rather resembles the reactions involving nucleophilic catalysis (reactions 1, 3, 6, 7). This would appear to be a rather tenuous comparison since there appears to be a considerable difference in Hammett ρ constants even between oxyanions (reaction 1) and neutral nitrogen nucleophiles (reactions 3 and 4). However, this is the only conclusion that may be drawn from the above data, tentative as it may be.

It is seen that there is a small difference in Hammett ρ constants between the correlations involving first- and second-order acylations of chymotrypsin. This difference of course results from the fact that there is a small but real substituent effect on the adsorption constant, K_m. Since the substituent effect on K_m is not large, it appears justified to discuss both the first-order and second-order acylations in the same context.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY, EVANSTON, ILL.]

The Mechanism of α-Chymotrypsin-catalyzed Hydrolyses[1-3]

BY MYRON L. BENDER[4]

RECEIVED NOVEMBER 6, 1961

Experimental evidence bearing on the mechanism of action of α-chymotrypsin has been summarized from previous papers in this series and the literature. Mechanistic proposals for α-chymotrypsin action have been developed on the basis of the following evidence: (1) the occurrence of a two-step catalytic mechanism in which the intermediate compound, the acyl-enzyme, is an alkyl ester; (2) the equivalence of the two catalytic steps of acylation and deacylation through experimental observations, and symmetry and microscopic reversibility arguments; (3) the nucleophilic character of each catalytic step; (4) the effect of pH on the rates of the individual steps, indicating the participation of a base or nucleophile in the catalytic process; (5) spectrophotometric and kinetic experiments indicating no bulidup of an intermediate containing the acyl group bound to imidazole in deacylation; and (6) the effect of deuterium oxide on the rates of the individual steps, indicating the occurrence of a rate-determining proton transfer. These mechanisms are given in eq. 7, 8 and 9.

A class of proteolytic enzymes may be defined as those enzymes which react uniquely with a labile phosphate derivative to produce an inactive phosphorylated enzyme in which the phosphorus atom resides on the hydroxyl group of a serine moiety of the enzyme. This class of enzymes has been called the "serine proteinases."[5] α-Chymotrypsin is the member of this class of enzymes most amenable to experimental investigation from the point of view of availability, purity, background of information and ease of experimentation. For these reasons, most of the mechanistic experiments described in previous papers were carried out with α-chymotrypsin. It is reasonable to believe that the discussion presented here for α-chymotrypsin will be pertinent as well for other members of the serine proteinase family such as trypsin, plasmin, thrombin and elastase.

Although a considerable amount of information concerning the mechanism of α-chymotrypsin-catalyzed hydrolyses is now available, the three-dimensional structure of the enzyme (protein) is not available. Even the sequential analysis of the structure of chymotrypsin has not as yet been completed. Therefore, any conclusions in this paper must be tempered by the realization that the all-important structural information is yet to come.

From a mechanistic viewpoint ignorance of the protein structure reduces to ignorance of the structure of the one active site in the molecule. The active site is probably small compared to the entire enzyme, since most of the substrates of α-chymotrypsin, even specific substrates, are quite small molecules compared to α-chymotrypsin itself, which has a molecular weight of 24,800.[6] Furthermore, several hydrolytic enzymes have been cleaved of appreciable fractions of their total bulk with little or no loss of catalytic activity.[7] The active site is pictured as a *region* among several chains which contribute nucleophiles, electrophiles and specificity loci to the totality called the active site. This paper will attempt to specify the catalysis carried out by this site.

Specificity and Binding.—Before proceeding to a consideration of the catalytic features of the α-chymotrypsin mechanism, it is of interest to consider the specificity exhibited by various kinds of substrates, including those used in the preceding investigations. It is well known that many poor substrates bind as well as good substrates. For example the association constant for the binding of the two members of an enantiomorphic pair with an enzyme will often be approximately the same, but only one will react subsequently to give products. This phenomenon may be easily explained by saying that the reactive substrate leads to a three-dimensional configuration on binding in which the susceptible linkage is positioned correctly with respect to the catalytic entities on the enzyme surface whereas the unreactive linkage, while bound, is incorrectly positioned. This explanation is based on the implicit assumptions that the binding of the substrate occurs to a relatively rigid enzyme surface and further that the binding is performed by groups on the enzyme

(1) This research was supported by grants from the National Institutes of Health.

(2) Paper XVI in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962).

(3) Some of this material was presented in preliminary form: M. L. Bender, G. R. Schonbaum, G. A. Hamilton and B. Zerner, *ibid.*, **83**, 1255 (1961).

(4) Alfred P. Sloan Foundation Research Fellow.

(5) B. S. Hartley, *Ann. Revs. Biochem.*, **29**, 45 (1960).

(6) P. E. Wilcox, J. Kraut, R. D. Wade and H. Neurath, *Biochim. et Biophys. Acta*, **24**, 72 (1957); P. E. Wilcox, E. Cohen and W. Tan, *J. Biol. Chem.*, **228**, 999 (1956).

(7) R. L. Hill and E. L. Smith, *Biochim. et Biophys. Acta*, **19**, 376 (1956); R. L. Hill and E. L. Smith, *J. Biol. Chem.*, **235**, 2332 (1960); G. E. Perlmann, *Nature*, **173**, 406 (1954).

TABLE I

KINETIC CONSTANTS OF SOME α-CHYMOTRYPSIN-CATALYZED REACTIONS[a]

	Substrate	K_m (app.), $M \times 10^{-3}$	K_m (acyl.), $M \times 10^{-3}$	k_{cat}, sec.$^{-1}$	Ref.
1	N-Acetyl-L-tyrosine ethyl ester	0.7		193	10
2	N-Acetyl-L-tryptophan ethyl ester	0.09		50.6	10
3	N-Acetyl-L-phenylalanine ethyl ester	1.1		173	14
4	N-Acetyl-L-valine methyl ester	108		0.15	11
5	N-Acetylglycine methyl ester	10		.008	11
6	Methyl hydrocinnamate[c]	3.9		.018	13
7	Methyl cinnamate[f]	2.05[d]	6.7 ± 0.6	.0073	17
8	*p*-Nitrophenyl cinnamate[e]		3–5[h]	.0125[b]	12
9	N-*trans*-Cinnamoylimidazole			.0125[b]	12
10	*p*-Nitrophenyl acetate[g]	0.04	5	.012	15
11	*p*-Nitrophenyl trimethylacetate	0.00057[d]	1.6 ± 0.5	.00013	16

[a] *p*H 7.9, 25°. [b] Assuming that deacylation is the rate-determining step of the catalytic sequence [c] 20% methanol–water. [d] K_m(app.) = K_m (acylation) k_3/k_2.[32,33] [e] 10% acetonitrile–water. [f] 3.2% acetonitrile–water. [g] 0.5 *M* KCl, 0.4% 1,4-dioxane. [h] Estimated value.[17]

and substrate other than the catalytic components.

Let us consider the α-chymotrypsin-catalyzed reactions in Table I. The variations of K_m with structure are difficult to interpret.[8] One problem is that it is not clear that K_m is an equilibrium constant in all cases.[9] If one assumes that specific substrates proceed through acylation and deacylation steps, a second and knottier problem arises: the measured K_m may be either K_m (acylation) or K_m (apparent) which is equal to K_m (acylation) k_3/k_2.[32,33] This problem will be the subject of a later paper. At this juncture a discussion of the variation of K_m with structure as a measure of relative binding abilities does not appear to be appropriate.

Even if measurement of the gross binding could be accomplished straightforwardly, determination of the stereospecificity of binding is a difficult measurement to envision. There is no method at present for determining the positioning of the reactive linkage of the substrate with respect to the catalytic entities of the enzyme. One can, however, infer from the catalytic rate constants which are the stereospecific bindings favorable to reaction, and define in this fashion a "kinetic specificity."

Stereospecificity of binding may be pictured as that binding which produces a rigid, correct fit between the reactive groups of the substrate and enzyme. The correctness is pictured in terms of the resemblance of the stereochemistry of the enzyme-substrate complex to the stereochemistry of the transition state,[18] and the rigidity is pictured in terms of a minimum of configurational entropy change between the enzyme-substrate complex and the transition state for the subsequent reaction.[19]

It is interesting to note that the non-specific substrates 7–11 in Table I possess $K_{m(app.)}$'s which are of the same order of magnitude as those of the specific substrates. One must therefore attribute the differences in catalytic rate constants between the specific and non-specific substrates to differences in the correctness and rigidity of binding.

It appears then that specificity may profitably be defined in terms of kinetic specificity rather than thermodynamic specificity, for the latter gives a deceptive picture of the catalytic aspect of an enzymatic reaction. If one does define specificity in kinetic terms, then one can say that the specificity of α-chymotrypsin for the cinnamate compounds is of the same order as that for acetylglycine methyl ester and for methyl hydrocinnamate. The compound of most interest in this respect is methyl cinnamate, for its leaving group is the same as that of the N-acetylglycine and hydrocinnamate esters. The catalytic rate constant of methyl cinnamate is 0.0073 compared to 0.008 and 0.018 sec.$^{-1}$ for N-acetylglycine methyl ester and methyl hydrocinnamate, respectively. (Furthermore the $K_{m(app.)}$'s for these three substrates are quite similar to one another, being 2.05, 3.9 and 10 × $10^{-3}M$, respectively.) There does not seem to be any discontinuity between specific and non-specific substrates of chymotrypsin. Rather there appears to be a continuum of catalytic rate constants covering a range of over 10^6 from the tyrosine, tryptophan

(8) A detailed discussion of the kinetic constants of a large number of substrates of α-chymotrypsin has recently been given: G. Hein and C. Niemann, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1341 (1961). See also a discussion of the steric and structural specificity of α-chymotrypsin in G. E. Hein and C. Niemann, *J. Am. Chem. Soc.*, **84**, in press (1962).

(9) K_m of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine ethyl ester is reported to be an equilibrium constant which makes it likely that all chymotrypsin K_m's are equilibrium constants; H. Gutfreund, *Disc. Faraday Soc.*, **17**, 220 (1954).

(10) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).

(11) R. B. Martin and C. Niemann, *J. Am. Chem. Soc.*, **80**, 1481 (1958); J. P. Wolf and C. Niemann, *ibid.*, **81**, 1012 (1959).

(12) M. L. Bender, G. R. Schonbaum and B. Zerner, *ibid.*, **84**, 2540 (1962).

(13) K. J. Laidler and M. L. Barnard, *Trans. Faraday Soc.*, **52**, 497 (1956); J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **182**, 577 (1950); k_{cat} has been recalculated on the basis of 24,800 for the molecular weight of α-chymotrypsin.

(14) B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955).

(15) E. Awad and H. Neurath quoted in H. Neurath and B. S. Hartley, *J. Cell. Comp. Physiol.*, **54**, Supp. 1, 184 (1959).

(16) M. L. Bender and G. A. Hamilton, *J. Am. Chem. Soc.*, **84**, 2570 (1962).

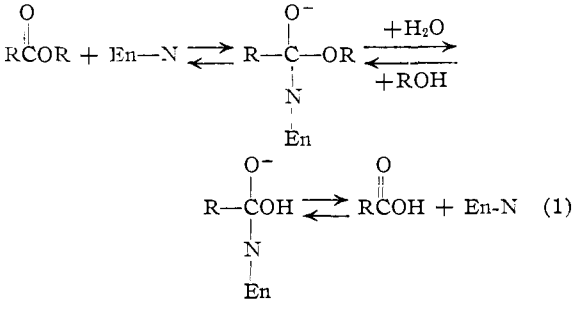
(17) M. L. Bender and B. Zerner, *ibid.*, **84**, 2550 (1962).

(18) It has been suggested that the "correct" configuration of an ester substrate resembles that of the more reactive lactone (T. C. Bruice, *J. Polymer Sci.*, **49**, 100 (1961)) but the lack of specificity for both R and R' in RCO_2R' does not support this idea.

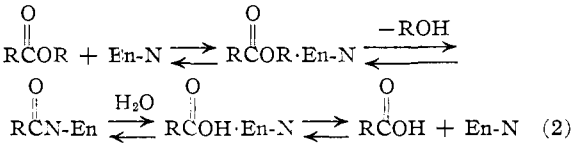
(19) Configurational entropy changes have proven to be a large factor in the rates of many intramolecular organic processes: M. L. Bender and M. C. Neveu, *J. Am. Chem. Soc.*, **80**, 5488 (1958) and T. C. Bruice and U. K. Pandit, *Proc. Natl. Acad. Sci., U. S.*, **46**, 402 (1960).

and phenylalanine compounds occupying the top positions to the trimethylacetate ester occupying the bottom position, with the cinnamates occupying a position somewhat below the middle.

The Acyl-enzyme Hypothesis.—The mechanisms postulated for catalysis by α-chymotrypsin fall into two families. One family of mechanisms proposes the formation of an enzyme-substrate (-co-substrate) complex followed by a one-step reaction[20] to produce the enzyme-products complex which then dissociates. A specific example of this general mechanism involves the reaction of a nucleophile on the enzyme with the substrate ester to form an enzyme-substrate complex in the form of a tetrahedral addition compound. This complex then reacts with water in an S_N2 displacement reaction to form a new tetrahedral addition compound, the enzyme-product complex which dissociates (eq. 1).[21] There are of course other special cases of this general mechanism.



The other family of mechanisms, which has been discussed in detail in the preceding papers, proposes the formation of an adsorptive enzyme-substrate complex, then an intra-complex reaction to produce an acyl-enzyme intermediate, and finally reaction of the acyl-enzyme with water to produce carboxylic acid and regenerate the enzyme (eq. 2).[22]



The two general mechanisms differ essentially in the question of whether an acyl-enzyme intermediate is formed. The second hypothesis treats the acyl-enzyme as an obligatory intermediate in the reaction scheme; on the other hand, the first hypothesis would admit the possibility of the formation of an acyl-enzyme only as a "blind alley" intermediate formed when a labile substrate (*e.g.*, a nitrophenyl ester) is used whose leaving group departs before nucleophilic attack by water can occur. However, a considerable body of evidence now indicates that eq. 2 is indeed the pathway not only for the reactions of labile substrates of α-chymotrypsin but also for specific substrates as well, and perhaps for all reactions of serine proteinases. This is the thesis of this paper and it will now be documented.

1. Isolation of Acyl-enzymes.—A number of dialkylphosphoryl-enzymes,[23] a dimethylcarbamyl-enzyme[24] and a host of acyl-enzymes[12,25-29] have been prepared and in many cases isolated. In all cases tested these compounds are stoichiometric compounds[30] which are enzymatically inactive. Several derivatives such as diisopropylphosphoryl-chymotrypsin and trimethylacetyl-α-chymotrypsin have been crystallized.[23,28] The phosphoryl, carbamyl or acyl group may be removed from the enzyme by a nucleophile with varying degrees of ease. This extensive family of similar enzymatic processes related to eq. 2 provides a solid chemical background for its applicability to the reactions of serine proteinases.

2. Spectrophotometric Detection of Acyl-enzymes.—Ample demonstration has been given in preceding papers that the formation and decomposition of acyl-enzyme intermediates may be detected spectrophotometrically during an enzymatic process without perturbation of the system. Such observations may be made at *p*H 7.8, usually spoken of as the *p*H optimum of the enzyme, as well as at the lower *p*H's which are usually used to isolate an acyl-enzyme. An acyl-enzyme has been spectrophotometrically detected in α-chymotrypsin[12] and trypsin-catalyzed[31] hydrolyses of labile substrates. More importantly, unequivocal demonstration of an acyl-enzyme has been achieved in the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate by spectrophotometric means.[17] The demonstration of the formation of an acyl-enzyme in the hydrolysis of this non-labile ester adds considerable generality to the acyl-enzyme hypothesis, and provides a definitive experiment contradicting the idea that acyl-enzymes are formed as intermediates only in reactions involving labile substrates.[31] In theory, spectrophotometric detection of acyl-enzyme intermediates in chymotrypsin-catalyzed reactions of even more specific substrates is possible. Two practical problems must first be met: fast times of reaction and small absorbance differences will probably be encountered in these reactions.

3. Stopped-flow Kinetics.—The quantitative determination of the kinetics of the initial rapid reaction (the pre-steady state, measuring acylation) and the subsequent slow reaction (the steady state,

(20) D. E. Koshland, Jr., in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, eds., Johns Hopkins Press, Baltimore, Md., 1954, p. 608; D. E. Koshland, Jr., in "The Enzymes," Vol. 1, 2nd Ed., P. D. Boyer, H. Lardy and K. Myrbäck, eds., Academic Press, Inc., New York, N. Y., 1959, p. 329.

(21) S. A. Bernhard and H. Gutfreund, Proc. Int. Symposium Enzyme Chemistry, Tokyo, Maruzen Co., 1958, p. 124; S. A. Bernhard, *J. Cell. Comp. Physiol.*, **54**, Supp. 1, 197 (1959).

(22) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **50**, 672 (1952); B. S. Hartley and B. A. Kilby, *ibid.*, **56**, 288 (1954).

(23) A. K. Balls and E. F. Jansen in "Advances in Enzymology," Vol. XIII, F. F. Nord, ed., Interscience Publishers, Inc., New York, N. Y., 1952, p. 321.

(24) I. B. Wilson, M. A. Hatch and S. Ginsburg, *J. Biol. Chem.*, **235**, 2312 (1960).

(25) A. K. Balls and F. L. Aldrich, *Proc. Natl. Acad. Sci., U. S.*, **41**, 190 (1955).

(26) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

(27) C. E. McDonald and A. K. Balls, *ibid.*, **227**, 727 (1957).

(28) A. K. Balls, C. E. McDonald and A. S. Brecher, Proc. Int. Symposium on Enzyme Chemistry, Tokyo, Maruzen, 1958, p. 392.

(29) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1957).

(30) An apparent exception to a stoichiometric reaction has been noted with a cholinesterase; J. A. Cohen and M. G. P. J. Warringa, *Biochim. et Biophys. Acta*, **11**, 52 (1953).

(31) M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, **84**, 2556 (1962).

measuring deacylation) in the α-chymotrypsin-catalyzed hydrolyses of *p*-nitrophenyl acetate are consistent with the scheme shown in eq. 2 (although no evidence is found for the enzyme-carboxylic acid product complex shown in eq. 2).[32,33] The stopped-flow method, used to follow the initial rapid reaction, has also been used to determine the kinetics of the acylation of α-chymotrypsin with 2,4-dinitrophenyl acetate,[33] and of the acylation of trypsin with *p*-nitrophenyl acetate.[34] The rate constants for these reactions are consistent with the isolation and spectrophotometric experiments described above. Although these kinetics do not measure a property of the acyl-enzyme, but rather a property of the system related to the acyl-enzyme, they have nevertheless proved to be a powerful factor in shaping thinking about the mechanism of α-chymotrypsin action in terms of eq. 2.

4. Relative Rates of Reaction.—One physical organic chemical argument for the existence of an intermediate is the observation of identical rates of reaction from two reactants which have only a portion of the molecule in common. In such a case it is reasonably concluded that a common intermediate is formed from each of the two reactants, involving that portion of the original reactants common to both. This argument is also applicable to enzymatic processes. N-α-Benzoyl-L-arginyl-trypsin has been postulated as a common intermediate in the trypsin-catalyzed hydrolysis of five esters of N-α-benzoyl-L-arginine which proceed with identical over-all rates.[31,35] Furthermore an N-acyl-L-tyrosyl-α-chymotrypsin has been postulated as a common intermediate in the α-chymotrypsin hydrolyses of some substrates whose relative rate constants cannot be explained on the basis of relative susceptibility to nucleophilic attack.[12,36] Finally it has been shown that the α-chymotrypsin-catalyzed hydrolyses of six *trans*-cinnamic acid derivatives proceed with identical rates of deacylation, indicating the formation of the common intermediate, *trans*-cinnamoyl-α-chymotrypsin, in these reactions.[12,17,37,40]

5. The Effect of Variables on the Classical Catalytic Rate Constant and the Deacylation Rate Constant.—The effect of variables on these two rate constants is detailed in a later section of this paper. The variables, including *p*H, deuterium oxide, solvent composition and structure of the substrate, are shown to lead to similar effects on the classical catalytic rate constant and on the deacylation rate constant. This parallelism has been spelled out in some detail with regard to *p*H effects,[41] deuterium oxide effects[16] and effects of structure on reactivity.[42] Aprotic solvents such as acetone and dioxane up to 20 volume per cent. have been shown to have no effect on the catalytic rate constant of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate.[43] We have found that 10% acetonitrile does not affect the rate constant of the deacylation of *trans*-cinnamoyl-α-chymotrypsin by more than 10%.[44] The only straightforward way to explain the parallelism of the effect of these four variables on the two rate constants is to say that the two processes are related to one another; specifically, that the deacylation reaction is a constituent part of the over-all catalytic process.

The arguments presented above constitute an imposing body of self-consistent evidence in favor of eq. 2 for the pathway of α-chymotrypsin-catalyzed hydrolyses (and also for catalyses by other serine proteinases).[45] The most important observation is the spectrophotometric detection of an acyl-enzyme intermediate in the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate. This experiment appears to be definitive and it may be asked what evidence exists which counters the above evidence. There is one such piece of evidence, concerned with the α-chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis of methyl hippurate.[48] The total rate of this enzymatic reaction is dependent on the hydroxylamine concentration, and is greater in the presence of hydroxylamine than in its absence. On the other hand, the dissociation constant for the methyl hippurate-enzyme complex is independent both of the concentration of hydroxylamine and of the particular reaction employed in its determination.

(32) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(33) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci., U. S.*, **42**, 719 (1956).

(34) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, **37**, 751 (1959).

(35) G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, **179**, 665 (1949).

(36) H. Gutfreund and B. R. Hammond, *Biochem. J.*, **73**, 526 (1959).

(37) Although the chymotrypsin- or trypsin-catalyzed hydrolysis of amides is considerably slower than that of the corresponding esters, the catalytic rate constants of the ficin- or papain-catalyzed hydrolyses of N-α-benzoyl-L-arginine ethyl ester and N-α-benzoyl-L-arginine amide are identical to one another.[38,39] This result clearly indicates that the ficin- and papain-catalyzed hydrolyses must involve the formation of acyl-enzyme intermediates whose subsequent reaction is rate-determining.

(38) S. A. Bernhard and H. Gutfreund, *Biochem. J.*, **63**, 61 (1956).

(39) J. R. Kimmel and E. L. Smith, *J. Biol. Chem.*, **207**, 515 (1954).

(40) I. B. Wilson and E. Cabib, *J. Am. Chem. Soc.*, **78**, 202 (1956), use an interesting variant of the above mechanistic argument. Linear Arrhenius plots of the maximum velocities were obtained for two poor substrates of acetylcholinesterase while concave plots were obtained for acetylcholine and dimethylaminoethyl acetate. These data were interpreted on the basis that acyl-enzyme intermediates were formed in all cases, but for the former two reactions the acylation step is rate controlling, while for the latter two reactions both steps are rate controlling leading to a curvature of the Arrhenius plot. This interpretation is interesting, but it should be remembered that activation parameters of enzymatic processes are subject to many perturbations.

(41) M. L. Bender, G. R. Schonbaum and B. Zerner, *ibid.*, **84**, 2562 (1962).

(42) M. L. Bender and K. Nakamura, *ibid.*, **84**, 2577 (1962).

(43) T. H. Applewhite, R. B. Martin and C. Niemann, *ibid.*, **80**, 1457 (1958).

(44) M. L. Bender and B. Zerner, unpublished observations.

(45) α-Chymotrypsin has been shown to catalyze the exchange of acetyl-L-phenylalanine methyl ester with methanol[46a] and the exchange of benzoyl-L-phenylalanine and acetyl-L-tryptophan with water.[46b] It has often been suggested that these exchange reactions constitute evidence for acyl-enzyme formation. It is true that it is easier to visualize a center of symmetry in eq. 2 than in eq. 1, but both reactions are capable of the symmetrical process necessary for an exchange reaction, and therefore one cannot use an exchange process as evidence for acyl-enzyme formation except under very special circumstances.[47]

(46) (a) M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960); (b) M. L. Bender and K. C. Kemp, *ibid.*, **79**, 116 (1957); D. Doherty and F. Vaslow, *ibid.*, **74**, 931 (1952); D. B. Sprinson and D. Rittenberg, *Nature*, **167**, 484 (1951).

(47) D. E. Koshland, *Disc. Faraday Soc.*, **20**, 142 (1955).

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

The latter fact constitutes a proof that this dissociation constant is an equilibrium constant; furthermore, the independence of binding constant on hydroxylamine concentration constitutes a proof that the binding of hydroxylamine (and water by analogy) is independent of the binding of the substrate. These results can be explained kinetically in terms of a ternary complex of enzyme, substrate and nucleophile.[49]

On the basis of these results and of a model system involving an aspartyl-seryl peptide, a mechanism for α-chymotrypsin-catalyzed hydrolyses has been proposed,[21] embodying eq. 1. As mentioned previously, acyl-enzyme formation is suggested as a blind alley intermediate, stemming from the decomposition of the original tetrahedral addition complex of substrate and enzyme through departure of the leaving group. Several arguments can be presented against this specific hypothesis. The rate-determining attack of a nucleophile such as water on a tetrahedral intermediate (an SN2 reaction), as in eq. 1, has no precedent in organic chemistry. Although orthoesters are hydrolyzed readily by acids, they ordinarily do not react with nucleophiles including hydroxide ion[51a]; for example, an orthoester can be separated from its corresponding carboxylic ester since the former is not attacked by 1% aqueous sodium hydroxide solution during the time that the latter compound is completely hydrolyzed.[51b] This indicates that if a choice must be made between a nucleophilic reaction involving an ester or an orthoester intermediate, the former is the one that would be favored for a facile enzymatic process. Furthermore, the rate-determining attack of water on the tetrahedral addition intermediate predicts no effect of deuterium oxide on the reaction, contrary to the observations made here.[16]

It appears to the present investigators that the evidence in favor of acyl-enzyme formation far outweighs that in favor of the opposite view with respect to directness of the evidence, the self-consistency of a large body of evidence and the occurrence of a reasonably definitive experiment in the observation of an acyl-enzyme in the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate. Although not completely proved, we shall use the acyl-enzyme hypothesis as one which is supported by the majority of the evidence and which gives the most consistent representation of the available experimental evidence.

The Equivalence of the Acylation and Deacylation Steps.—Assuming that the mechanism of α-chymotrypsin-catalyzed hydrolysis includes two catalytic steps, an acylation and a deacylation, the question arises as to the relationship of these two steps to each other. In previous papers in this series the effects of a number of variables on acylation rate constants (k_2 or k_2/K_m), deacylation rate constants (k_3) and over-all catalytic rate constants (k_{cat}) have been determined. It is of considerable interest to summarize these effects at this time, for these results lead to a consistent picture, namely of the equivalence of the acylation and deacylation reactions.

The effects of *p*H on the acylation and deacylation reactions are best interpreted in terms of the near equivalency of the apparent pK_a's involved in these two reactions.[41] It has been stated that the pK_a of the deacylation reaction is approximately 0.5 pK unit higher than that of the acylation reaction.[33] The results found in our investigations indicate that there may be a small difference in the two pK's but the difference appears to be smaller than 0.5 pK unit and is not much outside of experimental error. Furthermore a comparison of the apparent pK_a's of the acylation and deacylation steps with the apparent pK_a's of the catalytic steps of specific substrates shows all experimentally determined values to be reasonably consistent.[41] If indeed the hydrolyses of specific substrates proceed through the two catalytic steps of acylation and deacylation, then the apparent K of the catalytic step, measured in the classical manner, must be a complex constant[52,53]

$$K_{app} = (k_2 + k_3)/[(k_3/K') + (k_2/K'')] \qquad (3)$$

where K' and K'' are the ionization constants and k_2 and k_3 are the rate constants pertaining to the reactions of ES and ES', respectively. The ionization constants which have been measured in reactions involving discrete acylation and deacylation steps are the constants K' and K'', while the ionization constants measured in a classical kinetic approach correspond to the constant K_{app}. If any two of these three constants are equal to one another, it follows from eq. 3 that all three constants must be equal to one another. All determinations of ionization constants in chymotrypsin reactions, corresponding to either K_{app}, K' or K'', show the equality of these three constants. Since K' and K'' are equivalent to one another, the acylation and deacylation reactions appear to be subject to the same catalytic entities, as measured by the *p*H dependency of the reaction.

The effect of deuterium oxide on the acylation and deacylation reactions is practically the same.[16] Furthermore, the effect of deuterium oxide on k_{cat} is the same as its effect on k_2 or on k_3.[16] Therefore the same relationship exists with respect to deuterium oxide effects as with *p*H effects, and the acylation and deacylation reactions again appear to possess the same catalytic mechanism.

(49) As Bernhard, *et al.*,[48] state "The existence of this triple complex of enzyme, substrate and attacking reagent is strongly indicative of the absence of acylated enzyme intermediates as a *rate influencing* (this author's italics) species in this system." Further Bernhard[50] has shown that quasi-equilibrium is also maintained in similar experiments with benzoyl-DL-alanine methyl ester and benzoyl-DL-valine methyl ester. Significantly, however, quasi-equilibrium is not maintained with N-acetyl-L-tyrosine ethyl ester. This result was in fact predicted on the basis of a general theory for α-cymotrypsin-catalyzed reactions which we are currently developing. None of the results is inconsistent with the generality of the acyl-enzyme formation, and experimental work to show that "specific" substrates do indeed undergo reaction through an acyl-enzyme intermediate is being pursued in these laboratories at the present time and will form the substance of a future publication.

(50) S. A. Bernhard in Ch. 2, Vol. 1, "The Enzymes," P. D. Boyer, H. Lardy and K. Myrbäck, ed., Academic Press, Inc., New York, N. Y., 2nd ed., 1959.

(51) (a) J. N. Brönsted and W. F. K. Wynne-Jones, *Trans. Faraday Soc.*, **25**, 59 (1929); (b) H. Kwart and M. B. Price, *J. Am. Chem. Soc.*, **82**, 5123 (1960).

(52) R. M. Krupka and K. J. Laidler, *Trans. Faraday Soc.*, **56**, 1467 (1960).

(53) L. Peller and R. Alberty, *J. Am. Chem. Soc.*, **81**, 5907 (1959).

A comparison of the effect of structure of the acyl group on reactivity in the acylation and deacylation reactions is shown in Table II.

TABLE II

EFFECT OF STRUCTURE ON REACTIVITY IN THE ACYLATION AND DEACYLATION STEPS OF α-CHYMOTRYPSIN CATALYSIS

Substrate	$10^3 k_m$, M	k_2, sec.$^{-1}$	$10^3 k_3$, sec.$^{-1}$	Rel. k_{OH}[54]	Ref.
p-Nitrophenyl acetate	5	4	12	1	15
p-Nitrophenyl trimethylacetate	1.6	0.37	0.133	0.041	16

The trimethylacetate reaction is significantly slower than the acetate reaction in both cases, the ratios of rate constants of acetate/trimethylacetate being 10.8 and 90, respectively. It might have been expected that the two ratios would be identical to one another, but it is possible that the presence of the *p*-nitrophenyl group leads to a different positioning of the acyl group in the former reaction and therefore to a difference in the two ratios. At least qualitatively, relative reactivity in acylation and deacylation is the same. Furthermore, the catalytic rate constants in the α-chymotrypsin-catalyzed hydrolysis of two reasonably specific substrates, methyl hippurate and methyl hydrocinnamate, may be compared with the deacylation rate constants of hippuryl- and hydrocinnamoyl-α-chymotrypsins (formed in the reactions of the corresponding nitrophenyl esters) shown in Table III.

TABLE III

EFFECT OF STRUCTURE ON REACTIVITY IN SOME α-CHYMOTRYPSIN-CATALYZED REACTIONS

Acyl group	k_{cat} of methyl ester, sec.$^{-1}$	k_3 of *p*-nitrophenyl ester rel.[27]
Hippurate	0.19[43]	100
Hydrocinnamate	0.018[13]	16

It is seen that the effect of structure of the acyl group on the over-all catalytic reaction and on the deacylation step are similar. On the basis of the above data one may reasonably conclude that the structural parameters in acylation and deacylation point to a similar mechanistic process.

Organic aprotic solvents have been found to have essentially no effect on the acylation rate constant (k_2) of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate[55] or the deacylation rate constant (k_3) of the α-chymotrypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole.[44] These results parallel the lack of a solvent effect on the catalytic rate constant (k_{cat}) of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate.[43] Again it appears that acylation and deacylation must possess similar mechanistic pathways.

The three constants, the classical catalytic constant, the acylation constant and the deacylation constant, must form a set of interdependent quantities if the acyl-enzyme hypothesis is true. The mathematical relationship between the three constants is[32]

$$1/k_{cat} = 1/k_{acylation} + 1/k_{deacylation} \quad (4)$$

It is apparent from this relationship that if a variable affects two of the rate constants similarly, then it must also affect the third rate constant in the same manner. The data on *p*H effects, deuterium oxide effects, structural effects and solvents appear to conform to such an interdependent relationship, leading to a large set of consistent data which indicates the mechanistic equivalence of the acylation and deacylation steps.

Let us now consider the implication of this equivalence with respect to the mechanisms of these processes. Let us assume that acylation and deacylation correctly describe the catalytic processes, and that α-chymotrypsin-catalyzed isotopic exchange reactions proceed through the same steps as the hydrolysis reaction. Paralleling eq. 2 one can then write eq. 5 and 6 for the enzyme-catalyzed isotopic exchange of carboxylic acids[46b]

$$\mathrm{RCO^*H + En \rightleftharpoons RCO^*H\cdot En \underset{HO^*H}{\overset{k_2}{\rightleftharpoons}}}$$

$$\mathrm{RCEn \underset{k_{-3}}{\overset{k_3\ HOH}{\rightleftharpoons}} RCOH\cdot En \rightleftharpoons RCOH + En} \quad (5)$$

$$\mathrm{RCOMe^* + En \rightleftharpoons RCOMe^*\cdot En \underset{Me^*OH}{\overset{k_2}{\rightleftharpoons}}}$$

$$\mathrm{RCEn \underset{k_{-3}}{\overset{k_3\ MeOH}{\rightleftharpoons}} RCOMe\cdot En \rightleftharpoons RCOMe + En} \quad (6)$$

with $H_2{}^{18}O$ and the isotopic exchange of methyl-^{14}C esters with methanol,[46a] respectively. Equations 2, 5 and 6 are equivalent to one another except for the interchange of carboxylic acid for ester, and water for methanol in certain places. Neither of these changes is very profound, although it may be argued that methanol, which contains only one proton capable of hydrogen bridging, is significantly different from water, which contains two such protons. The isotopic exchange reactions are of course, symmetrical about the acyl-enzyme. From the principle of microscopic reversibility, one can therefore set step $k_2 = k_{-3}$ and step $k_3 = k_{-2}$, if one neglects isotope effects. From the principle of microscopic reversibility the transition state of the k_3 step must contain exactly the same microscopic components as the transition state of the k_{-3} step. Therefore the transition states of steps k_2 and k_3 must also be identical to one another. Thus all steps k_2, k_{-2}, k_3 and k_{-3} are equivalent to one another, passing through the same transition state. Since it is known that the acyl-enzyme RCOEn is an alkyl ester (of a serine hydroxyl group),[12] each transition state in eq. 6 can be described as consisting of an ester, an alcohol and the enzyme.[56,57]

(54) For ethyl esters: K. Kindler, *Ann.*, **452**, 90 (1927); *Ber.*, **69B**, 2792 (1936).

(55) Unpublished observations of Dr. R. J. Thomas, Dr. F. Kézdy and Dr. G. Clement.

(56) (a) There is apparently a difference in the pK_a's of methanol and the hydroxyl group of serine of about 2 pK units.[56b] However, this difference does not significantly affect the symmetry argument;

The equivalence of all steps can only have meaning if the chemical processes underlying these steps are equivalent. Thus the conclusions reached from the symmetry and microscopic reversibility arguments, as well as experimentally, are the same: that the acylation and deacylation processes are mechanistically identical to one another. Any mechanism therefore must satisfy not only the various pieces of information concerning each individual step, but also must satisfy the condition that the chemical processes of the two steps must be equivalent to one another according to the conditions outlined above for the isotopic exchange reaction.

The Mechanism of α-Chymotrypsin Action.—On the basis of the previous section, a consideration of either the acylation or deacylation reaction will lead to a complete description of the mechanism of α-chymotrypsin-catalyzed hydrolyses since the two are mechanistically equivalent to one another. For this reason it is advantageous to consider the deacylation of *trans*-cinnamoyl-α-chymotrypsin, since the most detailed and reliable information concerning any individual α-chymotrypsin-catalyzed step is available for this reaction.

The features of the deacylation of *trans*-cinnamoyl-α-chymotrypsin pertinent to mechanistic considerations consist of effects of *p*H, deuterium oxide and urea on the reaction. The deacylation reaction is dependent on a group with an apparent pK_a of 7.15 indicating the participation of a basic group on the enzyme surface in the deacylation process. This basic group may be the imidazole moiety of a histidine residue or an α-ammonium ion, since these are the only enzymatic groups with pK_a's around neutrality.[58,59]

It should be reiterated at this time that the constant of any pre-equilibrium occurring before the rate-determining step of the deacylation reaction will be absorbed into the apparent pK_a and may alter its value from one solely reflecting the ionization of a group on the enzyme surface.[60] This possibility, however, seems remote for several reasons. One is that the pre-equilibrium constant(s) in question might be expected to vary from substrate to substrate and thus the apparent pK_a should vary. This situation has apparently never been found in an α-chymotrypsin-catalyzed reaction. A second reason is that the magnitude of the equilibrium constant necessary to result in an apparent pK_a different from that of the pK_a of the ionizable group must be very large, in general, for those suggestions which have been made.[60] This restriction, although not an impossible condition, limits the applicability of the idea of hidden pre-equilibria.

The deacylation of *trans*-cinnamoyl-α-chymotrypsin is independent of *p*H from 8.5 to 12.9, indicating that no acidic groups of apparent pK_a less than 13.9 are involved in the reaction. This restriction eliminates the possible participation of general acids such as the phenolic group of a tyrosine residue (intrinsic pK_a 9.95),[58] the ε-ammonium ion of a lysine residue (intrinsic pK_a 10.2),[58] the guanidinium ion of an arginine residue ($pK_a \sim 13.5$),[61] and the alcoholic group of a serine or threonine residue (pK_a 13.6).[62] The data do not rule out, however, the possible participation of general acids whose pK_a's are above 13.9 such as an amide group, a peptide group or a water molecule.

On the basis of model studies,[63] the basic group with an apparent pK_a of 7.15 may participate in the deacylation as a general basic catalyst or as a nucleophilic catalyst. Imidazole has been shown to catalyze ester hydrolyses through either mechanism while an α-ammonium ion would be expected to catalyze an ester hydrolysis only through general basic catalysis. Criteria that have been used for the differentiation of these two mechanistic pathways include: (1) observation of the unstable intermediate formed in nucleophilic catalysis; (2) isotopic tracer experiments whose results may be rationalized only by the formation of an unstable intermediate of a nucleophilic catalysis; (3) deuterium oxide solvent effects which in a general basic catalysis will be larger than in a nucleophilic catalysis; and (4) the effect of structure of the leaving group on reactivity which is different in the two kinds of catalysis. 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.[12] These negative experiments unfortunately do not provide unambiguous information concerning the differentiation of nucleophilic and general basic catalysis, since, if an acylimidazole intermediate were formed, it would be expected to be present only in small concentration, from a thermodynamic consideration of the reactant and the intermediate. In the deacylation of *trans*-cinnamoyl-α-chymotrypsin, no isotopic tracer experiment has been devised to differentiate between nucleophilic and general basic catalysis. Nucleophilic catalysis alone cannot account for the deacylation mechanism, since the considerable effect of deuterium oxide on the deacylation rate con-

if eq. 6 were transformed to an exchange reaction of a trichloroethyl ester and trichloroethanol (which has approximately the same pK_a as the serine hydroxyl) the same kind of exchange reaction would be observed, and the symmetry would be complete; (b) T. C. Bruice, T. H. Fife, J. J. Bruno and N. E. Brandon, *Biochemistry*, **1**, 7 (1962).

(57) The equivalence of steps k_2 and k_3 assumes that in the transition states of these reactions the oxygen atom of the ester linkage (or of methanol) and the oxygen atom of the serine hydroxyl group are sterically equivalent to one another (with respect to the carbonyl carbon atom).

(58) C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.*, **78**, 5287 (1956), have analyzed the titration curve of ribonuclease, which contains very similar substituents to α-chymotrypsin, and have determined the following intrinsic pK_a's: imidazole, 6.5; and an α-ammonium ion, 7.8.

(59) Derived groups such as an oxazoline group or an acylated guanidine group have also been suggested: H. N. Rydon, *Nature*, **182**, 928 (1958); B. F. Erlanger, *Proc. Natl. Acad. Sci. U. S.*, **46**, 1430 (1960).

(60) T. C. Bruice and G. L. Schmir, *J. Am. Chem. Soc.*, **81**, 4552 (1959).

(61) Guanidination of the thirteen lysine residues of chymotrypsin has previously indicated that the ε-ammonium ions of lysine are not involved in the catalytic action; C. H. Chervenka and P. E. Wilcox, *J. Biol. Chem.*, **222**, 635 (1956).

(62) The pK_a of the model compound N-acetylserinamide has been found to be 13.6, significantly lower than that of ordinary alcohols.[56b]

(63) M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.*, **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).

stant may only be explained on the basis of a rate-determining proton transfer such as occurs in general basic catalysis.[16] Of course a combination of a nucleophilic and a general acidic catalysis could exhibit such a deuterium oxide effect, so that the isotope effect in itself does not rule out nucleophilic catalysis. Comparison of the Hammett ρ-constant for the effect of leaving groups in the acylation reaction with the ρ-constants for model reactions involving nucleophilic or general basic catalysis indicates that the acylation reaction resembles the former process.[42] Evidence for the differentiation of nucleophilic from general basic catalysis thus appears to be ambiguous. The conclusion derived from Hammett ρ-constants is probably the least reliable because of the tenuous assumptions on which it is based. The failure to observe an unstable intermediate is consistent with either mechanism. The deuterium oxide effect favors general basic catalysis, only if general acidic catalysis may be independently ruled out; independence of the deacylation rate constant up to pH 12.9 goes a long way toward meeting this condition, but does not do so absolutely. Therefore one may not unequivocally decide on nucleophilic or general basic catalysis at this time, although the writer leans toward general basic catalysis on the basis of the deuterium oxide experiments.

Studies of the effect of structure on reactivity, involving the alkyl portion of ester substrates, indicate that the dominating influence on the reactivity in both acylation and deacylation reactions reflects a nucleophilic attack at the carbonyl carbon atom of the ester.[42] This is further substantiated by the effect of the acyl portion of ester substrates, shown in Table II, where the relative enzymatic rate constants parallel the relative rates of alkaline hydrolysis.

The mechanistic features of the individual steps outlined above can now be fused into a mechanism of α-chymotrypsin action on the basis of the equivalence of the acylation and deacylation steps outlined in the previous section. It was pointed out there that in the isotopic exchange reaction involving the α-chymotrypsin-catalyzed methanolysis of a methyl ester, step k_2 must be equivalent to step k_{-3} and also step k_2 must be equivalent to step k_3. This in essence means that in this reaction all mechanistic processes of acylation and deacylation, both forward and reverse, must be equivalent. If this isotopic exchange reaction (eq. 6) is extrapolated to the normal hydrolytic reaction (eq. 2), the only change necessary is substitution of water for methanol in the deacylation step. Such a substitution would not affect the *enzymatic participants* in the catalytic processes, which of course must remain the same. Therefore we can use the highly symmetrical isotopic exchange reaction as a convenient way to discuss the mechanistic requirements of any α-chymotrypsin-catalyzed reaction.

Mechanisms of α-chymotrypsin action have been devised on the basis of the following factors: (1) the occurrence of a two-step catalytic mechanism in which the intermediate compound, the acyl-enzyme, is in alkyl ester (of a serine moiety); (2) the equivalence of the two catalytic steps of acylation and deacylation through experimental observations and symmetry and microscopic reversibility arguments; (3) the nucleophilic character of each catalytic step; (4) the effect of pH on the rates of the individual steps, indicating the participation of a base or nucleophile in the catalytic process; (5) spectrophotometric and kinetic experiments indicating no build-up of an intermediate containing an acyl group bound to imidazole in deacylation; and (6) the effect of deuterium oxide on the rates of the individual steps, indicating the occurrence of a rate-determining proton transfer in the catalysis.

Using the above criteria it is possible to rule out a number of mechanisms.[64] Several proposals have been made to the effect that general basic catalysis occurs in the acylation step while nucleophilic catalysis occurs in the deacylation step.[32,65,66] These proposals violate the principle of detailed balancing (sometimes called the principle of microscopic reversibility) in its most simple form when applied to eq. 6. Suggestions have been made that nucleophilic catalysis by an imidazole or an oxazoline group occurs in both the acylation and deacylation steps.[59,67] These proposals are inconsistent with the deuterium oxide isotope effects.

One mechanism meeting the above criteria employs two simultaneous catalytic functions such as a general acid and a general base. In eq. 7 all four reaction steps (2, −2, 3 and −3) are catalyzed by the same entities, combination of a general base (which removes a proton from the attacking nucleophile) and a general acid (which donates a proton to the leaving group). In acylation (step 2) B and HA are operative while in deacylation (step 3) the kinetically equivalent combination of BH^+ and A^- are operative. The scheme is completely symmetrical about the acyl-enzyme not only with regard to the reactants but also with regard to the catalytic entities. Furthermore, and more importantly, the scheme conforms to the requirement of the identity of the two *forward* steps of acylation and deacylation, both with regard to the reactants and also with regard to the catalytic entities (assuming the equivalence of (B and HA) with (BH^+ and A^-)). This scheme thus conforms to the symmetry requirements of the exchange reaction. It is suggested that this form of catalysis be called "conjugate catalysis," for a base and its conjugate acid are operative in the two catalytic steps, as are an acid and its conjugate base. General acidic–general basic catalysis similar to that shown in eq. 7 has been proposed by a number of workers previously, although the conclusions have resulted from lines of reasoning different from those presented here.[68−72] It should

(64) It is, of course, much easier to disprove a mechanism than to "prove" one.

(65) L. W. Cunningham, *Science*, **125**, 1145 (1957).

(66) G. H. Dixon and H. Neurath, *J. Am. Chem. Soc.*, **79**, 4558 (1957).

(67) F. H. Westheimer, *Proc. Natl. Acad. Sci. U. S.*, **43**, 969 (1957).

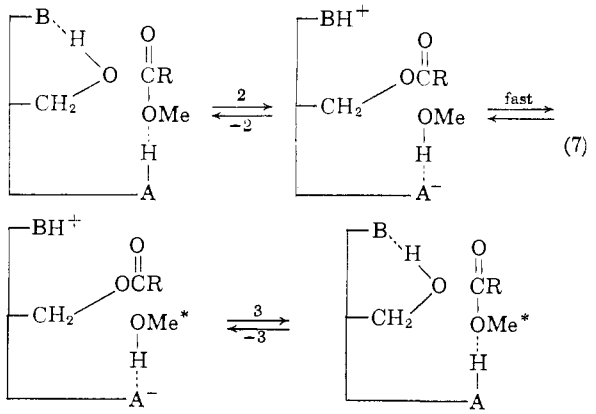
(68) K. J. Laidler, *Discussions Faraday Soc.*, **20**, 83 (1955); R. M. Krupka and K. J. Laidler, *J. Am. Chem. Soc.*, **83**, 1458 (1961).

(69) J. E. Scott, *Nature*, **172**, 777 (1953).

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

be pointed out that whereas the participation of the general base postulated in eq. 7 rests on a firm experimental basis, the participation of the general acid does not.

GENERAL ACIDIC–GENERAL BASIC (CONJUGATE) CATALYSIS

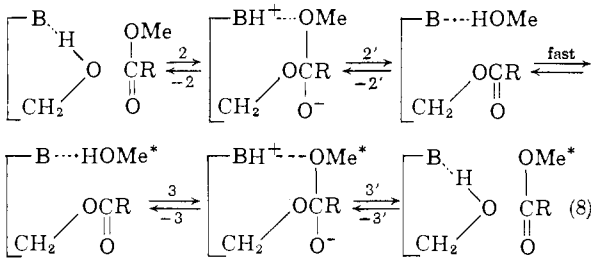


(7)

A mechanism involving general acidic–nucleophilic catalysis conforms to the symmetry requirements of the reaction as well as to the available experimental evidence of the individual steps, and is an additional mechanistic possibility whose over-all reaction is very similar to that of eq. 7.

A second group of mechanisms employs only one catalytic functionality, such as a general base. Several possibilities of this nature exist. One suggestion along these lines is that the tertiary nitrogen and the NH group of an imidazole moiety may serve as both the general acid and the general base.[73] This mechanism is of course a variant of the "conjugate catalysis" mechanism of eq. 7 in which HA and B are constituent parts of the same group. Another mechanism employing only one catalytic functionality is shown in eq. 8.[74] The

GENERAL BASIC CATALYSIS INVOLVING TETRAHEDRAL INTERMEDIATES

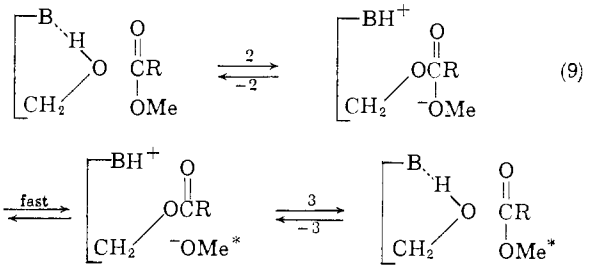


(8)

mechanism in eq. 7 omits consideration of the formation of tetrahedral addition intermediates in each of the steps 2 and 3, while eq. 8 takes this possibility into account. Present experimental evidence does not provide any clue to such a possibility, although it might be assumed by analogy with corresponding non-enzymatic reactions that such intermediates would be formed.[75] If indeed tetrahedral addition intermediates are formed in steps 2 and 3, B could serve as a general basic catalyst in the *formation* of the tetrahedral intermediate (steps 2, 3, −2′, −3′) and BH^+ could serve as a general acidic catalyst in its *decomposition* (steps 2′, 3′, −2, −3), in the acylation and deacylation steps, meeting the symmetry requirements of the reaction. If such tetrahedral intermediates have a reasonable lifetime, this mechanism is a distinct possibility.

Catalysis by a lone general base[76] not operating through a tetrahedral intermediate (eq. 9) also

GENERAL BASIC CATALYSIS



(9)

conforms to the mechanistic criteria set forth above. The base would remove a proton from the attacking alcohol in step 2 and its conjugate acid (BH^+) would donate a proton to the alkoxyl group of the ester in step 3. However, since the transition states of these processes are identical to one another, this mechanism conforms to the symmetry as well as the other requirements of the reaction.[77] It is conceivable that other mechanisms consistent with the experimental evidence also may be forthcoming.[78]

At the present time, 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 mechanisms embodied in eq. 7, 8 and 9 currently are being tested in experiments involving model systems and in further experiments with α-chymotrypsin.

Acknowledgment.—The author is pleased to acknowledge stimulating discussion and exacting criticism from his collaborators, Drs. G. A. Hamilton, E. T. Kaiser, G. R. Schonbaum and B. Zerner.

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

(72) B. R. Rabin, *Biochem. Soc. Symp.*, **No. 15**, 21 (1958).

(73) A suggestion of this kind has been made by M. A. Marini and G. P. Hess, *Nature*, **184**, 113 (1959).

(74) The author would like to acknowledge this suggestion of Dr. G. A. Hamilton. After this paper was submitted for publication, a mechanism of essentially this form was proposed: T. C. Bruice, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1924 (1961).

(75) M. L. Bender, *Chem. Revs.*, **60**, 53 (1960).

(76) Equation 9 is a slight variant of the mechanism given by T. Spencer and J. M. Sturtevant, *J. Am. Chem. Soc.*, **81**, 1874 (1959).

(77) This mechanism was incorrectly ruled out in ref. 3.

(78) See M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961) for a suggestion for the active site involving an adduct of the serine hydroxyl group and the imidazole ring.