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ALDEHYDIC TRANSITION STATE ANALOGUE INHIBITORS OF α-CHYMOTRYPSIN

by

Alexandra C. Cheerva

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

June

1978

LOYOLA UNIVERSITY MEDICAL CENTER

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VITA

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Richard M. Schultz and Alexandra C. Cheerva. "The Binding of a Non-Specific 'Transition State Analogue' to α-Chymotrypsin." FEBS Letters 50(1):47-49 (January, 1975).

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CHAPTER I

INTRODUCTION

The mechanisms responsible for enzyme catalysis are only slowly being elucidated. Up until quite recently, experimenters have been limited to the study of the interaction of enzymes and their substrates or substrate analogues in complexes which are, for the most part, analogous to the Michaelis complex (ES), a low-energy reversible association of enzyme and substrate. This low-energy Michaelis complex can be contrasted to that complex of highest energy along the reaction pathway from reactants to products, the transition state-enzyme complex (ES†). The direct study of the ES† complex is, by its very nature, almost impossible. Because of its high energy, there are necessarily quite few molecules in this state at one time. (The concentration of ES† may typically be 10^{-10} times the ES concentration.) However, the interactions between enzyme and transition state within this complex are of considerable interest in determining the exact mechanisms responsible for catalysis, and perhaps with this information more specific and more potent inhibitors for certain enzymes may be designed. This goal is obviously one of great interest in the design of drugs.

The use of transition state analogue theory may provide a means by which the interactions of transition state and enzyme may be studied. This theory predicts that the enzyme will bind the transi-

tion state far more tightly than the substrate in its ground state (1, 2). In theory, then, a stable substrate analogue, which has the structural features of the transition state for a given reaction, will also form a strong complex with the enzyme. This complex would more truly mimic the transition state structure than complexes between enzyme and substrate analogues of other types. A comparison can then be made of the enzyme-transition state complex interactions to those interactions present in the Michaelis complex or stable enzyme-substrate intermediates (e.g. an acyl enzyme) in order to gain more insight into the catalytic mechanism (1, 2). Investigation of the crystal structures of such enzyme-transition state complexes will also be of interest. Also, if a transition state analogue is found to bind tightly to the enzyme, strain is indicated in the catalytic process. If the enzyme binds to the transition state more tightly than to the substrate, then the Michaelis complex (ES) may be strained towards the enzyme-transition state complex (ES†). This implies that the active site of the enzyme is complementary in structure to the altered substrate in the transition state (S†) and therefore, a stable model of S† should fit exactly into that site on the enzyme.

Another benefit of the transition state analogue approach would be to pharmacology, in that very specific and very potent enzyme inhibitors can, in theory, be designed. One needs only a reasonable knowledge of the mechanism for a certain reaction and its transition state and a transition state analogue inhibitor can then be designed. This rational approach to the design of powerful new and specific

enzyme inhibitors should be of great value in the search for molecules which will produce specific physiological effects by interfering with certain enzymes in particular metabolic pathways (3).

STATEMENT OF THE PROBLEM

The purpose of this thesis is to synthesize and study the binding of molecules that geometrically mimic the proposed transition state for the α-chymotrypsin catalyzed reaction (all chymotrypsin is of bovine origin); and by this means: 1) to determine more specifically the mechanisms responsible for catalysis in α-chymotrypsin, and more generally in the serine proteases, and 2) to outline a pathway for the design of more specific and potent inhibitors for these enzymes, an objective of interest in drug design.

It is generally accepted that catalysis by α-chymotrypsin, as well as other serine and cysteine proteases, proceeds by a mechanism in which the enzyme is acylated at the active site serine or cysteine residue. The transition states for the acylation and deacylation of the serine or cysteine probably resemble a tetrahedral intermediate which is formed by the originally trigonal α-carbon atom of the substrate now tetrahedrally coordinated to the enzyme through the serine or cysteine residue (1, 3-6).

Certain peptide and amino aldehydes and boronic acid analogues have been proposed as transition state analogues for these acylation-deacylation reactions (3, 7-12). Aldehydes may bind to the enzyme active site serine or cysteine in a manner similar to that of substrates, forming a relatively stable hemiacetal structure. Complexes of this type are similar in structure to the tetrahedral species (1,3)

and are thus proposed as transition state analogues (1, 3, 7-12).

In this thesis, we wish to investigate the affinity of hydrocinnamaldehyde, a proposed transition state analogue of α-chymotrypsin catalyzed esterolysis (10), to the active site of α-chymotrypsin with respect to pH. Since hydrocinnamaldehyde is an analogue of a relatively nonspecific substrate of α-chymotrypsin, we have undertaken a synthesis of an aldehyde analogue of a more specific substrate. The attempted synthesis will be detailed herein.

CHAPTER II

REVIEW OF THE RELATED LITERATURE

THEORY OF THE TRANSITION STATE ANALOGUE APPROACH

One can view enzyme catalysis in terms of the activated complex or transition state. On the energy diagram (Figure 1), we see two pathways leading from reactants to products, one nonenzymatic, the other enzymatically catalyzed.

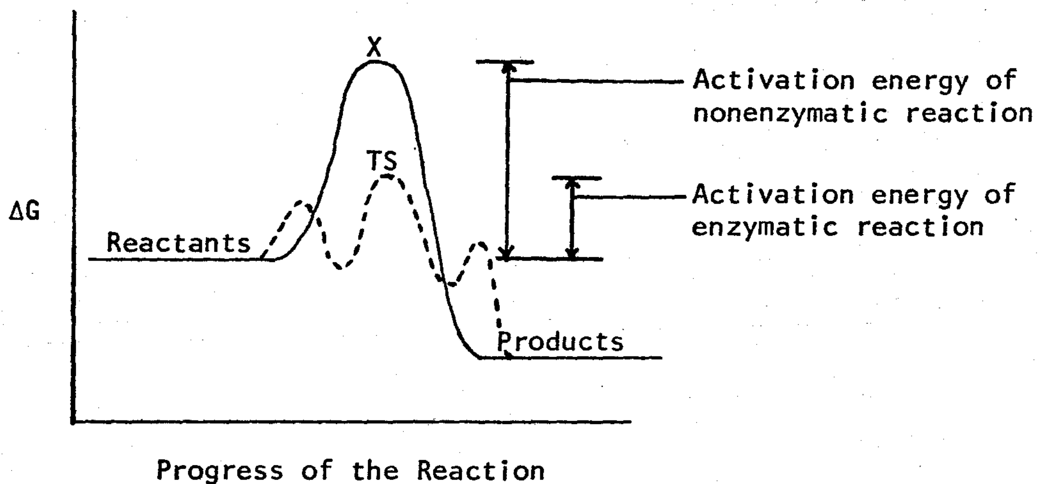


Figure 1. Energy diagram for a reaction, both uncatalyzed (——) and catalyzed by an enzyme (---).

There are high energy barriers in the nonenzymatic pathway which prevent the spontaneous conversion of reactants to products. A reactant molecule must acquire a substantial amount of energy to reach its transition state (X) before it can be converted to product. However, in the presence of enzyme, the reaction follows a different

pathway, reaching a different high energy transition state, and thus the energy barriers between reactants and products are reduced.

Linus Pauling first predicted that an enzyme has a strong power of attraction for the transition state of a reaction because of a complementarity of structure to the activated complex (13). This prediction meant that

> the activation energy for the reaction is less in the presence of the enzyme than in its absence, and accordingly that the reaction would be speeded up by the enzyme... The picture even presents us with ideas as to the nature of substances which would be effective inhibitors — they should resemble the activated complex (14).

This theory of enhanced binding of the transition state (13, 14) suggests that some enzymes may strain and distort the substrate toward the transition state during catalysis (15, 16). This catalysis by strain theory has led to the observation that some enzyme inhibitors owe their effectiveness to a resemblance to the transition state species (13, 16, 17).

The application of the transition state theory of reaction rates to enzymatic catalysis has been discussed by Lienard (1) and Wolfenden (2), and involves a discussion of the binding forces between enzyme and substrate during catalysis. The development of transition state theory for one-substrate reactions, two-substrate reactions and the special case involving covalent enzyme intermediates will be presented below. The general conclusion to be obtained from the discussion given below is that the transition state is probably bound very tightly to the enzyme.

1. Enzymatic Reactions Involving a Single Substrate

The unimolecular conversion of a substrate, S, to a product, P, may be schematically written as follows:

The substrate (S) must first gain enough energy to reach its transition state (S†) before it may be converted to product (P). The transition state is in equilibrium with the substrate, where K† is the equilibrium constant for the formation of the transition state. According to the

$$K\dagger = \frac{(S\dagger)}{(S)} \qquad (1)$$

transition state theory of reaction rates (18), the overall rate of any reaction is proportional to the concentration of the transition state form of the substrate, with the proportionality constant being the Boltzmann constant, k, times the absolute temperature, T, divided by Planck's constant, h.

Since, by equation 1, (S†) = K†·(S), we may substitute this expression for (S†) in equation 2. We find that the rate is now proportional to the concentration of substrate, with all constants now taken together in the term k_x, the measurable first-order rate constant for the reaction.

$$v = \frac{d(P)}{dt} = \frac{kT}{h}\cdot(S\dagger) = \frac{kTK\dagger}{h}\cdot(S) = k_x\cdot(S) \qquad (2)$$

Thus, K† can now be determined by simply multiplying the first-order rate constant, k_x, times h/kT. This equilibrium constant, K†,

is related to the difference between the free energy of the substrate and the transition state, $\Delta G\dagger$, by the usual thermodynamic equation, $\Delta G\dagger = -RT \cdot \ln K\dagger$, and so $\Delta G\dagger$ is also calculable from k_x. We now have laid the groundwork for the application of the theory.

The equilibria which describe a single substrate enzymatic reaction are:

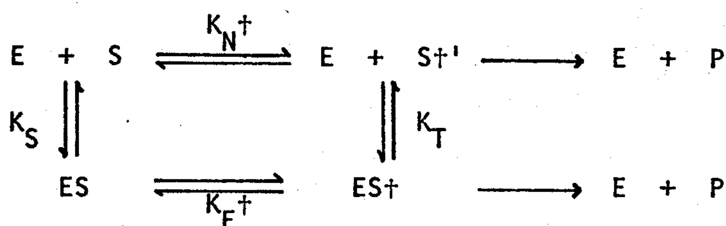


Figure 2. The equilibria describing a single substrate enzymatic reaction and its nonenzymatic counterpart.

In this scheme, K_S is the equilibrium constant for the association of the substrate, S, with the enzyme, E; $K_N\dagger$ and $K_E\dagger$ are equilibrium constants for the formation of the transition state of the nonenzymatic and enzymatic reactions, $S\dagger'$ and $ES\dagger$, respectively; K_T is the equilibrium constant for the binding of $S\dagger'$ to E to form $ES\dagger$. The expressions for these four equilibrium constants show that they are related by equation 3.

$$\frac{K_T}{K_S} = \frac{K_E\dagger}{K_N\dagger} = \frac{k_E}{k_N} \qquad (3)$$

Also, as was calculated above, $K_E\dagger$ is related to k_E, the first-order rate constant for the conversion of ES to EP, by the same factor (h/kT) that relates $K_N\dagger$ to k_N, the first-order rate constant for the

corresponding nonenzymatic reaction.

Thus, transition state theory yields the important conclusion that enzymatic catalysis, expressed by the ratio of first-order rate constants (k_E/k_N), is equivalent to tighter binding of the transition state than the substrate to the enzyme, expressed by the ratio, K_T/K_S (1, 2). The values that have been obtained for k_E/k_N suggest that the value for a typical enzymatic reaction will fall in the range of 10^8 to 10^{14} (2, 19, 20). Since K_S is usually in the range of 10^3 to 10^5 M^{-1}, the values expected for K_T are extremely large, of the order of 10^{15} M^{-1} (1).

This derivation has been made entirely on the basis of the transition state theory of reaction rates and does not depend on the mechanism of action of the enzyme or any particular enzyme-substrate interactions. Even though this theory gives no evidence for any conformational changes or attractive forces present in the enzyme, it does explain the substrate specificity of an enzyme. If a transition state structure fits more tightly into the enzyme active site, then the corresponding substrate will appear more reactive (3).

One can conclude by this development that enzymatic catalysis can be understood by describing the factors controlling the magnitudes of K_T and K_S. Lienhard (1) has considered these factors in terms of five categories:

a) changes in the basic structure of the transition state;

b) entropy changes;

c) interactions with the solvent water;

d) interactions with the enzyme; and

e) conformational changes of the enzyme.

Each of these categories will be considered in turn.

Transition state theory gives no information as to the structures of S† and S†'. They could have the same structure or they could be quite dissimilar. Regardless of the extent of similarity, equation 3 still holds. However, Lienhard (1) has come to the conclusion that the substrate probably has a similar structure in the transition state for both the enzymatic and nonenzymatic reactions. In most cases the mechanisms of enzymatic reactions and of the corresponding nonenzymatic reactions show a basic similarity in the bond making and breaking steps, thus indicating that S† and S†' are similar in structure and energy. In such cases, the value of K_T is not largely determined by changes in the transition state structure which affect its intrinsic energy. However, there may be exceptions to this conclusion, in which the mechanism of the enzymatic reaction is basically different from that of the nonenzymatic reaction.

In these exceptional cases, S† will generally be of greater energy than S†'; for if S† were of lower energy than S†', then the corresponding nonenzymatic reaction would prefer to use this alternative reaction pathway of lower energy. The effect of a basic change in reaction mechanism would be to increase the intrinsic energy of the enzymatic transition state and thus cause a decrease in the magnitude of K_T and also of the catalytic ratio, K_T/K_S. This effect may explain why there is usually a basic similarity between the mechanisms of an enzymatic reaction and of the corresponding nonenzymatic reaction,

for the enzyme could only decrease its enzymatic efficiency by choosing a different reaction pathway from the nonenzymatic reaction. Thus, the observed rate ratio can be considered only a minimum estimate of the rate enhancement possible if both enzymatic and nonenzymatic pathways involved essentially the same structure in the transition state.

The category of entropy change refers to the loss of entropy of S and S† upon their binding to the enzyme. In many nonenzymatic reactions the substrate's internal energy has been restricted upon going to the transition state and therefore S† has less internal rotational energy to lose than S upon binding to the enzyme. Where such a difference in entropy loss for the binding of S† and S to the enzyme exists, it contributes to the tighter binding of S† than of S, and thus contributes to catalysis, since catalysis actually requires an enhanced degree of binding (1, 2).

Differences in the interactions of S† and S with the solvent water can also have an effect on entropy change and thus on the ratio of K_T to K_S. For instance, if more molecules of solvating water are released upon the binding of S† to E than on the binding of S to E, then there is an entropic advantage for the formation of ES† (1, 2).

The interactions of substrate, transition state and enzyme all take place in aqueous solvent and are accompanied by disruption and reorganization of water molecules. Both S and S† interact with water and will release some water molecules upon binding to the enzyme. If S interacts with water more strongly than S†, then water

interactions favor tighter binding of S† to the enzyme, thus contributing to catalysis. If the reverse is true, water interactions hinder catalysis. Of course, the relative strengths of interaction with water of S and S† will vary depending upon the reaction in question (1, 2).

The fourth category of factors is concerned with the relative strengths of noncovalent interactions between the enzyme and S and S†. It is easy to see that if the enzyme active site is complementary in structure to the transition state structure, then it probably will not make optimal interactions with the ground-state structure of the substrate. For a particular reaction, it would be necessary to assess the relative binding strengths of S and S† in order to determine whether this factor contributes to or hinders the catalytic step (1, 2).

It is probable that, upon binding a substrate, some enzymes undergo a conformational change. This change must, in itself, be energetically unfavorable; if it were not, then the free enzyme would exist in that altered conformation of the ES complex. This conformational change necessarily introduces another equilibrium between E + S and ES; or, alternatively, between E + S† and ES†, depending on when the conformational change occurs. In both cases, the conformational changes will contribute to the ratio K_T/K_S, whether this is a contribution to tighter or weaker binding of S† (1).

2. Two-Substrate Reactions

The equilibria which describe a two-substrate enzymatic reaction

that proceeds by way of a ternary complex of the enzyme and both substrates, ES_1S_2, are shown below.

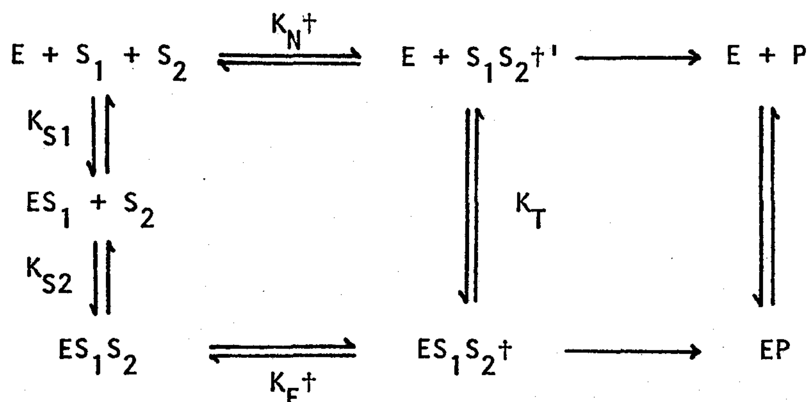


Figure 3. The equilibria describing a two-substrate enzymatic reaction proceeding through a noncovalent ternary complex of the enzyme and both substrates and its nonenzymatic counterpart.

The application of transition state theory to this scheme as before yields the relationship in equation 4, which equates the ratio between the binding constants for the transition state and the substrates to the ratio between the catalytic rate constant, k_E, and the second-order rate constant for the nonenzymatic reaction, k_N.

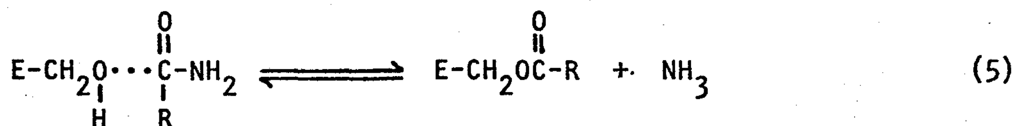
$$\frac{K_T}{K_{S1}K_{S2}} = \frac{K_E\dagger}{K_N\dagger} = \frac{k_E}{k_N} \qquad (4)$$

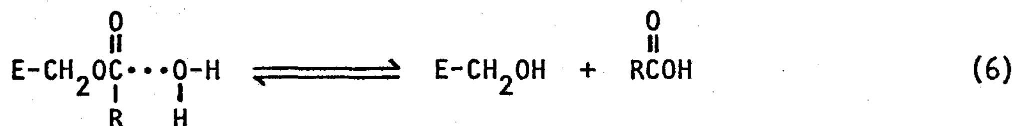
The factors that influence the binding ratio, $K_T/K_{S1}K_{S2}$, are the same ones discussed for one-substrate reactions. However, for two-substrate reactions, we may expect the contribution of entropy change to be much greater. The formation of the species ES_1S_2 is accompanied by the loss of translational and rotational entropy of

both S_1 and S_2, whereas the formation of $ES_1S_2\dagger$ is accompanied by the loss of translational and rotational entropy of only one species, $S_1S_2\dagger$. It has been estimated that this difference in the entropy changes contributes as much as a factor of 10^8 M in the value of $K_T/K_{S1}K_{S2}$ (1, 21).

3. Reactions Involving a Covalent Intermediate

Many enzymatic reactions proceed by way of intermediates in which the enzyme has formed a covalent bond with the substrate or a portion of the substrate. Upon further reaction, this intermediate then yields product(s) and in so doing regenerates free enzyme. An example of this type of reaction process is the chymotrypsin-catalyzed hydrolysis of acyl compounds, such as amides and esters. In the first step of the reaction, a specific seryl residue in the enzyme active site is acylated by the substrate to yield an acyl-enzyme intermediate (see equation 5). In the second step of the reaction, the acyl group is transferred from the seryl residue to water, regenerating free enzyme (see equation 6).





To apply the transition state theory to these reactions it is best to consider the enzyme to be both substrate and catalyst (1, 3).

The catalytic effect of the enzyme can then be estimated by comparing the enzymatic reaction to the nonenzymatic reaction between the substrate and the amino acid residue which is acylated in the enzyme.

For many proteolytic enzymes (including the acylation reaction of chymotrypsin), N-acetylserineamide (R'OH) will serve as the nonenzymatic reactant in place of the enzyme (1, 3).

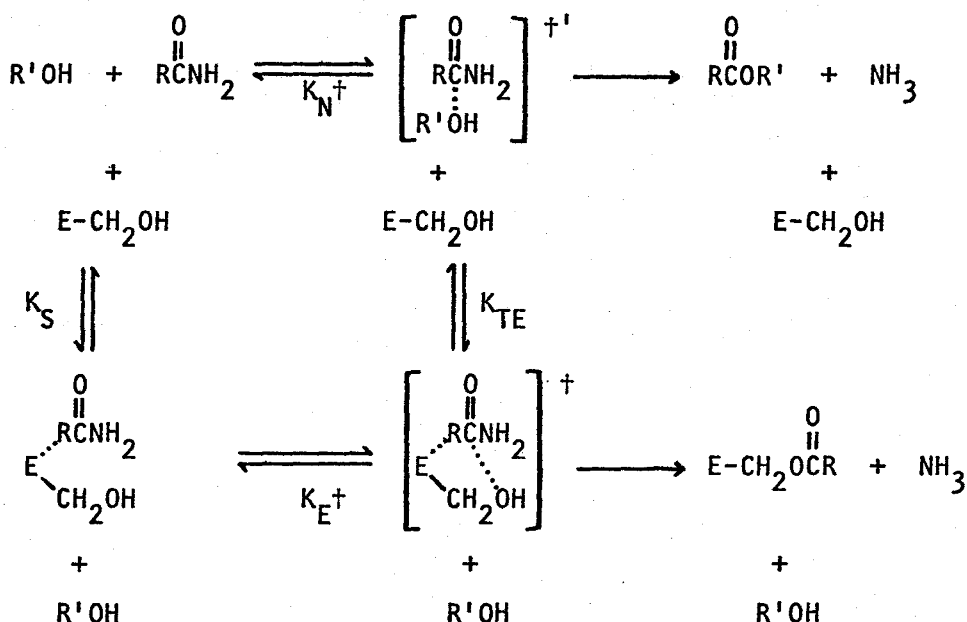


Figure 4. The equilibria describing a single substrate enzymatic reaction proceeding through a covalent intermediate and its noncovalent counterpart. (R'OH = N-acetylserineamide)

The displacement of N-acetylserineamide by the enzyme gives the equilibrium constant, K_{TE}, and the ratio shown in equation 7

$$\frac{K_{TE}}{K_S} = \frac{K_E^{\dagger}}{K_N^{\dagger}} = \frac{k_E}{k_N} \quad (7)$$

is thus a measure of the catalytic rate enhancement in the acylation step relative to the nonenzymatic reaction for acylserine formation.

This is then a measure of the increased stability of the transition state in the enzyme over that formed nonenzymatically. The magnitude of catalysis of the deacylation step may be calculated in a similar manner by a comparison of the rate of hydrolysis of the acyl-enzyme and the rate of hydrolysis of the O-acyl derivative of N-acetyl-serineamide. This approach to catalysis involving a covalent intermediate is simply that each step in the enzymatic reaction can be compared to a corresponding nonenzymatic reaction, in this instance, for acylation and deacylation of the enzyme (1, 3).

The ratio of K_{TE}/K_S will be perturbed by the same factors described for single-substrate reactions. However, on binding to the enzyme, the substrate loses translational and overall rotational entropy; whereas on binding of the transition state analogue in the exchange reaction of the above scheme, the enzyme merely displaces the N-acetylserineamide, releasing it to the surrounding medium while fixing the substrate to the surface of the enzyme. In the transition state interchange (K_{TE}), there is no net change in entropy since, on balance, there is no fixing of a species; two species are present on both sides of the reaction. Thus, the entropy changes greatly favor enhanced binding of the transition state to the enzyme (1).

4. The Magnitude of Transition State Analogue Binding Constants

Recently, a re-evaluation of the magnitude of transition state analogue binding constants has been undertaken by Schray and Klinman

(22), in which they consider the contribution to nucleophilic and acid-base catalysis of the anchoring of the appropriate amino acid residues at the enzyme active site.

As discussed on page 8, the transition state analogue theory developed for single-substrate reactions predicts that the binding ratio for the transition state/substrate is equal to the rate ratio of the enzymatic/nonenzymatic reaction ($K_T/K_S = k_E/k_N$, where K_T and K_S are the association constants for the enzyme to the transition state and to the substrate, respectively) (1, 2). The ratio k_E/k_N depends to a large extent on the nonenzymatic reaction chosen for comparison (see page 9) and this must include any amino acid residues in the enzyme which are involved in catalysis. Since entropy factors play a large role in enzymatic catalysis, we must consider the effect of situating residues involved in catalysis at the active site. The diagram below illustrates the importance of the catalytic residues located in the active site for single substrate reactions, and shows

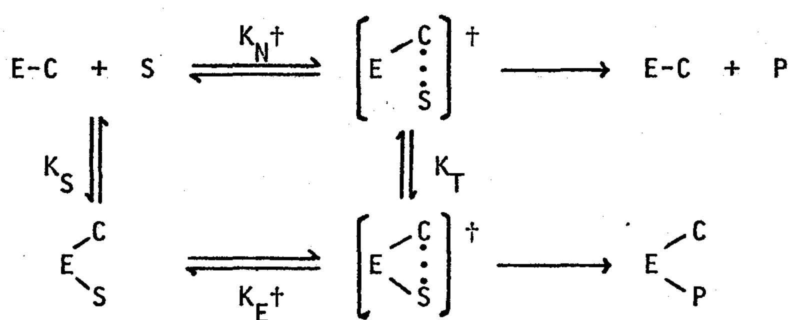


Figure 5. Anchoring of the catalytic residues at an enzyme active site.

a new choice for the nonenzymatic model reaction, that in which the catalytic residue(s) that participate(s) in the enzyme reaction also

are shown to react with the substrate in the model reaction. Accordingly, the catalytic group, C, is involved in both the enzymatic and nonenzymatic reactions, but the substrate is not bound to the enzyme active site in the nonenzymatic process.

This scheme predicts $k_E/k_N = K_T/K_S$ also, but K_T does not describe an association of the enzyme and transition state; rather, K_T describes the interaction between the enzyme and activated substrate that is already associated with catalytic residue(s), C. Therefore, the actual measured association constant, $K_{T(obs)}$, for the transition state (or analogue of) can be shown in the figure below to be a combination of equilibria, where K_D is an association constant between the substrate and the catalytic residues on the enzyme (22).

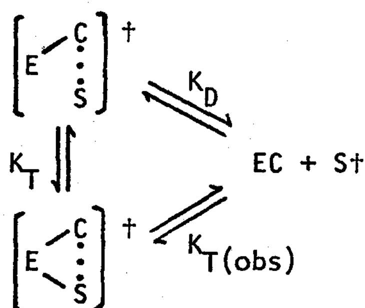


Figure 6. A consideration of entropy factors in the binding of the transition state to the enzyme active site.

Since $K_{T(obs)} = K_T \cdot K_D$,

$$\frac{K_{T(obs)}}{K_S} = K_D \cdot \frac{k_E}{k_N} .$$

Except in the case where $K_D = 1$, the absolute magnitude of K_D will determine whether the ratio of rate constants, k_E/k_N is an

underestimate (K_D<1M) or overestimate (K_D>1M) of the tighter binding of a transition state relative to a substrate. The magnitude of the association constant between the catalytic residues and the substrate, K_D, depends on both enthalpic and entropic factors. When there are no attractive forces between the catalytic residue (C) and the substrate (S), $K_{T(obs)}$ will approach K_S. This would be the case if enzymatic catalysis were simply the result of the favorable entropy factor of bringing the substrate and catalytic groups together at the enzyme active site. If, on the other hand, there were significant enthalpic forces such as attraction between the active site and $S^{\dagger}$, then enhanced binding of the transition state would be predicted (22).

This approach has predicted that the ratio $K_{T(obs)}/K_S$ will generally be different from the ratio of rate constants, k_E/k_N. This difference is due to a consideration of the entropy factors involved when catalytic residues are incorporated into the enzyme active site. This can lead to a possible explanation of the experimental fact that some "transition state analogues" have been found to bind less tightly than the theory predicts, although these differences might also be attributed to the imperfect nature of the analogues. This approach also suggests that transition state analogues studied thus far may be better analogues than previously believed.

TRANSITION STATE ANALOGUES OF SERINE AND CYSTEINE PROTEASES

The prediction that is made from transition state theory that an enzyme will bind far more strongly to the transition state of the corresponding nonenzymatic reaction than to the substrate itself cannot be tested directly, since by definition the transition state is an ephemeral and unstable species and therefore present in the lowest concentration. It can, however, be tested indirectly through the use of transition state analogues. A transition state analogue for a particular enzymatic reaction is a stable compound which resembles in structure the substrate part of the transition state. A number of such compounds have been synthesized and investigated (1-3, 23)

For most of these compounds, the ratio of the binding constant for the analogue to that for the substrate has a value between 10^2 and 10^5. This is obviously much lower than the values of 10^8 to 10^{14}, which are expected for K_T/K_S, $K_T/K_{S1}K_{S2}$ and K_{TE}/K_S. This fact has been explained a number of ways, most commonly as being due to the imperfect nature of the transition state analogues.

The enzyme elastase, one of the serine proteases, provides an example of an enzymatic reaction involving a covalent intermediate for which a potential transition state analogue has been prepared (7). The transition states for acylation and deacylation of the enzyme

resemble a tetrahedral complex with the active site serine. It has recently been shown by Thompson (7) that the aldehyde derived from a specific substrate for elastase is a powerful competitive inhibitor of the enzyme, and this effect has been reasonably explained by assuming that the aldehyde binds to elastase as a hemiacetal with the active site serine (3, 7). This hemiacetal would have a structure similar to the tetrahedral structure of the proposed transition state (see Figure 7).

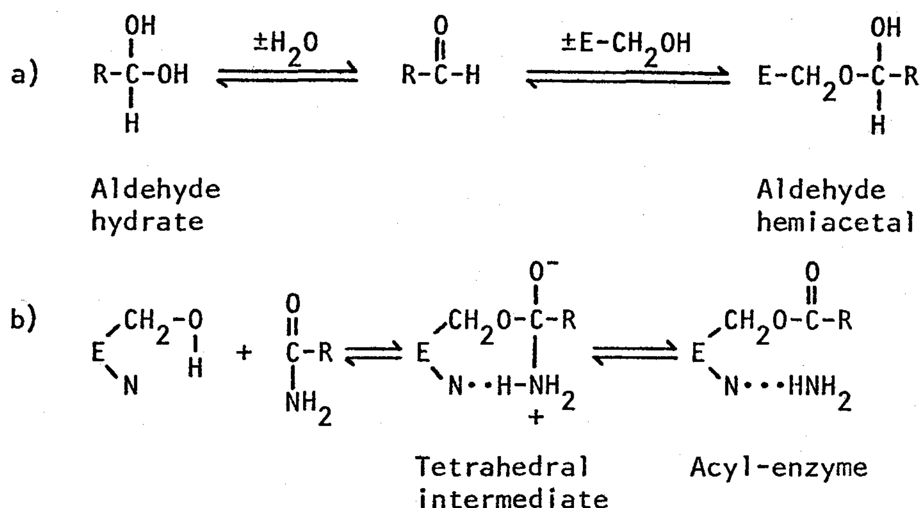


Figure 7. Elastase catalysis. a) Aldehyde reaction. b) Substrate reaction.

The hemiacetal is thought to be relatively stable. Aldehydes are unique among carbonyl compounds in that they prefer to exist as tetrahedral addition complexes and are frequently unstable with respect to their hydrates and hemiacetals in aqueous or alcoholic solutions. Particularly in the case of amino and small peptide aldehydes, it has been found that the ratio of aldehyde hydrate to free aldehyde is of the order of 10 (24-27). It is therefore reason-

able to expect the hemiacetal formed between aldehyde and enzyme to be a good transition state analogue for hydrolases acting through a tetrahedral intermediate as shown above.

In Table I are listed the K_I's of the peptide alcohols and aldehydes, as well as the kinetic parameters for hydrolysis of the corresponding amide substrates, all of which were investigated by Thompson (7). Immediately apparent are the extremely low values of K_I measured for the aldehydes. These enzyme-aldehyde complexes are the strongest yet observed between elastase and any peptide substrates or inhibitors. This seems consistent with the hypothesis that the aldehyde complexes are transition state analogues.

Table I. Kinetic Constants for Elastase-Catalyzed Hydrolysis of Peptide Amides and Elastase Binding of Peptide Alcohols and Aldehydes. (From Thompson (7)).

Peptide	K_I (mM)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1} \cdot sec^{-1}$)
Ac-Ala-Pro-Ala-NH_2		4.2	0.09	21
Ac-Ala-Pro-alaninol	7.0			
Ac-Ala-Pro-alaninal[a]	0.062			
Ac-Pro-Ala-Pro-Ala-NH_2		3.9	8.5	2200
Ac-Pro-Ala-Pro-alaninol	0.6			
Ac-Pro-Ala-Pro-alaninal[a]	0.0008			
Ac-Pro-Ala-Pro-alaninal[b]	0.002			

[a]At pH 7.00. [b]At pH 4.00.

We see a similar picture in another enzyme, papain, this time a cysteine rather than a serine protease, but which also acts via a tetrahedral intermediate. Westerik and Wolfenden (8) have found that

a common feature of the more effective inhibitors is the presence of an aldehyde group, with a side chain of the kind encountered in the acyl portion of substrates of papain (see Table II). Compared with benzoylaminoacetaldehyde (Compound I), the corresponding nitrile, carboxylic acid, alcohol, amide and methyl ester appear to be much less tightly bound.

Table II. Observed Dissociation Constants for Papain Complexes (From Westerik and Wolfenden (8)).

Compound		K_I (mM)	Analogous Ester (V_{max}/K_m) $(M^{-1} \cdot sec^{-1})$
Benzoylaminoacetaldehyde (I)		0.025	130
Carbobenzyloxyamino-acetaldehyde (II)		0.0072	380
Acetyl-L-phenylalaninyl-aminoacetaldehyde (III)		0.000046	170,000
$C_6H_5\text{-CONH-CH}_2\text{-C(=O)H}$	(I)	0.025	
$C_6H_5\text{-CONH-CH}_2\text{-C}\equiv\text{N}$		0.38	
$C_6H_5\text{-CONH-CH}_2\text{-CH}_2\text{OH}$		>1000	
$C_6H_5\text{-CONH-CH}_2\text{-C(=O)OH}$		17	
$C_6H_5\text{-CONH-CH}_2\text{-C(=O)O}^-$		830	
$C_6H_5\text{-CONH-CH}_2\text{-C(=O)O-CH(CH}_3)_2$	(K_S)	10	
$C_6H_5\text{-CONH-CH}_2\text{-C(=O)NH}_2$	(K_S)	202	

One possible structure for the papain-aldehyde complex is the

thiohemiacetal (see Figure 8), which is similar to the tetrahedral intermediate structure proposed as transition state for the acylation and deacylation reactions of the active site cysteine (3, 8, 28, 29).

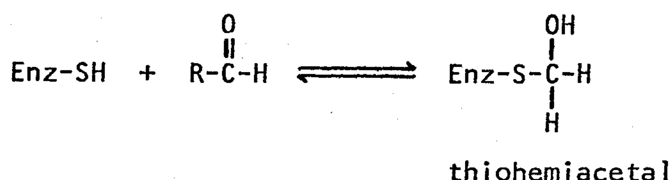


Figure 8. Thiohemiacetal formation.

Aldehydes are known to form covalent thiol adducts readily with the equilibria favoring adduct formation (3). The extremely tight binding of aldehydes to papain, in contrast to the relatively poorer binding of the corresponding carboxylic acids, amides, alcohols and ester derivatives, is consistent with the formation of a thiohemiacetal structure as an analogue for the metastable transition state of the cysteine catalyzed reaction.

The reaction of esters and amides with chymotrypsin also proceeds through a tetrahedral intermediate, in which the active site serine is covalently attached to the acyl portion of the substrate (30-33) (see Figure 9).

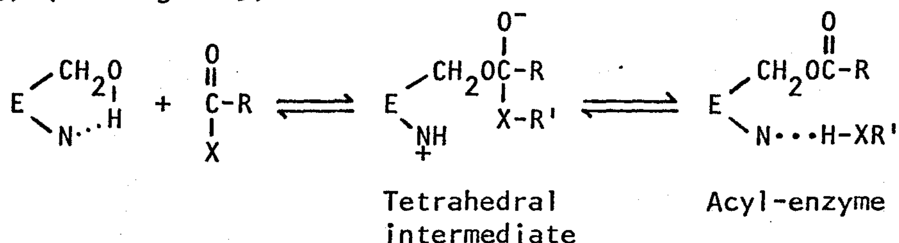


Figure 9. Acylation of the active site serine in chymotrypsin, proceeding through a tetrahedral intermediate.

Various boronic acids and aldehydes have recently been proposed

as transition state analogues for the acylation-deacylation reactions of chymotrypsin and related serine proteases (7-10, 12).

Boronic acids are known to ionize in aqueous solution with the addition of a hydroxide ion forming stable anionic tetrahedral adducts (34, 35) (see Figure 10). It is proposed that they may add to the active site serine of chymotrypsin in a similar manner (9) in order to form a tetrahedral species, not unlike the proposed transition state for chymotrypsin catalyzed reactions.

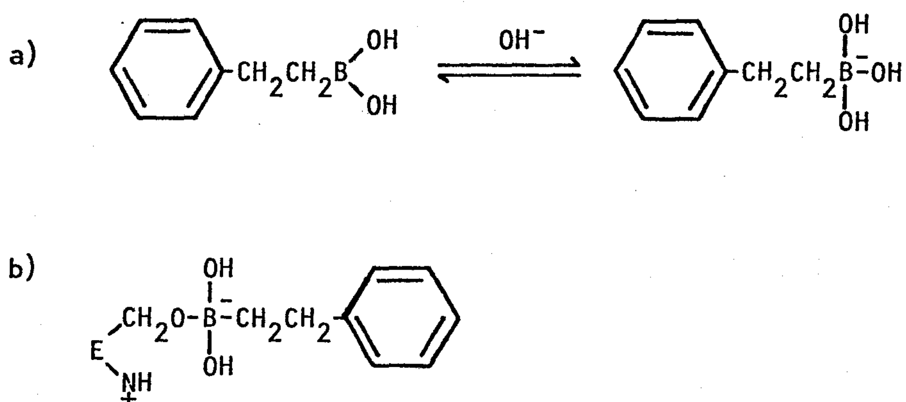


Figure 10. Boronic acids. a) Ionization in aqueous solution. b) Acylation of Serine-195 of Chymotrypsin.

Since chymotrypsin is specific for aromatic side chains (30), phenyl groups were used as part of the inhibitors investigated. The complex between 2-phenylethaneboronic acid and α-chymotrypsin was found to be quite strong; phenylethaneboronic acid binds about 150 times more tightly than hydrocinnamide, a noncovalent inhibitor with a similar structure (36) (see Figure 11).

Chymotrypsin is also inhibited by other boronic acids, but to a much lesser extent (11, 37). It is unlikely that the inhibition of

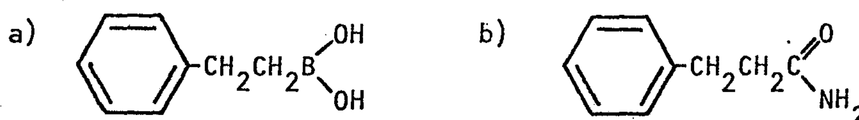


Figure 11. Chemical structures of inhibitors of chymotrypsin. a) 2-phenylethaneboronic acid. b) Hydrocinnamide.

chymotrypsin by the boronic acids is due to noncovalent binding, because the pH - K_I profiles for noncovalent inhibitors of a similar structure (hydrocinnamide and phenylethanesulfonic acid) are quite different from the pH - K_I profile of phenylethaneboronic acid (9). It is possible that boronic acids may form complexes with the enzyme other than that depicted in Figure 10, but some of these can be rules out (9). For a clearer picture of the nature of the complex between α-chymotrypsin and boronic acids, we must await the results of crystallographic studies. Such studies have already been done with subtilisin-boronic acid complexes and are reported below.

Subtilisin is a proteolytic enzyme which is in the same class of serine proteases as trypsin, chymotrypsin and elastase and appears to have a very similar mechanism of action (38, 39). This might be suspected since the structures of the active sites of these enzymes are very similar (40, 41). As might also be suspected, the proposed transition state for the subtilisin catalyzed reaction is also a tetrahedral intermediate similar to that of chymotrypsin.

Phenylethaneboronic acid and substituted benzeneboronic acids were found to be very good competitive inhibitors of subtilisin Carlsberg (42) and Novo (11). Benzeneboronic acid binds about 530 times more tightly than benzenesulfonic acid and about 230 times

more tightly than benzamide at pH 8.0 (42). Similar results are seen with phenylethaneboronic acid. This inhibition of subtilisin is dependent upon the ionization of a group at the enzyme active site with a pK_a near 6.6. Since the pH - K_I profiles for the boronic acids are quite different from those for the noncovalently bound inhibitors, sulfonic acids and amides, a structure similar to that in Figure 10 can tentatively be proposed for the inhibitory complex (3). Recent X-ray crystallographic studies done on subtilisin BPN' complexed with phenylethaneboronic acid and benzeneboronic acid have demonstrated that structures similar to Figure 10 are indeed present (3). In both cases, the boronic acids are tetrahedrally coordinated to the serine residue of the active site, although the aromatic ring of the benzene-boronic acid does not seem to extend into the hydrophobic cleft of the specificity site (a nonproductive association). This indicates that there is some nonspecific binding of these molecules at the active site. However, the structure proposed in Figure 10 seems to represent a good analogue of the metastable tetrahedral intermediate (transition state) for the reaction.

Boron acids also seem to be potential transition state analogues for acetylcholinesterase (1). This enzyme hydrolyzed acetylcholine by first going through a tetrahedral transition state during the acylation of the active site serine hydroxyl (43). The borinic acid analogue of the substrate (see Figure 12), acetylcholine, exhibits potent inhibition of the enzyme (1) and a possible structure for the enzyme-inhibitor complex involves the active site serine in

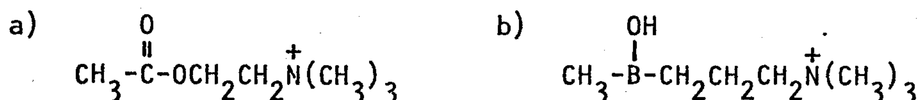


Figure 12. a) Acetylcholine. b) Borinic acid analogue of acetylcholine.

a structure similar to that of the tetrahedral intermediate (see Figure 13). Borinic acids also form stable tetrahedral adducts with

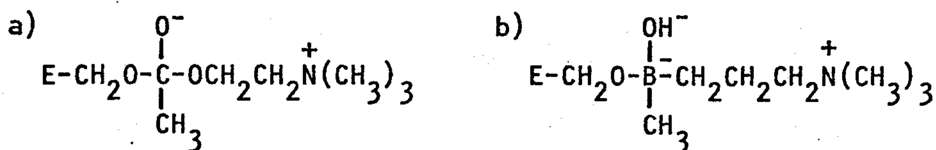


Figure 13. a) Enzyme-acetylcholine tetrahedral intermediate. b) Enzyme-borinic acid adduct.

oxygen atoms.

It was found that the borinic acid analogue binds four orders of magnitude tighter to acetylcholinesterase than does acetylcholine at pH 7.5 and 25°C (1); that is, $K_T/K_S = 10^4$. This is consistent with the prediction from transition state theory that an inhibitor-enzyme complex which mimics the metastable tetrahedral intermediate will be quite tightly bound with respect to the substrate-enzyme complex.

In the case of chymotrypsin, certain small, specific peptide aldehydes have been shown to exhibit potent inhibition on proteolytic activity (44). They are among the most efficient small molecular weight reversible inhibitors of α-chymotrypsin studied thus far, having $K_I = 10^{-5} - 10^{-6}$M. In each case, the presence of an aldehyde group seems to be essential for a good inhibitor. However, these peptides are not as effective as the elastase specific substrate aldehyde analogues, which had binding constants of $10^{-7} - 10^{-8}$M (7).

Indeed, the K_I value of N-Ac-L-Leu-L-Phenylalaninal is of the same order of magnitude as the noncovalent binding constant for the chymotrypsin specific ester substrate, N-Ac-L-Leu-L-Tyr-Methyl Ester, whose $K_S = 4 \times 10^{-5}M$ (44).

In each of these investigations, a covalent association between inhibitor and enzyme has been presumed. The complex between the boronic acids and subtilisin, however, is the only case in which the presence of such a covalent adduct is fairly certain, due to X-ray crystallographic studies. We can only infer from such information as the pH - K_I profiles that a covalent complex exists, especially for the amino and small peptide aldehyde inhibitors.

Recently, a study by Gorenstein (25) has presented direct evidence supporting the position that a nonspecific aldehyde substrate analogue, trans-cinnamaldehyde, binds to α-chymotrypsin as the free aldehyde. Specifically, using proton NMR studies on solutions of inhibitor and enzyme, it was found that the aldehydic proton chemical shift did not change in the presence of enzyme, thus ruling out binding as either the hemiacetal or aldehyde hydrate. In this case, trans-cinnamaldehyde cannot be considered an adequate transition state analogue for chymotrypsin catalyzed reactions. Whether or not this is the case for other amino and peptide aldehydes and other enzymes remains to be determined.

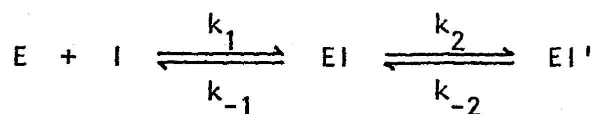
CHAPTER III

MATERIALS AND METHODS

KINETIC DERIVATION

We wish to investigate the affinity of hydrocinnamaldehyde to the active site of the hydrolase enzyme α-chymotrypsin between pH 4.5 and pH 8.3. Hydrocinnamaldehyde may bind to the active site either as the free aldehyde, in the hydrated form, or covalently attached to the serine-195 as a hemiacetal, with the sp^3 configuration which is similar to the tetrahedral intermediate or transition state of chymotrypsin catalyzed hydrolysis of ester and amide substrates (5, 7, 8, 31, 45). Thus, it may be a transition state analogue for hydrocinnamate methyl ester or hydrocinnamide, which are nonspecific substrates of chymotrypsin (46).

If hydrocinnamaldehyde binds as a hemiacetal to α-chymotrypsin, the binding will be a two-step process, similar to the reaction of substrates (31). In the first step, the aldehyde (I) may combine reversibly with the enzyme (E) to form the Michaelis complex (EI). In the second step a hemiacetal is formed with the active site serine-195 (EI') and this complex has the tetrahedral configuration proposed to be the enzyme-substrate transition state of chymotrypsin catalyzed reactions (5, 7, 8, 31, 45).



$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI'$$

For the following derivation, we may assume $k_{-1} >> k_2$. It follows that

$$K_I = \frac{(E)(I)}{(EI) + (EI')} \tag{1}$$

We need to find an expression for (EI').

$$\frac{(EI)}{(EI')} = \frac{k_{-2}}{k_2}$$

Therefore, $$(EI') = \frac{(EI) \cdot k_2}{k_{-2}} \tag{2}$$

We can also define a K_S,

$$\frac{(E)(I)}{(EI)} = \frac{k_{-1}}{k_1} = K_S$$

and thus derive an expression for (EI).

$$(EI) = \frac{(E)(I)}{K_S} \tag{3}$$

Substituting equations 2 and 3 into equation 1:

$$K_I = \frac{(E) \cdot (I)}{\dfrac{(E)(I)}{K_S} + \dfrac{(EI) \cdot k_2}{k_{-2}}}$$

$$K_I = \frac{(E)\cdot(I)}{\dfrac{(E)(I)}{K_S} + \dfrac{k_2}{k_{-2}}\left(\dfrac{(E)(I)}{K_S}\right)} = \frac{(E)\cdot(I)}{\dfrac{(E)(I)}{K_S}\left(1 + \dfrac{k_2}{k_{-2}}\right)}$$

$$K_I = \frac{K_S}{1 + k_2/k_{-2}} \tag{4}$$

If we assume that the formation of the hemiacetal (step k_2) is general base catalyzed and its decomposition (step k_{-2}) is general acid catalyzed, both by the imidazole of Histidine-57 (31, 46, 47), then the rate constants k_2 and k_{-2} may be rewritten as follows:

$$k_2 = \frac{k_{2(lim)}}{1 + (H^+)/K_a} \tag{5}$$

$$k_{-2} = \frac{k_{-2(lim)}}{1 + K_a/(H^+)} \tag{6}$$

Substituting equations 5 and 6 into equation 4:

$$K_I = \frac{K_S}{1 + \dfrac{\left(\dfrac{k_{2(lim)}}{1 + (H^+)/K_a}\right)}{\left(\dfrac{k_{-2(lim)}}{1 + K_a/(H^+)}\right)}}$$

$$K_I = \frac{K_S}{1 + \dfrac{\left(\dfrac{k_{2(lim)}}{(K_a + (H^+))/K_a}\right)}{\left(\dfrac{k_{-2(lim)}}{((H^+) + K_a)/(H^+)}\right)}}$$

$$K_I = \frac{K_S}{1 + \dfrac{\left(\dfrac{k_{2(lim)} \cdot K_a}{K_a + (H^+)}\right)}{\left(\dfrac{k_{-2(lim)} \cdot (H^+)}{(H^+) + K_a}\right)}}$$

$$K_I = \frac{K_S}{1 + \dfrac{k_{2(lim)}}{k_{-2(lim)}} \cdot \dfrac{K_a}{(H^+)}} \tag{7}$$

Now let

$$K' = \frac{k_{2(lim)}}{k_{-2(lim)}} \cdot K_a \tag{8}$$

for $\dfrac{k_{2(lim)}}{k_{-2(lim)}}$ is a constant.

Substituting equation 8 into equation 7:

$$K_I = \frac{K_S}{1 + K'/(H^+)} \tag{9}$$

It can be predicted from this equation that as (H^+) increases, the term $K'/(H^+)$ will become smaller and smaller, thus becoming less significant and K_I will approach K_S. However, with a small (H^+), the term $K'/(H^+)$ becomes very significant and K_I will decrease with respect to K_S.

In summary, the pH dependency shows that at low pH (high H^+ concentration) $K_{I(obs)}$ will approach K_S, and at high pH (low H^+ concentration) $K_{I(obs)}$ will be much less than K_S. In contrast, if the aldehyde only bound noncovalently to the enzyme, one would predict that the binding would be pH independent between pH 4.5 and pH 8.3, such as is found for the noncovalent binding of neutral compounds to the active site of α-chymotrypsin (48).

pH DEPENDENCY OF K_I OF HYDROCINNAMALDEHYDE

The pH dependency of K_I was investigated in order to test the prediction made above by equation 9 (see Chapter III, "Kinetic Derivation"). Binding constants (K_I) for hydrocinnamaldehyde to α-chymotrypsin will be obtained from the inhibition of N-acetyl-L-tyrosine ethyl ester hydrolysis by standard steady state techniques (49, 50) with solutions at 25°C, approximately 10% acetonitrile, approximately 0.1 _M_ NaCl, and approximately 0.05 _M_ in buffer component.

1. Materials

α-Chymotrypsin (three times crystallized) was obtained from Worthington Biochemical Corporation, Lot #CDI 2LX, 55 u/mg. N-acetyl-L-tyrosine ethyl ester (ATEE) was obtained from Sigma Chemical Corporation, m.p. 79-80°C, literature m.p. 79-80°C (49). A commercial preparation from Aldrich Chemical Corporation of β-phenyl-propionaldehyde (hydrocinnamaldehyde was purified by re-distillation at 81°C and 2.75 mm Hg.

All spectrophotometric readings were taken on a Heath 707 double beam recording spectrophotometer in a thermostatted cell compartment with 1 cm silica cuvettes (Beckman Instruments Inc., Pyrocell Manu-

facturing Company, Inc.).

2. N-Acetyl-L-Tyrosine Ethyl Ester Activity Assays

Activity assays for chymotrypsin were carried out with N-acetyl-L-tyrosine ethyl ester (ATEE). Decreases in absorbance at 237 nm were recorded with time. Cuvettes were thermostatted at 25°C. Procedures differed slightly at the different pH's due to decreased activity of the enzyme and decreased solubility of the inhibitor at certain pH ranges. The reference cell holder contained a cell with a window which could be adjusted manually to allow different amounts of light to penetrate. This was adjusted during the equilibration time so that the difference in absorbance was at the desired level.

At pH's 7.8, 7.2, 6.7 and 6.2, the procedure was as follows: In a cuvette was placed 2.7 ml of buffer (0.05 M phosphate, 0.1 M NaCl, appropriate pH), to which was added 0.25 ml acetonitrile (for the uninhibited reaction) or 0.25 ml inhibitor solution (1.91×10^{-2} M hydrocinnamaldehyde in acetonitrile) (for the inhibited reaction). Then 0.1 ml of substrate solution (5.1×10^{-2} M ATEE in acetonitrile) was added and the contents mixed manually. The solution was then allowed to equilibrate and a baseline was obtained. An aliquot of enzyme solution (0.025 ml, 7.2×10^{-5} M chymotrypsin) was added to the sample cell, the contents mixed manually and the decrease in absorbance at 237 nm was recorded within two seconds. The entire reaction was recorded until an endpoint was reached where the absor-

bance change with time was very near zero.

At pH 5.7 the procedure was as follows:

In a cuvette was placed 2.7 ml of buffer (0.05 _M_ acetate, 0.1 _M_ NaCl with 8.8×10^{-4}_M_ chymotrypsin, pH 5.7) to which was added 0.25 ml acetonitrile (for the uninhibited reaction) or 0.25 ml of inhibitor solution (1.91×10^{-2}_M_ hydrocinnamaldehyde in acetonitrile) (for the inhibited reaction). The contents were mixed manually, the solution was allowed to equilibrate and a baseline was obtained. An aliquot of substrate solution (0.1 ml, 5.1×10^{-2}_M_ ATEE in acetonitrile) was added to the sample cell, the contents mixed manually and the decrease in absorbance at 237 nm was recorded within two seconds. The entire reaction was recorded until an endpoint was reached where the absorbance change with time was very near zero.

At pH's 5.2, 5.0 and 4.5 the procedure was as follows:

In a cuvette was placed 2.6 ml of buffer (0.05 _M_ acetate, 0.1 _M_ NaCl, appropriate pH), to which was added 0.25 ml acetonitrile (for the uninhibited reaction) or 0.25 ml of inhibitor solution (1.79×10^{-2}_M_ hydrocinnamaldehyde in acetonitrile) (for the inhibited reaction). Then 0.1 ml substrate solution (5.1×10^{-2}_M_ ATEE in acetonitrile) was added and the contents mixed manually, after which the solution was allowed to equilibrate and a baseline was obtained. An aliquot of enzyme solution (0.1 ml, 2.0×10^{-4}_M_ chymotrypsin) was added to the sample cell, the contents mixed manually and the decrease in absorbance at 237 nm was recorded within two seconds. The entire reaction was recorded until an endpoint was reached where the absor-

bance change with time was very near zero.

At pH 8.3 the procedure was as follows:

In a cuvette was placed 2.7 ml of buffer (0.05 M pyrophosphate, 0.1 M NaCl, pH 8.3), to which was added 0.25 ml acetonitrile (for the uninhibited reaction) or 0.25 ml inhibitor solution (1.91×10^{-2}M hydrocinnamaldehyde in acetonitrile) (for the inhibited reaction). Then 0.1 ml of substrate solution (5.1×10^{-2}M ATEE in acetonitrile) was added and the contents mixed manually, after which the solution was allowed to equilibrate and a baseline was obtained. An aliquot of enzyme solution (0.025 ml, 7.2×10^{-5}M chymotrypsin) was added to the sample cell, the contents mixed manually and the decrease in absorbance at 237 nm was recorded within two seconds. The entire reaction was recorded until an endpoint was reached where the absorbance change with time was very near zero.

At each pH, at least three reactions were recorded for each uninhibited and each inhibited reaction.

ATTEMPTED SYNTHESIS OF N-ACETYL-L-PHENYLALANINAL

An attempt was next made to synthesize a more specific inhibitor of α-chymotrypsin, N-acetyl-L-phenylalaninal (VI), which was to be synthesized through the series of reactions outlined below:

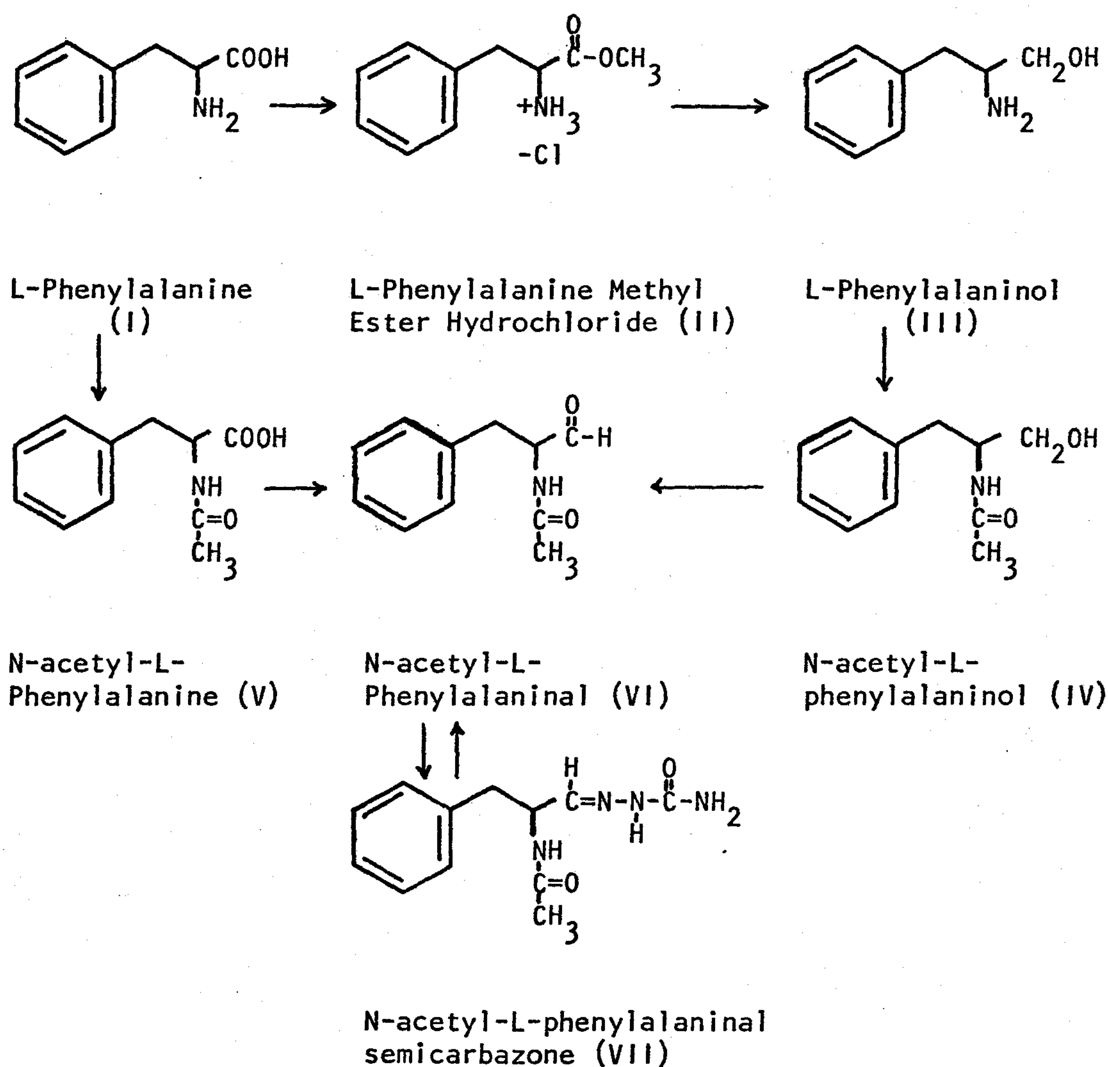


Figure 14. Synthesis of N-acetyl-L-phenylalaninal from L-Phenylalanine.

Phenylalanine and N-acetyl-L-phenylalanine were obtained from Sigma Chemical Corporation and Eastman Chemical Co. Lithium aluminum hydride was obtained from Matheson, Coleman and Bell. Lithium tri-tertiary-butoxy aluminum hydride, ethyl chloroformate, cinnamyl alcohol and cinnamaldehyde were obtained from Aldrich Chemical Co. Dicyclohexylcarbodiimide (DCCD) was obtained from Eastman Chemical Co. Phenylalaninol standard was obtained from Mann Research Laboratory. Seloxcette reagent was obtained from Ventrol Corporation, Alfa Products Division. All silica gel chromatography apparatus was obtained from Quantum Industries. All gases were obtained from Matheson and Liquid Carbonic. All solvents were obtained from Mallinckrodt Chemical Corp.

1. Phenylalanine Methyl Ester HCl (II) (51).

25 gm. phenylalanine (I) was dissolved with stirring in 375 ml methanol with HCl(g) bubbling through until all the phenylalanine was dissolved. The temperature was then reduced to 0°C with an ice-water bath for 30 minutes with HCl(g) still bubbling through. Then HCl(g) was bubbled through for an additional 3 hours at room temperature with constant stirring. The liquid was concentrated down to crystals by evaporation _in vacuo_. These were recrystallized from methanol. m.p. 159-161°C. Literature m.p. 159-161°C (52).

2. Phenylalaninol (III) (53).

L-phenylalanine methyl ester HCl (II) (15 g, 70 mmoles) was

added a little at a time during one hour to a vigorously stirring slurry of lithium aluminum hydride (7 g, 0.18 mmole) in tetrahydrofuran (250 ml). After stirring the mixture for a further 24 hours, distilled water was added dropwise (less then 50 ml) until the precipitate turned white. The precipitate was allowed to settle and the solution was first decanted and then filtered. The filtrate was slightly yellow and evaporation gave a more yellow oil and water mixture. The product should have been a colorless oil able to be triturated with diethyl ether and recrystallized from benzene to yield L-phenylalaninol. Trituration was done without success. A tlc was taken of the oil and matched with a phenylalaninol standard and the R_f values were quite close (0.70 for the oil and 0.73 for the standard in 3:1:1 Butanol:Acetic Acid:Water). Repeated crystallization attempts using various solvent systems did not yield any crystals. Each successive synthesis produced the same impurity, which showed up as a single spot of higher R_f than L-phenylalaninol when chromatographed in 3:1:1 Butanol:Acetic Acid:Water. This crude preparation was used in the subsequent step to synthesize N-acetyl-L-phenylalaninol (IV).

3. N-Acetyl-L-Phenylalaninol (IV).

To 1.3065 g of the crude preparation of L-phenylalaninol (III) in 30 ml $CHCl_3$ was added 0.56 ml pyridine and 0.66 ml acetic anhydride. This was stirred for 3 hours with a magnetic stirrer. The solution was yellow. After evaporation of the solvent, the yellow, oily

residue was dissolved in distilled water and then, after adding ice, the pH was increased to 10 by the addition of 1 N NaOH for about 15 minutes (to hydrolyze any secondary acetylation at the alcoholic hydroxyl group to form the ester). Then the solution was neutralized with HCl and Amberlite resin was added for about 30 minutes to remove any ions present. The solution was filtered and evaporated down to a yellow residue. This residue was then dissolved in about 250 ml ethyl acetate and the crystals that did not dissolve were filtered off (these were presumed to be NaCl). Upon evaporation, the residue was a yellow oil. The product was crystallized out of hot 2:1 ethyl acetate:hexanes. m.p. 100-101°C. IR was taken and is discussed below.

4. N-Acetyl-L-Phenylalaninal (VI).

This material was synthesized by a number of different procedures, attempting to maximize yield, purity and ease of synthesis.

a. Seloxcette oxidation - Seloxcette was obtained from Ventron Corporation and is composed of 50-58% chromium trioxide (CrO_3) intercalated into graphite. Seloxcette is said to be a reagent for the specific oxidation of alcohols to aldehydes or ketones, which will not over-oxidize to the carboxylic acid (54).

Following the directions given in the literature provided with the Seloxcette, and making a few modifications, the procedure was as follows:

N-acetyl-L-phenylalaninol (90 mg) was dissolved in 80 ml of

20% dimethylformamide (DMF) in toluene (due to insolubility of the alcohol in neat toluene), to which was added 500 mg Seloxcette (chromium trioxide intercalated in graphite) and the mixture was stirred at reflux for 20 hours. The reaction was followed by tlc in 9:1 $CHCl_3$:MeOH and successive tlc's showed the appearance of another species very close to the solvent front visualized with iodine and DNP positive. The solution was filtered and the filtrate evaporated. The residue was worked up by the bisulfite extraction procedure (55): The residue was partitioned between ethyl acetate and 0.1 M pH 7.5 phosphate buffer. The ethyl acetate layer was washed three times with buffer; the organic phase was dried over $MgSO_4$ and evaporated. This residue was dissolved in a small quantity of ethanol and distilled water was added to the cloud point. Then 5 ml of saturated aqueous sodium bisulfite ($NaHSO_3$) solution was added. The mixture was left to stand for 30 minutes and partially evaporated to approximately 2/3 volume. This was then extracted three times with diethyl ether. The aqueous phase was brought to pH 8.5 with 1 M Na_2CO_3 and extracted three times with ethyl acetate. The ethyl acetate fractions were pooled, dried over $MgSO_4$ and evaporated. Yield was only 6 mg of a white crystalline compound which, on tlc in 9:1 $CHCl_3$:MeOH, chromatographed as before very near the solvent front and was DNP positive. No other attempts at characterization have been made due to the small yield of the compound obtained by this method.

b. Phosphoric acid, Dimethyl Sulfoxide (DMSO) and Dicyclo-

hexylcarbodiimide (DCCD) oxidation (56, 57).

A solution of N-acetyl-L-phenylalaninol (2.0 g, 10.33 mmoles) and DCCD (6.3 g, 31 mmoles) in DMSO (10 ml) and benzene (10 ml) was mixed with a solution of H_3PO_4 (0.55 g, 5.6 mmoles) in DMSO (5 ml) under ice cooling for 30 minutes and the whole was allowed to stand at room temperature for 2 hours. Then a tlc was taken in 97:3 $CHCl_3$: MeOH and no starting material was seen to be present. The reaction was stopped after 3½ hours by the addition of a solution of oxalic acid (2.9 g) in MeOH (3 ml). This was stirred for an addition 2 hours at room temperature. Precipitates were filtered off and were washed with ethyl acetate. The filtrate and washings were combined, washed with saturated aqueous $NaHCO_3$ and H_2O, dried over $MgSO_4$ and evaporated to give a red-orange oil. Tlc's were done on all fractions of the above extraction procedure and were developed in 97:3 $CHCl_3$:MeOH, after which they were sprayed with DNP and KCN reagents (see below). In the ethyl acetate phase, a spot was seen which gave a yellow color with DNP-KCN migrating slightly higher than the alcohol and which was taken to be the aldehyde. This spot was not present in the aqueous fractions.

This material was then purified by passage over a silica gel column (Quantum Industries, 100 g. silica gel, 2.5 cm x 25 cm with 0.5 cm inlet). The column was first prepared by washing it with spectral grade $CHCl_3$ until the eluate was clear. The column was then washed with $CHCl_3$ (AR grade, 0.75% EtOH). Approximately 250 mg. of the crude mixture obtained from the oxidation procedure above was

layered on top of the silica gel column and eluted with $CHCl_3$ (0.75% EtOH) under 10 psi N_2 (dried) pressure. Fifty fractions were collected for 4 minutes each, after which 100% EtOH was pushed through the column to clean the rest of the material through it. This EtOH was also collected. The column was, after every use, dried overnight with air (first filtered through a drying tube).

All fractions were examined by tlc in 97:3 $CHCl_3$:MeOH and, when no spot corresponding to the aldehyde was seen, the EtOH wash was spotted. This revealed a spot corresponding to the aldehyde, plus a smear above and below it. The EtOH wash contained approximately 200 mg. material and this was re-layered on the above silica gel column in the same manner (after first wetting with solvent), but this time wetting and elution were done with 3% MeOH in $CHCl_3$. A seemingly good separation of the aldehyde spot from the lower R_f spots was achieved. Approximately 65 mg. of relatively pure aldehyde (one spot on tlc in 97:3 $CHCl_3$:MeOH) was recovered. This material was later used for IR and NMR analysis (see below).

This silica gel column chromatography method of purification was used subsequently to purify more reaction material; however, the method seemed capricious in that it gave good separation of the aldehyde spot from the impurities only some of the time and at other times no fractions contained pure aldehyde.

5. N-Acetyl-L-Phenylalaninal (VI).

An alternate method of synthesizing this compound is by

reduction from the acid. Two such methods were employed.

a. Reduction of the mixed anhydride (56).

To a solution of N-acetyl-L-phenylalanine (2.5 g, 12.1 mmoles) in 50 ml dry tetrahydrofuran (THF) was added triethylamine (Et_3N, 1.83 ml, 13.3 mmoles). Then a solution of ethyl chloroformate (1.06 ml., 13.3 mmoles) in 20 ml THF was added dropwise under ice cooling. Colorless precipitates were filtered off and washed with 30 ml dry THF and the filtrate and washings were combined and returned to the ice bath. Then acetic acid (1.2 ml) and 5% Pd-C (2.0 g) were added and H_2(g) was bubbled through the mixture for 8 hours at 3-5°C. Catalyst was filtered off and the filtrate was evaporated _in vacuo_ to give an oily, yellow residue.

This material was worked up by the bisulfite extraction procedure (55): The residue was partitioned between ethyl acetate and 0.1 _M_ pH 7.5 phosphate buffer. The ethyl acetate layer was washed three times with buffer. The organic phase was then dried and evaporated. The residue was dissolved in a small quantity of ethanol, and distilled water was added to the cloud point. Then 5 ml of saturated aqueous $NaHSO_3$ solution was added. This was left to stand at room temperature approximately 30 minutes and partially evaporated. The solution was then extracted three times with diethyl ether. The aqueous phase was brought to pH 8.5 with 1 _M_ Na_2CO_3 and extracted three times with ethyl acetate. The pooled ethyl acetate layers were dried and evaporated. Upon tlc in 97:3 $CHCl_3$:MeOH of all the fractions, a spot moving slightly higher than the alcohol was found

in the ethyl acetate residue and this was assigned to the aldehyde. This spot was also DNP positive, turning yellow after being sprayed with the DNP reagent. However, this material still was not pure or crystallizable.

b. Reduction of the carboxylic acid imidazolide (58).

To a solution of N-acetyl-L-phenylalanine (5 mmoles, 1.035 g) in THF (10 ml) cooled to 0°C in an ice bath was added carbodiimidazole (5.5 mmoles, 892 mg) and the resulting solution was stirred for 10 minutes at 0-5°C and then cooled to -20°C in a dry ice-CCl_4 bath. To the reaction mixture was added Lithium tri-tertiary-butoxy aluminum hydride (20 mmoles, 5.085 g) in THF (5 ml) over a period of 30 minutes, maintaining the temperature at -15 to -20°C and the reaction mixture was stirred for 20 minutes at -20°C. After decomposition of excess reagent by the addition of 15 ml of 5% NaOH, the mixture was warmed to room temperature, precipitates were filtered off and washed with THF (20 ml). The filtrate and washings were combined and washed five times with saturated saline solution. The organic phase was then dried over $MgSO_4$ and the solvent was evaporated to leave a pale yellow oil. On tlc in 97:3 $CHCl_3$:MeOH, two spots were uv positive, one at R_f = 0.25, corresponding to the alcohol, and one at R_f = 0.50, presumably the aldehyde, which turns yellow-orange with the DNP spray reagent.

This material was then run on a preparative tlc plate 200 μ thick (Quantum Ind.) in 95:5 $CHCl_3$MeOH solvent system. The spots were visualized under uv light and, while keeping the gel as wet

with solvent as possible, the spots were scraped off the plate and the gel was washed with $CHCl_3$. The solvent was evaporated and the residue remaining from the higher spot was used for an IR as an oil between two NaCl plates. The IR showed no aldehydic peak at 1740, but this would be the case if the aldehyde were in the hydrated form. This material (144 mg) was then dried over Na_2SO_4, evaporated and used for making a DNP derivative in solution (see below for IR and procedure for DNP derivative). This gave a very dirty looking reddish-brown precipitate. Its melting point was far above that of the literature value of the 2,4-dinitrophenylhydrazone of N-acetyl-L-phenylalaninal (200-201°C from (56)), so the synthesis seemed nonproductive.

6. N-Acetyl-L-Phenylalaninal Semicarbazone (VII).

Because of the unknown stability of the aldehyde, N-acetyl-L phenylalaninal, a method was investigated to "trap" the aldehyde as the semicarbazone derivative <u>in situ</u>, a compound which would be more easily crystallizable (59). This method was developed using commercially available cinnamaldehyde.

To 1 mmole of cinnamaldehyde, add 0.5 ml of semicarbazide reagent (0.444 g semicarbazide HCl in 2 ml H_2O) and 1 ml methanol (enough to produce a clear solution); then add pyridine (a few drops) and warm solution gently on a steam bath for a few minutes until crystals begin to separate. Filter and save crystals, m.p. 214-216°C.

This method was found not necessary, as the aldehyde was stable in organic solution up to three months.

7. Regeneration of aldehyde from its semicarbazone (59).

As in the above reaction, this method was worked out using cinnamaldehyde and was found not to be necessary in the synthesis of N-acetyl-L-phenylalaninal.

The aldehyde semicarbazone was dissolved in acetone and water and the pH was reduced to 1 with HCl. The solution was stirred for 2½ hours to hydrolyze the semicarbazone. The reaction was followed by tlc and a spot at the R_f characteristic of cinnamaldehyde was seen. The reaction was stirred for an additional 5 hours and then the solution was neutralized with NaOH. After filtering and evaporating, the residue was taken up in 1-2 ml $CHCl_3$ and purified by passing over a silica gel column eluted with $CHCl_3$.

8. Preparation of spray reagents for aldehyde identification (60).

DNP spray: 1 ml 36% hydrochloric acid is added to a solution of 100 mg 2,4-dinitrophenylhydrazine in 100 ml ethanol (96%).

After spraying tlc with the above spray reagent, the 2,4-DNP derivatives may be differentiated by subsequent spraying with a 0.2% solution of potassium ferricyanide in 2N hydrochloric acid. The 2,4-DNP derivatives of saturated ketones give a blue color immediately; those of saturated aldehydes react more slowly and turn olive green. The colors of the 2,4-DNP derivatives of unsaturated carbonyl compounds change only slowly or not at all. Cinnamaldehyde was found to react with a bright orange color after spraying with

the DNP reagent.

9. IR Studies.

All IR's were done on a Perkin-Elmer 337 Grating Infrared Spectrophotometer.

a. IR of the 2,4-dinitrophenylhydrazone of N-acetyl-L-phenylalaninal made by the reduction of the carboxylic acid imidazolide (see 5b) gave the following results: IR_{max}^{KBr} cm^{-1}: 3295 (NH), 1625, 1510 (amide), 1605 (C=N), 1550, 1331 (NO_2) (see Figure 15). These results are consistent with those found in the literature (56).

b. Comparison of IR's of N-acetyl-L-phenylalaninol and N-acetyl-L-phenylalaninal prepared by the phosphoric acid/DMSO/DCCD oxidation reaction (see 4b) showed basically the same spectrum, except for the introduction of a peak at 1740 cm^{-1} (-HC=O) in the aldehyde (see Figure 16).

10. NMR Studies.

NMR studies were done at Loyola University, Lake Shore Campus by Dr. David Crumrine on N-acetyl-L-phenylalaninal prepared by the phosphoric acid/DMSO/DCCD oxidation reaction (see 4b) and showed a pattern consistent with that found in the literature (56):

NMR (in $CDCl_3$) τ: 8.01 (3H, singlet, $-COC\underline{H_3}$), 2.72 (5H, singlet, $\underline{C_6H_5}-$), 0.30 (1H, singlet, $-C\underline{H}O$).

The NMR, however, indicated that the aldehyde was only 30-50% pure and perhaps had some contaminating hydrocarbons (see Figure 17).

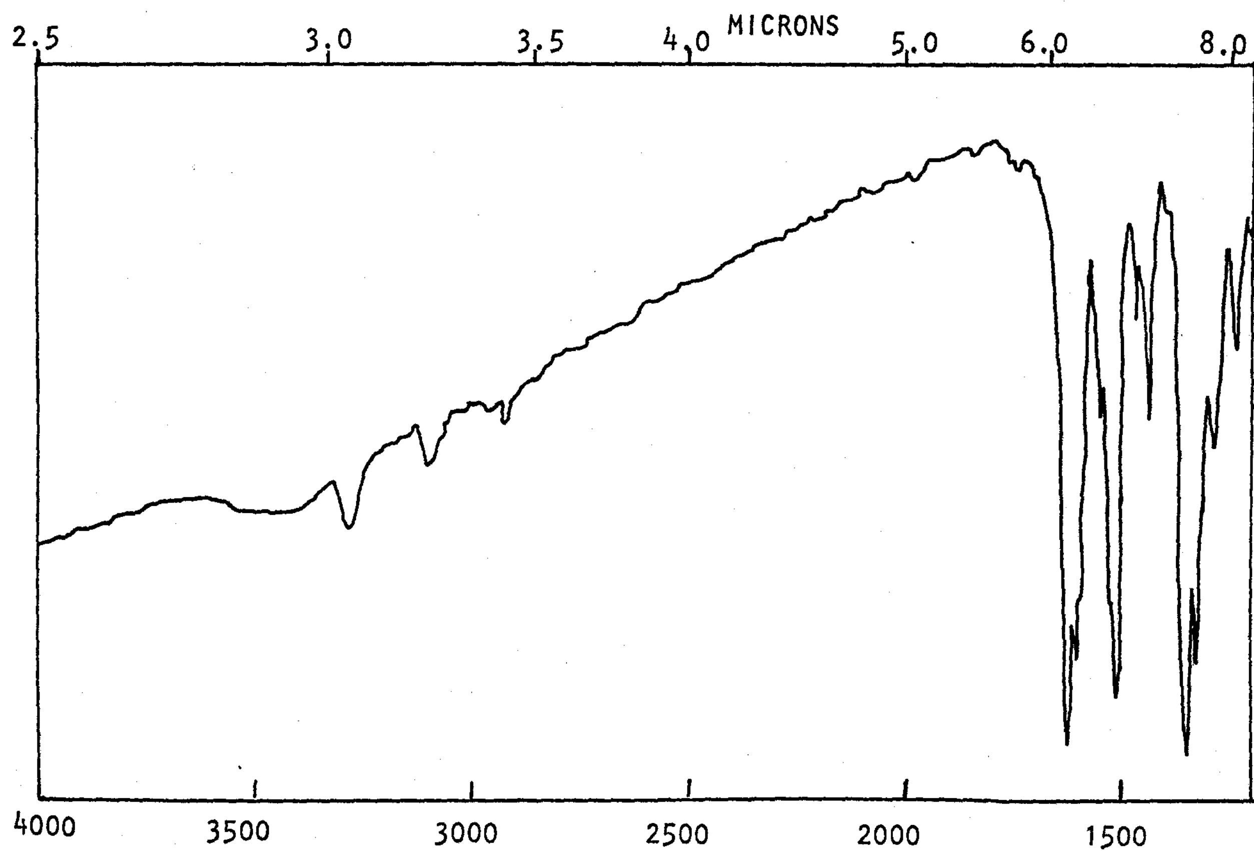
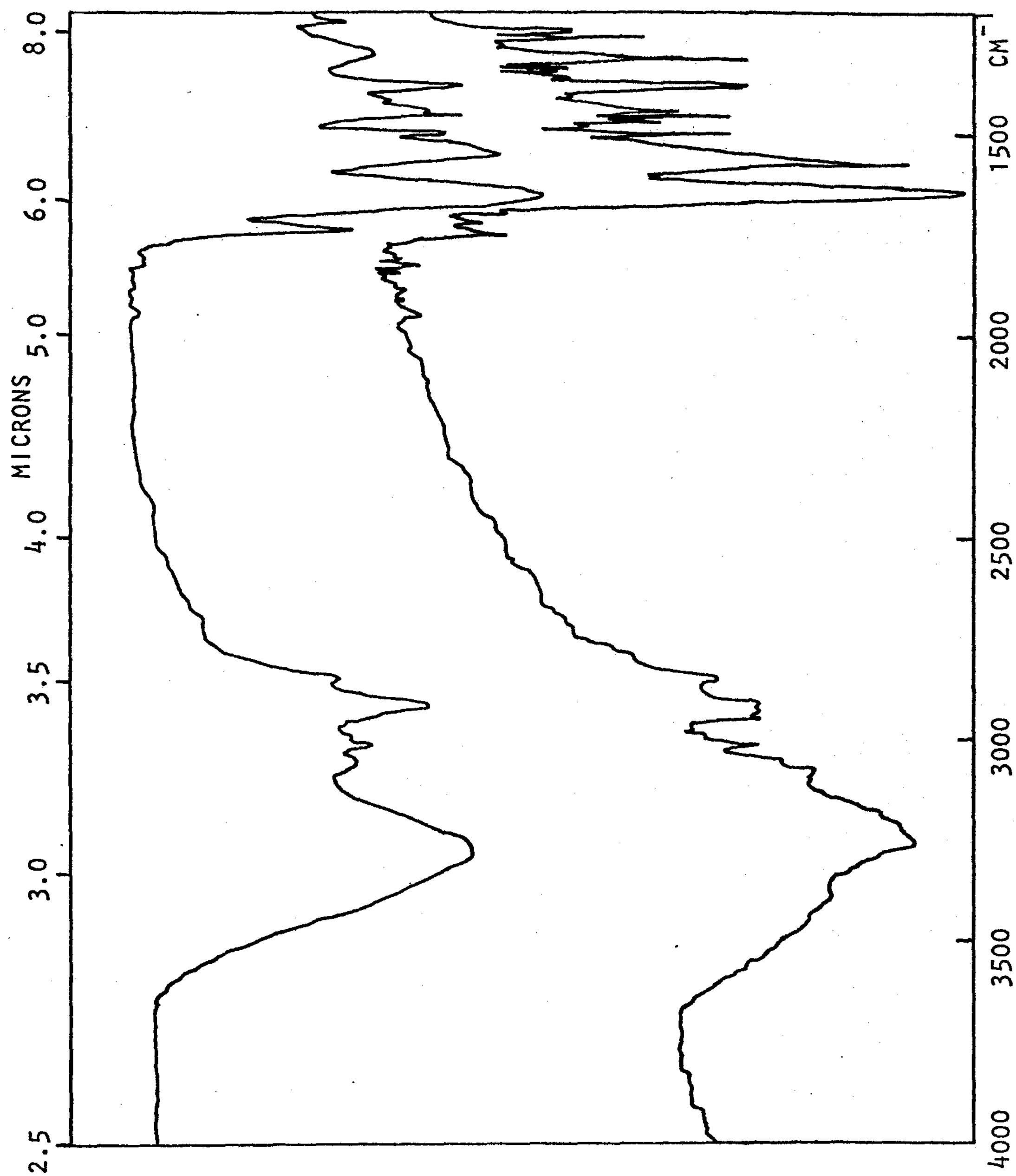


Figure 15. IR of the 2,4-Dinitrophenylhydrazone derivative of N-acetyl-L-phenylalaninal.

Figure 16. A Comparison of the IR's of N-acetyl-L-phenylalaninol (lower line) and N-acetyl-L-phenylalaninal (upper line).



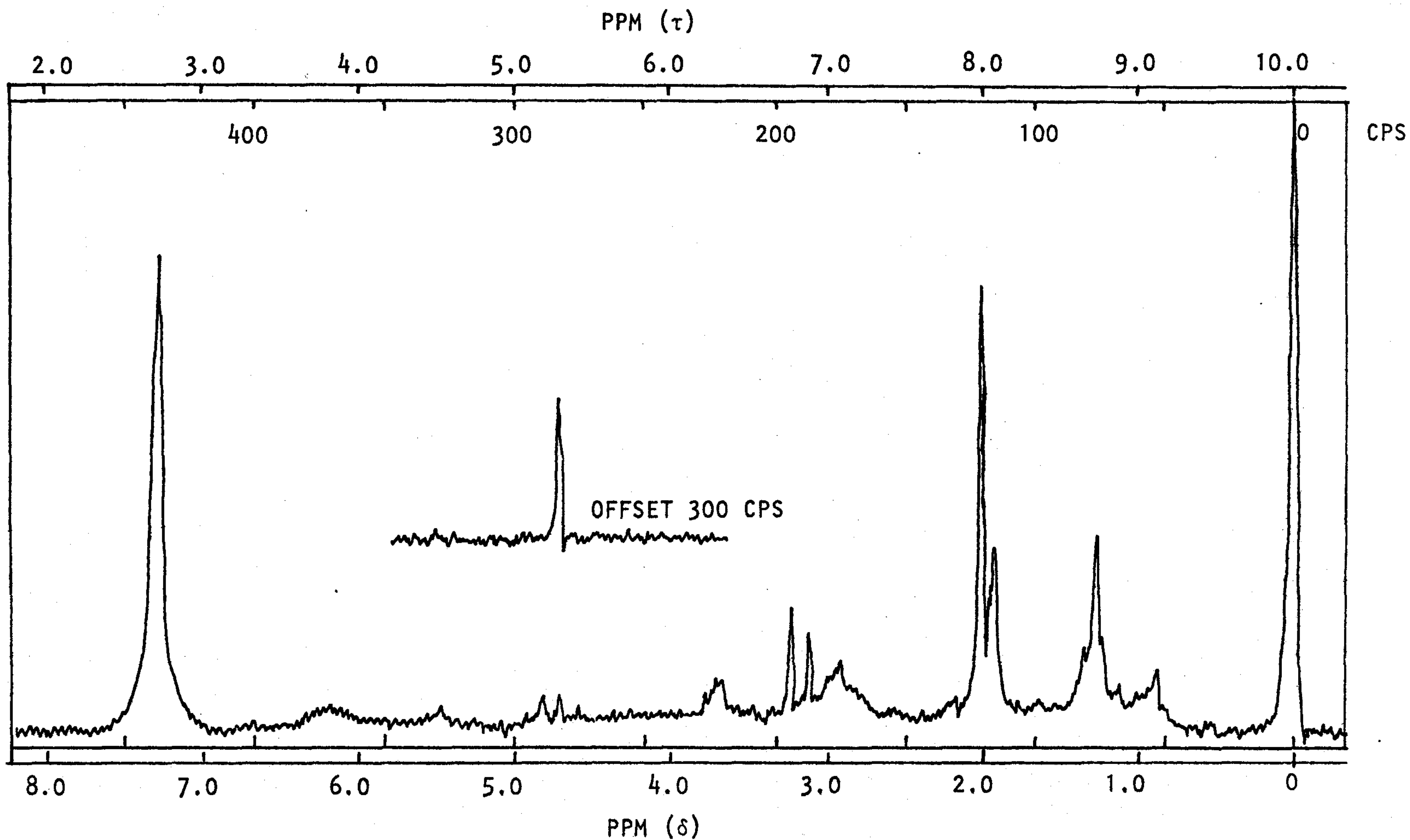


Figure 17. NMR of N-acetyl-L-phenylalaninal.

CHAPTER IV

RESULTS AND DISCUSSION

THE pH DEPENDENCY OF BINDING OF HYDROCINNAMALDEHYDE TO α-CHYMOTRYPSIN

All calculations were done on an Olivetti Programmable Calculator.

Lineweaver-Burke plots were drawn from the data collected in the ATEE assays at various pH's. The K_I at each pH was determined from the ratio of slopes of inhibited and noninhibited Lineweaver-Burke plots. Since the inhibition is assumed to be competitive, we may state the following:

$$K_{m(app)} = K_m(1 + (I)/K_I)$$

The ratio of the slopes of the inhibited and noninhibited plots can be simplified as follows:

$$M = \frac{slope_{(inh)}}{slope_{(non\text{-}inh)}} = \frac{K'_{m(app)}/k_{cat} \cdot V_{max}}{K_{m(app)}/k_{cat} \cdot V_{max}} = \frac{K'_{m(app)}}{K_{m(app)}}$$

$$M = \frac{K_m(1 + (I)/K_I)}{K_m} = 1 + \frac{(I)}{K_I}$$

$$K_I = \frac{(I)}{M - 1} \qquad (1)$$

Equation 1 was used to calculate the value of K_I at each pH, aver-

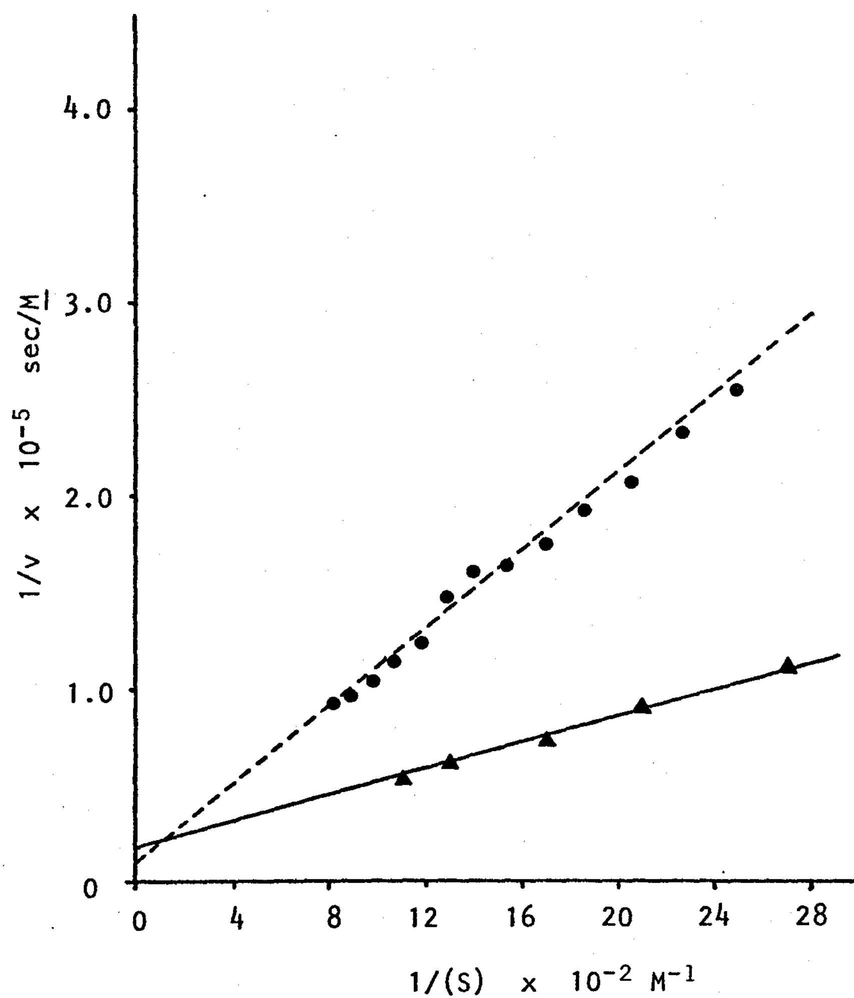


Figure 18. Lineweaver-Burke plot of ATEE hydrolysis at pH 7.8. Noninhibited reaction (——) and inhibited reaction (---).

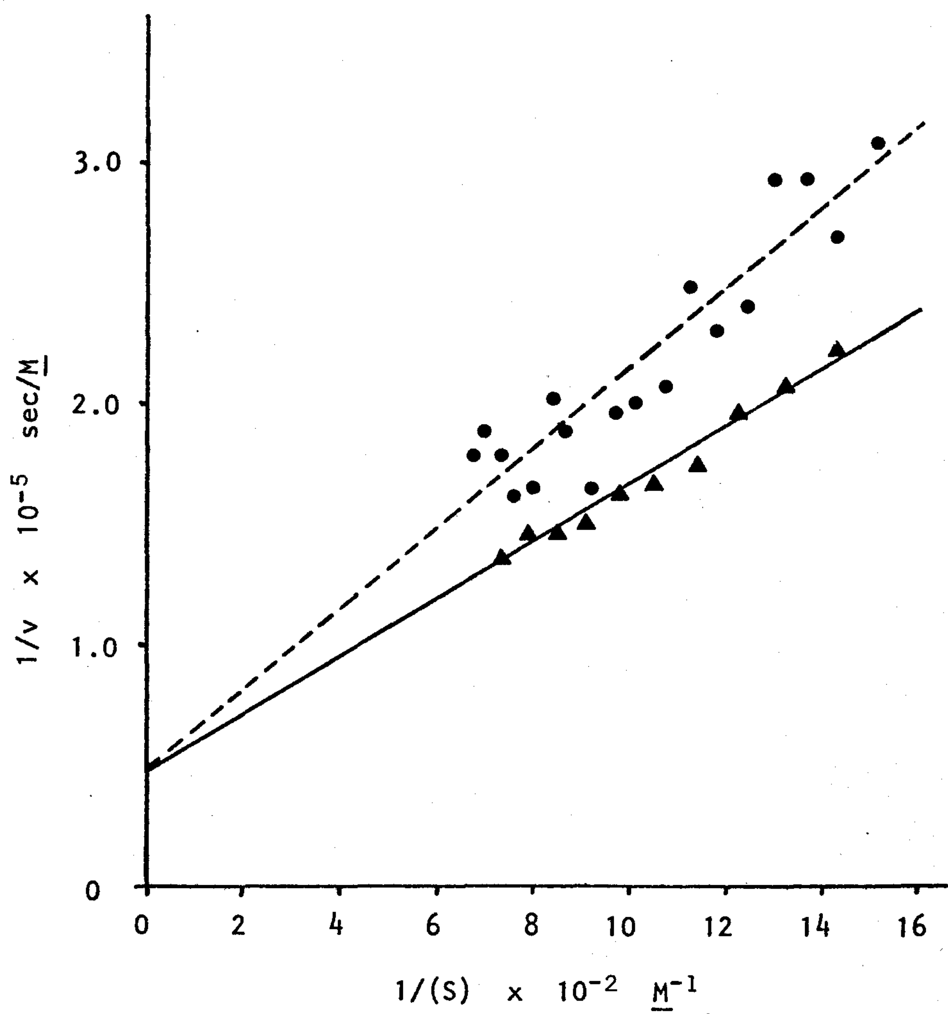


Figure 19. Lineweaver-Burke plot of ATEE hydrolysis at pH 4.7. Noninhibited reaction (——) and inhibited reaction (---).

aging the values from at least three reactions. The results are listed in Table III.

Theoretical curves were then drawn, using the equation derived previously in "Materials and Methods, Kinetic Derivation" (equation 9), and reproduced below.

$$K_I = \frac{K_S}{1 + K'/(H^+)} \quad \text{where} \quad K' = \frac{k_{2(lim)}}{k_{-2(lim)}} \times K_a \qquad (2)$$

Values were substituted into equation 2 for K_a, K_S, and $k_{2(lim)}/k_{-2(lim)}$ to attempt to get the best fit to the experimental data. Figure 20 shows the theoretical curve for K_I *vs*. pH based on equation 2 and values of $K_a = 10^{-7}$, $K_S = 5.5 \times 10^{-3}$*M* and $k_{2(lim)}/k_{-2(lim)} = 5$. The values for K_S and $k_{2(lim)}/k_{-2(lim)}$ are those chosen to give the best fir to the experimental points. The points on the graph are the experimental values of K_I obtained above (see Table III). A small deviation from the theoretical line is observed in the region of high pH (pH > 7) where the experimental values of K_I are slightly poorer than predicted. However, it has been previously reported that the binding of negatively charged molecules to the active site in α-chymotrypsin is slightly poorer above pH 7 than below, due to a repulsion by a negative charge of the active site above pH 7 where the imidazole group of histidine-57 is uncharged (48, 61). Thus, a slight deviation from the theoretical line above pH 7 might occur if a full or partial negative charge were present in the complexes EI or EI'. The presence of such a charge in the tetrahedral configuration of the hemiacetal

Table III. Binding Constants Obtained for the Binding of Hydrocinnamaldehyde to α-Chymotrypsin.

$K_I \times 10^3$, M	pH	Buffer
5.8 ± 0.3	4.5	Acetate
5.2 ± 1.4	5.0	Acetate
4.8 ± 0.7	5.2	Acetate
4.0 ± 0.3	5.7	Acetate
3.4 ± 0.1	6.2	Phosphate
1.8 ± 0.2	6.7	Phosphate
1.3 ± 0.2	7.2	Phosphate
0.79 ± 0.15[a]	7.8	Phosphate
0.62 ± 0.21[a]	8.3	Pyrophosphate

[a]Corrected for the increase in K_S due to a group in the enzyme of pK_a 8.7 (31).

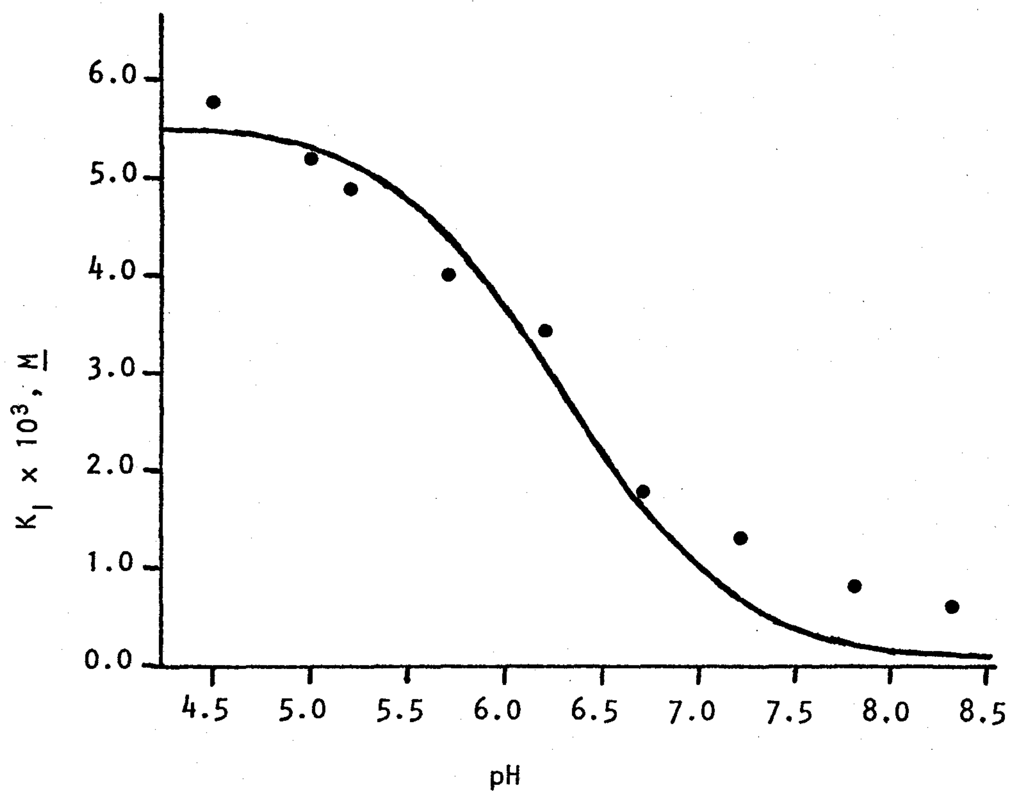


Figure 20. pH Dependency of K_I for hydrocinnamaldehyde binding to α-chymotrypsin.

or bound hydrated form of the aldehyde is not unexpected, since a few proposed mechanisms for chymotrypsin catalysis postulate a negative or partial negative charge in the tetrahedral transition state in substrate hydrolysis (45, 47).

Bender et al. (46) have argued that for N-acetyl-L-tryptophane amide the ratio of first order rate constants for the enzyme-catalyzed hydrolysis, k_e, to the nonenzymic hydrolysis proceeding through the same mechanism, k_n, is 10^8. Since hydrocinnamide is a nonspecific substrate of the enzyme, the ratio k_e/k_n will be smaller, approximately 10^5. Thus, if hydrocinnamaldehyde were a good transition state analogue for this substrate, the transition state theory would predict (1, 2) that the K_I for hydrocinnamaldehyde wo-ld be smaller than the K_S for hydrocinnamide by a factor of approximately 10^5. Surprisingly, the K_I found for hydrocinnamaldehyde at pH 7.8 is only 7 times better than the binding constant found for the substrate hydrocinnamide (62). In addition, the stability of EI' relative to EI ($k_{2(lim)}/k_{-2(lim)}$ = 5) is only twice that found for the aldehyde hydrate in water (K_h = 2.7 (26)).

Thus, it appears that the covalent hemiacetal intermediate (EI') for hydrocinnamaldehyde has a stability similar in magnitude to the stability of a hydrated aldehyde in solution, and that the binding of the hydrated tetrahedral form of hydrocinnamaldehyde (K_S) is not much better than that for hydrocinnamide. These results indicate that α-chymotrypsin does not show any particular binding strength to the sp^3 tetrahedral configuration as depicted by the hemiacetal

structure of this aldehyde inhibitor.

The differences between the relatively strong binding found previously with aldehyde analogues of papain and elastase substrates (7, 8) and the relatively poor binding found for hydrocinnamaldehyde to α-chymotrypsin in this work, may reflect the differences in substrate specificity as on the ratio $k_{2(lim)}/k_{-2(lim)}$. We may look at ester substrate hydrolysis by chymotrypsin for an analogy. Ester substrates form an sp^2 acylserine intermediate during catalysis by the enzyme (31, 46). The ratio $k_{acylation}/k_{deacylation}$ may be 10^3 times greater for specific substrates of α-chymotrypsin than for less specific substrates, due to the greater effect of specificity on $k_{acylation}$ than on $k_{deacylation}$ (46). We may infer that the ratio $k_{2(lim)}/k_{-2(lim)}$ may similarly vary with specificity by 10^3, and this factor is reflected in the value of K_I according to equation 2. A factor of 10^3 will explain the differences in K_I found for aldehyde analogues of specific substrates to elastase and papain and of hydrocinnamaldehyde to α-chymotrypsin.

Since this study was completed, a number of studies have been undertaken to investigate whether the amino aldehydes bind to α-chymotrypsin as the free aldehyde, the aldehyde hydrate or hemiacetal. As mentioned earlier in the "Review of Related Literature," a recent study by Gorenstein _et al_. (25) has presented direct NMR evidence that _trans_-cinnamaldehyde binds to α-chymotrypsin as the free aldehyde. A study by Breaux and Bender (63), using p-dimethylaminocinnamaldehyde, finds by uv spectrophotometric techniques that the predominant mode

of binding to α-chymotrypsin is noncovalent and K_I approximates K_S. However, two studies have been done which tend to support hemiacetal formation between hydrocinnamaldehyde and α-chymotrypsin as the major factor in the binding affinity. Lowe and Nurse (26) have detected hemiacetal formation between α-chymotrypsin and the inhibitor hydrocinnamaldehyde by NMR spectroscopy. A study by Schultz and Kennedy (27) has investigated the binding of N-benzoyl-L-phenylalaninal to α-chymotrypsin and dehydroalaninyl-chymotrypsin and shown that the binding is much poorer to dehydroalaninyl-chymotrypsin than to native. Thus, it appears that the serine-195 must be important in the binding of this aldehyde, suggesting hemiacetal formation.

SYNTHESIS OF N-ACETYL-L-PHENYLALANINAL

All reactions preceding the final step of aldehyde synthesis were worked out to a satisfactory level. However, of the four methods used to synthesize N-acetyl-L-phenylalaninal, none of them provided a product of satisfactory yield or purity for use in enzyme kinetic investigations. The Seloxcette oxidation procedure gave a yield which was much too small. The phosphoric acid/DMSO/DCCD oxidation method seemed to provide the purest compound after purification by silica gel column chromatography; however, this still was not more than 50% pure by NMR and not crystallizable. Neither of the two reductive procedures produced a compound of high enough yield or purity (even after silica gel chromatography) to allow positive identification of the aldehyde.

In this worker's hands, the synthesis of N-acetyl-L-phenylalaninal proved to be very difficult and even when a small quantity of aldehyde was recovered (i.e. after the phosphoric acid/DMSO/DCCD oxidation), the purification was extremely difficult. This is not surprising, however, since the one report of N-acetyl-L-phenylalaninal synthesis (56) also reported an impure oil as the final product. It remains for others to perfect this synthesis and to synthesize other aldehyde analogues of specific substrates of chymotrypsin, in order to pursue the elusive functions of enzymes as biological catalysts.

CHAPTER V

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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