Alpha-Chymotrypsin and the Nature of Enzyme Catalysis

The problem of enzyme catalysis is considered in terms of the behavior of a single hydrolytic enzyme.

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Enzymes are molecularly homogeneous proteins (*1*) recognized by their ability to accelerate (catalyze) certain chemical reactions. They share this property with other molecular species but differ from them in possessing a greater degree of selectivity (specificity) in both a structural and a stereochemical sense. Enzymes are further characterized by their effectiveness under essentially ambient conditions and by their ability to function either singly or as components of a multi-enzyme system. These properties permit enzymes to perform roles of singular importance in the development, maintenance, and reproduction of the living organism.

There is a large, and ever-increasing, number of known enzymes (*2*). However, in basic studies experimental considerations dictate the choice of enzyme to be studied. It is this feature of experimental accessibility, coupled with other desirable characteristics, that leads me, in this article, to center attention on a single hydrolytic enzyme, the bovine pancreatic proteinase α-chymotrypsin. In doing so I do not imply that all features of enzyme catalysis, even among the hydrolases, are to be found in α-chymotrypsin, although its properties include many that are common to all enzymes.

The Chymotrypsinogens

α-Chymotrypsin is one of a family of proteinases that arise from precursors (zymogens) known as chymotrypsinogens, which are enzymatically inactive (*3*). The existence of such zymogens provides the biologist with an answer to the question, How can proteinases be synthesized and stored in a protein environment? The zymogens are in an inactive form and are activated, when needed, by structural alteration of the inactive molecule at sites removed from those involved in their synthesis and storage. The availability of zymogens of several proteinases permits the chemist to isolate, purify, and otherwise manipulate these precursors, which, in contrast to the proteinases, are incapable of self-destruction.

There are two known bovine pancreatic chymotrypsinogens, A (*3*) and B (*4*). The two are present in bovine pancreas in relatively large and nearly equal amounts (*5, 6*). However, chymotrypsinogen A is more readily crystallized and purified than chymotrypsinogen B. This property has facilitated isolation of the former protein and has led to its availability in relatively large quantities and at modest cost. No crystalline zymogen is more accessible, and there are few crystalline proteins that can be obtained in a higher state of purity. The accessibility of bovine chymotrypsinogen A has fostered many studies on the structure of this zymogen. Many of the results have been summarized by Desnuelle and Rovery (*6*).

It appears (*6–8*) that bovine chymotrypsinogen A is a linear polypeptide of 240 to 250 α-amino acid residues cross-linked through five disulfide bridges. Its molecular weight is approximately 25,000; its nitrogen content is 16.5 percent; and its empirical formula, in terms of component α-amino acids and ammonia produced on hydrolysis, has been given as

$$Ala_{22} \cdot Arg_4 \cdot Asp_{22} \cdot (Cys/2)_{10} \cdot Glu_{14} \cdot Gly_{23} \cdot His_2 \cdot Ileu_{10} \cdot Leu_{19} \cdot Lys_{13} \cdot HOLys_1 \cdot Met_2 \cdot Phe_6 \cdot Pro_9 \cdot Ser_{27} \cdot Thr_{23} \cdot Try_4 \cdot Tyr_8 \cdot Val_{23} \cdot (NH_3)_{24}.$$

Ignoring the five disulfide bridges, we find that the single acyclic peptide chain begins, at the amino end, with a half-cystine residue and terminates, at the carboxyl end, with asparagine. Considerable information is available (*6*) regarding the sequence of α-amino acid residues, and a provisional sequence for a protein involving 242 residues has been suggested (*9*). It is likely that a precise but conformationally indeterminate structure of chymotrypsinogen A will be available shortly.

Recently Kraut *et al.* (*10*) described a three-dimensional Fourier synthesis at 5-angstrom resolution of chymotrypsinogen A, which, in their words, revealed "a molecule of approximately ellipsoidal shape with axes 50, 40 and 40 angstroms, but with an obvious hollow or depression. The chain conformation is complicated and appears to have little α-helix content in comparison with myoglobin and hemoglobin."

The Chymotrypsins

The transformation of chymotrypsinogen A to the various chymotrypsins is illustrated in Fig. 1. In Fig. 1 the positions of the disulfide bonds are those suggested by Brown and Hartley (*11*) for a chain of 250 residues.

It is evident from Fig. 1 that α-chymotrypsin is the product of the hydrolytic activation of chymotrypsinogen A, in which four peptide bonds are cleaved and two dipeptides are abstracted from the zymogen. It follows that α-chymotrypsin consists of three linear peptide chains which are inter- and intra-chain linked with five disulfide bonds.

The three peptide chains are identified as follows: chain A, amino terminal half-cystine, carboxyl terminal leucine; chain B, amino terminal isoleucine, carboxyl terminal tyrosine; and chain C, amino terminal alanine, carboxyl terminal asparagine. Meedom (*12*) has shown that chain A is a tridecapeptide and is linked to chains B and C through a single disulfide bond. Its complete sequence, beginning with the amino terminal half-cystine residue, is

$$\overset{+}{H_3}NCyS \cdot Gly \cdot Val \cdot Pro \cdot Ala \cdot Ileu \cdot Val \cdot Pro \cdot Glu(NH_2) \cdot Leu \cdot Ser \cdot Gly \cdot Leu\overline{CO_2}.$$

Comparable information is not at present available for chains B and C, which contain approximately 130 and 100

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α-amino acid residues, respectively (*6, 8*).

It is believed that π- and δ-chymotrypsin are more effective catalysts than α-chymotrypsin (*5*). While this belief eventually may be substantiated, the present evidence is not compelling, particularly with reference to maintenance of the difference with all types of substrates and under all reaction conditions. Very little is known about π- and δ-chymotrypsin-catalyzed reactions.

There are two crystalline chymotrypsins, β and γ (*3*), that have not been mentioned. These two enzymatically active proteins may be allomorphic forms of α-chymotrypsin, since all three are believed to have the same terminal α-amino acid residues. It has been noted (*13*) that γ-chymotrypsin is inactivated more rapidly at 25°C and *p*H 8.2 than α-chymotrypsin, although the kinetics of hydrolysis of benzoylglycine methyl ester by the two enzymes are indistinguishable. Other evidence (*5*) suggests that α-, β-, and γ-chymotrypsin have about the same enzymatic activity. The conclusion that they have the same specificity (*5*) is based on very limited and essentially qualitative information.

Alpha-Chymotrypsin

α-Chymotrypsin consists of three acyclic peptide chains linked with five intra- and inter-chain disulfide bonds. A tentative estimate of the composition of each chain is given in Table 1. As noted earlier, the sequence of chain A is known. Provisional sequences have been proposed for chains B and C, or portions of them (*9–12, 14*). Since more structural information is available for bovine pancreatic ribonuclease, which consists of a single peptide chain of 124 α-amino acid residues cross-linked with four disulfide bonds (*15*), it may be asked, Why the interest in the more complex α-chymotrypsin? Aside from the intrinsic attractiveness of the higher mountain, the answer is that far more is known about the properties of α-chymotrypsin as a catalyst, and additional information of this kind is more readily accessible. Solution of the problem of enzyme catalysis requires intimate and extensive knowledge of both the structure of the catalyst and the reactions that it catalyzes.

In an important series of experiments Balls and Jansen and their co-workers (*16*) noted that 1:1 stoichiometric reaction of diisopropylphosphofluoridate with α-chymotrypsin led to the formation of inactive enzyme, isolated as a crystalline protein, which contained one diisopropylphosphoryl residue per molecule. This and other evidence (*17, 18*) led to the view that α-chymotrypsin contains a single active center associated with a particular serine residue, since the inactive diisopropylphosphorylated enzyme, on hydrolysis, gave O-phosphorylserine (*19*). Similar experiments with *p*-nitrophenyl acetate (*20–22*) led to essentially the same conclusion. This *serine residue* is present in chain C in the sequence

• • • Asp • Ala • Met • Ileu • Cys/2 • Ala • Gly • Ala • Ser • Gly • Val • Ser • Ser • Cys/2 • Met • Gly • Asp • *Ser* • Gly • Gly • Pro • Leu • Val • Cys/2 • Lys • • •.

The presence of a histidine residue at the active center has been inferred from studies on the *p*H dependence of certain α-chymotrypsin-catalyzed reactions, and from experiments involving photo-oxidation of the enzyme (*17*). Recently Schoellmann and Shaw (*23*) obtained more direct evidence. It may be seen from Fig. 1 and Table 1 that the two histidine residues present in α-chymotrypsin are in chain B. Since the serine residue identified as being at the active center is in chain C, it is evident that the active center is an interchain feature dependent on the tertiary or three-dimensional structure of α-chymotrypsin.

There is considerable experimental support for the view that the serine and histidine residues at the active center are in close proximity to each other and are required for enzymatic activity. The evidence that other α-amino acid residues constitute a part of the active center and participate in the catalytic process is less clear.

Photo-oxidation, oxidation with hydrogen peroxide, or alkylation with iodoacetic acid leads to alteration of one or both of the methionine residues present in chain C. This structural change influences the level of catalytic activity of the enzyme but does not abolish the activity (*17, 24*). Since alteration of the structure of the methionine residue, three residues removed from the critical serine residue, does not abolish catalytic activity, the conclusion has been drawn that the methionine residue does not participate in the bond-breaking process (*17*). Explanation of the observed diminution in catalytic activity in terms of alteration of the tertiary structure of the enzyme molecule is plausible, but the evidence is not conclusive.

Effects similar to those noted for methionine have been associated with one of the several tryptophan residues (*17, 25*) and with a tyrosine residue (*26*). In addition, attention has been called to possible participation, in the catalytic process, of an aspartic acid (*27*) or arginine residue (*28*). Neither of these latter speculations was supported by any direct experimental evidence. However, recently such evidence has been presented for participation of a carboxyl group, possibly arising from aspartic acid, in the catalytic process (*29*).

The preceding concept of an active center focuses attention on but a part of the overall catalytic process. While useful in certain cases, it frequently is too restrictive. It fosters the erroneous idea that the overall catalytic process can be described for all substrates in terms of the simple summation of a set of presumed colligative properties of the enzyme. In reality the factors determining reactivity of a given system interact with each other to a marked degree and are not readily separable. It is more profitable to contemplate an active site which is defined, such as those regions of the enzyme molecule that are instrumental in promoting the hydrolysis, or synthesis, of amino acid and α-N-acylated α-amino acid alkyl esters, hydroxamides, amides, hydrazides, and so on, and of peptides (*30–32*). At present we cannot describe the

Table 1. Composition of α-chymotrypsin, in terms of α-amino acids obtained on hydrolysis. (*6–8*).

Amino acid residue	No. of residues			
	Chain A	Chain B	Chain C	Molecule
Alanine	1	11	10	22
Arginine		1	2	3
Aspartic*		13	9	21
Half-cystine	1	4	5	10
Glutamic†	1	10	4	14
Glycine	2	11	10	23
Histidine		2		2
Isoleucine	1	6	3	10
Leucine	2	9	8	19
Lysine‡		8	6	14
Methionine			2	2
Phenylalanine		6		6
Proline	2	3	4	9
Serine	1	14	11	26
Threonine		12	9	21
Tryptophan		2	2	4
Tyrosine		4	4	8
Valine	2	13	8	23
Total	13	129	97	237

* Present in protein as aspartyl and asparaginyl residues. † Present in protein as glutamyl and glutaminyl residues. ‡ One lysyl residue may be hydroxylysyl.

active site in molecular terms other than to say that it includes the active center already noted. Therefore, it is necessary that we infer features of the active site from kinetic studies of systems in which this enzyme functions as a catalyst.

Crystalline α-chymotrypsin prepared from chromatographically homogeneous chymotrypsinogen A usually is heterogeneous (*6*). It contains, in addition to α-chymotrypsin, the zymogen, other chymotrypsins, the corresponding denatured proteins, and peptides. The better commercial preparations of salt-free, thrice-recrystallized α-chymotrypsin, prepared by slow activation of thrice-recrystallized bovine chymotrypsinogen A, contain, on an anhydrous basis, 90 $\pm$ 5 percent of the desired enzyme. Preparations of greater purity can be obtained. Comparison of the kinetic properties of one such preparation with several of commercial origin, in all of which the same substrate is used, led to the conclusion that the various preparations were kinetically indistinguishable (*33*). Additional experiments of this kind are sorely needed, particularly with substrates of widely varying structures and reactivities.

A second kind of comparison also is required. As already noted, α-, β- and γ-chymotrypsin appear to be allomorphs. However, α- and γ-chymotrypsin differ with respect to stability (*13*), and α- and β- with respect to chromatographic behavior (*6*). It thus appears that interconversion among allomorphs proceeds at relatively slow rates. Although it has been observed that the kinetics of hydrolysis of benzoylglycine methyl ester by α- and γ-chymotrypsin are indistinguishable (*13*, *34*), there is no assurance that this situation will obtain for all substrates. If it does not, the results of many kinetic studies will have to be re-examined. If it does, it will demonstrate that the overall catalytic properties of an enzyme may be independent of the conformation of a part of the enzyme molecule, or that allomorphic enzymes of different conformation interact with a substrate to give a singular enzyme-substrate complex.

With α-chymotrypsin one is confronted with a molecular species which, under certain circumstances, can function both as a catalyst and as a substrate (*35*). Thus, it is important to conduct kinetic studies under conditions where self-destruction of the catalyst is minimized (*34*, *36*) or where the occurrence of self-destruction is recognized (*35*, *36*). Many kinetic studies, particularly with poorer substrates, have required such extended reaction times that one is uncertain not only about the amount of enzyme present but also about its nature.

Substrates of Alpha-Chymotrypsin

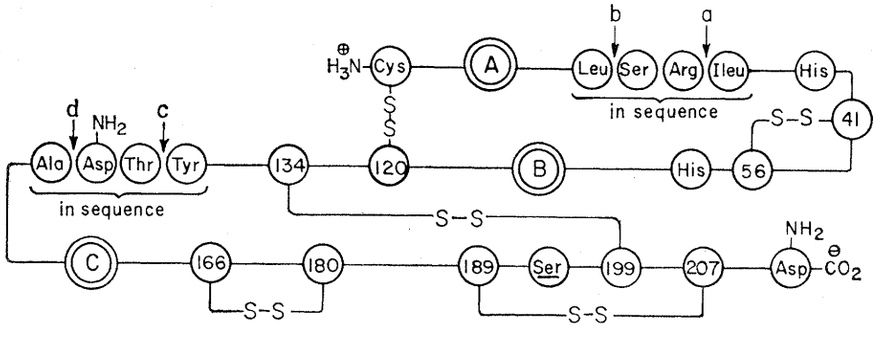
In the preceding discussion we have considered the question of what α-chymotrypsin is, as revealed by analytical procedures involving either direct examination or degradation of the molecule. Let us now turn to the alternative approach, in which one attempts to deduce the structural characteristics of the enzyme molecule from its behavior as a catalyst in dynamic systems.

α-Chymotrypsin, as its name implies, was first recognized by its ability to cause the clotting of milk (*37*), a process involving proteolysis. Furthermore, it has been known for a long time that many proteins are extensively hydrolyzed by this enzyme (*3*). However, in an earlier period when the primary structures of proteins were unknown and the methods of determining structure were primitive, one was faced with the dilemma of determining the mode of action of an enzyme with substrates of unknown structure. The discovery of low-molecular-weight synthetic substrates of known structure for the pancreatic proteinases, by Bergmann and his co-workers, was of inestimable value in approaching the problem of the mechanism of action of these enzymes and is responsible for much of the progress that has been made to date on this problem.

In 1937 Bergmann and Fruton (*38*) noted that carbobenzyloxyglycyl-L-tyrosylglycinamide,

$$C_6H_5CH_2OCONHCH_2CONHCH(CH_2C_6H_4OH)CONHCH_2CONH_2$$

was hydrolyzed in the presence of α-chymotrypsin to give carbobenzyloxyglycyl-L-tyrosine and glycinamide (the point of cleavage is indicated by the barred arrow). This synthetic substrate was the prototype from which several hundred were subsequently developed, largely by Bergmann, Fruton, Neurath, and Niemann and their co-workers (*39*). From experiments with substrates of this kind we now recognize that α-chymotrypsin is capable of catalyzing the hydrolysis of a very large number of compounds of the type $R_1CHR_2R_3$, where R_1 may be a hydrogen or halogen atom, a hydroxyl, acyloxy, or acyl group, an amino or ammonium group, an acylamino group, or a peptide or α-N-acylated peptide residue; R_2, a hydrogen atom, an alkyl, arylalkyl, or aryl group, or simple functional derivatives thereof; and R_3, a carboxyl group or functional derivatives thereof, in which the carbonyl oxygen atom is retained. It is important to recognize that all possible combinations of the groups R_1, R_2, and R_3 specified do not necessarily lead to compounds that are usable substrates of α-chymotrypsin. How-



Chymotrypsinogen A (not to scale)

Cleavage	Enzyme	Enzyme Product	Peptide Products
a	Trypsin	π-Chymotrypsin	
a + b	Chymotrypsin (b)	δ-Chymotrypsin	Ser Arg
a,b,c & d	Chymotrypsin (b,c,d)	α-Chymotrypsin	Ser Arg & Thr Asp(NH_2)
c & / or d	Chymotrypsin	Neochymotrypsins	Thr Asp(NH_2) (c & d)

Fig. 1. Formation of various chymotrypsins from chymotrypsinogen A.

ever, there are hundreds of compounds of this type that can be so used.

In addition to such substrates, which are structurally related to the more complex protein substrates, there are others that are atypical when judged by this criterion. In this latter group are the simple phenolic esters (for example, *p*-nitrophenyl acetate, *40*); the dialkylphosphofluoridates and analogous compounds (*41*); derivatives of the N,N-diarylcarbamic acids (for example, N,N-diphenylcarbamyl chloride, *42*); and the esters and amides of *trans*-cinnamic acid (for example, *trans*-cinnamoyl imidazole, *43*).

With such a very large number of known and potentially accessible substrates of α-chymotrypsin, one is overwhelmed by the wealth of probes that are available for determining how this enzyme functions as a catalyst. It may be argued that these numbers are deceptive because the basic reaction is simply one of hydrolysis, and that the large numbers arise from a monotonous elaboration on a single theme. I do not believe this view can be maintained, because it ignores one of the most dramatic features of enzyme catalysis—that is, a marked structural specificity.

While much useful information has been obtained from examining reaction systems containing model substrates of the types listed, it also is evident that practically all of these substrates have one serious drawback. They are conformationally indeterminate. The probing of the active site of an enzyme with conformationally indeterminate substrates leads to the same difficulties that are encountered in attempting to determine, with a piece of flexible tubing, the intimate details of a lock, which cannot be disassembled without being destroyed. It is for this reason that the discovery by Hein and Niemann (*44*), of a pair of conformationally constrained model substrates of α-chymotrypsin in D- and L-3-carbomethoxydihydroisocarbostyril is a particularly fortunate event. With substrates of this kind, or with others developed from these prototypes, one has, or can acquire, keys which can reveal intimate details of the lock.

Reaction Kinetics

The rates of hydrolysis for a large number of α-chymotrypsin-catalyzed hydrolyses of model substrates containing a single hydrolyzable bond, under conditions where all reaction parameters except enzyme and substrate concentrations are invariant, are described by the equation

$$-\mathrm{d}[S]/\mathrm{d}t = \mathrm{d}[P]/\mathrm{d}t = k_0[E][S]/(K_0 + [S]) \quad (1)$$

where $[S]$, $[P]$, and $[E]$ are the molar concentrations of substrate, products, and enzyme, respectively, and k_0 and K_0 are a pair of kinetic parameters. The parameter k_0 ordinarily is expressed in units of sec^{-1}, and K_0, in units of M or mM. Conditions can be found where the course of essentially all α-chymotrypsin-catalyzed reactions involving substrates of the kind mentioned are described by Eq. 1. Conversely, under some conditions there will be departures from Eq. 1.

If reaction is extensive, there may be competitive inhibition by one or more of the reaction products (*45*). The more effective competitive species generally are the α-N-acylated-α-amino acid carboxylate anions, whose *p*H-dependent enzyme-inhibitor dissociation constants increase markedly with increasing *p*H in the region from *p*H 5.5 to 9.5 and, at about *p*H 8, are substantially greater than the values of K_0 for the enzyme-substrate reaction (*46, 47*). Thus, prudence requires that the reaction be examined under conditions where the extent of reaction is limited and the *p*H of the reaction system is in the region of *p*H 8 (ordinarily the so-called *p*H optimum of the reaction system) if Eq. 1 is to be taken as the rate equation for the system.

α-Chymotrypsin, in common with many other proteins, is capable of association, and it is known that the kinetic properties of associated enzymes differ from those of the monomer (*32, 48*). The extent of association of monomeric α-chymotrypsin in aqueous solutions at 25°C is dependent upon the concentration of enzyme, the hydrogen ion concentration, and the ionic strength (*49*). It probably is also dependent upon temperature and upon the nature of the solvent when solvent systems other than water are used. In aqueous solutions, at 25°C, *p*H 7.9, and 0.1M concentration in sodium chloride, association of the enzyme is not a serious problem, provided the concentration of enzyme does not exceed 10^{-4} to $10^{-5}M$. A lower operational limit of 10^{-7} to $10^{-8}M$ is established by the fact that, as with all proteins, the enzyme is strongly adsorbed on glass surfaces and is present as surface-adsorbed enzyme rather than as molecularly dispersed monomer. Very little is known about the kinetic properties of surface-adsorbed enzyme other than that it retains its catalytic properties, at least in part. However, the kinetic behavior of systems in which the enzyme is surface-adsorbed and the substrate is molecularly dispersed are atypical when compared with systems in which both reactants are monomolecularly dispersed (*50*).

It has been suggested that enzymes may undergo conformational alteration (allosteric transition), with a concomitant change in their catalytic properties, when combined with compounds that are neither substrates nor reaction products (*51*). While transitions of this kind are unlikely in systems initially containing only enzyme and substrate, they may occur in systems containing an additional component, as in inhibition studies. It is possible that the apparent activation of the α-chymotrypsin-catalyzed hydrolysis of benzoyl- and *p*-aminobenzoylglycine methyl ester by micellarly dispersed 9-aminoacridinium cation, observed by Wallace (*52*), is an example of this phenomenon.

In the preceding paragraphs we have been concerned with situations where a change in the kinetic properties of the enzyme may lead to a departure from a first-order dependence on enzyme concentration—a dependence demanded by Eq. 1. Let us now turn to possible perturbations arising from a dependence upon substrate concentrations other than that indicated by Eq. 1.

If an enzyme-catalyzed reaction is examined over a wide range of substrate concentrations, either activation or inhibition by excess substrate may be observed (*53*). Activation by excess substrate was first seen in α-chymotrypsin-catalyzed reactions by Wolf and Niemann (*54*), who observed this phenomenon in the hydrolysis of acetylglycine methyl ester. Subsequent studies (*55*) have shown that activation by excess substrate is strikingly dependent upon the structure of the substrate, and to date such activation has been observed only with acetyl- and propionylglycine methyl ester and possibly with *p*-nitrophenyl acetate (*56*).

There are no known examples of inhibition of a chymotrypsin-catalyzed reaction by excess substrate. At one time it was believed that this phenomenon could be observed with nicotinylglycine methyl ester (*57*), but subsequent studies have shown that the apparent diminution of rate at substrate concentrations greater than 0.1M was

an artifact arising from overcorrection for the nonenzyme-catalyzed hydrolysis of substrate at high substrate concentration (*55, 58*). This experience provides a forceful example of the need for caution in interpreting the kinetics of enzyme-catalyzed reactions carried out at high substrate concentrations where artifacts arising from subtle systematic errors may be encountered (*58*).

The dependence of the rate of reaction upon substrate concentration specified by Eq. 1 requires the tacit assumption that the substrate is monomeric and has an activity coefficient of unity. The finding that Eq. 1 describes the reaction kinetics for dilute aqueous solutions and for many substrates affords ample support for this assumption. However, it must be anticipated that, with certain substrates, molecular or micellar aggregates will be formed even at relatively low substrate concentrations and thus give rise to a dependency upon substrate concentration other than that demanded by Eq. 1. While no unambiguous example of the α-chymotrypsin-catalyzed hydrolysis of a substrate capable of aggregation can be given, a counterpart has been found in inhibition studies with several acridine derivatives (*59*).

The rates of α-chymotrypsin-catalyzed reactions described by Eq. 1 are dependent upon reaction parameters other than enzyme and substrate concentrations. The additional parameters commonly include temperature, hydrogen ion concentration, presence or absence of added electrolytes, and solvent system. Other parameters may be involved in particular cases. These reaction parameters do not necessarily function independently, hence alteration in the magnitude of any one will frequently lead to perturbation of several others. Faced with such a complex situation we are limited at present to a qualitative or empirical description of the effects associated with these parameters. We are far from a rational quantitative description of any one of them and are still farther away from a grand rate equation applicable to all of them.

In conventional chemical reactions one ordinarily expects the rate of reaction to increase with increasing temperature, usually over a relatively wide range of temperatures. Unfortunately, with α-chymotrypsin and many other enzyme-catalyzed reactions, one of the reactants, the enzyme, is temperature-sensitive, with the result that one is limited in aqueous reaction systems to temperatures ranging from approximately 0° to 35°C. This experimental limitation is further aggravated by the modest overall temperature coefficients of these reactions (*60*). Thus, with reaction systems that are relatively insensitive to changes in temperature within the limited range accessible for study, and the clear need for resolution of the complex overall temperature coefficient into its separate components, it is not surprising that this paragraph begins with a recitation of experimental difficulties and ends with a confession of considerable ignorance.

The dependence of the rate of an α-chymotrypsin-catalyzed reaction upon hydrogen ion concentration is dramatic and readily determinable. In the simplest situation, where the rate of reaction at any given hydrogen ion concentration is described by Eq. 1, a change in hydrogen ion concentration may lead to alteration of the substrate, the enzyme, or the enzyme-substrate complexes.

The kinetic consequences of alteration of the substrate through an ionization process were considered by Hogness and Niemann (*61*), and subsequently more extensively by Friedenwald and Maengwyn-Davies (*62*). The solution of this problem is relatively unambiguous, and for the simplest case, where the substrate ionizes to give a species incapable of combining with the enzyme, all that is required is to modify Eq. 1 so that the substrate concentrations reflect the effective concentrations rather than the concentrations of all species. The only additional information required is the ionization constant of the substrate. The more complex cases include those where all substrate species are capable of reaction but at different rates, and where the rejected substrates are competitive or noncompetitive inhibitors (*62*, pp. 202–207).

Hydrogen ion dependencies arising from alteration of the enzyme or enzyme-substrate complexes present a far more complex situation, principally because the enzymes are ampholytes. In principle there are three ways in which a change in hydrogen ion concentration of the reaction system may alter the enzyme species: through localized ionization of groups at the active site (*62*, pp. 141–202; *63*), hydrogen-ion-induced conformational alteration of the active site, or a combination of these phenomena. In practically all studies conducted with α-chymotrypsin, attention has been focused on only the first of the three possibilities. Lack of space forbids a review of the extensive literature in which the hydrogen ion dependencies of α-chymotrypsin-catalyzed reactions are described.

In one study (*47*), notable for its extensive observations and for its freedom from reaction parameters tending to foster ambiguity, the α-chymotrypsin-catalyzed hydrolysis of benzoylglycine methyl ester was examined at 25.0°C in aqueous solutions 0.02, 0.20, and 1.0*M* in sodium chloride and over a *p*H range from 6.10 to 9.40. For these systems, replacement of the kinetic parameters k_o and K_o of Eq. 1 by the parameters specified in Eqs. 2 and 3,

$$k_o = k_o'/[1 + (K_{aes}/[H^+]) + ([H^+]/K_{bes})] \quad (2)$$

$$K_o = K_o'\{[1 + (K_{ae}/[H^+]) + ([H^+]/K_{be})] \div [1 + (K_{aes}/[H^+]) + ([H^+]/K_{bes})]\} \quad (3)$$

where $K_{ae} = ([E][H^+])/[EH]$, $K_{be} = ([EH][H^+])/[EH_2]$, $K_{aes} = ([ES][H^+])/[EHS]$, and $K_{bes} = ([EHS][H^+])/[EH_2S]$, gave a satisfactory rate equation after it was recognized that the reaction was being inhibited by benzoylglycinate anion. These results are compatible with any one of the three interpretations noted.

In a recent study (*64*) of the hydrogen ion dependence of the inhibition of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-leucine methyl ester by benzamide or formanilide it was observed that between *p*H 5.5 and 9.0 the *nature* of the inhibition process is *p*H-dependent. Only between *p*H 7.0 and 8.0 is the process fully competitive. At both extremes there is a more generalized process which results in a change in the comformation of the active site of enzyme, which in turn is reflected in the enzyme's ability to combine with substrate or modifier molecules.

Control of the hydrogen ion concentration of a reaction system through the use of a buffer or the maintenance of a constant ionic strength by means of a *p*H-stat (an electromechanical buffering device) requires that an electrolyte be added to the system. Unfortunately, these experimental benefits are purchased at the expense of introducing additional reaction parameters; this seems a dubious bargain until it is recalled that reaction systems of relatively high ionic strength also are required to avoid association of desired monomeric enzyme.

It is convenient to classify electrolyte effects as general or specific, the latter ordinarily being found at concentrations of less than $10^{-3}M$ and the former at concentrations of $10^{-2}M$ and up. Apart from the inhibiting effects of some metal ions, the most notable specific electrolyte effect is seen in enhancement of the rate of the α-chymotrypsin-catalyzed hydrolysis of several typical acylated α-amino acid methyl esters by calcium ion. In these cases the increased rate is achieved by an increase in the value of k_0, that of K_0 remaining essentially invariant (*65*). Since the magnitude of this effect is dependent upon the structure of the substrate (*65*), it is probable that the increase in rate arises from the formation of a ternary complex of calcium ion, enzyme, and substrate that is more reactive than the binary complex of enzyme and substrate, presumably because of improved orientation of substrate in the ternary complex.

With general electrolytes the observed effects are dependent to a minor degree on the nature of the electrolyte, particularly when it is univalent, and to a significant degree on the structure of the substrate. With some substrates an increase in concentration of electrolyte produces an increase in the kinetic parameter k_0, K_0 remaining invariant (*66*); with other substrates the inverse situation obtains (*67*), and with still others both kinetic parameters are affected, the magnitude of k_0 steadily increasing with increasing electrolyte concentration and that of K_0 first decreasing and then remaining essentially invariant (*68*). These results are at variance with the optimistic view that maintenance of reaction systems at a particular salt concentration will normalize the kinetic parameters k_0 and K_0 of Eq. 1 for all substrates.

The limited solubility in water of many substrates has led to the use of reaction systems in which both water and an organic solvent are present. In these cases information about substrate reactivity is purchased at a very high price because of the introduction of still another reaction parameter, one which perturbs all others. Distinction first must be made between protic and aprotic solvents, because the most common representatives of the first group are alcohols and hence might participate directly in the enzymic reaction. While such participation has been observed with methanol and other primary alcohols (*22, 69*), this phenomenon becomes less important with secondary alcohols and practically vanishes with tertiary alcohols because of their decreased reactivity (*22*). Thus, if the intent is to avoid direct participation in the enzymic reaction, aprotic dipolar organic solvents offer no advantages over the tertiary alcohols even in cases where the mechanism of reaction is such as to permit direct participation (*70*).

In earlier studies (*34, 71–73*) it was noted that addition of protic or dipolar aprotic organic solvents to aqueous reaction systems resulted in an increase in the kinetic parameter K_0, with k_0 either decreasing or remaining invariant. Organic solvents, such as *t*-amyl alcohol, ethyl ether, dioxane, and acetone, function as inhibitors of α-chymotrypsin-catalyzed reactions (*70, 74, 75*). However, to designate this inhibition fully competitive (*70, 74*) is incorrect (*75*). It is both competitive and noncompetitive (*75*), a characteristic compatible with the fact that the inhibition constant is dependent upon the structure of the substrate (*70, 75*). However, the effects associated with the presence of an organic solvent cannot be explained solely in terms of a conventional inhibition process. There is another factor that is responsible for the large increase in the value of K_0 usually observed when an organic solvent is added. While a linear relationship between $1/K_0$ and solvent concentration was observed with one reaction system (*71*), with others no such relationship was obtained (*34*). Barnard and Laidler (*72*) advocated that the dielectric constant of the medium be introduced as a parameter, a suggestion adopted by Clement and Bender (*70*) in their attempt to correlate rate with solvent composition in terms of the dielectric constant of the medium (the presence of enzyme and substrate were ignored) and fully competitive inhibition by the organic solvent component. Another approach was taken by Applewhite, Martin, and Niemann (*34*), who postulated that addition of organic solvent resulted in a decrease in the activity coefficient of the substrate, which they attempted to evaluate in terms of its solubility in the mixed solvent system relative to its solubility in water. On the basis of present knowledge, it appears that a process involving correction for a change (usually a decrease) in the activity coefficient of the substrate coupled with both competitive and noncompetitive inhibition of the reaction by the organic solvent component provides the most realistic representation of the nature of the solvent-reaction parameter. Although realistic, this representation is incomplete in that it ignores the effect of added organic solvent on reaction parameters such as hydrogen ion concentration and added electrolytes. However, it does recognize that the solvent-reaction parameter is markedly dependent upon the structure of the substrate.

In the preceding discussion we have been concerned with the kinetics of the simplest reaction systems in which α-chymotrypsin-catalyzed hydrolyses can be observed. Before becoming involved in the interpretation of these and more complex kinetic situations it is desirable that we consider two of the most prominent features of α-chymotrypsin-catalyzed reactions—that is, their stereochemical and structural specificity.

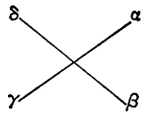
Stereochemical Specificity

The greater if not overwhelming reactivity of one member of a pair of optical antipodes is a characteristic of many enzyme-catalyzed reactions. For many years it was assumed that α-chymotrypsin-catalyzed reactions involving asymmetric substrates were necessarily limited to substrates possessing the same configuration as that of the L-α-amino acid residues present in proteins. This view of an obligatory absolute stereospecificity in favor of L-antipodes began to lose ground when it became evident that the stereochemical preference was relative rather than absolute (*44*), and it was negated when, in several instances, the D-antipodes were found to be the more reactive (*44, 76, 77*).

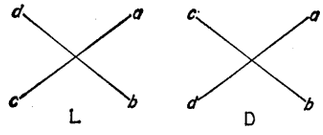
Stereochemical specificity has long been associated with the formation of diastereoisomeric enzyme-substrate complexes (*78*), and it is now possible to give a simple structural interpretation of the phenomenon of stereospecificity in terms of the nature of the enzyme-substrate complexes arising from interaction of enzyme with the two antipodes (*44, 76*). It is known that both antipodes can combine with the active site of α-chymotrypsin, the D-antipode frequently functioning as a fully competitive inhibitor of the hydrolysis of the L-antipode (*45*). Thus, with both antipodes capable of combination with the active site, in many cases with comparable facility (*79*), it is evident that the two complexes differ primarily only in the way in which the two anti-

podes are oriented at the active site (*45*).

In the simplest and most general representation (*44*), let it be assumed that the active site is asymmetric and can be represented by the notation



and that the two antipodes can be represented by all permutations of the notations



that do not lead to inversion on configuration. If we specify that productive reaction requires interaction of the structural component *a* with its complementary locus α, that optimal reactivity is achieved when *a* interacts optimally with α, *b* with β, *c* with γ, and *d* with δ, and that component *b* shall be no more bulky than a hydrogen atom, then for substrates of the type $R_1R_2CHR_3$, where $R_3 = a$, $R_2 = c$, and $R_1 = d$, it follows that the L-antipode will function as a substrate and that the D-antipode will function as a competitive inhibitor. However, when the structure of the substrate is altered so that *c* no longer interacts with γ and *d* no longer interacts with δ but, instead, *c* interacts with δ and *d* interacts with γ, then in the limiting case the D-antipode will function as an effective substrate and the L-antipode, as a competitive inhibitor. With all situations between the two limiting cases being possibilities, it is evident that relative stereospecificity, and preference for one or the other antipode, can be explained in terms of the foregoing model.

Structural Specificity

Definition of the structural specificity of α-chymotrypsin is dependent upon two sources of information. (i) With proteins used as substrates, largely qualitative information is obtained from the simultaneous identification of the structures of the substrates and the points of hydrolytic cleavage (*80, 81*). (ii) When simple model substrates of known and varied structures are employed, one obtains not only the same kind of information as from source i but also quantitative data capable of explaining the mechanism of structural specificity.

One feature of structural specificity pertains to the so-called specific side chain (*5*). For a peptide

$$-\text{NHCH}R_1\text{CONHCH}R_2\text{CONHCH}R_3\text{CO}-$$
$$\updownarrow$$

the point of hydrolytic cleavage (barred arrow) is associated primarily with the so-called specific side chain R_2, although it is unreasonable to conclude that the structural components R_1 and R_3 are without influence. From data obtained with protein substrates (*80, 81*) one is led to the conclusion that the side-chain specificity of α-chymotrypsin is extremely broad, that it is relative rather than absolute, and that the only restriction is that the group R_2 not bear a formal negative charge. The validity of this conclusion is supported by the observation that investigation of an increasing number of properly designed model substrates has resulted in increasing agreement in the information obtained from sources i and ii (*82*).

Explanation of the phenomenon of structural specificity was first approached from the premise that combination of substrate with the active site of the enzyme necessarily led to the formation of reaction products—that is, nonsubstrates did not combine. This view was shown to be incorrect when it was found that pairs of optical antipodes, with only one member of each pair capable of functioning as a substrate, combined with the active site with greater, equal, or lesser ability, depending upon the structures of the pairs (*30, 31, 79, 83*). From these and other data (*44, 84–87*) it became evident that, while combination is obligatory, orientation of the substrate at the active site of the enzyme frequently is the major factor in determining its reactivity (*30, 31, 44, 79, 83–87*). Undue attention to the role of the specific side chain R_2 in determining the structural specificity of α-chymotrypsin-catalyzed hydrolyses of model substrates of the type $R_1'\text{CONHCH}R_2\text{CO}R_3$ has tended to obscure the influence exerted by the other structural components in determining overall reactivity. The conception that overall reactivity can be viewed as arising from the contribution of the specific side chain R_2 to an invariant contribution arising from the presumed nonspecific substrate $R_1'\text{CONHCH}_2\text{CO}R_3$ is at odds with several observations that provide a suitable test of this hypothesis (*44, 87*).

A realistic approach to the definition of structural specificity has been proposed (*44, 85*). For substrates of the type $R_1\text{CH}R_2R_3$ it is assumed that combination with the active site of the enzyme proceeds not only through interaction of the structural components R_1, R_2, and R_3 with their presumed complementary loci, ρ_1, ρ_2, and ρ_3, at the active site but also through interactions arising from all permutations of these interactions—that is, through interaction of R_1 with ρ_2 and ρ_3, of R_2 with ρ_1 and ρ_3, and so on. For this situation, depending upon the nature of the components R_1, R_2, and R_3, productive combination is accompanied by nonproductive combinations that are fully competitive with the productive modes. Under these conditions the operational kinetic parameters k_o and K_o of Eq. 1 acquire the statistical significance given by Eqs. 4 and 5

$$k_o = [\sum_i^m (k_2/K_S)_i]/[\sum_i^m (1/K_S)_i + \sum_j^n (1/K_S)_j] \quad (4)$$

$$K_o = 1/[\sum_i^m (1/K_S)_i + \sum_j^n (1/K_S)_j] \quad (5)$$

where K_{Si} and K_{Sj} are the apparent dissociation constants of the productive and nonproductive complexes and k_{2i} represents the rate constants of the productive complexes.

The preceding hypothesis was developed for substrates of the type $R_1'\text{CONHCH}R_2\text{CO}R_3$, where R_1' is an alkyl, alkoxyl, or aryl group. It has been remarkably successful in accounting for a number of observations that had appeared anomalous (*44, 85, 87*), including frequent lack of correlation between the kinetic parameters K_o and k_o, and the inversion of antipodal specificity noted previously. It has proved to be of inestimable value in guiding the design of model substrates where enhancement of nonproductive combinations can obscure relationships associated with combination in a productive mode.

Although a very large number of observations will have to be made to evaluate the magnitude of all possible R-ρ interactions, the essential validity of the preceding hypothesis appears to have been established (*44, 85, 87*). Further support for this hypothesis and for its generality has been provided by its successful application in the case of the enzyme monoamine oxidase (*88*).

In the preceding interpretation it was

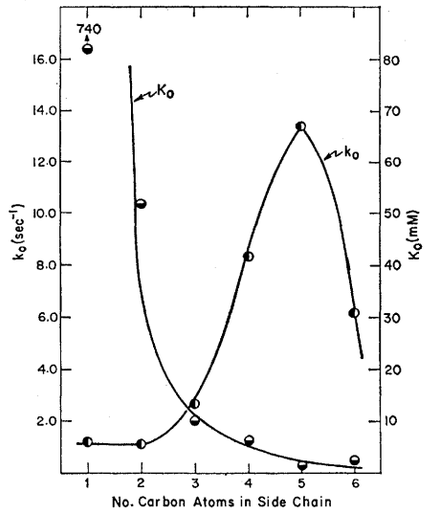


Fig. 2. The α-chymotrypsin-catalyzed hydrolysis of substrates of the type $CH_3CONHCH(CH_2)_nHCO_2CH_3$ for values of $n = 1$ to $n = 6$. The reactions were carried out in aqueous solutions at 25°C, *p*H 7.90, and 0.1*M* concentration in sodium chloride.

assumed that there were three combining loci at the active site of the enzyme. To forestall identification of these loci as points, it should be noted that a recent comparison of the kinetic parameters K_o and k_o of acetyl-L-alanine and acetyl-L-valine methyl esters with those of acetylglycyl-L-alanine and acetylglycyl-L-valine methyl esters has shown that extension of the α-acylamino component R_1, through incorporation of additional α-amino acid residues, leads to a considerable decrease in the kinetic parameter K_o and to an increase in k_o when the specific side chain R_2 is other than a hydrogen atom and R_3 is a methoxyl group (*89*). These observations clearly require an extended R_1-ρ_1 interaction and an active site of considerable dimensions. Relatively little is known about the consequences of extension of the R_3 component other than that the kinetic parameters K_o and k_o may vary with the nature of the ester (*87, 90*). However, these data suggest that the ρ_3 locus at the active site also is capable of extended interaction with respect to both binding and orientation of the substrate. Thus, with proteins it must be anticipated that the rate of hydrolysis of a peptide bond associated with a particular side chain will be perturbed by the nature of the α-amino acid residues present on both sides of the residue containing the hydrolyzable bond. How far this effect extends is not known.

Now that the dependence of over-all reactivity upon contributions from the structural components R_1 and R_3 is recognized, it is possible to assess more accurately the contributions arising from interactions involving the specific side chain component R_2. Although there is a region at the active site that is capable of effective combination with a large number of aromatic compounds (*59*), effective binding of model substrates is not necessarily dependent upon the presence of an arylalkyl component (*91*). However, the presence of such a component is associated with enhanced reactivity of the enzyme-substrate complex (*91*). From topographical studies, conducted with a set of aromatic inhibitors, it was concluded that the region of the active site with which these inhibitors combined resembled a long, curved, narrow valley (*59*). Other evidence supports this view. In a series of acetylated-L-α-amino acid methyl esters bearing normal alkyl side chains containing from one to six carbon atoms, the kinetic parameter K_o was observed to decrease from a high value of over 700 m*M* for the alanine derivative to a low value of 2.4 m*M* for the caprylic acid derivative. In contrast, the magnitude of the kinetic parameter k_o remained invariant for the first two members of the series, increased to a maximum value for the norleucine derivative, and then decreased with the next higher homolog (*89*). These data (see Fig. 2) suggest that while binding of substrate to the active site increases steadily with increasing chain length, probably through hydrophobic bonding, optimal orientation of the substrate proceeds in steps. For side chains containing two carbon atoms or less, there is relatively poor orientation; orientation then steadily improves with increasing chain length until a maximum of five carbon atoms is present. From this point on, steric factors intrude and lead to an increase in either the frequency or the number of nonproductive modes of combination.

Similar results are obtained when the two substrates α-N-acetyl-L-tyrosine methyl ester and α-N-acetyl-O-isopropyl-L-tyrosine methyl ester are compared. For these two substrates the magnitudes of the kinetic parameter K_o are comparable, a finding which implies that the two combine with the active site with equal facility (*92*). However, the rate parameter k_o for the latter substrate is approximately 10^{-4} that for the former. Again, a steric factor, which is without effect on binding, leads to a change in orientation of the substrate, which is now combined in largely unproductive modes.

The steric effects noted arise in large part from features of the active site. There are others that are generated by the structure of the substrate. Steric shielding of the reactive carbonyl group of substrates of the type $R_1CHR_2COR_3$ through β-branching is now well established (*86, 93*), as is the β- or ortho-compression (*94*) encountered in β,β or 2,6-disubstituted phenylalanine derivatives. An additional steric effect associated with replacement of the α-hydrogen atom by a bulkier group also has been noted (*95*).

Reaction Mechanism

Kinetic parameters do not lead directly to interpretations of reaction mechanisms. It is first necessary to interpret the kinetic parameters.

There is abundant evidence supporting the view, first advanced by Huang and Niemann (*45*), that for many model substrates of the type $R_1'CONHCHR_2COR_3$ the kinetic parameter K_o may be taken as an apparent enzyme-substrate dissociation constant (*96*). This finding does not imply that K_o is to be associated with a single process. On the contrary, it appears that, except for a few limiting cases, the constant K_o is a statistical kinetic parameter defined by Eq. 5. Generally it is not the dissociation constant of the productive enzyme-substrate complex but, instead, is the statistical summation of the dissociation constants of all enzyme-substrate complexes, both productive and nonproductive. The principal interpretative consequence of this situation is that, when nonproductive modes of combination are dominant, observed values of K_o do not reflect the apparent dissociation constants of the productive enzyme-substrate complexes. Therefore, kinetic interpretations based upon perturbation, or lack of perturbation, of an assumed apparent dissociation constant by a particular reaction parameter become ambiguous unless it can be shown that all combinations of enzyme and substrate are productive. The relatively low rates of reaction observed with simple model substrates—rates very much lower than those expected from a diffusion-controlled process—imply that this limiting case is rarely encountered. Similar considerations lead to the view that the rate parameter k_o generally is not the rate constant for

decomposition of the productive enzyme-substrate complex into reaction products but, instead, is the rate constant obtaining in the face of competitive and nonproductive combination of substrate with the active site of the enzyme, as implied in Eq. 4.

The kinetic parameters K_o and k_o describe the overall process, starting with isolated enzyme and substrate in their ground states and ending with enzyme and reaction products in the same states. The problem is, What happens in between?

One approach centers attention on the possibility that enzymatic catalysis is accompanied by, or arises in part from, conformational changes at the active site of the enzyme that are generated by combination with the substrate. This view is inherent in the so-called "rack" theory of Lumry and Eyring (*97*) and the "induced fit" theory of Koshland (*98*). Recently Havsteen and Hess and their co-workers (*99*) have examined systems, principally of α-chymotrypsin and diisopropylphosphofluoridate or *p*-nitrophenyl acetate, with the aid of kinetic, spectrophotometric, and spectropolarimetric techniques and have obtained evidence compatible with the proposition that the conformation of the active site in an enzyme-substrate complex may be different from that of the isolated enzyme. How it differs is not revealed, nor does it follow that a change in the conformation of the active site is a critical feature of the catalytic process responsible for the hydrolysis of proteins and the more typical model substrates. The fact that reaction parameters other than substrate may cause a change in conformation of the active site (*64*) adds to our confusion. While there is evidence that the active site may be mobile, it is not infinitely so. If it were, structural and stereochemical specificity would not be observed.

Another, and the most common, approach to the problem of the detailed mechanism of α-chymotrypsin-catalyzed reactions is based upon the assumption that the conformation of the active site is essentially invariant. At this stage of our knowledge it is not important that this assumption be completely valid. All that can be hoped for is a reasonable approximation to one or the other limiting situation—that is, a completely mobile or a completely immobile active site. The latter is the simpler situation and therefore deserves exploration as a possible limiting case.

It is an indubitable fact that the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate proceeds through formation of an acyl enzyme intermediate, in this case acetyl-α-chymotrypsin (*20, 56, 99-101*). However, the presence of such an intermediate in other α-chymotrypsin-catalyzed reactions (*43, 53, 90, 101-103*) becomes increasingly uncertain as the structures of the substrates depart from the structure of the example cited and approach those of the more reactive trifunctional model substrates of the type $R_1'CONHCHR_2$-COR_3. The claim that an acyl enzyme is an intermediate in all α-chymotrypsin-catalyzed reactions (*104*) is based upon arguments too insecure to be accepted with confidence. Recent tests of that proposition have not been definitive (*105*).

The present status of our understanding of the mechanism of the hydrolytic step is reflected in the conclusion (*103*) that "the efficient and specific catalysis of α-chymotrypsin appears to be carried out by a general base, or possibly a combination of functionalities such as a general acid and a general base or a general acid and a nucleophile. The facile reaction is due to precise stereochemical requirements including both the correct fit and rigidity of the substrate at the active site of the enzyme. . . ." The state of our knowledge is reflected, too, in the observation (*87*) that "an enzyme provides an intramolecular environment for a reaction that otherwise would be intermolecular, the rate of decomposition of the intermediate enzyme-substrate complex is dependent upon the degree to which a favorable geometry between the attacked and attacking groups can be achieved." The dependence of these conclusions upon structural and stereochemical specificity emphasizes the need for acquiring model substrates that will approximate limiting situations for these two reaction parameters before we embark on studies of the detailed mechanism of the hydrolytic step.

Conclusion

In 1906 Fischer (*106*) described the approach to elucidation of the chemical synthesis of a protein in terms that might be applied today to elucidation of the mechanism of action of an enzyme. The approach, he said, is that of a pedestrian who "seeks his way step by step with careful attentiveness and who must examine many roads until he has found the right one. On his long and troublesome travels, not only does he learn to recognize completely the geography and topography of the country, but also he becomes intimate with the tongue and culture of its inhabitants. When he finally has reached his goal, he is able to locate himself properly in every corner of the country, and this will be possible for other people also if he writes a book about it." As for elucidation of the mechanism of action of α-chymotrypsin, the pedestrian has barely begun his journey, and this review is his first letter home.

References and Notes

1. The monomeric protein may be simple or complex. The simple protein contains only covalently bonded α-amino acid residues and is a polypeptide. A complex protein contains, in addition, other components which frequently are noncovalently bonded to the simple protein and are dissociable.
2. *Report of the Commission on Enzymes of the International Union of Biochemistry* (Pergamon, Oxford, 1961).
3. J. H. Northrop, M. Kunitz, R. Herriott, *Crystalline Enzymes* (Columbia Univ. Press, New York, ed. 2, 1948).
4. C. K. Keith, A. Kazenko, M. Laskowski, *J. Biol. Chem.* **170**, 227 (1947).
5. P. Desnuelle, in *The Enzymes*, P. D. Boyer, H. Lardy, K. Myrbäck, Eds. (Academic Press, New York, ed. 2, 1960), vol. 4, pp. 93–110.
6. P. Desnuelle and M. Rovery, *Advan. Protein Chem.* **16**, 139 (1961).
7. Z. Zmrhal, *Collection Czech. Chem. Commun.* **27**, 2934 (1962).
8. D. Van Hoang, M. Rovery, A. Guidoni, P. Desnuelle, *Biochim. Biophys. Acta* **69**, 188 (1963).
9. B. S. Hartley, *Proc. Intern. Congr. Biochem. 5th, Moscow, 1961* (1963).
10. J. Kraut, L. C. Sieker, D. F. High, S. T. Frier, *Proc. Natl. Acad. Sci. U.S.* **48**, 1417 (1962).
11. J. R. Brown and B. S. Hartley, *Biochem. J.* **89**, 59P (1963).
12. B. Meedom, *Acta Chem. Scand.* **10**, 150, 881 (1956).
13. J. H. Lang, E. Frieden, E. Grunwald, *J. Am. Chem. Soc.* **80**, 4923 (1958).
14. B. S. Hartley, in *Proc. Symposium on Enzyme Models and Enzyme Structure, 1962* (Brookhaven National Laboratory, Upton, N.Y., 1962), pp. 85–99; B. Keil, B. Meloun, J. Vacécék, V. Kostha, Z. Prusik, F. Sorm, *Biochim. Biophys. Acta* **56**, 595 (1962).
15. D. G. Smyth, W. H. Stein, S. Moore, *J. Biol. Chem.* **238**, 227 (1963); C. B. Anfinsen, in *Proc. Symposium on Enzyme Models and Enzyme Systems, 1962* (Brookhaven National Laboratory, Upton, N.Y., 1962), pp. 184–197; K. Hofmann, F. Finn, W. Haas, M. J. Smithers, Y. Wolman, N. Yanaihara, *J. Am. Chem. Soc.* **85**, 833 (1963).
16. E. F. Jansen, M. D. Fellows-Nutting, R. Jang, A. K. Balls, *J. Biol. Chem.* **179**, 189, 201 (1949); E. F. Jansen and A. K. Balls, *ibid.* **194**, 721 (1952); E. F. Jansen, R. Jang, A. K. Balls, *ibid.* **196**, 247 (1952).
17. D. E. Koshland, Jr., D. H. Strumeyer, W. J. Ray, Jr., in *Proc. Symposium on Enzyme Models and Enzyme Systems, 1962* (Brookhaven National Laboratory, Upton, N.Y., 1962), pp. 101–119.
18. F. Sanger, *Proc. Chem. Soc.* **1963**, 77 (1963).
19. N. K. Schaffer, S. C. May, Jr., W. H. Summerson, *J. Biol. Chem.* **202**, 67 (1953); *ibid.* **206**, 201 (1954).
20. B. S. Hartley and B. A. Kilby, *Biochem. J.* **56**, 288 (1954).
21. R. A. Oosterbaan and M. E. Van Andrichem, *Biochim. Biophys. Acta* **27**, 423 (1958); A. K. Balls and H. N. Wood, *J. Biol. Chem.* **219**, 245 (1956).
22. C. E. McDonald and A. K. Balls, *J. Biol. Chem.* **221**, 993 (1956).

23. G. Schoellmann and E. Shaw, *Biochemistry* **2**, 252 (1963).
24. H. Schachter and G. H. Dixon, *Federation Proc.* **22**, 245 (1963).
25. B. Witkop, *Advan. Protein Chem.* **16**, 221 (1961).
26. S. K. Dube, O. A. Roholt, D. Pressman, *Federation Proc.* **22**, 245 (1963).
27. S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela, Y. Shalitin, *J. Am. Chem. Soc.* **84**, 2421 (1962).
28. B. F. Erlanger, *Proc. Natl. Acad. Sci. U.S.* **46**, 1430 (1960).
29. J. A. Stewart, H. S. Lee, J. E. Dobson, *J. Am. Chem. Soc.* **85**, 1537 (1963).
30. H. T. Huang and C. Niemann, *ibid.* **73**, 3223 (1951).
31. ———, *ibid.* **75**, 1395 (1953).
32. R. B. Martin and C. Niemann, *ibid.* **80**, 1473 (1958).
33. H. T. Huang and C. Niemann, *ibid.* **74**, 4713 (1952).
34. T. H. Applewhite, R. B. Martin, C. Niemann, *ibid.* **80**, 1457 (1958).
35. R. B. Martin and C. Niemann, *Biochim. Biophys. Acta* **26**, 634 (1957).
36. T. H. Applewhite, H. Waite, C. Niemann, *J. Am. Chem. Soc.* **80**, 1465 (1958).
37. H. M. Vernon, *J. Physiol. London* **28**, 448 (1902); **47**, 325 (1913–14).
38. M. Bergmann and J. S. Fruton, *J. Biol. Chem.* **118**, 405 (1937).
39. This literature is too extensive to be reviewed here.
40. B. S. Hartley and B. A. Kilby, *Biochem J.* **50**, 672 (1952).
41. A. K. Balls and E. F. Jansen, *Advan. Enzymol.* **13**, 321 (1952).
42. B. F. Erlanger and W. Cohen, *J. Am. Chem. Soc.* **85**, 348 (1963).
43. M. L. Bender, G. R. Schonbaum, B. Zerner, *ibid.* **84**, 2540, 2562 (1962).
44. G. E. Hein and C. Niemann, *ibid.*, pp. 4487, 4495.
45. H. T. Huang and C. Niemann, *ibid.* **73**, 1541 (1951).
46. R. R. Jennings, thesis, California Institute of Technology (1954).
47. T. P. Gordon, thesis, California Institute of Technology (1959).
48. K. A. Booman and C. Niemann, *Biochim. Biophys. Acta* **26**, 439 (1957).
49. See *32* for a brief summary of the pertinent literature.
50. R. L. Bixler and C. Niemann, *J. Am. Chem. Soc.* **81**, 1412 (1959).
51. J. Monod, J. P. Changeux, F. Jacob, *J. Mol. Biol.* **6**, 306 (1963).
52. R. A. Wallace, unpublished experiments.
53. M. Dixon and E. C. Webb, *Enzymes* (Academic Press, New York, 1958).
54. J. P. Wolf III and C. Niemann, *J. Am. Chem. Soc.* **81**, 1012 (1959).
55. J. P. Wolf III, R. A. Wallace, R. L. Peterson, unpublished experiments.
56. F. J. Kézdy and M. L. Bender, *Biochemistry* **1 (1962)**, 1097 (1962).
57. J. P. Wolf III, thesis, California Institute of Technology (1959).
58. H. R. Almond, Jr., and C. Niemann, *Biochim. Biophys. Acta* **44**, 143 (1960).
59. R. A. Wallace, A. N. Kurtz, C. Niemann, *Biochemistry* **2 (1963)**, 824 (1963).
60. N. M. Green and H. Neurath, in *The Proteins*, H. Neurath and K. C. Bailey, Eds. (Academic Press, New York, 1954), vol. 2, pt. B, pp. 1111–1115; C. Hamilton, unpublished experiments.
61. D. S. Hogness and C. Niemann, *J. Am. Chem. Soc.* **75**, 884 (1953).
62. J. S. Friedenwald and G. D. Maengwyn-Davies, in *Symposium on the Mechanism of Enzyme Action*, W. D. McElroy and H. B. Glass, Eds. (Johns Hopkins Press, Baltimore, 1954).
63. K. L. Laidler, *The Chemical Kinetics of Enzyme Action* (Oxford Univ. Press, New York, 1958), chap. 5.
64. A. Platt and C. Niemann, *Proc. Natl. Acad. Sci. U.S.* **50**, 817 (1963).
65. H. Waite, unpublished experiments.
66. H. J. Shine and C. Niemann, *J. Am. Chem. Soc.* **77**, 4275 (1955); R. A. Bernhard and C. Niemann, *ibid.* **79**, 4085 (1957); R. J. Kerr and C. Niemann, *ibid.* **80**, 1469 (1958).
67. A. N. Kurtz and C. Niemann, *Biochemistry* **1 (1962)**, 238 (1962).
68. R. B. Martin and C. Niemann, *J. Am. Chem. Soc.* **79**, 4814 (1957); *ibid.* **80**, 1481 (1958).
69. D. E. Koshland, Jr., and E. B. Herr, Jr., *J. Biol. Chem.* **228**, 1021 (1957); M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.* **82**, 3336 (1960).
70. G. E. Clement and M. L. Bender, *Biochemistry* **2 (1963)**, 836 (1963).
71. S. Kaufman and H. Neurath, *J. Biol. Chem.* **180**, 181 (1949).
72. M. L. Barnard and K. J. Laidler, *J. Am. Chem. Soc.* **74**, 6099 (1952).
73. R. B. Stein and K. J. Laidler, *Can. J. Chem.* **37**, 1272 (1959).
74. J. L. Miles, E. Morey, F. Crain, S. Gross, J. San Julian, W. J. Canady, *J. Biol. Chem.* **237**, 1319 (1962); J. L. Miles, D. A. Robinson, W. L. Canady, *Federation Proc.* **21**, 231 (1962).
75. W. Jackson and D. W. Thomas, unpublished experiments.
76. S. G. Cohen, J. Crossley, E. Khedouri, R. Zand, *J. Am. Chem. Soc.* **84**, 4163 (1962).
77. J. R. Rapp and C. Niemann, *ibid.* **85**, 1896 (1963).
78. L. Pasteur, *Compt. Rend.* **46**, 615 (1858); E. Fischer, *Chem. Ber.* **27**, 2985 (1894).
79. D. T. Manning and C. Niemann, *J. Am. Chem. Soc.* **80**, 1478 (1958); **81**, 747 (1959).
80. F. Sanger and H. Tuppy, *Biochem. J.* **49**, 481 (1951); F. Sanger and E. O. P. Thompson, *ibid.* **53**, 366 (1953); R. D. Shepherd, S. C. Willson, K. S. Howard, P. H. Ball, D. S. Davies, S. B. Davies, E. A. Eigner, N. E. Shakespeare, *J. Am. Chem. Soc.* **78**, 5067 (1956); H. H. Bromer, L. G. Sinu, O. K. Behrens, *ibid.* **79**, 2798 (1957); C. H. W. Hirs, *J. Biol. Chem.* **235**, 625 (1960); R. J. Hill and W. Konigsberg, *ibid.* **237**, 3151 (1962); F. A. Anderer, *Z. Naturforsch.* **17B**, 576 (1962); G. Braunitzer, V. Rudloff, N. Hilschmann, *Z. Physiol. Chem.* **331**, 1 (1963); W. A. Schroeder, J. R. Shelton, J. B. Shelton, J. Cormick, R. T. Jones, *Biochemistry* **2 (1963)**, 992 (1963).
81. J. Leonis, C. H. Li, D. Chung, *J. Am. Chem. Soc.* **81**, 419 (1959).
82. J. B. Jones, T. Kunitake, C. Hamilton, unpublished experiments.
83. H. J. Shine and C. Niemann, *J. Am. Chem. Soc.* **74**, 97 (1952); R. J. Foster, H. J. Shine, C. Niemann, *ibid.* **77**, 2378 (1955).
84. R. L. Bixler and C. Niemann, *ibid.* **80**, 2716 (1958); A. N. Kurtz and C. Niemann, *ibid.* **83**, 1879 (1961).
85. G. Hein and C. Niemann, *Proc. Natl. Acad. Sci. U.S.* **47**, 1341 (1961).
86. H. R. Waite and C. Niemann, *Biochemistry* **1 (1962)**, 250 (1962).
87. J. P. Wolf III and C. Niemann, *ibid.* **2 (1963)**, 82 (1963).
88. R. A. Zeller, *Ann. N.Y. Acad. Sci.* **107**, 811 (1963).
89. J. B. Jones and T. Kunitake, unpublished experiments.
90. R. P. Epaud and I. B. Wilson, *J. Biol. Chem.* **238**, 1718 (1963).
91. J. B. Jones and C. Niemann, *Biochemistry* **2 (1963)**, 498 (1963).
92. R. L. Peterson, K. Hubele, C. Niemann, *ibid.*, p. 942.
93. J. B. Jones and C. Niemann, *ibid.* **1 (1962)**, 1093 (1962).
94. H. I. Abrash and C. Niemann, *ibid.* **2 (1963)**, 947 (1963).
95. H. R. Almond, Jr., D. T. Manning, C. Niemann, *ibid.* **1 (1962)**, 243 (1962).
96. H. Neurath and B. S. Hartley, *J. Cellular Comp. Physiol.* **54**, suppl. 1, 179 (1959).
97. R. Lumry and H. Eyring, *J. Phys. Chem.* **58**, 110 (1954).
98. D. E. Koshland, Jr., in *The Enzymes*, P. D. Boyer, H. Lardy, K. Myrbäck, Eds. (Academic Press, New York, ed. 2, 1959), vol. 1, p. 305.
99. B. Labouesse, B. H. Havsteen, G. P. Hess, *Proc. Natl. Acad. Sci. U.S.* **48**, 2137 (1962).
100. H. Gutfreund and J. M. Sturdivant, *ibid.* **42**, 719 (1956); M. L. Bender, *Chem. Rev.* **60**, 53 (1960).
101. T. Spencer and J. M. Sturdivant, *J. Am. Chem. Soc.* **81**, 1874 (1959).
102. M. L. Bender and B. Zerner, *ibid.* **84**, 2550 (1962); M. L. Bender and G. A. Hamilton, *ibid.*, p. 2570; M. L. Bender and K. Nakamura, *ibid.*, p. 2577.
103. M. L. Bender, *ibid.* **84**, 2582 (1962).
104. B. Zerner and M. L. Bender, *ibid.* **85**, 356 (1963); M. L. Bender, G. E. Clement, F. J. Kézdy, B. Zerner, *ibid.*, p. 358.
105. M. Caplow and W. P. Jencks, *J. Biol. Chem.* **238**, PC 1907 (1963); R. M. Epand and I. B. Wilson, *ibid.*, p. PC 3138; M. Caplow and W. P. Jencks, *ibid.*, p. PC 3140; F. J. Kézdy, G. E. Clement, M. L. Bender, *ibid.*, p. PC 3141; M. L. Bender, G. E. Clement, C. R. Genter, F. J. Kézdy, *ibid.*, p. 3143.
106. E. Fischer, *Chem. Ber.* **39**, 530 (1906).