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The Current Status of the α-Chymotrypsin Mechanism[1]

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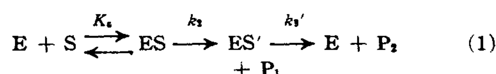
The evidence for the postulation of acyl–enzyme intermediates in α-chymotrypsin-catalyzed hydrolysis of specific ester, acid, and amide substrates is summarized. Individual rate constants determined from relative rate data, the effect of pH on the kinetics, alcoholysis data, and isotopic oxygen exchange data are compared. The over-all equilibrium constant of ester hydrolysis and other comparisons indicate the consistency of these data. The characteristics of the individual acylation and deacylation steps are discussed: (1) these steps may be characterized as nucleophilic reactions on the basis of studies on the effect of structure on reactivity; (2) two prototropic groups are seen kinetically in acylation, with pK_a's of 7 and 9, while one or two prototropic groups are seen kinetically in deacylation, one with a pK_a of 7 and the other a variable pK_a ranging from 8.3 to ∞; (3) the prototropic groups of acylation are tentatively identified as (two) imidazole(s) and an α-amino group of N-terminal isoleucine, respectively; (4) the prototropic groups of deacylation are tentatively identified as (two) imidazole group(s) and the nucleophile of deacylation, respectively; (5) the imidazole group(s) serve as catalyst for transferring protons in both acylation and deacylation on the basis of model studies and deuterium oxide isotope effects; (6) the serine hydroxyl group is acylated in the first step on the basis of present kinetic and spectrophotometric arguments and previous isolation studies; (7) the deacylation reaction is first order in water; and (8) no detectable intermediate may be observed in deacylation. The (intramolecular) deacylation of *m*-nitrobenzoyl-α-chymotrypsin is kinetically similar to the (intramolecular) nonenzymatic general base-catalyzed hydrolysis of *p*-nitrophenyl 5-nitrosalicylate. On the basis of the above data and the requirements of symmetry and microscopic reversibility, a mechanism for α-chymotrypsin catalysis is proposed embodied in eq. 10 and/or 11. This mechanism involving the formation and decomposition of tetrahedral addition intermediates in both acylation and deacylation describes the catalysis by imidazole(s) as involving both removal of a proton from the nucleophile (serine or water, respectively) and donation of a proton to the carbonyl oxygen atom. This cyclic process involving both imidazole group(s) is the one mechanism consistent with all known experimental data.

Introduction

The function of a mechanistic study is to identify the individual steps in a reaction and to describe the transition state of each step both stereochemically and electronically. Research in this laboratory has been pointed to the elucidation of the α-chymotrypsin mechanism using this frame of reference, and also the tacit assumption that there is a unique, discoverable, and describable mechanism which is related to the body of organic mechanistic theory.

The present paper attempts to summarize the facets of the preceding papers that bear on the α-chymotrypsin mechanism and thus to present the latest progress report in the continuing investigation of this problem. The progress since the last comprehensive installment[2,3] 2 years ago has not achieved its ultimate goal, but it has been finite, and justifies a further look at the problem of enzyme mechanism, as exemplified by α-chymotrypsin.

The Stepwise Process.—The acyl–enzyme mechanism which may be written as



$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3'} E + P_2 \quad (1)$$
$$+ P_1$$

where ES is the enzyme–substrate complex, ES′ is the acyl–enzyme, P_1 is the leaving group of the substrate, and P_2 is the carboxylate ion product, is consistent with a large body of data pertaining to nonspecific substrates[2] and also a large amount of data pertaining to specific substrates, presented in the present series of papers.

The direct evidence for the applicability of mechanism 1 to specific substrates includes: The acyl–enzyme intermediate has been demonstrated spectrophotometrically in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester at low pH.[4] A burst of *p*-nitrophenol has been demonstrated at low pH for the specific substrates N-acetyl-L-tryptophan *p*-nitrophenyl ester and N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester.[4] N-Acetyl-L-tryptophan reacts with α-chymotrypsin at low pH to form an appreciable equilibrium concentration of acyl-enzyme, as demonstrated spectrophotometrically and by the decrease in concentration of the normality of active sites in the solution.[4]

The indirect evidence for the application of mechanism 1 to the reactions of specific *ester* substrates includes: The catalytic rate constants of the α-chymotrypsin-catalyzed hydrolysis of the ethyl, methyl, and *p*-nitrophenyl esters of N-acetyl-L-tryptophan are essentially identical with one another, although the nucleophilic character of α-chymotrypsin reactions would predict at least a hundredfold difference in the relative rates of these substrates.[5] The pH dependence of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester can be quantitatively analyzed in terms of the bell-shaped and sigmoid pH–rate constant profiles of the individually determined acylation and deacylation steps, respectively, and the necessary change in rate-determining step which the mechanism and these profiles demand.[6] The kinetics of the α-chymotrypsin-catalyzed hydrolysis of specific substrates in the presence of added nucleophiles such as methanol may be successfully analyzed for all possible cases in terms of the competitive partitioning of acyl–enzyme intermediate by all the nucleophiles present in the system.[7]

(1) This research was supported by grants from the National Institutes of Health, the U. S. Atomic Energy Commission, and the National Science Foundation. Paper XXXII in the Series: The Mechanism of Action of Proteolytic Enzymes.

(2) M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962).

(3) *Cf.* M. L. Bender in "Mechanismen enzymatischer Reactionen," Springer Verlag, Berlin, 1964, p. 47.

(4) F. J. Kézdy, G. E. Clement, and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3690 (1964).

(5) B. Zerner, R. P. M. Bond, and M. L. Bender, *ibid.*, **86**, 3674 (1964).

(6) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *ibid.*, **86**, 3680 (1964).

(7) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kézdy, *ibid.*, **86**, 3697 (1964).

The indirect evidence for the application of mechanism 1 to the reactions of specific amide substrates includes the following. The rate of α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine amide and of N-acetyl-L-phenylalanine amide is not affected at all by 20% methanol.[7] Plots of k_{cat}/K_m(app) *vs.* pH for both N-acetyl-L-tryptophan amide and ethyl ester are identical in shape with one another, and also identical with the pH-k_2 profile of a discrete acylation step, indicating a common acylation process for all three substrates.[6] The synthesis of N-benzoyl-L-tyrosylglycylanilide from N-benzoyl-L-tyrosine and glycylanilide has been demonstrated.[8] Since α-chymotrypsin plus N-acetyl-L-tryptophan has been shown to produce N-acetyl-L-tryptophanyl-α-chymotrypsin,[4] N-benzoyl-L-tyrosine would be expected to form N-benzoyl-L-tyrosyl-α-chymotrypsin. Several acyl-enzymes have been shown to react with ammonia and amines such as hydroxylamine[7] and all acyl-enzymes would be expected to react with nucleophiles such as amines. Thus a good argument may be made for postulating that the pathway of the synthesis described above proceeds through the acyl-enzyme. The principle of microscopic reversibility demands that the reverse process must also be operative; thus the hydrolysis of an amide must proceed *via* an acyl-enzyme intermediate.

Rate Constants.—The hydrolysis of two esters of N-acetyl-L-tryptophan by α-chymotrypsin was analyzed by three different experimental approaches, resulting in the determination of the kinetic constants, k_2 and $k_3' = k_3[H_2O]$, in three independent ways.[5–7] A comparison of the results of these determinations is shown in Table I and shows a reasonable agreement between the three methods. The agreement of k_3' between the two esters is excellent, a necessary corollary of the acyl-enzyme hypothesis.[5] A variation of a factor of three in k_2 is found for the three values of the hydrolysis of the ethyl ester while a variation of about 40% in k_2 is found for the two values of the hydrolysis of the methyl ester. The authors believe that at this stage of the evolution of enzyme knowledge, enzyme purity, and enzyme kinetics, the agreement between the various methods of determination of the individual rate constants, k_2 and k_3', is satisfying indeed.

TABLE I

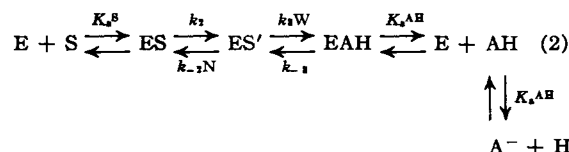
LIMITING RATE AND BINDING CONSTANTS FOR α-CHYMOTRYPSIN-CATALYZED REACTIONS OF N-ACETYL-L-TRYPTOPHAN DERIVATIVES[a,d]

Source	k_2, sec.$^{-1}$	k_3', sec.$^{-1}$	k_{-2}, M^{-1} sec.$^{-1}$	k_{-3}, sec.$^{-1}$	K_s, mM	Ref.
Ethyl ester						
Relative rate data	1120	48.5			2.52	5
pH data	1818	46.5			4.1	6
Ethanolysis data	627	51	54.5[b]		1.38	7
Oxygen-18 exchange data				323		4
Methyl ester						
Relative rate data	1160	48.5				5
Methanolysis data	857	45	32.5[c]		1.18	7
Oxygen-18 exchange data				323		4

[a] 0.8% (v./v.) acetonitrile–water. Data obtained at pH 7.00 or 7.65 were converted to limiting rate constants by using the equation $k_0 = k(1 + H/K)$ where K was taken to be 6.77 for k_2 and 6.86 for k_3 and k_4.[5] [b] Ethanolysis of the acyl–enzyme (denoted as k_4 previously[6]). [c] Methanolysis of the acyl–enzyme (denoted as k_4 previously[6]). [d] Errors in individual constants are described in the appropriate reference; errors tend to accumulate in the calculated k_2 values.

Table I also shows the comparison of k_2 and k_{-3}, reflecting the reaction of α-chymotrypsin with two N-acetyl-L-tryptophan esters and with N-acetyl-L-tryptophan. The ratio k_2/k_{-3} is ~3 for the methyl ester:acid ratio and ~4 for the ethyl ester:acid ratio. Comparable data for nonenzymatic reactions, from a comparison of the rates of the acid-catalyzed isotopic oxygen exchange of benzoic acids and the rates of the acid-catalyzed hydrolysis of the corresponding ethyl esters,[9] indicates that the ethyl ester:acid ratio varies from 2.2 to 3.4. The similarity of these ratios suggests that the mechanism of nonenzymatic acid-catalyzed reactions and enzyme-catalyzed reactions are quite similar to one another.

Equilibrium Constants.—The set of rate constants k_2, k_3', k_{-2}, and k_{-3} given in Table I are sufficient to define the kinetic parameters of the entire reversible ester hydrolysis, according to eq. 2, which is similar to



eq. 1, with the additional features that the carboxylic acid-enzyme product complex (EAH) is specified, from which the acid is dissociated and finally ionized. Also specified is step k_{-2}N, the alcoholysis of the acyl-enzyme, and step k_{-3}, the acylation of the enzyme by the free acid.

The enzyme-catalyzed ester hydrolysis must of course have the same over-all equilibrium constant as that of the acid-catalyzed reaction, or for that matter the uncatalyzed reactions.[10] Since, at equilibrium, each individual step must be in equilibrium,[10] the following equation may be written for the enzyme-catalyzed reaction.[11]

$$K = \frac{E_e S_e}{ES_e} \times \frac{ES_e}{N_e ES_e'} \times \frac{W_e ES_e'}{EAH_e} \times \frac{EAH_e}{E_e AH_e}$$
$$= (S_e \times W_e)/(AH_e \times N_e)$$
$$= (K_s^{S} \times k_{-2}k_{-3})/(K_s^{AH}k_2k_3) \qquad (3)$$

The equilibrium constant of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-phenylalanine methyl ester was measured at pH's varying from 1.79 to 4.82.[12a] A value of the equilibrium constant as de-

(8) O. Gawron, A. J. Glaid, III, R. E. Boyle, and G. Odstrchel, *Arch. Biochem. Biophys.*, **95**, 203 (1961).

(9) M. L. Bender, R. R. Stone, and R. S. Dewey, *J. Am. Chem. Soc.*, **78**, 319 (1956). A comparison of the aminolysis of esters (W. P. Jencks and J. Carriuolo, *ibid.*, **82**, 677 (1960)) and the aminolysis of acids (H. Morawetz and P. S. Otaki, *ibid.*, **85**, 463 (1963)), reactions which are hydroxide-ion catalyzed, leads to a similar conclusion.

(10) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1961, p. 211.

(11) *Cf.* J. B. S. Haldane, "Enzymes," Longmans Green and Co., London, 1930.

(12) (a) Unpublished experiments of Mr. P. D. Meyer in this laboratory; (b) H. M. Trimble and E. L. Richardson, *J. Am. Chem. Soc.*, **62**, 1018 (1940); recalculated from data reported in G. S. Parks and H. M. Huffman, "The Free Energies of Some Organic Compounds," The Chemical Catalog Co. (Reinhold Publishing Corp.), New York, N. Y., 1932, p. 177.

fined by eq. 3 of 5.46 ± 0.11 was found, which value is very close to the equilibrium constant of the acid-catalyzed esterification of many aliphatic esters of aliphatic acids as reported in the literature. Typical values of the equilibrium constant at 30° as defined above are ethyl acetate, 4.09; *n*-amyl acetate, 4.91; ethyl butyrate, 5.34; and *n*-amyl butyrate, 4.91.[12b]

We may calculate the equilibrium constant for the α-chymotrypsin-catalyzed hydrolyses of the methyl and ethyl esters of N-acetyl-L-tryptophan to test the validity and consistency of the individual constants of Table I, most of which have been determined independently.

1. **N-Acetyl-L-tryptophan Methyl Ester.**—The most reliable value for k_3' is 46.5 sec.$^{-1}$, the value of the ethyl ester and the average value of the methyl ester; since $k_3' = k_3[H_2O]$, $k_3 = 0.838\ M^{-1}$ sec.$^{-1}$. From the oxygen exchange data of N-acetyl-L-tryptophan, $k_{-3}/K_s^{AH} = 2.74 \times 10^5\ M^{-1}$ sec.$^{-1}$,[4] or $2.28 \times 10^5\ M^{-1}$ sec.$^{-1}$ in 0.8% acetonitrile–water.[13] The values of k_2, k_{-2}, and K_s are those listed in Table I from the treatment of the methanolysis data,[7] and were selected in order to have a self-consistent set of values. Thus

$$K_{\text{equil}} = \frac{(1.18 \times 10^{-3})(32.5)(2.38 \times 10^5)}{(857)(0.838)} = 12.2$$

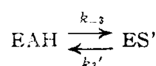
If the k_2 and K_s determined from the relative rate data are used, a similar value of the equilibrium constant is found. In view of the varied origin of the individual constants, the above value of the equilibrium constant is in reasonable agreement with the α-chymotrypsin-catalyzed equilibrium mentioned above and with the majority of ester hydrolysis equilibria recorded in the literature.

2. **N-Acetyl-L-tryptophan Ethyl Ester.**—For this calculation, the same values of k_3 and k_{-3}/K_s^{AH} were used as those listed above. The values of k_2, k_{-2}, and K_s are those listed in Table I from the treatment of the ethanolysis data, again selected in order to have a self-consistent set. Thus

$$K_{\text{equil}} = \frac{(1.38 \times 10^{-3})(54.5)(2.28 \times 10^5)}{(627)(0.838)} = 32.6$$

Although there is a considerable variation in the values of K_m and k_2 for this compound in Table I, the errors in these constants are probably compensatory, since another self-consistent set of these constants gives essentially the same equilibrium constant as that above. However, the k_{-2} for ethanolysis of the acyl-enzyme is larger than the k_{-2} for the methanolysis, in contradiction to all other known nucleophilic reactions at the carboxylic acid group, in which methanol is the more reactive of the two nucleophiles, including the reactions of *trans*-cinnamoyl-α-chymotrypsin.[14] Therefore it is not surprising that the equilibrium constant of the ethyl ester is higher than the expected value.

A final observation concerning the data of Table I concerns the equilibrium



$$\text{EAH} \underset{k_3'}{\overset{k_{-3}}{\rightleftharpoons}} \text{ES'}$$

This reaction can be profitably compared to a hydroxy acid–lactone equilibrium. The equilibrium constant (lactone)/(hydroxy acid) may be equated to ES'/EAH since the numerator is an ester formed in an intracomplex reaction while the denominator contains the free acid and alcohol forms in a suitable complex. The equilibrium constant (k_{-3}/k_3'), equal to 6.95, may be compared with the equilibrium values for the lactonization of γ-hydroxybutyric acid, 2.66, and that for the lactonization of γ-hydroxyvaleric acid, 13.86.[15] Thus the acyl-enzyme has the characteristics of a strainless five-membered ring.

The Scope and Limitations of the Acyl-Enzyme Mechanism.—Data presented in the previous papers of this series strongly indicate that an acyl-enzyme intermediate is formed in all reactions catalyzed by α-chymotrypsin. Two questions will be raised here: (1) is the acyl-enzyme intermediate unique to α-chymotrypsin reactions; and (2) is there any evidence pertaining to α-chymotrypsin reactions which is not in accord with such an intermediate?

The formation of an acyl-enzyme intermediate is a wide-spread phenomenon in the enzyme-catalyzed reactions of carboxylic acid derivatives, including reactions catalyzed by acetylcholinesterase,[16,17] trypsin,[18] elastase,[19] and papain.[20] The question may still be raised as to whether such intermediates are a necessary part of the pathway of the reaction of every substrate of every one of these enzymes, or more particularly whether acyl-enzyme intermediates are a necessary part of the pathway of the reaction of every substrate of α-chymotrypsin. If an acyl-enzyme intermediate can be demonstrated in the α-chymotrypsin-catalyzed reactions of a number of representative families of substrates, such as *p*-nitrophenyl esters, ethyl esters, acids, and amides, particularly of specific substrates, the reasonable conclusion can be reached that the intermediate is a general one. This has been the approach of the preceding papers, and the evidence mentioned before is reasonably complete, although not absolute.

Criticism of assigning mechanism to all reactions of α-chymotrypsin can still be given, at two levels. One criticism is to question the assignment of any discrete mechanism to reactions catalyzed by α-chymotrypsin. This criticism must be answered in a philosophical vein: if a compound has a discrete structure, its reactions must have a discrete mechanism. This statement does not require a unique mechanism, for many compounds exhibit multiple mechanisms of reaction; however, the number of mechanisms must be small and finite. Another kind of criticism is to point out that the present evidence is not all inclusive nor is all the available evidence positive. Certainly the reaction of no peptide, polypeptide, or protein with α-chymotrypsin has been investigated. One may say that these materials are the only real substrates of α-chymotrypsin and that all the preceding discussion is therefore irrelevant. However, since the α-chymo-

(13) Corrected for the presence of acetonitrile in the solution by means of the data of G. E. Clement and M. L. Bender, *Biochemistry*, **2**, 836 (1963).

(14) Unpublished observations of Mr. C. R. Gunter in this laboratory.

(15) A. Kailan, *Z. physik. Chem.*, **101**, 86 (1922).

(16) I. B. Wilson in "The Enzymes," P. D. Boyer, H. Lardy, and K. Myrback, Ed., 2nd Ed., Academic Press, Inc., New York, N. Y., 1960, Chapter 30.

(17) Unpublished observations of Mr. J. K. Stoops in this laboratory.

(18) M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, **84**, 2556 (1962), and references cited therein.

(19) Unpublished observations of Mr. T. H. Marshall in this laboratory.

(20) Unpublished observations of Mr. L. J. Brubacher in this laboratory.

trypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester is one thousand times faster than that of N-acetyl-L-tryptophan amide, it must be concluded from a kinetic standpoint that the former is the specific substrate of α-chymotrypsin, and thus we have indeed studied the most pertinent classes of compounds.

The difficulties of the acyl-enzyme hypothesis in incorporating the experimental data concerning the kinetics and partitionings in α-chymotrypsin-catalyzed reactions involving hydroxylamine and the enthusiasms of others for these data have been recounted earlier.[7] The present authors have a few lingering doubts: (1) Can the 10% difference between the partitioning of N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosinehydroxamic acid by hydroxylamine and water at equivalent enzyme concentration[21] be due to something other than the experimental difficulty of the observations? (2) How experimentally significant is the observation that in the α-chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis of N-acetyl-L-tyrosine ethyl ester the $K_s^{NH_2OH}$ is twice that of the $K_s^{H_2O}$ and the former is independent of hydroxylamine concentration?[22] (3) Is the dependency of the α-chymotrypsin-catalyzed reaction of N-acetyl-L-tyrosine with hydroxylamine on hydroxylamine concentration[21] consistent with the acyl-enzyme hypothesis? and (4) Can the data on the hydroxylaminolysis of methyl hippurate[22] be reconciled with the relative rate data of alkyl hippurates?[23] These doubts do not permit the unequivocal statement that the acyl-enzyme mechanism is completely proved. However, the present authors do not give much weight to these experiments with hydroxylamine for several reasons: (1) the proven complications of hydroxylamine reactions[24] and the possibilities of other unforeseen complications; (2) the indirect nature of the arguments; and (3) the possibility that the introduction of hydroxylamine may perturb the normal enzymatic mechanism.

The thermodynamic barrier to ester formation from relatively "low energy" amides and hydroxamides has been used as an argument to exclude the possibility of acyl-enzyme formation with amide and hydroxamide substrates and thus to view the acyl-enzyme as a scavenger for "energy-rich" acyl groups.[25] However, *discussion of mechanism must include kinetic concepts,* including the possibility of kinetic rather than thermodynamic control of a reaction.[26] For example, carboxylate ion is thermodynamically more stable than the corresponding methyl ester, but carboxylic acid derivatives react faster with methanol than with water (and faster with methoxide ion than with hydroxide ion)! This kinetic control is manifest both in nonenzymatic reactions[7] and also in the deacylation of *trans*-cinnamoyl-α-chymotrypsin with methanol-water solutions[7] as well as in all other known enzymatic deacylation reactions. Since the pathway of deacylation is kinetically rather than thermodynamically controlled, acylation should likewise be kinetically rather than thermodynamically controlled. Thus, an α-chymotrypsin-amide complex should react preferentially with the serine hydroxyl group to give the acyl-enzyme because of kinetic control, rather than react with a water molecule because of thermodynamic control.

Some unsolved problems include questions of multiple pathways or acyl-enzyme intermediates which may be blind alleys. However, the present uncertainties in defining the steps of α-chymotrypsin-catalyzed reactions are essentially no different from the uncertainties encountered in a consideration of the pathways of simple organic reactions, and it is therefore possible to proceed to a consideration of the individual steps of the enzymatic catalysis.

Characteristics of the Individual Steps. Nucleophilic Order of Reactivity.—In order to elucidate completely the mechanism of chymotrypsin action, the mechanisitic characteristics of each of the individual steps of the reaction must be probed. Those steps most amenable to investigation are those involving bond making and bond breaking, that is, the acylation and deacylation steps. These steps may be characterized first of all as nucleophilic reactions on the basis of studies on the effect of structure on reactivity. By minimizing differences in classical specificity and by introducing structural changes into the substrate that will result only in electronic effects on reactivity, mechanistic conclusions can be drawn concerning the acylation and deacylation steps. It has been found that the introduction of electron-withdrawing substituents into the substrate results in the facilitation of both the acylation[27] and deacylation[28] reactions. From the magnitude of this facilitation, it appears that the transition states of both reactions are similar and that both resemble the reactions of nucleophiles such as hydroxide ion with the corresponding substrates.

The Enzymatic Group Which Is Acylated.—Several pieces of evidence suggest the hydroxyl group of a serine moiety as the group which is acylated: (1) isolation experiments in which it is found that a dialkylphosphoryl or an acetyl group resides on the hydroxyl group of a serine moiety, after exhaustive hydrolysis to serine phosphate and/or after partial enzymatic hydrolysis to peptides containing the dialkylphosphoryl or acetyl group; (2) the spectrum of *trans*-cinnamoyl-α-chymotrypsin more closely resembles an ester of serine than any other possible species[2] and is almost identical with that of *trans*-cinnamoyl-N-acetylserineamide when the tertiary structure is disrupted by heating to 51°[29]; furthermore, the spectrum of furoyl-α-chymotrypsin closely resembles that of a furoyl ester[28]; (3) the rate of the nonenzymatic (alkaline) hydrolyses of *trans*-cinnamoyl-α-chymotrypsin and acetyl-α-chymotrypsin in 8 *M* urea is almost identical with the rate of alkaline hydrolysis of O-cinnamoyl-N-acetylserineamide[2] and N,O-diacetylserineamide,[30] re-

(21) M. Caplow and W. P. Jencks, *J. Biol. Chem.*, **238**, PC 3140 (1963); **239**, 1640 (1964).

(22) S. A. Bernhard, personal communication; S. A. Bernhard, W. C. Coles, and J. F. Nowell, *J. Am. Chem. Soc.*, **82**, 3043 (1960).

(23) R. M. Epand and I. B. Wilson, *J. Biol. Chem.*, **238**, 1718 (1963); personal communication from Professor I. B. Wilson indicates that the experimental data of ref. 22 is subject to revision.

(24) F. J. Kézdy, G. E. Clement, and M. L. Bender, *J. Biol. Chem.*, **238**, PC 3141 (1963).

(25) W. P. Jencks, *Ann. Rev. Biochem.*, **32**, 648 (1963).

(26) M. L. Bender, in "Technique of Organic Chemistry," 2nd Ed., Vol. 8, Part II, A. Weissberger, Ed., John Wiley and Sons, Inc., New York, N. Y., 1963, Chapter 25.

(27) M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962).

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

(29) J. Mercouroff and G. P. Hess, *Biochem. Biophys. Res. Commun.*, **11**, 283 (1963).

(30) B. M. Anderson, E. H. Cordes, and W. P. Jencks, *J. Biol. Chem.*, **236**, 455 (1961).

spectively; (4) the equilibrium constant k_{-3}/k_3' (*vide supra*) is that of a normal hydroxy acid–lactone equilibrium; and (5) the rate constant for the ethanolysis of N-acetyl-L-tryptophanyl-α-chymotrypsin is 54.5 M^{-1} sec.$^{-1}$ while the rate constant of the reaction of N-acetyl-L-tryptophan ethyl ester with the presumed serine hydroxyl of chymotrypsin (step k_2) is *ca.* 1000 sec.$^{-1}$; thus it would take ~20 M ethanol for the rate of the former second-order process to equal the rate of the latter first-order process, a relationship found frequently in the nonenzymatic hydrolysis of esters[31]; (6) an inactive monotosylated enzyme was converted *via* an elimination reaction to an inactive "anhydro" enzyme; the lack of activity of this modified serine residue indicates that the hydroxyl group of serine plays a positive role of acyl acceptor, and not a negative role.[32] Each of these arguments alone may be criticized, but when taken together they identify the group acylated as a serine hydroxyl group.

Ionizable Groups Involved in Acylation and Deacylation.—The pH dependence of α-chymotrypsin-catalyzed reactions indicate that several kinetically important ionizable groups must be considered.[4,6] One of these groups is operative in both acylation and deacylation reactions, a group with apparent pK_a of ~7. A second group of apparent pK_a ~9 is seen in acylation while a second-order group of variable pK_a is sometimes seen in deacylation. The identity and function of these groups will be discussed in turn.

The kinetic importance of the ionizable group of pK_a ~7 in its basic form has been noted by many workers and it is usually identified as the imidazole group of a histidine moiety of the enzyme. Imidazole is the only group on the enzyme with a pK_a around neutrality with the exception of the terminal α-ammonium ion. It must, however, be kept in mind that the kinetically determined pK_a is only an apparent constant which may be perturbed by a pre-equilibrium occurring before the rate-determining step. However, other lines of evidence support the supposition that imidazole is involved in the catalytic function of the enzyme. For example, when chymotrypsin was photooxidized in the presence of methylene blue, a decrease in activity occurred, eventually leading to complete inactivation after 4 moles of oxygen had been absorbed per mole of enzyme. From analysis of the resulting inactivated protein, it was found that 1 mole of histidine and 2.4 moles of tryptophan were oxidized.[33] A more recent photooxidation study, which was carried out using both rate and "all or none" assays, indicates that a histidine residue and a methionine residue[34] are photooxidized as inactivation occurs.[37] Furthermore, the compound L-1-tosylamido-2-phenylethyl chloromethyl ketone inactivates chymotrypsin, the haloketone moiety alkylating the enzyme. The inhibited enzyme shows on analysis the loss of a histidine residue[38]; a decapeptide containing the alkylated group is identical in composition with the known histidine-containing decapeptide. Finally, model studies indicate that imidazole is indeed an efficient catalyst of ester hydrolysis around neutrality.[39] In sum total, the evidence for the participation of an imidazole group of a histidine moiety in chymotrypsin catalysis seems to rest on firm ground.

Recent evidence indicates that the two histidine residues in chymotrypsin are very close in space to one another. The relationship found is -His-Phe-Cys-S-S-Cys-His-.[40] This fact would not be significant in itself, but the identical sequence is also found for two histidines in trypsin![41] In view of the many mechanistic similarities between chymotrypsin and trypsin it must be concluded that the finding of these identical sequences containing two histidines in close proximity must be mechanistically meaningful.[40,41]

The acidic group of pK_a ~9, seen solely in acylation, is more difficult to identify. The best evidence at the moment indicates that it is the α-ammonium group of the N-terminal isoleucine residue.[42] This conclusion is based on evidence involving a fully acetylated chymotrypsinogen molecule. Activation of this acetylated zymogen gave an acetylated δ-chymotrypsin containing one free amino group identified as the α-ammonium group of the N-terminal isoleucine residue; when this group is acetylated the enzyme is inactivated. Significantly, one may titrate this prototropic group in the acetylated δ-chymotrypsin (and thus it should be seen in acylation), but not in the DIP-acetylated δ-chymotrypsin (and thus it should not be present in deacylation). The reason for its disappearance in the acyl-(phosphoryl-) enzyme must be determined. Alternatively, the acidic group of pK_a 9 may be identified as the hydroxyl group of serine, since this group is present in acylation but not in deacylation.[43] However, the pK_a of a model serine hydroxyl group is 13.5,[44] and thus one must invoke some special characteristics for this hydroxyl group, which, significantly, are not seen when the similar hydroxylic compound, methanol, is used as the nucleophile (in the deacylation step). The effect of pH on the liberation of protons in the acylation reaction could in theory distinguish between these two possibilities. Unfortunately, the present evidence on this point is equivocal.[45-47]

(31) M. L. Bender, *Chem. Rev.*, **60**, 53 (1960).

(32) D. H. Strumeyer, W. N. White, and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U. S.*, **50**, 931 (1963).

(33) L. Weil and A. R. Buchert, *Federation Proc.*, **11**, 307 (1952).

(34) Other experiments indicate that the modification of a methionine residue near the active site leads to inhibition of enzyme acticity.[35,36] However, no evidence demands that the thiomethyl group of methionine participates in the catalytic action. Whereas an imidazole group will catalyze ester hydrolysis in a number of model systems, thioethers are not known to catalyze such hydrolyses. Whereas photooxidation of the imidazole group leads to an inert enzyme, photooxidation of the methionine residue leads to a partially active enzyme, indicating that the former but not the latter group is essential to chymotrypsin action.

(35) G. Gundlach and F. Turba, *Biochem. Z.*, **335**, 573 (1962).

(36) W. B. Lawson and H. J. Schramm, *J. Am. Chem. Soc.*, **84**, 2017 (1962).

(37) D. E. Koshland, Jr., D. H. Strumeyer, and W. J. Ray, Jr., *Brookhaven Symp. Biol.*, **15**, 101 (1962).

(38) G. Schoellman and E. Shaw, *Federation Proc.*, **21**, 232 (1962); *Biochem. Biophys. Res. Commun.*, **7**, 36 (1962); *Biochemistry*, **2**, 252 (1963); E. B. Ong, E. Shaw, and G. Schoellmann, *J. Am. Chem. Soc.*, **86**, 1271 (1964).

(39) M. L. Bender and B. W. Turnquest, *ibid.*, **79**, 1652, 1656 (1957); T. C. Bruice and G. L. Schmir, *ibid.*, **79**, 1663 (1957); W. P. Jencks and J. Carriuolo, *ibid.*, **83**, 1743 (1961).

(40) J. R. Brown and B. S. Hartley, *Biochem. J.*, **89**, 59P (1963); B. S. Hartley, *Nature*, **201**, 1284 (1964); B. Keil, Z. Prusik, and F. Sorm, *Biochim. Biophys. Acta*, **78**, 559 (1963).

(41) K. A. Walsh, D. L. Kauffman, K. S. V. S. Kumar, and H. Neurath, *Proc. Natl. Acad. Sci. U. S.*, **51**, 301 (1964).

(42) B. Labouesse, H. L. Oppenheimer, and G. P. Hess, *Biochem. Biophys. Res. Commun.*, **14**, 318 (1964); H. L. Oppenheimer, B. Labouesse, K. Carleson, and G. P. Hess, *Federation Proc.*, **23**, 315 (1964).

(43) The authors acknowledge this suggestion of Professor W. P. Jencks.

(44) T. C. Bruice, T. H. Fife, J. J. Bruno, and N. C. Brandon, *Biochemistry*, **1**, 7 (1962).

(45) B. F. Erlanger, H. Castleman, and A. G. Cooper, *J. Am. Chem. Soc.*, **85**, 1872 (1963).

The deacylation of both specific and nonspecific acyl-enzymes with water[6] and methanol[7] as nucleophiles has a sigmoid pH–k_3 profile which may be analyzed in terms of sole dependence of the enzymatic reaction on a base with a pK_a of ~7. However, deacylation reactions have been carried out using nucleophiles other than water or methanol. Three interesting deacylations involve isonitrosoacetone,[48] glycinehydroxamic acid, and phenylacetohydroxamic acid[49] as nucleophiles. These deacylations which involve methylisopropoxyphosphonyl-α-chymotrypsin and diethylphosphoryl-α-chymotrypsin, do not exhibit sigmoid pH–k_4 (deacylation) curves, but rather bell-shaped curves! The first reaction is quantitatively dependent on a base of pK_a 7 and on an acid of pK_a 8.3; the base of pK_a 7 is presumably the ubiquitous imidazole while the acid of pK_a 8.3 corresponds exactly in ionization constant to the nucleophile. Thus, this deacylation requires imidazole as a base and isonitrosoacetone as the un-ionized acid. The latter two deacylations may be qualitatively analyzed in the same terms, dependency on imidazole as a base and dependency on the nucleophiles as un-ionized acids (of pK_a 7.7 and 9.18, respectively). Thus all present deacylation pH data are consistent with the postulate that the *nucleophile must be in its protonated (or un-ionized) form.* In reality, the sigmoid pH–k_3 profile, observed from pH 2 to 13, implies a bell-shaped curve since the rate law may be written as $V = k_3(E_0)(H_2O)$ where E_0 is the enzyme molecule containing an unprotonated imidazole moiety, implying that when H_2O is ionized to OH^-, the reaction no longer occurs. Since water (and methanol) does not ionize in the observable enzymatic region, one cannot see the right-hand leg of the bell-shaped curve of deacylations by these nucleophiles. However, if a nucleophile is used which has an ionization constant below pH 12, as do those cited above, a bell-shaped curve, one of whose legs reflects the ionization of the nucleophile, should be observable in deacylation.[50] This discussion implies that the protonated (un-ionized) form of the serine hydroxyl group is involved in the acylation reaction.

Functions of the Groups of the Active Site.—In the previous sections, tentative identification of three component groups of the enzymatic active site have been given: the hydroxyl group of a serine moiety, the imidazole group(s) of histidine moiety(ies), and the α-ammonium ion of the N-terminal isoleucine moiety of the enzyme. The important question, of course, is not what these groups are, but rather what they do. The role for the serine hydroxyl group has already been specified as that group which is acylated by the substrate in the reaction described by eq. 1. The roles of the other two components of the active site will now be discussed.

The acylation reaction may be viewed as the microscopic reverse of the deacylation reaction. This is evident since the deacylation of N-acetyl-L-tryptophanyl-α-chymotrypsin and its microscopic reverse, the acylation of α-chymotrypsin by N-acetyl-L-tryptophan, have been demonstrated.[4] Other acylation and deacylation reactions which are the microscopic reverse of one another and which have been demonstrated include: (1) the acylation of α-chymotrypsin by N-acetyl-L-tryptophan methyl ester[4,5] and the methanolysis of N-acetyl-L-tryptophanyl-α-chymotrypsin[7]; (2) the acylation of α-chymotrypsin by N-acetyl-L-tryptophan ethyl ester and the ethanolysis of N-acetyl-L-tryptophanyl-α-chymotrypsin[5,7]; (3) the acylation of α-chymotrypsin by N-acetyl-L-phenylalanine methyl ester and the methanolysis of N-acetyl-L-phenylalanyl-α-chymotrypsin[5,7,51]; (4) the acylation of α-chymotrypsin by methyl cinnamate[52] and the methanolysis of *trans*-cinnamoyl-α-chymotrypsin.[7] These experiments based on direct spectrophotometric observations, isotopic exchange experiments, and kinetic analyses demand that the catalytic mechanisms of acylation and deacylation must be the microscopic reverse of one another. This dictum of course means that a catalytic group operative in acylation must also be operative in deacylation, a requirement which is satisfied by the imidazole moiety(ies) seen kinetically in both acylation and deacylation.

However, the α-ammonium ion of N-terminal isoleucine is seen kinetically in acylation, but not in deacylation. This group thus cannot be identified as a catalytic moiety, but must be identified as (1) the enzymatic group which is acylated or (2) a group responsible for conformational stabilization of the active site in acylation, but unnecessary in deacylation because of the covalent link of the acyl-enzyme.[53] The first possibility has already been ruled out by the identification of the serine hydroxyl group as the enzymatic group which is acylated. The second possibility implies that the conformation of the enzyme is pH dependent.

Evidence of the pH dependence of the enzyme conformation is seen in the pH dependence of the specific rotation of α-chymotrypsin[54,55] and in the pH dependence of the Moffitt a_0 parameter of α-chymotrypsin.[56] A crude analysis of the pH dependence of the specific rotation indicates that the rotation may be dependent on a carboxylate ion in its basic form and an ammonium ion in its acidic form, leading to the tentative hypothesis shown in Fig. 1 of a conformationally important electrostatic interaction between these two groups. This hypothesis is supported by (1) the observation of a time-dependent and pH-dependent reversible transformation of α-chymotrypsin from an inactive to an active form of the enzyme[57]; (2) the pH dependence of the specific rotation of acetylated δ-chymotrypsin which may be correlated with the ionization of the N-terminal ammonium ion of isoleucine[58]; (3) the pH dependence of the ultraviolet absorption spectrum of α-chymotrypsin[57]; and (4) the effect of pH dependence on the sedimentation coefficient of α-chymotrypsin.[59,60]

(46) D. E. Fahrney and A. M. Gold, *J. Am. Chem. Soc.*, **85**, 349 (1963).
(47) S. A. Bernhard, *Brookhaven Symp. Biol.*, **15**, 129 (1963).
(48) A. L. Green and J. D. Nicholls, *Biochem. J.*, **72**, 70 (1959).
(49) W. Cohen and B. F. Erlanger, *J. Am. Chem. Soc.*, **82**, 3928 (1960). *Cf.* W. Cohen, M. Lache, and B. F. Erlanger, *Biochemistry*, **1**, 686 (1962).
(50) This postulate is currently being tested further in this laboratory.
(51) M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960).
(52) M. L. Bender and B. Zerner, *ibid.*, **84**, 2550 (1962).
(53) M. L. Bender, G. E. Clement, F. J. Kézdy, and B. Zerner, *ibid.*, **85**, 357 (1963).
(54) H. Neurath, J. A. Rupley, and W. J. Dreyer, *Arch. Biochem. Biophys.*, **65**, 243 (1956).
(55) B. H. Havsteen and G. P. Hess, *J. Am. Chem. Soc.*, **85**, 791 (1963).
(56) H. Parker and R. Lumry, *ibid.*, **85**, 483 (1963).
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(58) G. P. Hess, personal communication.
(59) R. Egan, H. O. Michel, R. Schlueter, and B. J. Jandorf, *Arch. Biochem. Biophys.*, **66**, 366 (1957).
(60) I. Tinoco, *ibid.*, **68**, 367 (1957).

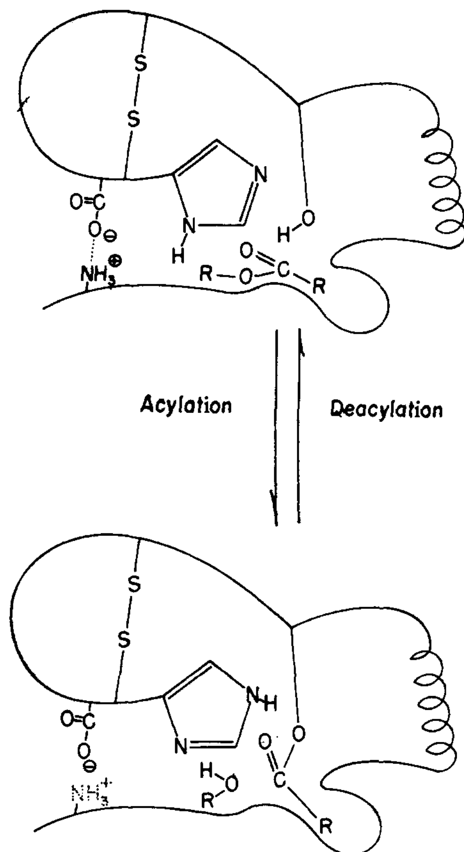
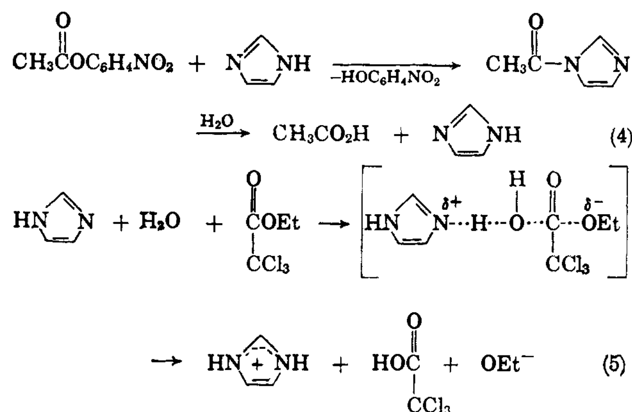


Fig. 1.—The participation of a conformationally important α-ammonium ion of N-terminal isoleucine in the acylation reaction.

The data necessary for a complete interpretation of the pH-dependent conformational changes occurring in α-chymotrypsin are far from complete, but the following tentative picture may be presented. The enzyme may exist in an active or an inactive conformation, the latter being able to form an inactive dimer, an "active site to active site" aggregate.[61] The monomers and dimer exist in equilibrium with one another, the dimer being in higher concentration at pH 2.7 than at pH 7. The monomer-dimer equilibrium between pH 2.7 and 7 is almost never seen in the acylation kinetics because of the swamping dependence of the reaction on the ionization of an imidazole group of pK_a 7.1, which changes the rate constant about five powers of ten from pH 2 to 7. Above pH 7, a conformational change may occur in the enzymatic active site, dependent on the α-ammonium ion of N-terminal isoleucine; it is seen kinetically in acylation, but not in deacylation because in acylation it is necessary to rigidify the specificity parts of the active site with respect to the catalytic components, while in deacylation, the covalent bond between the acyl group and the serine moiety rigidifies the various components of the active site, as shown in Fig. 1. It is the thesis of this discussion that the acylation step can reflect pH dependencies of both conformationally important groups and catalytically important groups of the enzyme, while deacylation can reflect only the latter. Future experiments must test this hypothesis.

The ionization with a pK_a $\sim$7 seen kinetically in acylation and deacylation is attributed to the involvement of an imidazole group(s) or a combination of imidazolium ion(s) and hydroxide ion in these reactions.[30] This ambiguity is a reflection of the usual ambiguity in the interpretation of pH-rate profiles. On the basis of model studies, the imidazole group(s) (or operationally, the basic group with an apparent pK_a of 7) then may participate in the acylation and deacylation reactions either as a nucleophilic catalyst,[39,40] whose gross mechanism is shown in eq. 4, or as a general basic catalyst, one of whose mechanistic descriptions is shown in eq. 5 (a variant of general basic catalysis is general acid-hydroxide ion catalysis).[41] The differentiation between these mechanisms



is not simple even in nonenzymatic systems. Criteria that have been used most successfully to distinguish between these pathways include: (1) observation of the unstable intermediate formed in nucleophilic catalysis; and (2) deuterium oxide solvent isotope effects. Both spectrophotometric and kinetic experiments involving the deacylation of *trans*-cinnamoyl-α-chymotrypsin indicated no observable buildup of an unstable intermediate containing the acyl group bound to imidazole, the presumed intermediate in a nucleophilic catalysis by an imidazole group.[2] These negative experiments unfortunately are ambiguous since a cinnamoylimidazole intermediate, if formed, would be expected thermodynamically to be present only in small concentration.

A positive clue to the role of imidazole in chymotrypsin catalysis is provided by the effects of deuterium oxide on the enzyme reaction. Let us first note the effect of deuterium oxide on the model reactions, eq. 4 and 5.[62] General basic catalysis carried out by imidazole (eq. 5) results in an isotope effect (k_{H_2O}/k_{D_2O}) of approximately 2–3, presumably caused by the rate-determining proton (deuteron) transfer in the catalysis. A general acid-hydroxide ion catalysis would presumably exhibit the same deuterium oxide isotope effect. However, nucleophilic catalysis carried out by imidazole (eq. 4) does not lead to an isotope effect (k_{H_2O}/k_{D_2O} = 1.0), there being no rate-determining proton transfer. In the deacylation of *trans*-cinnamoyl-α-chymotrypsin in deuterium oxide, two effects can be noted.[63] The rate constant in water is 2.5-fold greater than in D_2O. Furthermore, the whole rate curve is displaced about 0.6 pH unit to higher pH; that is, the pK_a of the basic group involved in the water reaction is 7.15 while that

(61) D. M. Blow, M. G. Rossmann, and B. A. Jeffery, *J. Mol. Biol.*, **8**, 65 (1964).

(62) M. L. Bender, E. J. Pollock, and M. C. Neveu, *J. Am. Chem. Soc.*, **84**, 595 (1962).

(63) M. L. Bender and G. A. Hamilton, *ibid.*, **84**, 2570 (1962).

of the D_2O reaction is 7.75. It is known that the pK_a of imidazole changes from 7.04 in water to 7.56 in D_2O; the ΔpK_a is thus consistent with imidazole participation. The same rate depression and pK_a change in deuterium oxide is noted in the acylation step with *p*-nitrophenyl trimethylacetate[63] and in the catalytic rate constants of the hydrolysis of acetyl-L-tryptophan ethyl ester (deacylation rate controlling) and of acetyl-L-tryptophan amide (acylation rate controlling).[5]

The data indicate that each of the rate constants k_2, k_3, and k_{cat} is decreased in D_2O relative to that in H_2O by a factor of 2 to 3. This factor is too large to be attributed only to solvation of ions produced in these steps. On the other hand, the factor of 2 to 3 is expected for a reaction involving a proton transfer in the rate-controlling step. The conclusion seems probable that in acylation and deacylation imidazole(s) of the enzyme does not act as a nucleophile, but rather as a general basic (general acidic–hydroxide ion) catalyst, carrying out a rate-determining proton transfer(s).

The deuterium oxide isotope effect in deacylation *per se* does not rule out a mechanism involving a nucleophilic attack by imidazole, producing no isotope effect, accompanied by general acid catalysis, *e.g.*, by water, which does produce an isotope effect. This possibility may be ruled out, however. Such a reaction must occur in two steps, the first involving the formation of an unstable acylimidazole intermediate and the second step the reaction of this compound with water. If the first step is rate determining, no kinetic dependence on a molecule of water will be observed, contrary to experiment[7]; if the second step is rate determining, the intermediate should be observable, contrary to experiment.[2]

Further evidence that imidazole is not acting as a nucleophile is seen in the calculated k_2's of the reactions of the ethyl, methyl, and *p*-nitrophenyl esters of N-acetyl-L-tryptophan with α-chymotrypsin, which exhibit an order resembling hydroxide ion reactions, but not resembling nucleophilic imidazole reactions.[5]

The best and sole model for the intramolecular general base-catalyzed deacylation reaction is the intramolecular general basic catalysis observed in the solvolytic reactions of *p*-nitrophenyl 5-nitrosalicylate.[64] The *o*-phenoxide ion of this molecule leads to an enhancement of solvolysis of approximately 30-fold with respect to the un-ionized ester. An analysis of the contribution of intramolecular general basic catalysis may be made by a comparison of the intramolecular general base-catalyzed deacylation of *m*-nitrobenzoyl-α-chymotrypsin (14×10^{-4} sec.$^{-1}$),[28] a nonspecific acyl-enzyme, and the intramolecular general basic catalysis in the hydrolysis of the comparable compound, *p*-nitrophenyl 5-nitrosalicylate (1.0×10^{-4} sec.$^{-1}$).[64] In both compounds a *m*-nitrobenzoate ester is being hydrolyzed. The intramolecular catalyst in the former reaction has a pK_a of 7 while the intramolecular catalyst in the latter reaction has a pK_a of 6; the leaving group in the former reaction has a pK_a of 13.5 while the leaving group in the latter reaction has a pK_a of 7; thus the better base in the former reaction and the better leaving group in the latter reaction should tend to compensate one another kinetically. The enzymatic intramolecular general basic catalysis is 14 times more efficient than the comparable nonenzymatic reaction. There are several explanations for this difference: (1) the placement of the general base in the enzyme may be stereochemically more favorable than in the nonenzymatic reaction; (2) the medium may be more favorable for the transfer of a proton in the enzymatic reaction[65]; and (3) general basic catalysis may not completely describe the system.

(64) M. L. Bender, F. J. Kézdy, and B. Zerner, *J. Am. Chem. Soc.*, **85**, 3017 (1963).

The Mechanism of α-Chymotrypsin-Catalyzed Hydrolyses.—Of the two catalytic steps in α-chymotrypsin reactions, deacylation is the step most amenable to mechanistic description because a large amount of mechanistic information has been gleaned from this step and, further, this step is not complicated by any prior complexing step, as is acylation. For this reason, we shall describe the deacylation step mechanistically, and then generalize the mechanism to the entire α-chymotrypsin process, since the acylation reaction is simply the microscopic reverse of deacylation.

The mechanistic information most pertinent to a description of the deacylation step includes: (1) the acyl-enzyme is an ester in which the acyl group of the substrate is attached to the hydroxyl group of a serine residue; (2) two bases of $pK_a \sim 7$ are kinetically involved in the deacylation[6,40,41]; (3) these bases are imidazole groups of histidine moieties as discussed above; (4) the deacylation reaction is a nucleophilic reaction as characterized by a Hammett ρ-constant of +2.1[28]; (5) the reaction is first order in the nucleophile water, as determined kinetically and by analogy with the kinetics of the methanolysis reaction[7]; (6) the nucleophile reacts in its protonated form[7,48,49]; (7) the rate-determining step of the deacylation includes a proton transfer, as reflected in the deuterium oxide isotope effects[6]; and (8) no detectable intermediate is observed in deacylation.[2]

The components of the transition state of the deacylation reaction must thus include the acyl-serine ester, at least one imidazole group, and a molecule of water. The rate-determining proton transfer must shift a proton from a water molecule, the only species containing an ionizable proton, to the imidazole base, the proton acceptor of kinetic importance. The nucleophilic attack at the carbonyl group of the ester by the water molecule is characterized by substituent effects resulting in a Hammett ρ of +2.1. This ρ is comparable to the ρ of hydroxide ion-catalyzed hydrolysis and alkoxide-catalyzed alcoholysis of esters, both of which are known to proceed through tetrahedral addition intermediates.[66] The greater reactivity of a carbamyl fluoride than of a carbamyl chloride[67] supports the hypothesis of a tetrahedral addition intermediate,[68] since this order of reactivity indicates either the existence of an electrophilic component of the reaction or that bond making takes place prior to bond breaking.[69,70]

A mechanism of deacylation, based on the information listed above, is shown in eq. 6. The postulation

(65) D. J. Cram, B. Rickborn, and G. R. Knox, *ibid.*, **82**, 6412 (1960).

(66) M. L. Bender, *ibid.*, **73**, 1626 (1951).

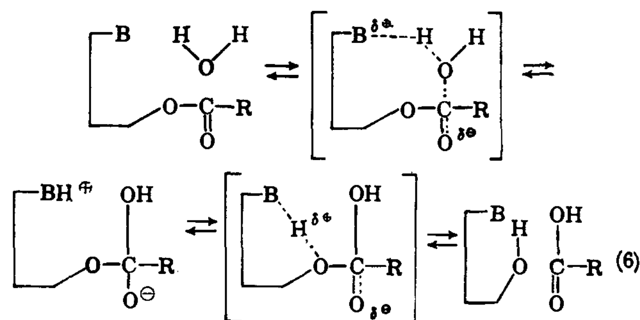
(67) H. P. Metzger and I. B. Wilson, *Federation Proc.*, **23**, 316 (1964).

(68) T. C. Bruice, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1924 (1961).

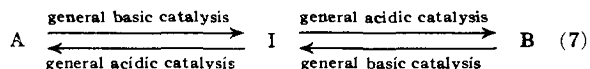
(69) J. F. Bunnett, E. W. Garbisch, Jr., and K. M. Pruitt, *J. Am. Chem. Soc.*, **79**, 385 (1957).

(70) Carbonyl oxygen exchange, analogous to that found in the saponification reaction, is now being investigated in the deacylation reaction.

of tetrahedral addition intermediates in the mechanism is supported not only by the arguments above but also by the principles of microscopic reversibility and by a



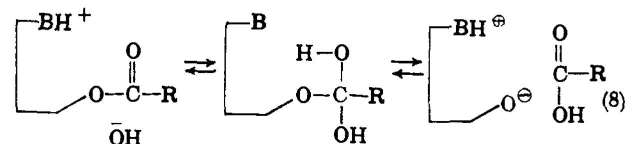
postulate dubbed "identical processes for identical reactants" which is difficult to prove rigorously, but which is superficially satisfying. The principle of microscropic reversibility requires that the microscopic reverse of a general basic catalysis is a general acidic catalysis, and thus eq. 6 may be described catalytically by eq. 7. This compatible system indicates that, in either direction, the process consists of a



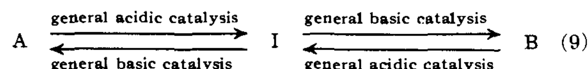
general basic catalysis followed by a general acidic catalysis. Such symmetry applies not only to hydrolysis of the acyl-enzyme, but also to alcoholysis of the acyl-enzyme. Consider the latter reaction: it consists of the reaction of an *ester* (acyl-enzyme) with an *alcohol* to produce an *ester* and an *alcohol* (serine of the enzyme). The reactants and products of this alcoholysis reaction are formally identical assuming the serine ester is identical with the substrate ester and the serine hydroxyl group is identical with the substrate hydroxyl group. If a tetrahedral addition intermediate were not present in such a reaction, the forward reaction of the ester and alcohol would consist of a general basic catalysis, whereas the reverse reaction of the ester and alcohol would consist of a general acidic catalysis, as required by microscopic reversibility. This difference in the catalytic process in the forward and reverse directions is not compatible with the identity of the reactants in the forward and reverse directions. This incompatibility may, however, be removed by introducing the tetrahedral addition intermediate as shown in eq. 6, which predicts for a transesterification reaction that not only the reactants, but also the catalytic processes, are identical in both directions.[71]

Equation 6 proposes two functions for imidazole in deacylation, general basic catalysis in the formation of the tetrahedral intermediate and general acidic catalysis in its decomposition. Presumably the former is the rate-determining step of the reaction, since the Hammett ρ and the relative reactivity of fluoride/chloride requires that bond making precede bond breaking in the transition state of the reaction.[72] The specification of general acidic catalysis by imidazolium ion in the decomposition of the tetrahedral intermediate is not a consequence of any direct experimental information, but rather a consequence of combination of the symmetry of the system and the principle of microscopic reversibility.

Equation 8 is a mechanism kinetically indistinguishable from that shown in eq. 6, since imidazole and water are kinetically indistinguishable from imidazolium ion



and hydroxide ion. Equation 8 may be described catalytically by eq. 9, the opposite of eq. 7. The reactants of eq. 8 are, of course, in equilibrium with the



reactants of eq. 6. The former species, however, are in much lower concentration at equilibrium at pH 8 than are the latter species. In the deacylation of N-acetyl-L-tyrosyl-α-chymotrypsin, the first-order rate constant at pH 8 is 190 $sec.^{-1}$.[51] This rate constant may be converted to a second-order constant by dividing by the concentration of water, 55 M, giving a rate constant for the deacylation in terms of the reaction of (free) imidazole with water of 3.45 M^{-1} $sec.^{-1}$. This rate constant for the reaction of imidazole with water may be transformed into a rate constant for the reaction of the imidazolium ion with hydroxide ion by using known equilibria leading to a value of 1.9×10^9 M^{-1} $sec.^{-1}$ for the rate constant of the deacylation of N-acetyl-L-tyrosyl-α-chymotrypsin *via* imidazolium ion and hydroxide ion; the rate constant of the acylation step would be even faster. These rate constants approximate those of diffusion-controlled reactions.[73] Since no covalent bond making or breaking process involving atoms larger than hydrogen is known to proceed at such a velocity, it is difficult to believe that eq. 8 describes the deacylation reaction. On the other hand, the first-order rate constant for the deacylation of N-acetyl-L-tyrosyl-α-chymotrypsin with 1 M water (3.45 $sec.^{-1}$) is not very different from the first-order rate constant for the hydrolysis of N-benzoyl-L-phenylalanine ethyl ester with 10 M sodium hydroxide (5 $sec.^{-1}$),[74] a relationship which is usually found when comparing comparable intramolecular and intermolecular reactions.[31] Thus, the deacylation is better described as a reaction of imidazole and water (eq. 6) rather than as a reaction of imidazolium ion and hydroxide ion (eq. 8).

Equation 6 satisfies all the data pertinent to the deacylation reaction with the exception that it does not predict a bell-shaped pH–k_3 profile, as has been found, but rather a double sigmoid, the first depending on the ionization of the imidazolium ion and the other depending on the ionization of the nucleophile. The mechanistic reason for the latter pH dependency is that the nucleophile of eq. 6 must be more active in its

(71) S. L. Johnson, *Tetrahedron Letters*, **23**, 1481 (1964), has given a similar argument to postulate the existence of a tetrahedral addition intermediate in general base-catalyzed alcoholysis of ethyl trifluoroacetate in ethanol-d_6.

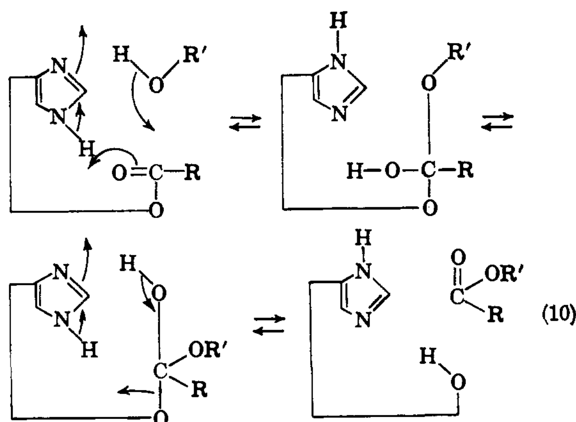
(72) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 197.

(73) M. Eigen and L. DeMaeyer in "Technique of Organic Chemistry," Vol. 8, 3rd Ed., A. Weissberger, Ed., John Wiley and Sons, Inc., New York, N. Y., 1963, Part II, p. 895.

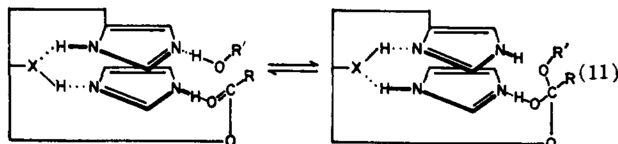
(74) M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.*, **77**, 4271 (1955); corrected to aqueous solution.

anionic form than its un-ionized form; *thus no catalytic function is given to the proton on the nucleophile.* The anionic form of the nucleophile may be postulated to be an unreactive species because it cannot approach the active site containing among other things an aspartate carboxylate ion. While this electrostatic effect might tend to reduce the apparent reactivity of the nucleophile as an anion, the high inherent nucleophilicity of anionic nucleophiles and the reasonably small electrostatic repulsion would add up to *some* reaction of the anionic form of the nucleophile. Present data indicate zero reactivity of the anionic form of the nucleophile.

A mechanism which does give a catalytic function to the proton of the nucleophile is embodied in eq. 10. This mechanism, in which the proton of the nucleophile serves as its own acid catalyst, leads to a bell-shaped curve for both acylation and deacylation reactions. Equation 10 agrees with all known experimental data pertinent to the α-chymotrypsin mechanism including: (1) pH dependencies; (2) the acyl-enzyme is a serine ester; (3) an imidazole group is involved in acylation and deacylation; (4) the acylation and deacylation reactions are nucleophilic reactions; (5) if the formation of the tetrahedral intermediate is the rate-determining step of each reaction, bond making must by definition be more important than bond breaking and conform to the observed Hammett ρ constants and fluoride/chloride reactivities; (6) a molecule of water is involved in deacylation; (7) no detectable intermediate is formed in deacylation since tetrahedral addition compounds are unstable; (8) a rate-determining proton transfer occurs in both acylation and deacylation; (9) there is no effect of ionic strength or dielectric constant on the rate constants of acylation or deacylation as predicted by the neutral character of all transition states; (10) the symmetry between the reactions of serine in the acylation step and water in the deacylation step is preserved; (11) the symmetry between the reactions of serine hydroxyl group and the leaving alkoxyl group in the acylation reaction is preserved; (12) the requirement of microscopic reversibility is met; (13) the mechanism is simple, straightforward, and utilizes the unique features of imidazole and thus the unique features of the enzyme; (14) the reaction has the attributes of a concerted reaction[75] which should enhance its kinetic efficacy; and (15) the maximal experimental rate constant of 38,300 sec $^{-1}$ (for the acylation of N-acetyl-L-tryptophan *p*-nitrophenyl ester)[5] is faster than the rate constant of a simple proton transfer from (55 *M*) water to imidazole (2300 sec.$^{-1}$) but comparable to the rate constant for such a proton transfer coupled with a second proton from the product conjugate acid.[76]



A negative aspect of eq. 10 is that the steric requirements of the reaction between the imidazole molecule, the substrate, and the serine hydroxyl group are not met. The proton transfers shown in eq. 10 should occur between the sp^2-orbitals of the two nitrogen atoms of imidazole and the respective oxygen atoms of the system. It is impossible to construct a model of eq. 10 in which protons may be colinearly transferred from one electronegative atom to the other. Furthermore, the participation of two (unprotonated) imidazole groups, strongly suggested by their juxtaposition in the sequence of both chymotrypsin and trypsin, has not been considered. The transformation of eq. 10 into a mechanism involving two imidazole groups is shown in eq. 11. In mechanism 11, the two imidazole rings of two histidine moieties of the enzyme are postulated to form a π-complex with one another. Other geometries do not meet stereochemical and mechanistic requirements. In a cyclic process analogous to eq. 10, a proton is abstracted from the nucleophile and added to the carbonyl oxygen atom through this π-complex, forming the tetrahedral intermediate. Thus not one but two imidazole groups serve as proton transfer agents, in a coupled fashion. Mechanism 11



has interesting consequences in addition to those already enumerated for mechanism 10: (1) the whole system, especially the two imidazole rings, must be sterically rigid demanding adherence to precise placement of the substrate (or acyl group), which requirement is seen in the conformational changes dependent on the α-ammonium ion of N-terminal isoleucine and the specificity discussed later[77]; (2) the pair of imidazoles is positioned suitably to act as proton transfer agent, but not positioned to act as a nucleophile; (3) the proton transfers and hydrogen bonds in mechanism 11 are reasonably colinear; and (4) the substrate may interact with one of the imidazole groups in the enzyme–substrate complex, which may result in a slight perturbation of the pK_a of the imidazole group, manifest in the slight dependence of the pK_a on the identity of the substrate and in the slight difference between the pK_a's of acylation and deacylation.[6,78,79]

(75) C. G. Swain and J. F. Brown, Jr., *J. Am. Chem. Soc.*, **74**, 2538 (1952).

(76) M. Eigen, *Pure Appl. Chem.*, **6**, 97 (1963).

(77) M. L. Bender, F. J. Kézdy, and C. R. Gunter, *J. Am. Chem. Soc.*, **86**, 3714 (1964).

(78) The pH dependence of the deacylation of N-acetyl-DL-tryptophan *p*-nitrophenyl ester from pH 7 to pH 2 shows a strict dependence on the ionization of a *single* basic group of pK_a 7.1.[4] The involvement of *two* basic groups of pK_a 7.1 predicts a slope of two for the log *k vs.* pH curve at a pH somewhat below the lower pK_a of the two groups. These phenomena may be reconciled by postulating that the pK_a of the second imidazole group of mechanism 11 is perturbed both electrostatically and *via* hydrogen bonding so that its pH dependence is not seen in our experimental range.

(79) The function of the two imidazoles in this mechanism should be contrasted with that of the two imidazoles in the proposed ribonuclease mechanism; F. H. Westheimer, *Adv. Enzymol.*, **24**, 441 (1962).

Many mechanisms have been proposed for catalysis by α-chymotrypsin using the imidazole and serine hydroxyl groups as features of the catalysis. These include: (1) nucleophilic catalysis by imidazole of acyl-enzyme formation and decomposition with[80] or without tetrahedral addition intermediates[48,81]; these suggestions are not consistent with the deuterium oxide solvent isotope effects; (2) general basic catalysis by imidazole of acyl-enzyme formation and decomposition[82,83]; this suggestion is not consistent with the pH dependence of the process; (3) general basic catalysis by imidazole involving tetrahedral addition intermediates[2,80,83]; this suggestion, like eq. 6, is not consistent with the pH dependence of the process; (4) general acidic catalysis by an unknown acid and general basic catalysis by imidazole of acyl-enzyme formation and decomposition[2,83-85]; this suggestion is inconsistent with the designation of the acidic pH dependency as the nucleophilic group; (5) general acidic catalysis by an unknown acid and nucleophilic catalysis by imidazole of acyl-enzyme formation and decomposition[2]; this suggestion is incompatible with the combination of dependence of deacylation on water concentration and the lack of an observable intermediate in deacylation, as mentioned earlier; (6) general basic catalysis by imidazole in acylation and nucleophilic catalysis by imidazole in deacylation[86-88]; this suggestion is incompatible with the principle of microscopic reversibility; (7) general acid-base catalysis by imidazole involving tetrahedral addition intermediates.[89-91] Of these seven suggestions, six are ruled out for the reasons mentioned above. The seventh suggestion is similar to that proposed here.

The mechanism of α-chymotrypsin catalysis suggested here is the product of as much concrete mechanistic information as has been found to date and can serve as a working hypothesis for the future.

(80) T. C. Bruice, *ibid.*, **47**, 1924 (1961).
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(82) T. Spencer and J. M. Sturtevant, *J. Am. Chem. Soc.*, **81**, 1874 (1959).
(83) B. M. Anderson, E. S. Cordes, and W. P. Jencks, *J. Biol. Chem.*, **236**, 455 (1961).
(84) K. J. Laidler, *Discussions Faraday Soc.*, **20**, 83 (1955).
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(86) L. W. Cunningham, *Science*, **125**, 1145 (1957).
(87) G. H. Dixon and H. Neurath, *J. Am. Chem. Soc.*, **79**, 4558 (1957).
(88) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).
(89) M. A. Marini and G. P. Hess, *J. Am. Chem. Soc.*, **82**, 5160 (1960).
(90) *Cf.* B. R. Rabin in "Mechanismen enzymatischer Reactionen," Springer Verlag, Berlin, 1964, p. 74.
(91) *Cf.* F. Bergmann, I. B. Wilson, and D. Nachmansohn, *J. Biol. Chem.*, **186**, 693 (1950).

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The Anatomy of an Enzymatic Catalysis. α-Chymotrypsin[1]

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The advantages of defining specificity in terms of kinetic specificity are presented. The kinetic specificity in the deacylation of a series of acyl-α-chymotrypsins shows an additive specificity of two parts of the acyl group. The temperature dependence of four deacylation reactions involving acyl-α-chymotrypsins of varying specificity was determined. The enthalpies of activation of this series are essentially constant whereas a wide variation in the entropies of activation is found, varying from -13.4 e.u. in the deacylation of a specific acyl-enzyme to -36 e.u. in the deacylation of a nonspecific acyl-enzyme. The kinetic specificity of deacylation (and acylation) is interpreted in terms of a variation in rotational entropy of activation, a specific substrate being rigidly fixed in a conformation resembling the transition state and a nonspecific substrate being free to rotate in many conformations. The standard free energy *vs.* reaction coordinate diagrams for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester and of N-acetyl-L-tryptophan amide show that the standard free energy of the acyl-enzyme intermediate, N-acetyl-L-tryptophanyl-α-chymotrypsin, is intermediate in energy between the reactant and the product. Thus, the acyl-enzyme intermediate is both kinetically and thermodynamically favored in the over-all pathway. Five factors can quantitatively account for the difference between the enzymatic and nonenzymatic (hydroxide ion catalyzed) rate constants of hydrolysis of N-acetyl-L-tryptophan amide: (1) the intramolecular character of the enzymatic process and the concomitant increase in effective concentration of the catalytic group(s); (2) general basic catalysis by imidazole; (3) the change in rate-determining step of the amide hydrolysis to an alcoholysis; (4) the freezing of the substrate in a conformation resembling the transition state; and (5) the general acidic catalysis by imidazole (Table V). This combination of rate factors can account for the enzymatic reactivity of α-chymotrypsin.

Introduction

The previous discussion has provided an over-all pathway for catalysis by α-chymotrypsin, and a catalytic mechanism for each step of the catalytic process.[2] The pathway involves, besides the usual enzyme-substrate complexes, the formation of a covalent intermediate, an acyl-enzyme. The catalytic mechanism of each step involves intracomplex or intramolecular bifunctional catalysis involving imidazole.

In the present paper an attempt is made to dissect the kinetic factors contributing to the difference between the enzymatic hydrolysis of an amide substrate (the usual physiological substrate of α-chymotrypsin) and a corresponding nonenzymatic hydrolysis of the same amide, taken as a saponification reaction at the same pH. In order to carry out a complete discussion, specific amide substrates must be used, and therefore our treatment will begin with a discussion of specificity, that is, the ability of an enzyme to catalyze selectively a particular reaction.

The specificity of enzyme reactions is usually discussed in terms of "fit" in the binding of a substrate to an enzyme, "fit" being a euphemism for a stereospecific maximization of the forces existing between two molecules. The classical theory of Emil Fischer[3] discussed

(1) This research was supported by grants from the National Institutes of Health. Paper XXXIII in the series: The Mechanism of Action of Proteolytic Enzymes.
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