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.

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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.
(2) M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).

specificity in these terms by means of a template-type pattern. One may consider specificity in terms of binding and thus in terms of relative Michaelis constants, but this procedure is open to many criticisms. One must first assume that the Michaelis constants are true equilibrium constants of binding, and do not contain any rate constants of subsequent steps. Such an assurance is difficult to obtain because of the difficulty in determining whether the Michaelis constant is an equilibrium constant (k_{-1}/k_1) or a steady-state constant ($(k_{-1} + k_2)/k_1$) and secondly whether it is a true equilibrium constant (k_{-1}/k_1) or an apparent equilibrium constant ($k_{-1}k_3/k_1(k_2 + k_3)$). Even if the above ambiguities could be overcome and measurement of the gross binding could be accomplished straightforwardly, determination of the stereospecificity of binding, including the problem of nonproductive binding,[4,5] is difficult to carry out. Finally it must be realized that binding of a substrate to a protein has no inherent relation to catalytic activity, for D-substrates bind very well to α-chymotrypsin, but for all practical purposes do not react; furthermore, substrates bind to chymotrypsinogen, and again no reaction is observed. The problem of interpretation of Michaelis constants is therefore a complicated one, and the use of Michaelis constants to define specificity is difficult at best.

On the other hand, one can infer stereospecific bindings favorable to reaction from the rate constants of the enzymatic process, and define in this fashion a "kinetic specificity." Catalysis implies kinetics; therefore specificity of catalysis may be defined in terms of a "kinetic specificity," the exceptional reactivity of the enzymatic process relative to that of the nonenzymatic reaction. The use of rates of α-chymotrypsin-catalyzed hydrolyses for this purpose does not eliminate complications of interpretation, unfortunately, for the two-step catalytic process discussed previously results in three possible rate constants: k_2, the rate constant of acylation; k_3, the rate constant of deacylation; and k_{cat}, the over-all catalytic rate constant defined as $1/k_{cat} = 1/k_2 + 1/k_3$. However, this complication has been resolved in enough cases of interest to make feasible the following discussion.[6]

Experimental

Materials.—The enzyme, the determination of the normality of its solution, the buffers, N-acetyl-L-tryptophan ethyl ester, N-acetyl-L-tyrosine ethyl ester, N-acetyl-DL-tryptophan *p*-nitrophenyl ester *trans*-cinnamoyl-α-chymotrypsin,[7] and have been described.[8,9] Acetyl-α-chymotrypsin was prepared in the following way. Five ml. of a stock α-chymotrypsin solution (3×10^{-3} *M*) was pipetted into pH 3.5 acetate buffer; 250 μl. of *p*-nitrophenyl acetate, 1.5×10^{-5} *M*, was added. The reaction was allowed to proceed for 24 hr. at 25° and 24 hr. at 5°, and was centrifuged at 13,000 r.p.m. for 1 hr. The liberated *p*-nitrophenol was removed by chromatography on a Sephadex column, made by equilibrating 1 g. of Sephadex G-25 in pH 3.5 acetate buffer for 60 hr. The amount of free α-chymotrypsin in the acetyl-α-chymotrypsin was shown to be less than 3% by titration with N-*trans*-cinnamoylimidazole. The acetyl-α-chymotrypsin was stored at 5° and used within 48 hr. of preparation. The product was shown to give the same deacylation rate constant as that prepared by the method of Balls and Wood[10] and that prepared at high pH with *p*-nitrophenyl acetate.[11]

Kinetic Measurements.—The determination of the k_{cat} of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester utilized the experimental methods and calculations described previously.[9] The kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L tyrosine ethyl ester was determined by measurement of initial rates of reaction at 300 mμ. The difference spectrum of N-acetyl-L-tyrosine ethyl ester *vs.* N-acetyl-L-tyrosine at pH 8.6 and 3.1% acetonitrile–water, $\Delta\epsilon_{300}$, = −113. The k_{cat}'s were calculated using Lineweaver and Burk[12] plots.

The kinetics of the deacylation of *trans*-cinnamoyl-α-chymotrypsin have been described previously.[7] One method of the determination of the deacylation of acetyl-α-chymotrypsin used the internal indicator of the hydrolysis of N-acetyl-L-tyrosine ethyl ester to monitor the reappearance of enzyme. The measurements were carried out in a Cary 14PM recording spectrophotometer according to Dixon and Neurath.[13] The first-order appearance of the free enzyme from the acyl–enzyme is seen in the increasing rate of hydrolysis of N-acetyl-L-tyrosine ethyl ester, which approaches a limiting value as the deacylation of the acyl–enzyme approaches completion.[13]

A second method of following the deacylation of acetyl-α-chymotrypsin does not involve an internal indicator of the kinetics but rather an external one. Acetyl-α-chymotrypsin was allowed to deacylate for a specified length of time at pH 8.6. The reaction was then stopped by adding sufficient hydrochloric acid to lower the pH to 1.5 where the acetyl group does not hydrolyze. After quenching at pH 1.5 (for periods of less than an hour, which lead to no significant denaturation), a rate assay of the amount of free enzyme was carried out at pH 5.0 using excess N-acetyl-DL-tryptophan *p*-nitrophenyl ester. The slope of the (zero-order) straight line obtained in the rate assay is proportional to the free enzyme concentration. From these zero-order rates, the rate constant of deacylation of acetyl-α-chymotrypsin may be obtained.

Results

The temperature dependence of the deacylation rate constant, k_3, of two acyl–enzymes, acetyl-α-chymotrypsin and *trans*-cinnamoyl-α-chymotrypsin, were measured, as shown in Table I and in Fig. 1. These deacylations were carried out at pH 8.6 to 8.8, in the pH-independent region of the pH–k_3 profile of these reactions, and thus any small temperature de-

TABLE I

TEMPERATURE DEPENDENCE OF THE DEACYLATION OF TWO ACYL-α-CHYMOTRYPSINS[a,e]

Temp., °C.	$k_3 \times 10^4$, sec.$^{-1}$	Temp., °C.	$k_3 \times 10^4$, sec.$^{-1}$	Temp., °C.	$k_3 \times 10^4$, sec.$^{-1}$
trans-Cinnamoyl-α-chymotrypsin[d]		Acetyl-α-chymotrypsin[b,d]		Acetyl-α-chymotrypsin[c,d]	
11.5	4.52	6.9	1.95	11.3	13.9
20	8.18	9.2	2.73	16.0	15.4
25	12.5	14.5	3.80	20.0	22.4
30	22.4	18.0	4.58	25.0	33.6
35	34.3	25.0	6.80	31.5	46.5
		27.9	9.85		

[a] pH of buffers at 25° was 8.6–8.8. [b] Deacylation in the absence of any added substrate. [c] Deacylation in the presence of 2.33×10^{-3} *M* N-acetyl-L-tyrosine ethyl ester. [d] 3.2% (v./v.) acetonitrile–water and 0.1 *M* Tris–HCl buffer. [e] The errors in the rate constants are ±5%.

(3) E. Fischer, *Ber.*, **27**, 2985 (1894); *Z. physiol. Chem.*, **26**, 60 (1898).
(4) H. T. Huang and C. Niemann, *J. Am. Chem. Soc.*, **74**, 4634, 5963 (1952); G. E. Hein and C. Niemann, *ibid.*, **84**, 4497 (1962).
(5) S. A. Bernhard and H. Gutfreund, "Proc. Intern. Symp. Enzyme Chemistry," Maruzen, Tokyo, 1957, p. 124.
(6) Nonproductive binding[4,5] may also complicate enzymatic rate constants. However, in the following discussion, only k_3 involving a covalently-bound substrate is considered, where nonproductive binding should be minimal. This problem will be considered in a future publication.
(7) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **4**, 2562 (1962).
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(11) F. J. Kézdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962).
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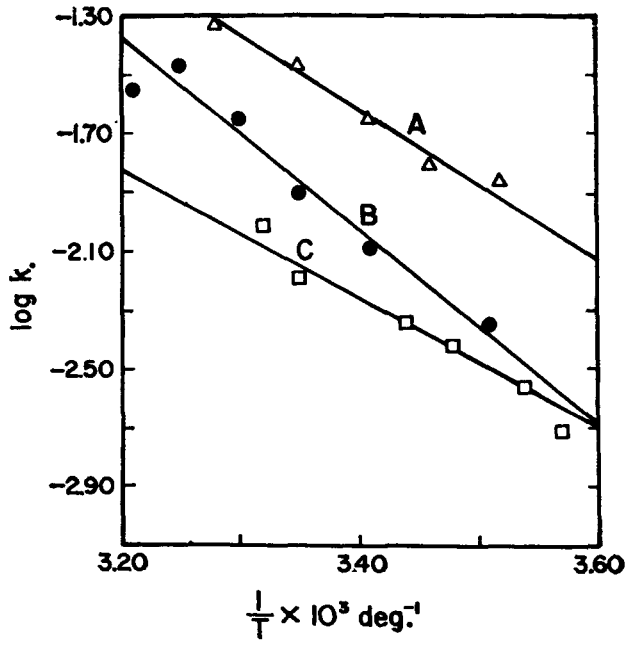

Fig. 1.—The kinetics of deacylation of some acyl–chymotrypsins at pH 8.6 to 8.8 in 3.2% (v./v.) acetonitrile–water: A, acetyl-α-chymotrypsin in the presence of 2.33×10^{-3} *M* N-acetyl-L-tyrosine ethyl ester; B, *trans*-cinnamoyl-α-chymotrypsin; C, acetyl-α-chymotrypsin.

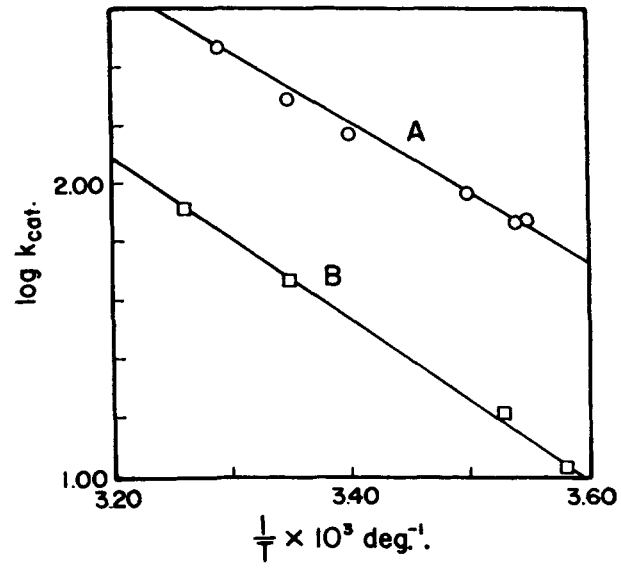

Fig. 2.—The kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester (A) and N-acetyl-L-tryptophan ethyl ester (B) at pH 8.55 to 8.70 in 1.8% (v./v.) acetonitrile–water.

pendence of the pK_a of the deacylation reaction will have no effect on these experiments.

The temperature dependence of the deacylation of acetyl-α-chymotrypsin was carried out using two different experimental procedures. The first involves the deacylation in the absence of any added substrate. The analysis of this reaction is performed on aliquots of the reaction mixture which are quenched by lowering the pH; the free enzyme of the aliquot is then measured by a rate assay at a low pH. The second method involves the deacylation in the presence of N-acetyl-L-tyrosine ethyl ester, a specific substrate of α-chymotrypsin, whose hydrolysis serves as a measure of the regeneration of free enzyme and thus as a measure of the deacylation reaction. The absolute magnitude of the deacylation rate constant in the latter procedure is about five times that in the former procedure at a given temperature. A similar increase in the rate constant of the deacylation of acetyl-α-chymotrypsin in the presence of indole has been noted.[14] In both these accelerations, the presence of a (portion of a) specific substrate appears to promote the deacylation reaction significantly.

The temperature dependence of the catalytic rate constants of the α-chymotrypsin-catalyzed hydrolysis of two specific substrates, N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tryptophan ethyl ester, as also measured, as shown in Table II and Fig. 2. These hydrolyses were also carried out in a pH independent region of pH 8.55 to 8.70 so that any small temperature dependence of the pK_a of the hydrolysis will have no effect on the results. The activation parameters, calculated from Fig. 1 and 2, are listed in Table IV.

Discussion

Table III presents kinetic constants of a representative group of α-chymotrypsin-catalyzed reactions of interest to the problem of specificity and binding. The catalytic rate constants are directly determined from experiment, using the Michaelis–Menten kinetic analysis. The individual rate constants, k_2 and k_3 were determined according to methods[15] discussed in detail in various papers of this series. The composite constant, k_{cat}, is not the constant of choice for a discussion of specificity. The acylation rate constant, k_2, describes the reaction of an adsorbed substrate molecule with the enzyme to give an acyl–enzyme and the first product (*e.g.*, alcohol or ammonia). Specificity may be discussed in terms of relative k_2 values, but such comparisons must be restricted to substrates containing similar leaving groups, such as methyl and ethyl esters, *p*-nitrophenyl esters, or the N-acylimidazoles. A consideration of the most extensive groups, the methyl and ethyl esters, shows a variation of 10^5-fold between the most reactive compound, N-acetyl-L-tyrosine ethyl ester, and the least reactive compound, methyl cinnamate.[16] There is a considerable kinetic specificity in these reactions, above that predicted

TABLE II

TEMPERATURE DEPENDENCE OF THE CATALYTIC RATE CONSTANTS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF TWO SPECIFIC SUBSTRATES[a,b]

Temp., °C.	k_{cat}, sec.$^{-1}$	Temp., °C.	k_{cat}, sec.$^{-1}$
N-Acetyl-L-tyrosine ethyl ester		N-Acetyl-L-tryptophan ethyl ester	
8.6	75.5	6.3	10.7
9.6	74.9	10.9	16.7
12.9	94.2	25.0	46.2
20.8	151	34.0	83.2
25.0	193		
31.0	295		

[a] pH of Tris–HCl buffer at 25° was 8.55–8.70; 1.8% (v./v.) acetonitrile–water. [b] The errors are ±5% in all cases.

(14) R. J. Foster, *J. Biol. Chem.*, **236**, 2461 (1961).

(15) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3669 (1964).

(16) The aryloxy group of *p*-nitrophenyl acetate must bind in approximately the same position as the *acyl* group of N-acetyl-L-tyrosine ethyl ester, since the binding of aromatic groups is of great importance. These two enzyme–substrate complexes are thus stereochemically different, and any comparison of the k_2 kinetic specificities of these two compounds are meaningless. Fortunately, this complication does not occur in the k_3 step.

TABLE III

KINETIC CONSTANTS OF SOME α-CHYMOTRYPSIN-CATALYZED REACTIONS

	Substrate	k_{cat}, sec.$^{-1}$	k_2, sec.$^{-1}$	k_3, sec.$^{-1}$	k_{OH^-} of ethyl ester × 10^3,[f] M^{-1} sec.$^{-1}$	Rel. k_3 (corrected)[g]	Ref.
1	N-Acetyl-L-tyrosine ethyl ester	193	4000	193[b]	76	3540	17
2	N-Acetyl-L-phenylalanine ethyl ester	173	2000	173[a]	76	3180	18
3	N-Acetyl-L-tryptophan ethyl ester	46.5	374	46.5	76	942	8
4	N-Acetylglycine methyl ester	0.013	0.013	2.29[d]	85	38.2	19
5	Methyl hippurate	.143	.192	0.567[c]	93	4.9	19
6	Methyl hydrocinnamate	.018	.020	.18[e]	7.7	32.4	24–26
7	Methyl cinnamate	.0073	.0282	.0125	1.19	14.7	20
8	*p*-Nitrophenyl cinnamate	.0125		.0125	1.19	14.7	21
9	N-*trans*-Cinnamoylimidazole	.0125		.0125	1.19	14.7	21
10	*p*-Nitrophenyl acetate	.0068	4.8	.0068	9.5	1.0	22, 28
11	*p*-Nitrophenyl trimethylacetate	.00013	0.37	.00013	0.0223	8.1	23
12	N-Benzoylimidazole			.0002	0.51	0.54	27
13	Methyl acetate	0 (?)	0 (?)	.0068	9.5	1.0	22

[a] The *p*-nitrophenyl and ethyl esters give essentially identical k_{cat} indicating that $k_{cat} = k_3$.[25] [b] The magnitude of K_m(app) indicates that $k_2/k_3 = 18$.[15] [c] Value determined from the turnover of the corresponding *p*-nitrophenyl ester. [d] Value determined from the turnover and presteady state of the corresponding *p*-nitrophenyl ester. [e] Values determined from the methanolysis action. [f] Estimated from data in the National Bureau of Standards Circular 510, "Tables of Chemical Kinetics," corrected to aqueous solution and 25°. [g] $(k_{3(i)})(k_{OH}(\text{acetate}))/(k_3(\text{acetate}))(k_{OH}(i))$.

from electronic and steric factors operating in the alkaline hydrolysis of the corresponding esters.

The deacylation rate constant, k_3, describes the reaction of the acyl–enzyme with water to produce the carboxylic acid and regenerate the enzyme. *All* k_3 values in Table III may be compared with one another, leading to an extensive list of data covering a range of over 10^6-fold in rate constant. In the acylation step, an adsorbed substrate molecule reacts while in the deacylation step a substrate molecule bound covalently to the enzyme reacts. There is still considerable kinetic specificity in the deacylation reaction; in fact, the range of rate constants is somewhat greater in the deacylation reaction involving a chemisorbed substrate than in the acylation reaction involving a physically adsorbed substrate. This similarity in kinetic specificities is expected if the acylation and deacylation reactions are mechanistically similar to one another.[2]

Since the data available for deacylation rate constants are more extensive than for acylation rate constants, let us consider the kinetic specificities exhibited in deacylation, k_3, and assume that such specificities are representative of both steps. Of the total spread in the relative rates of acylation and deacylation, a small part may be attributed to the electronic and steric factors manifest in the corresponding nucleophilic reactions. A correction has been made in Table III for these effects in the relative rates of deacylation, by using the relative saponification rate constants for the corresponding ethyl esters, on the basis that the Hammett ρ-constants (susceptibility to electronic effects) of the deacylation reaction and saponification reactions are almost identical.[27] The corrected rates have been normalized, setting the acetate derivative as 1. Of the million-fold variation seen in the deacylation series, about 10^2-fold must be attributed to normal electronic effects, (steric effects are not fully accounted for), leaving approximately a 10^4-fold rate enhancement which must be attributed to specificity by the enzyme (column relative k_3 (corrected) of Table III). Thus in deacylation, a complete spectrum of specificity is still seen.

The contributions to this specificity are interesting. Two rate comparisons involve the addition of an N-acetylamino group to the molecule, the relative k_3's of 4 *vs.* 10, and 2 *vs.* 6. These comparisons result in rate ratios of 38.2 and 98, respectively. Two other rate comparisons involve the addition of a benzyl group to the molecule, the relative k_3's of 6 *vs.* 10, and 2 *vs.* 4. These comparisons result in rate ratios of 32.4 and 83. The contributions of an N-acetylamino group and of a benzyl group to the specificity are thus independent of one another, within a factor of approximately two. These contributions indicate that specificity is a relative and not an absolute quantity. Further, the spectrum of specificity may be analyzed in terms of the independent contributions of two important components to the total specificity.

Let us assume that the kinetic specificity observed in deacylation is the full specificity exhibited by α-chymotrypsin and inquire how one may explain this phenomenon. The rate enhancement of 10^4-fold exhibited in the specificity by α-chymotrypsin is certainly explainable in chemical terms, since series of related reactions differing by as much as 10^{14}-fold are seen in simple organic chemical reactions.[29]

A simple, chemical explanation for the 10^4-fold spread in the deacylation rate constants is depicted in Fig. 3 which illustrates the idea that although the acyl groups of the acyl–enzyme are covalently attached to the oxygen atom of serine, the placement of the car-

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(25) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **85**, 356 (1963).
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(27) M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 883 (1962).
(28) G. H. Dixon, W. J. Dreyer, and H. Neurath, *J. Am. Chem. Soc.*, **78**, 4810 (1956).
(29) M. L. Bender in "Technique of Organic Chemistry," A. Weissberger, Ed., 2nd Ed., Vol. VIII, Part II, John Wiley and Sons, Inc., New York, N. Y., 1963, p. 1471.

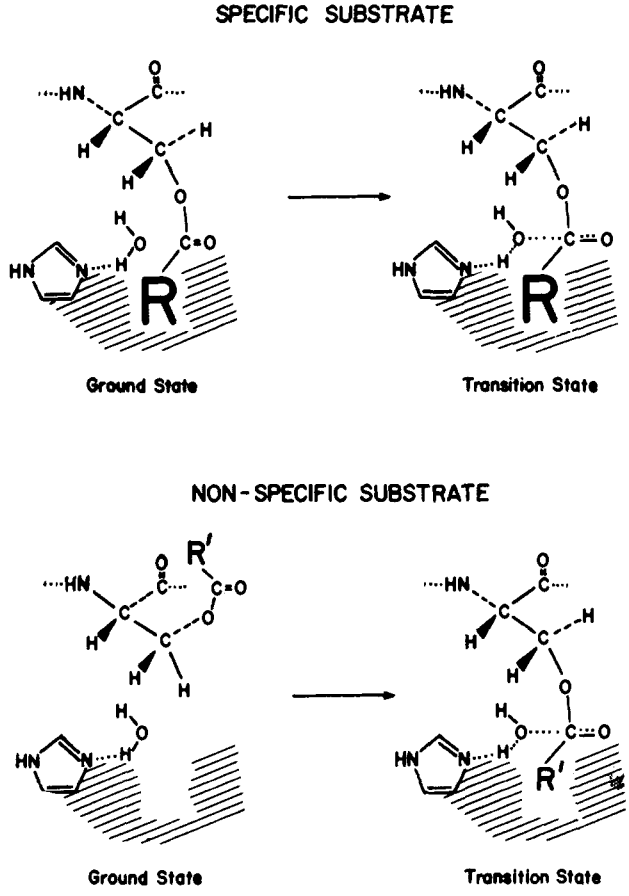

Fig. 3.—Specificity in the deacylation of acyl-α-chymotrypsins.

bonyl group of the ester in the ground state with respect to the water molecule and the catalytic entities of the protein is highly variable. Starting from the rigid polypeptide backbone of the protein (dotted lines from the NH and C=O groups) the side chain bearing the acyl group of the acyl enzyme may rotate around four single bonds in the ground state. In the transition state the rotation around these four bonds must be frozen so that the molecule may react with the water molecule and the catalytic entities on the enzyme surface. Specificity is then interpreted in terms of an interaction of the group *R* of the acyl group with the enzyme surface, *rigidifying* the whole set of bonds so that the carbonyl portion of the acyl group occupies the *correct* position for reaction, even in the ground state. Thus for a specific substrate, the reaction will be facile because the ground state of the acyl–enzyme resembles the transition state of the reaction, while for a nonspecific substrate the reaction will be slow because the ground state of the acyl–enzyme does not resemble the transition state.[30]

The requirement for the positioning of the carbonyl portion of the acyl group with respect to the catalytic components of the active site predicts that the specificity should be manifest in differences in the rotational entropy of activation measuring the correct positioning of the carbonyl group. The activation parameters of four deacylation reactions spanning the entire specificity spectrum have therefore been determined, as shown in Table IV.

(30) *Cf.* I. B. Wilson in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1954, p. 656.

TABLE IV

THE ACTIVATION PARAMETERS OF THE DEACYLATION OF SOME ACYL-α-CHYMOTRYPSINS[a,f]

Acyl–Enzyme	Rel. k_2 (corrected)[b]	ΔF^*, kcal./mole	ΔH^*, kcal./mole	$-T\Delta S^*$,[c] kcal./mole	ΔS^*, e.u.
N-Acetyl-L-tyrosyl–	3540	14.3	10.3	4.0[e]	−13.4
N-Acetyl-L-tryptophanyl–	942	17.9	12.0	5.9	−19.8
trans-Cinnamoyl–	14.7	20.1	11.2	8.9	−29.6
Acetyl–	1	20.4	9.7	10.7[d]	−35.9

[a] At pH 8.6 so that the temperature dependence of the ionization of pK_a 7 does not affect the results. [b] These values reflect the specificity imposed by the enzyme (see Table III). [c] $T\Delta S^*$ calculated at 298°K. [d] A ΔH^* of 15.1 and $T\Delta S^*$ of −4.7 kcal./mole have previously been reported for the deacylation of acetyl-α-chymotrypsin,[28] but these measurements were carried out in the presence of N-acetyl-L-tyrosine ethyl ester. [e] A ΔH^* of 10.9 and $T\Delta S^*$ of −3.2 kcal./mole were previously reported.[31] [f] Error analysis: ΔH^* ± 1 kcal.; ΔS^* ± 5 e.u.

The activation parameters for the deacylation of the specific acyl–enzymes, N-acetyl-L-tyrosyl– and N-acetyl-L-tryptophanyl–α-chymotrypsin, are based on the catalytic rate constants for the hydrolysis of the corresponding ethyl esters since deacylation is the rate-determining step of these hydrolyses.[8] The activation parameters of the deacylation of the relatively nonspecific acyl–enzymes, *trans*-cinnamoyl- and acetyl-α-chymotrypsin, were determined directly.

The acyl–enzymes in Table IV are arranged in order of descending specificity according to Table III. The enthalpies of activation are essentially constant for both specific and nonspecific substrates, whereas a more or less regular increase in $-T\Delta S^*$ occurs in going from specific to nonspecific substrates. Thus the kinetic specificity of deacylation is not caused by a difference in enthalpies of activation, but almost solely results from a large difference in entropies of activation. The difference in entropies of activation between the most specific and the least specific substrate is 23 e.u. The assignment of this difference in entropies of activation to differences in rotational entropies owing to torsional oscillations around single bonds is reasonable. Approximate calculations give values of 2.5 to 7.5 e.u. per single bond for the entropy associated with torsional oscillations.[32] Experimental determinations of the activation entropy associated with the freezing of rotation of one bond is 4 to 6 e.u.[33] Using the value of 6 e.u. per bond, it is thus calculated that zero to four bonds must be frozen in going from the ground state to the transition state in the various reactions of Table IV, a reasonable number. In terms of Fig. 3, none of the four single bonds which determine the position of the carbonyl group needs to be frozen in the activation process for a specific substrate since they are already correctly frozen in the ground state. On the other hand, all four of these single bonds need to be frozen in the activation process for a completely nonspecific substrate. Thus the entropy of activation of the latter process should be approximately 24 e.u. more negative than for the former process, as observed experimentally.

Both the kinetic parameters and the activation parameters of the deacylation of acetyl-α-chymotryp-

(31) J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **182**, 577 (1950).

(32) H. A. Scheraga, "Protein Structure," Academic Press, Inc., New York, N. Y., 1961, p. 40.

(33) E. G. Foster, A. C. Cope, and F. Daniels, *J. Am. Chem. Soc.*, **69**, 1893 (1947); P. D. Bartlett and R. R. Hiatt, *ibid.*, **80**, 1402 (1958).

sin differ depending on the presence of N-acetyl-L-tyrosine ethyl ester.[14,34] This observation may be explained on the basis of the binding of the phenolic group of the N-acetyl-L-tyrosine ethyl ester to the active site, leading to a partial fixation of the acetyl moiety, a lowering of the rotational entropy of activation, and thus an increase in the rate constant of the reaction. This picture is born out in the fact that the ΔS^* of deacylation of acetyl-α-chymotrypsin in the presence of N-acetyl-L-tyrosine ethyl ester is −27.2 e.u. while that in the absence of this compound is −35.9 e.u.

This discussion of the specificity of deacylation is unique in that it is based on reactions in which the substrate is covalently bound to the enzyme. This fact reduces the number of variables pertinent to the specificity, and provides a starting point for a detailed analysis of the meaning of specificity. The interpretation given here is essentially a modern physicochemical description of what Fischer called the lock and key theory, more recently called the template theory of specificity. Of course, there is no act of inserting a key in a lock in the deacylation step, but the requirement of a rigid correct fit between the substrate and enzyme, leading to the maximal rate of reaction, is implicit in both Fischer's early description and the present refinement.

The findings of an additive specificity pattern, that the specificity is related to the entropy of activation, and that the specificity as defined both in rate and in activation entropy terms may be enhanced by an added, extraneous molecule may possibly be interpreted in ways other than that presented here. One such interpretation is embodied in the "induced-fit" theory[35] which would explain the specificity of the deacylation step by varying degrees of induction of the correct conformation of the catalytic entities of the active site by various acyl groups. Likewise, the "induced-fit" theory would explain the increase in rate of deacylation by the addition of an extraneous reagent by its induction of a better conformation of the catalytic entities of the active site.

The specificity of deacylation may also be interpreted in terms of the introduction of strain into that part of the substrate which is to undergo reaction.[36,37] Presumably the binding forces available to the enzyme produce such a strain and an enhanced reaction, the magnitude of which would then be proportional to the magnitude of the binding forces and thus result in a variable specificity.

Distortions of the substrate to produce a reactive species have been discussed from two points of view, involving static and dynamic enzymes. Within the context of the transition state theory, distortion of the substrate must be viewed as a change in conformation stabilized by the enzyme which approximates the conformation of the transition state. Bruice has made an ingenious suggestion in the fremework of the distortion hypothesis to explain the efficiency of α-chymotrypsin,[38] based on the adsorption of an ester substrate on the enzyme in a lactone conformation. However, whereas

(34) *Cf.* T. Inagami and T. Murachi, *J. Biol. Chem.*, **238**, PC 1905 (1963).

(35) D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U. S.*, **44**, 98 (1958).

(36) R. Lumry in "The Enzymes," Vol. I, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, Inc., New York, N. Y., 1959, Chapter 4.

(37) W. P. Jencks, *Ann. Rev. Biochem.*, **32**, 658 (1963).

(38) T. C. Bruice, *J. Polymer Sci.*, **49**, 100 (1961).

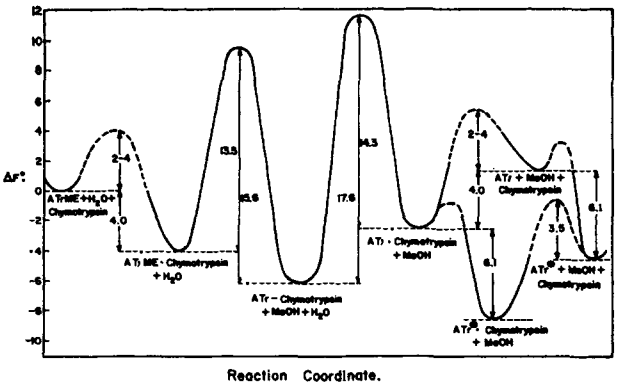

Fig. 4.—Standard free energy *vs.* reaction coordinate diagram for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester at 25°.

the *cis* form of an ester (a lactone) is exceptionally reactive, the *cis* form of an amide (a lactam) is not exceptionally reactive. Therefore, it is difficult to accept this specific suggestion as an important contributor to enzymatic efficiency.

It is clearly not possible to differentiate between the three suggestions given above to explain the specificity of deacylation. The detailed discussion of the rotational explanation of the specificity is given for two reasons. One is that to the present authors it is the simplest explanation requiring the least extrapolation from current chemical theory. The second is that it has not been described before in this form. The explanation of specificity given in this paper is satisfying in that it explains the magnitude of the activation entropy effect in a precise manner, whereas entropy changes in an induced fit might be much larger. However, definitive experiments to distinguish between these possibilities must still be sought.

Conclusions

At this time it is possible to discuss an over-all pathway, a mechanism of each step, and specificity of each step for catalysis by α-chymotrypsin. Let us now consider how these factors account for the kinetics of the hydrolysis of the specific amide substrate, N-acetyl-L-tryptophan amide.

First consider the α-chymotrypsin-catalyzed hydrolysis of the analogous specific ester substrate, N-acetyl-L-tryptophan methyl ester, all of whose individual rate constants have been determined.[2] The standard free energy *vs.* reaction coordinate diagram for this enzymatic process, Fig. 4, is well defined, there being only two minor areas of uncertainty—those representing the activation energies of the presumably diffusion-controlled reactions involving enzyme–substrate formation from enzyme and substrate, and enzyme–product formation from enzyme and product. In going from the methyl ester reactant to the carboxylate ion product, there are two intermediate energy minima, occurring at successively lower standard free energies, so that from reactant to product, each step in the process leads to a lower standard free energy.

The standard free energy *vs.* reaction coordinate diagram for the α-chymotrypsin-catalyzed hydrolysis of the corresponding amide, N-acetyl-L-tryptophan amide, Fig. 5, is normalized so that the product acid is identical in standard free energy in both Fig. 4 and 5.

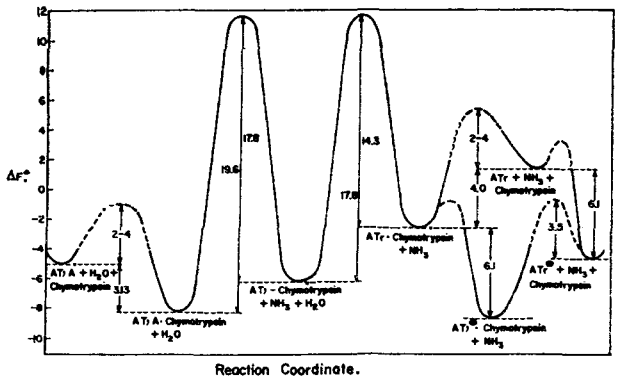

Fig. 5.—Standard free energy *vs.* reaction coordinate diagram for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide at 25°.

From the N-acetyl-L-tryptophanyl-α-chymotrypsin intermediate to the product, the diagrams for the hydrolysis of the methyl ester and amide are identical with one another. Representative amide and methyl ester pairs indicate that the amide is about 5 kcal./mole more stable than the corresponding ester.[39] The standard free energy of the amide with respect to the rest of the diagram is confirmed by the equilibrium measurements of amide formation, leading to a difference in free energy between the amide and the carboxylic acid of 6.4 kcal./mole,[40] which is also found when setting the amide 5 kcal./mole lower than that of the ester. Knowing this relationship, the standard free energies of activation of k_2 from the corresponding rate constant and of the K_s of the amide[19] completely fixes the standard free energy *vs.* reaction coordinate diagram for the amide hydrolysis. Again, the free energy of the important minima of the amide reaction, the acyl–enzyme intermediate, and the carboxylate ion product successively decreases. Thus, the acyl–enzyme intermediate is not a high energy intermediate with respect to the amide reactant, and one may say, not only kinetically[2] but also thermodynamically, that the acyl–enzyme intermediate is favored in the overall pathway of the reaction.

Let us now compare in some detail the nonenzymatic (hydroxide ion-catalyzed) hydrolysis and the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide. The second-order rate constant for the reaction of hydroxide ion with acetamide is $3.78 \times 10^{-5}\ M^{-1}$ sec.$^{-1}$ in water at 25°. From the hydrolytic rate constants of the esters N-acetyl-L-tryptophan ethyl ester and ethyl acetate, it is concluded that the N-acetyl-L-tryptophanyl substituent increases the saponification by eightfold, and therefore the second-order rate constant for the hydrolysis of the (unsubstituted) amide group of N-acetyl-L-tryptophan amide is $3 \times 10^{-4}\ M^{-1}$ sec.$^{-1}$.[41] The rate constant for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan amide at pH 8, the pH of maximal rate in water at 25°, is 4.4×10^{-2} sec.$^{-1}$.[19] Thus, the nonenzymatic and enzymatic reactions differ not only quantitatively, but also in kinetic form, the former being a second-order process and the latter being a first-order process. However, let us inquire as to the factors which account for the difference between the nonenzymatic and enzymatic processes.

The difference between the nonenzymatic and enzymatic processes must be attributed to more than one cause. Differences in reactivity in organic chemical reactions may frequently be expressed in terms of three (additive) contributions to the difference in standard free energies of activation: polar, steric, and resonance effects.[42] In seeking the explanation to the difference between two forms of catalysis, other factors than these will be involved, but certainly more than one factor must be considered.

The comparison of an intramolecular general basic catalysis by imidazole (the enzymatic process) with an intermolecular hydroxide ion catalysis (the nonenzymatic process) is a knotty problem. It may be resolved by (1) comparing the efficacy of intermolecular general basic catalysis by imidazole with intermolecular hydroxide ion catalysis, and by (2) using the hypothesis that an intramolecular general basic catalysis by imidazole is equivalent to intramolecular general basic catalysis by 10 *M* imidazole. The first step of this process is given by the rate constants of the hydrolysis of haloacetate esters catalyzed by hydroxide ion and by imidazole as an intermolecular general base. This comparison indicates that 1 *M* imidazole = $1.6 \times 10^{-6}\ M$ hydroxide ion.[43] The second step of this process depends on the observation, made many times before, that the *effective* concentration of an intramolecular catalytic group is the equivalent of *ca.* 10 *M* of the corresponding intermolecular catalyst.[44] On this basis, the second-order hydroxide ion-catalyzed hydrolysis of rate constant, $3 \times 10^{-4}\ M^{-1}$ sec.$^{-1}$, may be transformed into an intramolecular first-order imidazole-catalyzed hydrolysis of rate constant, 4.8×10^{-9} sec.$^{-1}$. That this transformation has meaning is seen in a calculation of the rate constant of the intramolecular general base-catalyzed hydrolysis of *p*-nitrophenyl 3-nitrosalicylate[45] from data on intermolecular reactions. In this reaction a phenoxide group of pK_a 6.02 is the intramolecular general basic catalyst. The rate constant of the hydroxide ion-catalyzed hydrolysis of *p*-nitrophenyl 3-nitrobenzoate is 21.2 M^{-1} sec.$^{-1}$. General basic catalysis by a base of pK_a 6.02 (mechanistically B + H_2O) is 0.58×10^{-6} less efficient than is catalysis by hydroxide ion.[43] Using this factor and the hypothesis that the effective concentration of intramolecular phenoxide ion is the equivalent of *ca.* 10 *M* of intermolecular phenoxide ion, the intramolecular rate constant for the hydrolysis of *p*-nitrophenyl 3-nitrosalicylate is calculated to be 1.26×10^{-4} sec.$^{-1}$. The experimental rate constant is 1×10^{-4} sec.$^{-1}$, in reasonable agreement. Thus, the calculated conversion of intermolecular hydroxide ion catalysis of N-acetyl-L-tryptophan amide hydrolysis to intramolecular general basic catalysis by imidazole has a solid analogy.

In comparing the nonenzymatic and enzymatic reactions, one compares a nonenzymatic reaction involving one intermediate, a tetrahedral addition com-

(39) F. H. Carpenter, *J. Am. Chem. Soc.*, **82**, 1111 (1960).

(40) H. Morawetz and P. S. Otaki, *ibid.*, **85**, 463 (1963).

(41) This constant cannot be measured directly since hydroxide ion cannot distinguish between the two amide linkages in the molecule.

(42) R. W. Taft, Jr., in "Steric Effects in Organic Chemistry," M. S. Newman, Ed., John Wiley and Sons, Inc., New York, N. Y., 1956, Chapter 13.

(43) W. P. Jencks and J. Carriuolo, *J. Am. Chem. Soc.*, **83**, 1746 (1961).

(44) M. L. Bender, *Chem. Rev.*, **60**, 53 (1960); *cf.* D. E. Koshland, Jr., *J. Theoret. Biol.*, **2**, 75 (1962).

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

pound,[46] to an enzymatic reaction involving at least three intermediates, two enzyme–substrate complexes and one enzyme–substrate compound and, if one counts possible tetrahedral intermediates, two more.[47] Thus one large activation process is distributed among several smaller activation processes. This description of catalysis has been often considered in the past; it is, however, interesting to see it come to light.

The chemistry which makes possible the stepwise pathway described above consists of both complex and compound formation between the enzyme and substrate. Complex formation between reactant and catalyst does not, in itself, facilitate reaction. If, however, complex formation not only lowers the ground-state energy but also lowers the transition-state energy (to a greater extent than the ground state), then complex formation may contribute to the over-all catalytic process.

Compound formation between the enzyme and the acyl group of the substrate is a key contributor to the stepwise process leading to the lowered multiple activation process. The acyl–enzyme intermediate is slightly lower than the amide substrate in standard free energy and is thus a reasonable intermediate in the catalytic process, from a thermodynamic point of view. However, a kinetic factor is also involved in the conversion of the amide substrate to the acyl–enzyme intermediate. In amide hydrolysis catalyzed by α-chymotrypsin, this step, an alcoholysis reaction since the nucleophile is a serine hydroxyl group, is the rate-determining step. On the other hand, in amide hydrolysis catalyzed by hydroxide ion, the rate-determining step is a hydrolysis. The *alcoholysis* of a carboxylic acid derivative, in general, occurs at a rate which is approximately 100-fold faster than the *hydrolysis* of the corresponding compound.[26] Thus, the conversion of the rate-determining step of the saponification of an amide from hydrolysis to alcoholysis should increase the rate constant by 10^2-fold. Implicit in this calculation is the assumption that the concentration of the serine hydroxyl group in acylation equals the concentration (55 *M*) of water in hydrolysis.

The enzyme imposes a kinetic specificity in its reactions, accounted for previously, in terms of a steric facilitation manifest in the lowered (rotational) entropy of activation. Whether one accepts this explanation or others, this rate enhancement amounts to 942 in the case of N-acetyl-L-tryptophanyl derivatives using the acyl group, acetate, as a standard. Although this calculation was carried out with deacylation rate constants, data indicate that this kinetic specificity is also operative in acylation. Thus in both acylation and deacylation steps, kinetic specificity will increase the rate constant of hydrolysis another factor of 10^3. This specificity factor is valid only on the assumption that the calculation made previously in converting the reaction from an intermolecular process to an intramolecular process produced an intramolecular process with complete freedom of rotation (or the equivalent in entropy).

Finally, consideration must be given to the fact that the enzymatic catalysis has the characteristics of both general basic and general acidic catalysis. Catalysis by the imidazole(s) on the enzyme is postulated to involve two proton transfers, one to an imidazole group and one from an imidazole group, as well as a nucleophilic attack, all occurring in a concerted cyclic process.[2] In the calculation, proton abstraction by imidazole (general basic catalysis) has been accounted for, but proton donation by imidazole (general acidic catalysis) has not. The effect of an intramolecular proton transfer on the hydrolysis of an ester may be seen in the hydrolyses of the compounds $CH_3CH_2C(O)SCH_2CH_2NH^+(CH_3)_2$ and $CH_3CH_2C(O)SCH_2CH_2N^+(CH_3)_3$. The former hydrolyzes 240 times faster than the latter,[48] which may be most reasonably interpreted in terms of the internal proton transfer which is available to the former but not the latter compound. Therefore a factor of 10^2-fold will be attributed to the general acidic catalysis function of imidazole in the enzymatic process, assuming additivity of the general basic and acidic components.

We have thus far considered five aspects of the enzymatic process: (1) its intramolecular character and the concomitant increase in effective concentration of the catalytic group(s); (2) the general basic catalysis by imidazole; (3) the change in rate-determining step of the hydrolysis of an amide to an alcoholysis; (4) the freezing of the substrate in a conformation resembling the transition state; and (5) the general acidic catalysis by imidazole. A summation of the effects of each factor on our comparison between the nonenzymatic and enzymatic processes is shown in Table V. It is seen that these five factors successfully account for the total enzymatic rate constant. The workings of the enzyme α-chymotrypsin can thus be discussed on a straightforward chemical basis, where no single factor is all-important and where each factor is based qualitatively on solid chemical analogy and each factor except the last is based quantitatively on solid chemical analogy. This combination produces an enzyme.

TABLE V

KINETIC FACTORS RESPONSIBLE FOR THE DIFFERENCE BETWEEN THE HYDROXIDE ION AND α-CHYMOTRYPSIN-CATALYZED HYDROLYSES OF N-ACETYL-L-TRYPTOPHAN AMIDE

Rate constant of hydroxide ion catalysis	$3 \times 10^{-4}\ M^{-1}$ sec.$^{-1}$
(1) Conversion to an intermolecular general base-catalyzed reaction involving imidazole (1.6×10^{-6})	$4.8 \times 10^{-10}\ M^{-1}$ sec.$^{-1}$
(2) Conversion to an intramolecular general base-catalyzed reaction involving imidazole (10 *M*)	4.8×10^{-9} sec.$^{-1}$
(3) Change in rate-determining step (10^2)	4.8×10^{-7} sec.$^{-1}$
(4) Freezing of the substrate specificity (10^3)	4.8×10^{-4} sec.$^{-1}$
(5) General acidic catalysis by imidazole (10^2)	4.8×10^{-2} sec.$^{-1}$
Total calcd. enzymatic rate on the basis of the above five factors	4.8×10^{-2} sec.$^{-1}$
Experimental rate constant	4.4×10^{-2} sec.$^{-1}$

(46) M. L. Bender, *J. Am. Chem. Soc.*, **73**, 1626 (1951).

(47) Tetrahedral addition intermediates have not been depicted in Fig. 4 and 5, but their omission does not affect the over-all conclusions.

(48) B. Hansen, *Acta Chem. Scand.*, **12**, 324 (1958).