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Associations of N-acyl Amino Aldehyde Analogs of Specific Substrates to the Active Site of Chymotrypsin

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ASSOCIATIONS OF N-ACYL AMINO ALDEHYDE ANALOGS OF SPECIFIC SUBSTRATES TO THE ACTIVE SITE OF CHYMOTRYPSIN

by

William Patrick Kennedy

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LIFE

William Patrick Kennedy is the son of Harold James and Jean Marie (Lucas) Kennedy. He was born on September 9, 1951, in Saginaw, Michigan.

William recieved a Catholic primary education at a local parish school, the Shrine of the Little Flower, in Royal Oak, Michigan. His secondary education was provided by Sacred Heart Seminary High School in Detroit, Michigan, where he graduated in June, 1969.

In September, 1969 he entered Oakland University, in Rochester, Michigan. There he held the position of class chairman and student curriculum advisor for an intra-university student body, Allport College.

In September, 1971 William transferred to Western Michigan University in Kalamazoo and in April, 1974 was awarded a Bachelor of Science degree in Chemistry with minors in Mathematics and Biology. While attending the University, he worked as an assistant animal caretaker for The Upjohn Company and as a volunteer research assistant.

In October, 1974 he took a position as Research Assistant in the Department of Nuclear Medicine at the University of Michigan Medical Center.

In July, 1976 William was granted a Basic Science Fellowship and began graduate work with the Department of Biochemistry and Biophysics at the Loyola University Medical Center in Maywood, Illinois. In October, 1978 he competitively sought and received a Schmitt Dissertation Fellowship. From June, 1978 to June, 1979 he also participated as an active member of the Computer Usage Committee at Loyola.

PUBLICATIONS

1. "Evidence for hemiacetal formation between N-acyl-L-phenylalaninals and α-chymotrypsin by cross-saturation nuclear magnetic resonance spectroscopy." R. Chen, D.G. Gorenstein, W.P. Kennedy, G. Lowe, D. Nurse and R.M. Schultz. Biochemistry 18, 921-926. (1979)
2. "Mechanism of association of a specific aldehyde transition state analog to the active site of α-chymotrypsin." W.P. Kennedy and R.M. Schultz. Biochemistry 18, 349-356. (1979).
3. op. cit., W.P. Kennedy and R.M. Schultz. Presented at the 176th meeting of the American Chemical Society, Biological Chemistry Section. Abstract no. 59. (1978).
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5. "Radio-iodinated pyridines- potential adrenocortical imaging agents." W.P. Kennedy and D. Wieland. Proceedings of the First International Symposium of Radiopharmaceutical Chemistry, Brookhaven National Laboratories. September, 1976.

6. "6β-I-131-iodomethyl-19-norcholest-5(10)-en-3β-ol (NP-59) concentrates in the adrenal gland better than I-131-iodocholesterol." S.D. Sarkar, W. Beierwaltes, R.D. Ice, G.P. Basmadjian, K.R. Hetzel, W.P. Kennedy and M.M. Mason. Journal of Nuclear Medicine 16, 1038-1042. (1975).

7. op. cit., Proceedings of the 22nd Annual Nuclear Medicine Conference. Journal of Nuclear Medicine 16, 565. (1975).

8. "Se-75-selenocholesterol, a new agent for adrenal imaging." S.D. Sarkar, W.P. Kennedy and W. Beierwaltes. Proceedings of the 22nd Annual Nuclear Medicine Conference. Journal of Nuclear Medicine 16, 565. (1975).

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CHAPTER I

INTRODUCTION

IA. Basic Concepts of Enzymatic Catalysis

Over the last several decades a substantial body of knowledge has accumulated on those features and mechanisms which contribute to the high efficiency by which enzymes catalyze biochemical reactions. Unfortunately, no clear understanding or complete thermodynamic quantification of enzymatic catalysis has emerged from these data for any one enzyme case.

Although the unique property of enzyme-substrate "complementarity" has been invoked for over a century to explain the favorable interactions between some specific site on the enzyme ("active site") and the unique chemical and three dimensional structure of a substrate, only recently have these integral forces been considered the driving force of catalysis(1-7). Accordingly, it is generally agreed that enzymatic catalysis principally involves a stereochemically precise and coherent arrangement of forces which constitute the re-

versible step of complexation between a substrate and its enzyme prior to and/or during the rate limiting step of catalysis (see figure 1). That a linkage must exist between the favorable free energies of binding that occur in the formation of an enzyme-substrate complex and the fast rates observed in an enzyme-catalyzed reaction is more clearly understood from a thermodynamic consideration of the process. It is widely accepted that the rate at which reactants (substrates) achieve thermodynamically defined equilibrium with products is dependent upon the free energy content of some minimum energy barrier that exists between them ("activation energy"). Since a catalyst accelerates the rate of reaction, then catalysis is manifested through a lowering of that activation energy barrier. In comparing a hypothetical unimolecular reaction as catalyzed by an enzyme with its uncatalyzed counterpart in solution (see figure 2), it is immediately evident that for catalysis to occur, the difference in the activation free energy of the enzymatic (G_E^*) versus non-enzymatic (G_N^*) reactions ($G_N^* - G_E^* = \Delta G_T^*$) must be greater than the favorable free energy associated with the binding process (ΔG_{ES}):

$$\Delta G_{ES} < \Delta G_T^* \qquad (1).$$

Many of the currently accepted hypotheses on the mechanisms of enzymatic catalysis argue that the favorable free energy associated with the non-covalent interactions between a substrate and its enzyme in the Michaelis complex is partially utilized to lower the activation energies of succeeding bond making and/or breaking (rate limiting) steps of the reaction(1-7). These "association-activation"

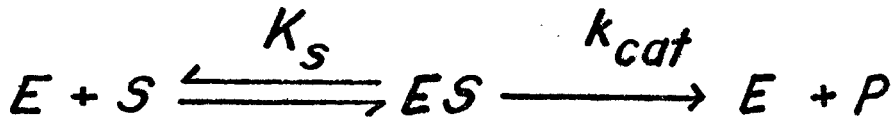

Figure 1. Kinetic Consequences of Enzyme-Substrate Complexation. Formation of an enzyme (E)-substrate (S) complex (ES) occurs prior to the rate limiting step of catalysis (k_{cat}) and product (P) release. Accordingly, the rate of substrate turnover is proportional to the concentration of ES:

$$-d[S]/dt = k_{cat}\,[ES]$$

where:

$$[ES] = [E][S]/K_S$$

(K_S is the so-called Michaelis constant. Also, the rate constant for the decomposition of ES back to E and S is assumed to be $> k_{cat}$.)

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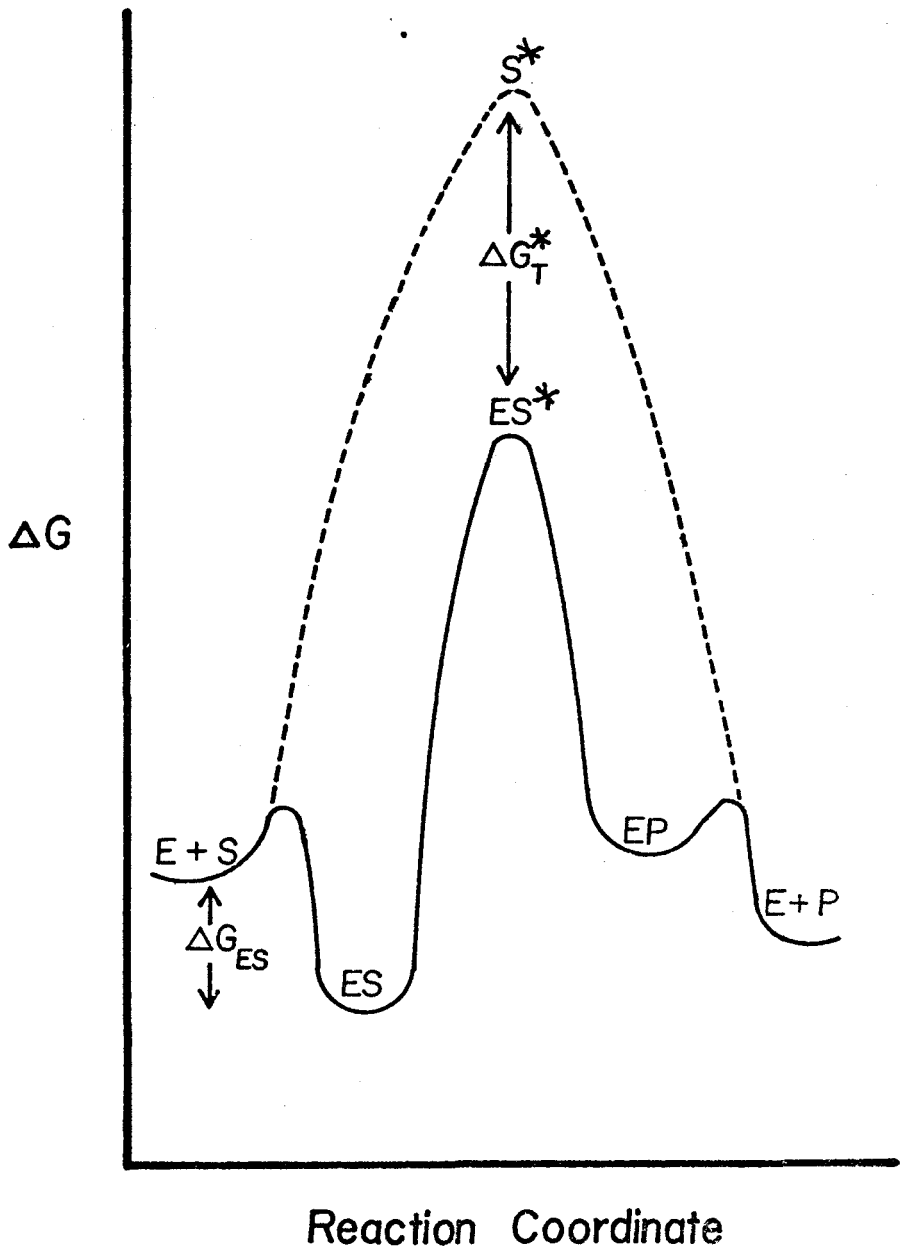
Figure 2. Difference Free Energy Versus Reaction Coordinate Diagram Comparing an Uncatalyzed with an Enzymatically Catalyzed Unimolecular Reaction.

S and P are the substrate and product, respectively, and ES and EP are their respective complexes with the enzyme. For catalysis to occur in the enzymatic case, the difference free energy between the enzymatically catalyzed "activated" state (ES*) and the activated state for the uncatalyzed reaction (S*) ($= \Delta G_T$) must be greater than the favorable free energy of S + E complexation (ΔG_{ES}).


S*
ΔG*T
ES*
ΔG
E + S
ΔGES
ES
EP
E+P
Reaction Coordinate

mechanisms, as coined by Schultz(8), attempt to explain the fast rates observed in enzymatically catalyzed reactions as being due to either:

i) a conformational change in the enzyme upon substrate association which incurs a precise orientation of the catalytic residues the active site with the sensitive bond(s) on the substrate ("induced-fit")(1,2);

ii) distortion of the substrate upon binding towards its "transition state" ("rack")(3-5); or,

iii) "propinquity" effects wherein the entropic demands of the reaction are satisfied prior to the rate limiting step, i.e. Michaelis complexation restricts translational and rotational degrees of freedom about the scissile bond on the substrate in close proximity with the catalytic residues in the active site of the enzyme(6,7) (For fuller review see references 9 and 10).

According to "association-activation" mechanisms, the observed standard free energy for the non-covalent associations of a substrate with an enzyme:

$$\Delta G_{obs} = -RT \ln (K'_S,obs) \qquad (2)$$

where K'_S is the association equilibrium constant (= $1/K_m$ where applicable), can be separated into terms describing the intrinsic binding energy (ΔG_i) and those energies used to lower the activation energy of succeeding and rate determining steps (ΔG_{aa}) (refer also to figure 3):

$$\Delta G_{obs} = \Delta G_i + \Delta G_{aa} \qquad (3).$$

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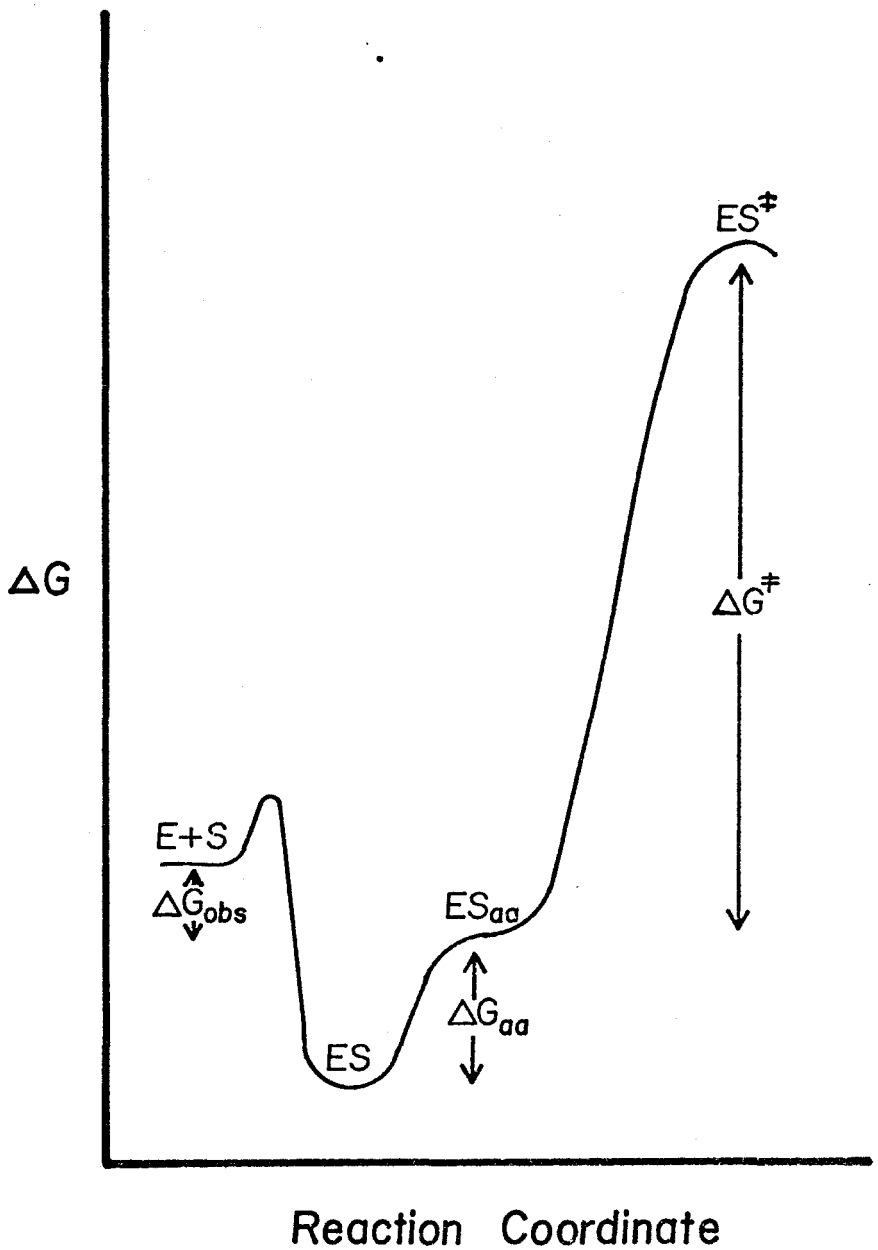
Figure 3. Diagrammatic Representation of the Contribution of "Association-Activation" Mechanisms to Enzymatic Catalysis.

Part of the favorable free energy of enzyme (E) - substrate (S) complexation (ΔGi) is purportedly utilized to activate the ground state Michaelis complex (ES to ESaa) towards the transition state ($ES^{\ddagger}$). Accordingly the observed difference free energy in the non-covalent interactions of the ES complexation ($\Delta Gobs$) is less favorable by the factor ΔGaa but the transition state for the subsequent, rate limiting step ($\Delta G^{\ddagger}$) is lowered by the contribution of ΔGaa.

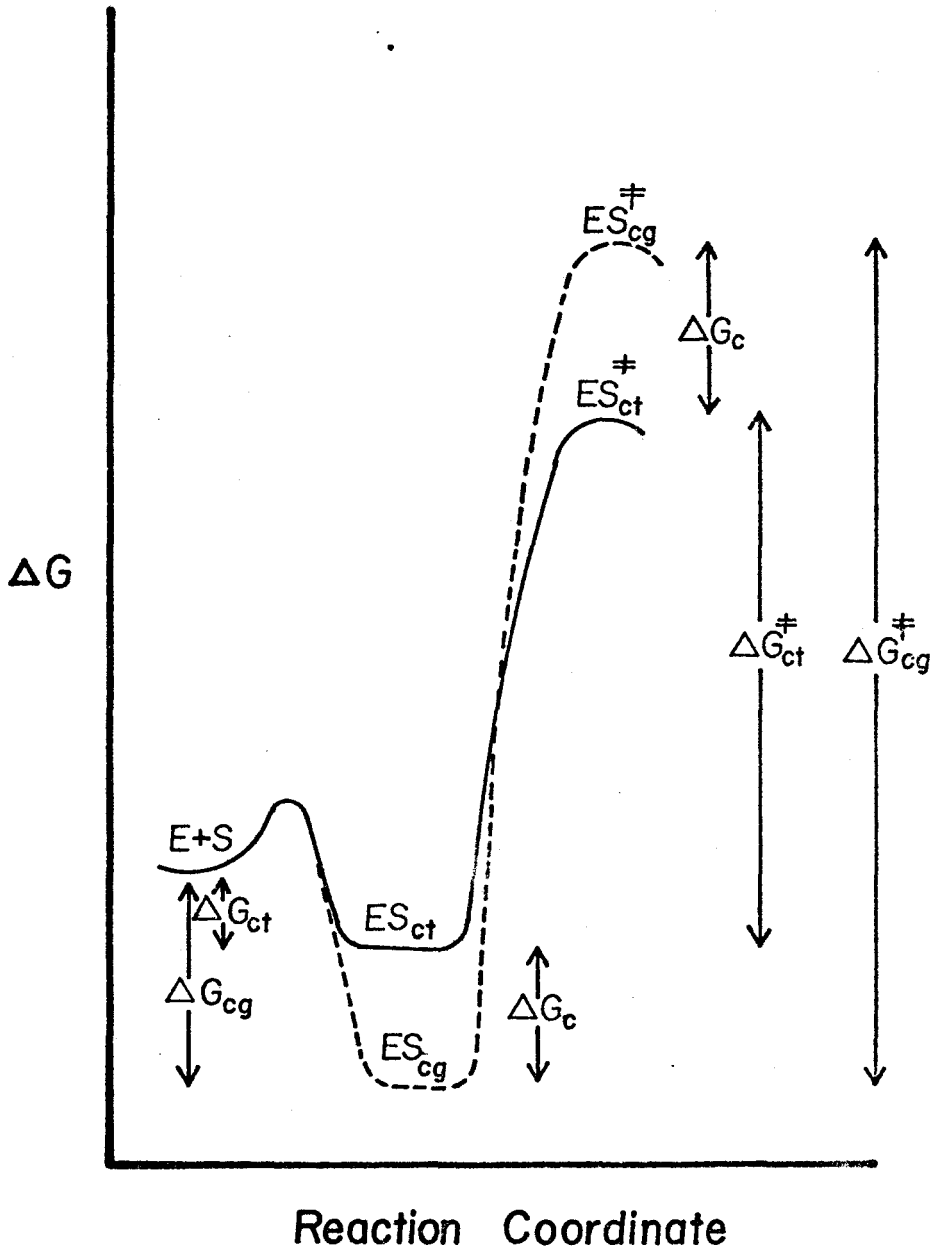

ΔG
ES‡
ΔG‡
E+S
ΔGobs
ESaa
ΔGaa
ES
Reaction Coordinate

Kinetically, "association-activation" mechanisms predict that specific substrates with high values of kcat should have poorer values of Km than less or non-specific substrates(9,11). Experimental data in support of that predicion, however, are generally not conclusive. Other hypotheses, such as non-productive binding, may explain the sometimes inverse relationship between kcat and Km. In addition, while "association-activation" mechanisms generally propose destabilization of the ground state of the substrate and/or enzyme to lower the activation energy of succeeding steps in the reaction at the expense of binding energies(1-8), the concept of transition state "complementarity", as originally suggested by Pauling(4,5) and recently discussed by Fersht(13), suggests that all of the potential energies associated with the binding of a substrate with the active site of an enzyme and its subsequent conversion to product will not be fully realized until the "transition state" is reached (see figure 4). Accordingly, the rate of an enzymatically catalyzed reaction may principally depend upon the efficiency of interactions occuring in the enzyme-substrate "transition state" complex. Substrate specificity will be manifested as an increase in kcat and the favorable energies of Michaelis complexation may be attributed to the similarity between those features on the substrate remote from the reactive bond(s) whose interactions with the enzyme are unperturbed as the transition state is formed.

LEGEND

Figure 4. Diagrammatic Representation of "Transition State Complementarity".

---- Represents the reaction pathway occuring when the enzyme's (E) binding site is complementary to substrate in its ground state configuration (S). ——— Represents the reaction pathway when E is complementary to substrate in its "transition state" structure ($S^{\ddagger}$). ΔGct and ΔGcg are the observed difference free energies of Michaelis complexation for the ground state (cg) and transition state (ct) complementarity situations, respectively. $\Delta G^{\ddagger}ct$ and $\Delta G^{\ddagger}cg$ are the respective free energies of activation. Note that $\Delta G^{\ddagger}ct$ is theoretically expected to be some 2 X ΔGc smaller than $\Delta G^{\ddagger}cg$.


ΔG
E+S
ES_ct
ES_cg
ES_cg‡
ES_ct‡
ΔG_ct
ΔG_cg
ΔG_c
ΔG_c
ΔG_ct‡
ΔG_cg‡
Reaction Coordinate

IB. Transition State Analogs as Probes of the Mechanisms of Enzymatic Catalysis

It may be inferred from the above discussion that enzymatic catalysis can be understood by describing those features and mechanisms intrinsic and differentiating the ground states and transition states of enzyme-substrate complexation. Unfortunately, attempts to study enzyme-substrate transition state complexes are precluded by their highly unstable and transient nature. Such complexes are calculated to exist at concentrations some 10^{10}-fold lower than respective ground state species and have a lifetime on the order of a covalent bond vibration 10^{-13} sec)(13).

Recently, however, Lienhard(15) and Wolfenden(16,17) have independently offered an approach for the study of enzyme-substrate transition states which attempts to supercede these inherent difficulties. Using a thermodynamic equilibrium scheme (see figure 5) which compares an enzymatically catalyzed unimolecular reaction with an appropriate non-catalyzed model, they find that enzymatic catalysis can be quantitatively defined as the ratio of equilibrium constants governing the hypothetically reversible association of a substrate in its transition structure ($S^{\ddagger}$) with the enzyme (E) ($=K_T$) versus that of the reversible complexation of substrate in its ground state configuration (S) with E ($=K\acute{S}$). That is, according to the transition state theory of chemical kinetics(14), the observable rate constant for a unimolecular reaction is equal to the product of the equilibrium constant governing the reversible formation of the tran-

sition state of a reactant from its corresponding ground state ($K^{\ddagger}$) times a universal rate constant, $k^{\ddagger}$ ($=kT/h$ where k is Boltzmann's constant, T is the absolute temperature in °Kelvin and h is Planck's constant) for the decomposition of the reactant's transition state to product(s). Accordingly, where enzymatic catalysis may be defined as the ratio of rate constants for the enzymatic (k_E) versus non-enzymatic (k_N) reactions, the relationship is derived:

$$k_E / k_N = k_E^{\ddagger} K_E^{\ddagger} / k_E^{\ddagger} K_N^{\ddagger} \qquad (4)$$

(note that the ratio of rate constants $k_E/k_N = 1$). A thermodynamic equilibrium cycle in figure 5 is thereby established so that:

$$k_E/k_N = K_E^{\ddagger}/K_N^{\ddagger} = K_T/K_S' \qquad (5)$$

Since $k_E/k_N \gg 1$, equation 5 requires that the transition state structure of the substrate be bound more tightly by the enzyme than its corresponding ground state structure. This prediction, although not new, led Lienhard(15) and Wolfenden(16,17) to suggest that if an inhibitor can successfully mimic a substrate in its transition state configuration, then it will reversibly associate with the enzyme and the equilibrium constant which describes that "transition state analog"-enzyme complex, K_I', will approach in value K_T. Since the ratio k_E/k_N is typically 10^8 to 10^{14} and K_S' is often in the range 10^3 to 10^5 $\underline{M}^{-1}$(9), then if enzymatic catalysis is entirely due to the tight binding of the transition state of the substrate, then KT, and by inference, K_I', are both predicted to be some 10^{10} to 10^{20} $\underline{M}^{-1}$.

In the last decade a variety of "transition state analogs" have

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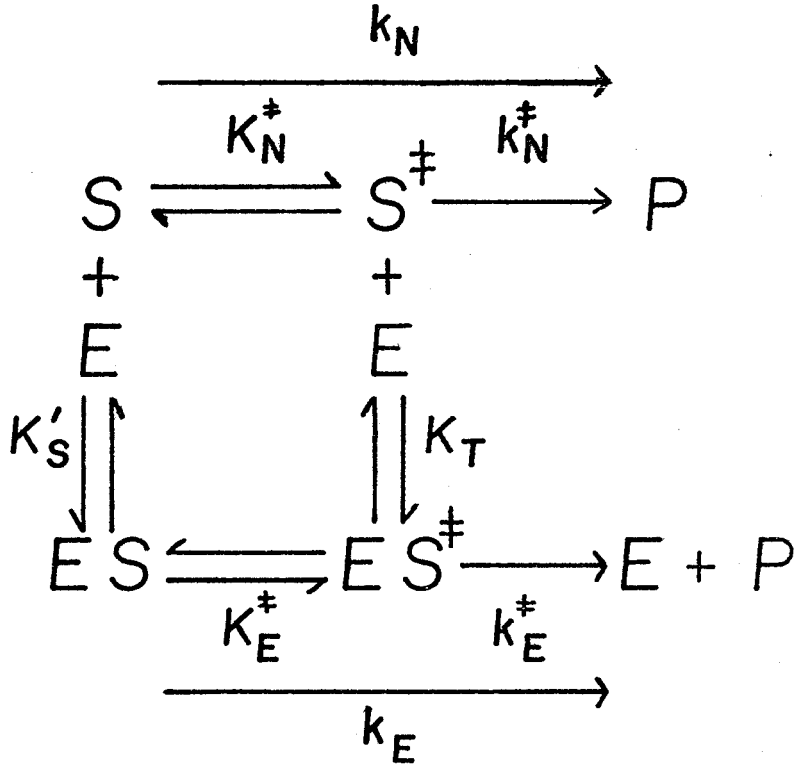
Figure 5. Schematic Representation of "Transition State Theory" as Applied to Enzymatic Catalysis.

See text for detail.


k_N
$K_N^{\ddagger}$
$k_N^{\ddagger}$
S
$S^{\ddagger}$
P
+
+
E
E
K_S'
K_T
E S
E $S^{\ddagger}$
E + P
$K_E^{\ddagger}$
$k_E^{\ddagger}$
k_E

been evaluated against their respective enzymes (see table 1 for some examples of analogs to the hydrolase enzymes). While in most cases these have demonstrated superior binding constants over those of respective substrates and thus support transition state analog theory, their relative affinities fall considerably short of the expected ratio of $K_I'/K_S' = 10^8$. The most obvious explanation for these discrepancies relates to the imperfect nature of the analog(15-17). Other, less obvious, practical and theoretical considerations, however, have been made in criticism of transition state analog theory.

In particular, Schray and Klinman(18) have discussed the entropic effects in juxtapositioning the catalytic residues in the enzyme's active site in close proximity with the substrate's sensitive bond(s). Page and Jencks(6) estimate these entropic factors to be highly significant. Schray and Klinman(18) argue that these entropic will not appear in the ratio K_I'/K_S' so that $K_T/K_S' \neq K_I'/K_S'$.

Several other considerations need also be included that may effect the relationship $K_I'/K_S' = K_T/K_S'$: the effects of ionization and/or solvation differences between the transition state analog's and substrate's complex with the enzyme; conformational changes in the enzyme which may accompany the binding of a substrate; possible differences in the transition state of the model versus enzymatic reaction; and, among others, differences in the chemical reactivities of the analog versus substrate. Leinhard(15) and Wolfenden(16,17) have considered many of problems but their potential effects were left unclear. Jencks(19) has argued that due to these difficulties, in most instances the binding

TABLE 1

COMPARISON OF PROPOSED TRANSITION STATE ANALOG INHIBITION CONSTANTS(K_I) WITH RESPECTIVE SUBSTRATE MICHAELIS CONSTANTS (K_m) TO SELECTED HYDROLASE ENZYMES[a]

Enzyme	Inhibitor, K_I, M or Substrate, K_m, M	K_I/K_m
Lysozyme	(N-acetylgalactosamine)$_4$-lactone, 3×10^{-7} (N-acetylgalactosamine)$_4$, 1×10^{-5}	10^2
α-Glucosidase(yeast)	1-aminoglucoside, 2.3×10^{-6} p-nitrophenyl-glucoside, 1.4×10^{-4}	10^2
β-Glucosidase(yeast)	1-aminogalactoside, 1.6×10^{-4} p-nitrophenyl-galactoside, 1.7×10^{-2}	10^2
Carboxypeptidase A	L-benzylsuccinate, 4.5×10^{-7} CBZ-L-Phe, 8×10^{-4}	10^3
Elastase	N-Acetyl-pro-ala-pro-alaninal, 8×10^{-7} N-Acetyl-pro-ala-pro-alaOMe, 1.5×10^{-3}	10^5
Papain	N-Acetyl-phe-glycinal, 4.6×10^{-8} N-Acetyl-phe-glyOMe, 3.2×10^{-5}	10^3
Subtilisin	Benzeneboronic acid, 8×10^{-4} N-Acetyl-tyrOEt, 9×10^{-2}	10^2
Amidase	Acetaldehyde-NH_3, 1.6×10^{-5} Acetamide, 8.3×10^{-4}	10^2

[a] From reference 17.

constant of a transition state analog versus that for a respective substrate's associations with the enzyme will not equal or even be close to the expected value of K_T/K_S'.

It is evident, then, that while good affinity of a putative "transition state analog" for its respective enzyme may indicate an analogy between its binding complex and the transition-state of enzyme-substrate reactions, lack of a significant ratio K_I/K_S does not necessarily rule out possible transition state-like associations, particularly if the aforementioned entropic factors are highly significant in the enzyme's mechanisms of catalysis. Accordingly, a detailed knowledge of the chemistry and mechanisms involved in the formation and stability of a putative transition state analog-enzyme complex must be obtained before any meaningful interpretations can be made with respect to its potential likeness to the transition states of the enzyme-substrate reaction.

IC. Goals of this Dissertation.

Aldehyde analogs of substrates to the homologous series of serine and cysteine proteases have recently been shown to form highly stable association complexes with their respective enzymes.(13,20-25) Their enhanced affinity for these enzymes over that for substrates has been attributed to possible transition state-like modes of association(13,15,17, 20) although the evidence for this prediction is indirect and conflicting. The experiments in this dissertation are designed to elucidate the probable modes and mechanisms of specific substrate aldehyde analog asso-

ciations with the protein hydrolase enzyme, chymotrypsin, in an effort to establish whether the stable aldehyde-enzyme complex is truly transition state-like. The participation of the catalytically essential residues in the active site of chymotrypsin on aldehyde associations will also be investigated.

CHAPTER II

REVIEW OF THE RELATED LITERATURE

IIA. Chymotrypsin- An Introductory Review

Chymotrypsin (Cht) is perhaps the most studied and best characterized of all the enzymes. Indeed, its initial purification and crystallization coincided with the beginnings of study on the specificity in and mechanisms of enzymatic catalysis(26).

Cht is synthesized in the acinar cells of mammalian pancreas as a single chain polypeptide in the form of its inactive zymogen chymotrypsinogen. It is secreted as such into the duodenum via the pancreatic duct. Its activation is well characterized(27). Both limited proteolysis by trypsin and autoactivation lead to the α-form which is the most stable conformation of Cht found in vivo. This "native" species consists of 241 amino acid residues in three polypeptide chains that are covalently interconnected by two interchain and three intrachain disulfide (cystine) bonds(28,29). The amino acid sequence and three dimensional structure of the enzyme have also

been determined(28,29). Cht catalyzes the hydrolysis of peptide bonds. The specificity of hydrolysis is on the carboxyl side of the aromatic amino acid residues: phenylalanine (phe), tyrosine (tyr) or tryptophane (trp)(30). It is one of several proteases found in the mamalian intestinal tract that are used to catalyze the digestion of dietary proteins into their constituent amino acids.

IIA-1. Specificity and Mechanisms of Chymotryptic Catalysis.

By the late 1960's Bender(31-34), Hess(35-37) and coworkers had accumulated sufficient kinetic and spectrophotometric evidence to substantiate the now commonly accepted minimum mechanism for the Cht-catalyzed hydrolysis of substrates (see figure 6). The first step (K_S) is formation of a reversible, non-covalent complex between natural peptide or synthetic amide or ester derivatives of pne, tyr or trp with Cht. In a second step (step k_2) nucleophilic attack at the substrate's carbonyl carbon by an active site residue on the enzyme results in the formation of an acyl-enzyme intermediate (ES') concommittant with the release of an alcohol or amine product (P_1). Chemical modification studies(38) and x-ray crystallographic investigations(39,40) have implicated the ser-195 γ-OH as the catalytically essential nucleophile in Cht's active site. In the subsequent and symmetrical step (step k_3), water acts as the nucleophile in the hydrolysis of ES' regenerating the free enzyme and releasing the acid product (P_2).

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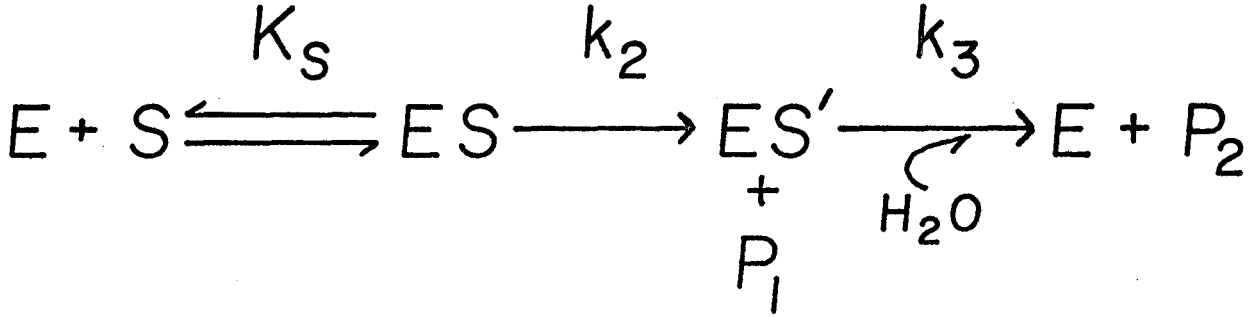
Figure 6. Minimum Mechanism Describing Cht-Catalyzed Substrate Hydrolysis.

See text for detail. Note that:

$$K_S = [E][S]/[ES]$$


K_S
k_2
k_3
$E + S \rightleftharpoons ES \longrightarrow ES' \longrightarrow E + P_2$
$+ P_1$
H_2O

The topology of the active site which contributes to the favorable interactions between Cht and specific substrates in the Michaelis and subsequent complexes have been probed by the solution studies of Niemann(30) and Cohen(41) and coworkers and the x-ray crystallographic investigations of Blow(39,40) and coworkers. Based on these studies, a model may be constructed(41) (see figure 7) representing the primary specificity region in Cht as composed of subsites complementary to those groups attached to the alpha carbon (C_α) of the specific ester substrate, N-acetyl-L-phe methyl ester.

The aromatic binding site (ar) is seen as a deeply invaginated, hydrophobic pocket capable of numerous contacts which sandwich the aromatic side chains of specific substrates into a distinct orientational plane that includes the methylene carbon (C_β)(39). This pocket is absent in the zymogen and is formed upon activation of the enzyme and subsequent rearrangements in the residues numbered 191 to 194(40).

The conformational angle X_1 which represents rotational degrees of freedom about the substrate's C_α-C_β bond may be restricted via a conveniently placed hydrogen bond between the substrate's acylamido nitrogen's proton and the carbonyl oxygen of a backbone ser-214 on Cht in the am subsite.

Binding of substrates in both the ar and am subsites may restrict translational and rotational motions in the substrate and orient the sensitive bond in close proximity with the catalytic (nucleophilic, n region) residues on the enzyme.

The h subsite, shown in figure 7, has been suggested to be the

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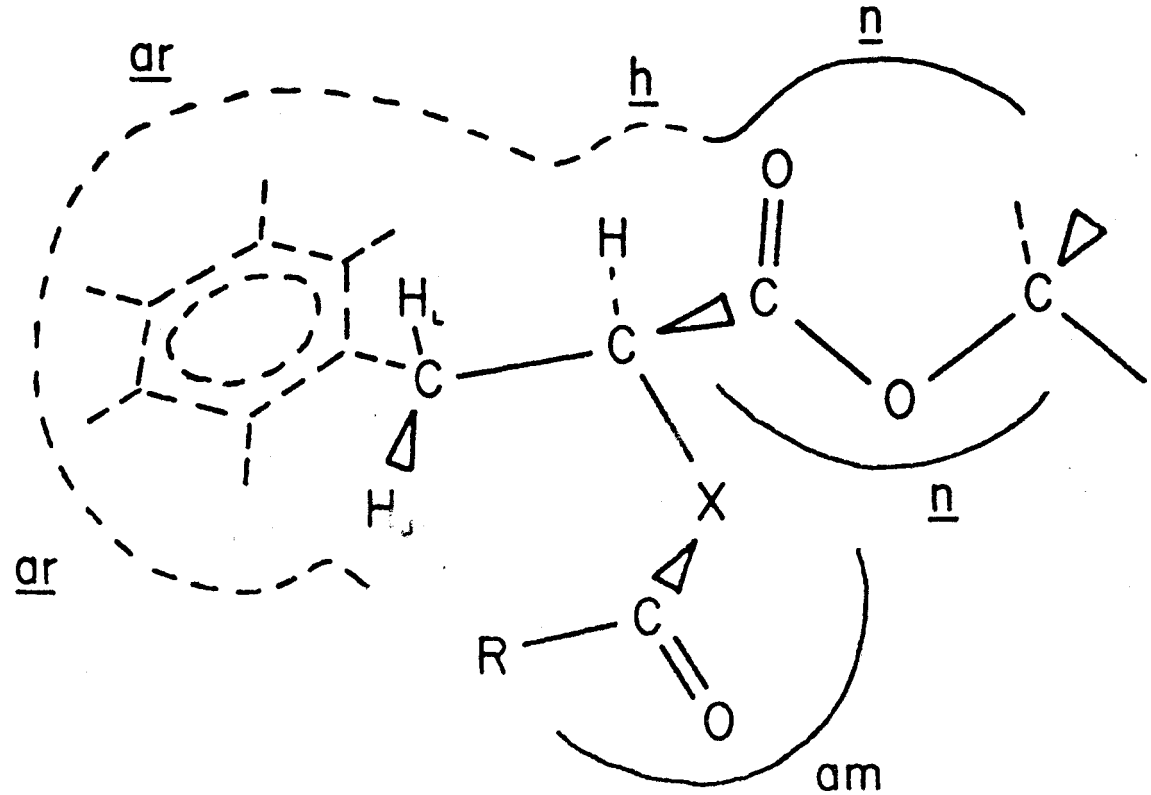
Figure 7. Topology of the Active Site of Chymotrypsin.

See text for detail.


ar
h
n
O
H
C
C
C
O
H
H
C
X
n
ar
C
R
O
am

source of distortion mechanisms in Cht-catalysis(42). Treadway and Schultz(43) have provided evidence in support of a small unfavorable interactions between a met-192 residue in the active site and small, pseudo-substrate's C_{α} hydrogen which contributes approximately 1 kcal/mole towards the lowering of the activation energy in the enzymatic reaction.

Finally, a fifth subsite, the "oxyanion hole", has been implicated as the source of transition state stabilization mechanisms from the x-ray crystallographic work of Robertus et al(44) and Paulos et al.(45) The focus of two hydrogen bond donors on the backbone of the Cht-like proteinase enzyme, subtilisin BPN', were suggested to be specific for the oxyanion on the tetrahedrally coordinated substrate's carbonyl carbon formed during the transition state of the acylation and deacylation steps of the reaction (see below for details). The stabilization of this tetrahedral configuration may be important in lowering the activation energy(44), but its quantitative effects remain unresolved. A similar feature in the active site of Cht is thought to exist with the acylamido nitrogen hydrogens of gly-193 and the ser-195(39,40) serving as hydrogen bond donors.

Thompson and Bauer(46) have recently attempted to quantitate the entropic effects associated with the binding of substrates in the ar region by systematically comparing the thermodynamics of non-specific versus specific amide substrate and alcohol and aldehyde substrate analog associations with Cht. Their analysis, based on the free energy of binding ($\Delta G^{\circ}obs$) concludes that interactions in the ar region upon Michaelis complexation reduces the entropic demands in

the subsequent acylation transition state by some -10 to -20 e.u./mole. These entropic factors are in agreement with earlier findings of Cohen et al(41) who compared the rates of comformationally restricted analogs with respective substrates and concluded that interactions with the am and ar binding sites in the Michaelis complex can appreciably effect the rates of substrate hydrolysis.

The thermodynamic data of Shiao(47) and Schultz et al(8,48) provide evidence in favor of an "induced fit" mechanism in Cht-catalyses. Their independent measurements of both the enthalpic (H°_{obs}) and entropic (ΔS°_{obs}) contributions to the free energy change, where:

$$\Delta G^\circ_{obs} = \Delta H^\circ_{obs} - T\Delta S^\circ_{obs} \qquad (6)$$

for the binding of "virtual" substrates and aromatic inhibitors with the ar region of the native enzyme showed each parameter to be large and negative. Protonation(47) or methylation(8,48) of the his-57 imidazole appears to block the "induced-fit" mechanism since the large and negative enthalpies and entropies of aromatic substrate and inhibitor non-covalent associations are ameliorated and reduced to values similar to those expected for the transfer of small molecules from an aqueous to a non-aqueous solvent (positive ΔS°_{obs} and ΔH°_{obs} near zero). (49) With ACht, however, the thermodynamics of substrate or inhibitor non-covalent associations are similar to the native enzyme.(48) Accordingly, it was proposed that some important linkage, most probably a conformational rearrangement in the enzyme principally involving the his-57 imidazole, exists between substrate binding in ar and the catalytic region (n) of Cht.(8,48) Con-

formational rearrangements in the enzyme upon substrate association have been observed by other methods(50-53), but their catalytic importance remains unclear.

IIA-2. The Tetrahedral Intermediate and its Relationship to the Transition State in Chymotryptic Catalyses.

Studies on non-enzymatic reactions which involve nucleophilic addition to the trigonally coordinated carbonyl carbon of ester or amide substrates conclude that some metastable, tetrahedrally coordinated intermediate is normally formed(54-57) (see figure 8). There exists considerable evidence in favor of the intermediacy of a similar intermediate in Cht-catalyzed reactions.

Bender et al(56) originally argued in favor of tetrahedral adduct formation in both steps k_2 and k_3 based on their symmetry and pH dependencies.(55,56) The unusual kinetics and pH dependencies of the hydrolysis of substituted anilide(58-60) and hydrazide(61,62) substrate analogs have also been explained in terms of the kinetics of tetrahedral intermediate formation and decomposition.

More direct evidence afforded by solution studies comes from work with thiol-ester (-SR) analogs of non-specific and specific substrates of Cht. Frankfater and Kezdy(63) originally observed that thiol acetate substrate analogs are hydrolyzed by Cht at a rate equivalent to respective oxygen ester counterparts even though the -SR moeity is a much better leaving group than -OR. A reasonable explanation for this behavior with the enzyme is that formation of the

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Figure 8. Acyl-Enzyme Formation Elaborated to Include Tetrahedral Intermediate Formation.

Assuming the reverse rate from acyl-enzyme (ES') to the tetrahedral intermediate (TI) is insignificant ($k_-' < k'$), then the acylation reaction rate is derived:

$$d[ES']/dt = k_2,obs[ES] = k'[TI] \qquad (1)$$

where: $$k_2,obs = k\ k'/(k_- + k') \qquad (2)$$

For $k' < k_-$ (decomposition of the TI rate limiting), then:

$$k_2,obs = k\ k'/k_- \qquad (3)$$

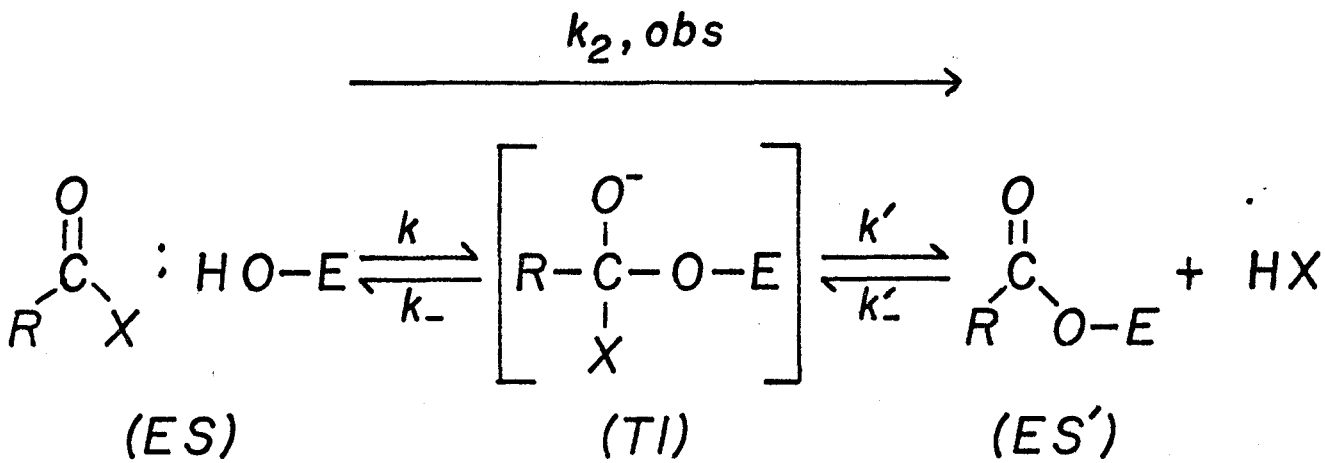
Given that k is general base catalyzed and its microscopic reverse, k_-, is general acid catalyzed (as well as k'), then:

$$k = k_{max} / (1+Ka/[H^+]) \qquad (4)$$

and,

$$k'\ (k_-) = k'_{max} / (1+[H^+]/Ka) \qquad (5)$$

where Ka is the acid dissociation constant for the acting general base-acid catalyst. Substitution of these latter two equations into equations 2 or 3 it can be seen that the overall rate constant k_2,obs will always appear general base catalyzed whether formation or decomposition of the TI is rate limiting.


k_2, obs
k
k_-
k'
k'_-
+ HX
(ES)
(TI)
(ES')

tetrahedral intermediate, the rates of which are equivalent for both thiol and oxygen esters, is rate limiting(63). Identical results have also been observed with specific substrate thiol and oxygen esters analogs(64).

In addition, Fink et al(65-67) have applied cryogenic, spectrophotometric methods for the study of substrate associations with Cht(65) and a related proteinase, papain(66,67). They observe additional intermediate formations post Michaelis complexation which are consistent with the formation and accumulation of a tetrahedral intermediate.

X-ray crystallography has also been used to observe the tetrahedral adduct. Huber et al(68) and Sweet et al(69) have apparently shown that the potentially reactive carbonyl carbon in natural, polypeptide inhibitors of trypsin are distorted towards an sp^3 tetrahedral configuration when complexed with the enzyme. Analogously, Paulos et al(45) have observed, x-ray crystallographically, a tetrahedral configuration about the carbonyl carbon of the chloromethyl ketone analog of carbobenzoxy-L-lysine when bound in the active site of the serine proteinase, subtilisn BPN'.

Although the tetrahedral intermediate certainly contains features of the transition state of Cht-catalyzed hydrolysis reactions, it cannot be considered the transition state since covalent bonds are fully formed. Some confusion remains as to which step, formation or decomposition of the tetrahedral intermediate corresponds to the rate determining step of peptide hydrolysis. Hirohara et al(64) have recently reviewed the current literature on this point and contend that

the transition state in the acylation step of Cht-catalyzed hydrolyses differ appreciably depending on the nature of the substrate's leaving group. Thiol and p-nitrophenyl ester substrates(63,64) are found to be rate limited in the formation of the tetrahedral intermediate while oxygen ester, amide or peptide substrates appear rate limited in the decomposition step(70,71). Although Nakagawa and Bender(72) have recently suggested that the tetrahedral adduct might not be formed in the acylation step of substrate amide hydrolysis by Cht, their arguments fail to explain an observed nitrogen isotope effect on the reaction(70,71) which supports formation of a tetrahedral adduct, decomposition of which is the rate determing step.

IIA-3. Role of the His-Asp Couple in Chymotryptic Catalyses.

Bender et al(73) have observed a deuterium isotope effect upon the rates of acylation and deacylation in substrate hydrolysis by Cht which is consistent with a rate limiting proton transfer in the reaction. Coupled with the kinetic arguments for tetrahedral intermediate formation (see formulations in figure 8), they argued in favor of a mechanism wherein generation of the ser-195 γ-O^- nucleophile and protonation of the leaving group in the respective steps k_2 and k_3 is facilitated by some base on the enzyme acting as a general base-acid catalyst.

Since titration of imidazole(74) or the histidine (his) imidazole(55) moeity both show a basic inflection about pH 7 (see table

TABLE 2

PROTONATION OF IMIDAZOLE[a] AND HISTIDINE[b] AROUND NEUTRAL pH

Derivative	pK_a
Imidazole	6.95
-1-methyl	7.25
-2-methyl	7.86
-4 or-5-methyl	7.52
-2,4 or -2,5-dimethyl	8.36
-2,4,5-trimethyl	8.86
Histidine	6.0
N-Acetyl-Histidine	7.05

[a]From reference 74

[b]From reference 55

2) similar to the catalytically essential pKa of 7(56), it is expected that some his imidazole on the enzyme participates as a base catalyst. Photo-oxidation(75) or chemical modification(76,77) of the his-57 imidazole in Cht resulted in a loss in its catalytic activity (78,79). It was not surprising, then, when Blow et al(80) identified, x-ray crystallographically, the $\varepsilon 2_N$-nitrogen of the his-57 imidazole side chain located in the nucleophilic (n) region of the active site of Cht proposedly hydrogen bonded to the ser-195 γ-OH. The same group identified an asp-102 carboxylate sidechain within hydrogen bonding distance with the $\delta 1_N$-nitrogen on the opposite side of that his-57 imidazole. Identical features have also been identified for most of the homologous series of serine proteinases including those from divergent evolutionary sources (for review see reference 81).

Blow proposed that the ser-195, his-57 and asp-102 side chains seemed nicely aligned to act in some concerted "charge relay" mechanism(42). Accordingly, attack of the ser-195 γ-OH at the substrate's scissile carbonyl carbon may occur simultaneous with the removal of its proton by the $\varepsilon 2_N$-nitrogen on the his-57 imidazole. The asp-102 was postulated to contribute to the general base-acid functions of the his-57 imidazole by stabilizing the incipient imidazolium cation occurring during tetrahedral intermediate formation.

From its inception, however, the "charge relay" system has been fraught with incongruities particularly in the assignment of the catalytically essential pKa of 7 to a particular component in the triad. Although some IR(82) and NMR(83) experiments have suggested

that the asp-102 carboxylate is the ultimate proton acceptor during formation of the tetrahedral intermediate and thereby assigned inverted pK_a's to the respective components, evidence to the contrary is now quite strong(84-86). In addition, a recent reexamination of the original electron density maps of Cht and related proteinases(87) reveals the putative ser-his hydrogen bond is severely distorted in the native enzyme, thereby disrupting the proposed charge relay mechanism. Rather, the $\varepsilon 2_N$-nitrogen is seen pointing towards the site occupied by the substrate's leaving group prior to acyl-enzyme formation or an incoming water molecule in the deacylation step.

Boland et al(88) have estimated that the contribution of a correctly oriented imidazole acting as a general base catalyst in the hydrolysis of esters can be as great as 10^5 to the overall rate of reaction. Accordingly, precise orientation of the his-57 imidazole in the active site of Cht is particularly important in its function as a general base or acid catalyst. This postulate is corroborated by the results of Henderson(78) who found that selective methylation of the $\varepsilon 2_N$-nitrogen on the his-57 imidazole lowers the catalytic efficiency of the enzyme by a factor of 10^5 to 10^6. The probable role of the his-asp hydrogen bond, then, besides maintaining the correct imidazole tautomer, is to freeze the imidazole into a position which is catalytically most advantageous. Steric constraints, however, disallow proper orientation of the imidazole's $\varepsilon 2_N$-nitrogen with both the ser-195 γ-OH and the leaving group or water site. Thus, its activity as a general base catalyst in the formation of the tetrahedral intermediate may be insignificant or even inhibited as

compared with its activity as a general acid catalyst, or vice versa, depending on the requirements of the reaction(89).

Bachovchin and Roberts(84) have enumerated several chemical advantages for orienting the imidazole towards the leaving group site. They argue that since breaking the carbon-nitrogen bond in the tetrahedral intermediate formed between the enzyme and a peptide or amide substrate is rate limiting(64,70,71), that step would be highly sensitive to general acid catalysis and orientation of the his-57 imidazole in close proximity would therefore be most advantageous. Also, since formation of the tetrahedral intermediate is rate limiting for ester hydrolysis(63,64) and, by inference, is rate limiting in decomposition of the acyl enzyme, proper orientation of the general base catalyst in proximity with an incoming water molecule for the deacylation step would be catalytically most advantageous. An even more speculative, yet intriguing, mechanism involving the imidazole has been suggested by Sutterthwait and Jencks(90). They propose that via an "induced fit"-like rearrangement in the enzyme's conformation occurring upon substrate association and tetrahedral adduct formation or simply by vacillation of the imidazole ring along the hydrogen bonding lines of the his-asp pair, the $\varepsilon 2_N$-nitrogen might be optimally oriented and then reoriented to act as both a good general base and general acid during formation and subsequent decomposition of the tetrahedral intermediate.

An alternative view is that the his-asp couple acts as a stationary relay(91) such that proton transfer from the serine δ-OH to the leaving group occurs via bent hydrogen bonds. Although Wang's

(91) pre-transition state mechanism has been shown to be unreasonable(59-63), Komiyama and Bender(72) have recently suggested that concerted proton relay by the imidazole during the transition state may be a viable alternative in specific substrate amide hydrolysis. Accordingly, the imidazole may be oriented intermediate between the ser δ-OH and the leaving group.

IIB. Aldehyde Derivatives of Specific Substrates as Transition State Analogs of Chymotryptic Catalyses.

Aldehyde analogs of specific substrate carboxylate products of serine and cysteine proteinases enzyme are found to be the most potent competitive inhibitors(13,20-25).

The leupeptins, which are fermentation products isolated from various species of Actinomycetes, were the first examples of competitive inhibition of serine or cysteine proteinases by peptide aldehydes(92-94). The leupeptins are predominately leucine containing peptides which are terminated (C-terminal) by an L-argininal residue. Their preferred inhibition of trypsin-like proteinases can be altered by replacement of the L-argininal moeity with aldehydic derivatives of L-phe, L-tyr or L-trp which results in the potent, competitive inhibition of proteinase enzymes of Cht-like specificity(95). Other N-acyl amino aldehyde analogs of specific substrates have been reported to strongly inhibit their respective serine or cysteine protein hydrolase enzymes: elastase(13,20,46), papain(24,25), a bacterial

amidase(26) and asparaginase(22).

It was Thompson(13,20) who originally suggested that the peptide aldehydes form hemi- or thiohemiacetal adducts with the active site nucleophile of respective enzymes (see figure 9). Furthermore, he argued that the enhanced stability of the complexes over that for the non-covalent associations of respective substrates is due to its structural resemblance to the transition state of substrate hydrolysis. He noted that substrate analogs bearing a tetrahedral orientation about the respective scissile carbonyl carbon of substrates, a feature which geometrically mimics the transition state occuring during substrate hydrolysis, is insufficient to enhance their binding over that for the non-covalent associations of substrates(13,20,46) (compare alcohol analog versus amide substrate binding constants in table 3). Using the arguments of "transition state analog theory" (15-17), then, the mechanisms of serine proteinase-catalyzed substrate hydrolyses do not include "distortion"(3-5) of the substrate, otherwise a better binding of the alcohol derivatives would be effected. More importantly, these results suggest that the similarly coordinated hydrate form of the peptide aldehyde (compound III, figure 9), the preferred form of peptide aldehydes in solution(96), should have no special affinity for respective enzymes. Accordingly, Thompson(13,20) argued that the strength of peptide aldehyde-serine or cysteine proteinase interactions requires formation of the covalent, hemiacetal complex.

Although the better affinity of peptide aldehydes for the serine or cysteine proteinases over that for respective substrate as-

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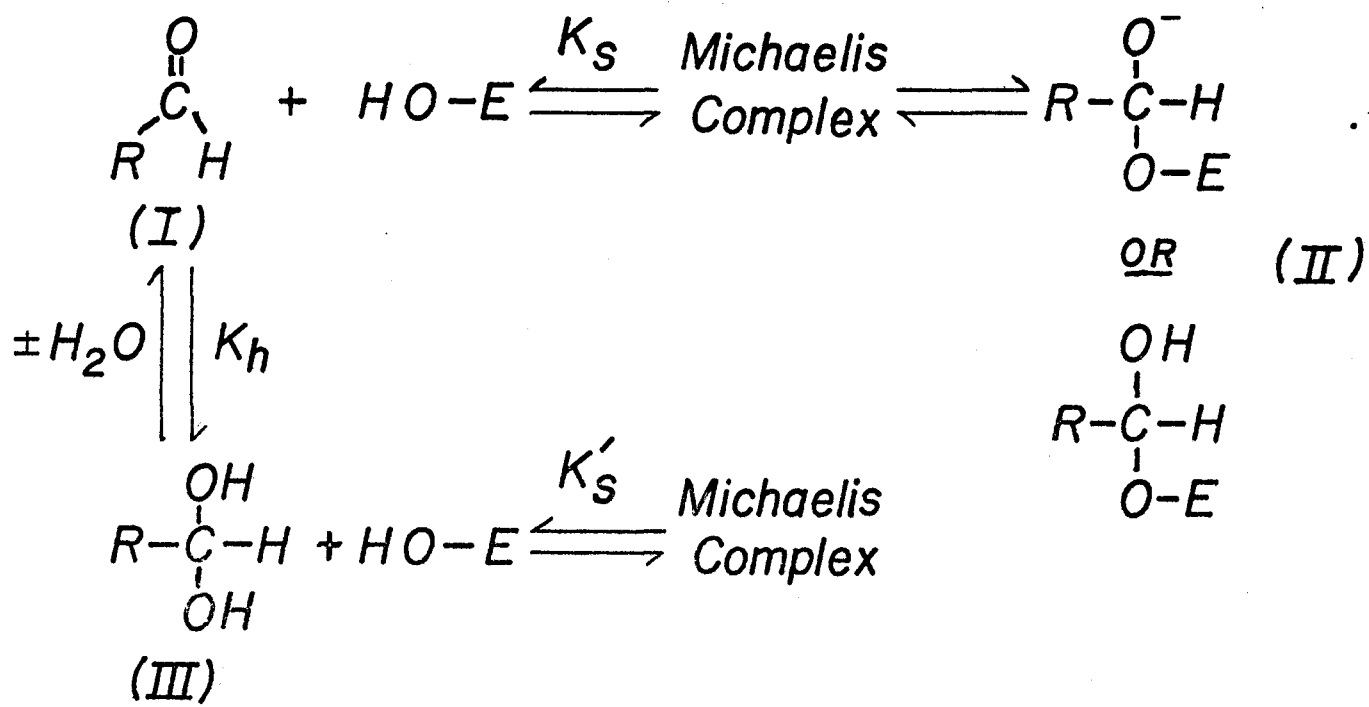
Figure 9. Possible Association Complexes Between Substrate Aldehyde Analogs and Serine Proteases.

The unhydrated aldehyde (compound I) or its hydrated counterpart (compound III) aldehydes can each non-covalently associate with the enzyme (E-OH) to form Michaelis complexes, as governed by the respective dissociation constants K_S and K_S'. The unhydrated aldehyde may further react with the enzyme's reactive serine nucleophile to form an anionic or neutral hemiacetal (compound II).


O
C
R H
(I)
+ HO-E
K_s
Michaelis Complex
O⁻
R-C-H
O-E
OR
(II)
±H₂O
K_h
OH
R-C-H
OH
(III)
+ HO-E
K_s'
Michaelis Complex
OH
R-C-H
O-E

sociations (see table 3) lends credence to the arguments of Thompson (13,20) and "transition state analog theory",(15-17) experimental evidence to support hemi- or thiohemiacetal formation and the resemblance of that complex to the transition state of substrate hydrolysis remain indirect and poorly resolved. Lewis and Wolfenden(23) have argued in favor of thiohemiacetal formation between benzaminoacetaldehyde and papain based on an observed deuterium isotope effect. For Cht, Schultz and Cheerva(97) argued that a hemiacetal complex is formed with the non-specific substrate aldehyde analog, hydrocinnamaldehyde, based on an observed pH dependency in its binding constant (K_I). Although earlier NMR(98) and UV(99) studies were unable to detect a hemiacetal between cinnamaldehyde or its chromophoric derivatives and Cht, Nurse and Lowe(100) confirmed the hydrocinnamaldehyde-Cht hemiacetal using a cross-saturation NMR technique. However, non-specific substrate aldehyde analogs have only a slightly better affinity for the enzyme than the non-covalent associations of respective substrates(97-100), contrary to the expectations of transition state analog theory. A foregoing conclusion, then, is that the transition state of Cht- and related proteinase-catalyzed hydrolyses, which may be mimiced by substrate aldehyde analogs, is highly dependent upon the favorable interactions occuring between the substrate and enzyme at sites distal from the reactive bond and residues, respectively (principally, the am and ar subsites and in secondary sites beyond the N-terminis of the specific substrate). Accordingly, a good transition state analog must contain the structural features of a specific substrate.

TABLE 3

BINDING CONSTANTS (K_I OR K_m) FOR SELECTED AMIDE SUBSTRATES AND ALCOHOL AND ALDEHYDE SUBSTRATE ANALOGS TO RESPECTIVE PROTEINASE ENZYMES

Compound	Enzyme	K_M or K_I, mM	Reference
Ac-ala-pro-ala-NH_2	Elastase	4.2	13
" " -ol	"	7.0	"
" " -al	"	0.062	"
Bz-gly-NH_2	Papain	202	22
" " -ol	"	$>10^3$	"
" " -al	"	0.025	"
Hydrocinnamide	Chymotrypsin		101
" " aldehyde	"	0.79	97
" at pH 4.5	"	5.8	"
Ac-leu-phe-al	"	0.018	99
" at pH 4.4	"	0.018	"

CHAPTER III

MATERIALS AND METHODS

IIIA. General Materials and Sources

The sources of uncommon chemical reagents and instrumentation utilized in this work are indicated throughout the text. All other chemicals and solvents were reagent grade or better as purchased through the Eastman Chemical Company, Scientific Products (S/P) or from the Malincrodt Chemical Works.

Dimethyl sulfoxide (DMSO), purchased from the Eastman Chemical Company, was twice redistilled under vacuum over lithium hydride (Matheson, Coleman and Bell) and stored under nitrogen at 4°C (solid form) until use.

Proflavin dihydrochloride dihydrate was obtained from the Mann Chemical Company. Its concentration in solution was routinely determined from its molecular absorbtivity coefficient of 3.66×10^4 ab-

sorbance units/M/cm(8).

Bovine pancreatic α-chymotrypsin (three times crystallized) was purchased from Worthington Biochemical Corporation. Lot #CDI-P693 (63 U/mg) was used exclusively throughout this work. The concentration of this enzyme or its modified forms was estimated from a molecular weight of 25,000 or accurately determined from its UV absorbance at 280 nm in pH 7.8 buffered solution using the molecular absorbtivity coefficient of 5×10^4 absorbance units/M/ cm(101).

A specific staining procedure was routinely utilized to identify the spots corresponding to an aldehyde or aldehyde hydrate on thin-layer chromatograms(TLC)(102). The developed and dried silica plate was initially sprayed with a solution containing 100 mg of 2,4-dinitrophenylhydrazine (DNP) (Aldrich) in 1 ml of 37% hydrochloric acid (conc HCl) and 99 ml of 95% ethanol and dried at 100°C in an oven. The resulting DNP derivatives were differentiated by subsequently spraying the plate with a solution of 0.2% potassium ferricyanide (Fisher) in 2N HCl. Upon drying, aldehydes appear as yellow to green spots, saturated ketones appear blue, and unsaturated carbonyls do not color at all.

III-B. Syntheses of N-Acyl Amino Ester, Alcohol and Aldehyde Derivatives of D- and L-Phenylalanine

The synthetic methods utilized to prepare the various N-acyl amino ester, alcohol and aldehyde derivatives of D- or L-phenyl-

alanine (phe) appear herein. Enantiomeric integrity was maintained throughout the scheme (see figure 10) as documented previously(21,95, 103-108). Derivatives of L-phe were preferentially prepared due to the stereospecific requirements of chymotrypsin(30,39-41). However, the D-enantiomer of N-benzoyl-phenylalaninal (BzPheal) was also prepared from D-phe in order to test the purity of the L-derivative and to evaluate whether the enzyme maintains its stereospecificity with aldehyde analogs of specific substrates. The α-amine of phe was N-protected with the acetyl or benzoyl group.

Indicated TLC Rf values (= distance traveled by a compound / distance traveled by the developing system) were obtained on Quantum UV_{240} sensitized 10 cm silica gel plates. Developing systems are reported as volume / volume (v/v).

Infrared (IR) spectra were recorded on a Perkin-Elmer model 337 IR-spectrometer. When useful, characteristic absorbance mazima in reciprocal centimeters (cm^{-1}) are included below. Full spectra of selected compounds may be viewed in the Appendix A.

^{1}H- nuclear magnetic resonance (NMR) spectra, when obtained, were recorded on a Varian FT-80A 80 megahertz spectrometer in an appropriate deuterated solvent. Characteristic chemical shifts from tetramethyl silane in parts per million (ppm) are included below. Full spectra may also be viewed in Appendix B. See also table 4 for selected physical constants.

III-I. Phenylalanine Methyl Ester Hydrochloride (PheOMe HCl).

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Figure 10. Scheme Depicting the Various Synthetic Derivatives of Phenylalanine Prepared in This Work.

See text for an explanation of the abbreviations used, reactants and conditions, etc.

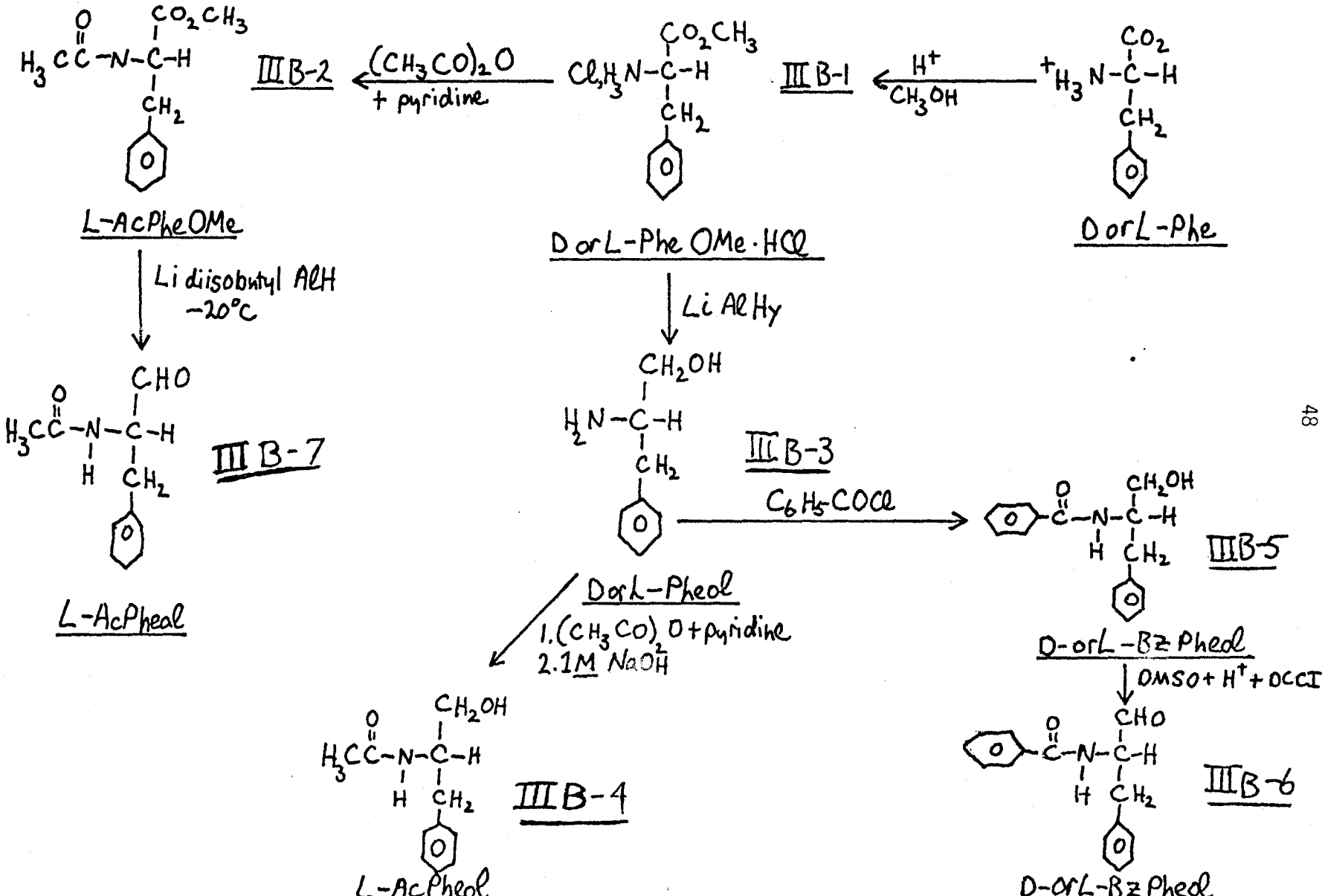
L-AcPheOMe
III B-2
(CH3CO)2O
+ pyridine
D or L-Phe OMe · HCl
III B-1
H+
CH3OH
D or L-Phe
Li diisobutyl AlH
−20°C
III B-7
L-AcPheal
Li Al Hy
III B-3
C6H5COCl
D or L-Pheol
1. (CH3CO)2O + pyridine
2. 1M NaOH
III B-4
L-AcPheol
III B-5
D-or L-Bz Pheol
DMSO + H+ + DCCI
III B-6
D-or L-Bz Pheol

TABLE 4

SELECTED PHYSICAL CONSTANTS FOR SYNTHETIC DERIVATIVES OF PHENYLALANINE PREPARED IN THIS WORK

Compound	Mol. Wt.[a]	m.p.°C[b]	TLC Rf (System)[c]
PheOMe•HCl (IIIB-1)[d]	215.68	162 - 163	0.1 (3/1/1 BuOHOHAc/H_2O)
AcPheOMe (IIIB-2)	221.25	89 - 90	0.9 (9/1 $CHCl_3$/MeOH)
Pheol (IIIB-3)[d]	151.20	90 - 91	0.4 (3/3/1 BuOH/HAc/H_2O)
AcPheol (IIIB-4)	193.31	101 - 102	0.4 (9/1 $CHCl_3$/MeOH)
BzPheol (IIIB-5)[d]	255.31	168 - 169	0.5 (98/2 $CHCl_3$/MeOH)
BzPheal (IIIB-6)[d]	253.29	141 - 142	0.6 (98/2 $CHCl_3$/MeOH)

[a]Molecular weights.
[b]Melting points as obtained in these syntheses.
[c]Thin layer chromotography (TLC) Rf values obtained on Quantum UV_{240} sensitized 10 cm silica gel plates; developing systems are v/v.
[d]Identical values obtained in the synthesis of the D-enantiomer.

D- or L-phe was converted to its corresponding methyl ester hydrochloride by standard procedures(103). Anhydrous HCl gas (Matheson) was bubbled through a vigorously stirring slurry of 20 g (0.12 moles) finely divided phe (Eastman) in 300 ml of anhydrous methanol (MeOH) until the solute was completely dissolved. Under constant addition of HCl, the mixture was cooled to 0°C in an ice bath, stirred for 30 min., and stirred at room temperature for an additional 3 hrs. The solvents were then rotary evaporated under vacuum and the resulting solids recrystallized from MeOH giving 23.4 g (89%) fine, white needles. (m.p. 162-163°C, m.p. lit.(104) 162-163°C; TLC Rf: 0.1 in 3/1/1 butanol (BuOH)/acetic acid (HAc/ H_2O).

IIIB-2. N-Acetyl-Phenylalanine Methyl Ester (AcPheOMe).

N-acetylation of PheOMe HCl (compound IIIB-1) was also carried out by standard procedures(103). PheOMe HCl (5.4 g;25 mmoles) was dissolved in 50 ml of anhydrous MeOH and 3.0 ml (37.5 mmoles) of dry pyridine (Eastman) and cooled to 0°C in an ice bath. Acetic anhydride (3.5 ml; 37.5 mmoles) (Eastman) was then added dropwise, the solution allowed to slowly warm to room temperature and finally stirred for an additional 3 hrs. The solvents were rotary evaporated under vacuum and the resulting oil dissolved in 150 ml of chloroform ($CHCl_3$). The organic layer was washed 3 times with 0.1 N HCl to remove excess pyridine, 2 times with saturated, aqueous sodium bicarbonate ($NaHCO_3$), once with saturated saline, dried over sodium sul-

fate (Na_2SO_4) and filtered. The solvents were rotary evaporated under vacuum and the product crystallized from MeOH / ethyl ether: 5.1 g (92%) recovered. (m.p. 89-90°C, m.p. lit.(105) 90-91°C; TLC Rf: 0.9 in 9/1 $CHCl_3$/MeOH; IR: 3300 cm^{-1} (NH), 1750 cm^{-1} (ester carbonyl), 1640 cm^{-1} (amide-I) and 1530 cm^{-1} (amide-II))

IIIB-3. Phenylalaninol (Pheol).

Reduction of the methyl ester of D- or L-phe (compound IIIB-1) to the corresponding alcohol was performed by the methods of Jones et al.(106). PheOMe HCl (10.8g; 50 mmoles) was added a little at a time over about an hour to a vigorously stirring slurry of 5.0 g lithium tetrahydroaluminate (Matheson, Coleman and Bell) in 200 ml of dry tetrahydrofuran (THF). The reaction was quenched after 24 hrs. by dropwise addition of 5 ml 1 N NaOH in 7 ml THF followed 30 min. later with 15 ml H_2O. The resulting white precipitates were filtered and the filtrate concentrated under vacuum. The residues were taken up into 300 ml of ethyl acetate (EtOAc) and the organic layer washed with saturated, aqueous $NaHCO_3$ and saturated saline, dried over Na_2SO_4 and filtered. The solvents were rotary evaporated under vacuum and the product recrystallized from benzene (Bz) yielding 5.9 g (78%). (m.p. 90-91°C, m.p. lit.(106) 91-92°C; TLC Rf: 0.4 in 3/1/1 BuOH/HAc/H2O).

IIIB-4. N-Acetyl-Phenylalaninol (AcPheol).

N-acetylation of Pheol (compound IIIB-3) was carried out by the methods of Jones et al(106) and Greenstein and Winitz(103). Pheol (2.27 g; 15 mmoles) was dissolved in 100 ml of anhydrous MeOH and 1.8 ml (22.5 mmoles) dry pyridine (Eastman), cooled to 0°C in an ice bath and treated dropwise with 2.1 ml (22.5 mmoles) acetic anhydride (Eastman). The solution was allowed to slowly warm to room temperature and stir for an additional 3 hrs. TLC indicated complete conversion of the starting materials to the N- and N,O- acylated products. To hydrolyze the unwanted ester, the mixture was again cooled to 0°C in an ice bath, brought to and maintained at pH 10-11 with 1 N NaOH for 30 min. and finally neutralized to pH 7 with 1 M sulfuric acid. The MeOH solvents were rotary evaporated under vacuum and the resulting oils extracted into $CHCl_3$. The organic layer was washed 3 times with 0.1 N HCl, 2 times with saturated, aqueous $NaHCO_3$ and saturated saline, dried over Na_2SO_4 and filtered. The solvents were again rotary evaporated under vacuum and crystals (2.4 g, 86%) were obtained from Bz-hexanes. (m.p. 101-102°C, m.p. lit.(106) 100-102°C; TLC: Rf 0.4 in 9/1 $CHCl_3$/MeOH; IR: 3270- 3170 cm^{-1} (NH,OH), 1640 cm^{-1} (amide-I) and 1530 cm^{-1} (amideII)).

IIIB-5. N-Benzoyl-Phenylalaninol (BzPheol).

The N-benzoylated derivative of D- or L-Pheol (compound IIIB-3) was prepared by methods similar to those employed for N-acetylation. Pheol (2.27 g; 15 mmoles) was dissolved in 100 ml of anhydrous MeOH and 1.8 ml (22.5 mmoles) dry pyridine (Eastman), cooled to 0°C in an ice bath and to it was added dropwise 1.8 ml (22.5 mmoles) benzoyl chloride (Eastman). The reaction was slowly warmed to room temperature and stirred for an additional 3 hrs. The benzoyl ester was hydrolyzed by treatment of the recooled solution with 1 M NaOH to pH 10 - 11 as was earlier discussed for compound IIIB-4. After neutralization to pH 7 with 1 M sulfuric acid, the MeOH was removed by rotary evaporation and the residue taken up into 200 ml of EtOAc. The organic layer was washed 3 times with 0.1 HCl, 2 times with saturated aqueous $NaHCO_3$ and saturated saline, dried over Na_2SO_4 and filtered. The solvents were concentrated under vacuum and 2.4 g (68%) of sharp, colorless needles deposited upon cooling. (m.p. 168-169°C, m.p. lit.(107) 169°C; TLC Rf: 0.5 in 9/1 $CHCl_3$/MeOH; IR: 3360-3260 cm^{-1}(NH,OH), 1620 cm^{-1}(amide-I) and 1530 cm^{-1} (amide-II)).

IIIB-6. N-Benzoyl-Phenylalaninal (BzPheal).

Mild oxidation of the compound IIIB-5 (BzPheol) to the aldehyde was performed by the general methods of Thompson(21). A solution of 1.1 g (4.2 mmoles) of BzPheol and 4.0 g (20 mmoles) of 1-ethyl(3,3 dimethylaminopropyl) carbodiimide hydrochloride (Aldrich) in 25 ml of doubly distilled DMSO (Eastman) was strongly stirred for 10 min. then

treated dropwise with 2 ml of a 2 M anhydrous phosphoric acid solution in DMSO. After 3 hrs., 150 ml of a 0.1 M sodium phosphate buffer, pH 8, was added and the aqueous layer extracted twice with 100 ml of EtOAc. The organic layer was then washed with saturated, aqueous $NaHCO_3$ and saturated saline, dried over Na_2SO_4, filtered and the solvents rotary evaporated under vacuum. The BzPheal (492 mg; 45% yield) was crystalized from Bz-hexanes. (m.p. 141-142°C, m.p. lit.(108) 143-144°C; TLC Rf: 0.6 in 98/2 $CHCl_3$/MeOH (2,4-dinitrophenylhydrazine positive); IR: 3320 cm^{-1}(NH), 1620 cm^{-1}(amide -I), 1530 cm^{-1}(amide-II), 1741 cm^{-1}(CHO); ^{1}H-NMR($CDCl_3$): 1H singlet at 6.7 ppm (CHO)).

The D-enantiomer of BzPheal, also prepared from D-phe by similar methods, was found to have the same properties as the L-derivative. The mixed melting point of equal parts D- and L-BzPheal was 118-120°C.

IIIB-7. N-Acetyl-Phenylalaninal (AcPheal).

Mild reduction of the compound IIIB-2 (AcPheOMe) to the aldehyde was carried out by the general methods of Ito et al(95) as modified by Lowe and his coworkers(109). AcPheOMe (2.1 g; 9.5 mmoles) was dissolved in 50 ml of 1,2-dimethoxyethane (Matheson, Coleman and Bell) and cooled to -60°C in a dry-ice/ethanol bath. The solution was treated dropwise, over an hour period with 20 ml (20% in toluene) di-isobutylaluminohydride (Aldrich) and the reaction allowed to stir for an additional 2 hrs. before adding 100 ml of 2 N HCl and allowing

it to warm to 4°C with stirring in a cold room. In the cold, the aqueous phase was then seperated and extracted twice with EtOAc (50 ml) and then five times with MeOH-free $CHCl_3$. The $CHCl_3$ extracts were combined, washed with saturated saline, dried over magnesium chloride, filtered and the solvents rotary evaporated under vacuum to yield a white solid (723 mg, 40%) which appeared on TLC to be mainly aldehyde. Pure solids were afforded from EtOAc/ethyl ether/petroleum ether. (m.p. 105-107°C, m.p. lit.(109) 107-110°C; TLC Rf: 0.5 in 9/1 $CHCl_3$/MeOH (2,4-dinitro-phenylhydrazine postive); IR: 3320 cm^{-1} (NH), 1640 cm^{-1} (amide-I), 1530 cm^{-1} (amide-II) and 1740 cm^{-1} (CHO); ^{1}H-NMR: 1H singlet at 6.8 ppm (CHO)).

IIIC. pH and Temperature Dependencies of the Dissociation Constants of Inhibition: N-Acyl Amino Alcohol and Aldehyde Derivatives of Phenylalanine to Chymotrypsin.

IIIC-1. General Discussion of the Steady-State Techniques Employed.

Two spectrophotometric methods and a method employing a pH-stat technique have been utilized to quantitatively assess the dissociation constants for inhibition (K_I) of the N-acyl amnio alcohol and aldehyde derivatives of L-phenylalanine to the native enzyme Cht. Strict competitive inhibition of the enzyme by these general class of inhibitors has been previously demonstrated.(13,20)

IIIC-1a. Inhibition of N-Benzoyl-L-Tyrosine p-Nitroanilide Hydrolysis.

The specific substrate amide analog, N-benzoyl-L-tyrosine p-nitro-anilide (BzTpNA) has been shown to exhibit Michaelis-Menten type kinetics of hydrolysis by Cht.(58). It is a particularly attractive substrate for use in a competitive assay due to the strong visible light absorbing properties of the product of hydrolysis, p-nitroaniline (pNA) (Δm_{390}= 11,500)(58), and the substrate's convenient rate of turnover such that pseudo-first order conditions may be utilized. Accordingly, where $[Cht]_o < [BzTpNA]_o \ll K_m$ (K_m, the Michaelis constant for enzyme-sustrate associations prior to the rate determining step for product formation,= 0.6 mM at pH 7.8)(58), then the rate of pNA release (d[pNA]/dt = v) is:

$$v = kobs\ [BzTpNA] \qquad (7)$$

where:

$$kobs = kcat[Cht]/Km \qquad (8)$$

The course of the reaction can be followed spectrophotometrically to completion by observing the increase in absorbance due to pNA release at 390 nm. If first order conditions are met, a plot of ln(end point absorbance - absorbance at time=t) versus time is a straight line of slope = $-k_{obs}$ ($time^{-1}$).

Addition of a competitive inhibitor to the reaction will affect the substrate's K_m:(110)

$$K_{m,apparent} = K_{m,true}\left(1+\frac{[I]}{K_I}\right) \qquad (9)$$

A direct comparison can be made on the pseudo-first order rate constants for the uninhibited (k_u) and inhibited (k_i) reactions if carried out under the same conditions and [Cht]. After substitution of equation 9 into equation 8, it is seen that:

$$K_I = \frac{[I]}{\frac{k_u}{k_i} - 1} \qquad (10)$$

A similar relationship can be derived for the initial rates of the uninhibited (v_u) and inhibited (v_i) reactions, under identical conditions and [Cht] and [BzTpNA] when the time course of the reaction is slow. Then:

$$K_I = \frac{[I]}{\frac{v_u}{v_i} - 1} \qquad (11)$$

IIIC-1b. Proflavin Displacement Assay.

Hess and others(35-37,111-113) found the chromophore, proflavin, to reversibly associate with Cht forming a strong 1:1 noncovalent complex that is competitively displaced at diffusion controlled rates by active site directed substrates and inhibitors.(52,-112) Further, these same properties are found for forms of Cht in which the catalytically essential residues have been modified.(8,48,-78) In all cases, the enzyme-proflavin complex strongly absorbs at a visible wavelength that is significantly red-shifted from the absor-

bance maxima for free proflavin (λ_{max} = 465 nm and λ_{max} = 444 nm, respectively).(36,113) The parameters governing the EP complex (K_p, Δm_{465}) are therefore ascertainable for a given set of solution conditions from a characteristic shift in the absorbance at 465 nm upon addition of enzyme. Further, the binding constants for the association of active site directed substrates or inhibitors with the enzyme can be readily calculated from a spectrophotometrically observed displacement (decrease in absorbance at 465 nm) of the EP complex.(37)

The equilibrium dissociation constant governing the EP complex (K_p) and its Δm_{465} (defined as the difference molecular absorbtivity between the EP complex and free proflavin at 465 nm) were obtained in the following manner. Difference absorbance values at 465 nm (ΔA_{465}) are obtained from solutions containing constant [P] and a range of [E] such that $[P]_o < [E]_o$ and $[E] >> [EP]$, against a reference solution containing no enzyme. For these conditions, it has been shown that:(113,114)

$$\Delta A_{465} = \frac{\Delta m_{465} [E]_o [P]_o}{K_p + [E]_o} \qquad (12)$$

The inverse of equation 12 takes a linear form:

$$\frac{1}{\Delta A_{465}} = \frac{K_p}{\Delta m_{465} [P]_o} X \frac{1}{[E]_o} + \frac{1}{\Delta m_{465} [P]_o} \qquad (13)$$

The double reciprocal plot of $1/A_{465}$ versus $1/[E]$ yields Δm_{465} from the inverse of the intercept and K_p from the

slope/intercept of the line.

The equilibrium dissociation constants for the binding of inhibitors to Cht and its modified forms can be rendered from the following procedure and relationships (for an extensive review see reference 37). ΔA_{465} values are again obtained from difference spectra of solutions containing inhibitor, enzyme and proflavin where $[I]_o = K_I$, $[E]_o$ and $[P]_o = K_p$, and $[I]_o > [E]_o$ against a reference solution containing no enzyme. From the values for K_p and Δm_{465} obtained earlier for similar solution conditions, the ΔA_{465} value gives knowledge of both [EP] and $[E]_{free}$:

$$[EP] = \frac{\Delta A_{465}}{\Delta m_{465}} = \frac{[E]_{free}[P]_{free}}{K_p} \tag{14}$$

Since:

$$[P]_{free} = [P]_o - [EP] = [P]_o - \frac{\Delta A_{465}}{\Delta m_{465}} \tag{15}$$

$$[E]_{free} = \left[K_p\left(\frac{\Delta A_{465}}{\Delta m_{465}}\right)\right] \Big/ \left[[P]_o - \left(\frac{\Delta A_{465}}{\Delta m_{465}}\right)\right] = \gamma \tag{16}$$

Further, in the presence of proflavin and the inhibitor:

$$[E]_o = [E]_{free} + [EP] + [EI] \tag{17}$$

so that,

$$[EI] = [E]_o - \gamma - \frac{\Delta A_{465}}{\Delta m_{465}} \tag{18}$$

Proper substitution of equations 17 and 18 into the relationship:

$$K_I = \frac{[E]_{free}\,[I]_{free}}{[EI]} \qquad (19)$$

noting that if $[I]_o$ is not >> [EI] so that $[I]_{free} = [I]_o$ then $[I]_{free} = [I]_o - [EI]$, and therefore,

$$K_I = \frac{\gamma\,[I]_o - [E]_o - \gamma - \frac{\Delta A_{465}}{\Delta m_{465}}}{[E]_o - \gamma - \frac{\Delta A_{465}}{\Delta m_{465}}} \qquad (20)$$

Under conditions where proflavin-inhibitor interactions occur, the gathered ΔA_{465} values need be corrected for the inhibitor-proflavin complex contribution.(37) Fortunately, the N-acyl amino aldehyde derivatives of L-phenylalanine possess K_I's several orders orders of magnitude below the concentration of inhibitor necessary to significantly interact with proflavin, uncomplicating the calculations.

IIIC-1c. pH Stat Assay.

An alternative method for determining equilibrium dissociation constants for the inhibition of Cht takes advantage of the net production of acid when ester substrates (S) are hydrolyzed by the enzyme. The course of the reaction, then, can be followed by the rate at which a base of known normality is added to maintain a constant solution pH. Reactions can be carried to completion and recorded as a function of base added per unit time. Velocity at $[S]_t$

for a constant time increment dt are calculated in the region where Michaelis-Menten kinetics are obeyed ($[S]_t$ = 3-5 to 1/3-1/5 times $K_{m,apparent}$) and are appropriately plotted to obtain the parameters $K_{m,apparent}$ and $V_{max,apparent}$. Accordingly, since the amount of base added at any time = t (b_t) is proportional to the amount of substrate hydrolyzed, then, letting:

$$C_1 = \frac{(b_{t=\infty} - b_{t=0})}{[S]_{t=0}} \tag{21}$$

$$[S]_t = \frac{(b_{t=\infty} - b_t)}{C_1} \tag{22}$$

The velocity of turnover (v_t) at $[S]_t$ is calculated as:

$$v_t = \frac{([S]_{t-dt} - [S]_{t+dt})}{2dt} \tag{23}$$

Dilution of the [S] and [E] over the course of the reaction by the addition of base necessitates the use of a correction factor: allowing,

$$C_2 = \frac{([S]_{t=0})(\text{reaction volume}, t=0)}{(\underline{N}_{base})(b_{t=\infty} - b_{t=0})} \tag{24}$$

then the dilution correction factor (dcf) is:

$$dcf = 1 + \frac{(b_{t=0} + b_t)\, C_2}{(\text{reaction volume}, t=0)} \tag{25}$$

The corrected $[S]_t$ are then,

$$[S]_{t,c} = [S]_t \times dcf \qquad (26)$$

and are substituted into equation's 22 and 23. Also, the corrected velocities to account for dilution of the enzyme is:

$$v_{t,c} = v_t \times dcf \qquad (27)$$

The inhibition constant K_I can then be calculated from an analysis of its effects on the apparent K_m of the substrate, as seen in the equation 9 described earlier.

IIIC-2. Binding Constants for BzPheol and D- and L- BzPheal at 25°C.

The equilibrium dissociation constants for the binding of BzPheol and BzPheal to Cht at its catalytic optimum pH of 7.8 and the pH dependency of BzPheal's K_I over the pH range 3-8 were determined at 25°C against the substrate BzTpNA by the general methods discussed earlier (IIIC-1a). The rates of pNA release were followed and recorded on a GCA/McPherson dual beam spectrophotometer model 707 equipped with a thermostatted cuvette holder in the sample beam against air in the reference beam. K_I values were calculated by comparing the pseudo-first order or initial rates of BzTpNA hydrolysis for duplicate runs at two concentrations of inhibitor (K_I and 3 X K_I) versus triplicate runs containing no inhibitor as carried out on the same day using the same stock solution of enzyme. In all these experiments, [BzTpNA] = 3-4 X 10^{-5} M so as to give a

baseline to endpoint deflection of 0.3-0.4 absorbance units while maintaining the pseudo-first order relationships [S] << K_m.

Due to their limited solubilities, the substrate and inhibitors were initially dissolved in redistilled DMSO and then added to the appropriate aqueous buffer. Stock solutions of enzyme were prepared in 10^{-3} M HCl to eliminate losses in activity due to auto- catalysis.

The most commonly used precedure for these sets of experiments was as follows: the appropriate amount of BzTpNA in DMSO was added to a solution containing the appropriate amounts of buffer salts and sodium chloride in degassed, distilled, deionized H_2O, adjusted to the desired pH with dilute solutions of HCl or NaOH (typically 1N) and finally diluted to the proper volume to give a stock solution of substrate containing 0.05 M buffer (sodium phosphate at pH's 5.5-8.0 or sodium acetate at pH's 5.5 and below), 0.1 M in sodium chloride and 10% (v/v) DMSO. To 3.0 ml of this substrate-buffer solution in a 1 cm cuvette was then added 100 µl of DMSO alone or the appropriate concentration of inhibitor in DMSO and the mixture incubated at 25°C in the sample cell holder of the spectrophotometer while a baseline was recorded at 390 nm (typically, for 3-5 min.). To it, then, was added and rapidly mixed 100 µl of the stock solution of enzyme and the reaction recorded. Final solution conditions were 0.047 M in buffer, 0.094 M sodium chloride and 12.5% (v/v) DMSO at 25°C. Pseudo first order rate constants were calculated from a computer assisted least squares line regression analysis of the data points as selected from the reaction curves and representing 3-6 half-lives of the reac-

tion. Initial rates were hand calculated as absorbance change per unit time from reaction traces recorded on the most sensitive machine scale of 0 to 0.1 absorbance units.

IIIC-2a. Binding Constants for BzPheol and BzPheal at pH 7.8 and 25°C.

Due to its insufficient solubility at concentrations above 2 mM the K_I for BzPheol to Cht could only be estimated at pH 7.8 and 25°C as per the above conditions and methods. This difficulty pre-empted its use as the standard for non-covalent associations of the aldehyde analog to the enzyme.

For both the D- and L-enantiomers of BzPheal, the standard assay was carried out using [Cht] = 5×10^{-6} M (giving an uninhibited reaction half-time of 2 min.) [L-BzPheal] = 3.13 or 10.3×10^{-5} M or [D-BzPheal] = 0.1 or 1.1 mM.

To evaluate the effects of DMSO on the K_I at pH 7.8, a second experimental technique was employed. To 3.0 ml of a 0.05 M sodium phosphate, 0.1 M sodium chloride buffer at pH 7.8 in a 1 cm cuvette was added 100 l of BzTpNA (3×10^{-5} M) in DMSO and the mixture incubated at 25°C while a baseline was obtained at 390 nm. An aliquot (50 μl) of Cht was then added, rapidly mixed and the reaction followed to completion ([E] = 5×10^{-5} M). Final solution conditions were 0.047 M sodium phosphate, 0.094 M sodium chloride and 3.1% DMSO (v/v) at pH 7.8 and 25°C.

IIIC-2b. pH Dependency of Binding: BzPheal to Cht.

The pH dependency of the K_I for the L-enantiomer of BzPheal was determined over the pH range 3-8 at 0.5 pH intervals by the aforementioned BzTpNA standard assay at [BzPheal] = K_I or 3 X K_I. By appropriate increases in the concentration of enzyme, at pH's above 5.0 the reactions were followed to completion and the pseudo-first order rate constants obtained and compared. At pH 5.0 and below, the initial rates were compared for a single day and the experiment repeated on several occasions (see table 5) at various enzyme concentrations. No apparent dependence of K_I on the enzyme concentration was observed so that all the individual values of K_I at a particular pH were included in the mean and standard deviation reported.

Further, at pH 5.5 the effect of the buffer salt on K_I was evaluated. No significant change in the K_I for the sodium phosphate versus sodium acetate buffer was observed.

Finally, the effect of pre-incubating BzPheal with the enzyme on K_I was evaluated at pH 3.5. To a stock solution of 2 X 10^{-5} M enzyme in the appropriate acetate buffer was added DMSO or BzPheal in DMSO (to 10.3 X 10^{-5} M) and the solution allowed to stand at 25°C for 30 min. To 3.1 ml of this solution was then added 100 μl of the substrate in DMSO and the initial rate of hydrolysis followed at 390 nm. No significant difference between the K_I so derived and the K_I calculated by the earlier methods was detected.

TABLE 5

BUFFER COMPONENT AND ENZYME CONCENTRATIONS USED AND THE FREQUENCY OF K_I DETERMINATIONS: pH DEPENDENCE OF BZPHEAL'S ASSOCIATION WITH CHT[a]

pH	Buffer Component	[Cht] X 10^5, M	Occasions	Determinations
3.0	Sodium Acetate	2.2, 2.6	2	7
3.5	"	1.2, 2.6(2)	4	10
4.0	"	0.66, 1.4, 2.2, 2.6(2)	5	15
4.5	"	1.3, 2.2, 2.3	3	10
5.0	"	0.67, 1.1, 2.4	3	8
5.5	"	1.1(2)	2	6
5.5	Sodium Phosphate	1.1	1	4
6.0	"	1.3	1	4
6.5	"	1.1	1	4
7.0	"	0.2	1	4
7.5	"	0.1	1	4
8.0	"	0.2	1	4

[a]Solution conditions were: 0.05 M buffer component, 0.1 M sodium chloride and 12.5% of DMSO at 25°C.

III-3. Temperature Dependency of Binding: BzPheal and AcPheal Over the Range 5° to 35°C.

The temperature dependencies of the equilibrium dissociation constants of inhibition for BzPheal and AcPheal to Cht were determined by the proflavin displacement assay as described earlier (III-1b), and the general methods of Schultz et al.(8)

A Tm analyzer (Beckman 139000-W) modified for placement in the sample compartment of a Cary-15 dual beam spectrophotometer was utilized to facilitate the collection of ΔA_{465} data between 5°C and 35°C. The Tm analyzer consisted of an electrically heated, water cooled cuvette holder, an automatic temperature programmer, and a temperature bridge connected to a platinum resistance probe which measured the temperature of the sample solution in the cuvette. Temperature versus ΔA_{465} readings were plotted directly on a Hewlett-Packard X-Y recorder. When cooling, the sample chamber of the spectrophotometer was flushed with N_2 to prevent moisture condensation on the cuvette faces. The rate of sample heating was 0.5°C per min. All complete difference spectra were recorded over the range 540 to 420 nm.

In a typical experiment, a proflavin-buffer stock solution containing 3.5×10^{-5} M proflavin (P), 0.05 M sodium phosphate dibasic and 0.1 M sodium chloride in distilled, deionized, degassed H_2O was adjusted to pH 7.8 with a similar solution containing the monobasic form of sodium phosphate, filtered through a Millipore 0.3μ membrane and the actual concentration of proflavin determined

from its difference absorbance at 444 nm against a buffer reference. A difference baseline spectra was obtained for 3.0 ml of this same solution in each of two well-matched 1 cm cuvettes to which had been added 100 µl of BzPheal in DMSO or in which had been previously dissolved an appropriate amount of AcPheal. To the sample cell was then added 100 µl of the enzyme in 10^{-3} M HCl and an equal volume of 10^{-3} M HCl added to the reference cell and a difference spectra taken with the temperature of the solution carefully noted. Finally, the sample cell was cooled to 5°C and the relative absorbance at 465 nm versus temperature from 5° to 35°C recorded upon activation of the Tm analyzer program.

For BzPheal, duplicate runs on five separate occasions were performed where: [BzPheal] = 5.1 X 10^{-5} M, [Cht] = 1.95 - 2.97 X 10^{-5} M, [P] = 3.0 - 3.35 X 10^{-5} M in the cuvette and for the solution conditions: 0.047 M sodium phosphate, 0.094 M sodium chloride and 3% (v/v) DMSO at pH 7.8.

With AcPheal, duplicate runs on two occasions were completed where [AcPheal] = 7.01 - 7.09 X 10^{-4} M, [Cht] = 2.35 - 2.84 X 10^{-5} M, [P] = 3.92 - 4.79 X 10^{-5} M for the solution conditions: 0.048 M sodium phosphate, 0.097 M sodium chloride at pH 7.8.

The relative absorbance values at 465 nm at 5°C intervals from 5° to 35°C were converted to actual ΔA_{465} data as per the value obtained earlier at room temperature and corrected for the intrinsic temperature dependency of the ΔA_{465} for the inhibitor-proflavin solution containing no enzyme. From prior knowledge of the equilibrium dissociation constant governing the enzyme-proflavin com-

plex (K_p) and its corresponding difference molecular absorbtivity (Δm_{465}) at each selected temperature (courtesy of John R. Peters and Dr. Richard M. Schultz, see table 6), the K_I's were calculated according to the equations provided earlier (IIIC-1b).

IIIC-4. Binding of AcPheol Over the Temperature Range 10° to 40°C

The temperature dependency of the equilibrium dissociation constant of inhibition (K_I) for AcPheol to native Cht was determined from an analysis of its competitive inhibition of N-acetyl-L-phenylalanine methyl ester hydrolysis by the enzyme as followed on a pH stat (for a general discussion of the technique, see IIIC-1c). The Radiometer pH-stat aparatus employed consisted of a model TTT-2 pH meter/titrator, an ABU-11 automatic burette equipped with a 0.2500 ml barrel, an SBR-2c chart recorder and a type G2222B glass versus KCl reference electrode set emersed in a magnetically stirred and thermally jacketed 10 ml reaction vessel. The temperature was maintained with a Lauda K2-R constant temperature bath and monitored with a Yellow Springs Instruments model 46 TUC telethermometer via a probe mounted directly in the reaction solution. The pH meter was calibrated to pH 7.0 prior to each day's set of runs using a standard solution (Scientific Products) equilibrated to the proper temperature. Triplicate runs in the presence of 3-5 inhibitor concentrations at a particular temperature were performed on a single day using the same stock solution of enzyme. A 0.1000 $\pm$ 0.0001 N NaOH

TABLE 6

TEMPERATURE DEPENDENCE OF THE BINDING DISSOCIATION CONSTANT AND DIFFERENCE MOLECULAR ABSORBTIVITY AT 465 NM FOR THE CHT-PROFLAVIN COMPLEX AT pH 7.8[a]

T^oC	K_{p_5}[b] $X10^5$, M	ΔM_{465}[b] $X10^4$	CORR.[c]	T^oC	K_{p_5}[b] $X10^5$, M	ΔM_{465}[d] $X10^4$	CORR.[c]
5.1	1.06	2.45	+.0155	5.0	1.86	1.67	+.0009
11.0	1.73	2.32	+.0088	10.0	2.07	1.61	+.0024
20.0	2.02	2.30	+.0010	15.0	2.45	1.61	+.0039
25.0	2.70	2.23	0.0	20.0	3.13	1.57	+.0022
27.8	3.09	2.20	0.0	25.0	3.69	1.54	0.0
30.2	3.50	2.20	0.0	30.0	4.84	1.52	-.0039
35.0	4.43	2.18	-.0008	35.0	6.05	1.41	-.0095

[a] Data courtesy of J.R. Peters and R.M. Schultz.
[b] Solution conditions were: 0.05 M sodium phosphate and 0.1 M sodium chloride.
[c] Baseline correction (ΔA_{465}) for the intrinsic temperature dependency of the solution containing proflavin and inhibitor alone.
[d] Solution conditions were: 0.05 M sodium phosphate, 0.1 M sodium chloride, and 5% (v/v) DMSO.

titrant (Fisher) was used throughout.

A typical reaction was carried out as follows: the substrate and inhibitor were weighed together in a 5 ml volumetric flask such that [AcPheol] = 0.25 to 4 X K_I (between 0.01 and 0.1 M) and [APME] = 3 to 5 X K_m,apparent (between 3 and 20 mM) and dissolved just prior to use with heating in an aqueous buffer containing 0.0025 M sodium phosphate (dibasic) and 0.2 M sodium chloride previously adjusted to pH 7.8 with dilute HCl. A 1.0 ml aliquot of this solution was then placed in the glass reaction vessel, equilibrated to the desired temperature, adjusted to pH 7.8 or slightly above by manual addition of the 0.1 N NaOH titrant and the pH-stat apparatus activated so as to maintain the reaction mix at pH 7.8 by a controlled addition of the base via the automatic burette. A 3-5 min. baseline was obtained to detect H_2O rate of hydrolysis of the ester (none detected throughout). An aliquot (100 μl) of enzyme in 10^{-3} M HCl was then added to the reaction and the amount of base added to neutralize the evolved acid product of hydrolysis followed as a function of time on the chart recorder. By appropriate adjustment of the [Cht], the reactions were followed to completion (typically a half-time of 2 min. for the least inhibited reactions). K_m,apparent values were calculated from the slope and intercept of a computer assisted least squares linear regression of the double reciprocal plot of the dilution corrected data points ($1/v_{t,c}$ versus $1/[S]_{t,c}$) as selected from a manual plot of the transformed reaction curve and representing the Michaelis-Menten region ([S]t = 3-5 to 1/3-1/5 X K_m,apparent) according to the equations outlined

in section IIIC-1c. A computer assisted least squares linear regression of the secondary plot: mean $K_{m,apparent}$ versus [AcPheol], yielded K_I from the y-intercept/slope of the line.

IIIC-5. Temperature Dependence of the Hydration Constant for AcPheal.

The temperature dependence of the hydration constant (K_d) for the nucleophilic addition of deuterium oxide (D_2O) to the aldehydic carbonyl carbon of AcPheal over the temperature range 11° to 30°C was determined by ^{1}H-NMR for solution conditions similar to those employed in the study of AcPheal's association to native and MCht (0.05 M sodium phosphate, 0.1 M sodium chloride at pD = 7.4 which corresponds to a pH of 7.8). Spectra were recorded at each of the desired temperatures on two separate occasions. The peaks corresponding to the free aldehyde (singlet at 9.6 ppm) and the gem-diol (doublet about 5.2 ppm) were subsequently integrated and the hydration constant (K_d) calculated from the relationship:

$$K_d = \text{hydrate integral} / \text{aldehydic integral} \qquad (28)$$

Representative NMR spectra are offered in figure 11.

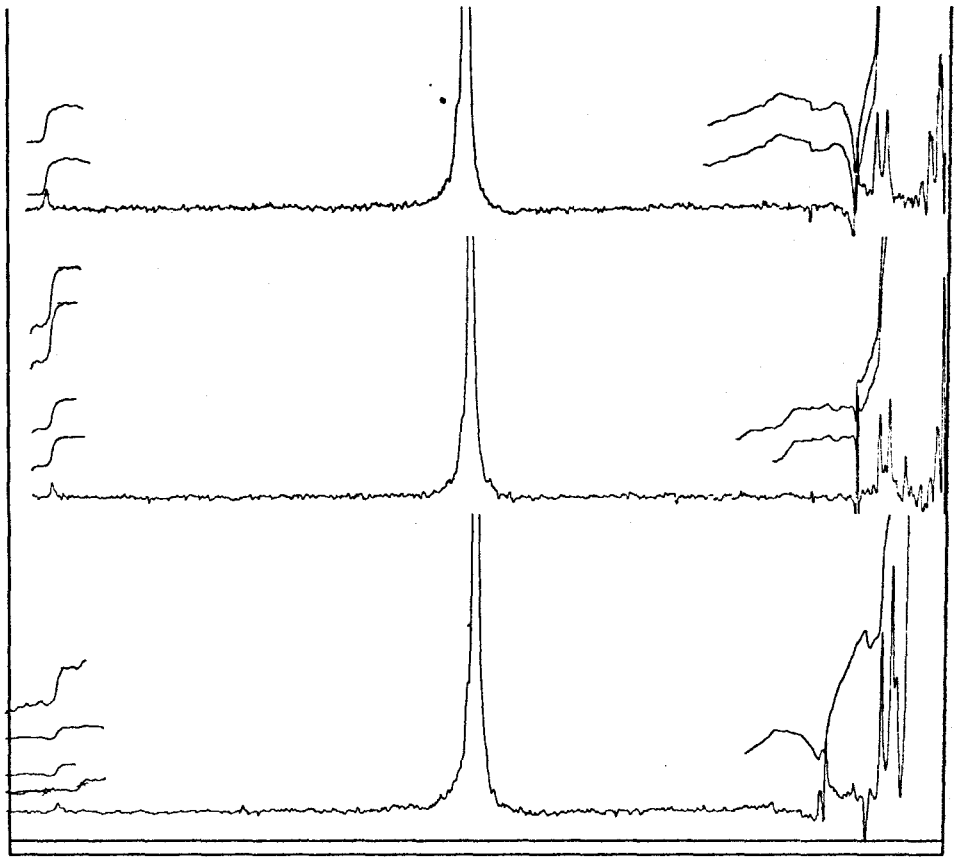

Figure 11. ^{1}H-NMR Spectra of AcPheal's Aldehydic, Benzyl, and Hydrate Protons.

Spectral signals and respective integrations are:

top: 10°C;

middle: 20°C; and

bottom: 30°C.

IIID. Dissociation Constants of Inhibition: N-Acyl Amino Aldehyde Derivatives of Phenylalanine to Forms of Chymotrypsin in Which Catalytically Essential Residues are Modified.

IIID-1. Synthesis of Dehydroalaninyl-195-Cht (ACht) and $\varepsilon 2_N$-Methyl-histidinyl-57-Cht (MCht).

Dehydroalaninyl-195-Cht (ACht) was prepared by acylation of the serine-195 hydroxyl side chain of Cht with phenylmethanesulfonyl fluoride (PMSF,Pierce), at pH 7.8, followed by a base induced Hoffman elimination at pH 13. The product was subsequently dialyzed, lyophilized and purified on an affinity column of lima bean trypsin inhibitor (Worthington) covalently bound to a Sepharose 4B resin (Pharmacia) as per the methods of Schultz et al(8) as modified from the procedures of Ako et al.(38)

$\varepsilon 2_N$-methylhistidinyl-57-Cht (MCht) was prepared by alkylation of the $\varepsilon 2_N$-nitrogen of the histidine-57 imidazole with methyl p-nitrobenzene sulfonate (Pierce) followed by purification (after dialysis and lyophilization) over an affinity column of turkey ova mucoid trypsin inhibitor (Sigma) covalently bound to a Sepharose 4B resin (Pharmacia) as described by Ryan and Feeney(77) and modified by Schultz et al.(8)

Both modified forms of the enzyme were stored at -20°C as their lyophilized powders prior to use. Further, each demonstrated < 1% of

the activity of an equivalent of the native enzyme as measured spectrophotometrically utilizing N-acetyl-L-tyrosine ethyl ester as substrate.(115)

IIID-2. Binding of BzPheal to ACht at pH 7.8 and 25°C.

As was the case for the association of BzPheol to Cht, BzPheal's poor binding to ACht and insufficient solubility above 2mM in aqueous buffer containing 12.5% (v/v) DMSO allowed only an estimate of its dissociation constant K_I as determined by the proflavin displacement procedure described earlier (IIIC-1b). The ACht was dissolved in 10 ml of an aqueous buffer containing 0.05 M sodium phosphate, 0.1 M sodium chloride and 10%(v/v) DMSO at pH 7.8, treated with 30 μl of a 2 mg/ml aqueous solution of PMSF, filtered through a Millipore 0.3μ membrane, and a baseline scan of 3.0 ml against 3.0 ml of buffer alone in 1 cm well-matched cuvettes was obtained on a Cary-15 dual beam recording spectrophotometer over 540 to 420 nm. To each was added 100 μl of proflavin in buffer and the solutions were rescanned. The cuvettes contained: $[ACht]_o = 2.13 \times 10^{-4}$ M, (sample cell only), $[P]_o = 3.69 \times 10^{-5}$ M, $[PMSF] = 3.2 \times 10^{-5}$ M at pH 7.8 and 25°C. To each cell was then added 100 μl of BzPheal in DMSO and the solutions rescanned. The altered conditions were: $[ACht]_o = 2.06 \times 10^{-4}$ M (sample cell only), $[P]_o = 3.57 \times 10^{-5}$ M and $[BzPheal]_o = 2 \times 10^{-3}$ M with 0.048 M sodium phosphate, 0.1 M sodium chloride and 12.5% (v/v) DMSO at pH 7.8 and 25°C. Duplicate

runs were performed on the same day.

IIID-3. Binding of BzPheal to MCht at pH 7.8 and 4.0 and 25°C

The binding dissociation constants for BzPheal to MCht at pH's 7.8 and 4.0 were also determined by the proflavin displacement assay as described earlier (III-1b). Prior to those experiments containing inhibitor, the equilibrium dissociation constant for the enzyme-proflavin complex and its respective molecular absorbtivity coefficient at 465 nm (Δm_{465}) were determined at pH 7.8 and 4.0 for the standard solution conditions and 5%(v/v) DMSO as previously discussed (IIIC-1b). Accordingly, a stock solution of proflavin in 0.05 M buffer salts, 0.1 M sodium chloride and 5% DMSO was prepared in distilled, deionized, degassed H_2O and adjusted to the desired pH with dilute HCl. Aliquots (3.0 ml) were placed in two well-matched 1 cm cuvettes and a baseline scan over 540 to 420 nm taken on a GCA/McPherson model 707 dual beam recording spectrophotometer. To the sample cell was then added sequential aliquots (50 or 100 μl) of a stock solution of MCht in proflavin-buffer taking ΔA_{465} readings after each addition. At pH 7.8, the conditions in the sample cuvette were: $[MCht]_o$ = 1.74 to 9.66 X 10^{-5} M, $[P]_o$ = 3.78 X 10^{-6} M with 0.05 M sodium phosphate, 0.1 M sodium chloride and 5% (v/v) DMSO. At pH 4.0, the solution conditions were: $[MCht]_o$ = 0.938 to 6.73 X 10^{-5} M, $[P]_o$ = 4.83 X 10^{-6} M, and 0.05 M sodium acetate, 0.1 M sodium chloride and 5% (v/v) DMSO. A computer

assisted least squares linear regression of the double reciprocal plot: $1/\Delta A_{465}$ versus 1/[MCht] revealed these following values: at pH 4.0, $K_P = 1.72 \pm .07 \times 10^{-4}$ M and $\Delta m_{465} = 2.2 \pm .06 \times 10^4$ abs/M/cm; and at pH 7.8, $K_P = 4.73 \pm .24 \times 10^{-5}$ M and $\Delta m_{465} = 2.47 \pm .12 \times 10^4$ abs/M/cm.

The binding constants of BzPheal to MCht were then determined. To 3.0 ml of a stock solution of proflavin in the standard aqueous buffer was added 100 μl of BzPheal in DMSO and a baseline spectra obtained over 540 to 420 nm on a Cary-15 dual beam recording spectrophotometer. To the sample cell was then added 100 μl of MCht in buffer and an equal volume of buffer to the reference beam and the solution rescanned. Calculation of the K_I's proceeded as described earlier (IIIC-1b). Duplicate runs at two concentrations of BzPheal were completed at pH 7.8: $[MCht]_o = 4.41$ or 1.61×10^{-5} M, $[P]_o = 3.56$ or 2.54×10^{-5} M and $[BzPheal]_o = 0.5$ to 10.3×10^{-5} M with 0.048 M sodium phosphate, 0.097 M sodium chloride, and 5% DMSO at 25°C. At pH 4.0, the experiments were performed in triplicate for two concentrations of BzPheal: $[BzPheal]_o = 1.99$ and 0.99×10^{-5} M, $[MCht]_o = 2.17 \times 10^{-5}$ M, $[P]_o = 2.23 \times 10^{-5}$ M, with 0.048 M sodium acetate, 0.097 M sodium chloride and 5% DMSO at 25°C.

IIID-4. Temperature Dependency of Binding: AcPheal to MCht at 7.8 Over the Range 5° to 35°C.

The temperature dependency of the dissociation constant for the

binding of AcPheal to MCht was determined by the proflavin displacement assay over the temperature range 5° to 35°C under similar conditions to those employed to determine AcPheal's temperature dependence of association to Cht (IIIC-3). Final solution conditions were: $[P]_o = 3.94 \times 10^{-5}$ M, $[MCht]_o = 2.88 \times 10^{-5}$ M, and $[AcPheal]_o = 9.11 \times 10^{-5}$ M with 0.05 M sodium phosphate and 0.1 M sodium chloride at pH 7.8. The values for K_p and Δm_{465} for these solution conditions are shown in table 7, courtesy of Dr. R.M. Schultz and J.R. Peters.

III-E. Pre-Steady State Associations: BzPheal to Cht and MCht.

By convenient analogy to the determination of those rate constants controlling acylation and deacylation of Cht by amide or ester substrates,(35-37) the rate constants for the formation and decomposition of the putative hemiacetal can be studied by a proflavin displacement assay. Since the equilibria governing aldehyde and proflavin non-covalent associations with the enzyme (K_S and K_p) are established almost instantaneously (should approach diffusion controlled rates(35,64) then any transient perturbation in the established concentration of an enzyme-proflavin complex (EP) upon addition of the aldehyde must be due to further complexation between aldehyde and enzyme. If a simple two step association process is

TABLE 7

TEMPERATURE DEPENDENCE OF THE BINDING DISSOCIATION CONSTANT AND DIFFERENCE MOLECULAR ABSORBTIVITY FOR THE MCHT-PROFLAVIN COMPLEX AT pH 7.8[a]

T°C	$K_p \times 10^5$, M	Δm_{465}
5.0	1.57	2.49
11.0	1.74	2.45
16.0	2.08	2.42
20.0	2.42	2.39
25.0	3.05	2.38
30.0	4.00	2.39
35.0	5.40	2.41

[a]Data courtesy of R.M. Schultz and J.R. Peters. Solution conditions were: 0.05 M sodium phosphate, 0.1 M sodium chloride at pH 7.8.

assumed:

$$E + al \underset{}{\overset{K_s}{\rightleftharpoons}} MC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H \qquad (29)$$

then the rate of disappearance of the EP complex (d[EP]/dt) is related to the formation of the putative hemiacetal (H) as:

$$(d[EP]/dt) = k_2[MC] + k_{-2}[H] \qquad (30)$$

where the rate constant for the formation of the hemiacetal is k_2 and its decomposition to the Michaelis Complex (MC) is k_{-2}. According to Himoe et al,(36) solution of equation 30 for [EP], [MC] and [H] and integrating reveals a pseudo-first order rate constant for the observed transient under the condition $[P]_o, [al]_o > [E]_o$:

$$k_{obs} = \frac{k_2[al]_0}{K_s\left(1 + \frac{[P]_o}{K_p}\right) + [al]_0} + k_{-2} \qquad (31)$$

At $[al]_o \ll K_S\ (1 + [I]/K_p)$, the equation 31 reduces to the linear relationship:

$$k_{obs} = \frac{k_2[al]_0}{K_s\left(1 + \frac{[P]_o}{K_p}\right)} + k_{-2} \qquad (32)$$

The pseudo-first order rate constant k_{obs} is calculated as the -slope of the line ln(absorbance at steady state - absorbance at time=t) versus time. The parameters k_2/K_S and k_{-2} can then be derived from the slope and y-intercept, respectively, of a plot of k_{obs} versus $[al]_o$ for a range of aldehyde concentrations.

The values for the dissociation constant governing the EP complex (K_p) over the pH range 3-8 were calculated according to the findings of Marini and Caplow.(118) They had previously shown K_p could be calculated at a particular pH from a knowledge of

binding dissociation constants for the conjugate acid (K_S) and (K_S') forms and the respective pKa:

$$K_p = \frac{K_s' K_a + K_s [H^+]}{K_a + [H^+]} \qquad (33)$$

Their data afforded: $K_S' = 3.25 \times 10^{-5}$ M, $K_S = 12.19 \times 10^{-5}$ M, at a pKa of 5.6, further noting that the molecular absorbtivity coefficient changed little from a value of 1.74×10^4 abs/M/cm over the pH range 4-8. The ratio of K_p values found at pH 7.8 (see IIIC-3) and at pH 4.0 (as determined here by the methods described in IIID-3) for both the binding of proflavin to MCht and Cht under the conditions utilized in these stopped-flow experiments were found to be identical to the ratio of K_p values at pH 4.0 and 7.8 by Marini and Caplow.(118) Accordingly, the value for K_p applied to equation 32 was calculated from equation 33 times the ratio: K_p determined in this work / K_p determined by Marini and Caplow,(118) each at pH 7.8. These are summarized in table 8.

An Aminco (courtesy of Dr. D. Gorenstein) and Durrum (courtesy of Dr. F. Kezdy) stopped-flow spectrophotometer systems were employed to observe and record EP relaxation transients at 465 nm upon rapid mixing with BzPheal. Accordingly, a stock solution of proflavin (P) in degassed aqueous buffer containing 0.05 M buffer salts (sodium phosphate at pH's above 5.5 and sodium acetate at pH 5.5 and below) and 0.1 M sodium chloride was prepared, adjusted to the proper pH, filtered (Millipore 0.3μ membrane) and to 5 ml lots were added 250 μl of BzPheal in DMSO or DMSO alone. To another 25 ml of the pro-

TABLE 8

pH DEPENDENCY OF BINDING: PROFLAVIN TO CHT FROM THE DATA OF MARINI AND CAPLOW[a]

pH	K_p,calc. X 10^5, M[b]	K_p X 10^5, M to Cht[c]	K_p X 10^5, M to MCht[c]
4.0	11.97	12.3[d]	17.2[d]
4.5	11.53	11.9	16.5
5.0	10.40	10.7	14.9
5.5	8.23	8.48	11.8
6.0	5.80	5.97	8.29
6.5	4.25	4.38	6.08
7.0	3.59	3.70	5.13
7.5	3.36	3.46	4.80
7.8	3.31	3.41[d]	4.73[d]
8.0	2.29	3.39	4.70

[a]Reference 118.
[b]Calculated according to equation 33.
[c]K_p,calc. X 1.03 for Cht; X 1.43 for MCht.
[d]Values for K_p as determined in this work for the solution conditions: 0.05 M buffer component (sodium acetate or sodium phosphate), 0.1 M sodium chloride and 25°C.

flavin buffer was added the appropriate amount of enzyme and dissolved just prior to use. At the beginning of each experimental day, the stopped-flow system was flushed with distilled, deionized, degassed H_2O and the 0 to 100% transmission of light at 465 nm adjusted to give a 0 to 10 volts full scale deflection on the system's phosphorous storage oscilloscope. Equal volumes (approximately 200 μl) of solutions, one containing proflavin and the other containing enzyme and proflavin, were then rapidly mixed and a baseline noted. Finally, a solution containing BzPheal and proflavin was rapidly mixed with the enzyme proflavin solution and the resulting transient recorded on a time base of proper speed to observe the establishment of the steady state. On the Aminco system, the transient was recorded as the change in per cent transmission versus time while the Durrum system recorded as absorbance change per unit time. Triplicate runs at each of four [BzPheal] at a particular pH were completed on a single day with the same enzyme solution, the oscilloscope tracings photographed, and the mean k_{obs} values calculated from first order plots as described above. Secondary plots at each pH were then constructed as k_{obs} versus [BzPheal] to yield the k_2/K_S and k_{-2} values. Solutions in the stopped-flow cuvette after mixing were 5 X 10^{-5} M in proflavin, 1 X 10^{-5} M in native Cht or 5 X 10^{-6} M in MCht, 0.09 M sodium chloride and 0.047 M sodium phosphate or sodium acetate and 5% (v/v) DMSO, [BzPheal] was varied between 2 and 8 X 10^{-4} M. Experiments utilizing Cht were performed at 0.5 pH units between pH 4 and 6.5 (k_{obs} being too fast to observe by stopped-flow at pH's > 6.5),

and those with MCht were determined at 0.5 pH units between pH 4 and 8.

To be certain the rate of proflavin displacement by the aldehyde was independent of the enzyme concentration, these same experimental conditions were repeated at pH 5.0 using 1 and 0.5 X 10^{-5} M Cht. No significant difference in the rate constants obtained nor in the slopes of the plots k_{obs} versus [BzPheal] were observed.

III-F. Temperature Dependency of the Relative Fluorescent Yield of Cht in the Presence of Saturating Concentrations of BzPheal.

The temperature dependence of the steady-state fluorescent yield of Cht alone and in the presence of saturating concentrations of BzPheal at pH 7.8 relative to an L-tryptophane standard were determined by the methods of Kim and Lumry.(50) Where the fluores- yield (Q) is defined as the ratio of light quanta absorbed (A) that emitted as fluoresence (I), then the fluorescent yield of a substance relative to some standard is:

$$Q/Q_{ref} = A/A_{ref} \times I_{ref}/I \qquad (34)$$

For the conditions and standard utilized in these experiments, it was arbitrarily assumed that $Q_{ref} = 1.00$, A = absorbance of the solution at the wavelength of excitation and I = area under the spectral emmission peak.(50)

An Aminco-Bowman D223 scanning spectrophotofluorimeter equipped with a circuit in the photomultiplier which automatically compensates for fluctuations in the zenon lamp light source, a thermostatted cuvette holder, and a mechanical stirring device were utilized. Desired temperatures were monitored with a Yellow Springs Instrument model 46 TUC telethermometer via a temperature probe mounted directed in the sample solution and maintained with a Lauda temperature bath. Absorbance measurements were made at 290 nm (λ of excitation) in a GCA/McPherson dual beam spectrophotometer against the suitable buffer reference. Emmission spectra were routinely recorded on a Houston Instruments Omnigraphics-2000 X-Y recorder over the range 200-500 nm. These fluoresence scans tended to be reproducible only on a single day's run due to heavy machine use. Thus, scans repeated on additional days or at different temperatures were corelated by comparison of a stock solution of L-tryptophane (trp) reference scanned at the commencement of each experiment.

The fluorencent yields on the following solutions at temperatures between 5° and 40°C were determined:

1) 2×10^{-5} M standard in distilled, deionized, degassed H_2O at pH 6.7;
2) 2×10^{-5} M trp + 1 mM BzPheal in pH 7.8 buffer*;
3) 4×10^{-6} M Cht in pH 7.8 buffer*; and
4) 4×10^{-6} M Cht + 1 mM BzPheal in pH 7.8 buffer*;

In a typical experiment, then, 3.0 ml of the trp standard or 3.0 ml of a degassed aqueous buffer* treated with 100 μl of BzPheal in DMSO or DMSO alone was preincubated to the desired temperature

with stirring and to it added, where appropriate, 100 μl of a stock solution of trp or Cht in 10^{-3} M HCl and the emmission spectra taken and recorded. Solutions containing Cht alone were scanned at only one temperature and the corresponding absorbance at 290 nm quickly taken thereafter due to the rapid rate of autocatalysis at pH 7.8. Other solutions were adjusted to the next temperature and re-scanned (warming and cooling directions both applied), continuing this process throughout the temperature range. Absorbance values at 290 nm were found to have no temperature dependence, so the value obtained at room temperature was utilized. Final solution conditions were, where applicable: 0.047 M sodium phosphate, 0.097 M sodium chloride and 3.1% DMSO at pH 7.8. Fluorescent yields were determined by hand integration of the emmission spectra over the range 300 - 500 nm with a Keuffel and Esser Co. model 4236M planimeter and were compared with the standard's fluorescence.

CHAPTER IV

RESULTS

IVA. Steady-State Associations of N-Acyl Amino Alcohol and Aldehyde Derivatives of Phenylalanine to Native and Active Site Modified Forms of Chymotrypsin

Standard steady-state kinetic assays(110) and proflavin displacement experiments(8,35-37) have been successfully utilized to study the equilibrium parameters which characterize the associations of N-acyl amino alcohol and aldehyde derivatives of L-phenylalanine (phe) to native and active site modified forms of Cht. Since the N-benzoyl-phe derivatives (BzPheol and BzPheal) are more easily prepared than their N-acetylated counterparts (AcPheol and AcPheal), they have been preferentially used for the materially expensive mechanism studies. However, the general insolubility of the N-benzoylated compounds and the sparsity of appropriate data in the literature on their respective substrate associations with the the enzyme precludes their use as good models for the thermodynamic studies.

Hence, the N-acetylated derivatives, which were synthesized or obtained as a generous gift from Dr. G. Lowe (Oxford Univ.), were utilized in the latter experiments.

The N-benzoyl-L-tyrosine p-nitroanilide (BzTpNA) assay(58) was successfully applied to determine the equilibrium dissociation constant of inhibition (K_I) for BzPheal to Cht. Typical first order plots representing the ln (change in the observed amount of p-nitroaniline released) versus time in the absence and presence of $[BzPheal]_o = K_I,obs$ and $3 \times K_I,obs$ at pH 7.8 and 25°C are presented in the figure 12. A calculated value of 2.6×10^{-5} M (3% DMSO cosolvent) for the K_I,obs so obtained is corroborated by that value determined under similar conditions using the proflavin displacement assay. These results are consistent with those expected for a competitive inhibitor.

The necessary use of DMSO cosolvent to solubilize sufficient amounts of BzPheal has a decided effect upon its observed binding constant to Cht and its hydration constant in solution. Increasing the DMSO content from 3.1% to 12.5% (v/v) results in a 35% increase in the K_I,obs (from 2.6 to 3.5×10^{-5} M at pH 7.8 and 25° C) and Chen et al(109) found K_h to decrease from 9 to 6 when the DMSO content is increased from 20% to 25% (v/v). Although these effects are significant, they remain nonetheless predictable as demonstrated by the work of Fink et al(114) such that the mechanistic interpretations derived from the association of BzPheal to Cht are qualitatively if not quantitatively accurate. In addition, attempts to accurately determine the binding constant for the N-benzoylated

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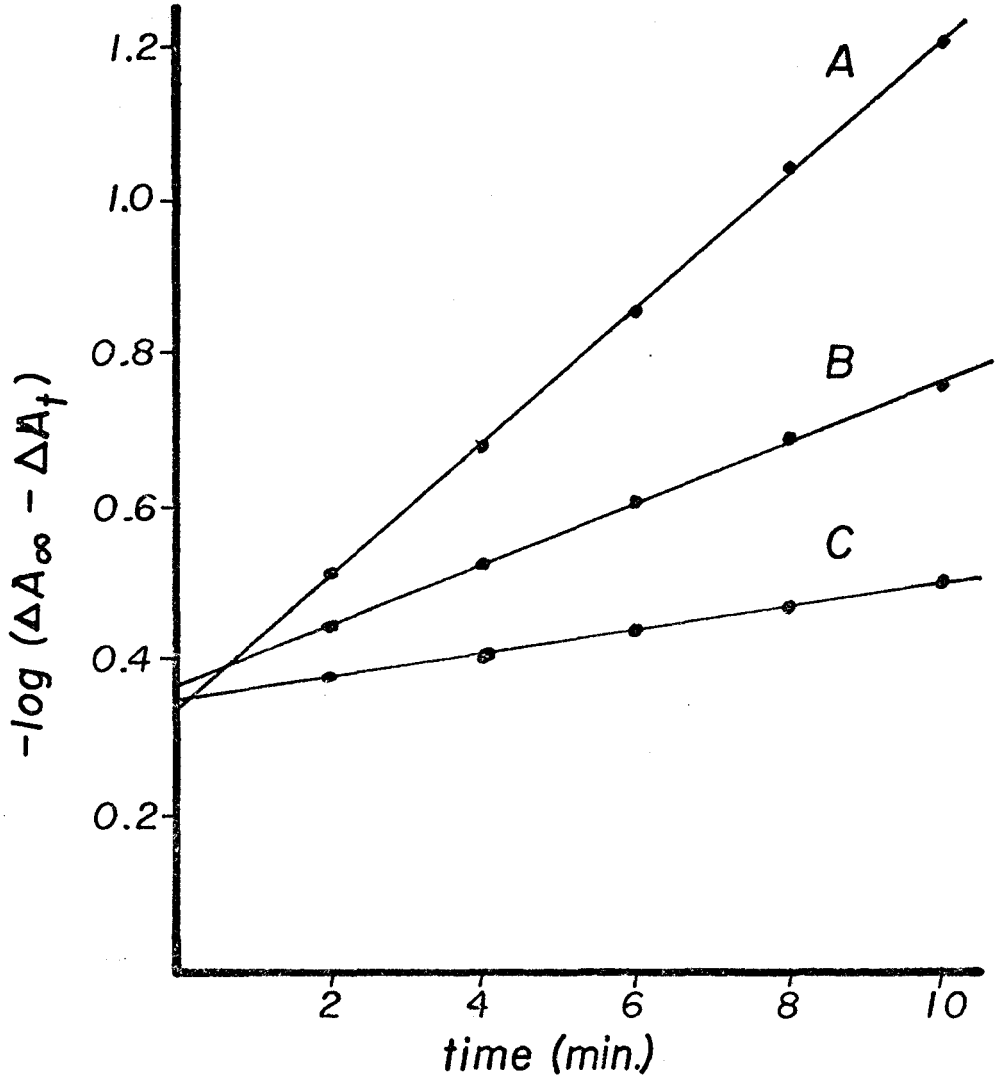
Figure 12. Typical First Order Plots of the Inhibition of the Cht-Catalyzed Hydrolysis of BzTpNA by BzPheal.

Conditions were: $[Cht]_o = 2.5 \times 10^{-6}$ M, $[BzTpNA]_o = 3.5 \times 10^{-5}$ M in 0.09 M sodium chloride, 0.047 M sodium phosphate and 3.1% DMSO at pH 7.8 and 25°C.

Plot A: $[BzPheal]_o = 0$;

B: " $= 3.4 \times 10^{-5}$ M; and,

C: " $= 0.1$ mM.


1.2
1.0
0.8
0.6
0.4
0.2
$-\log(\Delta A_\infty - \Delta A_t)$
A
B
C
2
4
6
8
10
time (min.)

alcohol and amide derivatives of phe were abandoned due to solubility limitations even when the DMSO content was raised to 20%. Thus, the reported values in this work represent minimum estimates.

For reasons that are as yet inexplicable, the BzTpNA assay yielded erratic, unreasonable binding constants for the associations of N-acetylated phe substrates and inhibitors to Cht. These inconsistencies remain a subject for future investigation and will not be considered further in this work. Fortunately, the concentrations of AcPheal necessary to significantly displace proflavin from the active site of Cht or MCht were sufficiently dilute so that those technical difficulties associated with correcting for its interactions with proflavin alone have been avoided. Such would not be the case for AcPheol, however. Accordingly, the pH-stat assay was alternatively applied. Representative double reciprocal plots of the inverse rate of the N-acetyl-L-phe methyl ester (AcPheOMe) substrate hydrolysis by Cht versus its inverse concentration at pH 7.8 and 25°C in the presence of successively increasing concentrations of AcPheol are shown in the figure 13. These results are also consistent with those expected for a competitive inhibitor.

The equilibrium dissociation constants observed for the steady-state associations of the N-acyl amino alcohol and aldehyde derivatives of L-phe to Cht (K_I,obs values) are compared with those values of K_m observed for structurally related substrates in table 9. To the native enzyme, the binding constants of AcPheal and BzPheal at pH 7.8 and 25°C are decidedly better (some 70 to 200-fold smaller K_I,obs) than those observed for the amide or alcohol deriv-

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Figure 13. Inhibition of the Cht-Catalyzed Hydrolysis of AcPheOMe by AcPheol.

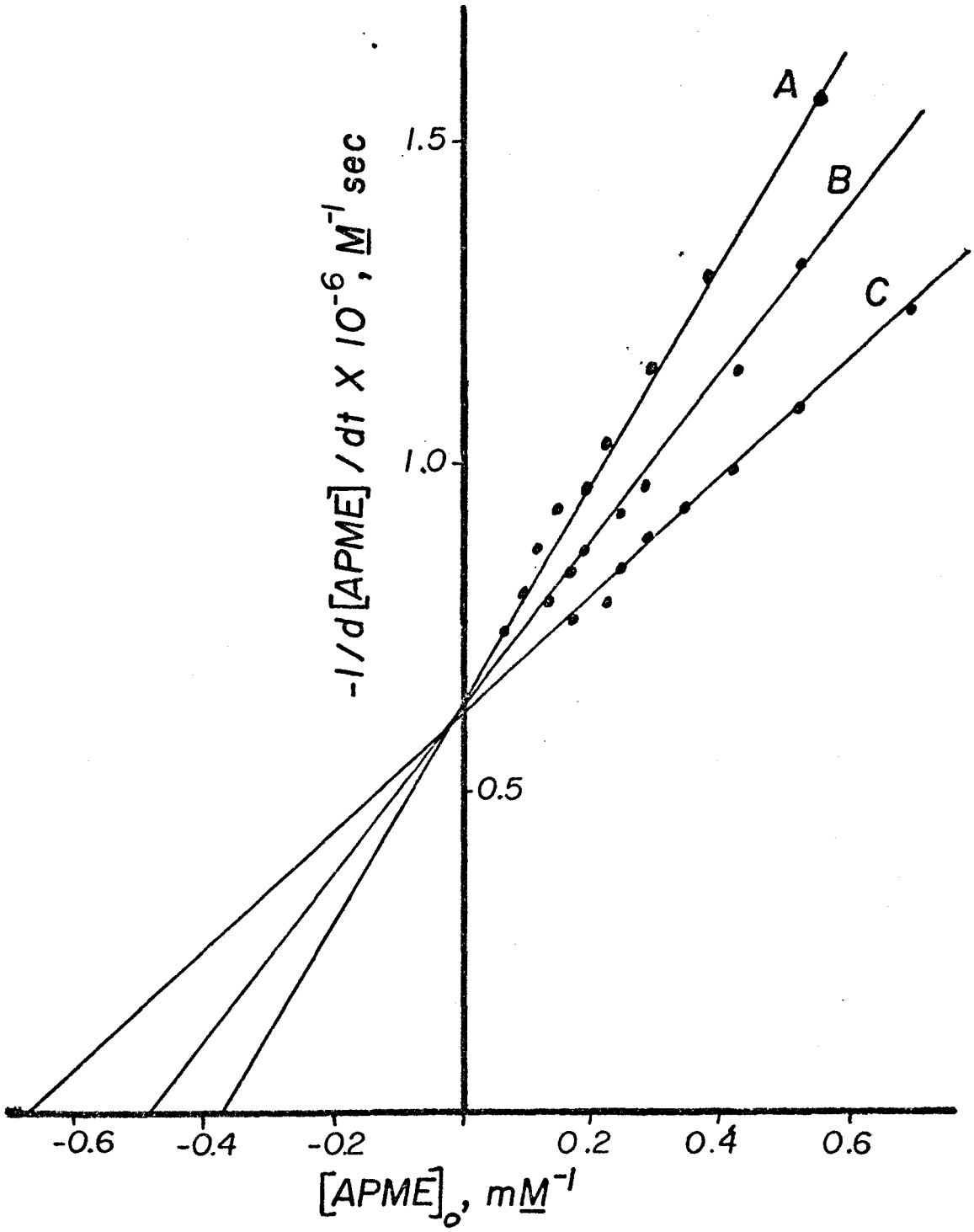
Solution conditions were: $[Cht]_o = 1.3 \times 10^{-7}$ M and $[AcPheOMe]_o = 8$ mM in 0.15 M sodium chloride and 0.0025 M sodium phosphate at pH 7.8 and 25°C.

$[AcPheol]_o$ was:

Plot A: 80 mM;

B: 51 mM; and

C: 25 mM.


A
B
C
1.5
1.0
0.5
-1/d[APME]/dt X 10⁻⁶, M⁻¹ sec
-0.6
-0.4
-0.2
0.2
0.4
0.6
[APME]₀, mM⁻¹

atives. Although varying somewhat in magnitude, these results confirm the potent inhibition of Cht by aldehyde analogs of specific substrates as reported in the earlier literature and suggest possible "transition state"-like modes of association. In addition, the binding constant of D-BzPheal (1.1 ± 0.2 mM) is several orders of magnitude poorer than the respective L-stereoisomer indicating that the enzyme retains its stereospecificity for the aldehyde analogs.

As was anticipated by Thompson(13,20,46) and determined in this work (see following sections) and by recent NMR(109) experiments, the trigonally coordinated forms of AcPheal and BzPheal preferentially associate with Cht, while the tetrahedrally coordinated hydrate forms poorly bind to the enzyme with K_I values approaching those observed for the alcohol derivatives. The intrinsic inhibition constant due to the associations of the unhydrated aldehyde component (K_I,al) can be calculated from a knowledge of the hydration constant (K_h) where:

$$K_h = [alOH_2] / [al] \qquad (35)$$

($alOH_2$ and al being the hydrated and unhydrated forms of the aldehyde respectively), such that:

$$K_I,al = K_I,obs / (1 + K_h). \qquad (36)$$

The values at 25°C of K_h = 10 for BzPheal (5% DMSO cosolvent) found by Chen et al(109) and K_h = 9.7 for AcPheal determined in this work (see following sections) are in good agreement with those values previously reported for the hydration of N-acyl amino aldehydes.(96) The appropriately corrected values of K_I,obs are presented in parentheses in table 9. Accordingly, the intrinsic binding constants

TABLE 9

EQUILIBRIUM DISSOCIATION CONSTANTS OBSERVED FOR BINDING OF THE N-ACYL AMINO ALCOHOL AND ALDEHYDE DERIVATIVES OF PHENYLALANINE TO NATIVE AND ACTIVE SITE MODIFIED FORMS OF CHYMOTRYPSIN AS COMPARED WITH STRUCTURALLY RELATED SUBSTRATES

Derivative	Enzyme Form	K_I or K_m (mM)	Cosolvent (v/v)	Reference
AcPheol	Cht	56 ± 4	-	this work
$AcPheNH_2$	"	31 ± 1	-	a
AcPheOME	"	1.1 ± 0.1	-	this work
AcPheal	"	0.45 ± 0.04 (0.042)	-	"
"	MCht	0.081 ± 0.002 (0.0076)	-	"
BzPheol	Cht	2.0	DMSO (12.5%)	"
$BzPheNH_2$	"	3.5	MeOH (3%)	117
BzPheal	"	0.036 ± 0.002(0.0033)	DMSO (12.5%)	this work
"	"	0.026 ± 0.001(0.0024)	" (3.1%)	"
"	MCht	0.0035 ± 0.0004(0.00032)	" (5%)	"
"	ACht	2.0	" (12.5%)	"
D-BzPheal	Cht	1.1 ± 0.2	" (12.5%)	"

a) Courtesy of J. H. Huff and Dr. R.M. Schultz

for the unhydrated aldehyde components of AcPheal and BzPheal are found to be some 10^3 to 10^4-fold better than respective substrates.

In the case of ACht, the catalytically essential ser-195 -OH is removed.(38) If covalent addition of that nucleophile to the aldehydic carbonyl carbon of BzPheal is required for its good binding to the native enzyme, then its association with ACht should be drastically diminished. Figure 14 demonstrates that this is indeed the result. At the maximally soluble concentration of BzPheal which is approximately 100-fold in excess of that necessary to half-saturate the native enzyme, proflavin displacement from ACht is only minimally effected.

In contrast, the affinities of AcPheal and BzPheal for MCht at pH 7.8 and 25°C (see table 9) are actually enhanced over that observed for the native enzyme, being some 5 to 6 times better. These results are somewhat surprising in that methylation of the ^{2}N-nitrogen of the his-57 imidazole side chain(76,77) should disrupt its participation in the transition states of Cht catalyses.(78,79) Since the rates of ester substrate hydrolyses by MCht are some 10^5 to 10^6 times slower than with the native enzyme,(78,79) transition state analog theory(14-17) predicts a much poorer binding of specific substrate aldehyde analogs to MCht if their complexes indeed mimic the transition state. That the reverse is true implies that aldehyde analogs in general are not "exact" " transition state analogs".

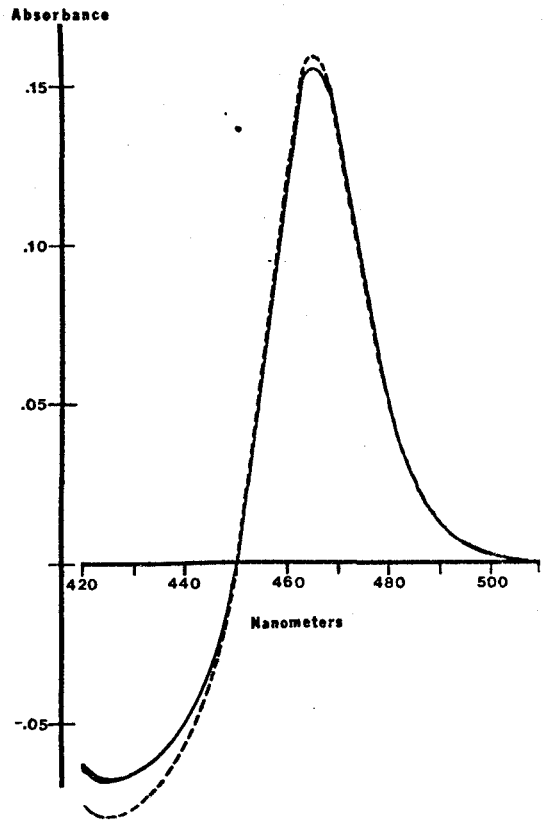


Figure 14. Difference Spectra of Proflavin Binding to ACht in the Absence and Presence of BzPheal.

Solid line is the observed absorbance spectra in the absence of BzPheal and dotted line is that observed with $[BzPheal]_o$ = 2 m<u>M</u>.Conditions were: $[ACht]_o$ = 0.21 m<u>M</u>, $[Proflavin]_o$ = 3.6 X 10^{-5} <u>M</u> in 0.1 <u>M</u> sodium chloride 0.05 <u>M</u> sodium phosphate and 12.5% DMSO at pH 7.8 and 25°C.

IVB. Mechanisms of Specific Substrate Aldehyde Analog Associations With Native Chymotrypsin.

IVB-1. pH Dependency of Binding: BzPheal to Cht

Although it seems clear from the ACht experiment that specific substrate aldehyde analogs covalently associate with Cht, the mechanisms of the reaction, particularly the role of the his-57 imidazole in the formation and stability of the adduct required further investigation.

By analogy to methylation, protonation of the $\varepsilon 2_N$-nitrogen of the his-57 imidazole side chain or another component of the his-57: asp-102 hydrogen bonded couple should disrupt its participation as a general base-acid catalyst in the transition states of Cht mediated reactions. Schultz and Cheerva(97) have previously argued that the pH dependency of the binding constant for the nonspecific substrate aldehyde analog, hydrocinnamaldehyde, to Cht might be mechanistically interpreted. Accordingly, where the equation 37 can be readily derived showing the dependence of K_I,al on terms describing non-covalent interactions between the unhydrated aldehyde and the enzyme (K_S,al) and the ratio of rate constants for the formation (k_2) and decomposition (k_{-2}) of the covalent adduct:

$$K_I,al = K_S,al \; / \; (1 + k_2/k_{-2}) \qquad (37)$$

if addition of the ser-195 δ-O^- nucleophile to the aldehydic carbonyl carbon of specific substrate aldehyde analogs is catalyzed by a mechanism similar to that which is commonly believed to occur in the

acylation step of substrate hydrolysis by Cht (see figures 6 and 8),(31-34) then formation of the adduct will require the basic form of the his-57 imidazole side chain and decomposition, by microscopic reversibility, will require the corresponding acid form of the general base-acid catalyst. Proper manipulation of terms describing the pH dependency of k_2 and k_{-2} (see also equations included in figure 8) reveals a relationship which predicts that as the solution pH is lowered from the catalytically optimum pH 8 and below the apparent acid dissociation constant of the his-57 imidazole (K_a 10^{-7}), $K_{I,al}$ should approach K_S(97)(note $k_{2,lim}$ and $k_{-2,lim}$ are the maximal, pH independent rate constants):

$$K_{I,al} = \frac{K_S}{1 + \frac{k_{2,lim}}{k_{-2,lim}} X \frac{K_a}{[H^+]}} \qquad (38)$$

The hydrocinnamaldehyde results of Schultz and Cheerva(97) tended to confirm the predictions of equation 38. If the associations of BzPheal are similar, then its $K_{I,al}$ is predicted to increase by a factor of 10^3 to 10^4 on going from pH 8 to pH 3.

As presented in table 10 and graphically illustrated in figure 15, the $K_{I,al}$ for BzPheal to Cht (determined by the BzTpNA assay) was found to increase by only a factor of 4 (from 3.88 to 13.2 X 10^{-6} M) over the pH range 8-3. The slight increase about the apparent pKa of 4.5 most probably reflects simple perturbations in K_S with pH as have been previously described for other neutral sub-

TABLE 10

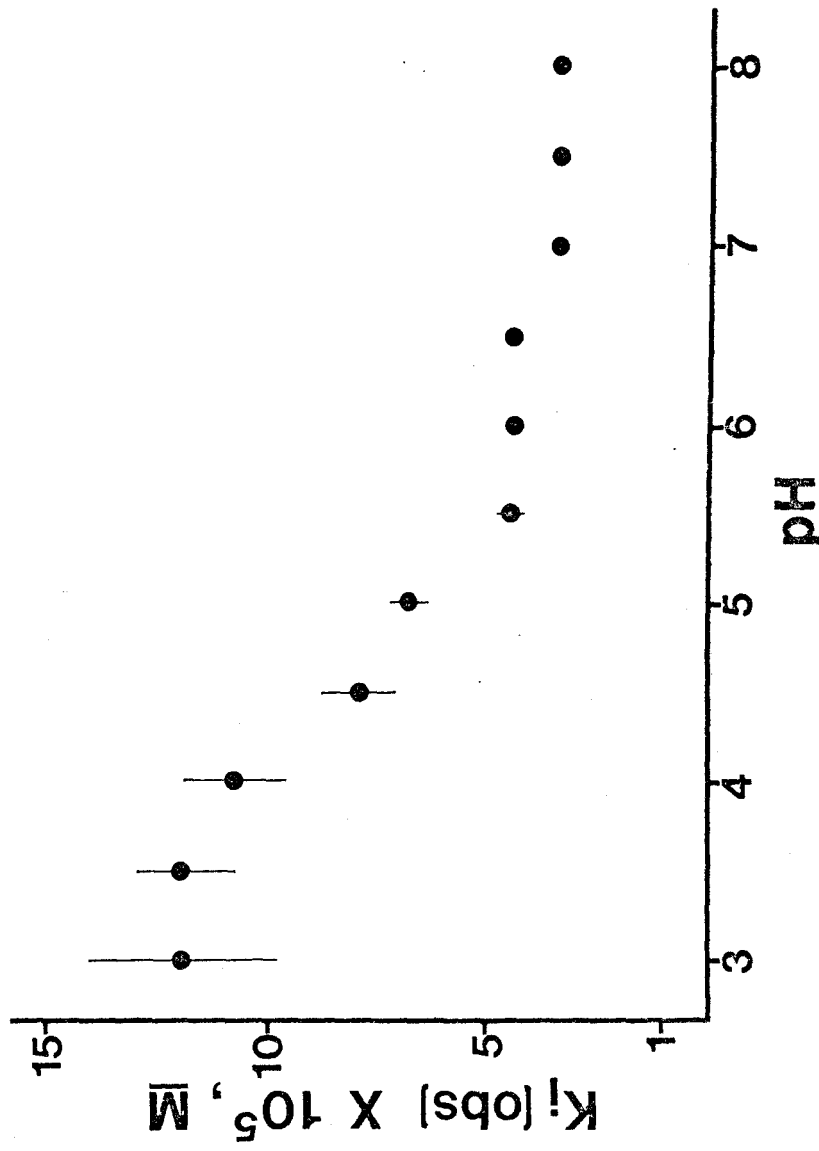
pH DEPENDENCY OF BINDING: BZPHEAL TO CHT[a]

pH	K_I,al X 10^6, M	Buffer Component
8.0	3.88 ± 0.04	Phosphate
7.5	3.91 ± 0.34	"
7.0	3.87 ± 0.28	"
6.5	4.96 ± 0.29	"
6.0	4.99 ± 0.29	"
5.5	5.19 ± 0.12	"
5.5	4.96 ± 0.16	Acetate
5.0	7.60 ± 0.73	"
4.5	8.82 ± 1.39	"
4.0	11.95 ± 1.97	"
3.5	13.07 ± 1.67	"
3.0	13.15 ± 2.33	"

[a] K_I values obtained against the substrate BzTpNA at 25°C and the solution conditions: 0.09 M sodium chloride, 0.047 M buffer component and 12.5% DMSO at the appropriate pH. Values corrected for the hydration of BzPheal.

LEGEND

Figure 15. pH dependency of Binding: BzPheal to Native Cht. Values determined using the BzTpNA assay. See text for details.


k_i (obs) X 10^5, M
15
10
5
1
3
4
5
6
7
8
pH

strates and inhibitors of Cht.(61) Clearly, the equation 38 does not describe the interactions of BzPheal with native Cht unless an extremely good K_S (< 0.1 mM) and a poor ratio of rate constants (k_2/k_{-2} < 10) are assumed. These assumptions, however, are unreasonable in light of the ACht results and the observed binding constants for the alcohol and amide derivaties of BzPhe which may be taken as minimum estimates of K_S for the aldehyde.

Rather, the equation 38 was derived on the assumption that the stable hemiacetal is anionic.(97) The lack in a significant pH dependence in K_I,al for BzPheal to Cht suggests the possibility that two protons are transferred in the covalent steps k_2 and k_{-2} such that the stable hemiacetal formed between specific substrate aldehyde analogs and Cht are neutral. Whether in the case of hydrocinnamaldehyde, the hemiacetal adduct, which has been shown to be formed by NMR studies,(100,109) remains anionic requires further investigation.

Several possible mechanisms can be conceived by which Cht will catalyze the formation of the neutral hemiacetal. Each rely upon the assumption that the his-57 imidazole is active as a general base-acid catalyst in the formation and decomposition of the adduct. In order to test that hypothesis, stopped-flow spectrophotometry was employed to quantitate and follow the pH dependence of the individual rate constants in the covalent reaction.

IVB-2. Pre-Steady State Associations of BzPheal with Cht.

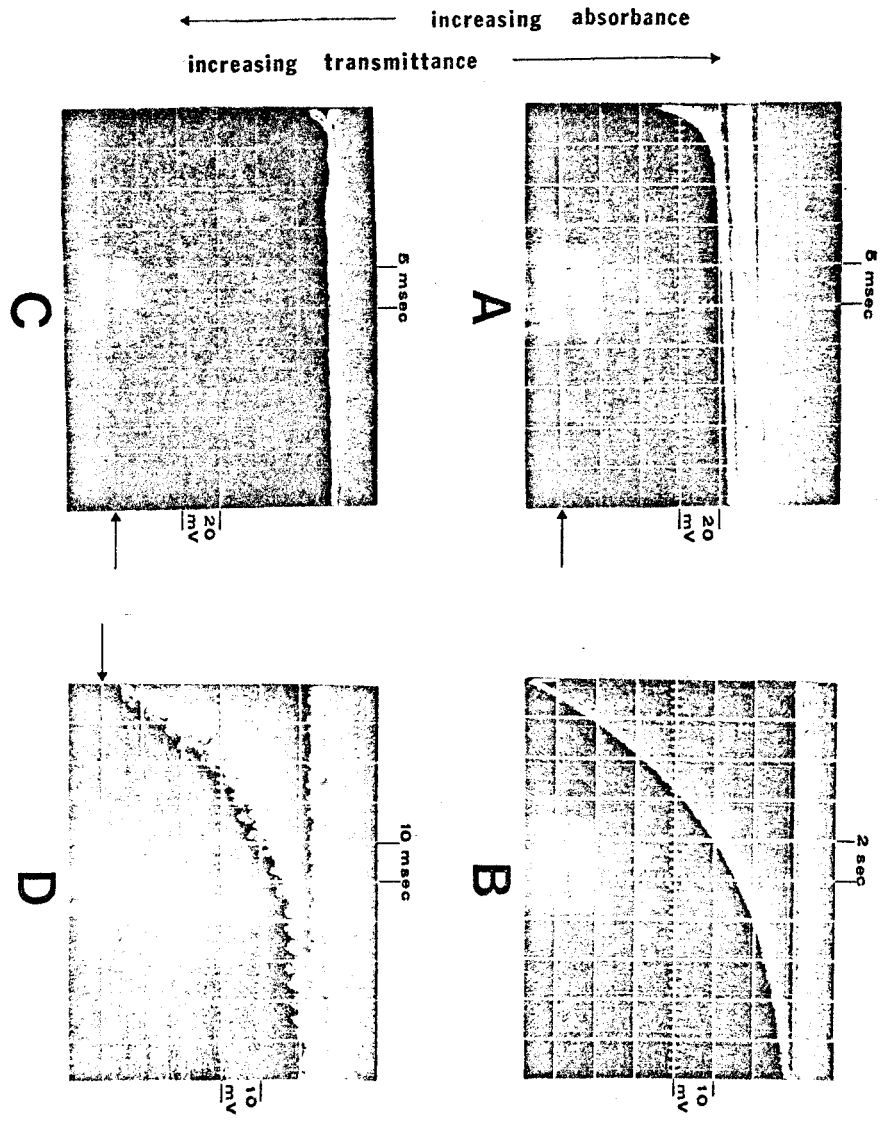
The diffusion controlled rates of substrate and inhibitor non-covalent associations with Cht have been calculated to be some 0.6 to 2 X 10^8 $\underline{M}^{-1}$ sec^{-1}(35,64) which are well out of the range of rates observable by stopped-flow spectrophotometry. Any observable transient corresponding to the displacement of a pre-established proflavin-Cht complex upon rapid mixing with BzPheal, then, is strong indication that accumulation of additional complexes, probably the covalent, hemiacetal adduct, between the aldehyde and enzyme is effected. In addition, if hemiacetal formation is the major contributor to the stability of BzPheal-Cht interactions (i.e. $K_S,al >> K_I,al$), then at [BzPheal] $<< K_S,al$ the observed transient should account for 100% of the proflavin displaced.

In light of these expectations, the initial results obtained upon rapidly mixing 5 X 10^{-5} $\underline{M}$ BzPheal with 1 X 10^{-5} $\underline{M}$ Cht in the presence of 5 X 10^{-5} $\underline{M}$ proflavin were perplexing. As shown in the figure 16A and its expansion in figure 16B, a biphasic displacement is observed wherein 90% of the proflavin-Cht complex is instantaneously eliminated with the remaining 10% disappearing with an apparent first order rate constant of about 0.3 sec^{-1}. It is believed that these results are evidence in support of the preferential association of the unhydrated aldehyde form of BzPheal with Cht. By increasing [BzPheal] 10-fold, a factor similar to its hydration constant, (K_h),(109) the slower transient disappears as shown in figure 16C. Accordingly, at pH 7.8, the rate of hemiacetal formation is too fast to be observed and the slow, observed rate at limiting concentrations of the unhydrated aldehyde is due to the rate limiting

LEGEND

Figure 16. Representative Oscilloscope Tracings of the Stopped-Flow Observed Transient Displacement of Proflavin from the Active Site of Cht by BzPheal.

See text for detail.


increasing absorbance
increasing transmittance
A
B
C
D
5 msec
2 sec
10 msec
20 mV
10 mV

transformation of the gem diol to free aldehyde form of BzPheal in solution. Concentrations of BzPheal in successive experiments were therefore adjusted to eliminate the effects of hydration on the observable rates.

At solution pH's 6.5 and below, the rates of BzPheal covalent associations with native Cht become observable. And, as predicted above, when the pseudo-first order transients are extrapolated to time=0, they are found to account for 100% of the displacement reaction (see representative oscilloscope tracing in figure 16D). The rate constants obtained from a first order analysis of the recorded oscilloscope tracings (k_{obs}) for the competitive displacement of proflavin from Cht by BzPheal over the pH range 3 to 6.5 are given in table 11 and represent the mean and standard deviation of a minimum of three determinations at each of three to five concentrations of BzPheal at the indicated solutions pH's.

By appropriate use of the expressions given in the Materials and Methods (see equation 31 and 31), the observed second order rate constants for the formation of the putative hemiacetal between BzPheal and Cht are calculated and shown in the table 12. Note that the observed data has been adjusted to account for hydration of the aldehyde by allowing:

$$(k_2/K_S)al = (k_2/K_S)obs \times (1 + K_h) \qquad (39)$$

where K_h = 10 under these standard solution conditions and 5% DMSO cosolvent. Unfortunately, the rate constants for the decomposition of the hemiacetal adduct at the various pH's could not be accurately calculated from the secondary plots of k_{obs} versus $[BzPheal]_o$

TABLE 11

OBSERVED FIRST ORDER RATE CONSTANTS FOR THE PRE-STEADY STATE ASSOCIATION OF BZPHEAL TO NATIVE CHT OVER THE pH RANGE 3.0 to 6.5[a]

	$[BzPheal]_o \times 10^4$, M					
	8.0	6.0	5.0	4.0	3.0	2.0
pH	k_{obs}, sec^{-1}					
6.5	-	-	171 ± 28	156 ± 34	133 ± 26	-
6.0	152 ± 17	-	92.3 ± 1.8	79.7 ± 2.9	50.5 ± 1.4	41.8 ± 1.2
5.5	146 ± 10	107 ± 8	-	70.5 ± 6.6	55.6 ± 3.7	-
5.0	123 ± 14	99.2 ± 4.3	82.8 ± 5.6	71.2 ± 6.6	49.8 ± 4.3	-
4.5	-	38.9 ± 3.0	32.1 ± 2.2	23.9 ± 1.0	-	13.4 ± 0.8
4.0	39.8 ± 1.1	-	26.4 ± 1.3	19.2 ± 0.8	14.5 ± 1.4	10.4 ± 1.0
3.0	-	-	2.99 + 0.05	2.43 ± 0.12	1.84 ± 0.08	1.16 ± 0.11

[a]For the solution conditions: $[Cht]_o = 1.0 \times 10^{-5}$ M, $[Proflavin]_o = 4.8 \times 10^{-5}$ M, in 0.048 M sodium phosphate or sodium acetate buffer, 0.095 M sodium chloride and 5% DMSO at 25°C.

TABLE 12

KINETIC CONSTANTS CALCULATED FROM THE OBSERVED PRE-STEADY STATE ASSOCIATIONS OF BZPHEAL WITH CHT.

pH	(k_2/K_S)obs X 10^{-4}, $\underline{M}^{-1}$ sec^{-1}	(k_2/K_S)calc X 10^{-5}, $\underline{M}^{-1}sec^{-1}$	K_I,al X 10^6, $\underline{M}$	k_{-2}, sec^{-1}	k_2 X 10^{-3}, sec^{-1}
6.5	61.4 ± 5.0	67.5 ± 5.5	3.31	22.3 ± 2.2	30.4 ± 2.5
6.0	33.3 ± 0.9	36.6 ± 1.0	3.34	12.2 ± 0.8	16.5 ± 0.4
5.5	28.9 ± 0.8	31.8 ± 0.9	3.46	11.0 ± 0.7	14.3 ± 0.4
5.0	20.7 ± 1.3	22.8 ± 1.4	5.07	11.6 ± 0.8	10.3 ± 0.6
4.5	9.18 ± 0.60	10.1 ± 0.7	5.89	5.9 ± 0.4	4.55 ± 0.3
4.0	6.04 ± 0.31	6.64 ± 0.34	7.97	5.3 ± 0.3	2.99 ± 0.15
3.0	0.868 ± 0.032	0.955 ± 0.035	8.77	0.83 ± 0.04	0.430 ± 0.016
8.0	(148)	(163)	2.59	(42)	(73)

(see the representative plot in figure 17; remaining plots are given in Appendix C) as previously expected since the y-intercepts were near zero in the coordinate system. However, k_{-2} can be alternatively calculated from a knowledge of K_I,al at the appropriate pH and a rearrangement of equation 37. Since the ratio (k_2/k_{-2}) is clearly >> 1 over the pH range 3 to 8, then:

$$k_{-2} = K_I,al \times (k_2/K_S)al \quad (40)$$

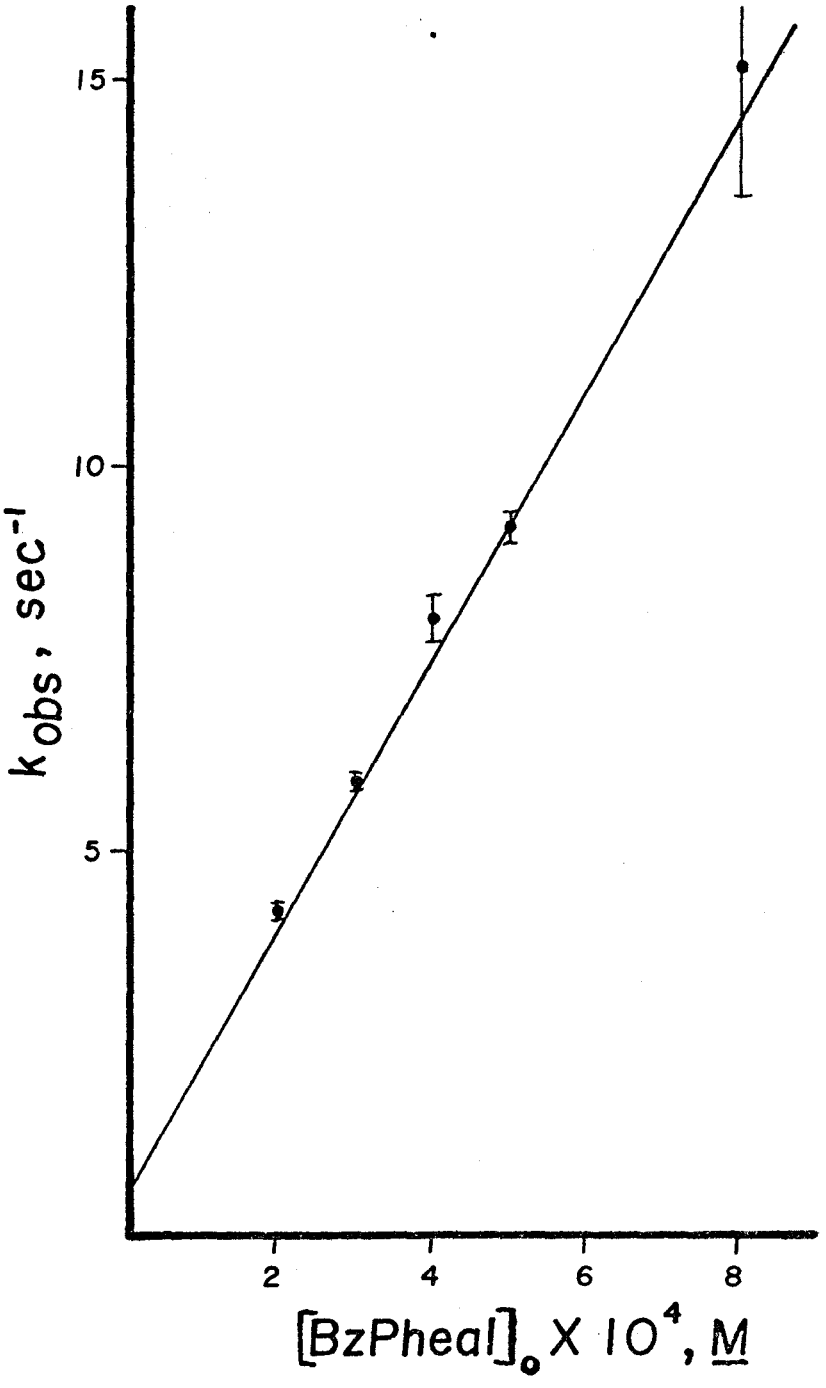
The values of K_I,al given in table 12 have been extracted from table 10, and corrected for the difference in the DMSO cosolvent concentrations between experiments: estimated to be some 20% lower in the 5% DMSO used here than in the earlier results obtained in 12.5% DMSO. In addition, k_2 has been estimated from the $(k_2/K_S)al$ data and are presented in table 12 and where a K_S of 4.5 mM has been assumed. That value of K_S is based on the K_m observed for the amide substrate $BzPheNH_2$(117) and was corrected for the DMSO cosolvent effects.

A graphical representation of the pH dependency of $(k_2/K_S)al$ for the association of BzPheal to Cht is presented in figure 18. Although it is clear that the rates of hemiacetal formation are base catalyzed, the sigmoidality in the data about pH 5 is not anticipated by a mechanism wherein a single base of pKa 7 controls the rate of covalent bond formation as is established in the case of substrate acylation by Cht.(31-34) The larger than expected rates are apparently real. At pH 4 the $(k_2/K_S)al$ value is an order of magnitude greater than that expected to result from small perturbations in K_S.(61) Further, while another study attributed similar anoma-

LEGEND

Figure 17. Representative Secondary Plot of the Stopped-Flow Observed Pre-Steady State Associations of BzPheal to Cht.

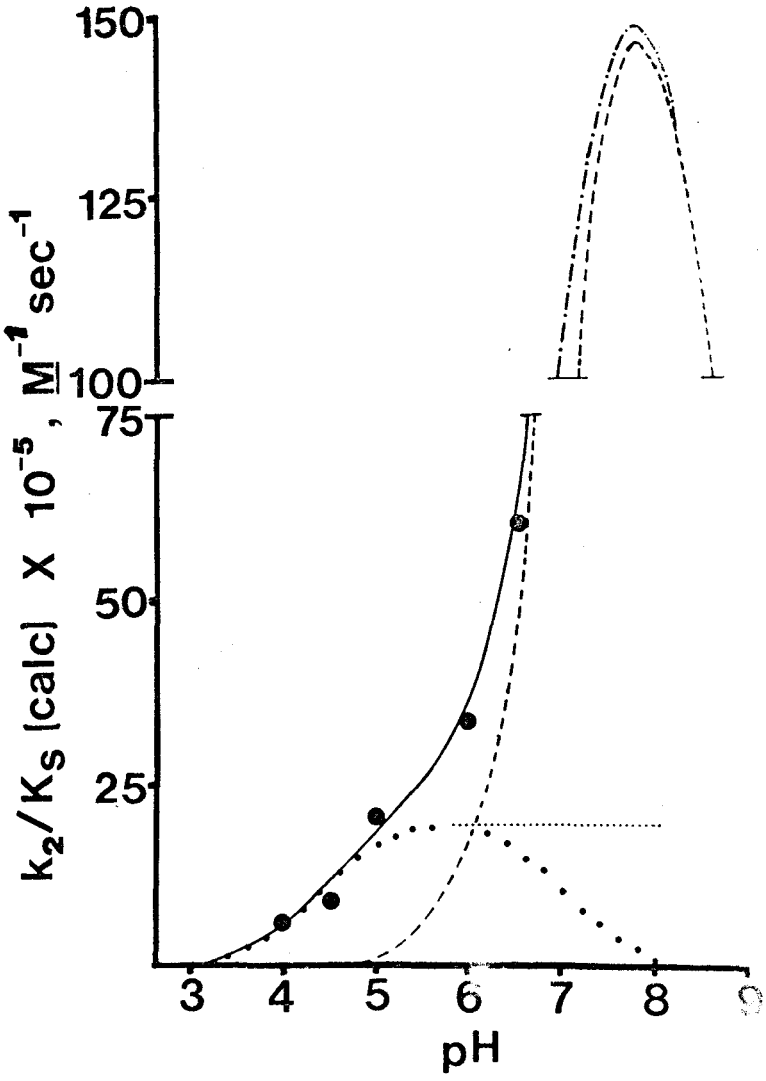
From the data in table 11, values at pH 6.0.


15
10
5
k_{obs}, sec^{-1}
2
4
6
8
$[BzPheal]_o \times 10^4$, M

LEGEND

Figure 18. pH Dependency of the Calculated Second Order Rate Constant for the Pre-Steady State Associations of BzPheal with Native Cht.

(●) Are the (k_2/K_S)calc values taken from table 12. ——— Represents the calculated line for base catalyzed hemiacetal formation by two groups on the enzyme of pKa's 7.0 and 4.4, respectively. – – – – Represents the contribution of the dibasic form (pKa 7) of Cht to the reaction, and, ····· indicates the rate contribution by the monobasic form (pKa 4.4)(the extension is the limiting value of (k_2/K_S)calc for the monobasic form). ------- Represents the calculated second order rate constants extrapolated into the pH region where hemiacetal formation is too fast to be observed using stopped-flow methods.


150
125
100
75
50
25
k_2/K_S (calc) X 10^{-5}, M^{-1} sec^{-1}
3
4
5
6
7
8
9
pH

lies observed in the kinetics of substrate analog hydrolysis to dimerization of the enzyme,(63) the above experiments were performed at [Cht] much lower than that necessary to observe polymerization.(118, 119) In addition, proflavin does not bind to the Cht dimer or polymer forms(52,119) such that the net effect upon addition of BzPheal and possible disruption of the dimer would be to decrease the apparent rates of proflavin displacement. As added proof, the $(k_2/K_S)al$ values at pH 5.5 were reevaluated and found to be identical with the original data for a $[Cht]_o$ 1/2 the original concentration used.

A computer assisted curve fitting revealed that the data in table 12 best conforms to a mechanism which includes general base catalysis by two groups on the native enzyme, and the equation 41 is derived:

$$\left(\frac{k_2}{K_s}\right)_{calc} = \frac{\left[\dfrac{\left(\frac{k_2}{K_s}\right)al,lim2}{1+[H^+]/K_{a2}+\frac{[H^+]^2}{K_{a1}K_{a2}}} + \dfrac{\left(\frac{k_2}{K_s}\right)al,lim1}{1+K_{a2}/[H^+]+[H^+]/K_{a1}}\right]}{1+[H^+]/K_{a3}+K_{a4}/[H^+]} \quad (41)$$

Ka3 and Ka4 refer to the acid dissociation constants of the γ-COOH of the asp-194 and the α-NH_3^+ of the ile-16, respectively, which have been previously shown to control substrate's ability to initially associate with the enzyme.(35,120) $(k_2/K_S)al,liml$ and $(k_2/K_S)al,lim2$ are the maximal second order rate constants for the monobasic and dibasic forms of Cht, respectively. The best fit of equation 41, as shown by the solid line in figure 18 was calculated using a pKal = 4.4 and pKa2 = 7.0 at $(k_2/K_S)al,liml$ = 24 X

10^6 $\underline{M}^{-1}$ and $(k_2/K_S)al,lim2 = 21 \times 10^7$ $\underline{M}^{-1}sec^{-1}$.

The dashed line in figure 18 represents the contribution of the monobasic form of Cht to the observed $(k_2/K_S)al$ and the dotted line refers to the contribution by the dibasic form of the enzyme to the observed rate. In addition, based on the equation 41, a value of $(k_2/K_S)al$ at pH 8 is calculated and reported in parentheses in table 12.

The appearance of the second pKa of 4.4 in the base catalyzed rates of hemiacetal formation between BzPheal and Cht is quite surprising and reasonable explanations for these results are elusive. Although a mechanism wherein the asp-102 carboxylate and the his-57 imidazole are of inverted pKa's (7.0 and 4.4, respectively) might be invoked as an explanation,(82,83) several recent NMR studies strongly support normal pKa's for these respective components. A more detailed analysis is offered in the Discussion Section.

IVB-3. Pre-Steady State Associations of BzPheal with MCht.

It is intuitively expected, based on the observed binding constants for BzPheal and AcPheal to MCht, that specific substrate aldehyde analogs covalently associate with the modified enzyme as hemiacetals. Stopped flow experiments run similar to those with the native enzyme bear out the prediction. Relaxation transients for the displacement of a pre-established proflavin-MCht complex by BzPheal were observable over the pH range 4 through 8 and conformed to the pseudo-first order equations offered in the Materials and Methods

Section. The calculated parameters as prepared by the methods discussed in the last section (see Appendix D for secondary plots) are given in tables 13 and 14. The steady state values of $K_{I,al}$ for BzPheal to MCht were obtained only at pH 7.8 and 4.0 so that only the respective k_{-2} values were calculable.

As illustrated in figure 19 and the Dixon(110) plot in figure 20, the pH dependency of $(k_2/K_S)al$ for BzPheal to MCht conforms to a mechanism in which hemiacetal formation is catalyzed by a single base of pKa=6.8 and a $(k_2/K_S)al,lim = 23 \times 10^6$ $\underline{M}^{-1}sec^{-1}$ (data also corrected for the influence of pH on the conformational transition in the enzyme due to protonation of the ile-16:asp-194 salt bridge as mentioned previously). These results are not suprising in that MCht has been previously shown to general base catalyze the hydrolysis of specific substrates with an apparent pKa similar to that of the native enzyme.(78,79) What is surprising is that MCht catalyzed hemiacetal formation with an apparent rate constant only about one order of magnitude slower than the native enzyme at pH 7.8 (19.6 versus 163 $\times 10^6$ $\underline{M}^{-1}$ sec^{-1}, respectively) while substrate hydrolysis is effected by the modified enzyme at rates 10^5 to 10^6 slower.(78,79)

IVC. Thermodynamics of Association of the N-Acyl Amino Alcohol and Aldehyde Derivatives of Phenylalanine to Cht and MCht

TABLE 13

OBSERVED FIRST ORDER RATE CONSTANTS FOR THE PRE-STEADY STATE ASSOCIATIONS OF BZPHEAL WITH MCHT OVER THE pH RANGE 4.0 to 7.8[a].

	$[BzPheal]_o \times 10^4$, M					
pH	8.0	6.0	5.0	4.0	3.0	2.1
	k_{obs} sec^{-1}					
7.8	74.3 ± 3.6	59.5 ± 3.0	51.3 ± 3.1	38.7 ± 2.9	34.7 ± 3.6	21.6 ± 1.2
7.5	70.0 ± 4.9	55.2 ± 3.8	-	35.8 ± 2.8	30.2 ± 1.1	-
7.0	64.6 ± 1.3	47.1 ± 1.4	-	34.4 ± 1.1	27.5 ± 1.2	-
6.5	28.2 ± 2.0	19.9 ± 1.2	-	-	8.69 ± 0.91	-
6.0	20.3 ± 2.6	-	12.6 ± 1.2	11.0 ± 0.7	-	6.14 ± 0.21
5.5	4.38 ± 0.47	3.23 ± 0.26	-	2.22 ± 0.11	1.79 ± 0.08	-
5.0	2.09 ± 0.11	1.68 ± 0.08	-	1.12 ± 0.08	0.904 + 0.091	-
4.0	0.818 ± 0.06	0.635 ± 0.05	-	0.473 ± 0.03	0.347 ± 0.02	-

[a]For $[MCht]_o = 5 \times 10^{-6}$ M, $[Prof\,in]_o = 5 \times 10^{-5}$ M at 25°C in 0.047 M sodium phosphate or sodium acetate, 0.095 M sodium chloride and 5% DMSO.

TABLE 14

KINETIC CONSTANTS CALCULATED FROM THE OBSERVED PRE-STEADY STATE ASSOCIATIONS OF BZPHEAL WITH MCHT.

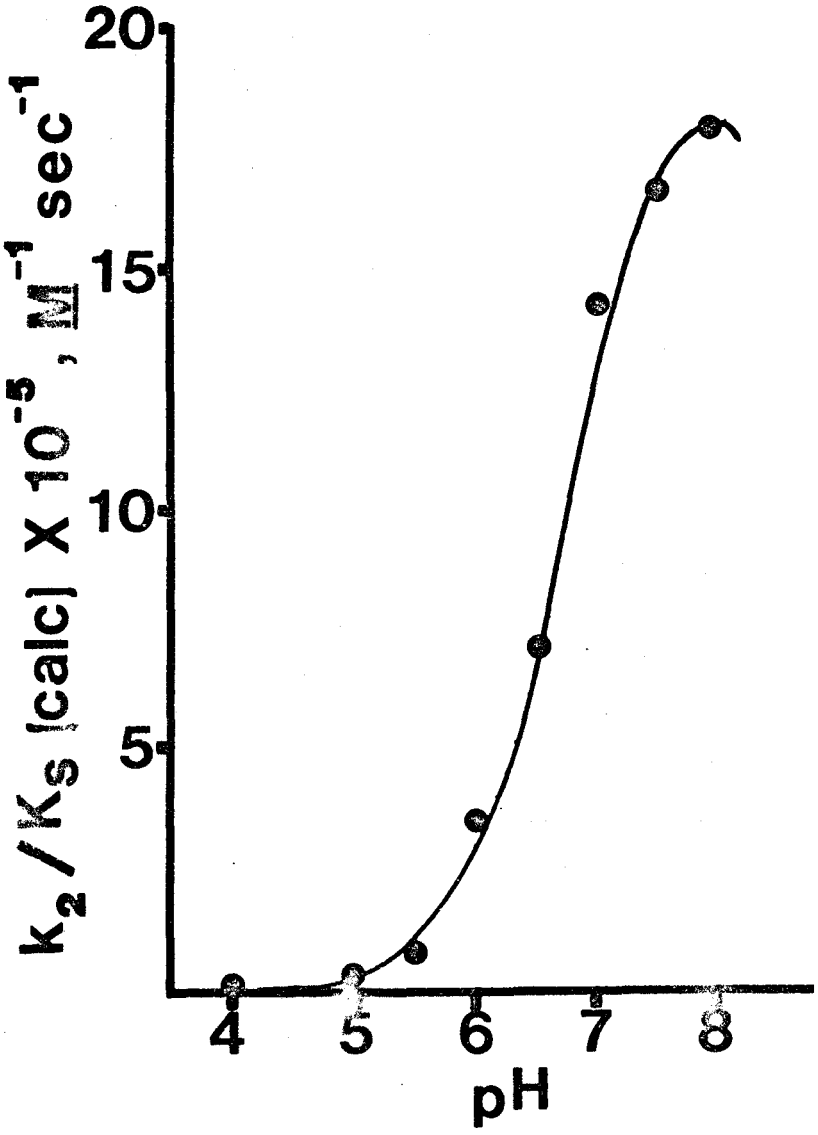
pH	(k_2/K_S)obs X 10^{-4}, $\underline{M}^{-1}sec^{-1}$	(k_2/K_S)calc X 10^{-5}, $\underline{M}^{-1}sec^{-1}$	$K_{I,al}$ X 10^{7}, $\underline{M}$	k_{-2} sec^{-1}	k_2 X 10^{-3}, sec
7.8	17.8 ± 1.0	19.6 ± 1.1	3.85 ± 0.44	0.75 ± 0.03	8.8
7.5	16.6 ± 0.9	18.3 ± 1.0			8.2
7.0	14.3 ± 0.8	15.7 ± 0.9			7.1
6.5	7.09 ± 0.18	7.80 ± 0.2			3.5
6.0	3.76 ± 0.14	4.14 ± 0.15			1.9
5.5	0.739 ± 0.025	0.813 ± 0.028			0.37
5.0	0.310 ± 0.011	0.341 ± 0.012			0.15
4.0	0.118 ± 0.006	0.130 ± 0.007	12.9 ± 2.5	0.016 ± 0.004	0.059

LEGEND

Figure 19. pH Dependency of the Calculated Second Order Rate Constant for the Pre-Steady State Associations of BzPheal with MCht.

(●) Are the (k_2/K_S)calc values taken from table 14.

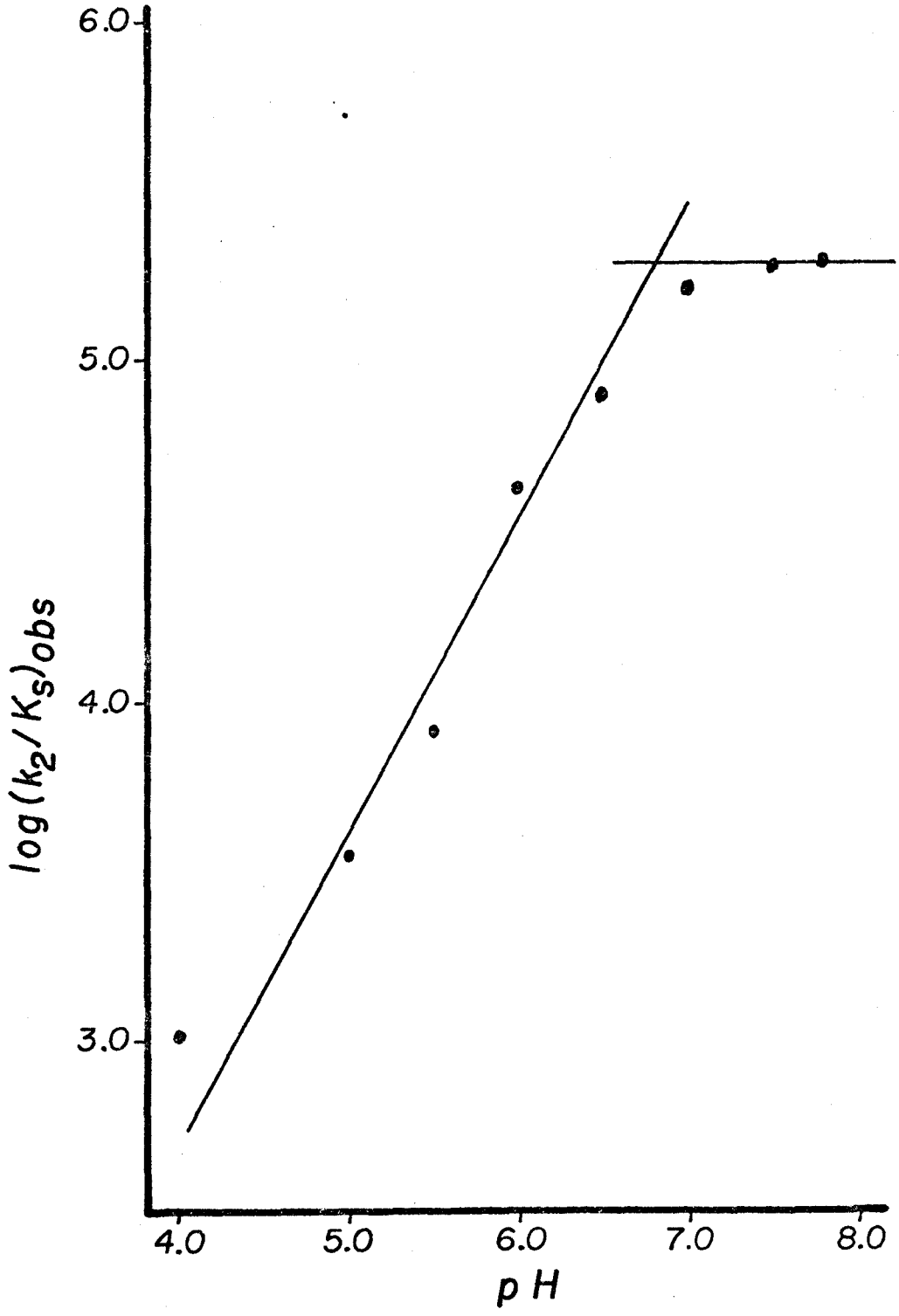
——— Represents the calculated line assuming base catalysis of hemiacetal formation by a single group of pKa 6.8.


20
15
10
5
k_2 / K_S [calc] X 10^{-5}, M^{-1} sec^{-1}
4
5
6
7
8
pH

LEGEND

Figure 20. Dixon Plot of the Observed Second Order Rate Constant Versus pH of the Pre-Steady State Associations of BzPheal with MCht.

Log plot of the data shown in figure 19 as taken from table 14.


6.0
5.0
4.0
3.0
log (k2/Ks)obs
4.0
5.0
6.0
7.0
8.0
p H

IVC-1. Temperature Dependence of the Hydration Constant for AcPheal.

The hydration constants (K_d) for AcPheal in buffered (0.05 M sodium phosphate, 0.1 M sodium chloride) D_2O at pD 7.4 (corresponding to a pH of 7.8) were obtained by appropriate integration of the ^1H-NMR spectral peaks associated with the hydrated and unhydrated aldehydic carbonyl proton over the temperature range 11° to 30°C. The values given in table 15 represent the means and standard deviations of duplicate experiments run on two occasions at each of the indicated temperatures. Although a similar set of experiments were attempted using BzPheal, its insufficient solubility in aqueous media (including 10% DMSO) proved technically unbearable and the study was abandoned. Since both BzPheal and AcPheal are similarly hydrated at 25°C (K_h 10)(109), it is assumed that the observed K_h values for AcPheal over the temperature range studied are applicable to BzPheal to a good approximation.

A Van't Hoff plot of the K_d data for AcPheal is found to be linear as shown in figure 21 and a standard enthalpy ($\Delta H°$) of -12.6 ± 1.3 kcal/mole is calculated from the corresponding slope and its standard error, respectively, of a least squares fit. Others have observed a linear temperature dependence in the hydration constant for aliphatic aldehydes in both D_2O and H_2O.(121,122) The corresponding slopes were found to be parallel, differing only by a constant isotope effect (K_h/K_d) of about 0.8 (see table 16). This correction factor is accordingly used in table 15 to convert the K_d values for AcPheal to hydration constants applicable to aqueous

TABLE 15

TEMPERATURE DEPENDENCE OF THE HYDRATION OF ACPHEAL

T, °C	K_d[a]	K_h[b]
5.0	-	44.3[c]
11.0	38.4 ± 4.2	27.4 + 3.4
15.0	23.0 ± 2.1	20.1 ± 1.7
20.0	16.6 ± 2.2	13.9 ± 1.8
25.0	11.1 ± 1.8	9.7 ± 1.4
28.0	-	8.0[c]
30.0	9.5 ± 0.7	6.8 ± 0.6
35.0	-	4.9[c]

[a]Values determined by ^{1}H-NMR on D_2O solutions containing 1 mM AcPheal, 0.05 M sodium phosphate and 0.1 M sodium chloride at pD = 7.4.

[b]From $K_h = K_d \times 0.8$.

[c]Calculated by extrapolation of the line ln (K_d) versus 1/T,°K.

LEGEND

Figure 21. Van't Hoff Plot of the Temperature Dependence of Hydration of AcPheal.

See text.

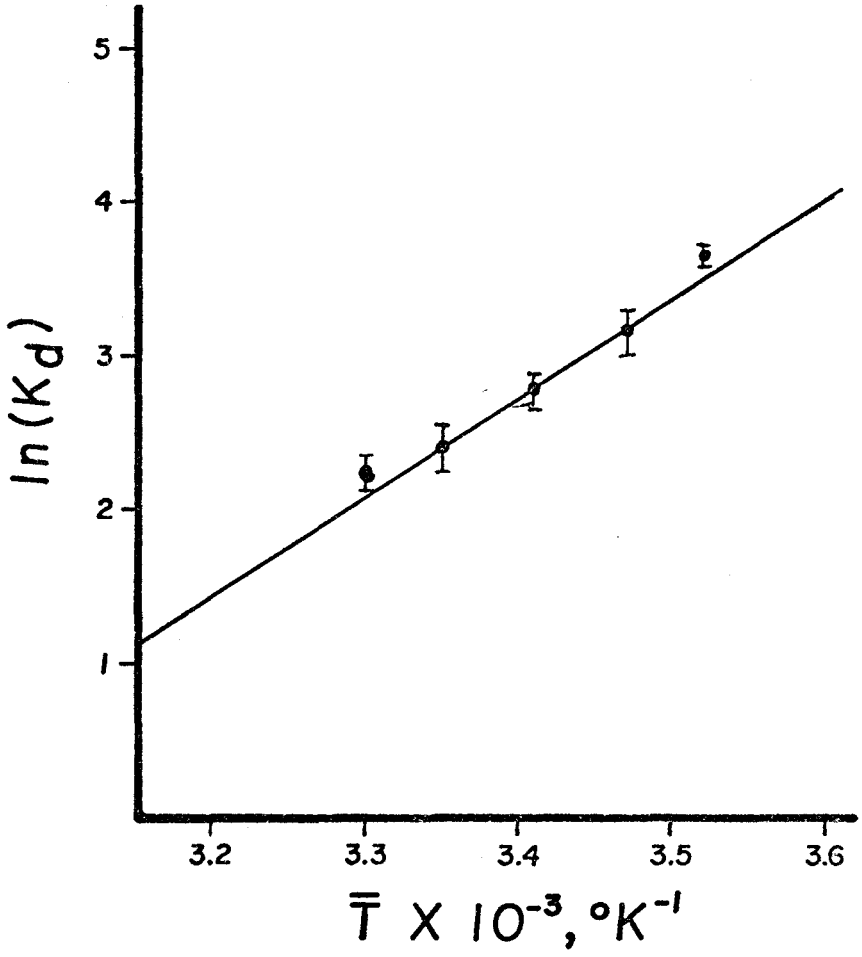

5
4
3
2
1
ln(Kd)
3.2
3.3
3.4
3.5
3.6
T̄ X 10⁻³, °K⁻¹

TABLE 16

THERMODYNAMICS OF HYDRATION: ALIPHATIC ALDEHYDES IN H_2O AND D_2O[b]

Compound	Solvent	K_h or K_d (25°C)	$\Delta H°$, kcal/mole	$\Delta S°$, e.u./mole
H_2O	H_2O	103	-5.7	-5.4
CH_3CHO	"	0.93	-5.7	-19.2
CH_3CH_2CHO	"	0.69	-6.5	-22.5
CCl_3CHO	"	2.8×10^4	14.0	-26.6
CH_3CHO	D_2O	1.11	-5.5	-18.4
CH_3CH_2CHO	"	0.8	-6.2 2	-21.3
CCl_3CHO	"	3.3×10^4	-14.0	-26.2

[a]From Reference 121

[b]From Reference 124

solutions (K_h's). The thermodynamics for the water reaction at 25°C, then, is characterized by a standard free energy ($\Delta G°$) of -1.35 ± 0.08 kcal/mole and entropy ($\Delta S°$) of -37.7 ± 4.5 entropy units/mole (e.u./mole; cal/°K/mole).

IVC-2 Temperature Dependence of the Inhibition Constants of AcPheol and AcPheal to Cht at pH 7.8.

Standard kinetic assays(110) following (on a pH-stat) the hydrolysis of AcPheOMe by Cht in the presence of increasing concentrations of AcPheol were performed at pH 7.8 over the temperature range 10° to 40°C. Triplicate runs at each of three to five concentrations of AcPheol were carried out at the specified temperatures and the corresponding K_I values shown in table 17 were calculated by the methods previously discussed (secondary plots of K_m,apparent for AcPheOMe versus [AcPheol] given in appendix E).

The temperature dependence of the observed inhibition constant (K_I,obs) for AcPheal to Cht was determined using the proflavin displacement assay.(8,35-37) The data presented in table 18 represent the means and standard deviations of four independent displacement experiments. Appropriate use of the hydration constants (K_h's) given in table 17 allows the K_I,obs data to be corrected for the intrinsic association of the unhydrated aldehydic form of AcPheal to Cht (K_I,al) as are also presented in table 18.

Van't Hoff plots constructed from these data for AcPheol and

TABLE 17

TEMPERATURE DEPENDENCE OF BINDING:

ACPHEOL TO NATIVE CHT[a]

T, °C	K_I, mM
10	56.0 ± 4.5
17	53.9 ± 5.3
25	55.9 + 4.4
32	47.8 ± 5.9
40	35.4 ± 3.9

[a]Values determined using competitive kinetics against AcPheOMe. Solution conditions were: 0.0025 M sodium phosphate, 0.16 M sodium chloride at pH 7.8.

TABLE 18

TEMPERATURE DEPENDENCE OF BINDING:

ACPHEAL TO NATIVE CHT[a]

T, °C	K_I,obs X 10^4,M[a]	K_I,al X 10^5,M[b]
5	1.92 ± 0.16	0.42
20	3.28 ± 0.30	2.20 ± 0.35
25	4.50 ± 0.39	4.21 ± 0.73
28	5.04 + 0.35	5.63
30	5.57 ± 0.42	7.10 ± 0.82
35	6.19 ± 0.34	10.6

[a]Solution conditions were: 0.05 M sodium phosphate and 0.1 M sodium chloride at pH 7.8 using the proflavin displacement assay.

[b]Calculated from the hydration constant data in table 15 where:

$$K_I,al = K_I,obs / (1 + K_h);$$ and,

standard deviation reflects uncertainty in both K_I,obs and K_h where calculable.

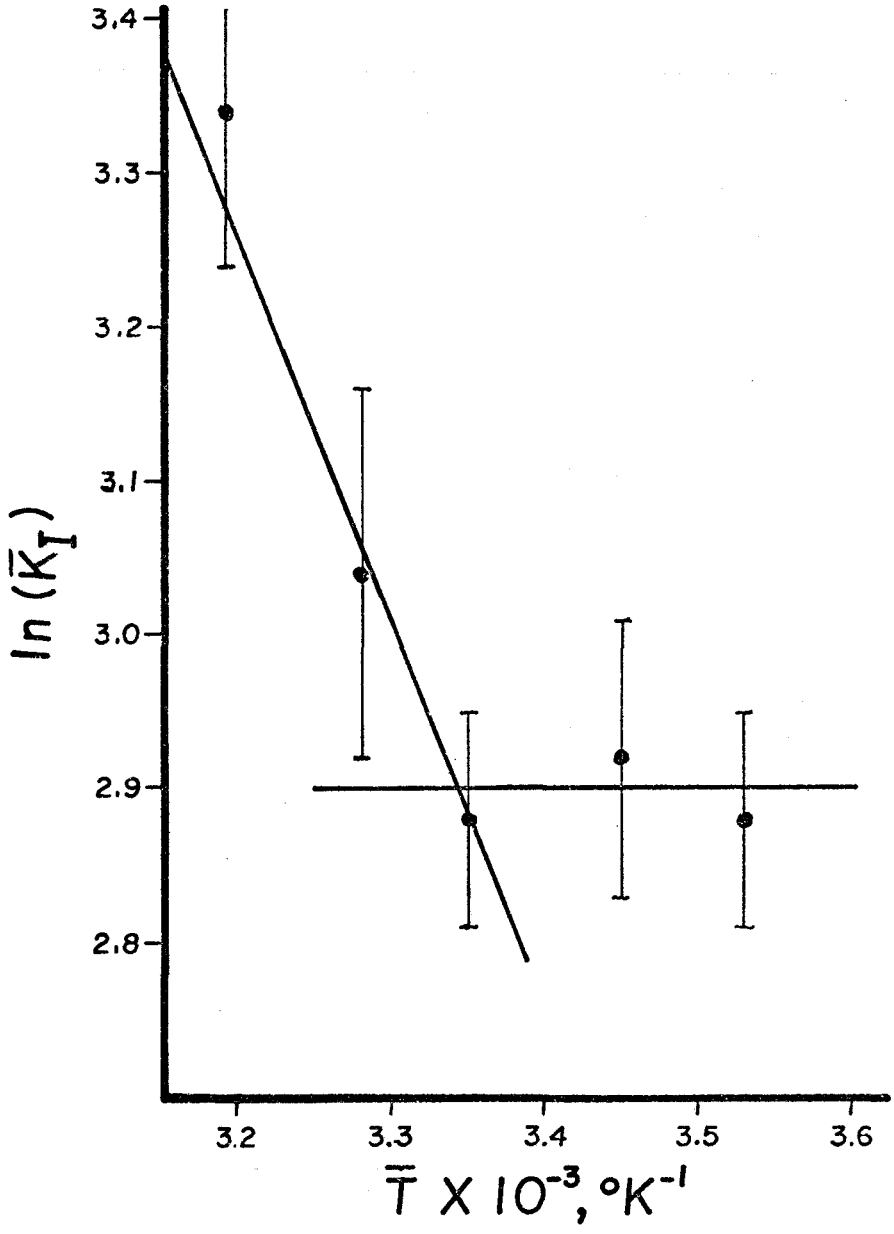
AcPheal associations with Cht are shown in figures 22 and 23, respectively. The discontinuity in the Van't Hoff plot for AcPheol has been observed for other substrates and inhibitors of Cht(8,50,51,123, 124) and attributed to the existence of at least two temperature dependent conformational forms of the enzyme.(8,50,51,123,124) Although only minimal differences in Cht turnover rates, specificity for trp or tyr substrates and inhibitors and thermodynamic data were found between these two forms,(123) it is clear that the discontinuity in figure 22, perhaps characteristic of small, phe-based substrate and/or inhibitor non-covalent interactions, cannot be ignored. Accordingly, the differential affinity of AcPheol for the two conformational forms of Cht are enthalpically characterized by the two least squares lines drawn in figure 22 revealing a ΔH°_h = +5.7 $\pm$ 0.9 kcal/mole and ΔH°_l = 0 for the high (h) and low (l) temperature forms, respectively. Where ΔG°,obs at 25°C = -1.7 $\pm$ 0.2 kcal/mole, then the corresponding entropic components are ΔS°_h = +25 $\pm$ 5 e.u./mole and ΔS°_l = +6 $\pm$ 1 e.u./mole. Regardless of the discontinuity, the thermodynamics of AcPheol associations with Cht reflect those expected for the transfer of small, hydrophobic molecules from an aqueous to a non-aqueous medium(49) (entropy driven: ΔH° near zero or positive offset by a favorable, i.e. positive, ΔS°). These results are in conflict with earlier thermodynamic studies which showed the non-covalent associations of substrates and inhibitors with the specificity pocket of native Cht to be enthalpically favorable ($-\Delta H^\circ$) and entropically unfavorable ($-\Delta S^\circ$).(8,47,48)

In contrast with its alcohol counterpart, the Van't Hoff plot

LEGEND

Figure 22. Van't Hoff Plot of the Temperature Dependence of Binding: AcPheol to Cht.

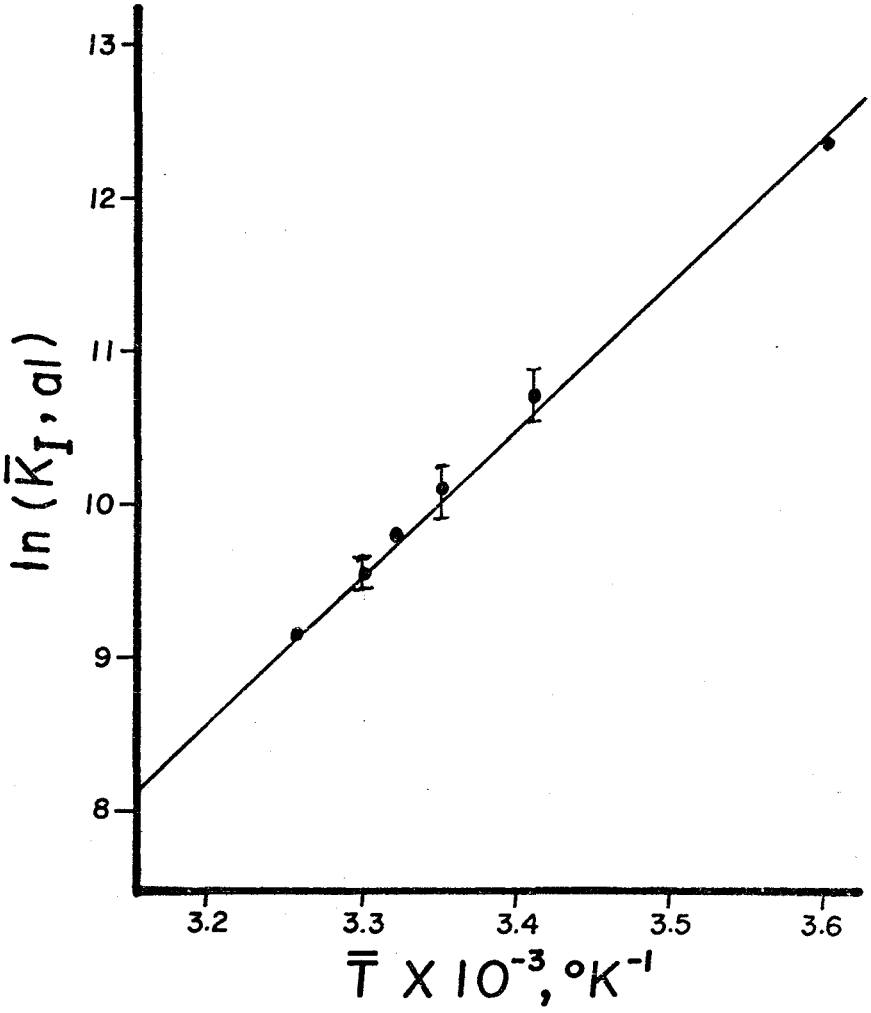
See text.


3.4
3.3
3.2
3.1
3.0
2.9
2.8
ln ($\bar{K}_T$)
3.2
3.3
3.4
3.5
3.6
$\bar{T}$ X 10^{-3}, °K^{-1}

LEGEND

Figure 23. Van't Hoff Plot of the Temperature Dependence of Binding: AcPheal to Cht.

See text.


13
12
11
10
9
8
ln ($\bar{K}_I$, al)
3.2
3.3
3.4
3.5
3.6
$\bar{T}$ X 10^{-3}, °K^{-1}

of the associations of AcPheal with Cht is found to be linear over the temperature range studied. These results suggest that the stability of the aldehyde-enzyme complex is either insensitive to conformational changes in the enzyme or that the adduct stabilizes a particular conformation in the protein. More importantly, the stability of the AcPheal-Cht complex compared to its hydrate model in solution is found to be principally due to a large and favorable enthalpy of association (ΔH°_{al} = 18.7 $\pm$ 0.4 kcal/mole versus a ΔH° for hydration = -12.6 $\pm$ 1.3 kcal/mole) since the respective entropic components are nearly identical (ΔS°_{al} = -43 $\pm$ 2 e.u./mole versus ΔS° for hydration =-38 $\pm$ 5 e.u./mole). The similarity between the thermodynamic parameters for the hydrate model and those for AcPheal-Cht interactions are also clear evidence for covalent bond formation in the latter case, i.e. hemiacetal adduct formation.

IVC-3. Temperature Dependency of Binding: AcPheal to MCht

The temperature dependency of the inhibition constant for the association of the free aldehydic component of AcPheal with MCht at pH 7.8 was determined by the proflavin displacement assay(8,35-37) and calculated from the relationships described earlier. The respective observed (K_I,obs) and calculated aldehydic (K_I,al) binding constants are given in table 19 and represent the means and standard deviations of 3 independent experiments. From the Van't Hoff plot shown in figure 24 an enthalpy of association (ΔH°_{al}) of -15.9 $\pm$ 0.6 kcal/mole is calculated. Again, where ΔG°_{al} = -39 $\pm$ e.u./mole, the stability of the hemiacetal between AcPheal and MCht com-

TABLE 19

TEMPERATURE DEPENDENCE OF BINDING:

ACPHEAL TO MCHT[a]

T, °C	K_I,obs X 10^5, M[a]	K_I,al X 10^6, M[b]
5	5.87 ± 0.61	1.30
11	5.73 ± 0.54	2.02 ± 0.31
15	6.34 ± 0.52	3.00 ± 0.35
20	6.78 + 0.58	4.55 ± 0.71
25	8.05 ± 0.83	7.54 ± 0.34
30	9.53 ± 0.84	12.2 ± 0.52
35	12.4 ± 0.92	21.2

[a]Solution conditions were: 0.05 M sodium phosphate, 0.1 M sodium chloride and pH 7.8 using the proflavin displacement assay.

[b]Calculated from the hydration constant data in table 15, where:

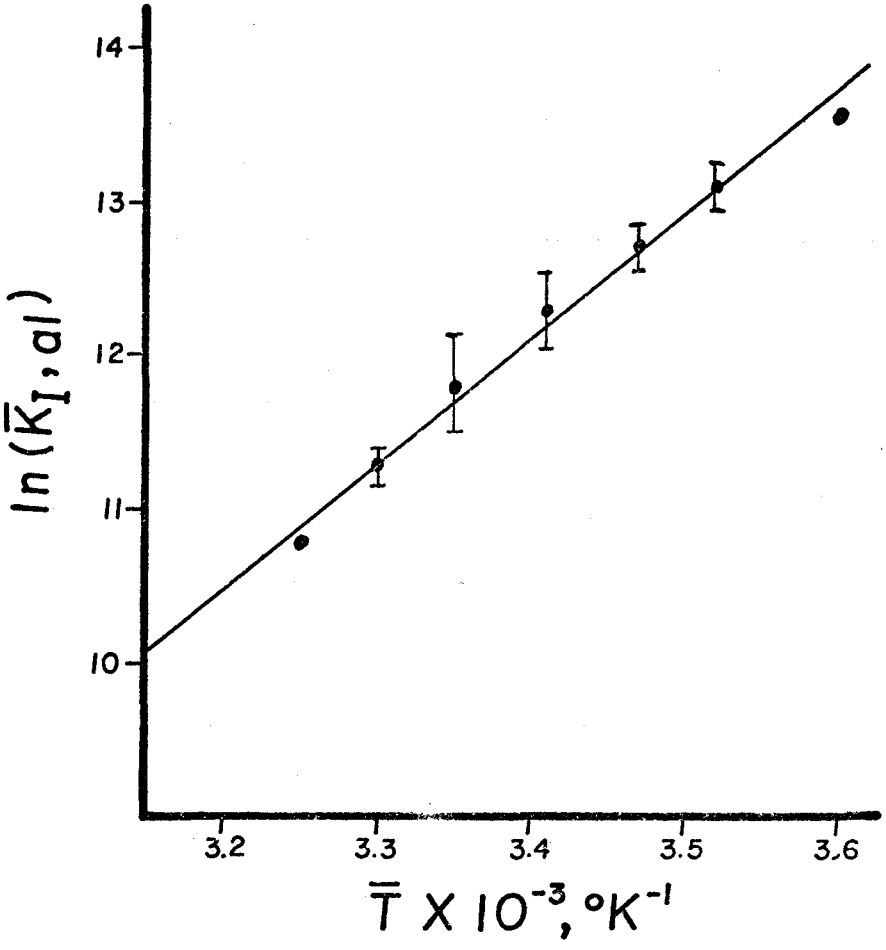
$$K_I, = K_I,obs \; / \; (1 + K_h) \text{ and,}$$

standard deviation reflects uncertainty in both K_I,obs and K_h where calculable.

LEGEND

Figure 24. Van't Hoff Plot of the Temperature Dependence of Binding: AcPheal to MCht.

See text.


14
13
12
11
10
$\ln(\bar{K}_I, al)$
3.2
3.3
3.4
3.5
3.6
$\bar{T} \times 10^{-3}, °K^{-1}$

pared with the hydrate model is principally due to the large and favorable enthalpy of association. Compared with the reaction involving the native enzyme, however, the association of AcPheal to MCht is slightly less favorable enthalpically ($\Delta\Delta H^{\circ}_{al}$ = +2.5 kcal/mole) which is more than compensated for by a favorable entropy ($\Delta\Delta S^{\circ}_{al}$ = +13 e.u./mole).

IVC-4. Temperature Dependence of the Inhibition Constant and Relative Fluorescence of BzPheal to Cht at pH 7.8.

The observed binding constants (K_I,obs) for BzPheal to Cht were also determined by the proflavin displacement assay(8,35-37) over the temperature range 5° to 35°C. The values presented in table 20 represent the means and standard deviations of seven independent runs, and the intrinsic binding constants of the free aldehydic component of BzPheal to the enzyme (K_I,al) were calculated from the hydration data for AcPheal as previously discussed. The value at 25°C is notably in agreement with that value obtained by competitive kinetic means.

A Van't Hoff plot of these data, shown in figure 25 is found to be linear over the temperature range 5° to 25° and the slope of the corresponding least squares line drawn in the figure 25 reveals a H_{al} for the reaction = -17.3 $\pm$ 0.7 kcal/mole. The ΔS°_{al}, then, is calculated to be -32.4 $\pm$ 5.6 e.u./mole where ΔG°_{al} = -7.64 $\pm$ 0.08 kcal/mole. In agreement with the results for AcPheal,

TABLE 20

TEMPERATURE DEPENDENCE OF BINDING:

BZPHEAL TO NATIVE CHT[a]

T, °C	K_I,obs X 10^5, M[a]	K_I,al X 10^6, M[a]
5.0	1.40 ± 0.15	0.31
10.0	1.75 ± 0.18	0.62
15.0	2.09 ± 0.15	0.99 ± 0.11
20.0	2.66 ± 0.09	1.79 ± 0.24
25.0	2.68 ± 0.14	2.50 ± 0.38
30.0	2.68 ± 0.42	3.44 ± 0.62
35.0	2.16 ± 0.62	3.66

[a]Solution conditions were: 0.05 M sodium phosphate, 0.1 M sodium chloride and 3% DMSO at pH 7.8 using the proflavin displacement assay.

[b]Calculated from the hydration constant data in table 15, where:

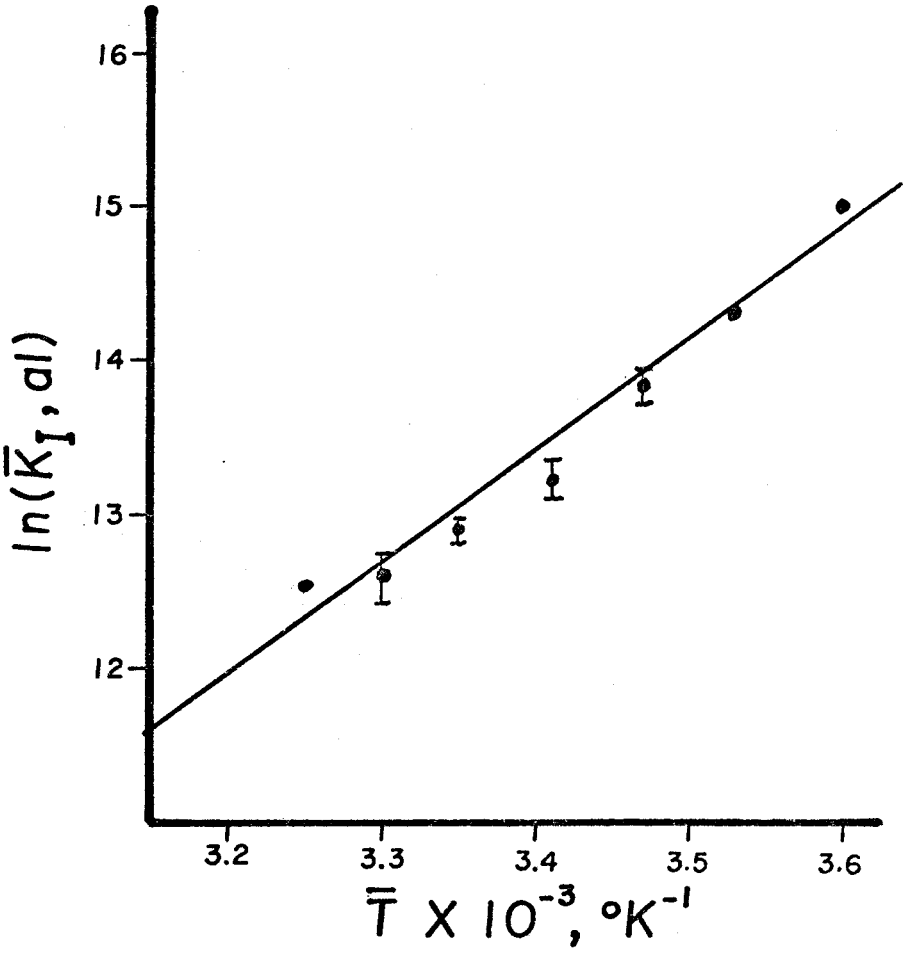
$$K_I,al = K_I,obs / (1 + K_h);$$ and,

standard deviation reflects uncertainty in both K_I,obs and K_h where calculable.

LEGEND

Figure 25. Van't Hoff Plot of the Temperature Dependence of Binding: BzPheal to Cht.

See text.


16
15
14
13
12
$\ln(\bar{K}_I, al)$
3.2
3.3
3.4
3.5
3.6
$\bar{T} \times 10^{-3}, °K^{-1}$

the stability of the BzPheal-Cht hemiacetal adduct is principally due to the large and favorable enthalpy of association.

Although an undue amount of uncertainty exists in the data points above 25°C (>30%), they clearly do not conform to the Van't Hoff plot for the line 5° to 25°C, within a standard deviation. To assess whether or not the apparent discontinuity represents a sensitivity of the binding constant of BzPheal for the two or more temperature dependent conformational forms of Cht mentioned earlier, the original fluorescence studies of Kim and Lumry(50) were repeated and compared with the relative steady-state fluorescence of the enzyme in the presence of saturating concentrations of BzPheal.

The temperature dependence of the relative fluorescent yields of solutions containing Cht in the absence and presence of saturating concentrations of BzPheal (~1 mM) and/or 5% DMSO were compared with an L-trp standard (in pH 6.7 H_2O). There was no discernable shift in the wavelength of maximum fluorescent intensity for the respective indole components (350 nm) between solutions containing enzyme (fluorescence almost solely due to trp residues)(125,126) or L-trp in the presence or absence of DMSO or BzPheal. In addition, the absorbances of the various solutions at the wavelength of excitation (290 nm) were found to be independent of temperature. Daily fluctuations in the fluorescent yields, however, were observed. Accordingly, a fluorescence emmision spectra of the L-trp standard (in pH 6.7 H_2O) was prepared on each day of experimentation at 25°C and the temperature dependence of the relative quantum yields of the sample solutions were determined as follows: a triplicate assay of the relative

fluorescent yields (RF), generally defined as the integral of the fluorescent emission spectra over the range 300 to 500 nm (I) divided by the solution's absorbance at the wavelength of excitation (290 nm) at 25°C (A),(50,124,125) of the L-trp standard was prepared on a single day over the temperature range 5° to 35°C. A plot of the observed RF values versus 1/T°K was found linear over that temperature range in agreement with the results of Kim and Lumry(50) (see figure 26). A least squares line is thereby drawn in the figure 26 and the respective slope (= 2.84 X 10^6, K^{-1}) and intercept (= 8900) used to calculate the corresponding RF values for the standard at each temperature of comparison with subsequent experiments. Fluorescent spectra were then recorded and integrated for the sample solutions over the temperature range 5° to 40°C and their absorbances at 290 nm and 25°C recorded. Relative quantum yields (Q) were calculated according to a modified relationship of Kim and Lumry:(50)

$$Q_{sample} = \frac{RF_{sample,T°C}}{RF'_{std,T°C}} X \frac{RF_{std,25°C}}{RF'_{std,25°C}} X Q_{std} \qquad (42)$$

where RF' denotes the relative fluorescence of the standard at T°C calculated from the standard curve described above and Q_{std} is assumed = 1.00.

The collected data are offered in table 21 and represent the means and standard deviations in a minimum of three determinations at each temperature. The fluorescent intensities and absorbances of solutions containing Cht alone (± 5% DMSO) increased significantly on standing at pH 7.8 due to autocatalytic degradation of the protein.

LEGEND

Figure 26. Temperature Dependence of the Relative Fluorescence of L-Tryptophane

See text for detail.

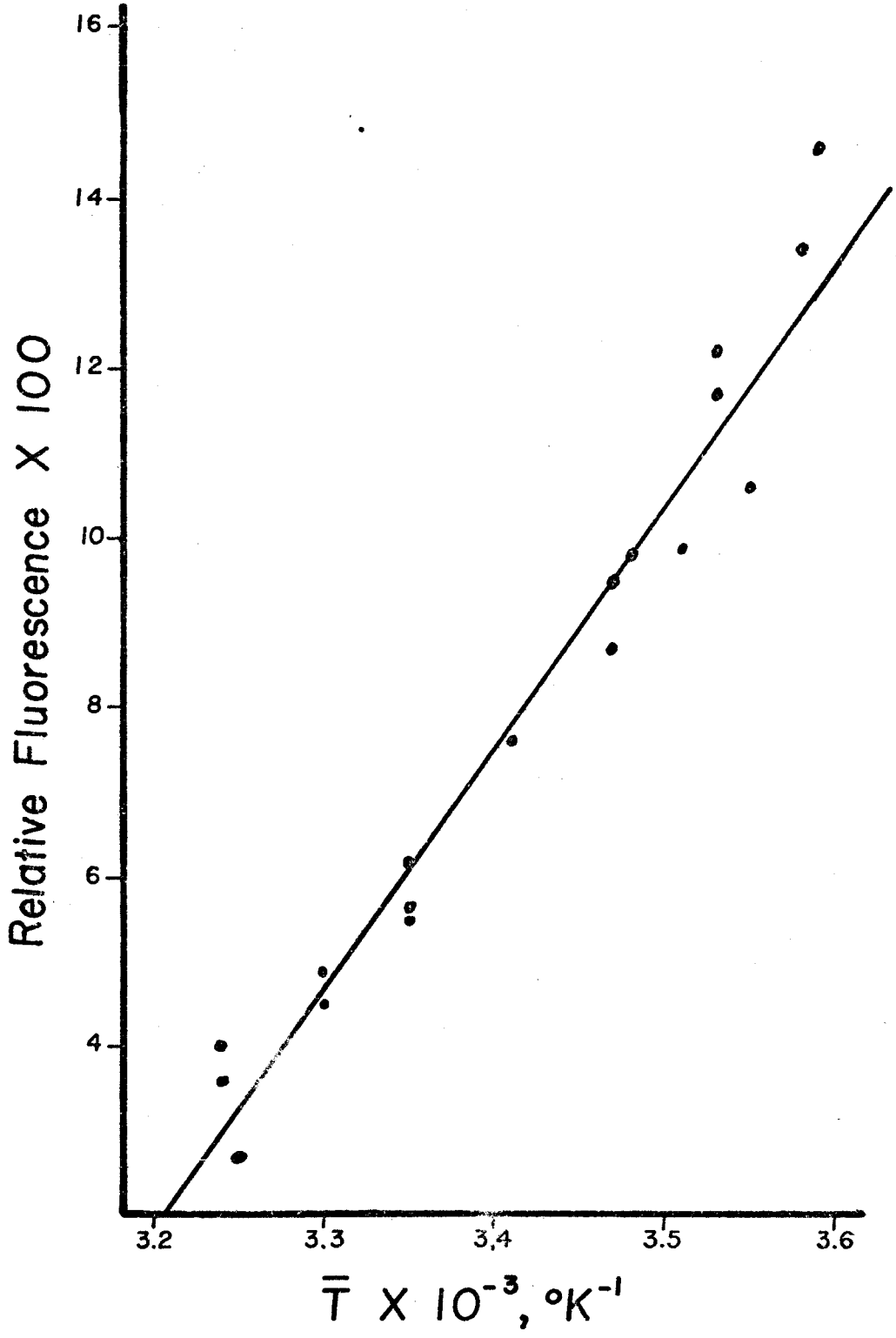

16
14
12
10
8
6
4
Relative Fluorescence X 100
3.2
3.3
3.4
3.5
3.6
$\bar{T}$ X 10^{-3}, $^{o}K^{-1}$

TABLE 21

TEMPERATURE DEPENDENCE OF THE RELATIVE QUANTUM YIELDS OF L-TRYPTOPHANE AND CHT IN THE ABSENCE AND PRESENCE OF BZPHEAL

T°C	L-trp + BzPheal[a]	Cht alone[b]	Cht + DMSO[a]	Cht + BzPneal + DMSO[a]
5.5	0.589 ± 0.013	-	0.278 ± 0.016	0.277 ± 0.020
7.0	-	-	0.364 ± 0.010	-
10.0	0.572 ± 0.029	-	-	0.297 ± 0.024
15.0	0.559 ± 0.007	0.428 ± 0.010	0.432 ± 0.044	0.307 ± 0.020
20.0	0.552 ± 0.041	0.481 ± 0.015	0.504 ± 0.037	0.360 ± 0.012
25.0	0.596 ± 0.022	0.605 ± 0.004	0.559 ± 0.020	0.412 ± 0.031
30.0	0.586 ± 0.013	0.696 ± 0.013	0.682 ± 0.008	0.493 ± 0.030
35.0	0.599 ± 0.016	0.975 ± 0.021	0.973 ± 0.023	0.644 ± 0.018
41.0	-	-	1.07 ± 0.01	0.788 ± 0.049

[a]Solution conditions were: 0.05 M sodium phosphate, 0.1 M sodium chloride and 3% DMSO at pH 7.8.

[b]Solution conditions were: 0.05 M sodium phosphate, 0.1 M sodium chloride and pH 7.8.

Accordingly, fluorescent spectra and absorbances were obtained on individual solutions at each temperature in a minimum time interval (<10 min). Conversely, solutions containing L-trp ± BzPheal or Cht + BzPheal were found to be stable for long periods (enzyme>99% inhibited) such that the temperature could be sequentially adjusted and the fluorescent spectras obtained on a single solution.

The data in table 21 are plotted against 1/T°K as shown in the composite figure 27. The relative quantum yield of L-trp is found to be unaltered in the presence of BzPheal. Nor is the relative quantum yield of the enzyme perturbed by addition of BzPheal and/or DMSO over the temperature range 5° - 40°C. The apparent shifts (vertical) in the respective plots are entirely attributable to the contribution of BzPheal to the absorbance of the solution at 290 nm studied in its presence and therefore diminished RF values as compared to solutions studied in its absence. The results graphically presented in figure 27 are essentially identical to those presented by Kim and Lumry.(50) If their original predictions are correct, then the temperature dependent conformational transition in Cht about 25°C is unaltered by the covalent associations of BzPheal.

LEGEND

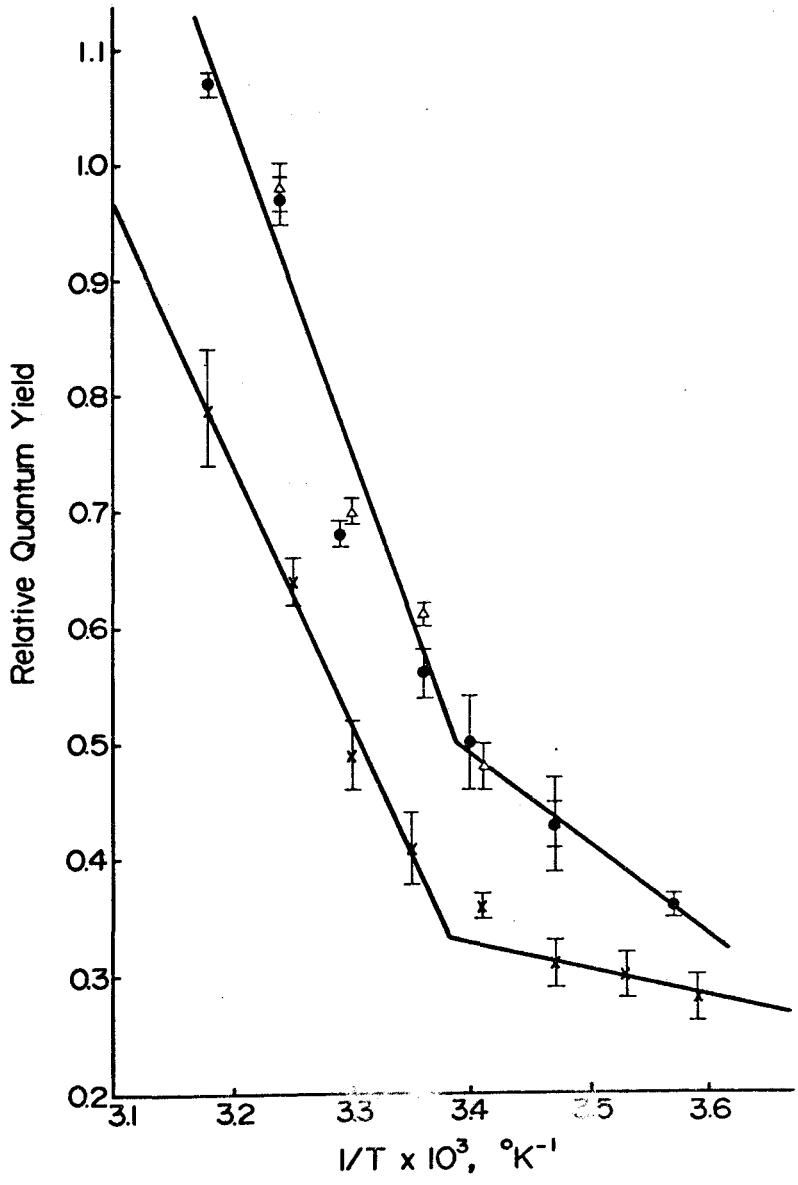
Figure 27. Temperature Dependence of the Relative Quantum Yield of Cht in the Absence and Presence of BzPheal

All data are relative to an L-tryptophane standard.

(Δ) Represents those values for Cht alone.

(●) Represents those values for Cht with 3% DMSO.

(X) Represents those values for Cht in the presence of 1 mM BzPheal (3% DMSO). Lines were drawn from a least squares regression analysis of the data. Solution conditions were: 0.1 M sodium chloride and 0.05 M sodium phosphate at pH 7.8.


Relative Quantum Yield
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
3.1
3.2
3.3
3.4
3.5
3.6
1/T x 10³, °K⁻¹

CHAPTER V

DISCUSSION

VA. Modes and Mechanisms of Specific Substrate Aldehyde Analog Associations With Native and Active Site Modified Forms of Chymotrypsin.

VA-1. The Hemiacetal Adduct.

Overwhelming evidence is now available to support the original prediction of Thompson(13,20) that specific and some non-specific substrate aldehyde analogs reversibly associate with the serine (-OH) or cysteine (-SH) nucleophiles in the active site of respective protein hydrolase enzymes forming hemi- or thiohemiacetals. Further, it is found that covalent adduct formation is the major contributor to the strength of the complex. In this work, hemiacetal formation between the ser-195 γ-OH in Cht or MCht and the aldehydes AcPheal or BzPheal has been demonstrated by the following observations:

i) Removal of the ser-195 γ-OH in the case of ACht results in

a drastic 10^3-fold increase in the observed dissociation constant of inhibition (K_I) for BzPheal as compared with the native enzyme;

ii) Although the alcohol derivatives, AcPheol and BzPheol, contain an sp^3 (tetrahedral) geometry about the carbon which corresponds to the carbonyl carbon on respective substrates that is involved in the hydrolysis reaction, that "transition state"-like configuration does not enhance their affinity for Cht over substrates. It is inferred by these results that the similarly coordinated hydrate forms of BzPheal or AcPheal show no special affinity for the enzyme over that observed for the non-covalent interactions of substrates;

iii) The observable pre-steady state associations of BzPheal with Cht at solution pH's below 7 and with MCht over the pH range 3 through 8 constitute 100% of the proflavin displacement reaction at $[BzPheal]_o \ll K_I$ for the respective alcohol or K_S for the respective amide counterpart. These results are best explained by a mechanism wherein a covalent bond, presumably the hemiacetal, is formed and is essential for the stability of BzPheal-Cht complexation. In addition, at concentrations of the unhydrated (aldehydic) component of BzPheal equal to or less than that for the native enzyme at pH 7.8, a rate limiting dehydration of the aldehyde is observed which is consistent with the preferable association of the free aldehyde with Cht since only that form can react to form the stable hemiacetal; and,

iv) The thermodynamics of AcPheal and BzPheal associations with Cht or MCht at pH 7.8 are consistent with covalent bond formation, i.e. the observed enthalpic and entropic values are similar to those observed for the addition of water to the aldehydic carbonyl carbon (each large and negative) while the non-covalent associations of substrates and inhibitors with the enzyme (AcPheol studied in this work) are characterized by a positive ΔS° and ΔH° near zero (if not positive).

The findings of others, as presented in the literature during the time interval coincident with the preparation of this dissertation, provide further support for the formation of hemi- or thiohemiacetals between specific and some non-specific substrate aldehyde analogs and the serine or cysteine proteinases:

i) Chen et al(109) have observed an NMR signal which corresponds to the hemiacetal adduct between AcPheal and Cht. Cross saturation of that signal results in a lowering of the unhydrated aldehydic signal but not the hydrate. Accordingly, the free aldehyde is preferably bound and hemiacetal formation is effected. Identical results were obtained for AcPheal to MCht. Earlier work by Lowe and coworkers(100,127) showed the association of the non-specific substrate aldehyde analog hydrocinnamaldehyde with Cht(100) and a papain-benzoylaminoacetaldehyde complex(127) to correspond with hemi- or thiohemiacetal adducts; and,

ii) Brayer et al(128) have presented x-ray crystallographic

evidence which shows a hemiacetal adduct between N-acetyl-pro-ala- pro-pheal and the active site of the serine proteinase from Streptomyces Grises.

VA-2. Mechanisms of Hemiacetal Formation.

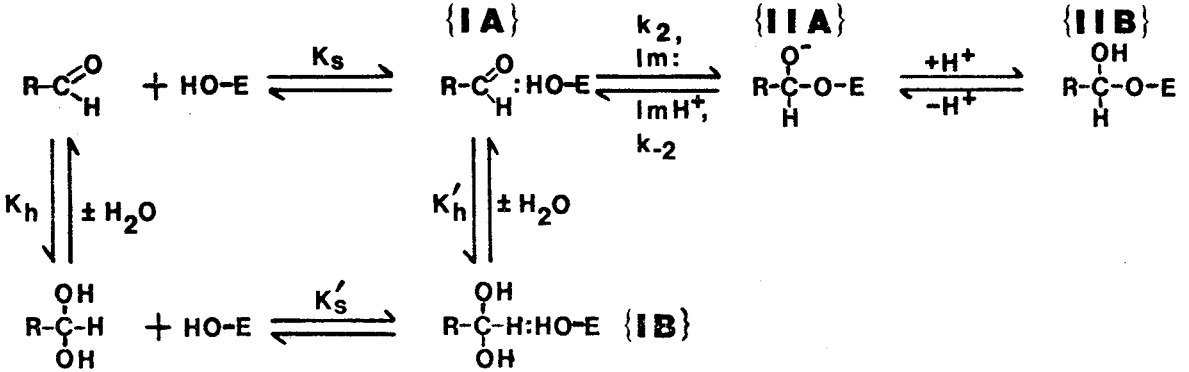
While the excellent binding of the unhydrated form of BzPheal to Cht clearly necessitates the "active" ser-195 γ-OH on the enzyme, a study of the participation of the his-57 imidazole in the formation and stability of the hemiacetal adduct has led to rather startling results. Neither protonation nor methylation of the $\varepsilon 2_N$-nitrogen on the imidazole ring, which has been shown to participate as a general base-acid catalyst in the transition states of Cht mediated substrate hydrolysis reactions,(31-34,56,80) diminishes the strength of BzPheal-Cht interactions. The rates of hemiacetal formation for both the native and methylated enzyme, however, about pH 7 are clearly general base catalyzed, being dependent on the operational normality of some basic group on the enzyme of a pK_a which corresponds to the his-57 imidazole (~7). A reasonable explanation for these pre-steady state and steady state results is that the stable end-product of the reaction between BzPheal and Cht or MCht is a neutral hemiacetal (compound IIB, figure 28). Accordingly, two protons are transferred in the covalent step k_2 in figure 28.

The initial rate limiting proton transfer in the covalent reaction between BzPheal and Cht or MCht about pH 7 is presumably general

LEGEND

Figure 28. Mechanisms of Specific Substrate Aldehyde Analog Associations with Cht or MCht.

See text for detail.


{IA}
{IIA}
{IIB}
{IB}
Ks
k2,
Im:
ImH+,
k-2
+H+
-H+
Kh
± H2O
K'h
± H2O
K's
HO-E

base catalyzed by a mechanism similar to that proposed for the formation of the tetrahedral intermediate in the acylation step of substrate hydrolysis by Cht.(31-34,56) The second proton transfer which neutralizes the incipient oxyanion on the aldehydic carbonyl oxygen, however, may result from several possible, kinetically indistinguishable mechanisms: i) In a concerted mechanism, the his-57 imidazole may act to both abstract the δ-OH proton from the ser-195 and simultaneously donate that proton to the developing oxyanion on the aldehyde generating the neutral hemiacetal in a single step; or,

ii) The same events in (i) may occur in a stepwise manner so that covalent bond formation is complete prior to protonation of the hemiacetal oxyanion;

In both the above cases, formation and decomposition of the neutral hemiacetal adduct between BzPheal and Cht or MCnt require the basic form of the acting general base such that both formation and decomposition of the adduct are each subject to base catalysis. As a result, hydrogen ion induced alterations in the rate constant k_2 are mirrored by those in k_{-2} (see figure 28) so that their pH dependency terms cancel in the ratio k_2/k_{-2} which controls the strength of the hemiacetal adduct (see equation 37 and arguments in figure 8).

According to current hypotheses on the stereochemistry of Cht mediated reactions,(39-41) however, protonation of the developing or developed hemiacetal oxyanion by the $\varepsilon 2_N$-nitrogen of the his-57

imidazole requires that oxyanion be placed or rotated into the site normally occupied by a substrate's leaving group rather than in the "oxyanion hole" which is proposed to stabilize the tetrahedral transition state in the Cht mechanism.(39,40,44,45) A recent x-ray crystallographic investigation found the aldehydic hemiacetal hydroxl group of a specific substrate aldehyde analog to be closely associated with the "oxyanion hole" of the Streptomyces Grises protease, SPGA.(127) Accordingly, a third mechanism wherein neutralization of the insipient oxyanionic hemiacetal is solvent mediated might be a mechanism operating in the covalent step k_2. The rate constant for formation of the hemiacetal will therefore be general base-specific acid catalyzed and its microscopic reverse specific base-general acid catalyzed. Since specific base-general acid catalysis is kinetically indistinguishable from general base catalysis,(9) both of the pH dependency terms for the rate constants k_2 and k_{-2} will appear identical and thus cancel in the ratio k_2/k_{-2} which controls the affinity of BzPheal associations with Cht or MCht.

In light of the above discussion, the mechanistic interpretations made from the pH dependency of the non-specific substrate aldehyde analog, hydrocinnamaldehyde, associations with Cht by Schultz and Cheerva(97) are suspect. Their results supported the anionic form of the hemiacetal as being the stable association adduct. However, only a 7-fold better binding is achieved by hydrocinnamaldehyde over that demonstrated by respective substrates (see table 3). If the observed increase in its K_I at low pH is due to small perturbations in K_S (note the 4-fold increase in the $K_{I,obs}$ for BzPheal)

rather than in the ratio k_2/k_{-2}, the mechanistic interpretations would be quite different. This discrepancy may be resolved if the pH dependency of the non-covalent associations of hydrocinnamide is studied to assess the effects of pH on K_S.

VA-3. Role of the His-Asp Couple in Hemiacetal Formation.

Current hypotheses on the mechanisms and groups responsible for the efficiency of Cht mediated substrate hydrolysis reactions assign an essential role to the his-57 $\delta 1_N$-nitrogen-asp-102 carboxylate hydrogen bond.(39,40.84-88) Its functions in maintaining the correct imidazole tautomer and steric deposition of the $\varepsilon 2_N$-nitrogen in addition to possibly stabilizing the incipient imidazolium cation which appears post formation of the putative tetrahedral intermediate in substrate reactions are each thought to be essential for efficient general base-acid catalysis by imidazole in the enzyme's active site. Accordingly, it is reasonable to assume that the pH dependency of the pseudo-second order rate constants for the formation of a hemiacetal between BzPheal and Cht or MCht (k_2/K_S,al) might reflect the protonic states of the his-asp couple. Assignment of the observed pKa's (7.0 and 4.4 with Cht and 6.8 for MCht) to the individual components and a correlation of those assignments with reasonable mechanisms for the general base catalyzed step of hemiacetal formation, however, is not very clear-cut.

One explanation for these data is based on recent spectral

studies which argue that the his-57 imidazole and the asp-102 carboxylate are of inverted pK_a's in the enzyme (~4 and ~7, respectively) compared with their counterparts in solution.(82,83) Initial protonation of the asp-102 carboxylate in the native enzyme, then, may disrupt the his-asp hydrogen bond allowing the neutral imidazole to freely rotate about its C_α-C_β bond and remain active as a general base (see figure 29) until it becomes protonated about the pK_a of 4. The rates of substrate hydrolysis will appear governed by a single base of pK_a 7 (although anomalies about the pK_a of 4 have been reported)(61,63) since the steric requirements in the transition state of the reaction will not be met. The general base catalyzed rates of hemiacetal formation, on the other hand, are found to be relatively insensitive to the steric deposition of the his-57 imidazole as evidenced by the only 10-fold poorer rates of BzPheal associations with the native enzyme versus MCht at pH 7.8 assuming the methylated imidazole is also free to rotate (see arguments below). Substrate turnover, on the other hand, is effected at rates some 10^5 to 10^6 times slower by the modified enzyme.(78-79)

Several discrepancies, however, make the above an improbable explanation for the additional pK_a of 4 observed in the pH dependency of the association of BzPheal to Cht. Foremost, assigment of inverted pK_a's to the respective components of the his-asp couple in the native enzyme remains highly controversial if not entirely speculative in light of the most recent NMR experiments of Markley et al and others.(84-88) Regardless of these assignments, the ability of the his-57 imidazole in Cht to freely rotate allowing its unpro-

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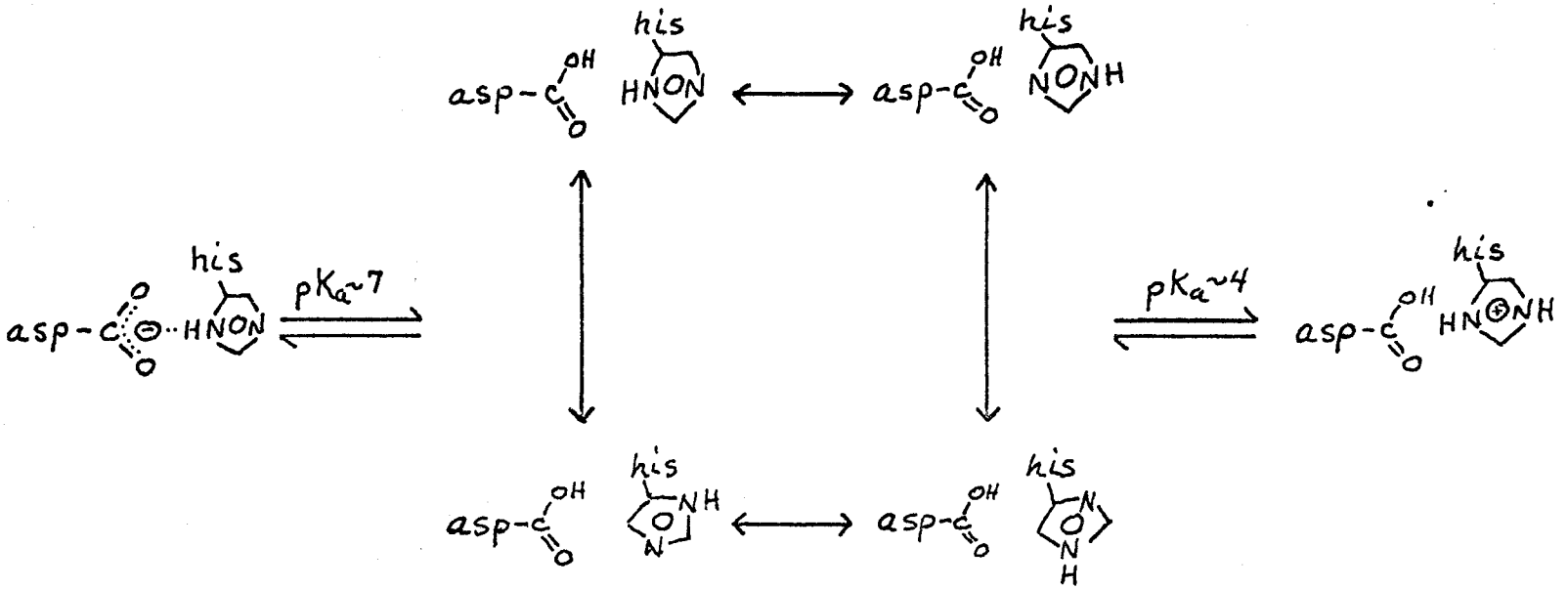
Figure 29. pH Transitions for the His-Asp Couple in Cht Assuming Inverted pKa's and Rotational Freedom about the Imidazole's C_α-C_β Bond.

The neutral imidazole ring may be free to rotate as well as exist as both tautomeric forms in Cht's active site if the putative his-57 $^{\delta 1}N$-nitrogen:asp-194 γ-COOH hydrogen bond is disrupted at pH's below 7.0.


asp-C(=O)-O
his
HNON
pKa~7
asp-C(=O)-OH
his
HNON
asp-C(=O)-OH
his
NONH
asp-C(=O)-OH
his
NH
ON
asp-C(=O)-OH
his
N
ON
H
pKa~4
asp-C(=O)-OH
his
HN⊕NH

tonated nitrogen to act as a general base catalyst (if inverted pK_a's are assumed) is also questionable. This author is not aware of any x-ray crystallographic studies on Cht at low pH (usually prepared at pH 4.2) where the imidazole is not seen hydrogen bonded to the asp-102 carboxylate.

An alternative explanation of the pH dependency data on the presteady state associations of BzPheal to Cht or MCht may be made from the arguments of Byers and Koshland(79) who suggest that the methylated $\varepsilon 2_N$-nitrogen on the his-57 imidazole in MCht acts as the general base in ester substrate hydrolysis. By analogy, the protonated $\varepsilon 2_N$-nitrogen in the native enzyme at pH's below 7 might also act as a general base. Yet, the arguments of Byers and Koshland(79) also contain serious discrepancies which, to this author, make it an improbable explanation for the association kinetics of specific substrate aldehyde analogs with Cht or MCht. Although reasonable Bronsted coefficients may be calculated to explain the lowered effects of general base catalysis by the methylated imidazole, the pKa of that group is estimated to be some -6.9.(79) While their arguments against a mechanism based on unassisted nucleophilic attack by the serine anion at pH's below 10 are convincing, Byers and Koshland (79) failed to accurately consider general base catalysis by the $\delta 1_N$-nitrogen on the methylated his-57 imidazole as a model for catalysis by MCht. The pK_a of that nitrogen is identical to the rate controlling pK_a observed in MCht mediated reactions.(72) Their arguments hinged principally on the x-ray crystallographic structures of MCht prepared by Wright et al(129) which showed the

$\delta 1_N$-nitrogen on the methylated imidazole in MCht remaining hydrogen bonded to the asp-102 carboxylate side chain. Byers and Koshland(79) failed to consider, however, that the x-ray structures were prepared at pH 4.2, a condition wherein that $\delta 1_N$-nitrogen is expected to be protonated allowing the hydrogen bond to be formed. At pH's above the pK_a 7 the his-asp hydrogen bond in MCht is not expected to exist between an unprotonated nitrogen and an anionic carboxylate. Indeed, Wright et al(129) clearly suggest that rotation of the methylated imidazole may well be expected at neutral pH's. In addition, Byers and Koshland's(79) arguments against general base catalysis by the rotated imidazole based on steric constraints can be equally used as an explanation for the poor catalytic abilities of the modified enzyme. Boland et al(88) have found that general base catalysis by a correctly oriented imidazole may contribute a factor of 10^5 to 10^6 to the overall rate of a hydrolysis reaction- a factor which is identical to the rate decrease observed for ester substrate hydrolysis by MCht in which the active general base is not correctly oriented.(78)

Accordingly, the most probable mechanism responsible for the general base catalyzed rates of association of BzPheal to MCht involves the participation of the rotated $\delta 1_N$-nitrogen of the his-57 imidazole ring. An alternative mechanism needs be considered, however, to account for the base catalysis observed in the rates of association of BzPheal with the mono-protonated (low pH form) enzyme.

Probable mechanisms can be formulated from that which has been shown to occur in the acid catalyzed formation of a hemiacetal be-

tween aldehydes and alcohols in solution(121,122,130) (see figure 30). The rate determing step in the reaction shown in figure 30 is not well known.(121,122,130) More importantly, the overall rates of hemiacetal formation by this acid catalyzed mechanism are not consistent with those observed for the association of BzPheal with Cht at low pH. The solution reaction is slower and increases with decreasing pH as opposed to the decreasing rates of hemiacetal formation with the enzyme at decreasing pH. It can be postulated, however, that the active site his-57 imidazolium cation in Cht at pH < 7 is well oriented to act as a general acid and catalyze oxonium-carbonium ion formation and promote nucleophilic attack at the aldehydic carbonyl carbonyl carbon by the ser-195 γ-OH.

Figure 31 summarizes several possible mechanisms by which hemiacetal adduct formation between specific substrate aldenyde analogs and Cht might occur at low pH. Steps 1, 2 and 3 depict the pathway leading from the non-covalent aldehyde-enzyme complex (I) to the neutral hemiacetal complex (III) which is analogous to the reaction in figure 30. It may be expected, however, that the mechanism leading from the stabilized oxonium-carbonium ion complex (II) to the hemiacetal complex (III) proceeds in a manner similar to that postulated for hemiacetal formation at pH's above 7 (see section VA-2, cases i and ii). Accordingly, abstraction of the ser-195 γ-OH proton and its subsequent nucleophilic attack at the aldehydic oxonium-carbonium ion may be general base catalyzed by the $\varepsilon 2_N$-nitrogen on the his-57 imidazole in a sequential (steps 4 and 5) or a concerted (step 6) process. Alternatively, the neutral complex (III) may be formed

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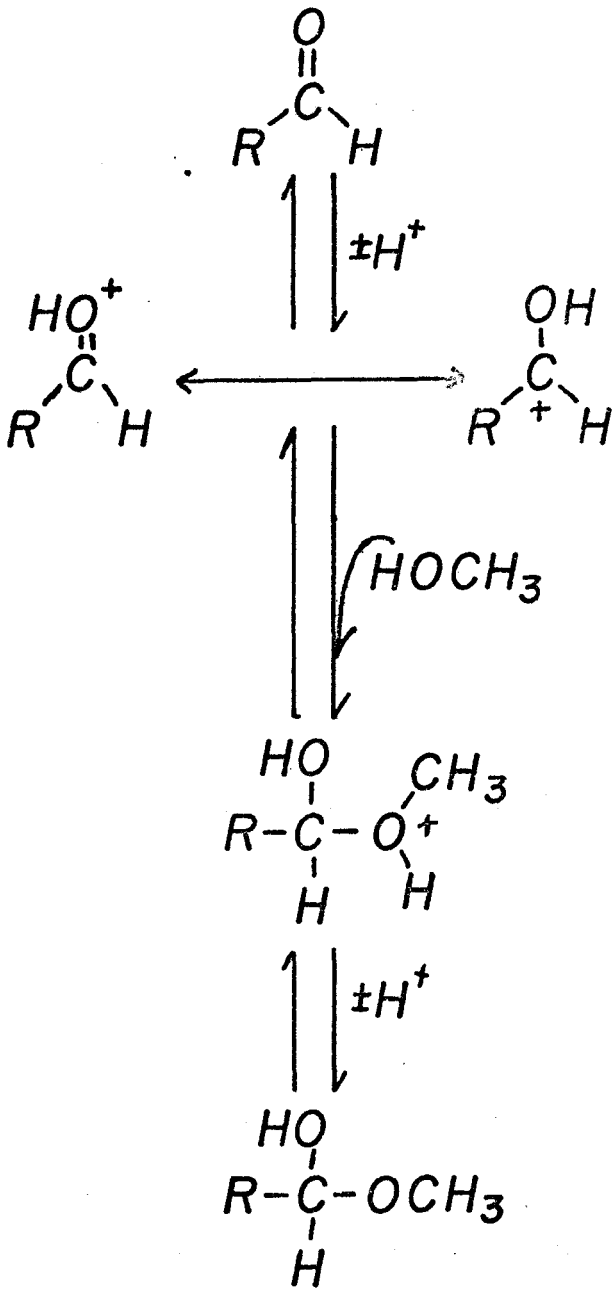
Figure 30. Mechanism of Acid Catalyzed Hemiacetal Formation between Aldehydes and Methanol.

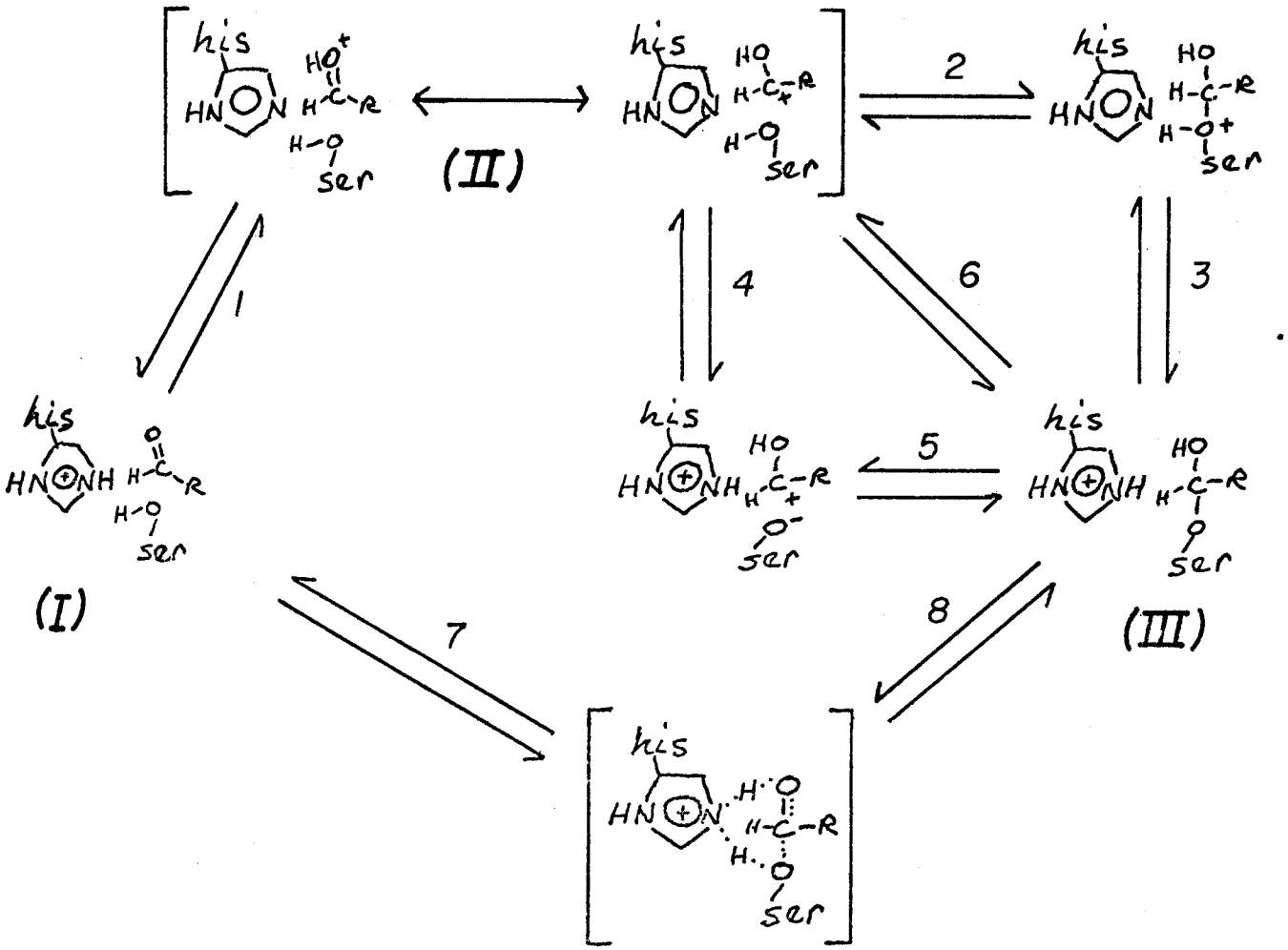
From reference 130.


O
C
R
H
±H+
HO+
C
R
H
OH
C+
R
H
HOCH3
HO
CH3
R–C–O+
H
H
±H+
HO
R–C–OCH3
H

LEGEND

Figure 31. Possible Mechanisms for Hemiacetal Formation between Substrate Aldehydes Analogs and Cht at Low pH.

See text for detail.


his
HN
N
HO
H-C-R
H-O
ser
(II)
2
1
4
6
3
5
(I)
(III)
7
8

in a single step involving concerted proton transfers as mediated by the his-57 imidazole simultaneous with nucleophilic attack by the ser-195 γ-O^- (steps 7 and 8).

These mechanisms are speculative and cannot be verified by the present data. However, regardless of which of the several pathways is involved in the reaction, the catalytic participation of the $\varepsilon 2_N$-nitrogen on the his-57 imidazole ring is clearly indicated. Otherwise, the rates of hemiacetal formation between BzPheal and Cht by a mechanism involving acid catalysis by solvent would increase with decreasing pH. In addition, the rates of hemiacetal formation between BzPheal and MCht at low pH would be identical to those found with the native enzyme. Rather, the decreasing rates of hemiacetal formation about the pK_a of 4.4 between BzPheal and Cht can be attributed to disruption but more probably a reorientation of the his-asp hydrogen bond. The pKa of the asp-102 carboxylate and the observed pK_a are identical. Precise orientation of the $\varepsilon 2_N$nitrogen on the his-57 imidazole ring in close proximity with the aldehydic carbonyl oxygen and the ser-195 γ-OH is also expected to have a decided effect upon the rates of hemiacetal formation.(88) If, at pH's below 4, the his-asp hydrogen bond is disrupted allowing free rotation of the imidazole ring or is simply reoriented such that its catalytically active orientation is perturbed, the rates of hemiacetal formation will therefore decrease. Accordingly, the second pK_a found in the pH dependency of the rates of association of BzPheal to Cht may be assigned to the asp-102 carboxylate.

VB. Thermodynamics of N-Acyl Amino Aldehyde Associations with Cht and MCht.

A major goal in studying the physical-chemical properties of enzymatic catalysis is to understand the thermodynamics associated with each step along the reaction pathway. The use of transition state analogs is compelling with respect to this goal since, according to transition state analog theory,(15-18) the thermodynamics corresponding with a transition state analog-enzyme complex are directly related to the thermodynamics of respective enzyme-substrate transition states. Conversely, studying the thermodynamics governing the association of a proposed transition state analog with the enzyme may lend important information as to how closely that analog-enzyme complex reflects the enzyme-substrate transition state.

In general, however, interpretations based on the observed thermodynamics of ligand (substrate)-protein (enzyme) complexation are speculative due to the complexity of inter- and intramolecular interactions and solvation differences between the free and bound states.(49,131,132) An appropriate approach often taken to overcome these problems is to study the free energy differences between the binding and reactions of a series of similarly coordinated substrates and substrate analogs with the enzyme. Yet, it has frequently been observed that potentially significant catalytic events may occur with little apparent change in free energy (ΔG) since the respective en-

thalpic (ΔH) and entropic (ΔS) changes, where:

$$\Delta G = \Delta H - T\Delta S \qquad (43)$$

although significant in themselves, may be compensatory.(48,133) Indeed, such compensation phenomena in the enthalpy and entropy have been suggested to play an important role in lowering the activation energy of enzyme-substrate transition state complexes.(48,133,134) Furthermore, it has been argued that the enthalpy and entropy parameters for the associations and reactions of the various enzyme substrate complexes need be individually assessed in order to differentiate amongst the various mechanisms proposed to significantly contribute to the fast rates observed in enzyme-catalyzed reactions.(8, 48,132)

By the above reasoning, this work has determined the standard (1 M, 25°C, pH 7.8) free energy (ΔG°_{obs}), enthalpy (ΔH°_{obs}) and entropy (ΔS°_{obs}) values governing the AcPheal-Cht, BzPheal-Cht and AcPheal-MCht complexes. And, for comparison, the respective thermodynamics of a solution model- the hydration of AcPheal (data also used to correct the aldehyde-enzyme values for hydration)- and the non-covalent interactions of AcPheol with Cht have also been determined. These data are summarized in table 22.

It seems clear from the sharp contrast in the enthalpy and entropy values observed for N-acyl amino aldehyde versus alcohol- interactions with Cht ($\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$ both large and negative) and their similarity to the hydration model that a covalent hemiacetal adduct is formed between the unhydrated forms of BzPheal or AcPheal and Cht. Analogously, the entropy and enthalpy observed for the formation of

TABLE 22

STANDARD FREE ENERGY, ENTHALPY AND ENTROPY VALUES OBTAINED IN THIS WORK[a]

Reaction	ΔG^{o},obs kcal/mole	ΔH^{o},obs kcal/mole	ΔS^{o},obs e.u./mole
AcPheal + H_2O	-1.4 ± 0.1	-12.6 ± 1.3	-38 ± 5
AcPheol + Cht	-1.7 ± 0.2	+5.7 ± 0.9 0	+25 ± 5[e] +6 ± 1
AcPheal + Cht	-6.0 ± 0.1	-18.7 ± 0.4	-43 ± 2
AcPheal + MCht	-7.0 ± 0.1	-15.9 ± 0.6	-30 ± 1
BzPheal + Cht	-7.6 ± 0.1	-17.3 ± 0.7	-32 ± 6

[a]At pH 7.8 and 25° C; solution conditions were throughout: 0.1 M sodium chloride and 0.05 M sodium phosphate.
[b]ΔG°_{obs} calculated from observed association constants as per equation 32; aldehyde-enzyme values corrected for hydration.
[c]ΔH°_{obs} values calculated from Van't Hoff plots over the temperature range 5° to 40°C; aldehyde-enzyme data corrected for hydration.
[d]ΔS°_{obs} calculated from the free energy and enthalpy values.
[e]To the high temperature conformational form of Cht (see text).
[f]To the low temperature conformational form of Cht (see text).

the AcPheal-MCht complex also support the kinetic results that a hemiacetal is the stable adduct.

While in both enzyme cases hemiacetal formation is enthalpically favored over hydration ($\Delta\Delta H^\circ$ = 3 to 6 kcal/mole), the greater stability of the AcPheal-MCht hemiacetal over its counterpart with the native enzyme is noticeably due to a more favorable entropy ($\Delta\Delta S^\circ$ = 13 e.u./mole) of binding which more than offsets the slightly less favorable enthalpy ($\Delta\Delta H^\circ$ = +3 kcal/mole). Schultz and coworkers(8, 48) have recently argued that these thermodynamic differences commonly observed in the enthalpy and entropy for the noncovalent interactions of "virtual" substrates and aromatic inhibitors with the native versus modified enzyme (see tables 23 and 24) are indicative of a greater degree of motional freedom in the latter complex. Their finding that the faster rates of α-bromoaceto- phenone alkylation of the active site met-192 residue in MCht than with native Cht also corrleates with a greater degree of motional freedom in the modifying reagent-MCht Michaelis complex since the alkylation reaction proceeds from a binding mode other than normally assumed by substrates undergoing catalysis.(48) Similarly, covalent adducts with MCht have been found to be "looser" than in the native enzyme. Fastrez and Houyet (135) have shown that deacylation of the AcPheMCht acyl-enzyme complex proceeds slower than in Cht solely due to a more negative entropy of activation for deacylation (ΔS° = +15 e.u./mole). Calculations by Byers and Koshland(79) and x-ray crystallographic(129) data have also been put forth as evidence for a "looser" association complex between substrates and substrate analogs and MCht.

TABLE 23

THERMODYNAMIC VALUES FOR THE NON-COVALENT ASSOCIATIONS OF AROMATIC INHIBITORS TO NATIVE AND ACTIVE SITE MODIFIED FORMS OF CHT[a]

Inhibitor	Enzyme Form	ΔH^{o},obs kcal/mole	ΔS^{o},obs e.u./mole
Proflavin	Cht	-8.0	-6.0
	MCht	-9.1	-9.8
	ACht-III[b]	-5.4	+1.5
L-AcTrp	Cht	-9.1	-21
	MCht	-4.3	-6.4
	ACht-III[b]	-7.7	-12.2
D-AcTrp	Cht	-10.0	-8.0
	MCht	-5.2	-23
Indole	Cht	-15.2	-37

[a]At pH 7.8 and 25°C.
[b]Non-lyophilized sample. See reference 48 for detail.

TABLE 24

ENTHALPIC AND ENTROPIC DIFFERENCES BETWEEN NATIVE AND METHYL OR PROTONATED CHYMOTRYPSIN - INHIBITOR COMPLEXES

Inhibitor	$\Delta\Delta H^\circ$, kcal/mole	$\Delta\Delta S^\circ$, kcal/mole
MCht versus Cht[a]:		
Proflavin	+5.0	+17
L-AcTrp	+10.4	+33
D-AcTrp	+7.3	+24
AcPheal	+2.8	+13
HCht versus Cht[b]:		
Hydrocinnamate	+9.9	+33
Indole	+8.8	+30
D-AcTrp	+14	+46

[a] $\Delta\Delta H^\circ$ represent: ΔH°,obs to MCht $-\Delta H^\circ$,obs to Cht; similar for $\Delta\Delta S^\circ$.
[b] H° represent: ΔH°,obs to HCht $-\Delta H^\circ$,obs to Cht; similar for $\Delta\Delta S^\circ$; HCht is the low pH form of the enzyme.

Association-activation mechanisms argue that strain, motional restrictions and/or conformational changes in the enzyme and/or substrate may be induced in the Michaelis complex and act to lower the activation energy of succeeding bond making and/or breaking steps which are commonly rate limiting.(1-8) Intrinsic to each mechanism is the thermodynamic prediction that the entropy and/or enthalpy for the non-covalent associations of substrates with the enzyme will be observably different from that expected for simple transfer processes or "loose" ligand-protein interactions. Although the tendency in the comparative data between AcPheal-Cht and AcPheal- MCht hemiacetals is similar to that observed for the non-covalent interactions of substrates and inhibitors ($\Delta\Delta H°$ and $\Delta\Delta S°$ positive), these data do not necessarily support interpretations based on the occurrence of "association-activation" mechanisms.(8,48) While the entropic factor is highly significant ($\Delta\Delta S°$ = 13 3.u./mole), the change in enthalpy ($\Delta\Delta H°$ < 3 kcal/mole) is small compared to values for other inhibitor associations (see table 23) and may simply indicate a difference in solvation between the respective hemiacetals. Moreover, the thermodynamics of AcPheol associations with Cht versus those for other substrates and inhibitors show that the enthalpy and entropy of association of AcPheol with Cht appears anomalous. Perhaps "association-activation" mechanisms are not induced when small, phe-based substrates or inhibitors initially associate with the enzyme, or else, the C-terminal hydroxyl of AcPheol may be differently solvated than respective substrates causing nonproductive associations.

The AcPheol-Cht complex is characterized by an enthalpy and en-

tropy which are similar to values found for the transfer of small, hydrophobic molecules from an aqueous to non-aqueous solvent.(49) If these non-covalent interactions are indicative of the non-covalent complex between AcPheal and Cht, then the thermodynamics of hemiacetal formation can be calculated from the difference free energy, enthalpy and entropy between the AcPheol- and AcPheal-Cht data. It is seen that the favorable thermodynamics associated with hemiacetal formation is entirely attributed to the covalent step. Accordingly, AcPheal may initially associate with Cht as a "loose" encounter complex which is "trapped" as hemiacetal formation proceeds. Interactions between features on the hemiacetal and specific subsites in the enzyme's active site (am, ar, "oxyanion hole, etc.) may act to stabilize that hemiacetal once it is formed, as "transition state complementarity" mechanism(4,5,12) would argue, rather than contribute to the activation of the ground state Michaelis complex, as per "association-activation" mechanisms.(1-8)

The incongruities between the thermodynamic data presented in this work and those appearing in the recent literature and their respective interpretations are disturbing. There appears, however, to be a major area of difficulty that needs to be resolved before these and subsequent thermodynamic data with Cht can be confidently interpreted. Cht undergoes a temperature induced, cooperative conformational change about a transition temperature of 25°C, as has been demonstrated by a variety of physical chemical techniques.(50,51,123-126) The origins and effects of this conformational change on the catalytic efficiency, and hence the apparent thermodynamics of Cht-

mediated hydrolyses is not well understood. Nor has it been shown that the occurences and degrees of the often observed discontinuities in the Van't Hoff and Arrhenius plots of the temperature dependence of association and reactions of the various substrates and inhibitors to Cht relates to this temperature dependent conformational change. Such discontinuities vary considerably- from sharp, inverted V-shaped plots like those of Adams and Swart(124) who studied the thermodynamics of non-specific p-nitrophenyl ester substrate hydrolysis by Cht, to the almost imperceptible anomaly in the plots of Rajender et al(123) describing the thermodynamics of N-AcTrp ethyl ester hydrolysis by Cht.

If the interpretation of the steady-state fluorescence of Cht by Kim and Lumry(50,51) are correct, the similar results obtained in this work (see figure 27) in the presence of saturating concentrations of BzPheal suggest that the hemiacetal adduct does not influence the temperature dependent substate transition. By inference, it is expected that formation of the AcPheol-Cht non-covalent complex also does not affect a conformational shift in the enzyme. There is no data, however, which demonstrates whether other substrates and/or inhibitors are capable of stabilizing a particular conformational form of Cht although Kim and Lumry(50,51) suggest that the high temperature form is the predominate species in specific substrate associations. If other substrates and/or inhibitors do indeed induce the conformational shift to the higher temperature form, then the apparent thermodynamics of association are expected to reflect that process. Appropriate corrections for the process, if it is catalyti-

cally unimportant, may drastically alter interpretations based on the thermodynamics of substrate-Cht interactions.

VC. Specific N-Acyl Substrate Aldehyde Analog-Cht Hemiacetal Complexes: Analogs of the Proposed Transition State?

It seems quite clear from the foregoing discussion on the mechanisms of BzPheal association with Cht and MCht that the stable hemiacetal complex does not mimic the proposed transition states of Cht-catalyzed substrate hydrolyses. The most damaging evidence against the stability of the BzPheal-Cht hemiacetal complex being "transition state"-like is the lack of participation of the his-57 imidazole in its formation and stability. Although the basic form (unprotonated) of the his-57 imidazole contributes to the fast (catalyzed) rates of hemiacetal formation, the lack of a significant pH dependency in K_I,al and the high affinity of BzPheal for MCht demonstrate that an "active" his-57 imidazole is not required for the high affinity of specific N-acyl substrate aldehyde analogs for the enzyme. A priori, since the his-57 imidazole participates in currently accepted transition state structures for Cht-catalyzed hydrolyses(31-34,56,80), its participation is thereby deemed essential for the high affinity of a "good" transition state analog. Accordingly, the BzPheal-Cht complex is not conformationally similar to the proposed transition complex. A similar conclusion has been presented by Byers and Koshland(79) based on the higher affinity of phenylethaneboronic acid to MCht than to Cht.

In addition, the BzPheal-Cht hemiacetal complex has been found in this work to structurally differ from proposed transition state structures. In the latter case, an incipient oxyanion is generally accepted to occur as a feature on the substrate's carbonyl carbon which may favorably interact with the "oxyanion hole" thereby stabilizing while conformationally restraining the scissile bond during formation of the tetrahedral intermediate. The BzPheal-Cht hemiacetal, on the other hand, is found to be neutral and not necessarily conformationally restrained.

SUMMARY

The mechanisms and thermodynamics governing the associations of the N-acyl amino substrate aldehyde analogs, N-acetyl-L- and N-benzoyl-L-phenylalaninal (AcPheal and BzPheal, respectively), to native -chymotrypsin (Cht) and its active site modified forms, $\varepsilon 2_{N}$-methylhistidinyl-57-Cht (MCht) and dehydroalaninyl-195-Cht (ACht), have been investigated. Equilibrium binding and pre-steady state kinetic results confirm earlier predictions that the unhydrated aldehydic forms of AcPheal and BzPheal predominately associate as covalent, hemiacetal adducts with the active site ser-195 γ-OH nucleophile in the native enzyme. Appropriate corrections of the observed binding dissociation constants (K_I,obs) for the hydration of AcPheal or BzPheal in aqueous solution reveals the respective hemiacetal adducts to be some 10^3 to 10^4-fold more stable (K_I, al = 2.4 X 10^{-6} M for BzPheal and 4.2 X 10^{-5} M for AcPheal) than respective substrate or inhibitor non-covalent complexes with Cht. These findings support hypotheses which attribute the high affinity of specific substrate aldehyde analogs for the serine or cysteine proteinase enzymes to "transition state"-like modes of association. However, "transition state analog" theory predicts that the

affinity of a "transition state analog" for the active site modified forms of the enzyme should be much poorer than with the native (active) enzyme. Yet, neither protonation nor methylation (MCht) of the enzyme's active site His-57 imidazole side chain, which is currently proposed to intimately participate in the transition state of Cht-catalyses, diminishes the stability of the AcPheal or BzPheal hemiacetal complex. Rather, the K_I,al's for AcPheal and BzPheal to MCht (7.6×10^{-6} M and 3.2×10^{-7} M, respectively) are some 6 to 8-fold better than those to the native enzyme and the association of BzPheal to Cht at pH 3 is only some 4-fold poorer than at pH 8. Furthermore, the lack of a significant pH dependency in K_I,obs for BzPheal to Cht is best explained by a mechanism of association in which the stable hemiacetal complex is neutral. Currently proposed substrate "transition state" structures, on the other hand, argue for an oxyanionic carbonyl oxygen on the tetrahedrally coordinated carbon in the transition state. Since the hemiacetal is structurally different from the prposed substrate transition state and since an integral basic histidine imidazole is not essential for the stability of the complex, it appears that the AcPheal- or the BzPheal-Cht hemiacetal adducts are not "exact" "transition state analogs".

The pH dependency of the second order rate constants (k_2/K_S)al for the associations of the unhydrated form of BzPheal with Cht and MCht have been determined. In native Cht, the fast rates of hemiacetal formation appear dependent upon two basic groups of pKa 4.4 and 7.0 for which the limiting rate constants, (k_2/K_S)al,lim = 2.3×10^7 $M^{-1}sec^{-1}$. Although the data

cannot support any one hypothesis, possible mechanisms involving the participation of the active site His-57 imidazole and Asp-102 hydrogen bonded couple, which may account for the base catalyzed rates of BzPheal-Cht and -MCht hemiacetal formations, are extensively discussed.

The standard enthalpies and entropies for hemiacetal formation for the AcPheal- and BzPheal-Cht and AcPheal-MCht complexes have been determined. Comparison of the thermodynamics governing AcPheal-Cht complexation versus those for AcPheal's hydration in aqueous solution show that the energetic preference for aldehyde-enzyme complexation over the model water reaction is attributed to a more favorable enthalpy term ($\Delta\Delta H°$>-6 kcal/mole while $\Delta\Delta S°$<4 e.u./mole). In addition, by correction of the enthalpy and entropy data for AcPneal-Cht noncovalent interactions using the respective thermodynamics of its alcohol counterpart, AcPheol, it appears that the favorable energies of hemiacetal formation (primarily enthalpic) are acquired in the covalent step. These results are discussed in terms of "association-activation" mechanisms. Finally, the better affinity of AcPheal for MCht versus Cht is found to correlate with a more favorable (positive) entropy of binding. "Transition state analog" theory predicts that the more "loosely" bound complex with the modified versus native enzyme should be detrimental to the affinity of a "transition state analog". That the converse is true for the AcPheal-MCht versus AcPheal-Cht complexes is contrary to these expectations.

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APPENDIX A

Appendix A. IR Spectra of Selected Compounds Prepared in this Work.

Spectra were recorded on a Perkin-Elmer model 337 IR-spectrometer. The spectra included here are, in sequence:

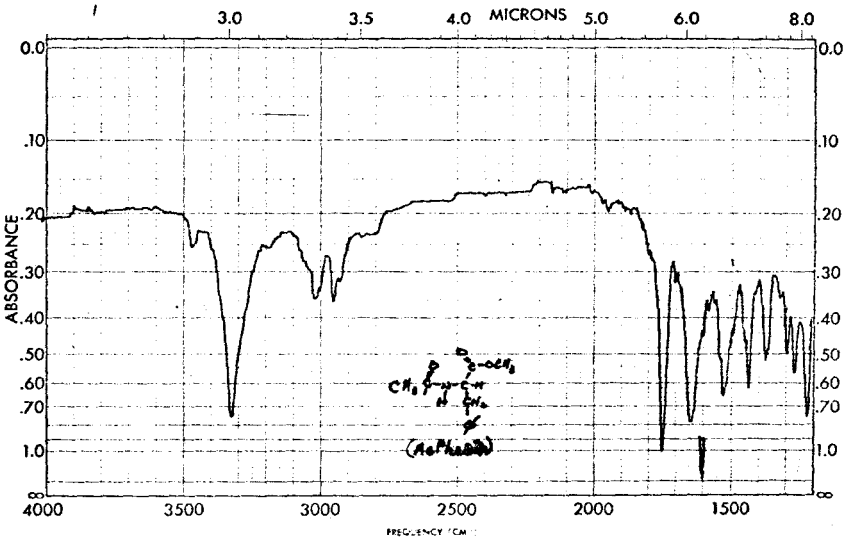
I. AcPheOMe;

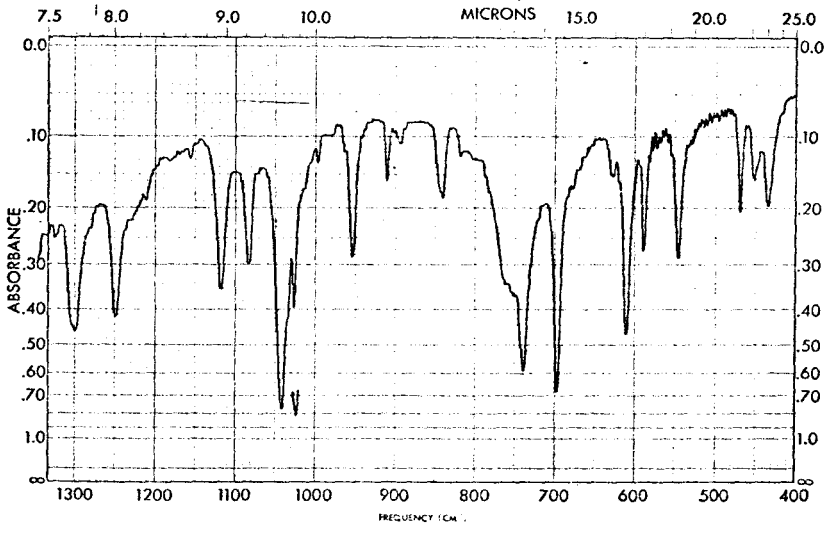
II. AcPheol;

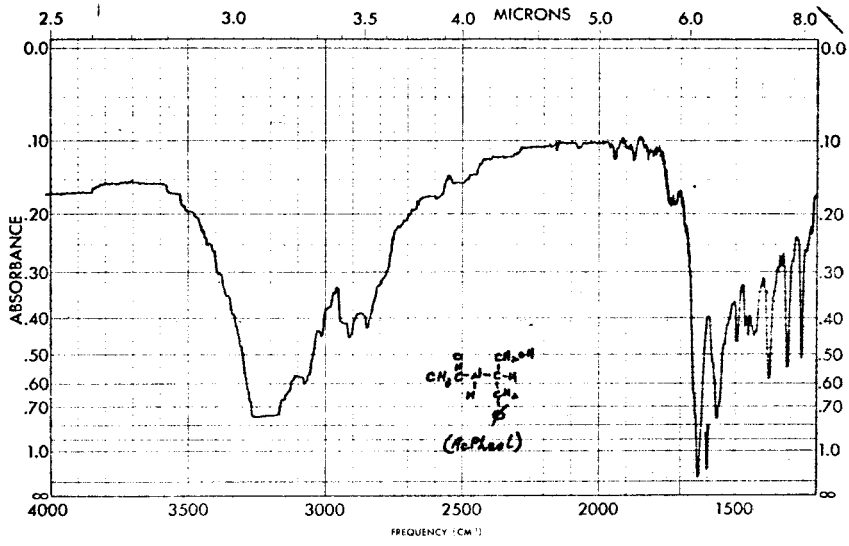
III. AcPheal;

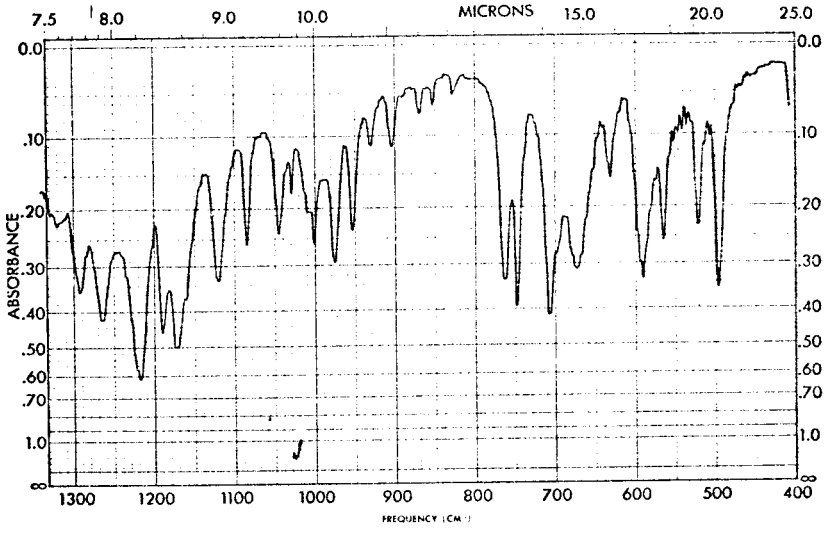
IV. BzPheol; and,

V. BzPheal.


3.0
3.5
4.0
MICRONS
5.0
6.0
8.0
ABSORBANCE
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
4000
3500
3000
2500
2000
1500
FREQUENCY (CM⁻¹)


7.5
8.0
9.0
10.0
MICRONS
15.0
20.0
25.0
ABSORBANCE
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)


2.5
3.0
3.5
4.0
MICRONS
5.0
6.0
8.0
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
ABSORBANCE
4000
3500
3000
2500
2000
1500
FREQUENCY (CM⁻¹)
(As Phase)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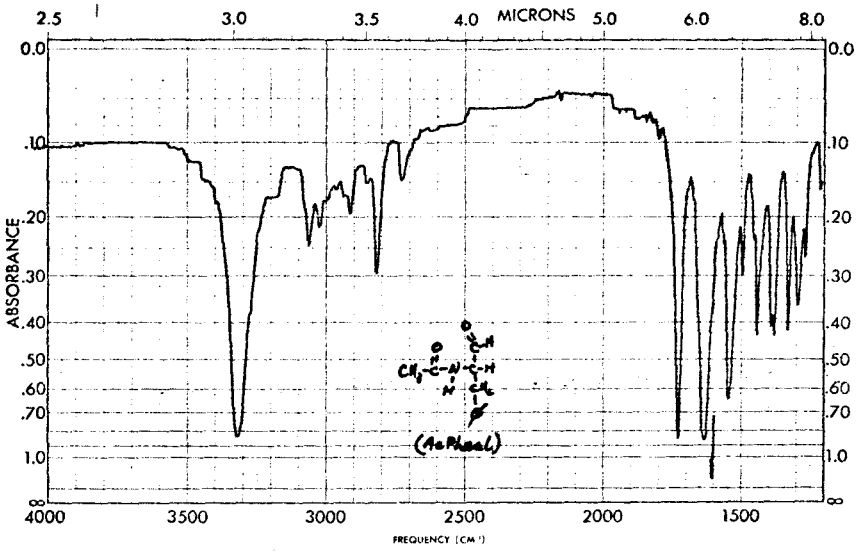
9.0
10.0
MICRONS
15.0
20.0
25.0
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
ABSORBANCE
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)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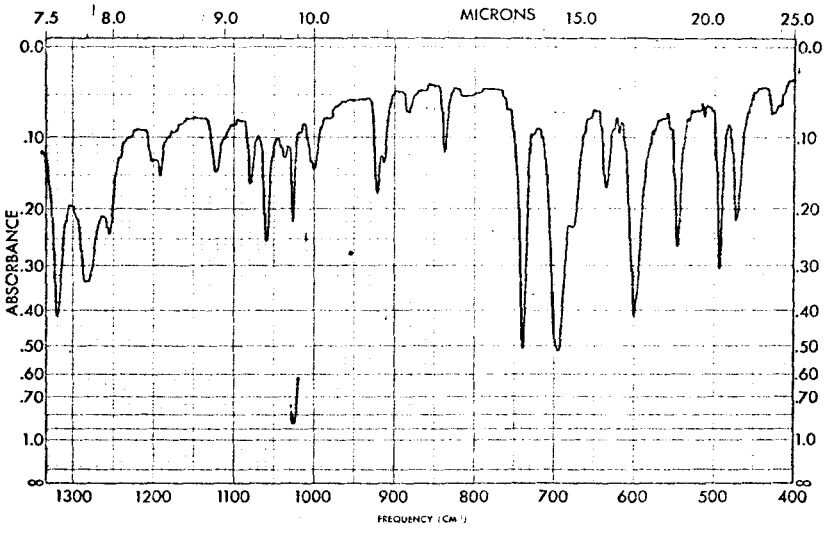
4.0
MICRONS
5.0
6.0
8.0
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
ABSORBANCE
4000
3500
3000
2500
2000
1500
FREQUENCY (CM⁻¹)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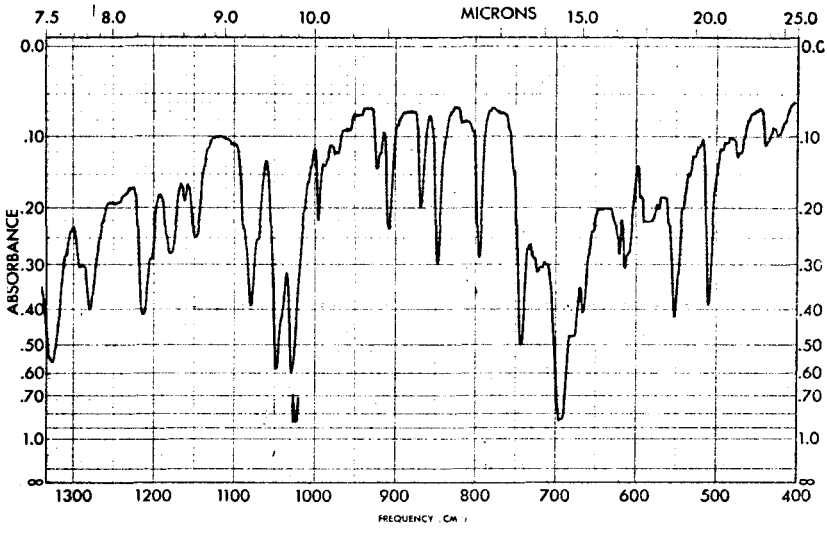
8.0
9.0
10.0
MICRONS
15.0
20.0
25.0
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
ABSORBANCE
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)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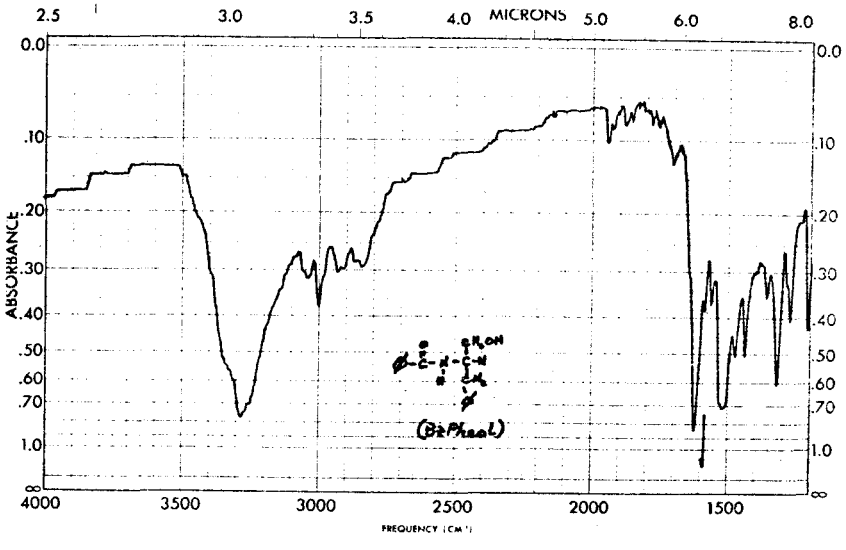
8.0
9.0
10.0
MICRONS
15.0
20.0
25.0
ABSORBANCE
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)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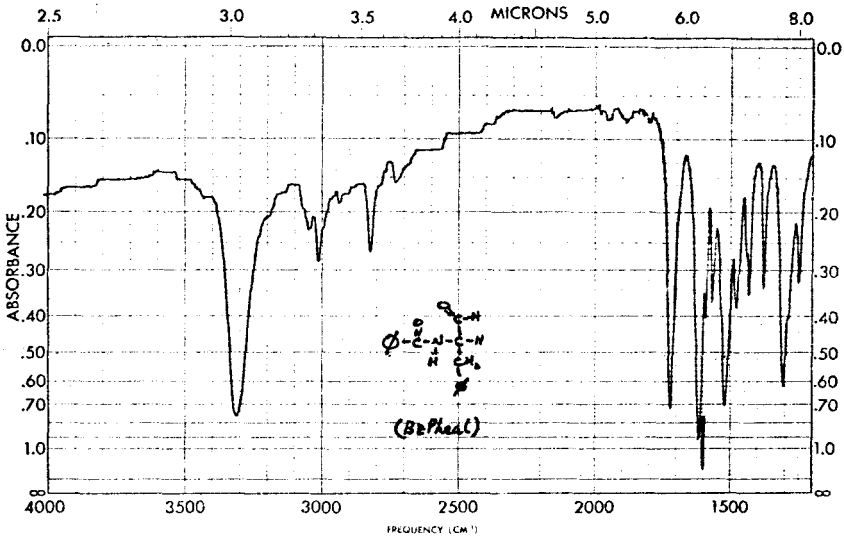
3.5
4.0
MICRONS
5.0
6.0
8.0
ABSORBANCE
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
4000
3500
3000
2500
2000
1500
FREQUENCY (CM⁻¹)
(BisPhenol)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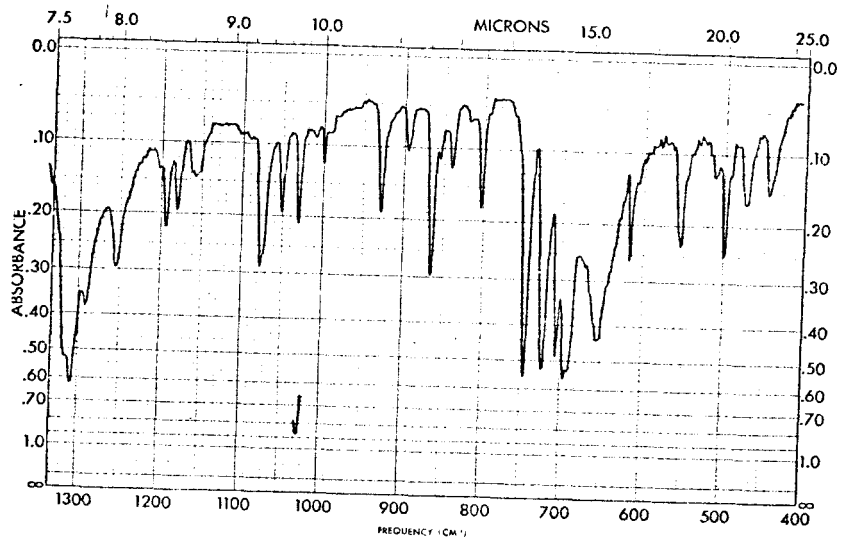
3.5
4.0
MICRONS
5.0
6.0
8.0
ABSORBANCE
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
4000
3500
3000
2500
2000
1500
FREQUENCY (CM⁻¹)
(BzPhtal)


7.5
8.0
9.0
10.0
MICRONS
15.0
20.0
25.0
ABSORBANCE
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)

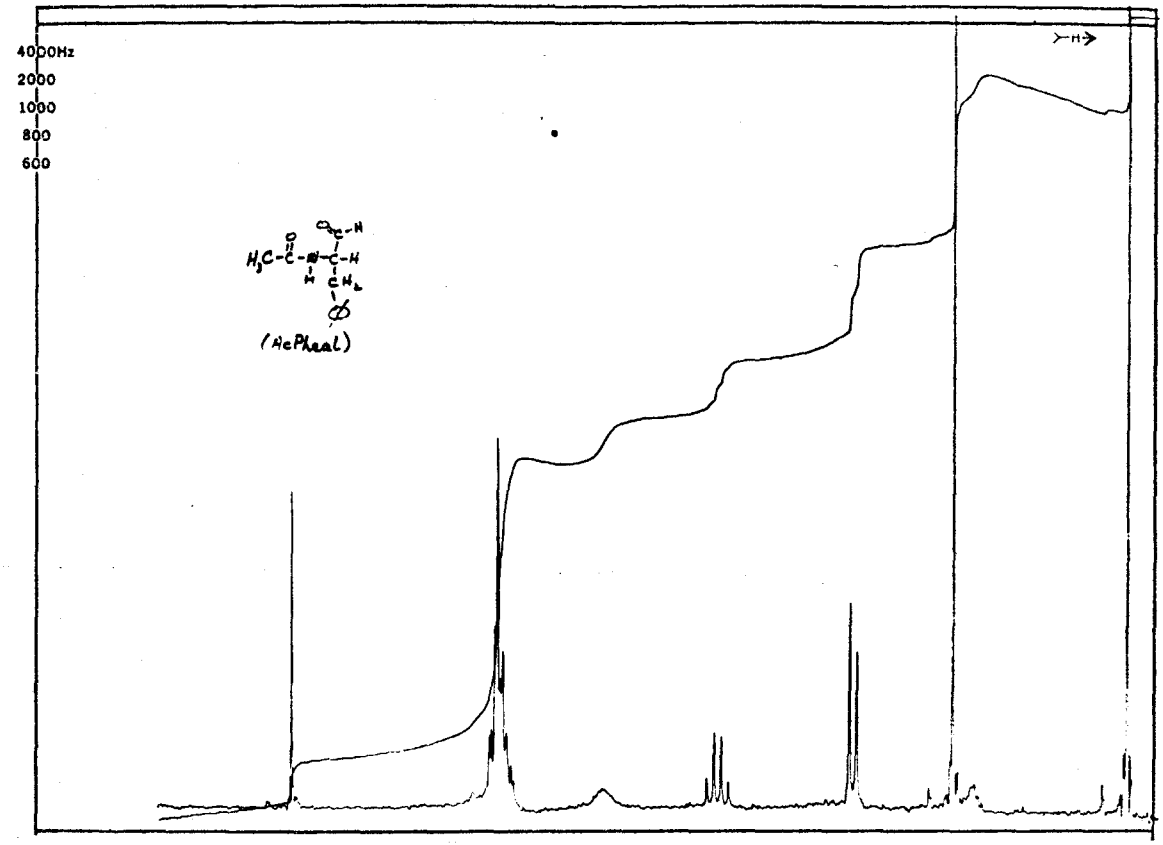
APPENDIX B

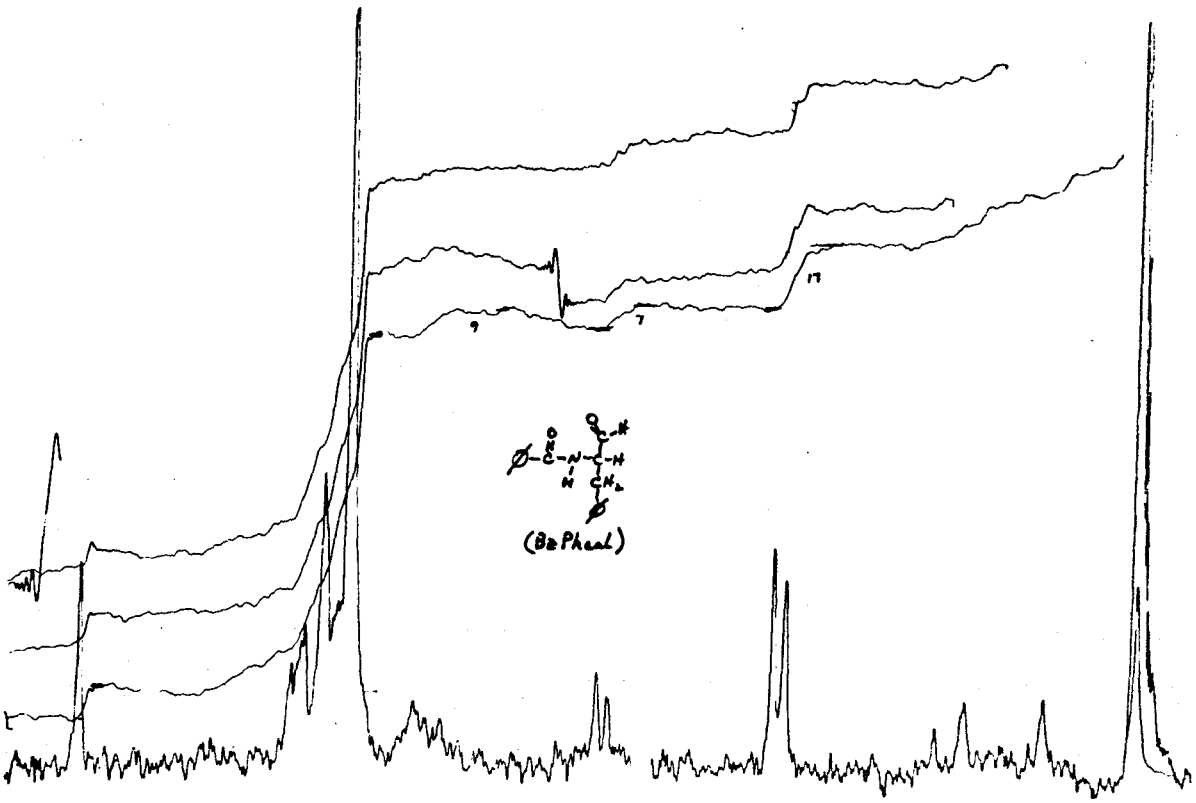
Appendix B. ^{1}H-NMR Spectra of AcPheal and BzPheal in $CDCl_3$.

Spectra were recorded on a Varian FT80A 80 megahertz nuclear magnetic resonance spectrometer using tetramethylsilane as the reference signal. Spectra on the following page are:

Top: AcPheal; and

Bottom: BzPheal.


4000Hz
2000
1000
800
600
(AcPheaL)


9
7
19
(BzPheaL)

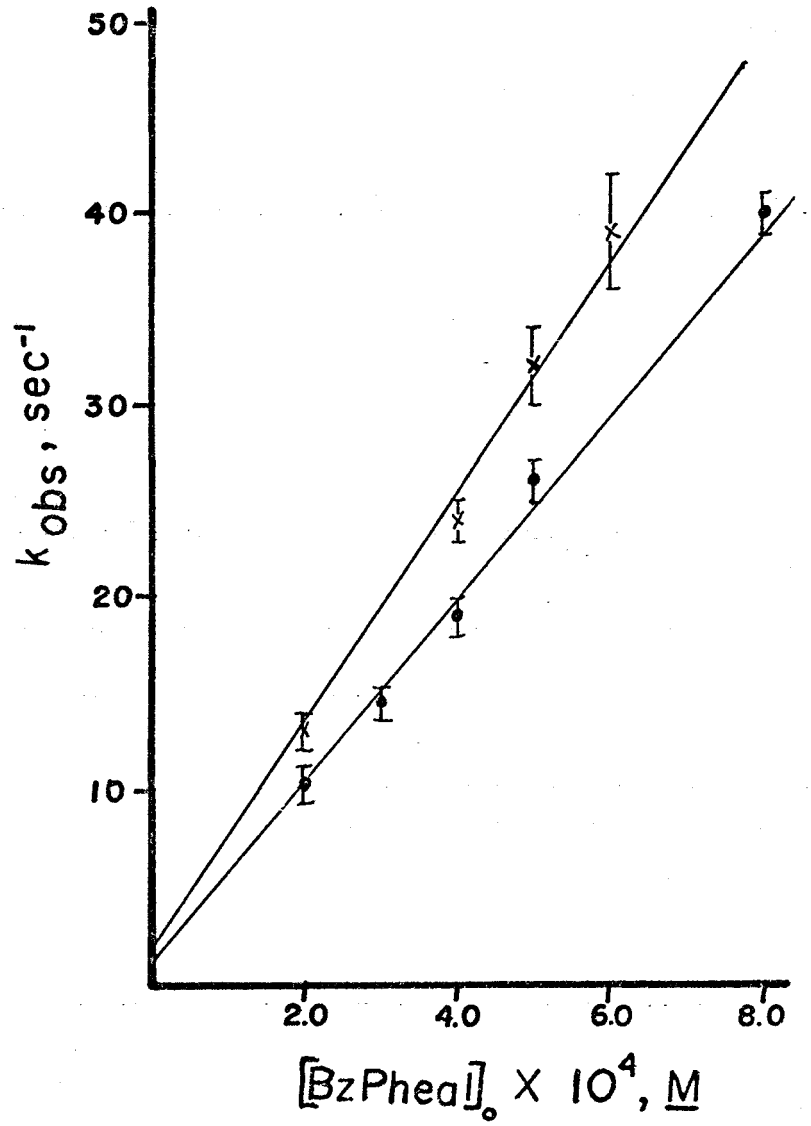
APPENDIX C

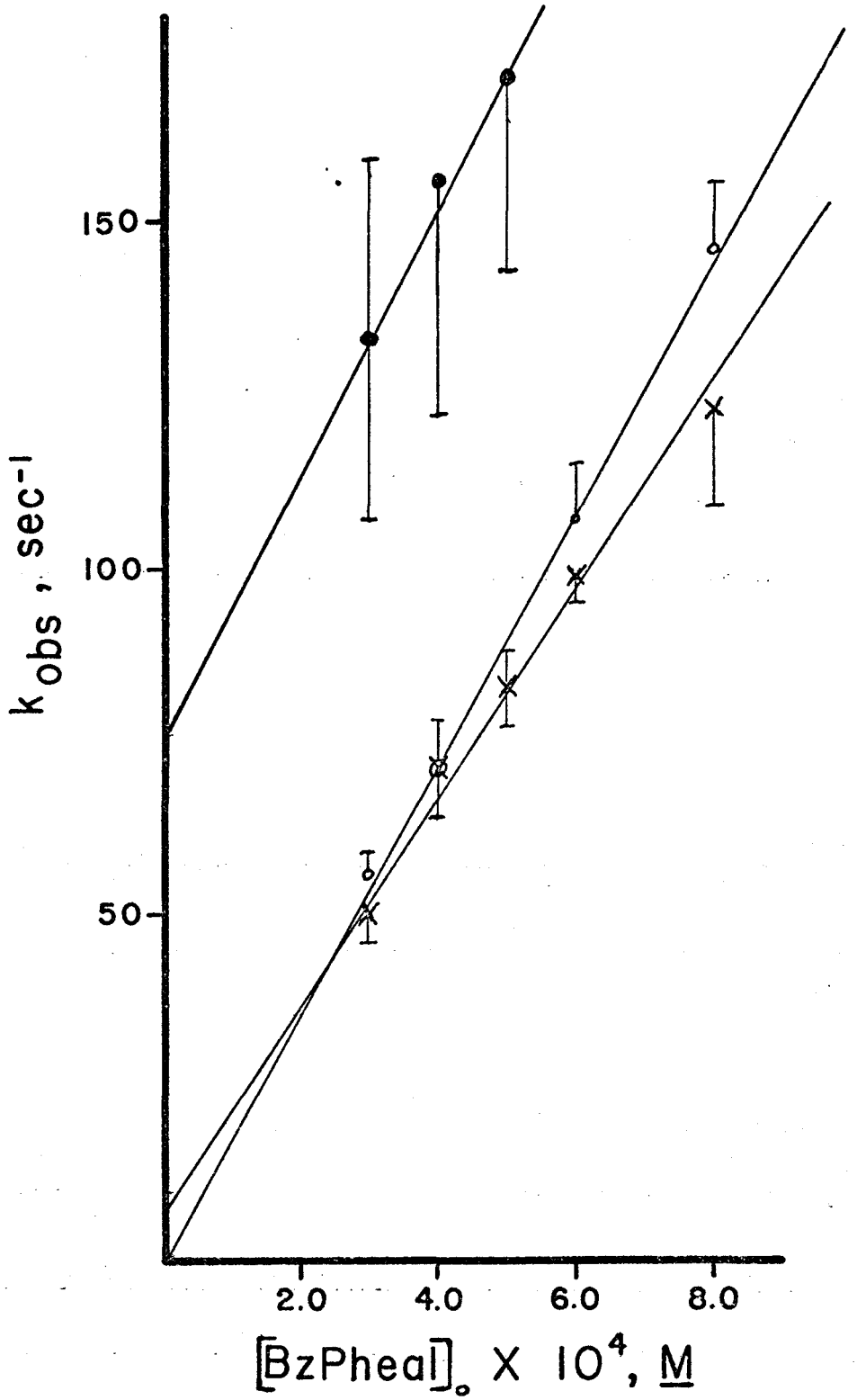
Appendix C. Secondary Plots from the Observed Pre-Steady State Association Rate Constants for BzPheal to Cnt.

Refer to table 11 for the pertinent k_{obs} versus [BzPheal] data at each pH. Plots on successive pages are:

I. At pH 4.0 (= ●) and pH 4.5 (= X); and,

II. At pH 5.0 (= X), pH 5.5 (= o) and pH 6.5 (= ●).


50
40
30
20
10
k_{obs}, sec^{-1}
2.0
4.0
6.0
8.0
$[BzPheaI]_o \times 10^4$, M


150
100
50
k_{obs}, sec^{-1}
2.0
4.0
6.0
8.0
$[BzPheal]_o \times 10^4$, M

APPENDIX D

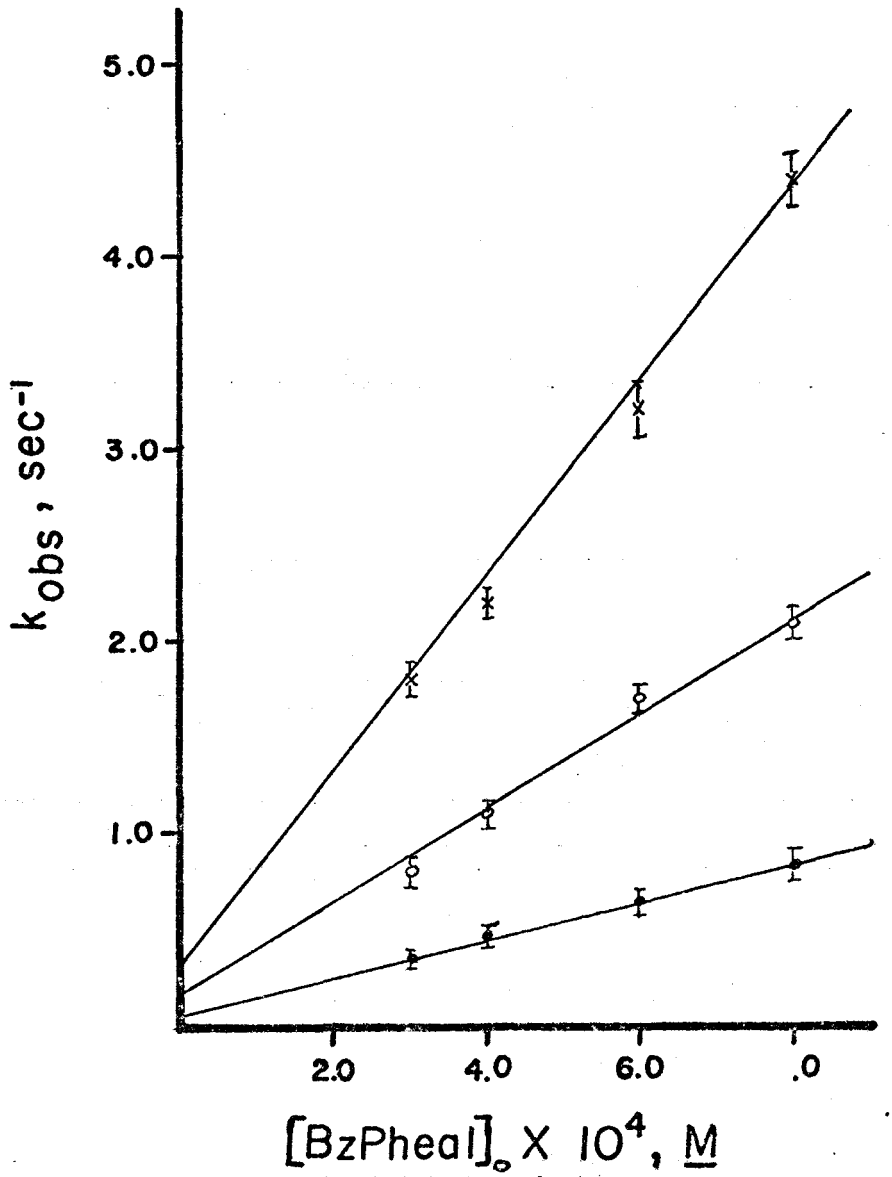
Appendix D. Secondary Plots from the Observed Pre-Steady State Association Rate Constants for BzPheal to MCht.

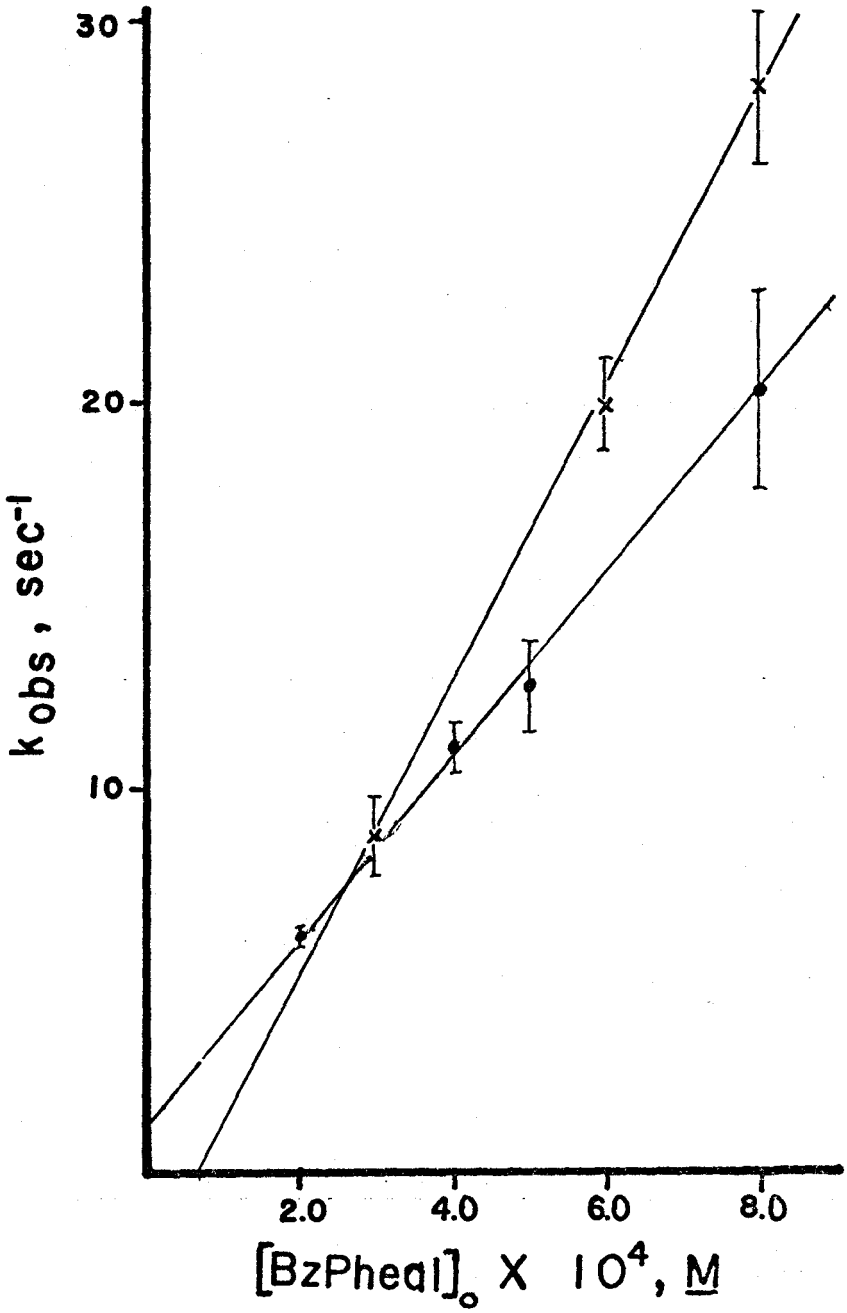
Refer to table 13 for the pertinent k_{obs} versus [BzPheal] data at each pH. Plots on successive pages are:

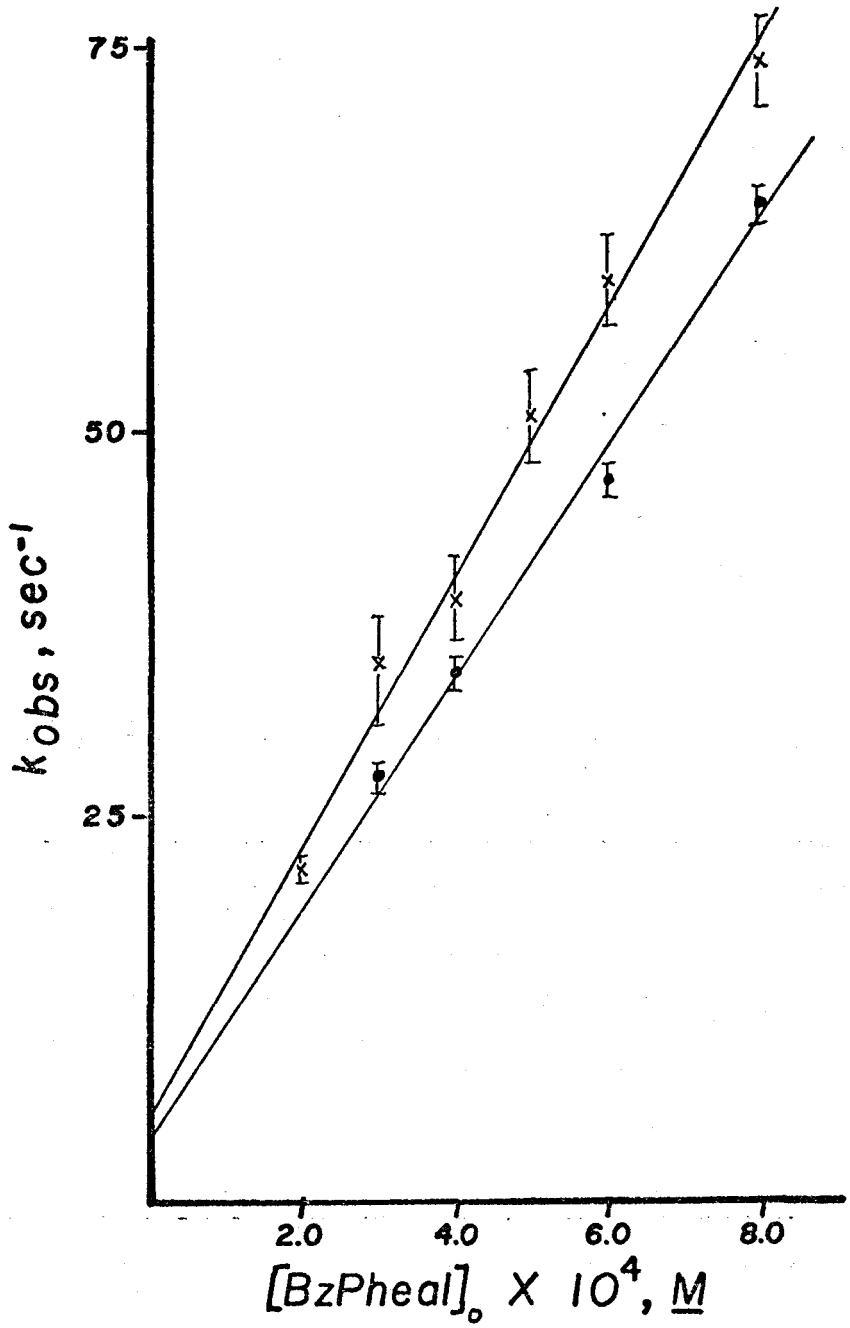
I. At pH 4.0 (= ●), pH 5.0 (= o) and pH 5.5 (= x);

II. At pH 6.0 (= ●), pH 6.5 (= x); and

III. At pH 7.0 (= ●) and pH 7.8 (= x).


5.0
4.0
3.0
2.0
1.0
k_{obs}, sec^{-1}
2.0
4.0
6.0
.0
$[BzPheal]_o \times 10^4$, M


30
20
10
k_{obs}, sec^{-1}
2.0
4.0
6.0
8.0
$[BzPheal]_o \times 10^4$, M


75
50
25
k_{obs}, sec^{-1}
2.0
4.0
6.0
8.0
$[BzPheal]_o \times 10^4$, M

APPENDIX E

Appendix E. Temperature Dependency of the Inhibition of Cht-Catalyzed Hydrolysis of AcPheOMe by AcPheol.

From the values of K_m,apparent versus [AcPheOMe] listed in the table on the next page, the following plots were constructed (in sequence):

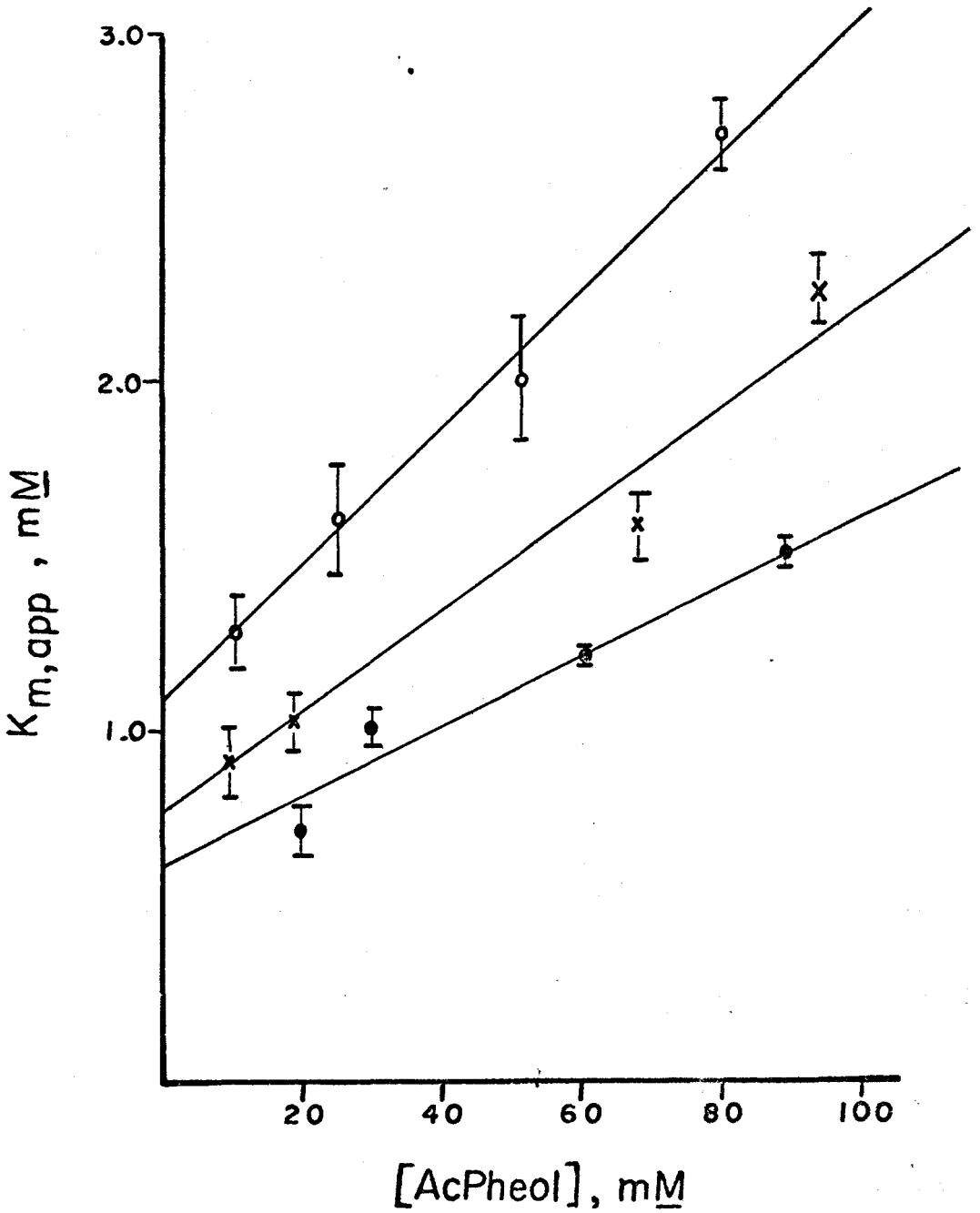
I. At 10°C (= ●), 17°C (= X) and 25°C (= o); and

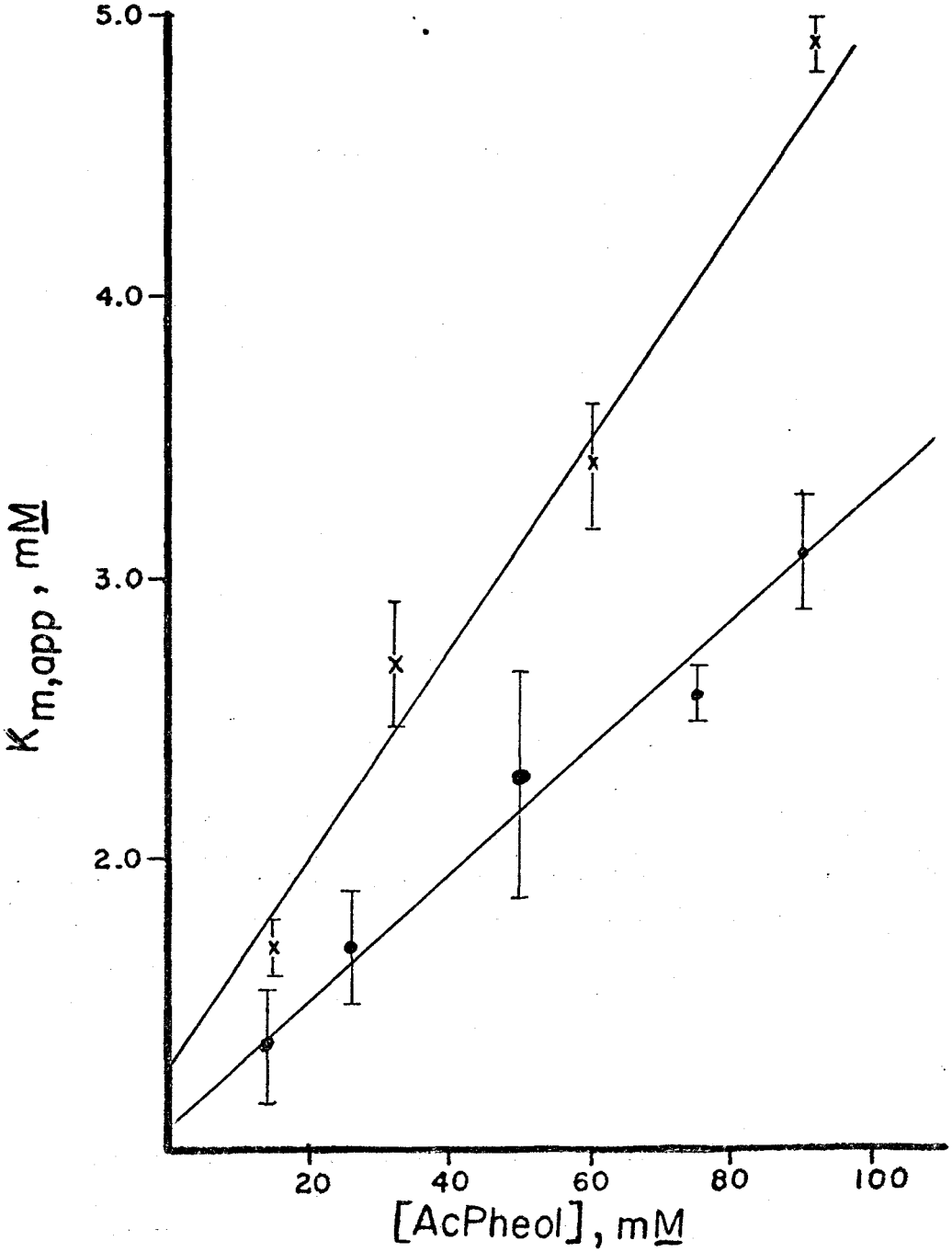
II. At 32°C (= ●) and 40°C (= X).

TABLE

INHIBITION OF CHT-CATALYZED HYDROLYSIS OF ACPHEOME BY ACPHEOL

T°C	[AcPheol], mM	Km, app, mM
10°	19.5	0.705 ± 0.073
	30.2	0.992 ± 0.050
	60.5	1.21 ± 0.02
	89.2	1.50 ± 0.04
17°	9.1	0.904 ± 0.119
	18.7	1.10 ± 0.08
	68.4	1.58 ± 0.14
	93.5	2.23 ± 0.10
25°	10.1	1.27 ± 0.13
	25.3	1.61 ± 0.16
	50.9	2.00 ± 0.19
	80.3	2.67 ± 0.09
32°	13.7	1.30 ± 0.13
	25.6	1.68 ± 0.04
	49.6	2.28 ± 0.47
	75.1	2.61 ± 0.23
	90.1	3.11 ± 0.21
40°	15.0	1.71 ± 0.07
	32.0	2.72 ± 0.22
	60.3	3.39 ± 0.22
	92.2	4.92 ± 0.11


3.0
2.0
1.0
$K_{m,app}$, mM
20
40
60
80
100
[AcPheol], mM


5.0
4.0
3.0
2.0
$K_{m,app}$, mM
20
40
60
80
100
[AcPheol], mM

APPROVAL SHEET

The dissertation submitted by William P. Kennedy
has been read and approved by the following committee:

Dr. Richard M. Schultz, Director
Associate Professor, Biochemistry and Biophysics, Loyola

Dr. Allen Frankfater
Associate Professor, Biochemistry and Biophysics, Loyola

Dr. Marvin Stodolsky
Associate Professor, Microbiology, Loyola

Dr. Abraham Rosenberg
Professor, Biochemistry and Biophysics, Loyola

Dr. David G. Gorenstein
Professor, Chemistry, University of Illinois, Circle Campus

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 16, 1980
Date

Richard M. Schultz
Director's Signature