium proton transfer, but such a pre-transition state transfer would not be consistent with the observed solvent isotope effects of approximately two for the acylation by p-nitrophenyl acetate and p-nitrophenyl thiolacetate, and also call into question the identical pH dependence for all observed acylation reactions.

The low rhos observed for specific phenyl esters could be rationalized as the result of electrophilic catalysis (40) occurring only in the catalytic hydrolysis of specific ester substrates due to orientational specificity.

It is of note that recent studies of isotope effects in mixed H_2O and D_2O solvents have called into question the existence of a coupled proton relay in the catalytic mechanism of chymotrypsin (107).

The above alternate explanation of the magnitudes of the leaving group rho and β_{lg} values for two series of non-specific ester substrates entails speculation and some contradiction, but until a more detailed and direct determination of the pK_a values of the active site components is made it cannot be ignored.

A Comparison of the Rate Enhancements of the Acylation of Chymotrypsin over Nucleophilic Heterolysis for the Ester Substrates p-Nitrophenyl Benzoate and p-Nitrophenyl Acetate. The operational second-order rate constant for acylation, k_2/K_s, determined in this and a previous study (38) is a composite of the true first-order acylation rate constant, k_2, and the dissociation constant for the Michaelis complex, K_s. If K_s could be estimated with some certainty, k_2

could be calculated directly. A comparison of the computed k_2 with the second order rate constants for the nucleophilic heterolysis of the same ester substrates will give an indication of the enzymatic rate enhancement over nucleophilic attack. The acylation reactions of p-nitrophenyl benzoate and p-nitrophenyl acetate along with their nucleophilic heterolysis reactions with imidazole and hydroxide ion were chosen for such a comparison. The values of k_2/K_s (pH independent), k_{Im}, and k_{OH} are collected in Table 4-6.

Using assumptions outlined previously the dissociation constant for substituted phenyl esters can be estimated to be in the range of 10^{-3} to 10^{-2} M (38). The resulting range for k_2 will be 2.2 - 22 sec^{-1}. Using this range as a theoretical k_{obs} the concentration of imidazole necessary to result in a k_{obs} in the above range can be calculated.

$$k_{obs} = k_{Im}(Im)$$

$$2.2 - 22 = 2 \times 10^{-3} \ (Im)$$

$$(Im) = 1100 - 11{,}000 \ M$$

Therefore, a theoretical rate enhancement of 1100 to 11,000 over imidazole is determined. This

Table 4-6. Second-Order Rate Constants for the Reactions of Nucleophiles with p-Nitrophenyl Benzoate and p-Nitrophenyl Acetate at 25° C.

Nucleophile	$k(M^{-1} sec^{-1})$ p-Nitrophenyl Benzoate	$k(M^{-1} sec^{-1})$ p-Nitrophenyl Acetate
Imidazole	0.002[a]	0.58[d]
OH^-	0.79[b]	9.5[d]
Chymotrypsin	2170[c]	1550[e]

(a) Reference 32.

(b) Reference 63.

(c) This study.

(d) Reference 109.

(e) Reference 82.

number differs significantly from the value given earlier (38).

A similar calculation can be made for acylation by p-nitrophenyl acetate. Again assuming $K_s = 10^{-3} - 10^{-2}$, the estimated range of the first-order rate constant for acylation is 1.5 - 15 sec^{-1}. This compares favorably with k_2 values for acylation by p-nitrophenyl acetate in 20% isopropanol, 3.3 sec^{-1} (16), and 4% acetonitrile, 3.7 sec^{-1} (77). The rate enhancement over imidazole, in terms of concentration of imidazole necessary to achieve an observed first-order rate constant in the above range is 2.7 - 27.

If one were to assume that the acylation of the enzyme proceeds by the same mechanism for both esters, nucleophilic attack by the imidazole of His 57, one might expect similar rate enhancements over non-enzymatic nucleophilic imidazole attack. This is seen not to be the case. The acylation by p-nitrophenyl benzoate is enhanced by an approximately 400 fold greater extent that p-nitrophenyl acetate. One possible conclusion to be drawn is that the acylation by these two esters proceeds by two different mechanisms depending upon the mode of binding of the substrate to the enzyme.

A comparison of the theoretical rate enhancements over nucleophilic attack by hydroxide ion will be of use

if it is assumed that the hydroxyl group of Ser 195 is activated to the same extent in the acylation by both esters. The absolute magnitude of each rate enhancement will have no physical significance because the oxygen of the serine will only have a partial negative charge, but the relative values for the two esters might indicate if serine can be the nucleophile in both cases. For p-nitrophenyl acetate, using $k_2 = 3\ sec^{-1}$, a theoretical rate enhancement of 0.3 can be estimated. If it is assumed that p-nitrophenyl benzoate binds at least as well as p-nitrophenyl acetate, $k_2 \geq 2.2\ sec^{-1}$, and a theoretical rate enhancement of approximately 3 is found. Because of uncertainties about the true binding constant, K_s, these two enhancements are within reasonable approximation of one another.

While comparison of rate enhancements of nucleophilic attack by imidazole tentatively support the previously presented evidence for two different mechanisms for acylation, the results obtained for hydroxide ion, if assumptions about the degree of activation of Ser 195 and the similarity of binding constants are accepted, present conflicting evidence in favor of similar mechanisms.

The Effect of Dioxane Inhibition upon the Acylation of Chymotrypsin by some Nonspecific p-Nitrophenyl Esters. Kinetic studies of acylation by nonspecific p-nitrophenyl ester substrates have shown that the rate of acylation, expressed as the complex rate constant k_2/K_s, is maximal for compounds having an acyl side chain length of approximately 10 A° (41), presumably as a consequence of a combination of enhanced hydrophobic binding in the "tosyl hole", which would be expressed in K_s, and favorable juxtaposition of the reactive carbonyl group of the ester and the Ser 195 and His 57 moieties of the enzyme active site, which would be expressed in k_2. X-ray studies have determined the "tosyl hole" to be 10 - 12 A° in depth (37), which would be consistent with the above description of most efficient binding. Therefore, assuming a general-base mechanism for acylation, an optimal ester substrate will have its side chain bound to the enzyme in the "tosyl hole" with the ester carbonyl aligned for attack by the activated hydroxyl of Ser 195.

It has been suggested in this study that phenyl benzoates may bind to the active site in a unique manner which renders the imidazole of His 57 the primary nucleophile upon acylation. Examination of a model of the active site of chymotrypsin has demonstrated that if

either the acyl or leaving group portion of phenyl benzoate esters were placed in the "tosyl hole" the imidazole of His 57 was too far removed from the ester carbonyl for nucleophilic attack (108). If this is the case it is possible that the benzoyl moiety does not bind efficiently in the "tosyl hole". The inhibitor dioxane (75), which has been shown to occupy the "tosyl hole", may, therefore, affect the acylation by p-nitrophenyl benzoate to a lesser degree than acylation by a homologous series of esters with longer acyl side chains, such as p-nitrophenyl phenylacetate and p-nitrophenyl hydrocinnamate, which because of increased hydrophobic interactions would bind more strongly in the "tosyl hole". Previous kinetic studies have shown that in this series the p-nitrophenyl hydrocinnamate, with a side chain length of 9.7 A°, acylates most rapidly as a result of efficient hydrophobic binding (41).

The acylation of chymotrypsin by p-nitrophenyl acetate, p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, and p-nitrophenyl hydrocinnamate was studied using 5% v/v acetonitrile and 5% v/v dioxane as cosolvents at pH 8.00, which is in the pH independent region of the pH-rate profile (38,81)(Table 3-23). The acylation rate constants for p-nitrophenyl phenyl-

acetate and p-nitrophenyl hydrocinnamate in 5% v/v acetonitrile, 5.46×10^4 M^{-1} sec^{-1} and 4.98×10^5 M^{-1} sec^{-1} respectively, compare favorably with the pH independent rate constants for the same two esters determined previously in 4.5% v/v acetonitrile, 5.6×10^4 M^{-1} sec^{-1} and 5.0×10^5 M^{-1} sec^{-1} respectively (41).

Acylation rate constants for the esters p-nitrophenyl p-methylbenzoate and p-nitrophenyl p-ethylbenzoate have been determined at pH 7.0 and are 2.03×10^3 M^{-1} sec^{-1} and 9.33×10^3 M^{-1} sec^{-1} respectively. Values of k_2/K_s at pH 8.0 can be approximated by multiplying the values determined at pH 7.0 by 1.6 (a factor determined from the pH-rate profile of several p-nitrophenyl benzoates), and are 3.2×10^3 M^{-1} sec^{-1} and 14.9×10^3 M^{-1} sec^{-1} respectively. The acyl side chain lengths of each of the two pairs of esters, p-nitrophenyl phenylacetate and p-nitrophenyl p-methylbenzoate, and p-nitrophenyl hydrocinnamate and p-nitrophenyl p-ethylbenzoate, would be approximately the same. But it can be seen that acylation by the two esters with terminal phenyl groups are accelerated by factors of 16 and 33 respectively over the acylation by the p-methylbenzoate and the p-ethylbenzoate. Therefore, it is likely that either the terminal phenyl groups are more effective for hydrophobic binding or the benzoates exhibit a different mode of binding.

The ratios of second-order acylation rate constants for the esters p-nitrophenyl acetate, p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, and p-nitrophenyl hydrocinnamate, in acetonitrile and dioxane cosolvents, presented in Table 3-23, are 2.15, 1.39, 2.89, and 2.52, respectively. It can be seen that the inhibition ratio for p-nitrophenyl benzoate is considerably less than those for the other three esters. This difference may indeed speak for a different mode of binding for p-nitrophenyl benzoate, with less dependence upon binding in the "tosyl hole".

In an attempt to rationalize the magnitude of the depression of the acylation rate constant in dioxane cosolvent for p-nitrophenyl hydrocinnamate, which was chosen because of its hydrophobic acyl side chain, the following assumptions were made.

1) The hydrophobic side chain p-nitrophenyl hydrocinnamate is bound in the "tosyl hole" during acylation.

2) Any inhibition by dioxane is the result of competition with the ester side chain for the "tosyl hole".

3) Acetonitrile does not compete for the "tosyl hole".

The inhibition constant, K_1, for dioxane has

been found to be 0.32 M at pH 7.8 (110). From the known concentration of dioxane and enzyme the concentrations of enzyme inhibitor complex and free enzyme were calculated as 3.20×10^{-5} M and 1.84×10^{-5} M respectively. Therefore, in 5% v/v dioxane the effective concentration of enzyme was 1.84×10^{-5} M. Since it was assumed that acetonitrile does not bind in the "tosyl hole", the effective concentration of enzyme in 5% v/v acetonitrile would be equal to the initial enzyme concentration determined spectrophotometrically, 5.18×10^{-5} M. From the values of effective enzyme concentration and the first-order k_{obs} values for the acylation by p-nitrophenyl hydrocinnamate in acetonitrile and dioxane cosolvents (25.8 sec^{-1} and 9.98 sec^{-1} respectively) second-order acylation rate constants were determined from the equation

$$k_2/K_s = \frac{k_{obs}}{[E]_{effective}}$$

Values of 4.98×10^5 M^{-1} sec^{-1} and 5.42×10^5 M^{-1} sec^{-1} were calculated for the acylation reactions in acetonitrile and dioxane cosolvents respectively assuming the absence of competitive inhibition by dioxane. The similarity of these two rate constants can be used to rationalize the ratio of acylation rate

constants found in the acetonitrile and dioxane co-solvents.

Another approximate treatment can be used which attempts to take into account any common medium effects caused by the two cosolvents. The K_i for acetonitrile has been reported to be 0.83 at pH 7.8 (110). Assuming that inhibition by acetonitrile is nonspecific and that dioxane exhibits the same nonspecific inhibition along with specific competitive inhibition of the "tosyl hole", a set of rate constants normalized for the absence of this nonspecific inhibition can be calculated using the acetonitrile inhibition constant. The theoretical concentrations of free enzyme using K_i = 0.83 for both the acetonitrile and dioxane systems are 2.38×10^{-5} M and 3.01×10^{-5} M respectively. Therefore, calculated theoretical second-order rate constants for no inhibition in each case are 10.8×10^5 M^{-1} sec^{-1} and 3.3×10^5 M^{-1} sec^{-1} respectively. A theoretical k_{obs} for the dioxane system in the absence of nonspecific inhibition can be calculated to be 15.2 sec^{-1} from the following equation

$$k_{obs} = [E_o] \times k_2/K_s$$

Now, using the dioxane K_i and assuming specific competitive inhibition by dioxane, a theoretical k_2/K_s in the absence of such inhibition was calculated to be 8.3×10^5 M^{-1} sec^{-1}. This assumption is only approximate because some medium effects would be included in the K_i for dioxane. Nevertheless a rough correspondence is seen between the two theoretical rate constants calculated for the absence of cosolvent inhibition, 10.8×10^5 M^{-1} sec^{-1} and 8.3×10^5 M^{-1} sec^{-1} for the acetonitrile and dioxane cases respectively.

Both sets of calculations are fraught with assumptions and speculation, but both present a consistent picture of specific competitive inhibition by dioxane and rationalize the differences in rate constants determined in the presence of the two cosolvents. It must be pointed out that since both dioxane and acetonitrile are components of the solvent system medium effects such as the dielectric and differences in solvation and viscosity must be considered although they are not readily quantifiable. Also, recently Bell (75) has reported a K_i for dioxane of 10 M for the acylation by p-nitrophenyl acetate, which is significantly different from the value reported by Bender (110) and would alter the results of the above interpretation. Since Bell did not report a K_i for acetonitrile this differen-

ce in reported values cannot be evaluated.

It is of note that a similar ratio of acetonitrile and dioxane cosolvent rate constants for acylation by p-nitrophenyl p-nitrobenzoate was found to be 2.89 at pH 8.0. This value is significantly different from the value obtained for p-nitrophenyl benzoate and similar to the value predicted on the basis of relative inhibition constants. This discrepancy is not readily explicable, but it is unlikely to be the result of steric variations since both esters fall on a Hammett rho line in both cosolvents for the same series of esters. Therefore, the low inhibition ratio found for p-nitrophenyl benzoate cannot be used as unequivocal proof of a unique binding mode for phenyl benzoates.

Solvent Deuterium Isotope Effects Upon the Acylation of Chymotrypsin by a Homologous Series of p-Nitrophenyl Esters in Acetonitrile and Dioxane Cosolvents. The efficiency of binding to the chymotrypsin active site in the "tosyl hole" increases in the series p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, and p-nitrophenyl hydrocinnamate (41). In order to detect a possible change in acylation mechanism over this series the solvent deuterium isotope effects upon acylation were determined with 5% v/v acetonitrile cosolvent. Isotope effects of 1.30, 1.3, and 2.0 (Ta-

ble 3-21) were found for p-nitrophenyl benzoate, p-nitrophenyl phenyl acetate, and p-nitrophenyl hydrocinnamate respectively. An isotope effect of approximately 2 for p-nitrophenyl hydrocinnamate is consistent with the hydrocinnamoyl side chain being optimally bound in the "tosyl hole" presumably orienting the ester carbonyl for attack by the activated hydroxyl of Ser 195. The low isotope effect for acylation by p-nitrophenyl benzoate has previously been attributed to direct nucleophilic attack by the imidazole of His 57 to give an acylimidazole intermediate. These two extremes in isotope effect would seem to be consistent with unique binding of the benzoate leading to initial acylation at His 57 and strong specific binding of the hydrocinnamate firmly in the "tosyl hole" leading to acylation of the serine though a general-base activation mechanism. The isotope effect of 1.3 for acylation by p-nitrophenyl phenylacetate would appear to be consistent with an acylimidazole intermediate, but there is no other supporting evidence for this speculation.

Approximate solvent deuterium isotope effects for these three esters were determined using 5% v/v dioxane as the cosolvent. Acylation rate constants were determined at pH 8.00 in H_2O and pD 8.40 in D_2O. Since these pH and pD values are in the pH independent region of the pH-rate profile for acylation (100)

approximate isotope effects can be determined from the resulting rate constants. Isotope effects of 1.7, 1.7, and 2.0 were observed for p-nitrophenyl benzoate, p-nitrophenyl phenylacetate, and p-nitrophenyl hydrocinnamate respectively. The isotope effects for the benzoate and phenylacetate increased while that for the hydrocinnamate remained constant. It is possible to speculate that for phenyl benzoates there is a multiplicity of potential acylation mechanisms with one involving rate-determining proton transfer becoming predominant in the dioxane inhibited system. This interpretation will be discussed below along with structure-reactivity data obtained with dioxane as the cosolvent.

In conclusion the variation of the solvent deuterium isotope effect upon acylation with the length of the ester side chain and consequent "tosyl hole" binding in acetonitrile cosolvent is consistent with a variation of acylation mechanism with the binding mode in this series of substrates.

The Effect of Dioxane upon Structure-Reactivity Correlations for the Acylation of Chymotrypsin by Phenyl Benzoates. The study of the effect of the variation of substituents on the acyl portion of a series of p-nitrophenyl benzoates upon the rate of acylation was carried out using 5% v/v dioxane as the

cosolvent. A rho for acyl group variation of 0.60 was determined (Figure 3-12). A previous study carried out in 5% v/v acetonitrile yielded a rho of 0.97 for the same series of substrates (38). The magnitudes of both rho values are consistent with a transition state in which there is no net change in charge, which could indicate the nucleophilicity of the imidazole of His 57. The fact there is a difference between the rho values in acetonitrile and dioxane is of interest in light of the difference in the isotope effects for p-nitrophenyl benzoate in acetonitrile and dioxane co-solvents. In dioxane the isotope effect increases to approximately 1.7 from 1.30 in acetonitrile. This may signal the increasing prominence of a mechanism involving a proton transfer. It is possible to speculate that this proton transfer is the result of electrophilic assistance by an unknown chymotrypsin moiety at the carbonyl oxygen of the ester (40). A combination of mechanisms for the acylation by phenyl benzoates with an increasing contribution of one which exhibits electrophilic catalysis would be consistent with the observed increase in isotope effect and decrease in acyl group rho in dioxane cosolvent. Nucleophilicity of imidazole could be invoked with or without electrophilic catalysis.

The acylation rate-constant for p-chlorophenyl p-nitrobenzoate in 5% v/v dioxane at pH 7.6 is 167 M^{-1} sec^{-1}. Multiplying the acylation rate constant for p-nitrophenyl p-nitrobenzoate at pH 8.0 (5% v/v dioxane) by 0.93 (a factor determined from the pH-rate profile for the acylation of chymotrypsin by p-nitrophenyl p-nitrobenzoate in 5% v/v acetonitrile) yields an approximate rate constant of 4200 M^{-1} sec^{-1} for acylation at pH 7.6. From these two values an approximate leaving group rho value for acylation by phenyl p-nitrobenzoates in 5% v/v dioxane is found to be 1.8. This value remains consistent with the intermediacy of an acylimidazole species. In order to accommodate this value with electrophilic assistance at the carbonyl a large degree of leaving group bond breaking must occur in the transition state, which is the case for nucleophilic heterolysis of esters by neutral nitrogen nucleophiles (96).

The observed magnitudes of the rho values obtained for the acylation by phenyl benzoates in 5% v/v dioxane remain consistent with the acylimidazole hypothesis. The participation of electrophilic assistance at the carbonyl oxygen does not alter this conclusion but pending more concrete evidence such participation remains speculative.

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PART II.

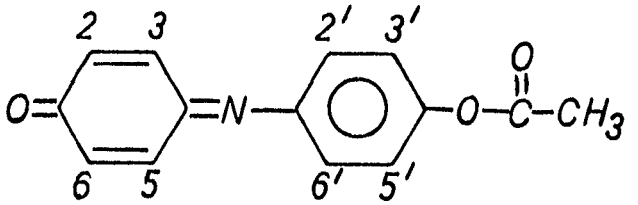
THE RAPID HETEROLYSIS OF INDOPHENYL ACETATE BY α_1-ACID GLYCOPROTEIN IN THE COMMERCIAL PREPARATION OF HORSE SERUM CHOLINESTERASE

INTRODUCTION

Cholinesterases are a class of enzymes which possess catalytic specificity in the hydrolysis of esters of the quaternary aminoalcohol, choline. There are at least two distinct varieties of the enzyme, acetylcholinesterase, which has greatest specificity toward acetylcholine, and butyrylcholinesterase or serum cholinesterase, which exhibits greatest activity toward butyrylcholine (1). Both enzymes are also characterized by their sensitivity to inhibition by organophosphates, carbamates, and quaternary ammonium salts (1,2,3). While acetylcholinesterase has been implicated in the mechanism of the transmission of neural impulses (4,5), the physiological function of serum cholinesterases remains unclear. It is possible to speculate that the function of serum cholinesterase is to control the metabolism of choline esters.

Indophenyl acetate (IPA) and its halogenated derivatives were found to be useful monitors of cholinesterase activity because of the intense color of the phenolic products of hydrolysis (6). Acetylcholinesterase was found to hydrolyze only IPA whereas horse serum cholinesterase exhibited less specificity by hydrolyzing the ring chlorinated and brominated derivatives also. The rates of horse serum cholinesterase

catalyzed hydrolyses of 2,6-dibromo, 2,6-dichloro, and unsubstituted indophenyl acetates paralleled their alkaline hydrolysis rates, but the steric effects of the 3',5'-dihalo substituents outweighed their polar effects and consequently these acetates were hydrolyzed by the enzyme less rapidly than IPA (6).



Subsequently it was reported that acetylcholinesterase, which had been inhibited in activity toward acetylcholine by the N,N-dimethyl-2-phenylaziridinium ion, exhibited increased activity with IPA (7). This behavior was rationalized by the hypothetical existence of a multiplicity of active sites on the enzyme. One was thought to be blocked or allosterically deactivated by aziridinium binding while another, presumably the site at which IPA was hydrolyzed, was activated. O'Brien also found that acetylcholinesterase as well as serum cholinesterase hydrolyzed halogenated indophenyl acetates in contradiction to the earlier reports (8).

Studies have confirmed the enhanced rate of hydrolysis of 2,6-dichloroindophenyl acetate over IPA

by horse serum cholinesterase, and have determined the optimal pH for the reaction with both substrates to be 8.0. The steady-state hydrolyses of these two esters were also found to obey Michaelis-Menten kinetics (9).

These earlier, sometimes contradictory studies prompted our interest in the catalytic mechanism of the reaction of horse serum cholinesterase with indophenyl acetate. An acylenzyme intermediate has been proposed in the mechanism of cholinesterase catalysis (10), and an attempt to detect the formation of this intermediate using IPA as a substrate was an initial objective of this study.

In preliminary studies in this laboratory the reaction of IPA with the commercial preparation of horse serum cholinesterase was examined in a stopped-flow spectrophotometer. A biphasic liberation of indophenolate anion was observed, which consisted of a rapid first-order step followed by a much slower steady-state (11). This observed biphasic reaction was consistent with the proposed general mechanistic scheme for the cholinesterase catalyzed cleavage of esters as outlined in Equation 1-1.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}S \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P \qquad (1\text{-}1)$$

E-S is the Michaelis complex and ES' is the acylated enzyme (10,12,13). The implications of this mechanism are clear; if deacylation of the ES' species is the rate-determining step in the turnover, then under suitable conditions a biphasic release of product should obtain. While IPA is a non-specific substrate of cholinesterases, the examination of the presteady-state behavior upon hydrolysis might provide an insight into the mechanism of this physiologically important class of enzymes.

The horse serum cholinesterase used in this study was prepared by a modified Strelitz procedure (14) based upon fractionation with ammonium sulphate. Subsequent studies have led to the isolation of the pure enzyme indicating that the Strelitz preparation was quite impure, and the molecular weight per active site was estimated to be in the range of 120,000 - 141,000 (15,16).

The validity of the mechanism outlined in Equation 1-1 was tested by the examination of the enzyme and substrate concentration dependence of the first-order rate constant for the presteady-state liberation of indophenol. In order to assess the hypothesis of a multiplicity of active sites on the enzyme the effects of several specific cholinesterase inhibitors on the presteady-state behavior with IPA was examined.

The implications of the impurity of the commercial preparation of horse serum cholinesterase cannot be overlooked. If the impurities are simply inactive protein material the mechanistic conclusions drawn from the kinetic studies would not be altered. On the other hand, if it can be demonstrated that these impurities exhibit esterase activity toward IPA it would become desirable to isolate and identify these components. This characterization process would require separation of the commercial preparation into its various constituents and testing each for esterolytic activity toward IPA. Various analytical techniques could then be employed in an attempt to identify and characterize any esteratic components.

EXPERIMENTAL SECTION

Materials and Equipment. Horse serum cholinesterase was obtained from Nutritional Biochemical Corporation in partially purified form with an indicated activity of 4 units/mg based upon the hydrolysis of acetylcholine. Horse serum albumin (Cohn Fraction V) and horse serum glycoprotein (Cohn Fraction VI) were also obtained from Nutritional Biochemical Corporation. Human serum glycoprotein was purchased from Miles Laboratories. Eserine, butyrylthiocholine iodide, and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Company, and Sephadex G-200 was obtained from Pharmacia Fine Chemicals. Paraoxon (diethyl-p-nitrophenyl phosphate) was from Chemical Services, Westchester, Pa. Phosphate buffers were freshly prepared from glass distilled water and reagent grade chemicals. Methanol was spectrograde and ethanol was commercially anhydrous.

Indophenyl acetate (IPA) was prepared according to the method of Kramer and Gamson (17) and was recrystallized several times from ether-petroleum ether, mp 117-118° (lit. mp 115-118°). p-Nitrophenyl acetate (pNPA) was prepared from p-nitrophenol and acetic

anhydride and was recrystallized twice from ethanol, mp 78-79° (lit. mp 79.5-80°) (18). o-Nitrophenyl dimethyl carbamate (oNPDMC) was prepared by the method of Bender (18), mp 56.7-57.5° (lit. mp 56.7-57°).

Spectrophotometric determinations of activity with IPA and pNPA were carried out in either a Durrum-Gibson stopped-flow spectrophotometer or a Cary 14 recording spectrophotometer. The Cary 14 was also used for spectrophotometric assays of the gel filtration separations of the commercial horse serum cholinesterase preparations.

All computerized data analyses were carried out using the University of New Hampshire's IBM 360 Model 50 computer.

Kinetics of the Hydrolysis of Indophenyl Acetate by the Commercial Preparation of Horse Serum Cholinesterase. The reaction of indophenyl acetate (IPA) with the commercial preparation of horse serum cholinesterase (BuChE) was monitored in the Gibson-Durrum stopped-flow spectrophotometer by following the appearance of the indophenolate anion at 675 nm, a wavelength at which there is no IPA absorbance. The visible spectra of IPA and indophenolate anion are given in Figure 2-1. Beer's law was confirmed for the indophenolate anion in 0.05 M NaOH (3% methanol). The molar absorptivity of the indophenolate species at 675 nm was found to be $1.81 \pm 0.02 \times 10^4$ M^{-1} cm^{-1}.

Figure 2-1. The Visible Spectra of IPA and Indophenolate Anion.

—·—·—· 1.15×10^{-4} M IPA, pH 6.0, 0.1 M phosphate; 3% v/v methanol.

——— 1.45×10^{-5} M Indophenolate Anion, 0.05 M NaOH, 3% v/v methanol.

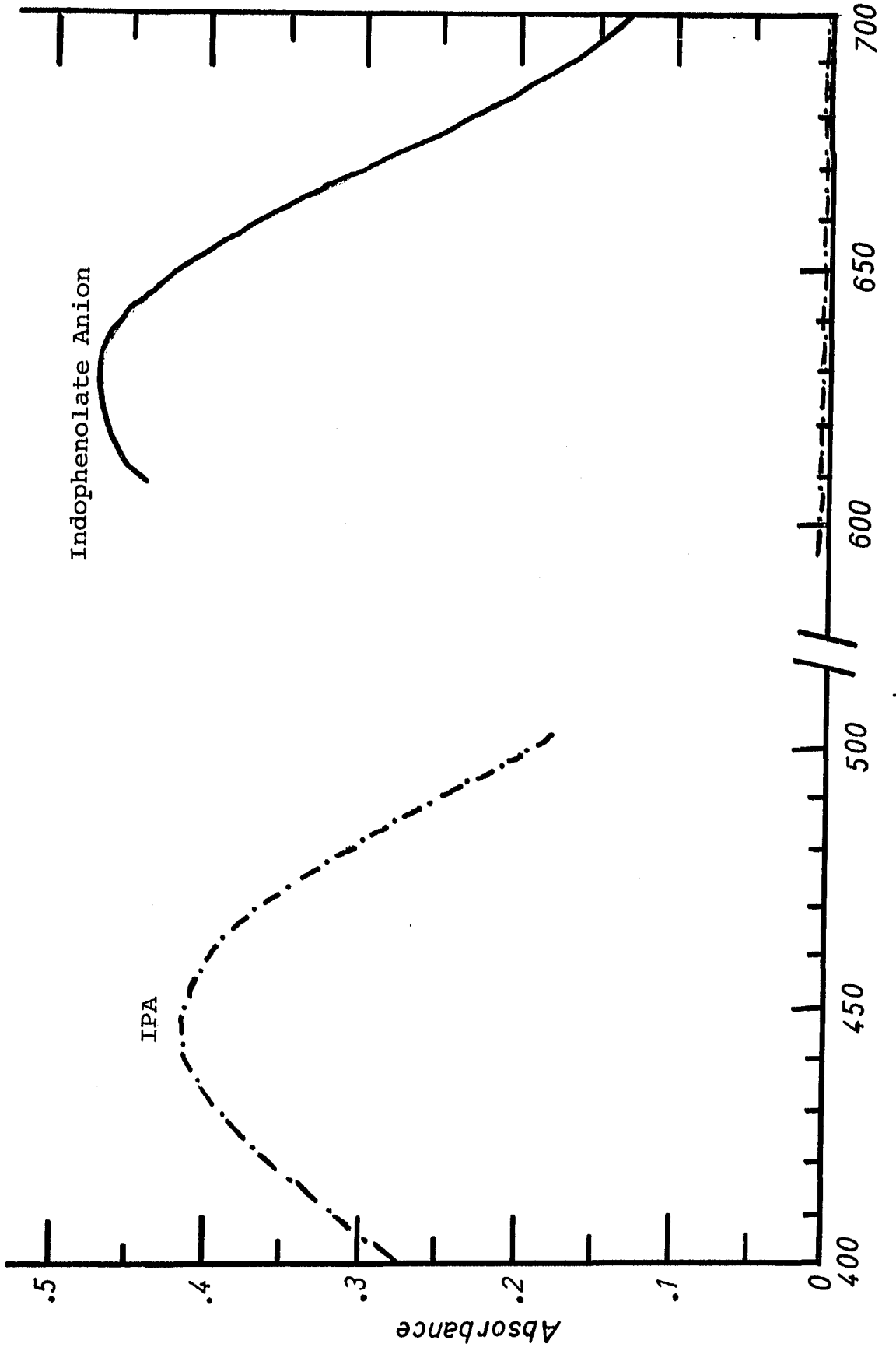


Figure 2-1

A typical run was carried out in the following manner. A stock solution of IPA was prepared in methanol or ethanol. Substrate solutions were prepared by adding aliquots of the stock solution to the appropriate phosphate buffer with the alcohol concentrations adjusted to 3% v/v in order to facilitate solution of the organic substrate. These solutions were prepared immediately prior to each run to minimize hydrolysis of the substrate by the phosphate buffer. Enzyme solutions were prepared by dissolving precisely weighed amounts of the commercial preparation of BuChE in the buffer also 3% v/v alcohol. Alcohol concentrations in the substrate and enzyme solutions were made equal in order to minimize undesirable post-mixing perturbations in the cuvette of the stopped-flow instrument. Enzyme concentrations were given in terms of mg/ml because the molar concentrations were unknown.

An absolute infinity for the transient step for a given series of runs was determined on the 0-100% transmittance scale. An infinity line for each burst was obtained by drawing a tangent to the steady-state portion of the curve at the magnification used. Measurements from the photographs of the oscilloscope traces were converted to absorbance units, and the data tested for first-order kinetics by plotting the logarithm of $A_\infty - A_t$ versus time, where A_∞ and A_t are the absorbance values at infinite time and time $\underline{t}$ respect-

ively. Straight lines were obtained in each case for at least three half-lives of the reaction Program TRDATA was used to perform these operations.

The Kinetics of the Transient Phase of IPA Hydrolysis by the Commercial Preparation of BuChE in the Presence of Inhibitors and Competitive Substrates. The kinetics of the initial burst of indophenolate anion upon reaction of IPA with commercial BuChE were determined as a function of added inhibitors and competitive substrates. Two approaches were followed. 1) The enzyme solution was incubated with an inhibitor and then reacted with IPA; and 2) the IPA solution was prepared with the addition of an inhibitor or competitive substrate and then reacted with the uninhibited BuChE preparation. All runs were carried out in phosphate buffers with methanol cosolvent. The reactions were monitored in the stopped-flow spectrophotometer following the appearance of indophenolate anion at 675 nm.

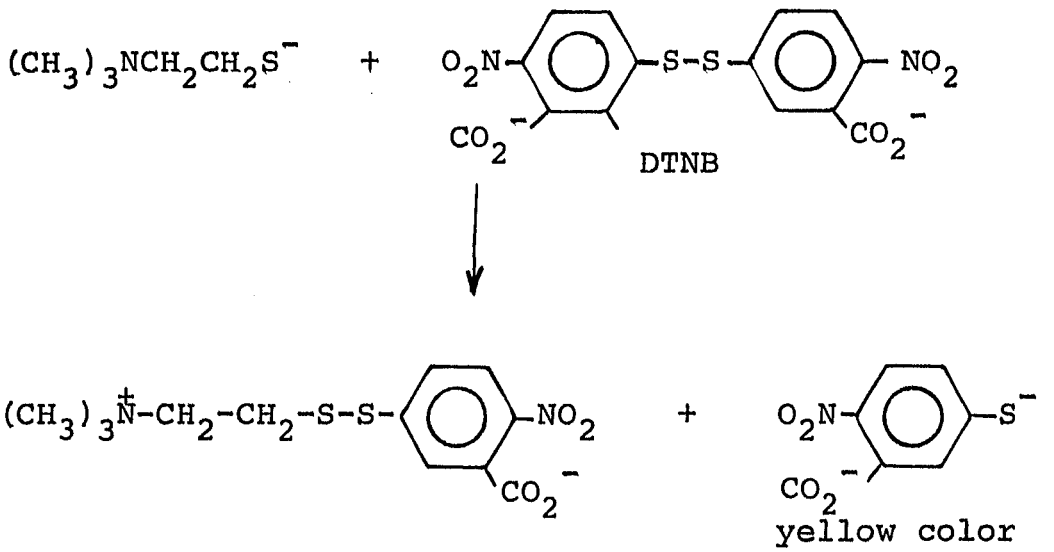
Fractionation of the Commercial Preparation of BuChE on Sephadex G-200. Sephadex is a chromatographic material capable of separating substances according to molecular size. Molecules larger than the pores of the swollen Sephadex pass directly through the gel without interference, whereas smaller molecules are absorbed by the gel particles to varying degrees depending on size and shape. The first step of a published purification of BuChE entailed the Sephadex G-200 fraction-

ation of the Strelitz preparation (15); therefore, this purification step was undertaken.

A typical fractionation is outlined below. A 55 X 2.5 cm column of Sephadex G-200 was prepared and equilibrated with pH 7.00 buffer (0.02 M phosphate; 0.02% NaN_3) for three days. 250 mg of Commercial BuChE in 3.0 ml of buffer was applied to the top of the column and was eluted with the equilibrating buffer at the rate of 6 ml/hour. Fractions of 2.3 ml were collected at room temperature (15).

The collected fractions were assayed for protein spectrophotometrically by determination of the absorbance at 280 nm in the Cary 14 spectrophotometer. The fractions were also assayed for cholinesterase activity with butyryl thiocholine iodide (BuSCh) and Ellman's indicator, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (19). The fractions were also assayed for steady-state activity with IPA.

Determination of Cholinesterase Activity with BuSCh. Cholinesterase hydrolyzes butyrylthiocholine to yield thiocholine and butyric acid. Thiocholine reacts with DTNB to yield a mixed disulfide with a deep yellow color. (Scheme I) Use has been made of this reaction for a colorimetric assay of cholinesterase (19). A modification of this method was used to monitor the fractions obtained from the gel filtration of commercial BuChE for cholinesterase activity.



Scheme I

The cholinesterase activity will be a function of the initial rate of appearance of thiocholine as measured by DTNB indicator. Stock solutions of DTNB (20 mg/ml) and BuSCh (10 mg/ml) were prepared in methanol. Substrate solutions were prepared in 10 ml volumes by the addition of 0.10 ml DTNB, 0.10 ml BuSCh, and 0.10 ml methanol to a 10 ml volumetric flask and filling with pH 8.0 buffer (0.002 M phosphate). The resulting concentrations of reagents were 5.05×10^{-4} M DTNB and 3.15×10^{-4} M BuSCh.

Into each of the two cuvettes 2.0 ml of substrate solution were pipetted. One cuvette was placed in the reference compartment. To the other 5 µl of the protein fraction was added and the reaction was monitored at

405 nm. The initial rates were determined as the total absorbance change per minute (Table 2-1).

Determination of Steady-State Activity with IPA. Substrate solutions of IPA were prepared in pH 8.0 buffer (0.02 M phosphate, 5% v/v methanol). The concentration of substrate was 1.03×10^{-4} M. Two ml of substrate were pipeted into each of two cuvettes and the spectrophotometer was zeroed at 675 nm. 50 µl of the protein fraction from the gel filtration added to the reaction cuvette and the rate of appearance of indophenolate anion was monitored at 675 nm. The initial rates were recorded as the total absorbance change per minute (Table 2-2).

The above determinations were carried out for the separation outlined above. A composite graph of the fraction number versus A_{280}, initial rate with BuSCH, and initial rate with IPA is presented in Figure 2-2.

Monitoring for Burst Activity in the Fractions of the Sephadex Separation of Commercial BuChE. The magnitude of the absorbance change at 675 nm monitored in the Cary 14 upon the reaction of IPA with the pooled "burst active" fractions was used as a criterion for burst activity. The magnitude of the burst was recorded under the following conditions:

Table 2-1. Results of the Butyrylthiocholine Assay of the Commercial BuChE Fractionation.

Fraction No.	ΔA/ Min 405 nm
30	0.014
35	0.324
39	0.254
40	0.178
42	0.172
44	0.154
46	0.078
48	0.048
50	0.014
52	0.012
54	0
56	0
60	0

Table 2-2. Results of the IPA Assay of the Commercial BuChE Fractionation.

Fraction No.	ΔA/ Min 675 nm
35	0.079
37	0.067
39	0.052
42	0.035
45	0.025
48	0.010
50	0
52	0
59	0

Figure 2-2. Sephadex Separation III

○ , Absorbance at 280 nm.

X , Cholinesterase Activity Determined from the Hydrolysis of BuSCh using DTNB Indicator (ΔA/min).

△ , Steady-State Activity with IPA (ΔA/min).

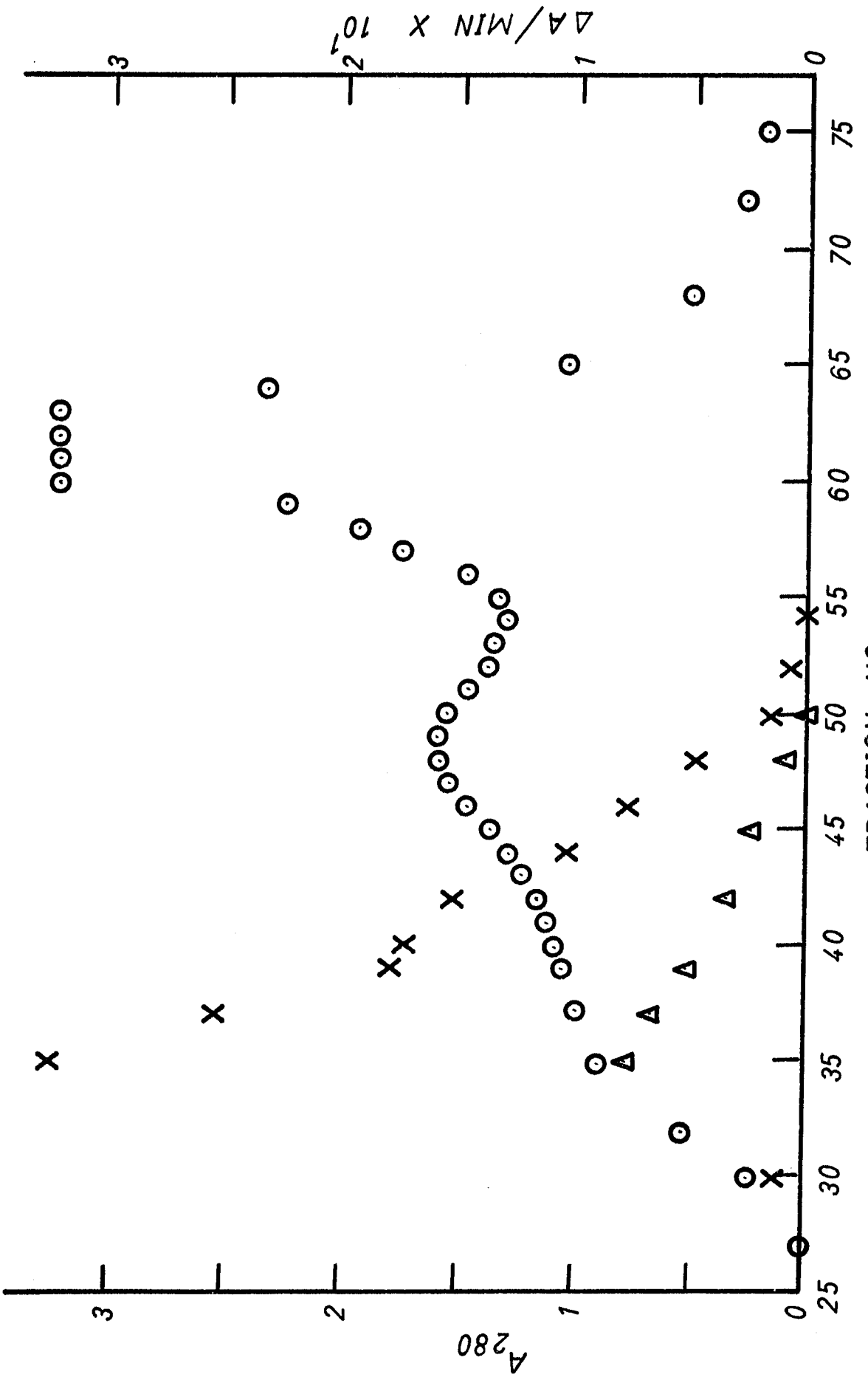


Figure 2-1

1) Pooled active fraction + IPA

2) Eserine inhibited pooled active fraction + IPA

3) Heat treated pooled active fraction + IPA

4) Pooled active fraction 8 M in urea + IPA

5) Pooled active fraction + pNPA

A typical determination was made by pipeting 2.0 ml of IPA (pNPA) solution in pH 7.8 (0.02 M phosphate, 3% v/v methanol) buffer into each of two cuvettes. To the reference cuvette 0.25 ml of an appropriately treated (e.g. eserine inhibited) non-protein fraction from the gel filtration was added. These early fractions were buffer solution which had been equilibrated on the column and collected in the first five fractions of the eluant before protein material was collected. 0.25 ml of the appropriately treated pooled burst active fraction is added to the reaction cuvette and the net absorbance at 675 (400) nm is recorded versus time. Extrapolation of the curve to zero time gives the magnitude of the burst as ΔA.

A sample of horse serum albumin (Cohn Fraction V) was also tested in this manner for burst activity. A solution of 4 mg/ml of the preparation was prepared in phosphate buffer (0.02 M), pH 7.8. This yielded a

solution with an absorbance at 280 nm approximating that of the pooled active fractions. 2.0 ml of 1.77 x 10^{-4} M IPA was added to each cuvette, to the reference cuvette was added 0.25 ml buffer, and to the reaction cuvette was added 0.25 ml of the albumin solution. The release of indophenolate anion was followed at 675 nm.

The Kinetics of the Reaction of the Pooled Burst Active Fractions with IPA. The kinetics of the reaction of the burst active component of the fractionation of commercial BuChE with IPA were studied using the Durrum-Gibson stopped-flow spectrophotometer. The substrate solutions were prepared from stock solutions of IPA in methanol. The enzyme solutions were prepared by adding a given volume of the pooled active fractions to buffer and adjusting the alcohol concentration. The concentration of enzyme is given as ml of pooled active fractions in 10 ml. The reactions were monitored at 675 nm and the rate constants determined as outlined above.

The Effect of Diethyl-p-Nitrophenyl Phosphate (Paraoxon) upon Burst Activity with IPA and pNPA. 3.0 ml of pooled burst active fractions of a gel filtration of commercial BuChE was inhibited with 1.0 x 10^{-5} M eserine to eliminate any residual cholinesterase activity. The protein solution was then separated into two 1.50 ml portions with one being made 1.85 x 10^{-5} M in paraoxon.

Substrate solutions were prepared in 0.067 M phosphate buffer (pH 7.6, 3% v/v methanol). The IPA and pNPA concentrations were 2.49 x 10^{-4} M and 3.31 x 10^{-4} M respectively. In order to study the effect of paraoxon upon the burst of the phenolate moiety the bursts were monitored first with paraoxon-free protein solution and then with the paraoxon treated solution.

A typical experiment was carried out in the following manner. 2.0 ml of substrate was added to each of two cuvettes and the spectrophotometer was zeroed at the appropriate wavelength (400 nm for pNPA and 675 nm for IPA). To the reference cuvette was added 0.2 ml of inactive early fraction from the gel filtration, and to the reaction cuvette was added 0.2 ml of the protein solution. The release of phenolate was monitored as a function of time. The magnitude of the burst was used as a criterion for the effect of paraoxon on the activity of the pooled burst active fractions.

In order to determine if paraoxon was cleaved by the protein, blanks were run with buffer in place of substrate in order to detect any residual absorbance at 400 nm. Cleavage of the paraoxon would have resulted in the liberation of p-nitrophenol which would have been detected by its absorbance at 400 nm.

It should be noted that extreme caution should

be exercised when working with paraoxon because of its toxicity due to its potent anticholinesterase activity.

Analysis of the Burst Active Fractions of the Gel Filtration of Commercial BuChE. The pooled burst active fractions of a gel filtration of BuChE were dialyzed against distilled deionized water (2 x 1 l). A sample was analyzed by polyacrylamide gel electrophoresis for homogeneity (3.5% polyacrylamide, pH 9.5) (20).

The remaining dialyzed sample was lyophilized and a sample was analyzed for amino acid composition (Beckman Amino Acid Analyzer, Department of Biochemistry, University of New Hampshire). Another sample was analyzed for carbon, hydrogen, and nitrogen content.

3.48 mg of the lyophilized preparation was dissolved in 2.0 ml of water yielding a solution 0.174% in protein. The absorbance at 280 nm was determined. The absorbance of a 1% solution was calculated from this value.

Samples of the lyophilized preparation were also analyzed by polyacrylamide gel electrophoresis (3.5 and 7% polyacrylamide, pH 9.5) (21).

Hydrolysis of IPA by Bovine Serum Glycoprotein (Cohn Fraction VI). The reaction of bovine serum glycoprotein with IPA was followed in the stopped-flow

spectrophotometer by monitoring the release of indophenolate anion at 675 nm. Substrate solutions were prepared immediately prior to each run in pH 8.0 buffer (0.1 M phosphate, 3% v/v methanol). Protein solutions were prepared by dissolving accurately weighed quantities of bovine glycoprotein in the buffer. Protein concentrations were given as mg/ml of glycoprotein. The k_{obs} of the resulting first-order burst was determined as outlined previously using program TRDATA.

RESULTS

The Reaction of Commercial BuChE with IPA.

Preliminary studies of the reaction of the commercial preparation of horse serum cholinesterase (BuChE) with indophenyl acetate (IPA) indicated a biphasic release of indophenolate anion determined spectrophotometrically (Figure 3-1). A rapid first-order liberation followed by a slower steady-state was observed. The dependence of the first-order rate constant, k_{obs}, for the transient phase as a function of initial IPA concentration, $[S_o]$, and initial enzyme concentration, $[E_o]$, was determined. These values are collected in Tables 3-1 through 3-4. k_{obs} was found to be independent of $[S_o]$ over a large range of concentrations and directly dependent upon $[E_o]$.

The total change in absorbance for the burst and k_{obs} for the first-order process increase as the initial enzyme concentration is increased. This relationship is detailed in Table 3-5.

The Reaction of Commercial BuChE with IPA in the Presence of Cholinesterase Inhibitors and Competitive Substrates.

The k_{obs} and the magnitude of the transient phase of the reaction of commercial BuChE with IPA was studied with the enzyme incubated separately

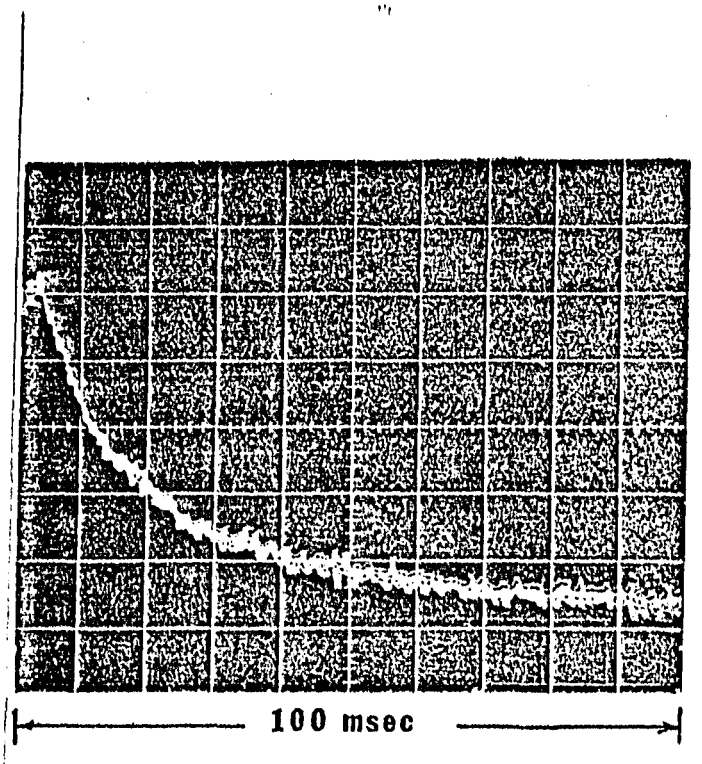


Figure 3-1. Spectrophotometric record at 675 nm of stopped-flow observation after mixing BuChE and indophenyl acetate in 0.02 M phosphate buffer, pH 7.9 (3% v/v methanol). The concentrations upon mixing were 1.0 mg/ml for BuChE and 7.0×10^{-5} M for IPA. The k_{obs} for this run was 88.1 sec^{-1} and was determined in the manner outlined in the text.

TABLE 3-1. Dependence of k_{obs} upon $[S_o]$ for the Transient Phase of the Commercial BuChE Catalyzed Hydrolysis of IPA.[a]

$[S_o] \times 10^5$ M	$[E_o]$ mg/ml	k_{obs}	(STD DEV) sec^{-1}
0.7	0.5	59.6	(2.1)
0.9	0.5	53.9	(7.5)
1.3	0.5	63.4	(6.0)
2.0	0.5	50.9	(6.9)
3.0	0.5	54.1	(4.2)
4.0	0.5	54.7	(5.5)
5.0	0.5	51.1	(3.7)
6.0	0.5	52.3	(4.6)
7.0	0.5	57.3	(2.3)
8.0	0.5	58.5	(3.2)
10.0	0.5	56.8	(6.5)

(a) The reactions were run in 0.05 M phosphate buffer, pH 7.9, 3% v/v ethanol, 25.0° C.

TABLE 3-2. Dependence of k_{obs} upon $[S_o]$ for the Transient Phase of the Commercial BuChE Catalyzed Hydrolysis of IPA.[a]

$[S_o] \times 10^5$ M	$[E_o]$ mg/ml	k_{obs}	(STD DEV) sec^{-1}
3.0	1.0	98.6	(5.4)
7.0	1.0	92.0	(4.3)
10.0	1.0	90.2	(6.2)

(a) The reactions were run in 0.02 M phosphate buffer, pH 7.9, 3% v/v methanol, 25.0° C.

TABLE 3-3. Dependence of k_{obs} upon $[S_o]$ for the Transient Phase of the Commercial BuChE Catalyzed Hydrolysis of IPA.[a]

$[S_o] \times 10^5$ M	$[E_o]$ mg/ml	k_{obs}	(STD DEV) sec^{-1}
5.0	0.5	63.8	(7.2)
10.0	0.5	55.2	(2.4)
20.0	0.5	54.8	(4.6)

(a) The reactions were run in 0.05 M phosphate buffer, pH 7.42, 3% v/v methanol, 25.0° C.

TABLE 3-4. Dependence of k_{obs} upon $[E_o]$ for the Transient Phase of the Commercial BuChE Catalyzed Hydrolysis of IPA.[a]

$[E_o]$ mg/ml	$[S_o] \times 10^5$ M	k_{obs}	(STD DEV) sec^{-1}
0.5	7.0	51.3	(2.1)
1.0	7.0	92.0	(2.6)
2.0	7.0	204	(21)
3.0	7.0	198	(18)
4.0	7.0	335	(39)

(a) The reactions were run in 0.02 M phosphate buffer, pH 7.93, 3% v/v methanol, 25.0° C.

TABLE 3-5. The Dependence of k_{obs} and the Absorbance Change (ΔA) upon $[E_o]$ for the Transient Phase of the Commercial BuChE Catalyzed Hydrolysis of IPA.[a]

$[E_o]$ mg/ml	$[S_o] \times 10^5$ M	k_{obs} (STD DEV) sec^{-1}	ΔA
0.2	7.0	36.2 (8.2)	0.0101
0.4	7.0	56.1 (3.1)	0.0175
0.6	7.0	64.4 (4.6)	0.0276
0.8	7.0	74.0 (5.6)	0.0326
1.0	7.0	92.2 (8.2)	0.0355

(a) The reactions were run in 0.02 M phosphate buffer, pH 7.98, 3% v/v methanol, 25.0° C.

with the potent cholinesterase inhibitors eserine, o-nitrophenyl dimethylcarbamate (oNPDMC), and tetrabutyl ammonium iodide (TBAI). The results are collected in Table 3-6. The k_{obs} and ΔA are seen to be unaffected by the presence of these cholinesterase inhibitors.

Substrate solutions were incubated separately with the inhibitors eserine and oNPDMC and the cholinesterase substrates butyrylthiocholine iodide (BuSCh) and p-nitrophenyl acetate (pNPA). The effect of these added reagents upon k_{obs} was examined and it was found that k_{obs} was unaffected by the presence of inhibitors or competitive cholinesterase substrates. These results are collected in Table 3-7.

The Separation of the Commercial Preparation of Horse Serum Cholinesterase by Sephadex Gel Chromatography. The commercial preparation of BuChE was fractionated on a column of Sephadex G-200 using 0.02 M phosphate buffer (pH 7.0; 0.02% NaN_3) as the eluant. Three different separations were carried out and are designated separations I-III. All three separations were assayed for protein spectrophotometrically by the determination of the absorbance at 280 nm for each fraction. Fraction sizes for separations I and II were 3.5 ml and for separation III 2.3 ml. In each case two major protein components were found. The first and smaller of the two contained the cholinester-

TABLE 3-6. The Effect of Cholinesterase Inhibitors upon k_{obs} and ΔA for the Transient Phase of the Commercial BuChE Catalyzed Hydrolysis of IPA.[a]

Inhibitor	$[I_o] \times 10^4$ M	$[S_o] \times 10^5$ M	$[E_o]$ mg/ml	k_{obs} (STD DEV) sec^{-1}	ΔA
None		7.0	1.0	94.7 (5.0)	0.0359
Eserine	0.5	7.0	1.0	86.0 (2.5)	0.0368
TBAI	5.0	7.0	1.0	90.7 (1.2)	0.0339
oNPDMC	5.0	7.0	1.0	98.5 (5.9)	0.0323

(a) The reactions were run in 0.02 M phosphate buffer, pH 7.9, 3% v/v methanol, 25.0° C

TABLE 3-7. The Effect of Incubation of the Substrate Solution with Cholinesterase Inhibitors and Substrates upon k_{obs} for the Transient Phase of the Commercial BuChE Catalyzed Hydrolysis of IPA.[a]

Added Inhibitor or Substrate	$[I_o] \times 10^5$ M	$[S_o] \times 10^5$	$[E_o]$ mg/ml	k_{obs} (STD DEV) sec^{-1}
None	--	7.0	0.6	60.7 (3.0)
Eserine	7.0	7.0	0.6	62.2 (3.9)
oNPDMC	7.0	7.0	0.6	62.1 (3.0)
BuSCh	7.0	7.0	0.6	63.6 (2.1)
pNPA	7.0	7.0	0.6	56.7 (2.6)

(a) The reactions were run in 0.02 M phosphate buffer, pH 7.9, 3% v/v methanol, 25.0° C.

ase activity as determined by the steady-state activity with BuSCh and IPA. The second and major components contained all of the burst activity with IPA. The fractions containing the maximum protein content in the second component were pooled and used in subsequent studies of the burst active component.

Graphs of the fraction number versus the absorbance at 280 nm for each separation are presented in Figure 2-2, 3-2, and 3-3. Also for separation I the steady-state activity of the various fractions with IPA as measured by the initial rate of release of indophenolate ion at 675 nm is included in Figure 3-2. In Figure 3-3 the activity of the fractions of separation II with BuSCh using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) indicator is shown. Figure 2-2 shows both the steady-state activity with IPA and BuSCh for separation III. The steady-state activity with IPA directly parallels the cholinesterase activity with BuSCh.

Analysis of the Burst Activity of the Pooled Burst Active Fractions (43-48) of Separation I. Aliquots of pooled fractions 43-48 were reacted with IPA and pNPA substrate solutions in the Cary 14 recording spectrophotometer using appropriate blanks. The reactions were monitored at 675 and 400 nm respectively. Reaction of the pooled fractions, both untreated and inhibited with 10^{-4} M eserine, with 1.77×10^{-4} M IPA

Figure 3-2. Sephadex Separation I

○ , Absorbance at 280 nm.

X , Steady-State Activity with IPA (ΔA/min).

Figure 3-3. Sephadex Separation II

○ , Absorbance at 280 nm.

X , Cholinesterase Activity Determined from the Hydrolysis of BuSCh using DTNB Indicator (ΔA/min).

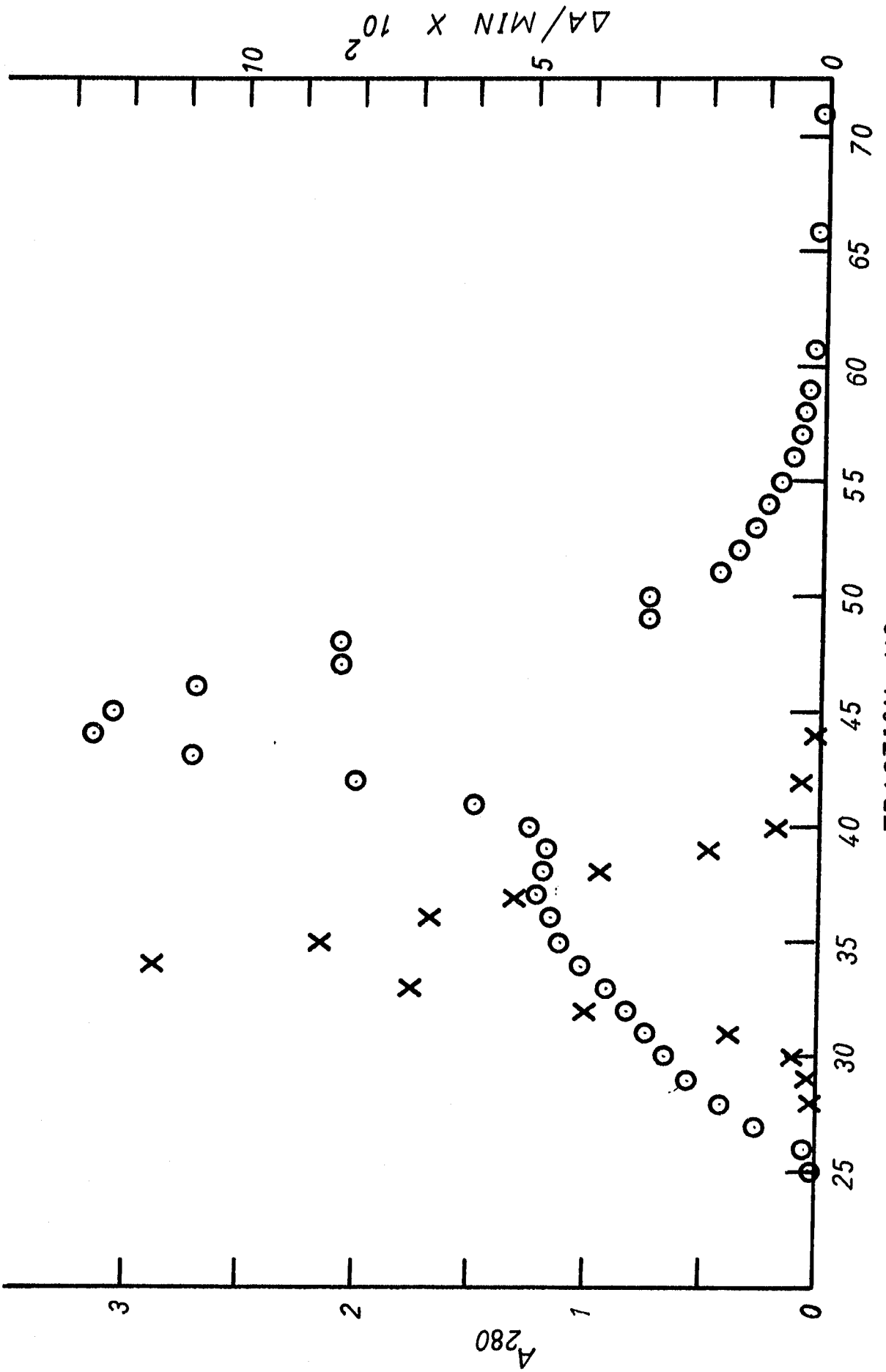


Figure 3-2

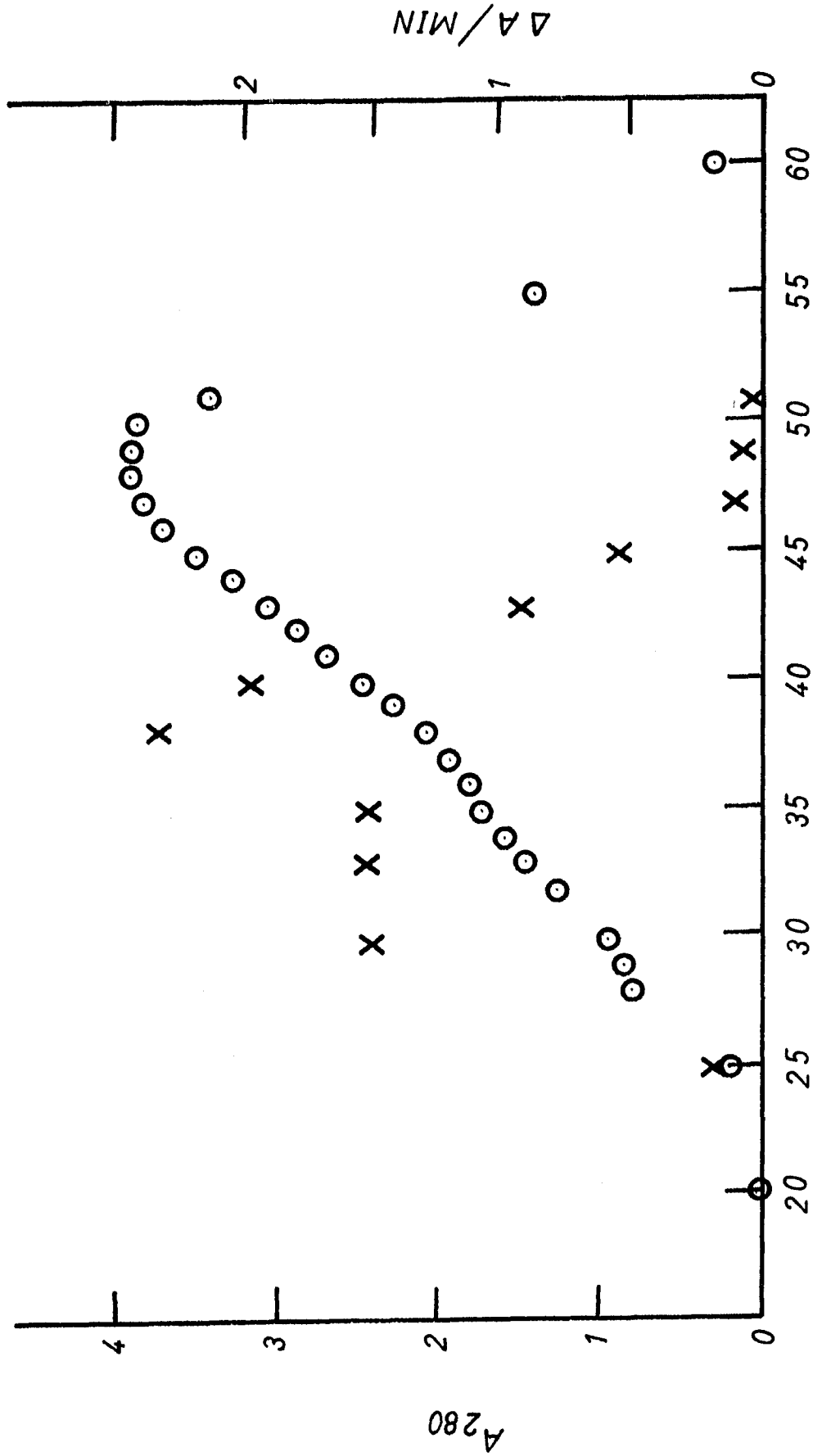


Figure 3-3

resulted in an instantaneous and reproducible absorbance change of 0.032 ± 0.001 followed by an extremely slow steady-state. Reaction of the same volume of pooled fractions with 1.77×10^{-4} and 7.36×10^{-4} M pNPA resulted in a slow observable burst, the magnitude of which was dependent upon $[S_o]$ indicating only partial saturation of the active sites. The absorbance change of the burst with 1.77×10^{-4} M pNPA was 0.047 and with 7.36×10^{-4} M pNPA was 0.081. All reactions were run at pH 7.80 in 0.02 M phosphate buffer, 3% v/v methanol.

From the known pK_a values of indophenol and p-nitrophenol, the molar absorptivities of the phenolate anions, and the pH of the solutions, the concentrations of liberated phenol can be calculated. Assuming a 1::1 stoichiometry of the reaction of the esters with the burst active component a determination of the molarity of the "active sites" can be determined. The results of these calculations are presented in Table 3-8. The concentration of active sites was found to be the same from the bursts with IPA and the 7.36×10^{-4} M pNPA and was approximately 5.4×10^{-6} M.

Two experiments were carried out in order to determine the necessity of conformational integrity of the protein for burst activity with IPA. Heating a sample at 70° C for one hour and cooling to room temperature before reaction had no effect upon the magnitude

TABLE 3-8. Determination of the Concentration of Active Sites in an Aliquot of Pooled Active Fraction 43-48 of Separation I by Titration with IPA and pNPA.[a]

Ester	$[S_o] \times 10^4$ M	λnm	pH	pK_a Phenol	$\varepsilon \times 10^4$ M^{-1}	ΔA	Calculated $[E_o] \times 10^6$ M
IPA	1.77	675	7.80	8.1[b]	1.81	0.032	5.4
pNPA	1.77	400	7.80	7.14[c]	1.83[d]	0.047	3.13
pNPA	7.36	400	7.80	7.14[c]	1.83[d]	0.081	5.39

(a) The reactions were run by the addition of 0.25 ml of pooled active fractions 43-48 to 2.0 ml of substrate solution. The pH was measured after each reaction, 7.80 ± 0.03, 0.02 M phosphate, 3% v/v methanol, 25.0° C.

(b) Reference 17.

(c) Reference 22.

(d) Reference 23.

of the burst measured at 675 nm ($\Delta A = 0.030$, same conditions as above). Treatment of the sample with 8 M urea, a potent protein denaturant, rendered the burst component completely inactive toward IPA.

As an allied experiment the reaction of horse serum albumin (Cohn Fraction V) with IPA was examined. A solution of the albumin was made so that its absorbance at 280 nm approximated that for the pooled active fractions ($A_{280} = 2.4$). The reaction of this solution with 1.77×10^{-4} M IPA was monitored at 675 nm in the same manner as the burst active pooled fractions. A very slow liberation of indophenolate was observed, but no burst was detected.

The first-order k_{obs} of the rapid hydrolysis of IPA by pooled fraction 43-48 was examined as a function of both $[S_o]$ and $[E_o]$. The kinetic runs were carried out in the stopped-flow spectrophotometer. $[E_o]$ was given as the volume of pooled fractions 43-48 in 10 ml of enzyme solution. As with the commercial preparation of BuChE, k_{obs} was found to be independent of $[S_o]$ and directly dependent upon $[E_o]$. These results are compiled in Tables 3-9 and 3-10.

TABLE 3-9. The Dependence of k_{obs} upon $[S_o]$ for the Transient Phase of the Hydrolysis of IPA Catalyzed by Pooled Fractions 43-48 of Separation I.[a]

$[S_o] \times 10^5$ M	$[E_o]$[b]	k_{obs} (STD DEV) sec^{-1}
7.0	1.0	59.1 (6.9)
10.0	1.0	61.5 (9.5)
30.0	1.0	50.2 (3.1)
50.0	1.0	53.1 (4.9)

(a) The reactions were run in 0.02 M phosphate buffer, pH 8.0, 10% v/v methanol, 25.0° C.

(b) The enzyme concentration is expressed in ml of pooled fractions 43-48 in 10 ml enzyme solution.

TABLE 3-10. The Dependence of k_{obs} upon $[E_o]$ for the Transient Phase of the Hydrolysis of IPA Catalyzed by Pooled Fractions 43-48 of Separation I.[a]

$[E_o]$[b]	$[S_o] \times 10^5$ M	k_{obs} (STD DEV) sec^{-1}
0.5	10.0	37.9 (2.9)
1.0	10.0	47.3 (5.1)
2.0	10.0	59.1 (4.8)

(a) The reactions were run in 0.02 M phosphate buffer, pH 8.0, 3% v/v methanol, 25.0° C.

(b) The enzyme concentration is expressed in ml of pooled fractions 43-48 in 10 ml of enzyme solution.

The Effect of Paraoxon upon the Ability of the Burst Active Fractions of the Chromatographic Separation of Commercial BuChE to Hydrolyze IPA and pNPA. Fractions 45-52 of Sepadex separation II were pooled for the study of the effect of paraoxon upon the burst active component. The pooled fractions were made 1.0×10^{-5} M in eserine in order to inhibit any residual cholinesterase activity. The sample was then divided into two parts, one being incubated with 1.85×10^{-5} M paraoxon. Each of the two protein samples were reacted with IPA and pNPA (2.49×10^{-4} M and 3.01×10^{-4} M respectively, 0.067 M phosphate, pH 7.6) in the Cary 14 using appropriate blanks. The magnitudes of any resulting bursts were noted. It was found that the presence of paraoxon did not affect the existence or reduce the magnitudes of the bursts. Supporting data are collected in Table 3-11. It was also observed that the eserine inhibited pooled fractions did not hydrolyze paraoxon. It was assumed there was no significant interaction between eserine and paraoxon.

Analysis of the Burst Active Component of the Chromatographic Separation of Commercial BuChE. Fractions 60-63 of Sephadex separation III were pooled and dialyzed against two one liter changes of water. A sample of the dialysate was analyzed by polyacrylamide gel electrophoresis (3.5%, pH 9.5). A single homogeneous band was obtained. The remaining dialysate

TABLE 3-11. The Effect of Paraoxon upon the Magnitude of the Bursts of Liberated Phenolate Anions.[a]

Ester	[Paraoxon] x 10^5 M	[S_o] x 10^4 M	λnm	ΔA
IPA	--	2.49	675	0.029
IPA	1.85	2.49	675	0.028
pNPA	--	3.01	400	0.048
pNPA	1.85	3.01	400	0.049

(a) Reactions were initiated by the addition of 0.2 ml of eserine inhibited pooled fractions 45-52 of Separation II to 2.0 ml of substrate solution, 0.067 M phosphate, pH 7.6, 25.0° C.

was lyophilized to yield a solid sample. A portion of the solid sample was analyzed for amino acid content. Since the precise molecular weight of the sample and percent sugar content was unknown only the relative amounts of the constituent amino acids could be determined. The results of the amino acid analysis are presented in Table 3-12. It is noteworthy that the presence of glucosamine was found in the hydrolysate indicating the burst active component may be a glycoprotein. If it is assumed that the least prevalent amino acid as indicated by the amino acid analysis, methionine, is represented by 1 mole/mole of protein, an approximate number of residues can be assigned for each amino acid. These numbers of residues are collected in Table 3-12. The lyophilized sample was analyzed for carbon, hydrogen, and nitrogen content. The resulting percentages were found to be: C, 46.81; H, 6.44; N, 12.87.

The absorbance of a 0.174% by weight solution of lyophilized preparation at 280 nm was found to be 1.513. The absorbance of a 1% solution at 280 nm was therefore calculated to be 8.69. The absorbances of 1% solutions of horse serum albumin and bovine serum albumin were determined to be 5.14 and 5.96 respectively. Electrophoresis of a solution of the lyophilized sample (polyacrylamide gel, 3.5 and 7%, pH 9.5) showed one major band and two minor bands.

TABLE 3-12. Results of the Amino Acid Analysis of the Lyophilized Sample of Pooled Active Fractions 60-63 of Separation III.[a]

Amino Acid	Concentration µmol/ml	Relative Number of Residues
Lysine	0.2947	19
Histidine	0.1066	7
Arginine	0.1389	9
Aspartic Acid	0.3516	22
Threonine	0.2426	15
Serine	0.2556	16
Glutamic Acid	0.4196	26
Proline	0.1790	11
Glycine	0.2192	14
Alanine	0.2933	18
Valine	0.2146	14
Methionine	0.0159	1
Isoleucine	0.1263	8
Leucine	0.3167	20
Tyrosine	0.0965	6
Phenylalanine	0.1919	12
Cysteine[b]	----	--
Glucosamine[b]	----	--

(a) Amino acid analysis was carried out in the Department of Biochemistry of the University of New Hampshire.

(b) Cysteine and Glucosamine were detected in the hydrolysate but the molar concentrations were not able to be determined.

The Kinetics of the Reaction of Bovine Serum Glycoprotein (Cohn Fraction VI) with IPA. The reaction of bovine serum glycoprotein with IPA was examined in the stopped-flow spectrophotometer. A rapid liberation of indophenolate anion followed by a very slow steady-state was observed. The first-order k_{obs} for the transient phase was independent of $[S_o]$ over the range $[S_o]$ = 2.0 to 20 x 10^{-5} M. The supporting data are presented in Table 3-13.

The Reaction of Human Serum Glycoprotein with IPA. The reaction of human serum glycoprotein was studied in the stopped-flow spectrophotometer. IPA (8.0 x 10^{-5} M) was reacted with the glycoprotein (0.6 mg/ml) in pH 8.0 (0.1 M phosphate, 3% v/v methanol) buffer. No transient phase liberation of indophenolate anion was detected.

TABLE 3-13. The Dependence of k_{obs} upon $[S_o]$ for the Transient Phase of the Reaction of Bovine Serum Glycoprotein (Cohn Fraction VI) with IPA.[a]

$[S_o] \times 10^5$ M	$[E_o]$ mg/ml	k_{obs} (STD DEV) sec^{-1}
2.0	0.6	66.9 (6.0)
5.0	0.6	60.3 (2.3)
8.0	0.6	57.6 (1.3)
20.0	0.6	58.1 (3.7)

(a) The reactions were run in 0.10 M phosphate buffer, pH 8.0, 3% v/v methanol, 25.0° C.

DISCUSSION

The following reaction scheme has been used to describe the reaction of butyrylcholinesterase (BuChE) with substrates and organophosphorous inhibitors (10).

$$E + AB \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EAB \xrightarrow{k_2} EA + B \xrightarrow{k_3} E + A \qquad (I)$$

Under the limiting assumptions of initial substrate concentration, $[S_o]$, being in excess of the initial enzyme concentration, $[E_o]$, and $k_2 >> k_3$, reaction Scheme I implies a biphasic reaction (24) consisting of a rapid first-order liberation of moiety B with a subsequent steady-state. We have undertaken a study of the kinetics of the reaction between the commercial preparation of BuChE and IPA with particular interest in the pre-steady-state portion of the reaction as might be detected in a stopped-flow spectrophotometer. It is generally accepted that for the cholinesterase catalyzed hydrolysis of specific substrates such as acetylcholine k_2 is rate determining (7), but for non-specific substrates such as IPA the rate determining step might become deacylation; k_3 is the characteristic rate constant.

In preliminary studies with the commercial preparation of the enzyme and IPA a biphasic release of indophenolate anion was observed (Figure 3-1). While the precise molar concentration of the enzyme solutions were not able to be determined, crude estimates based upon available values of the molecular weight per active site, 120,000 - 141,000 (15,16) indicate that $[S_o]$ exceeds $[E_o]$ at all values of $[S_o]$.

The kinetic equations for the general mechanism shown in reaction Scheme I have been derived (24,25) and have been applied, for example, in the studies of the chymotrypsin catalyzed hydrolysis of specific substrates (26) and non-specific substrates (23). These equations predict a dependence of the first-order rate constant for the burst process, k_{obs}, on $[S_o]$ when $[S_o] >> [E_o]$ and $[S_o]\ (k_2 + k_3) >> k_3 k_{m(app)}$, according to the following equation.

$$\frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{K_s}{k_2[S_o]} \qquad (4\text{-}1)$$

(K_s in Equation 4-1 is the dissociation constant for the enzyme substrate complex, EAB). This relationship does not obtain in our system.

The first-order rate constant, k_{obs}, for the initial burst of indophenolate anion remained essentially constant over a large range of initial substrate concentrations (Tables 3-1 through 3-3). The concentration of the buffer in the range studied had no effect upon k_{obs}, and lowering the pH to 7.4 and changing the cosolvent from ethanol to methanol also had no effect upon k_{obs} (Table 3-3). Holding the IPA initial concentration constant and varying $[E_o]$, a direct dependence of k_{obs} on $[E_o]$ was observed (Table 3-4). As $[E_o]$ was increased the reaction became quite rapid resulting in greater experimental uncertainty in k_{obs}. Varying $[E_o]$ at constant $[S_o]$, k_{obs} and the total absorbance change of the initial burst of indophenolate increased in a parallel fashion (Table 3-5).

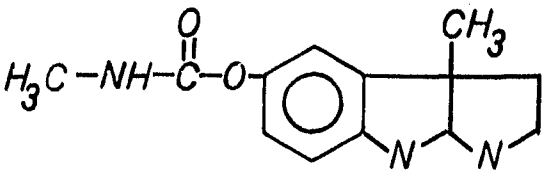
By assuming $[S_o] >> K_s$, Equation 4-1 would reduce to $k_{obs} = k_2$ and the invariance of k_{obs} with $[S_o]$ would be expected. But in the range of initial concentrations used this is considered an unlikely possibility. Also, if k_{obs} were indeed k_2 an increase in $[E_o]$, and therefore an increase in the concentration of EAB, would be expected to increase the rate of the reaction according to

$$\text{rate} = k_2 [EAB],$$

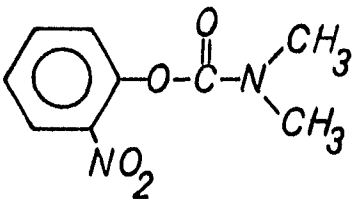
but the first-order rate constant would not change. This would be in direct contradiction to the observed dependence of k_{obs} on $[E_o]$. The observed kinetics also cannot be explained by a direct second-order reaction of enzyme with IPA.

The fact that the commercial preparation was only partially purified coupled with the anomalous kinetics of the transient phase led to experiments to determine if the burst was caused by "impurities". Samples of the enzyme were incubated separately with eserine (I), (10), o-nitrophenyl dimethylcarbamate (II) (18), and tetrabutyl ammonium iodide (III) (10), all potent inhibitors of cholinesterase. The incubated enzyme solutions were reacted with IPA in the stopped-flow spectrophotometer. None had any perceptible effect upon the magnitude of k_{obs} or the total absorbance change of the burst (Table 3-6). IPA substrate solutions were also incubated separately with eserine, o-nitrophenyl dimethylcarbamate, and the competitive BuChE substrates butyrylthiocholine and p-nitrophenyl acetate, and no effect was observed on k_{obs} for the initial burst of indophenolate (Table 3-7).

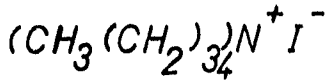
It became clear that either a non-cholinesteratic site within the enzyme of another component of the sample was responsible for the burst activity with IPA.



I



II



III

Therefore, the commercial preparation was fractionated on a column of Sephadex G-200 (15), and the isolated constituents were analyzed for burst activity with IPA. The fractionation yielded two distinct components (Figures 2-2,3-2,3-3). The protein contained in the first peak had the majority of the cholinesterase activity as determined by monitoring the hydrolysis of butyrylthiocholine spectrophotometrically using DTNB as an indicator and also by monitoring the steady-state hydrolysis of IPA, while the components eluted in the second peak contained all of the burst activity with

IPA. While the latter components also contained slight activity with butyrylthiocholine, all such activity could be eliminated by incubation with eserine leaving the burst with IPA unaffected. Fractions from the absorbance maxima of the second peak were pooled and subsequent studies were made using aliquots from these pooled fractions.

Experiments were carried out in a recording spectrophotometer in order to study the burst process in the non-cholinesterase component of the commercial preparation of the enzyme. The net magnitude of the burst with IPA, as measured by the deflection from zero absorbance at 675 nm was strictly reproducible. When p-nitrophenyl acetate (pNPA), which exhibited no burst in stopped-flow experiments, was the substrate a slow burst of p-nitrophenolate anion monitored at 400 nm was observed. The magnitude of the slow burst with pNPA was dependent on the initial concentration of substrate in the range of concentrations studied (1.77×10^{-4} to 7.36×10^{-4} M) possibly indicating only partial saturation of the active sites at the lower concentrations. Using the bursts with IPA and pNPA as titrations for the active sites, nearly identical molar concentrations of active sites are calculated for the bursts with IPA and the 7.36×10^{-4} M pNPA (5.54×10^{-6} M and 5.39×10^{-6} M respectively)(Table 3-8).

From these data the approximate molecular weight of the burst active component can be calculated. A solid sample of the component was subsequently isolated and the absorbance at 280 nm of a 1% solution was determined to be 8.69. From this value and the absorbance at 280 nm for the pooled active fractions used in the titration (2.425) a concentration of 0.309 mg/ml was calculated for the titration sample. From the molar concentration determined by the titration with IPA (5.54×10^{-6} M) an approximate molecular weight of 56,000 is determined.

In order to determine the relationship between the conformational integrity of the protein and the burst process a sample was heated before reaction with IPA. This treatment did not diminish the magnitude of the burst. The possibility of rapid renaturation of the protein (27) does not allow unequivocal statement of the relationship. But 8 M urea rendered the protein totally inactive with IPA leading to the conclusion that conformational integrity is a prerequisite for the burst process.

Previous reports have discussed the catalytic activity of serum albumins (28,29), and the possibility of serum albumin, a ubiquitous constituent of serum, being the burst active component must be considered. Consequently a sample of horse serum albumin (Cohn

Fraction V) was obtained and its activity with IPA was examined. A slow net hydrolysis was observed, but no burst was detected. Therefore, it was concluded that the burst active "impurity" in the commercial preparation was not serum albumin.

Studies on mammalian serum have shown the presence of uncharacterized esterases which have been classified as arylesterases (30). Also, recent work has described the presence of one of these esterases in rabbit serum which shows catalytic specificity for paraoxon (diethyl p-nitrophenyl phosphate) (31). Experiments carried out with the isolated burst active fractions indicated that paraoxon is neither hydrolyzed by the action of the horse serum protein nor does it inhibit the activity observed with IPA and pNPA (Table 3-11). Since the burst active component neither hydrolyzes nor is inhibited by paraoxon its classification as a serum arylesterase (32) is rendered difficult.

Stopped-flow experiments were carried out with the burst active pooled fractions with IPA. As with the unfractionated preparation, k_{obs} for the first-order burst was found to be independent of $[S_o]$ (Table 3-8) and directly dependent on $[E_o]$ (Table 3-9).

The thrust of the investigation at this point became an attempt to determine the identity of the burst active component. Experiments have shown that

the component is not serum albumin or a readily characterized serum arylesterase, but mammalian serum contains a wide diversity of proteins and glycoproteins (33). An approach was followed which entailed determining several properties of the unknown sample, such as homogeneity, molecular weight, amino acid composition, nitrogen content, and absorbance data, and comparing these with properties of known constituents of serum. It was hoped that such an approach would yield the identity of the burst active fraction.

A sample of the burst active component was found to be homogeneous by polyacrylamide gel electrophoresis. The absorbance of a 1% solution of the sample at 280 nm was found to be 8.69. A comparison of this value with similar values for purified human plasma proteins showed a similarity with the value for α_1-acid glycoprotein (8.9) (34). This type of comparison across species can be partially justified by examination of the absorbances at 280 nm of 1% solutions of horse and bovine serum albumins (5.14 and 5.96 respectively) compared with that for human serum albumin (5.8) (34). Also, the molecular weight of the protein determined from titration with IPA, 56,000, is similar to the value given for human serum α_1-acid glycoprotein, 45,000 (34).

A sample of the lyophilized material was analyzed for amino acid content. Besides the normal complement of amino acids, glucosamine was detected in the hydrolysate, which was consistent with the sample being a glycoprotein. An approximate determination was made of the numbers of the invidual amino acid residues in one molecule. Two assumptions were made : 1) The preparation was homogeneous, and 2) The residue showing the lowest concentration in the hydrolysate represented 1 residue/molecule. The second assumption is speculative, but at least the relative numbers of residues can be determined. Table 4-1 shows the number of residues per molecule thus calculated and compares these with similar values determined for α_1-acid glycoproteins isolated from man, ox, rat and sheep. A rough parallel can be seen by this comparison, particularly the common high concentrations of Glu, Asp, and Leu residues.

The nitrogen content of the lyophilized sample was determined to be 12.87%, which would be low for pure protein material. Nitrogen content of acid glycoproteins has been found to be in the range of 10.4 to 12.9% (35) and α_1-acid glycoprotein from human serum has 10.7% nitrogen (36). Seromucoid, a substance containing a large per cent of α_1-acid glycoprotein, isolated from horse serum had a nitrogen content of 13% (37).

Table 4-1. Amino Acid Composition of α_1-Acid Glycoprotein from Various Sources Compared with the Amino Acid Composition of the Burst Active Component from Horse Serum.

Residues per Mole

Amino Acid	Man[a]	Ox[a]	Rat[a]	Sheep[a]	Horse[b]
Ala	9-15	17	20	13	18
Arg	9-10	11	15	10	9
Asp	22-30	22	39	18	22
Cys	2-4	7	5	3	--
Glu	30-36	35	42	27	26
Gly	5-9	9	13	8	14
His	3-7	5	7	7	7
Ile	9-14	12	10	10	10
Leu	15-19	15	30	13	20
Lys	14-19	20	28	16	19
Met	2-3	2	5	2	1
Phe	9-12	26	22	8	12
Pro	8-12	7	14	10	11
Ser	4-11	13	13	7	16
Thr	9-22	14	28	12	15
Try	2-4	2	--	--	--
Tyr	5-11	11	26	11	6
Val	6-11	9	10	7	14

(a) Abstracted from data, reference 35.

(b) This study.

Subsequent polyacrylamide gel electrophoresis of the lyophilized sample revealed three components, one major and two minor. The electrophoretic homogeneity of acid glycoproteins remains unclear. Studies have shown seven bands for purified α_1-acid glycoproteins in starch-gel electrophoresis (38). After removal of the sialic acid component three bands were observed which were identical in the presence of immune goat serum (38). Therefore, the inconsistency of the electrophoretic homogeneity of our sample before and after lyophilization cannot unequivocally mitigate against chemical homogeneity.

A significant point of favor in α_1-acid glycoprotein being the burst active component is the result obtained upon heating the burst active fraction. The burst activity was insensitive to heat treatment. One of the most salient properties of α_1-acid glycoprotein is its stability in boiling water (36,39). Schmid (36) reported the sedimentation coefficient of α_1-acid glycoprotein from human plasma to be unchanged after heating at 100° C.

A sample of bovine glycoprotein (Cohn Fraction VI) was obtained and its reaction with IPA was monitored in the stopped-flow spectrophotometer. Cohn Fraction VI (33) of human plasma contains albumin and α and β glycoproteins, among which the major component

(59%) is α_1-acid glycoprotein (36). It is assumed here that approximately the same distribution obtains in the bovine Fraction VI.

The reaction of bovine Fraction VI with IPA yielded a rapid first-order liberation of indophenolate anion followed by a very slow steady-state. The first-order k_{obs} for the rapid reaction was independent of $[S_o]$ as was seen to be the case with the reactions of commercial horse serum cholinesterase and its isolated burst active fraction (Table 3-13). For the reaction in which $[E_o]$ = 0.6 mg/ml and $[S_o] = 2.0 \times 10^{-4}$ M at pH 8.0 the total absorbance change for the burst was measured to be 0.0596. From the pK_a of the indophenol (8.1) and the molar absorptivity of indophenolate anion at 675 nm the total amount of liberated indophenol was calculated to be 7.9×10^{-6} M. Using the distribution given by Schmid (36) and a molecular weight of α_1-acid glycoprotein of approximately 45,000 (34) the molar concentration of α_1-acid glycoprotein in a 0.6 mg/ml solution will be approximately 8×10^{-6} M. This value compares favorably with the concentration of active sites which is determined by the liberation of indophenol in the rapid burst.

A sample of human glycoprotein (Fraction VI) was reacted with IPA but no rapid reaction was observed. This observation is confusing but may speak to some

species differentiation in the activity with IPA. It is likely that the activity with IPA is the result of a fortuitous confluence of amino acid residues rather than a true physiological enzymatic reaction. The rapid single turnover with IPA could indicate inhibition of the active site of an enzyme by acetylation. This is unlikely since enzymes with esterase activity are readily deacetylated. Therefore, the proposition that the α_1-acid glycoprotein reaction with IPA is a fortuitous circumstance rather than a true enzymatic reaction is reinforced. The constituent amino acids of α_1-acid glycoprotein differ across species (Table 4-1), therefore, the lack of IPA activity in human glycoprotein may be explained by its unique amino acid backbone.

Finally, it is possible that the activity with IPA is an artifact of a low molecular weight contaminant of these preparations. However, this is unlikely for a number of reasons. A small molecule, relative to α_1-acid glycoprotein, would have been separated by the Sephadex filtration. The experiment on urea denaturation speaks for the necessity of a three dimensional protein structure for activity. Also, dialysis of a sample of commercial horse serum cholinesterase did not remove the burst component. Therefore, available evidence favors a high molecular weight protein component,

which has been tentatively identified as serum α_1-acid glycoprotein, being the burst active material.

The tentative identification of the burst active component does not aid in the explanation of the anomalous kinetics of the burst process. There have been no previous reports of esterase activity by α_1-acid glycoprotein. It is possible to speculate that complex conformational interactions in the substrate or glycoprotein contribute to the complex kinetics, but a full explanation is not forthcoming from the available data. However, it should be possible to use the rapid burst activity with IPA as a analytical probe for α_1-acid glycoprotein from bovine and horse serum.

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