THE MODE OF ACTION OF THE CRYSTALLINE PANCREATIC PROTEOLYTIC ENZYMES

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CONTENTS

I. Introduction

The term "proteolytic enzymes" is used herein to denote those enzymes which catalyze the hydrolysis of internal or terminal peptide bonds in peptides or proteins. This ability to catalyze the hydrolysis of substrates of their own kind places the proteolytic enzymes in a unique position among proteins in general, and among enzymes in particular. Since the proteolytic enzymes appear to be devoid of a prosthetic group of nonprotein character to which their specificity may be ascribed, their catalytic function must be associated with a unique configuration of the amino acid residues within these protein molecules.

Despite recent rapid strides, the amino acid analysis of the proteins has not yet sufficiently progressed to yield the complete stereochemical structure of any protein in terms of the sequence of the constituent amino acid residues. The prospects for determining the nature of the catalytic centers of the proteinases by direct analytical procedures appear, therefore, remote. However, an indirect approach to this problem lies in the determination of the structural requirements of specific substrates for these enzymes, positive and negative results being of equal importance in such an analysis. The ultimate aim of this approach is to deduce, from the structure of the substrates, the complementary structures on the enzyme surface which are most compatible with combination and activation of the model substrates.

This approach is neither novel nor restricted to proteolytic enzymes. Its force has, perhaps, been most keenly felt in the field of immunochemistry, as exemplified by the pioneer work of Landsteiner (170) on the specificity of serologic reactions, and its subsequent extension by Pauling and coworkers (210, 215, 216). There, as here, however, the ultimate limit of resolution lies in the fact that even if the structure of the active centers can be elucidated, and duplicated by analysis, such a model would probably be devoid of biological activity. For, as with other manifestations of biological activity which are mediated by specific proteins, enzymatic activity will most likely be found to be dependent not only on the integrity of an active center but on that of the protein surface as a whole.

The experimental approach to the specificity and mode of action of the proteolytic enzymes has been rendered possible by the pioneer work of two groups of distinguished investigators: that of Northrop, Kunitz, and Herriott (207) and of Anson (12) on the purification and crystallization of the pancreatic proteinases; and that of Bergmann and coworkers (21, 22, 23, 27, 88), who have shown that for some of these enzymes synthetic peptides and peptide derivatives can take the place of the immensely more complex protein substrates. The application of quantitative kinetic measurements to these well-defined enzyme–substrate systems may be regarded as the third prerequisite for the present approach.

Studies on the metabolic requirements of microörganisms have shown that the cellular activities can be retarded and even completely inhibited by structural analogs of specific metabolites (284, 290). It was obvious that analogous studies on the effect of structural analogs of specific substrates for proteolytic enzymes would eventually result in the recognition of specific inhibitors for these enzymes. This was recently accomplished for two of the present enzymes, i.e., carboxypeptidase (254) and chymotrypsin (138). It was also found that the susceptible peptide bond can be replaced by the ester bond and that the resulting esters are hydrolyzed perhaps even more rapidly than the parent peptides, provided the remaining structural requirements of substrates are fulfilled (232). Conversely, if the peptide bond is replaced by a group of higher bond strength, such as the ketone group, or entirely omitted, the resulting compounds behave as potent inhibitors for the enzymes (134).

It is of historical interest that the hydrolysis of amino acid esters by crude pancreatic extracts was first demonstrated by Otto Warburg in 1905 (280) and was used by him and others for the enzymatic resolution of racemic mixtures of amino acids (6, 281). It should be noted, however, that since these investigations preceded by decades the isolation of the pancreatic proteases, no unequivocal proof was provided in this early work that the enzymatic hydrolysis of these amino acid esters was mediated by proteolytic enzymes. The demonstration of a specific esterase activity of trypsin (232), subsequently also extended to chymotrypsin and carboxypeptidase (48, 138, 254), is of recent origin.

The present review is limited to the three crystalline proteinases of the pancreas, i.e., trypsin, chymotrypsin, and carboxypeptidase, which exemplify the behavior of endopeptidases and exopeptidases, respectively. This restriction was made not only in the interest of economy of space but also because quantitative measurements with highly purified preparations of these proteolytic enzymes minimize the doubt that the properties ascribed to any one enzyme may actually be those of other enzymes, present as adventitious impurities.

This review is further limited to a consideration of the action of these enzymes on synthetic substrates of known chemical structure and configuration. It is to be recognized, however, that the range of specificities of some of these enzymes toward protein substrates may be wider than that determined from specificity measurements toward low-molecular-weight substrates. This expectation arises from the greater affinity of the enzyme for the larger protein substrate and the

less critical steric requirements in the formation of the enzyme–substrate complex.

The interaction between enzyme and substrate may be considered as a specific case of binding of a low-molecular-weight compound by proteins. It will be shown elsewhere in this review (page 94) that the kinetics of enzymatic reactions may be derived in part from the equations proposed for the statistical binding of ions and molecules by proteins. Considered from another viewpoint, it should be recognized that enzymatic reactions represent just one type of catalytic reactions and, accordingly, are interpretable in terms of the principles of catalysis in general, which have been so brilliantly explored and formulated by H. S. Taylor (265).

II. The Pancreatic Proteases

No attempt will be made to review the early literature on the pancreatic proteases. From present knowledge it is clear that studies carried out on unfractionated extracts of the complex mixture of zymogens, active enzymes, and inhibitors present in pancreatic tissue could only give rise to ambiguous and contradictory results. References to early studies in this field are given by Northrop and Kunitz (206) and by Kunitz and Northrop (166).

The modern history of the pancreatic proteases began in 1931, when Northrop and Kunitz (204) announced the crystallization of trypsin. Within seven years these investigators and their colleagues at the Rockefeller Institute for Medical Research also isolated in crystalline form trypsinogen, the inactive precursor of trypsin, α-, β-, and γ- chymotrypsins, their inactive zymogen, chymotrypsinogen, pancreatic trypsin inhibitor, and carboxypeptidase. The mechanism of activation of these enzymes and the action of pancreatic trypsin inhibitor were elucidated. Of equal importance with this brilliant experimental work was the demonstration that these enzymes are themselves proteins and that their activity depends entirely upon their molecular integrity. This work has been summarized by Northrop, Kunitz, and Herriott (207).

A. Preparation of Enzymes and Inhibitors

Crystalline trypsin was first prepared from the fraction of commercial "trypsin" which was soluble in quarter-saturated ammonium sulfate but insoluble in half-saturated ammonium sulfate. When a cold saturated solution of this fraction in ammonium sulfate was warmed slowly to 25°C., square platelets of trypsin appeared (204).

This procedure was soon modified (206) in that an acidified pancreatic exudate was used as the source material. The activity of the product was increased twofold by utilizing the observation that trypsin is reversibly denatured by heat while contaminating proteins are irreversibly denatured.

In 1933 Kunitz and Northrop (162) announced the crystallization of chymotrypsinogen from an acid extract of fresh pancreas in quarter-saturated ammonium sulfate at pH 5. This crystalline protein was activated to chymotrypsin by small amounts of trypsin. The chymotrypsin formed was isolated in crystalline form.

In the following year Kunitz and Northrop (164) reported that the chymotrypsinogen *mother liquor* could be activated by enterokinase or autocatalytically in concentrated salt solutions. From this mother liquor they isolated trypsinogen (165) and demonstrated that it could be activated by enterokinase or by trypsin to yield trypsin identical with that previously isolated. In 1937 a complete scheme for the isolation of trypsinogen, trypsin, chymotrypsinogen and chymotrypsin, trypsin inhibitor, and trypsin–inhibitor compound was described (167).

Kunitz (161) has shown that salt-free chymotrypsinogen and chymotrypsin can be crystallized from dilute ethanol solutions and Jacobsen (124) found that concentrated salt-free solutions of chymotrypsinogen crystallize at pH 5 to form needles and at pH 3 to form a mixture of needles and rhombohedrons.

Kunitz' report of the isolation of β- and γ-chymotrypsins, one of the most remarkable contributions in the field of protein chemistry, was published in 1938 (151). It was observed that only about one-half the activity of a solution of chymotrypsinogen, activated by trypsin, could be isolated as crystalline chymotrypsin. When the chymotrypsin mother liquor, or a solution of chymotrypsin itself, was allowed to stand for several days, needle-shaped prisms appeared. These crystals were found to be a solid solution of two active enzymes with inactive protein. Crude β-chymotrypsin crystals were purified by allowing the enzyme to digest the inactive protein contaminant.

From experience in this laboratory, one hundred beef pancreas glands yield 150 g. of crystalline chymotrypsinogen of which, after eight recrystallizations, 100 g. remains. This can be activated to yield 48 g. of crystalline chymotrypsin. The yield of crystalline trypsinogen is 64 g., which can be activated to yield 35 g. of crystalline trypsin. These weights refer to filter cakes, dried as thoroughly as is possible by vacuum filtration.

Recently Jacobsen (124) has reported that when chymotrypsinogen is activated at 0°C., using much larger amounts of trypsin than were used by Kunitz and Northrop (167), the resulting solution has a specific activity about 50 per cent greater than that of α-chymotrypsin. From a kinetic analysis of the activation process it was postulated that a new enzyme, π-chymotrypsin, is formed by the tryptic hydrolysis of one peptide bond of chymotrypsinogen. The activity of π-chymotrypsin is about twice that of α-chymotrypsin. π-Chymotrypsin is destroyed by two competing reactions: The first is a tryptic hydrolysis of one peptide bond to form a second new enzyme, δ-chymotrypsin, which has a specific activity about 1.5 times that of α-chymotrypsin and which represents the maximum measurable specific activity of the system following rapid activation. The second reaction is an autolytic or spontaneous hydrolysis of about three peptide bonds to form α-chymotrypsin. Neither π-chymotrypsin nor δ-chymotrypsin was isolated.

Keith, Kazenko, and Laskowski (141) isolated a new zymogen, B-chymotrypsinogen, from the fraction of an acid extract of pancreas which was soluble between 0.2 and 0.4 saturation with ammonium sulfate. When a concentrated solution of this fraction at pH 6.5 was adjusted to pH 5.5, crystals resembling the long prisms of chymotrypsinogen were formed. Dialysis of the mother

liquor from these crystals against pH 5.5 acetate buffer resulted in the formation of crystalline plates. These two crystalline forms were interconvertible.

B-Chymotrypsinogen has some proteolytic activity toward casein, but its activity is increased twenty- to thirty-fold upon activation by trypsin or by enterokinase. The rate of activation by trypsin shows little dependence on pH between pH 5 and 9.

Brown, Shupe, and Laskowski (50) succeeded in crystallizing B-chymotrypsin by dialyzing the activated protein against pH 5.5 acetate buffer. If the solution was initially alkaline to pH 5.5, short needles were formed; if initially acid to this pH, long prisms appeared. These crystals had unchanged activity through three recrystallizations. α-Chymotrypsin does not crystallize under the conditions used for B-chymotrypsin, but chymotrypsinogen does crystallize under the conditions used for B-chymotrypsinogen, except that plates are never formed.

Other reports of the isolation of crystalline pancreatic proteases are those of Tazawa (266) and Hofmann and Bergmann (112). Northrop and Herriott (203) believe the enzyme which Tazawa called trypsin to be chymotrypsin. Hofmann and Bergmann (113) later withdrew their claim of a new pancreatic protease, an error in analytical procedures having led them to an erroneous conclusion.

Carboxypeptidase (carboxypolypeptidase in the older literature) was crystallized by Anson (11, 12) in 1935, both from beef pancreatic exudate and from an extract of the whole pancreas. The state of knowledge of this enzyme at about that time has been reviewed by Bergmann (22) and by Waldschmidt-Leitz (278). The preparation of this enzyme, according to Anson's procedure, entails the following major steps: (*1*) Spontaneous autolysis in the refrigerator for the conversion of procarboxypeptidase to the active form. (*2*) Acidification to pH 4.6, followed by 2 hr. heating at 37°C. to aid in the precipitation of a coagulum which is removed. (*3*) Tenfold dilution of the filtrate with water, which results in the precipitation of a euglobulin which is collected after several hours of standing. (*4*) Extraction of the euglobulin precipitate with barium hydroxide at about pH 9, followed by gradual acidification of the clear extract with acetic acid until crystallization begins, usually at about pH 8.3. Recrystallization is accomplished by dissolving the crystals by the gradual addition of 0.1 *N* sodium hydroxide, followed by the addition of acetic acid.

This method has been subsequently modified by Putnam and Neurath (218), who have found that the redissolution of the first crystalline precipitate is greatly aided by the use of lithium hydroxide in place of sodium hydroxide, and by carrying out this procedure in the cold. An alternative method of recrystallization recommended by these authors consists in dissolving the crystals in 5 per cent lithium chloride, and dialyzing this solution first against 2 per cent sodium chloride and then against tap water and distilled water. With this latter method, large tabular crystals were obtained, in contrast to the short needles described by Anson. Smith and coworkers (245) have found that the insolubility of the contaminating proteins in solutions of 0.2 ionic strength affords a more rapid purification than repeated crystallization.

Recent experiments in this laboratory (194) have indicated that the yield of

carboxypeptidase can be greatly increased by observing the following precautions: (*1*) The pH of the freshly collected pancreatic juice is adjusted to 7.8, and the solution is incubated at 37°C. for approximately 1 hr. for maximum activation of procarboxypeptidase. (*2*) In contrast to Anson's procedure, the activated juice is adjusted to pH 4.6, immediately diluted tenfold with cold distilled water, and the granular euglobulin precipitate collected after 2 hr. This procedure is recommended, since activity measurements have shown that at pH 4.6 rapid inactivation of the enzyme occurs. (*3*) The euglobulin precipitate is extracted with barium hydroxide at pH 6, the enzymatically inactive, extracted material discarded, and the remaining euglobulin precipitate extracted repeatedly with barium hydroxide, in the cold, at pH 10.4, each extract yielding, upon acidification, a crystalline precipitate of carboxypeptidase. Activity measurements on activated juice indicated considerable variations from batch to batch, the average content being, approximately, 1.5 g. of carboxypeptidase per liter of pancreatic exudate, of which approximately 25 per cent is obtained as the final purified product.

Anson has stated (12) that thrice-recrystallized carboxypeptidase is free of dipeptidase and aminopolypeptidase activity and that the six-times-recrystallized enzyme is free of amylase but contains a minute trace of trypsin. Ågren and Hammarsten have reported that carboxypeptidase can be separated into three enzymes (8) and that trypsin can be removed from carboxypeptidase by electrophoretic separation (9). Anson has replied (14) that these impurities are readily removed by recrystallization with no loss in carboxypeptidase activity.

Although the zymogen of carboxypeptidase has not been isolated in pure form, Anson (13) has presented evidence for the existence of inactive procarboxypeptidase.

Several trypsin inhibitors have been isolated from biological materials in crystalline form. Kunitz (157) has isolated one factor from soybean meal by the following procedure: Defatted soybean meal is extracted with 0.25 *N* sulfuric acid and some impurities are adsorbed on small amounts of bentonite. The active protein is then adsorbed on a larger amount of bentonite. The inhibitor is eluted with dilute pyridine and the pyridine removed by dialysis. The dialysate is adjusted to pH 5.3 and a precipitate of inert protein is removed. The inhibitor protein is precipitated at pH 4.65 in the cold and redissolved at 36°C. at pH 5.1 to a concentrated solution. On standing at 36°C. needles and hexagonal plates appear. The protein can be recrystallized from dilute alcohol. This inhibitor reacts stoichiometrically with trypsin over a wide pH range. The trypsin–inhibitor compound has been isolated and its properties studied in some detail (159).

Tauber and coworkers (263, 264) have recently isolated a crystalline trypsin inhibitor from the lima bean. The initial step is a slight modification of the procedure of Kunitz (157) for the isolation of the soybean inhibitor. After the pyridine elution the inhibitor is precipitated with ammonium sulfate, dialyzed free of salt, and precipitated with acetone or lyophilized. The precipitate is then dissolved in 1 per cent sodium chloride, and impurities are removed by adsorp-

tion on a small amount of bentonite. One-fifth volume of acetone is added to the filtrate and colorless octahedra appear on standing in the cold. Recrystallization is performed by dissolving the crystals in 1 per cent sodium chloride and adding acetone to turbidity.

Kazal and coworkers (140) have isolated a pancreatic trypsin inhibitor from the supernatant solution remaining after the precipitation of insulin. After sodium chloride fractionation, dialysis, and ethanol fractionation, needle-like crystals were obtained at pH 3.25 in cold 2.5 per cent trichloroacetic acid. If the material was recrystallized from water adjusted to pH 3.25, mixed hexagonal plates and needles were obtained. About 75.3 micrograms of twice-recrystallized inhibitor was recovered from 1 kg. of frozen pancreas. Although this material could not be crystallized by the method of Kunitz and Northrop (167), the presence of three electrophoretic components in this material leaves open the possibility that inhibition is due to the same component in both preparations.

Although many studies have been carried out on the antitryptic factors of egg white (65) and of various legumes (39, 40), particularly with reference to the effect of these substances on nutrition, only those materials which have been obtained in pure form are considered in this review. It should be noted, however, that Bowman (41, 42) has found that extracts of both navy beans and soybeans contain more than one trypsin inhibitor. The inhibitor isolated by Kunitz (157) from the soybean appears to be different from that studied by Ham and Sandstedt (101). Lineweaver and Murray (180) have shown that the trypsin inhibitor of egg white is associated with the ovomucoid fraction.

B. ACTIVATION OF THE ENZYMES

At one time it was believed that the activation of the zymogens of the pancreatic proteases consists in the combination of zymogen with activator to form an active enzyme. However, evidence such as that presented by Bates and Koch (20) and by Guillaumie (99), who found the rate of activation of trypsinogen to be proportional to the amount of enterokinase added, supported the view that the reaction was enzymatic in nature. A clear understanding of these processes had to await the isolation of the materials in pure form. It is now known, from the researches of Kunitz, that activation of chymotrypsinogen and trypsinogen is an enzymatic process. A brief summary of the current knowledge of these processes is shown in table 1. Kunitz (153) has reviewed the kinetic studies from which the nature of most of these reactions was deduced.

MacDonald and Kunitz (185) have studied the effect of a great number of ions on the autocatalytic conversion of trypsinogen to trypsin. Some ions increase the rate of formation of inert protein, some increase the rate of formation of trypsin, some increase both rates, and others decrease both rates. Calcium salts in concentrations greater than 0.02 *M* completely repress the formation of inert protein (186).

The *penicillium* kinase discovered by Kunitz (152) appears in the culture medium late in the growth phase. It has a molecular weight of 40,000 and is inactivated between 50° and 60°C. It has a slight proteolytic activity at 35°C., but no detectable activity at 5–10°C.

TABLE 1

Activation of precursors of pancreatic enzymes

PRECURSOR	ACTIVATOR	NATURE OF REACTION	OPTIMUM pH	REMARKS	REFERENCES
Trypsinogen.....	Swine enterokinase	Catalytic	5.2–6	Good yields are obtained only at a slightly acid pH or in very dilute solutions	(155, 156)
Trypsinogen.....	Trypsin	Autocatalytic	7–9	Two competing reactions; one yields trypsin, the other inert protein	(154)
Trypsinogen.....	Trypsin + calcium salt	Autocatalytic	7–9	The reaction forming inert protein is completely suppressed by dilute solutions of calcium salts	(185, 186)
Trypsinogen.....	*Penicillium* kinase	Catalytic	3.4	Quantitative yields, since there is no autolysis in this pH range	(152)
Chymotrypsinogen..	*Penicillium* kinase	Catalytic	3.4	This reaction goes only 2 per cent as fast as the activation of trypsinogen	(152)
Chymotrypsinogen..	Large amounts of trypsin	Catalytic	7–9	At 0°C. products are π- and δ-chymotrypsins	(124)
Chymotrypsinogen..	Trace of trypsin	Catalytic	8.5	Product is α-chymotrypsin	(166)
α-Chymotrypsin.....	α-Chymotrypsin	Autolytic	4	After 1 month products are β- and γ-chymotrypsins with inert protein	(151)
α-Chymotrypsin.....	α-Chymotrypsin	Autolytic	8	After 1 hr. at 35°C. product is γ-chymotrypsin	(151)
Chymotrypsinogen..			None	Slow spontaneous activation, *ca.* 1 per cent per month at 5°C.	(166)
Procarboxypeptidase.....	Trypsin	Catalytic		Precursor has not been isolated in pure form	(13)

β-Chymotrypsin cannot be converted into γ-chymotrypsin (151). The reactions forming these enzymes from α-chymotrypsin are simultaneous and not progressive.

Kunitz and Northrop (166) found that five to six amino groups are released per mole in the activation of chymotrypsinogen to α-chymotrypsin (using a molecular weight of 36,000). Butler (55) confirmed this result and found the activation energy to be 16,300 cal. per mole. Jacobsen (124) found four amino groups per mole released during this activation. Butler's interpretations were criticized on the basis that no account was taken of the formation of π-chymotrypsin or of the difference in activity between π- and α-chymotrypsins (124).

Evidence that peptide chains are opened in the activation process also comes from the work of Woolley and Sprince (256, 292), who have found that tryptic hydrolysates of the zymogens have different contents of strepogenin, a peptide promoting the growth of *L. casei*, than do hydrolysates of the active enzymes. Taking the activity of a standard liver concentrate as unity, the activity of trypsinogen is 30, of trypsin 13, of chymotrypsinogen 2, of α-chymotrypsin 16, and of γ-chymotrypsin 9. A hydrolysate of insulin, the richest source of strepogenin, has 40 units of activity. An acid hydrolysate of pancreatic trypsin inhibitor has no activity. Woolley (291) has further reported that "pancreatin" digests of dinitrophenylinsulin and of dinitrophenyltrypsinogen each contain two long peptides of identical composition.

C. PROPERTIES OF ENZYMES AND INHIBITORS

A summary of the available data on some of the properties of the pancreatic enzymes and of their precursors and inhibitors is given in table 2. The properties of trypsinogen have not been extensively studied, since this protein can be recrystallized only in the presence of large amounts of a trypsin inhibitor.

Although the pancreatic proteases have been available in pure form for many years, only carboxypeptidase has been completely studied by modern physical methods. Studies of the other enzymes were begun in the laboratory of J. W. Williams, but this work was interrupted by the war and was not resumed (109, 285). The initial results obtained in this laboratory in a study of the molecular weights of the pancreatic proteases are included in table 2.

With the exception of those for carboxypeptidase (218) and chymotrypsinogen (109, 228), the diffusion constants shown in table 2 were determined by the porous disk method and should be checked by free diffusion techniques. Oncley (208) has suggested that the original value for α-chymotrypsin should be multiplied by the factor 1.11. The corrected value is also shown in table 2.

The values shown for the molecular weights of chymotrypsinogen and α-chymotrypsin show a much greater variation than is the case for most well-characterized materials. From the results of Kunitz (166) and of Jacobsen (124), it appears that the molecular weights of these two proteins and also that of δ-chymotrypsin must be virtually identical. Since from x-ray measurements the weight of the unit cell of α-chymotrypsin, corrected for residual water, is 108,000 (82), the molecular weights of these proteins must be some simple fraction of this

TABLE 2

Properties of pancreatic enzymes and of their precursors and inhibitors

PROTEIN	CRYSTAL FORM	NITROGEN	DIFFUSION CONSTANT*	SEDIMENTATION CONSTANT	MOLECULAR WEIGHT		pH OF ISOELECTRIC POINT	PURITY‡	pH OF MAXIMUM STABILITY
					Osmotic pressure	Other methods†			
		per cent	*d or D*	*Svedberg units*				*per cent*	
Trypsinogen	Triangular prisms (167)	15.3 (167)							
Trypsin	Square platelets (204); short prisms (206)	16.13 (167)	$d^{6°}$ = 0.023 (166) $d^{5°}$ = 0.020 (224)		36,500 (166)		7-8 (205)		2.3 at 30°C. (163)
Chymotrypsinogen	Long square prisms (166); rhombohedrons (124)	15.8 (166,124) 16.18 (43)	$d^{6°}$ = 0.039 (166) $D^{20°}$ = 7.9 (109) $D^{20°}$ = 8.9 (228)	2.5 (228) 3.1 (109)	36,500 (166)	36,800 (A) (43) 38,000 (SD) (109) 25,300 (SD) (228)	9.5 (10)	>95 (sol) (168, 54)	
α-Chymotrypsin	Rhombohedrons (166); monoclinic twins (124); needles (151)	15.83 (151) 16.06 (44)	$d^{6°}$ = 0.038 (166) $D^{20°}$ = 7.1 (208)	*ca.* 2.5 (227)	41,000 (166) 40,000 (151) 27,000 (19, 126)	32,000 (X) (37) 54,000 (X) (81, 82)	8.1-8.6 (10)	>95 (sol) (151)	3-3.5 at 37°C. (166)
β-Chymotrypsin	Rosettes of fine needles (151)	15.81 (151) 16.24 (44)			30,000 (151)			85-90 (sol) (151)	3-3.5 at 37°C. (151)
γ-Chymotrypsin	Bipyramidal polyhedrons (151)	15.69 (151) 16.00 (44)		*ca.* 2.3 (227)	27,000 (151)	30,100 (X) (82)		98 (sol) (151)	3-3.5 at 37°C. (151)
Pancreatic inhibitor	Square prisms (167)	11.25 (167)			6,000 (167)				
Trypsin-pancreatic inhibitor compound	Multifaced hexagons (167)	15.4 (167)			40,000 (167)				3-7 at 6°C. (167)
Soybean trypsin inhibitor	Hexagonal plates (157)	16.74 (158)	$d^{24°}$ = 0.07-0.08 (158)		24,000 ± 3,000 (158)		4.5 (158)	*ca.* 100 (sol) (158)	1-12 below 40°C. (158)
Trypsin-soybean inhibitor compound	Rosettes of needles (159)						5-5.2 (159)		*ca.* 2-11 below 30°C. (159)
Carboxypeptidase	Needles or boat-shaped prisms (12); hexagons (218)	14.4 (12)	$D^{25°}$ = 9.95 (218)	3.07 (245)		33,800 (SD) (245)§ 31,600 (VD) (218)	5.95 (218)	95-100 (E) (218); (S) (245)	6.5-8 at 5°C. (195)

* d is the diffusion constant expressed in square centimeters per day, and D is the diffusion constant expressed in square centimeters per second × 10^7. The superscripts denote temperature (°C.)

† The letters in parentheses denote the following methods for determining molecular weights: A = chemical analysis, S = sedimentation analysis, D = diffusion measurements, X = x-ray, and V = viscosity measurements.

‡ (sol) denotes solubility method; (E) denotes electrophoresis; (S) denotes sedimentation method.

§ Dr. F. W. Putnam has called to our attention that Smith, Brown, and Hanson (245) did not apply the full temperature correction to the diffusion constant used for this molecular weight calculation. The fully corrected value of $D^{20°}$ is 8.67 instead of 8.82, corresponding to a molecular weight of 34,300.

value. The choice of n, the number of molecules per unit cell, depends upon the best non-x-ray molecular weight determination. Perutz, as quoted by Fankuchen (81, 82), suggests that n is 2. However, from sedimentation and diffusion measurements made in this laboratory the molecular weight of chymotrypsinogen appears to be 25,300 (228), a value which would require that the number of molecules per unit cell be four.

A possible explanation of the discrepancy between the results obtained by sedimentation and diffusion and by the other determinations shown in table 2 lies in the fact that chymotrypsinogen and α-, γ-, and δ-chymotrypsins all tend to associate in solution. This tendency is so slight with chymotrypsinogen that it was overlooked in initial sedimentation measurements (227). A reinvestigation of the sedimentation rate at low concentrations confirmed the observation that a slight degree of dissociation occurs on dilution (228).

In the case of α-chymotrypsin, however, the observed sedimentation rate varies markedly with concentration and pH (227). A plot of $S_{20,w}$ against concentration for three pH values between pH 3.86 and pH 6.20 indicates that high pH values and low protein concentrations favor dissociation. Points determined at high concentrations fall on a single line, irrespective of pH, and this line extrapolates to a value of $S_{20,w} = 3.5$ Svedberg units at zero protein concentration. A single point, determined by Cecil with a Svedberg centrifuge (57), agrees reasonably well with determinations made in this laboratory.

These observations have been confirmed by viscosity measurements which indicate that the specific viscosity increment of the associated form of α-chymotrypsin is 0.058 while that of the dissociated form is 0.041, a value identical with that found for chymotrypsinogen (231). Since these viscosity increments indicate that the axial ratio of the associated form is about twice that of the dissociated form, the postulate derived from sedimentation data, that a simple reversible dimerization is involved, seems to be confirmed. Since β-phenylpropionic acid, a powerful competitive inhibitor of chymotrypsin, has no effect upon the monomer–dimer equilibrium (231), it appears that the enzymatically active centers of the molecule are not responsible for the observed dimerization reaction.

Although a complete study of the sedimentation behavior of δ-chymotrypsin has not been made, at low protein concentrations $S_{20,w}$ has been shown to increase with concentration. $S_{20,w}$, extrapolated to zero concentration, is 2.5 Svedberg units (230). In the ultracentrifuge γ-chymotrypsin behaves similarly to α-chymotrypsin, with the exception that the points determined at high concentrations fall on different lines for varying pH values, the sedimentation rate decreasing more rapidly with increasing concentration at lower pH values. Pedersen (211) has suggested that this phenomenon may be a reflection of different degrees of hydration of the dimer at different acidities. Extrapolation of these lines to zero concentration does, however, lead to a common intercept for all pH values at $S_{20,w} = 3.2$ Svedberg units. $S_{20,w}$ for the monomer is of the order of 2.3–2.4 Svedberg units. Results essentially similar to those just described for the chymotrypsins have also been obtained in preliminary sedimentation

measurements of trypsin (228). Extrapolation of the sedimentation constants, obtained at low protein concentrations, to zero concentration yields a value of approximately 1.8 Svedberg units.

Recently Jansen *et al.* (127) have reported a molecular weight of 27,000 for α-chymotrypsin. This determination was based upon osmotic pressure measurements carried out within the range of 0.5–1.0 per cent protein at pH 5.25. This was confirmed by similar measurements made on chymotrypsin inhibited by diisopropyl fluophosphate (DFP) (19). Further, it was found that 1 mole of DFP reacts stoichiometrically with 27,000 g. of chymotrypsin (*vide infra*) (126).

On the basis of the present evidence it appears that in the range of concentrations used for enzymatic measurements, trypsin and the chymotrypsins exist in the monomeric molecular form.

Recent determinations of the isoelectric points of chymotrypsinogen (pH 9.5) and of α-chymotrypsin (pH 8.1–8.6) by Anderson and Alberty (10), using the Tiselius apparatus, are in wide disagreement with the determinations made by measuring the microelectrophoretic migration of coated collodion particles. The values found by the latter method were pH 5 and 5.4 for the isoelectric points of chymotrypsinogen and of α-chymotrypsin, respectively (166); similar values were found for β- and γ-chymotrypsins (151).

Although the results of Anderson and Alberty (10) have been confirmed by other investigators, using the Tiselius method (148, 149), and by titration data (228), it is of interest that chymotrypsinogen, unlike many proteins (217), does not precipitate at its isoelectric point, i.e., pH 9.5, in the presence of sodium dodecyl sulfate, but does precipitate at about pH 5.4 (228). Further, it has been found that electrophoresis in acetate buffers at pH 5 results in *very marked* spreading of the descending boundary with concomitant sharpening of the ascending boundary (228). Since measurements made in the Tiselius apparatus usually agree well with measurements made by the microelectrophoretic technique (7), these observations leave open the possibility that the dimerization reaction, postulated from sedimentation studies, may influence the observed isoelectric point of these proteins.

It has been reported (148, 149) that B-chymotrypsinogen and B-chymotrypsin are monophoretic in the Tiselius apparatus and that two peaks appear in mixtures of α-chymotrypsinogen and B-chymotrypsinogen. The isoelectric point of B-chymotrypsinogen was found to be pH 5.2, while that of B-chymotrypsin was 4.7, in buffers of 0.1 ionic strength. Solubility studies indicated that the "B-proteins" contain only a small amount of impurity.

One of the most remarkable properties of trypsin, chymotrypsinogen, α-chymotrypsin, the crystalline soybean trypsin inhibitor and, in view of its great heat stability, possibly also of the crystalline lima bean inhibitor is their reversible inactivation by heat. Northrop (200) demonstrated that if trypsin is heated to boiling between pH 1 and pH 7 and quickly cooled, there is no loss of soluble protein and no loss of activity. This reaction was studied (163) in relation to the stability of trypsin. The reversible inactivation occurs practically instantaneously when the temperature is raised or when the solution is made strongly alka-

line. The reversibly inactivated protein is insoluble in 0.5 *M* salt solutions at pH 2, while the native active protein is completely soluble under these conditions. The reversibly inactivated protein is in equilibrium with the active protein and reverts to the active protein if the solution is allowed to stand at pH 2 at 20°C. for several hours. The inactivation reaction is reversible for only a short time, and prolonged exposure to high temperatures or to high pH values results in the appearance of irreversibly denatured protein.

The stability behavior of trypsin is further complicated by the fact that on the acid side of pH 2 the protein is irreversibly transformed into acid-denatured protein, while in the pH range 2–9 the inactive protein which is formed is digested by the active enzyme. From pH 9 to pH 12 the processes of inactivation and hydrolysis of the inactive protein are accompanied by alkali denaturation, and at pH 13 the latter reaction predominates.

Anson and Mirsky (15) have studied the equilibrium between native and reversibly inactivated trypsin and have found the equilibrium to depend upon pH, temperature, and alcohol concentration. The heat of the reversible inactivation reaction in 0.01 *N* hydrochloric acid was found to be 67,000 cal. per mole. Stearn (259) has used the data of Pace (209) for the rate of inactivation of trypsin together with the equilibrium constants of Anson and Mirsky to evaluate the energetic constants for the inactivation and reactivation of trypsin. This procedure is open to the objection that the measurements of Pace were not made on crystalline trypsin.

The most elegant study of the reversible heat inactivation of these proteins was made by Kunitz on crystalline soybean inhibitor (160). It was established that the reactivated protein was identical with the untreated inhibitor in its ability to inhibit trypsin, resistance to digestion by pepsin, solubility, and crystallizability. The kinetic and thermodynamic constants for the inactivation and reactivation reactions were evaluated in 0.0025 *M* hydrochloric acid between 30° and 65°C. The heat of reaction for the inactivation was found to be 57,000 cal. per mole, while the entropy of reaction was found to be 180 entropy units (E.U.). The heat and entropy of activation for the inactivation reaction are 55.4 kilocal. per mole and 95 E.U., respectively, while the corresponding values for the reactivation reaction are -1.9 and -84, respectively.

The inactivation of trypsin (269, 277), chymotrypsin (83) and soybean trypsin inhibitor (133) by ultraviolet irradiation has been studied, and the effect of radiant energy on enzymes has recently been reviewed (187). One of the most interesting studies of this type is that of Dale (69), who found that when carboxypeptidase was irradiated with x-rays in the presence of a substrate (the peptic digest of edestin used by Anson), the enzyme was completely protected against a radiation dosage sufficient to cause 85 per cent inactivation in the absence of the substrate.

It is of historical interest that Corey and Wyckoff (67) used chymotrypsinogen crystals to demonstrate that the x-ray diffraction characteristics of protein crystals are such that protein crystals must be regarded as true crystals of very large molecules. Northrop (201) quotes Wyckoff's judgement that x-ray diffrac-

tion patterns of chymotrypsinogen and of chymotrypsin resemble each other no more than do any two dissimilar proteins. Jacobsen (124) has pointed out that this statement was based upon studies of different crystal forms of the two proteins and suggests that diffraction patterns of needle-shaped prisms of the two proteins might reveal a similarity.

Ten Broeck (267) found an immunological similarity between bovine chymotrypsinogen and chymotrypsin in that cross-reactions with chymotrypsin appeared in some guinea pigs sensitized to chymotrypsinogen. In contrast, complete enzyme and species specificity held for swine and bovine trypsin and either bovine chymotrypsinogen or chymotrypsin. Similarity between the chymotrypsins is indicated by the observation that the precipitin reaction fails to distinguish α-, β-, and γ-chymotrypsins (207).

Brand and Kassel (45, 46) determined the content of several amino acids in chymotrypsinogen, and Brand (43) has reported the amino acid composition of chymotrypsinogen and partial analyses of α-, β-, and γ-chymotrypsins (44). Although the chymotrypsins showed no striking differences in the amino acids reported, it is of interest that chymotrypsinogen contains 10.4 g. of leucine per 100 g. of protein, while α-chymotrypsin contains only 9.1 per cent of this amino acid.

The values shown in table 2 for the molecular weight of carboxypeptidase, determined by a combination of diffusion–viscosity and diffusion–sedimentation determinations, indicate good agreement between these determinations. Putnam and Neurath (218) calculated the axial ratio to be 2.1, assuming 30 per cent hydration. Smith and Hanson (246, 247) have presented evidence that carboxypeptidase is a metal protein, the enzymatic activity depending upon the presence of very tightly bound magnesium. This conclusion is based on the inhibition of the enzyme by anions which combine with magnesium and on the spectrographic identification of magnesium in the ash of the enzyme. The inhibition studies will be discussed in connection with the kinetics of carboxypeptidase (page 140), but it should be pointed out that because of the ubiquity of magnesium and the high sensitivity of spectrographic methods, the identification of this metal in the ash of the protein constitutes only presumptive evidence for its presence as part of the carboxypeptidase molecule.

III. Kinetics of Enzymatic Reactions

A. GENERAL FORMULATION

It would be beyond the scope of the present review to consider in detail the subject of the kinetics of enzymatic reactions. This problem has been adequately considered in reviews (100, 192, 258, 271, 288, 289) and original publications (49, 58, 96, 179, 190, 259, 261, 272) and will be dealt with herein as an aid in the interpretation of experimental results.

The overall kinetics of enzymatic reactions is usually determined by measurements of the rate of disappearance of the substrate (or of the rate of formation of the reaction products), with time as the independent variable. When enzyme activity is to be determined, it is sometimes expedient to select time as the

dependent variable and to express enzyme activity as the time required for a given fraction of the initial substrate concentration to disappear (271).

Except for systems in which either the reaction products (*cf.* 179) or the substrate itself (16, 17, 18, 100, 296) cause inhibition (pages 93 and 94), enzymatic reactions are generally characterized by two features: (*1*) At constant initial substrate concentration the rate is proportional to enzyme concentration, at least over a relatively wide range (261). (*2*) At constant enzyme concentration, the reaction rate increases with increasing initial substrate concentration according to the equation of a rectangular hyperbola, approaching asymptotically a maximum value. These relations have been recognized early in the study of enzymatic reactions and have led Michaelis and Menten (190) to postulate the existence of an intermediate enzyme–substrate complex. This theory, with its subsequent extensions by Van Slyke and Cullum (272), Briggs and Haldane (49), and others (58, 100, 271, 279), has proven a firm foundation in the interpretation of the kinetics of enzymatic reactions and will be the point of departure for the present discussion of the problem.

If E denotes free enzyme, S, free substrate, ES, the enzyme–substrate complex, and P, the reaction products, the reaction may be represented by:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ES) \xrightarrow{k_3} E + P \tag{1}$$

where k_1, k_2, and k_3 are the rate constants for the reactions denoted by the respective arrows.

The rate of formation of ES is given by

$$dp/dt = k_1(e - p)a - (k_2 + k_3)p \tag{2}$$

where e, p, and a denote, respectively, the total concentration of enzyme, and the concentrations of ES and S.

The rate of disappearance of the substrate is given by

$$da/dt = -k_1(e - p)a + k_2p \tag{3}$$

The rate of the overall reaction is given by summation of equations 2 and 3:

$$d(p + a)/dt = -k_3p \tag{4}$$

A complete solution of equation 4 was obtained by Chance (58) with the aid of the differential analyzer and has been given in graphical form. It suffices for the present discussion to consider the restricted conditions usually met with in hydrolytic enzymatic reactions. This condition is that of the steady state, i.e., the concentration of ES (i.e., p), is constant, or else the rate of change of p is negligibly small as compared to the rate of change of a (58, 100). For specific systems it has been shown by Chance (58, 59) that this assumption is valid and that it applies not only to the initial phase of the reaction but to any transient portion of the rate curves, provided this portion is sufficiently narrow for the velocity to be a linear function of time.

Accordingly, for $dp/dt = 0$, equations 2 and 3 reduce to

$$-da/dt = k_3p \tag{5}$$

Hence, the overall reaction velocity is proportional only to the concentration of the enzyme–substrate complex, p. It also follows from equation 2 that, since $dp/dt = 0$,

$$k_1(e - p)a = (k_2 + k_3)p \tag{6}$$

and

$$\frac{k_2 + k_3}{k_1} = \frac{(e - p)a}{p} = \frac{(E)(S)}{(ES)} = K_m \tag{7}$$

Solving equation 7 for p, and substituting this value into equation 5, one obtains the overall reaction velocity as a function of enzyme and substrate concentrations:

$$v = -da/dt = \frac{k_3 \cdot a \cdot e}{K_m + a} \tag{8}$$

Equation 8 is formally identical with that of Michaelis and Menten (190) but differs in the interpretation of K_m (49, 58, 77, 100, 138, 279). Since, according to the mass law, the concentration of ES increases as a is increased, the velocity, v, increases hyperbolically with increasing substrate concentration to a maximum value, V_{max}, which is reached when all of the enzyme is bound in ES, i.e., when $e \simeq p$. Under these conditions equation 8 becomes

$$v = \frac{V_{max}\, a}{K_m + a} \tag{9}$$

where

$$V_{max} = k_3 \cdot e \tag{10}$$

A linear relation between v and a is obtained by plotting the reciprocal form of equation 9 (179),

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{a} + \frac{1}{V_{max}} \tag{11}$$

the slope of the straight line being K_m/V_{max}, and the ordinate intercept, $1/V_{max}$. A higher precision can be obtained when rearranged forms of equation 11 are plotted, such as a/v *versus* a (179, 288) or, as proposed by Eadie (74), when v is plotted *versus* v/a, according to equation 12:

$$v = V_{max} - K_m \frac{v}{a} \tag{12}$$

This plot has the advantage that the slope, K_m, appears as a regression coefficient and that V_{max} and K_m appear in separate terms.

B. DETERMINATION OF REACTION VELOCITIES

If the rate of the overall reaction is measured in terms of the rate of disappearance of the substrate, equation 5 applies, provided, as already stated, that $dp/dt \simeq 0$. This approximation requires that the initial or transient portion of the velocity curve be sufficiently narrow for the velocity, v, to be a linear function

of time, t. Since the error in the experimental determination of the reaction rate is higher the smaller the time interval, it is necessary to extrapolate the velocities from a larger portion of the velocity curve to $t = 0$. This extrapolation is unequivocal for zero-order reactions, in which the velocity–time relation is linear throughout the entire course of the reaction, but in all other cases it depends on the nature of the function f in the general equation

$$v = -\mathrm{d}a/\mathrm{d}t = k \cdot f(a) \tag{13}$$

where k is the rate constant and $f(a)$ is a function of the substrate concentration. The actual nature of this function and the physical significance of the corresponding order of reaction are of no consequence for the calculation of initial reaction velocities, *provided the form of the function does not change as a given condition*, e.g., enzyme or substrate concentration, pH, or temperature, *is varied*. Although this problem has been elegantly analyzed and illustrated by Bodansky (38), it has not always been fully appreciated in the interpretation of experimental data. Although the kinetics of enzymatic reactions cannot usually be characterized by first-order reaction constants which are independent of substrate concentration, the initial velocity can often be obtained for each initial substrate concentration by extrapolation of a first-order reaction plot (136, 137, 138, 252). Since, in the case of first-order reaction kinetics, $-\mathrm{d}a/\mathrm{d}t = k \cdot a$, where k is the first-order reaction constant corrected to unit enzyme concentration, equation 11 assumes the form (137):

$$\frac{1}{ka} = \frac{K_m}{k_3}\frac{1}{a} + \frac{1}{k_3} \tag{14}$$

However, caution and discretion have to be exercised in any such extrapolation, particularly if the enzymatic reaction is inhibited either by the reaction products (107, 181, 229) or by the substrate itself. These restricted cases will be considered elsewhere in this review (page 93).

C. THE ORDER OF REACTION

The overall order of reaction governing the system represented by equation 1 will depend on the relative magnitudes of the three reaction constants, k_1, k_2, and k_3, and on initial enzyme and substrate concentrations. If the three reaction constants have finite values, the order of reaction will be given by integration of equation 8:

$$k_3 \cdot e \cdot t = 2.3K_m \log \frac{a_0}{a} + (a_0 - a) \tag{15}$$

This equation was first derived by Van Slyke and Cullen (272) for the restricted condition of $k_2 \ll k_3$, and was given in the general form by Walker and Schmidt (279) and by Elkins-Kaufman and Neurath (77). It is evidently a composite first-order and zero-order expression, the contribution of the two respective terms depending on the magnitude of K_m and on initial substrate concentration (77). Adherence to these kinetics of the hydrolysis rates of specific substrates by

proteolytic enzymes has been convincingly demonstrated for several such systems (77, 138, 139, 252). It has also been shown that interpretation of the rate measurements according to conventional first-order reaction kinetics yields rate constants which increase with increasing degree of hydrolysis and with decreasing initial substrate concentration (77). While the latter effect would likewise result if the reaction were of any order but were inhibited by reaction products (181), the fact remains that the reaction order represented by equation 15 is a necessary requirement of the Michaelis–Menten theory.

It is apparent from equation 15 that reactions will tend to approximate those of first-order kinetics under conditions which favor predominance of the first right-hand member of equation 15 and, conversely, that zero-order reaction kinetics will be approximated as the second term becomes predominant. These limiting conditions may be described as follows:

1. Apparent first-order reaction kinetics

If the rate of formation of ES is considerably slower than the rate of decomposition (see equation 1), enzyme–substrate complex formation becomes the rate-determining step. Under these conditions, the overall velocity is given by equation 3. However, in the initial phase of the reaction, $p \simeq 0$, and $(e - p) \simeq e$. Hence,

$$v = -\mathrm{d}a/\mathrm{d}t = k_1 \cdot e \cdot a \tag{16}$$

Equation 16 is the expression for a second-order reaction but since e = constant, it reduces to a first-order expression.

While in regions of low substrate concentration first-order reaction kinetics is approximated, provided K_m is sufficiently large (77, 271), it is important to realize that such a kinetic interpretation is of limited value in the characterization of an enzymatic system. Interpretation of reaction velocities, determined at a single, arbitrary, initial substrate concentration, by first-order reaction kinetics has been a matter of routine in the investigations by Bergmann and coworkers (23, 27) and has been propagated since (70, 104, 241, 257). The term "proteolytic coefficient," defined as the first-order reaction constant calculated from decimal logarithms (usually determined in 0.05 M substrate concentration), per milligram of enzyme nitrogen per milliliter (118) has been used to express enzymatic activity, to compare the activity of the same enzyme toward several structurally related substrates, and to compare the activity of two or more enzymes toward the same substrates (23, 27, 28, 70, 118, 241, 257). The limited validity of such interpretations is evident from the preceding considerations of the dependence of approximated first-order reaction constants on K_m and initial substrate concentration, which have been clearly established by experiments (77, 138). Thus the overlapping and crossing of curves, obtained when the proteolytic coefficients for the hydrolysis of two or more substrates by the same enzyme are plotted against initial substrate concentration, will obviously result in varying ratios of these coefficients (138, 252). A careful analysis of the hydrolysis of three specific substrates by α- and δ-chymotrypsins, respectively (230), has cast similar doubt on the strict validity of the concept of "homospecificity" (23, 118).

If first-order reaction constants are extrapolated to zero initial substrate concentration, the resulting value, k^{1st}, is related to K_m and k_3 (272) by

$$k^{1st} = \frac{k_3}{K_m} \tag{17}$$

and the corresponding "maximum proteolytic coefficient," C_{max} (77, 138) is, accordingly

$$C_{max} = \frac{k_3}{2.3K_m} \tag{18}$$

2. Zero-order reaction kinetics

If the rate of formation of ES is considerably faster than the rate of disappearance of substrate, i.e.,

$$k_1/k_2 \gg k_3 \qquad \text{and} \qquad e \simeq p \tag{19}$$

the overall reaction velocity becomes

$$v = -\mathrm{d}a/\mathrm{d}t = k_3 \cdot p \simeq k_3 \cdot e \tag{20}$$

Equation 20 is that for a zero-order reaction, since e = constant, and yields on integration:

$$k_3 \cdot e \cdot t = (a_0 - a) \tag{21}$$

Adherence to equation 21 has been observed for the tryptic hydrolysis of a series of esters of benzoyl-L-arginine (229, 232), as well as for the initial phase of the hydrolysis of benzoyl-L-argininamide by trypsin. In the latter instance, however, the apparent order of the reaction increases as the reaction proceeds, owing to the inhibition by one of the reaction products, i.e., benzoyl-L-arginine (107, 229). With certain restrictions, zero-order reaction kinetics applies also to the hydrolysis of certain specific esters by carboxypeptidase (252, 254). It is evident that conditions which tend to increase k_3 more than k_1/k_2 (change in pH, temperature, etc.) will tend to shift the kinetics of such systems toward that described by equation 15, by increasing the contribution of the first-order term. While, theoretically, the order of the reaction of any enzymatic system may be shifted toward zero-order kinetics, by increasing the initial substrate concentration so that $a \gg K_m$, and conversely toward first-order kinetics if $a \ll K_m$, the experimental realization of these conditions is restricted by the solubility of the substrate and by the resolving power of the analytical methods, respectively.

D. THE SIGNIFICANCE OF K_m

According to the original theory of Michaelis and Menten (190) and subsequent interpretations by others (179), K_m defines the equilibrium for the reversible formation of ES from E and S (equation 1), regardless of the disturbance of the equilibrium by the subsequent decomposition of ES into E and P. In this case, K_m defines a thermodynamic equilibrium which is characteristic of the

affinity of the substrate for the enzyme. However, kinetic measurements which are based solely on the rate of disappearance of the substrate never yield k_2/k_1 since k_3 must have a finite value. While the equation

$$K_m = \frac{(E)(S)}{(ES)} = \frac{k_2 + k_3}{k_1} \tag{22}$$

bears formal resemblance to an equilibrium expression, it actually represents the condition for the steady state in two consecutive reactions, the first one of which is reversible (58, 77, 100, 138, 253, 271, 279). In the limiting case of $k_3 \ll k_2$, the graphically determined value of K_m becomes k_2/k_1, whereas, conversely, for $k_3 \gg k_2$, K_m will converge toward k_3/k_1 (253). In all other, most frequent, cases K_m will always be a composite function of all three reaction constants. However, comparison of K_m and k_3 values for the hydrolysis of two or more substrates by the same enzyme yields a qualitative measure of their relative affinities for the enzyme. Thus, if for two substrates, S_1 and S_2, their respective K_m values are in inverse order of their respective k_3 values, such as

$$(K_m)_1 \gtrless (K_m)_2 \qquad \text{and} \qquad (k_3)_1 \lessgtr (k_3)_2$$

the corresponding enzyme–substrate affinities, k_2/k_1, will be greater for S_1 than for S_2. This type of analysis has been used to compare the relative affinities of a series of substrates for chymotrypsin (251) and for carboxypeptidase (252).

It is worthy of note that simultaneous kinetic measurements of the rate of formation of ES and of the rate of disappearance of the substrate have shown that in certain oxidative enzyme systems, the approximation $k_2 \rightarrow 0$ applies (58, 59, 60, 61). Rate measurements of the chymotryptic hydrolysis of acetyl-L-tyrosinamide in solutions of varying methanol concentration have been similarly interpreted (136).

The concept of the formation of an intermediate enzyme–substrate complex, ES, may be logically extended to a series of such complexes (62, 259, 268). One of these, i.e., the activated complex (ES)*, will be considered in the interpretation of the reaction constant k_3 by the transition state theory (page 89). The formation of complexes between E or ES with ions, inhibitors, and reaction products will be considered elsewhere in this review (pages 94, 91, and 93). The formulation of the reaction kinetics for a process involving several intermediate complexes has been given by Christiansen (63) and by Burton (53).

The graphical determination of K_m is essentially based on the formulation of equations 9 to 12. A thorough analysis of this determination, with due consideration of the stoichiometric relations and of the effects of inhibitors, has been given in the classical paper by Lineweaver and Burk (179).

E. THE SIGNIFICANCE OF k_3

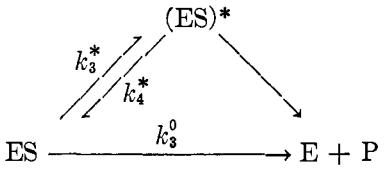
According to the present formulation of the reaction mechanism, k_3 denotes the specific rate constant for the conversion of the enzyme–substrate complex, ES, into reaction products, P, and free enzyme, E, as represented by equation 1. According to equation 5,

$$k_3 = \frac{v}{p} \tag{23}$$

As enzyme concentrations are usually expressed in milligrams of enzyme nitrogen per milliliter, and time in minutes, the dimensions of k_3 are moles per liter per minute per milligram per milliliter. If both substrate and enzyme concentrations are expressed in moles per liter, and time in seconds, the dimensions of k_3 become sec.$^{-1}$, i.e.,

$$k_3^0 = k_3 \frac{\text{enzyme molecular weight} \times \text{fraction N}}{60} \tag{24}$$

In isolated systems containing a single enzyme and a single substrate, hydrolysis by crystalline pancreatic proteases is experimentally essentially irreversible. Accordingly, in the classical sense, k_3^0 represents a specific rate constant for an essentially irreversible process. However, according to the transition state theory (95, 258, 259), k_3^0 may be related to an equilibrium constant, denoted herein as K_3^*, for the reversible interconversion of the enzyme substrate complex, ES, to its activated form, (ES)*, where (ES)* represents, by definition, the state at the top of the highest energy barrier in the conversion of ES to the final reaction products, E and P:



(25)

The formal relation between k_3^0 and K_3^* is

$$k_3^0 = \kappa \frac{RT}{Nh} K_3^* \tag{26}$$

where R is the gas constant, T the absolute temperature, N the Avogadro number, h Planck's constant, and κ a transmission coefficient, usually assumed to be close to unity. The frequency factor, RT/Nh, represents the rate of passage of the activated complex over the energy barrier, and at 25°C. has a value of 6.25×10^{12}.

The breakdown of (ES)* into E and P is never a rate-determining step since, for $\kappa = 1$, every molecule which passes the activated state will spontaneously decompose into E and P, the rate of the reaction being simply equal to the concentration of the activated complex multiplied by the frequency of crossing the barrier. Since, in the present case, k_3^0 is the reaction rate per unit concentration of ES, as defined by equations 23 and 24, and $K_3^* = (ES)^*/(ES)$, equation 26 permits the calculation of (ES)* from experimentally determined values of k_3.

For values of k_3^0 ranging from about 10^{-2} to 10^2, as have been found for the hydrolysis of specific substrates by crystalline pancreatic proteases, K_3^* varies from 10^{-14} to 10^{-10}, RT/Nh being of the order of 10^{12}. It follows, therefore, that

the quantum-mechanical equilibrium between ES and (ES)* is shifted far in the direction toward ES and that $k_4^* \gg k_3^*$, these two reaction constants being simply related to each other by (see equation 25):

$$K_3^* = k_3^*/k_4^* \tag{27}$$

If the activated complex (ES)* were omitted from the formulation, the equilibrium concentration of ES could only be a rate-determining factor if $(k_2 + k_3) \gg k_1$. Since, however, $1/K_m$ is of the order of 10 to 10^6, instead of 10^{-10} to 10^{-14}, the concentration of (ES)* is evidently only a small fraction of that of ES (51).

Application of the transition state theory is not necessarily limited to the reaction step characterized by k_3 but may be similarly extended to any single phase of the reaction, provided the corresponding reaction constants can be determined. Thus, the formation of (ES) from E and S may be likewise considered to involve the formation of an activated complex (253). Calculation of the corresponding equilibrium constant, K_1^*, is, however, predicated on the evaluation of k_1, which is generally indeterminate in the enzymatic systems under discussion.

F. INHIBITION

Studies of the inhibition of enzymatic reactions have played a dominant role in the elucidation of the specificity and of the kinetics of enzymatic systems. The present discussion will be limited to the effects of inhibitors on the kinetics of proteolytic reactions, whereas the interpretation of such data in terms of the specificities of these enzymes will be considered separately for each enzyme (pages 107, 122, and 137).

The kinetics of inhibition depend on the postulated nature of the interaction of the inhibitory compounds with the other components of the enzymatic system (179). From the viewpoint of enzyme specificity, the most important type of inhibition is the "*competitive*" type where, presumably, substrate and inhibitor compete for the same combining sites of the enzyme surface (75, 179, 288), as represented below:

$$\begin{aligned} &E + S \rightleftharpoons ES \rightarrow E + P \\ &E + I \rightleftharpoons EI \end{aligned} \tag{28}$$

I and EI denote, respectively, free inhibitor and the enzyme–inhibitor complex. The degree of inhibition depends on both inhibitor and substrate concentrations, according to:

$$\frac{1}{v_i} = \frac{1}{V_{max}} + \left[1 + \frac{(I)}{K_i}\right] \frac{K_m}{V_{max}} \frac{1}{a} \tag{29}$$

where v_i is the initial velocity in the presence of the inhibitor, K_i is the dissociation constant of the enzyme–inhibitor complex, and (I) is the inhibitor concentration, both in moles per liter. If a plot of equation 29 is compared to that of equation 11, the resulting straight lines will be found to intersect at the same ordinate intercept (V_{max} remaining constant), whereas the slope will be increased by the quantity $(1 + (I)/K_i)$.

In strictly "*noncompetitive*" inhibition, the inhibitor is assumed to combine with catalytically inactive sites on the enzyme surface, and the system will be described by three reactions, i.e., those given in equation 28 and that in equation 30,

$$ES + I \rightleftharpoons (ESI) \text{ inactive} \tag{30}$$

where the corresponding dissociation constant, K_{esi}, is assumed to be equal to K_i. The kinetic equation is:

$$\frac{1}{v_i} = \left[1 + \frac{(I)}{K_i}\right]\left[\frac{1}{V^i_{max}} + \frac{K_m}{V^i_{max}} \cdot \frac{1}{a}\right] \tag{31}$$

where V^i_{max} is the maximum velocity in the presence of the inhibitor. It is apparent from equation 31 that a comparison of a plot of this equation with that of equation 11 will result in an increase of both the slope and the ordinate intercept by the factor $(1 + (I)/K_i)$ (75, 179).

In addition to strictly "competitive" and "noncompetitive" inhibition, the so-called "*uncompetitive*" inhibition (75) has been formulated for the case that the inhibitor combines with ES but not with E. A plot according to equation 31 will also be linear, but in comparison to a similar plot in the absence of the inhibitor (equation 11), the slope may be found to change by a factor less than the intercept, and in the limiting case, the slope will remain unchanged whereas the ordinate intercept increases by $(1 + (I)/K_i)$. A complete discussion of these types of inhibition has been given by Ebersole, Guttentag, and Wilson (75).

Regardless of the type of inhibition, the degree of inhibition actually observed depends on the ratio K_m/K_i, inhibition decreasing as this ratio increases (*cf.* 134, 137).

It is to be recognized that the nature of the combination of the inhibitor with the enzyme may be affected by the absolute concentration of the inhibitor in the system. Thus, the inhibition of carboxypeptidase by butyrate and chloroacetate (78), and of trypsin by benzoyl-L-arginine (229), changes from an apparently "uncompetitive" type toward the "competitive" type as the inhibitor concentration is increased, presumably because the first increments are bound by catalytically inactive sites on the enzyme surface, whereas additional increments are subsequently also bound by the catalytically active sites ("*indeterminate*" type of inhibition). This situation is analogous to that of the binding of ions and molecules by proteins, which requires more than one parameter (i.e., intrinsic binding constant) for the quantitative description of the binding curve (131).

Compounds capable of causing the inhibition of enzymatic reactions may be divided into the following categories: (*1*) structural analogs of specific substrates or products of the enzymatic reaction; (*2*) the substrate itself; (*3*) nonspecific organic inhibitors; (*4*) ions. Inhibitors belonging to group 1 are usually of the "competitive" type (equation 29), whereas members of groups 2 to 4 may give rise to various types of inhibition, depending on the nature of their interaction with the enzyme. Inhibition by reaction products is a relatively frequent phenomenon (86, 96, 107, 117, 179, 181, 229, 261).

The kinetic equations for an enzymatic reaction which is inhibited by the reaction products can be readily derived from the following formulation of the process (86, 107, 117, 179, 181):

$$\begin{array}{ccccccccc} \mathrm{E} & + & \mathrm{S} & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & \mathrm{ES} & \xrightarrow{k_3} & \mathrm{E} & + & \mathrm{P} \\ (e-p-q) & & a & & p & & & & (a_0-a) \\ \mathrm{E} & + & \mathrm{I} & \underset{k_5}{\overset{k_4}{\rightleftharpoons}} & \mathrm{EI} & & & & \\ (e-p-q) & & (a_0-a) & & q & & & & \end{array} \tag{32}$$

where the lower case symbols denote concentrations of the respective components. Steady-state conditions (86, 107) require that:

$$\begin{aligned} K_m &= \frac{k_2 + k_3}{k_1} = \frac{(e-p-q)\cdot a}{p} \\ K_i &= \frac{k_5}{k_4} = \frac{(a_0-a)(e-p-q)}{q} \end{aligned} \tag{33}$$

Rearrangement and mutual substitution of equations 33 yield:

$$\begin{aligned} q &= \frac{(a_0-a)(e-p)}{K_i + (a_0-a)} \\ p &= \frac{e\cdot a\cdot K_i}{K_m(K_i + a_0 - a) + aK_i} \end{aligned} \tag{34}$$

Since $v = -\mathrm{d}a/\mathrm{d}t$, and letting $K_i/K_m = r$, one obtains

$$v = -\mathrm{d}a/\mathrm{d}t = k_3 p = \frac{k_3\cdot e\cdot a}{K_m + \frac{a_0}{r} + \frac{a(1-r)}{r}} \tag{35}$$

For $t = 0$, i.e., when the *initial* velocity is being determined, or for $r = 1$, i.e., when the apparent enzyme–substrate affinity is the same as the enzyme–reaction product affinity (107), equation 35 reduces to

$$v = \frac{k_3\cdot e\cdot a_0}{K_m + a_0} \tag{36}$$

which, as is to be expected, is identical with the Michaelis–Menten equation (equation 8).

In the presence of inhibitory reaction products, the course of the reaction is given by integration of equation 35:

$$k_3\cdot e\cdot t = 2.3\left(K_m + \frac{a_0}{r}\right)\log\frac{a_0}{a} - \frac{(1-r)}{r}(a_0 - a) \tag{37}$$

Equation 37 bears formal relation to equation 15 and reduces to the latter for $r \to \infty$. For the specific case of $r = 1$, equation 37 reduces to a first-order reaction equation but the resulting value of k_3 will still be a function of initial substrate

concentration. Except for this unique condition, the reaction will proceed more slowly than required by first-order reaction kinetics if $r < 1$, and more rapidly if $r > 1$.

Equation 37 has been tested for the inhibition of the tryptic hydrolysis of benzoyl-L-argininamide by benzoyl-L-arginine (107) and it was believed that in this case $r = 0.5$ ($K_m = 2K_i$). Subsequent work (229) has provided evidence that in this case inhibition is not of the simple competitive type. The course of the reaction of the chymotryptic hydrolysis of *N*-acetyl-L-tyrosylglycinamide may be described by equation 37, assuming inhibition by the reaction products (181) but, as the authors themselves state, the data fit equally well the integrated Michaelis–Menten equation (equation 15).

It has been recently shown (107) that the linear equations of Klotz and coworkers (144, 147) for the multiple binding of one ion species by proteins can be applied to the derivation of a velocity equation which is essentially identical with that of Michaelis and Menten (equation 11). An equation similar to equation 35 can be analogously derived from the equations of Klotz, Triwush, and Walker (145) for the competitive binding of two ion species. It is to be anticipated that the active work on the binding of ions and small molecules by proteins, now in rapid progress (131, 146, 223, 276), will be extended and applied to the interaction of enzymes with substrates and inhibitors.

The *inhibition* of enzymatic reactions *by the substrate* itself (16, 17, 18, 100, 169, 296) appears to be a relatively infrequent phenomenon but has been recently convincingly demonstrated for two unrelated enzyme systems, i.e., acetylcholine esterase–acetylcholine (16, 17, 18) and urease–urea (169). In the former case, the phenomenon was impressively documented by the bell-shaped curves obtained when initial reaction velocity was plotted against initial substrate concentration. The apparent inhibition of urease by its substrate was explained in terms of a model in which urea and water molecules occupy neighboring sites on the enzyme surface. At high urea concentrations the substrate becomes adsorbed on both sites, thereby inhibiting the reaction (169). In the case of carboxypeptidase, too, evidence has been provided for the inhibition of the reaction by a specific substrate, i.e., hippuryl-β-phenyllactate (252) (see page 135).

It has been recently claimed that, in common with many oxidation enzymes (282, 283) and certain dipeptidases (128, 240, 242, 244), carboxypeptidase is a metal-containing enzyme and that the enzymatic activity is *inhibited* by *ions* (105) which have a high affinity for the metal which, supposedly, is magnesium (246). In fact, the inhibition by anions such as orthophosphate, pyrophosphate, citrate, and oxalate was the most direct evidence for the postulated role of magnesium in the enzymatic activity of carboxypeptidase, and has played a dominant role in the formulation of a theory according to which the metal forms chelate complexes with the substrate (243). This theory, as far as it concerns carboxypeptidase, is not supported by subsequent experiments carried out in the laboratory of the present authors (194), since the inhibition by orthophosphate is of different nature than was proposed by Smith and coworkers (105) (see page 140). None of the ions which affects the kinetics of hydrolysis by carboxypeptidase (105) inhibits either trypsin (105) or chymotrypsin (248).

G. THE EFFECT OF pH

The large dependence of the velocity of enzymatic reactions on the hydrogen-ion concentration of the solution was explained by Michaelis and Davidsohn (189) on the assumption that only the "uncharged" (isoelectric) form of the enzyme molecule was enzymatically active. Since, however, it was later shown that the isoelectric point of many enzymes does not coincide with their pH optimum, this simple concept was subsequently revised by assuming that the activity depends on the ionization of certain groups on the enzyme molecule (100). The theory was further extended by Northrop (99) and Willstätter *et al.* (286), so as to include the effect of pH on the ionization of both the enzyme and the substrate, and by Kunitz and Northrop (163), who have shown that in the case of one of the enzymes studied by Northrop, i.e., trypsin, the decrease of enzymatic activity in higher pH regions may be ascribed, in part, to the irreversible inactivation of the enzyme. A careful study by Johnson, Johnson, and Peterson (129) of the pH dependence of the splitting of triglycine by intestinal aminopeptidase was interpreted by the postulate that the acid region of the pH–activity curve depends on the apparent pK of the ionization of the enzyme, whereas the shape of the curve in the alkaline region is determined by the pK of the peptide (pK_2 = 7.71). Similar studies of the enzymatic activity of intestinal leucyl peptidase, an enzyme activated by magnesium, have led to the conclusion that the rate of hydrolysis of leucylglycylglycine depends on the concentration of both the substrate anion and the enzyme–magnesium complex (129). Recent studies of the effect of pH on the activity of carboxypeptidase (195, 196) have shown that the specific rate constant, k_3, varies markedly between pH 6.0 and 9.0, although the change in ionization of the substrate (carbobenzoxyglycyl-L-phenylalanine) within this pH range is insignificant.

A detailed analysis of the effect of pH on the rates of enzymatic reactions requires an evaluation of the pH effect on both rate-determining kinetic constants, k_3 and K_m. A mathematical formulation has been given by Walker and Schmidt (279) in their analysis of the effect of pH on the activity of histidase. Considering that hydrogen ions may combine reversibly either with the enzyme, E, the substrate, S, or the enzyme–substrate complex, ES, according to the equation

$$K_X = \frac{(H^+)(X)}{(HX)} \tag{38}$$

where X represents either E, S, or ES, and HX represents the enzymatically *inactive* form, the course of the reaction was described by the following equation (using the symbols of the present paper):

$$\frac{1}{\left(1 + \frac{(H^+)}{K_{HES}}\right)} k_3^0 \cdot e \cdot t = \frac{\left(1 + \frac{(H^+)}{K_{HE}}\right)\left(1 + \frac{(H^+)}{K_{HS}}\right)}{\left(1 + \frac{(H^+)}{K_{HES}}\right)} 2.3K_m \log \frac{a_0}{(a_0 - a)} + (a_0 - a) \tag{39}$$

K_{HE}, K_{HS}, and K_{HES} denote, respectively, the dissociation constants of the complex formed between hydrogen ions and E, S, and ES. The acid branch, but not the alkaline branch, of the hydrolysis of histidine by histidase could be reasonably well represented by equation 39, on the assumption that the rate-limiting effect of hydrogen ions is their combination with ES. The contribution of the hydrogen-ion concentration to the reaction velocity is apparent from comparison of equations 39 and 15.

More recently, Bull and Currie (51) attempted to interpret the influence of pH on the peptic hydrolysis of egg albumin by assuming the hydrogen ions to combine with ES, according to the following formulations:

$$E + S \underset{K_1}{\rightleftharpoons} ES; \qquad ES + H^+ \underset{K_2}{\rightleftharpoons}$$

$$(H^+ES) \underset{K_3}{\rightleftharpoons} (H^+ES)^* \rightarrow E + P \quad (40)$$

where K_1, K_2, and K_3 are the dissociation constants for the reversible reactions indicated by the corresponding arrows, and $K = K_1 \times K_2 \times K_3$. At constant temperature the resulting kinetic equation reduces essentially to that of Walker and Schmidt (279) for the case that hydrogen ions combine with ES rather than with E or S. If the effect of temperature is interpreted according to the transition-state theory, the following relation between the initial reaction velocity, v, the frequency factor, kT/h, the total molar enzyme concentration, E_0, the hydrogen-ion concentration, H, and the substrate concentration, S, was shown to apply:

$$\frac{1}{v} = \frac{h}{\kappa k T E_0}\left[\frac{K}{HS} + \frac{K}{K_1 H} + \frac{K}{K_1 K_2} + 1\right] \quad (41)$$

From various plots of this equation the individual dissociation constants K_1, K_2, and K_3 were evaluated, yielding, at 25°C., $K_1 = 6.6 \times 10^{-4}$, $K_2 = 1.7 \times 10^{-2}$, and $K_3 = 4.9 \times 10^{12}$. Comparison of K_1 and K_3 indicated, as was to be expected, that the concentration of the activated complex, $(H^+ES)^*$, was only a small fraction of that of the Michaelis complex, (ES); K_2, the dissociation constant of (H^+ES) was numerically comparable to the ionization constant of α-carboxyl groups in amino acids. While Bull and Currie's approach is unquestionably attractive, the supporting data were admittedly derived from a narrow acid pH region. It is also apparent that in their derivations, the pair of specific rate constants of consecutive steps appear in separate terms throughout, whereas according to considerations set forth in this review (page 89) a steady-state constant (K_m) always contains explicitly a rate constant for the subsequent activation reaction, k_3. The effect of pH on the activity of the pancreatic proteases will be considered separately for each enzyme.

H. THE INFLUENCE OF TEMPERATURE

Enzymatic reactions have a characteristically high temperature coefficient which corresponds, approximately, to a two- to three-fold increase in reaction velocity per 10°C. temperature increment within the temperature range in which

the enzyme retains its full integrity. The proteolytic enzymes occupy in this respect no exceptional position.

The temperature effect is conventionally expressed by the Arrhenius equation

$$k' = C \cdot e^{-\mu/RT} \tag{42}$$

where k' is the specific reaction constant for the overall reaction, and μ, often denoted also as A or ΔE, is the critical temperature increment (Arrhenius activation energy (192, 288)). μ is graphically determined from a plot of log k' against the reciprocal absolute temperature, the resulting slope of the straight line being equal to $\mu/2.3R$. The nature and order of the reaction constant, k', is inconsequential for the calculation of μ provided the temperature increment, ΔT, is sufficiently narrow to yield a linear relation when equation 42 is being plotted. Discontinuous changes in the curves resulting from Arrhenius plots have been claimed, even to the extent of changes in the sign of the slope (52, 68, 235, 239), and have been associated with a shift in the rate-determining step in simultaneous reactions at specific "critical" temperatures (for a theoretical discussion, see reference 259). This interpretation has been recently challenged by Kistiakowsky and Lumry (143) on the grounds that curvatures in the Arrhenius plots, exceeding about 100 cal. per degree, and discontinuous changes are incompatible with kinetic or statistical interpretations of reactions in homogeneous media, and that it is actually impossible to prove from rate measurements alone a discontinuity in the activation energy.

According to the collision theory, the integration constant C in equation 42 is interpreted as a measure of the probability that collision between molecules which have acquired a minimum energy of μ will yield a reaction, the fraction of molecules having this energy being $e^{-\mu/RT}$ (111, 191). In view of the critical analysis of this theory by Eyring and Stearn (79, 258, 259, 260), the collision theory will not be considered herein in the interpretation of the temperature dependence of enzymatic reactions (for a review, see reference 235).

The transition-state theory (95, 258, 259) appears more firmly rooted and more realistic, since it ascribes thermodynamic significance to the velocity constant, k_3, through its conjugate equilibrium constant, K_3^*. In contrast to the collision theory, it distributes the energy term, μ or ΔE, between both the entropy of activation and the free energy of activation. In the following discussion the effect of temperature on the specific activation rate, k_3, and on the steady-state constant, K_m, will be considered separately, whereas the interpretation of the experimental results will be deferred to a later section of this review (page 141).

1. The effect of temperature on reaction rates

In accordance with the preceding discussion of the significance of k_3^0 (page 90), the rate-limiting step described by this constant is assumed to be that of the activation of ES, with no specific interpretation of the nature of the activation process. The relation between k_3^0, temperature, and energetic constants is

$$k_3^0 = \kappa \frac{RT}{Nh} \exp(\Delta S^*/R) \exp(-\Delta H^*/RT) \tag{43}$$

where RT/Nh is, as before, the frequency factor, κ the transmission coefficient, R the gas constant, and T the absolute temperature;ΔS^* is the entropy of activation and ΔH^* the heat of activation.

The free energy of activation is given by

$$\Delta F^* = -RT \log K^* = -RT \log \frac{k_3^0}{Nh/RT} \tag{44}$$

or by

$$\Delta F^* = \Delta H^* - T\Delta S^* \tag{45}$$

ΔH^* is related to the Arrhenius activation energy by

$$\Delta H^* = \Delta E - RT \tag{46}$$

ΔE being determined by equation 42, which on integration yields:

$$\log \frac{k_3''}{k_3'} = \frac{\Delta E}{R}(1/T' - 1/T'') \tag{47}$$

Accordingly, interpretation of measurements of the temperature dependence of k_3 by equations 43 to 46 yields values for the three energetic constants, ΔH^*, ΔF^*, and ΔS^*.

The application of these formal relations in the interpretation of enzymatic reactions (56, 138, 143, 207, 229, 232, 253, 259) requires recognition of certain experimentally intangible restrictions. One of these, and perhaps the most important one, is the definition of the standard state in the calculation of ΔF^* and of ΔS^*. As pointed out by Eyring and Stearn (80), the only valid basis of reference for a standard state is that which includes all substances which are involved in the equilibrium between the normal and activated states. "Suppressing any of these, amounts to treating as unit activity the actual activity of the suppressed substance at which k' (k_3) is measured, and thus may involve a different standard state for each measurement." The most conspicuous variable in enzymatic systems, and in the comparison of these with nonenzymatically catalyzed reactions, is the hydrogen-ion concentration. Measurements at the "pH optimum" of a proteolytic enzyme are not necessarily a valid basis of reference, since the specific rate constant k_3 need not have reached a maximum value at that pH (page 136). Moreover, although within the pH–activity range of an enzyme the change in ionization of the peptide, amide, or ester substrates may be negligible, proton transfer during the activation process may be a rate-contributing factor. These considerations apply equally to those cases in which the rate of the enzymatic activity is affected by buffer ions.

The interpretation of the free energy and entropy of activation is likewise restricted by the unknown number of active sites per enzyme molecule, n. It is usually assumed that $n = 1$. If $n > 1$, the actual rate constant, k_3^0, will be n times smaller, the free energy of activation, ΔF^*, will be less by $RT \ln n$, and the entropy of activation will be more negative by $-R \ln n$ (143).

2. The effect of temperature on K_m

Interpretation of the temperature dependence of experimentally determined values of K_m is rendered difficult by want of an explicit physical definition of this constant (259) (page 88). If K_m were to express simply the reversible formation of ES from E and S, regardless of the shift in this equilibrium resulting from the conversion of ES into (ES)*, the temperature dependence of K_m could be interpreted by the van't Hoff equation. The resulting values of ΔH, ΔF, and ΔS would have classical thermodynamic significance since, under the stated conditions, $K_m = k_2/k_1$. Conversely, if $k_2 \ll k_3$, the graphically determined value of K_m will approximate k_3/k_1, and a plot of log K_m against reciprocal temperature will yield the quantity ΔE, which is the difference between two heats of activation (253), i.e.,

$$\Delta E = \Delta H^* - \Delta H_1^* \tag{48}$$

where ΔH^* is, as before, the heat of activation corresponding to the step (ES) $\xrightarrow{k_3}$ (ES)*, and ΔH_1^* is the heat of activation for the formation of the *transition* complex in the reaction E + S $\xrightarrow{k_1}$ (ES).

Under all other circumstances, K_m is $\left(\frac{k_2}{k_1} + \frac{k_3}{k_1}\right)$ and a plot of log K_m against reciprocal absolute temperature yields as slope a composite function (253) which contains implicitly both the classical heat of reaction, ΔH, and the heat of activation ($\Delta E = \Delta H^* - \Delta H_1^*$). Since there is no obvious physical model for such a function, attempts to accord thermodynamic significance to the temperature dependence of K_m (138, 142, 235, 239) have to be viewed with great reservation, unless it can be shown that for any of the systems one of the two limiting conditions described above applies.

IV. Measurements of Enzymatic Activity

A variety of analytical methods is available for the quantitative determination of hydrolysis rates of specific substrates by proteolytic enzymes. Most of these have originated as procedures in the quantitative analysis of proteins, peptides, and amino acids, and have been reviewed by Martin and Synge (184). The more prominent methods which have been employed in enzymatic analyses are summarized in table 3.

The most commonly used, but by no means the most accurate, methods are the volumetric titrations in media containing alcohol, formaldehyde, or acetone. The advantages offered by the relative simplicity and rapidity of performance of these titration methods are outweighed by their limited accuracy and specificity, particularly when the determination of the end point is based on the visual observation of the color change of a single indicator (219, 274). In the alcohol titration of Willstätter and Waldschmidt-Leitz (287) and of Grassmann and Heyde (97), an additional error may arise from the absorption of atmospheric carbon dioxide by the alkaline solution required for the complete titration of the

ammonium groups (approximately pH 12). While this error may be appreciably reduced if the titration is carried out in an atmosphere of nitrogen (218), this precaution has been seldom observed; moreover, it may result in surface denaturation and precipitation of the enzyme with concomitant fading of the indicator color (thymolphthalein).

TABLE 3

Analytical methods for the determination of the hydrolysis of synthetic substrates by proteolytic enzymes

METHOD	ENZYMATIC SUBSTRATES	GROUP DETERMINED	ANALYTICAL REACTION PRODUCT	REFERENCES
Alcohol titration	Peptides, amides	$-NH_3^+$	$-NH_2$	(287, 97)
Formol titration	Peptides, amides	$-NH_3^+$	$-N(CH_2OH)_2$	(255, 172, 173, 174, 72, 73, 234, 122)
Acetone titration	Peptides, amides	$-COO^-$	$-COOH$	(175, 293)
Gasometric nitrous acid	Peptides	$-NH_3^+$	N_2	(270)
Dilatometric	Peptides, amides	$-COO^-$, $-NH_3^+$	Volume contraction	(123, 176, 177, 178)
Ammonia determination (Conway)	Amides	NH_4^+	NH_4^+	(66, 232)
Gasometric ninhydrin	Exopeptides	Amino acid	CO_2	(183, 218, 273)
Titrimetric ninhydrin	Exopeptides	Amino acid	CO_2	(275)
Colorimetric ninhydrin	Exopeptides	Amino acid	Colored complex	(71, 193, 225, 226)
Colorimetric ferric chloride	Hydroxamides	Hydroxamides	Colored complex	(121)
Enzymatic decarboxylation	Exopeptides	Tyrosine	CO_2	(86, 294)
Enzymatic oxidative deamination	Exopeptides	Amino acid	O_2	(108, 222, 295)
Copper precipitation	Peptides	Amino acid	Cu	(214)
Aqueous titration (potentiometric)	Esters	$-COO^-$	H_2O	(232)

Determination of the liberated amino groups in the presence of formaldehyde (72, 73, 172, 173, 174, 234, 255) is less equivocal, particularly when the glass electrode (73, 234) is used for the determination of the end point, which is reached in less alkaline solutions (approximately pH 9) than in the alcohol titration.

A potentiometric formaldehyde titration (18 per cent formaldehyde) has been recently proposed by Iselin and Niemann (122), in conjunction with the monovalent tris(hydroxymethyl)aminomethane hydrochloride buffer ($pK = 8.1$). This buffer is distinguished by an excellent buffer capacity within the pH range of enzymatic measurements with chymotrypsin and trypsin, and does not participate in the reaction between chymotrypsin and nicotinyl-L-tryptophanamide or nicotinyl-L-phenylalaninamide.

The titration of the liberated carboxyl groups in the presence of acetone (175) likewise circumvents the alkali error, since the titration is carried out with standard acid, using naphthyl red as indicator (pH 3.5). The accuracy of this method has been increased by spectrophotometric determination of the color change (293) and this modified technic has been shown to be useful in the determination of enzymatic hydrolysis rates (293). However, since most proteolytic enzymes have a pH optimum on the alkaline side of neutrality, the large blank values arising from the titration of the buffer solution may be considered to be detrimental to the application of the acetone titration method to the present enzymatic systems.

The manometric nitrous acid method of Van Slyke (270) has been of limited use in the study of proteolytic enzymes, primarily because it is a time-consuming procedure which, moreover, is not strictly specific for α-amino groups.

Another method which is applicable to the determination of hydrolysis rates of peptide groups is the dilatometric method of Linderstrøm-Lang and coworkers (123, 176, 177, 178), which is based on the volume contraction which ensues when ionized carboxylate and ammonium groups are created during the hydrolysis of the uncharged peptide group. In the hands of these authors, this method has been shown to operate with a high degree of precision. The rigorous experimental technics required in the use of this method have limited its application.

The determination of the hydrolysis rates of amides has never been a problem, since several accurate methods for the determination of ammonia have long been known. The microdiffusion method of Conway (66), modified by the replacement of standard hydrochloric acid by boric acid as a trapping medium for ammonia, has been shown to be a convenient and accurate means to this end (232).

When terminal amino acids are the reaction products of peptide hydrolysis, the reaction of the free amino acids with ninhydrin appears to excel all previous methods as a quantitative and specific procedure for the determination of hydrolysis rates. The manometric determination of carbon dioxide, as described by Van Slyke and coworkers (183, 273), or its titrimetric (275) variation has been shown to operate with a high degree of precision if previously standardized toward the specific amino acid which is to be determined. Comparative measurements of the gasometric ninhydrin method and of the alcohol titration method in the presence of nitrogen, in the enzymatic analysis of carboxypeptidase (218), subsequently led to the preferential use of the former in quantitative kinetic measurements (197).

Although the collection of aliquots is not a time-limiting factor in the manometric ninhydrin method, the entire procedure is time-consuming and somewhat

laborious. The spectrophotometric determination of the complex resulting in the reaction of ninhydrin with amino acids has been recently described by two groups of investigators. In Schwert's (226) modification of Harding and MacLean's (71) procedure, the color is developed in the presence of pyridine at about pH 8 and read in the spectrophotometer at 572 mμ. In this procedure, ammonia does not contribute to the color intensity; however, the calibration line does not pass through the origin but intersects with the 100 per cent transmission axis at 0.3 micromole of amino acid (phenylalanine), which is the lower limit of resolving power of the method. A more extensive investigation by Moore and Stein (193), directed toward the quantitative determination of a large series of amino acids and related compounds, has led to the colorimetric analysis of the colored complex after boiling of the amino acids with ninhydrin in methyl cellosolve in the presence of a reducing agent (stannous chloride) at pH 5 (citrate buffer), dilution being carried out with aqueous 1-propanol. This method determines also ammonia and amino groups other than those contributed by free amino acids. Although the color yield from a given amino acid is constant, the different amino acids do not all give the same percentage yield of the blue product. Moore and Stein's method has been shown to be eminently suitable for the measurements of hydrolysis rates of substrates which are devoid of free amino groups and which yield free amino acids as reaction products (194, 195, 255).

Moore and Stein's method has been adapted to the determination of hydrolysis rates of substrates which contain a free amino group and which on hydrolysis give rise to more than one amino acid (225). With the use of standard solutions, calibration curves were constructed from which the per cent hydrolysis of a synthetic peptide (L-leucylglycylglycine) may be estimated without prior determination of the extinction curves of the individual components of the hydrolysis mixture (L-leucylglycylglycine, glycylglycine, and L-leucine). If this method should fulfill its initial promise, it should be generally applicable to the determination of hydrolysis rates of peptides and amides.

As a result of the observation that chymotrypsin catalyzes the hydrolysis of *N*-acyl amino acid hydroxamides, a colorimetric method for the determination of chymotrypsin activity has been recently developed, based on the reaction of the unhydrolyzed substrates, in acid aqueous methanol, with ferric chloride (121). This method was found to be sufficiently sensitive to allow hydrolysis rates to be determined in initial substrate concentrations of 0.0025 *M*.

An attractive method of analysis, albeit of limited application, has been described by Zamecnik and Stephenson (294) and applied by Frantz and Stephenson (86) to the kinetic study of the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine. In this method, the reaction product, L-tyrosine, is decarboxylated by bacterial tyrosine decarboxylase, and carbon dioxide is subsequently determined manometrically in the Barcroft–Warburg apparatus. This method is a variation of that previously proposed by Herken and Erxleben (108) and by Zeller (222, 295), who employed amino acid oxidase for the oxidative deamination of the liberated amino acids and determined the oxygen uptake manometrically.

The different combining ratios of peptides and amino acids, respectively, with

cupric ions were used by Pope and Stevens (214) for measuring the enzymatic hydrolysis of representative peptides.

The enzymatic hydrolysis of esters of amino acid derivatives is conveniently measured by determining the amount of alkali required to compensate for the incipient pH shift caused by the liberation of the carboxyl group. This titration is carried out in aqueous solutions at the pH of enzymatic hydrolysis, a pH meter being used as null instrument (232).

INCUBATION TIME AND EXTENT OF HYDROLYSIS

The theoretical relation of time and extent of enzymatic hydrolysis to enzyme and substrate concentrations has been considered in a preceding section of this review (page 83). It may not be amiss to consider herein these relations as they pertain to the design of enzymatic experiments.

When the kinetic course of an enzymatic reaction is to be determined, the rate of the reaction should be sufficiently slow to enable accurate definition of the relation between time and degree of hydrolysis. This is of particular importance when, for reasons already given (page 86), the entire course of the reaction cannot be expressed by a single function and when the reaction velocity at zero time has to be extrapolated from the initial portion of the rate curve. Such determinations have to be supplemented by an additional experiment in which the rate of the reaction is sufficiently increased to determine the maximum extent of enzymatic hydrolysis. This latter information is a prerequisite for the former for the determination of the chemical nature and optical specificity of the enzymatic reaction. In either case, the rate of the reaction is usually adjusted by the proper choice of enzyme concentration and/or of temperature.

An upper limit to an enzymatic reaction time is usually imposed by the stability of the enzyme and by spontaneous hydrolysis of the substrate. While specific substrates exert a protective action on enzymes (71), it is to be recognized that the proteinases, such as trypsin, are extremely labile in the absence of the substrate, this enzyme losing 50 per cent of its initial activity within 60 min. at pH 7.8 and 25°C. (232). Although proper control experiments should provide a safeguard against these inherent sources of error, there is no apparent need for extending enzymatic hydrolyses over a period of 24 hr. or longer, particularly at temperatures which are favorable for bacterial growth (25° to 40°C.). The frequent practice of ascribing significance to limited degrees of hydrolysis (of the order of 10 to 20 per cent), observed during prolonged incubation (24 hr. or more), is apt to leave the interpretation of such data open to conjecture. A timely warning has been recently voiced by Fodor, Price, and Greenstein (84) in justifying their selection of a maximum period of 8 hr. in the enzymatic incubation of saturated and unsaturated tripeptides: "It is probable in several instances, that, had the incubation period been extended well beyond the period chosen, the relatively resistant bonds would have been completely hydrolyzed, and perhaps even those apparently completely resistant bonds in certain substrates might have been at least measurably attacked. It is somewhat judicious, however, to set some sort of a limit to what an enzyme might reasonably be expected to

do, and we are reluctant to ascribe authentic enzymatic reactivity to a reaction which, under otherwise optimum conditions, requires an undue amount of time."

V. The Specificity of Trypsin

A. SPECIFICITY

The investigations of Bergmann and his colleagues established the nature of the specific amino acid residues for trypsin substrates and demonstrated the effects of some modifications in these substrates. This topic has been reviewed by Bergmann and Fruton (27). The substrates used were exclusively amides of *N*-substituted amino acids and the bond hydrolyzed was in every case an amide bond. Recent investigations have shown that, contrary to the impression previously held, trypsin can also hydrolyze ester bonds and that other polar groups can replace peptide and amide bonds as secondary structural elements. These results are summarized in table 4.

1. The typical amino acid residue, R′

As shown in table 4, arginine and lysine are the typical amino acid residues for trypsin substrates. Masking of the terminal cationic groups of these amino acids by carbobenzoxylation causes a loss of substrate activity. The failure of the norvaline or norleucine analogs to act as substrates indicates that it is the loss of the charge at this point rather than an unfavorable steric effect of the masking group which causes the loss of activity.

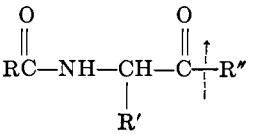
Bergmann, Fruton, and Pollok (30) demonstrated that histidine cannot replace arginine or lysine in trypsin substrates, since α-benzoyl-L-histidinamide and α-hippuryl-L-histidinamide are inactive. Benzoylglycinamide, α-benzoyl-L-tyrosinamide, carbobenzoxy-L-isoglutamine, and α-benzoyl-L-tyrosylglycinamide, the latter being a typical chymotrypsin substrate, were also shown to be unaffected by trypsin.

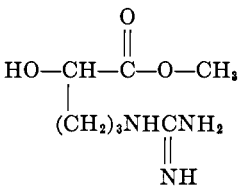
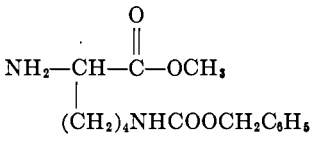
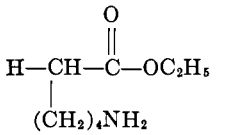
The following dipeptides have been shown not to be hydrolyzed by trypsin (206): DL-leucyl-L-tyrosine, leucylglycylglycine, chloroacetyl-L-leucine, glycylalanine, glycylglycine, glycylaspartic acid, and chloroacetyl-L-tyrosine, a typical carboxypeptidase substrate.

It has been claimed (2) that several tetrapeptides containing two glycine, one tyrosine, and one alanine residue, and tripeptides of glycine and tyrosine are hydrolyzed by "tryptic protease" prepared according to the method of Waldschmidt-Leitz. However, the finding that this enzyme preparation hydrolyzes α-hippuryl-L-lysinamide to hippuric acid, lysine, and ammonia (31), while crystalline trypsin splits this substrate only at the amide bond to yield α-hippuryl-L-lysine and ammonia (113), suggests that the hydrolysis of the tri- and tetrapeptides may have been due to other enzymes than trypsin. On the other hand, the report that "tryptic protease" fails to hydrolyze L-pseudoleucyl-L-tyrosine (4) must be accepted as a property of trypsin, since trypsin is undoubtedly present in this preparation.

TABLE 4

Specificity of trypsin

Amino acid derivatives hydrolyzed by trypsin: 

	$RC(=O)-$	$-NH-CH(R'')-C(=O)-$	R″	REFERENCES
1.....	Benzoyl	arginine	amide	(29, 30)
2.....	Hippuryl	arginine	amide	(115)
3.....	*p*-Toluenesulfonyl	arginine	amide	(232)
4.....	*p*-Toluenesulfonyl	arginine	methyl ester	(232)
5.....	Benzoyl	arginine	methyl ester	(232)
6.....	Benzoyl	arginine	ethyl ester	(229)
7.....	Benzoyl	arginine	isopropyl ester	(229)
8.....	Benzoyl	arginine	cyclohexyl ester	(229)
9.....	Benzoyl	arginine	benzyl ester	(229)
10.....	Benzoyl	arginine	α-glyceryl ester	(229)
11.....	Benzoyl	lysine	amide	(113)
12.....	Hippuryl	lysine	amide	(113)
	Related compound hydrolyzed by trypsin			
13.....				(251)
	Related compounds not hydrolyzed by trypsin			
14.....	Benzoyl	ε-carbobenzoxyarginine	amide	(113)
15.....	Hippuryl	ε-carbobenzoxyarginine	amide	(113)
16.....	Benzoyl	norleucine	ethyl ester	(250)
17.....	Benzoyl	norvaline	ethyl ester	(250)
18.....				(232)
19.....				(250)

The report of Bergmann, Zervas, and Fruton (33) that carbobenzoxyglycylglutamylglycine ethyl ester is hydrolyzed by trypsin to carbobenzoxyglycine and glutamylglycine ethyl ester can probably be discounted, since these investigators did not pursue the topic further and later came to regard the basic amino acid residues as characteristic of trypsin substrates.

A few studies of the hydrolysis of more complex synthetic peptides, containing arginine or lysine, have been made. Plentl and Page (213) have shown that L-tyrosyl-L-lysyl-L-glutamyl-L-tyrosine is hydrolyzed by trypsin to L-tyrosyl-L-lysine and L-glutamyl-L-tyrosine, and Katchalski, Grossfeld, and Frankel (132) have reported that polylysine, containing about 32 lysine residues polymerized through α-linkages, is hydrolyzed by trypsin. This observation has been confirmed by Brand, Plentl, and Erlanger (47), who further found that poly-D-lysine and poly-L-ornithine were resistant to tryptic action. On the other hand, in copolymers of arginine and lysine, a greater number of bonds was opened by trypsin than could be accounted for by the arginine content of these polymers if it is assumed that only the bonds involving an arginine carboxyl could be opened.

2. The "secondary peptide" or equivalent group, R

Considerable confusion, arising in large part from inadequate analytical methods, has attended some of the earlier studies on the influence of various substituents in this position. Although it has been reported that α-*p*-toluenesulfonyl-L-argininamide and α-benzoyl-L-lysinamide are resistant to tryptic action, both of these findings were later denied (113, 229). In fact, introduction of the *p*-toluenesulfonyl group in place of the benzoyl group promotes hydrolysis (see table 5). Further, the failure of trypsin to hydrolyze α-hippuryl-L-lysinamide was used as evidence for the existence of heterotrypsin (29). It was later established that this substrate is readily hydrolyzed by trypsin and that heterotrypsin was impure trypsin (113).

Although all of the substrates studied by Bergmann and his coworkers have a "secondary peptide" bond in this position, Snoke and Neurath (251) have shown that this group can be replaced by a hydroxyl group as in methyl α-hydroxy-δ-guanidinovalerate (table 4). However, the rate of hydrolysis of this compound is lower than that of α-benzoyl-L-arginine methyl ester. Further, since α-hippuryl-L-argininamide and α-hippuryl-L-lysinamide are hydrolyzed at about twice the rate of the corresponding benzoyl analogs at a single substrate concentration (table 5), this effect can be ascribed to the greater number of polar groups introduced at this position. If the polar group is omitted, as in ethyl ϵ-aminocaproate (table 4), activity is lost. Niemann has undertaken an investigation of the effect of substituents at this position on the rate of hydrolysis (198).

Although the effect of a positively charged free amino group in the α-position has not been determined, there is evidence that a negative charge in the vicinity of the susceptible bond tends to retard tryptic hydrolysis, since L-tyrosyl-L-lysyl-L-glutamyl-L-tyrosine is hydrolyzed very slowly, while L-tyrosyl-L-lysyl-L-glutamic acid is not hydrolyzed at all (213).

3. The hydrolyzable bond, R″

As is evident from the preceding discussion, the susceptible bond may be a peptide, an amide, or an ester bond. In general, probably in part as a result of lower bond strength, esters are hydrolyzed more rapidly than are the corresponding amides (*cf.* table 5). As a result, some esters whose amide analogs would be expected to be resistant to hydrolysis are hydrolyzed at a very slow rate by trypsin. Examples of this class are L-tyrosine ethyl ester, L-phenylalanine ethyl ester, and *N*-carbobenzoxy-*O*-acetyl-L-tyrosylglycine ethyl ester. On the other hand, glycine ethyl ester, carbobenzoxyglycyl-DL-phenylalanine ethyl ester, and carbobenzoxyglycyl-L-tyrosine ethyl ester are not hydrolyzed at all. As would be anticipated, trypsin has no "true" esterase activity and ethyl butyrate and butyl acetate are not hydrolyzed (232).

The minimum structural requirements for trypsin substrates may be summarized as follows:

1. A positive charge separated from the susceptible bond by a chain not shorter than that of arginine or lysine. The maximum distance of separation has not been established.
2. A polar group in the α-position to the carbonyl carbon atom of the susceptible bond. Although all polar groups studied have been uncharged, it appears that a *negative* charge at this point would be unfavorable for hydrolysis. The effect of a *positively* charged α-substituent is unknown. Further, although studies of the optical specificity of trypsin have not been carried out, it appears, by analogy with the other enzymes studied, that the α-carbon must be of the L-configuration.
3. The susceptible peptide, amide, or ester bond.

B. KINETIC STUDIES

The kinetic studies which have been made on the hydrolysis of synthetic substrates by trypsin are summarized in table 5.

Although it was reported that the tryptic hydrolysis of benzoyl-L-argininamide (56, 115, 232), hippuryl-L-lysinamide, and hippuryl-L-argininamide (115) follows first-order kinetics at an initial substrate concentration of 0.05 *M*, this conclusion has been challenged by Harmon and Niemann (107) and by Schwert and Eisenberg (229). These workers demonstrated that K_m for benzoyl-L-argininamide is so small that at substrate concentrations of the order of 0.01 *M* the reaction initially follows zero-order kinetics and that deviations from zero-order kinetics, previously interpreted as adherence to first-order kinetics, are the result of inhibition by benzoyl-L-arginine. This inhibition was shown to be of the "indeterminate" type, being essentially noncompetitive at low concentrations of benzoyl-L-arginine but becoming more competitive as the concentration of benzoyl-L-arginine was increased.

In contrast, the trypsin-catalyzed hydrolysis of esters of benzoyl-L-arginine follows zero-order kinetics and is not influenced by the addition of benzoyl-L-

arginine. This behavior is a reflection of the very high affinity of these esters for the enzyme (see table 5). Further, all esters of benzoyl-L-arginine which have been studied are hydrolyzed at the same rate by trypsin, steric and chemical effects playing no role in the hydrolysis rate. Between 3° and 25°C. the tempera-

TABLE 5

Kinetic studies with trypsin at 25°C.

SUBSTRATE	pH	k^{1st}	k_3	K_m	REFERENCES
		min.$^{-1}$/*mg.* N/*ml.*	*moles/liter/min./mg.* N/*ml.*	*M*	
0.05 *M* α-benzoylargininamide	7.5	8.8×10^{-2}			(115)
0.05 *M* α-benzoylglycylargininamide	7.5	9.2×10^{-1}			(115)
0.05 *M* α-benzoyllysinamide	7.4	4.6×10^{-2}			(93)
0.05 *M* α-benzoylglycyllysinamide	7.5	5.1×10^{-1}			(115)
0.002–0.05 *M* α-benzoylargininamide	7.7		2.2×10^{-3}*	2.1×10^{-3}*	(107)
0.0125–0.05 *M* α-benzoylargininamide	7.8		3.8×10^{-3}†		
0.05 *M* α-toluenesulfonylargininamide	7.8	1.3×10^{-1}			(232)
10^{-4} *M* L-tryosyl-L-lysyl-L-glutamyl-L-tyrosine	7.8	3×10^{-4}			(213)
0.0007–0.03 *M* α-benzoylarginine methyl ester	8.0		2.5×10^{-1}	$\sim 8 \times 10^{-5}$	(232, 229)
ethyl ester	8.0		2.5×10^{-1}	$\sim 8 \times 10^{-5}$	(229)
isopropyl ester	8.0		2.5×10^{-1}	$\sim 8 \times 10^{-5}$	(229)
cyclohexyl ester	8.0		2.5×10^{-1}	$\sim 8 \times 10^{-5}$	(229)
benzyl ester	8.0		2.5×10^{-1}	$\sim 8 \times 10^{-5}$	(229)
-α-glyceryl ester	8.0		2.5×10^{-1}	$\sim 8 \times 10^{-5}$	(229)
0.006 *M* α-toluenesulfonylarginine methyl ester	8.0		1.8		(232)
0.01 *M* methyl α-hydroxy-δ-guanidinovalerate	8.0		1.4×10^{-1}		(250)

* Calculated by Harmon and Niemann (107) assuming competitive inhibition.

† Calculated from data of Schwert and Eisenberg (229), using initial slopes of reaction curves and assuming zero-order kinetics in this concentration range.

ture coefficient is identical for the hydrolysis of these esters (229). The observed zero-order kinetics for amide and ester substrates of trypsin, indicating a very high rate of combination between enzyme and substrate, together with the essentiality of a positively charged group in trypsin substrates, suggests that the combination between enzyme and substrate is essentially electrostatic in character.

The shapes and maxima of the pH–activity curves for the tryptic hydrolysis of casein (163), benzoyl-L-argininamide (30), benzoyl-L-arginine methyl ester, and *p*-toluenesulfonyl-L-arginine methyl ester (232) show great similarity. This finding, together with the observation of a parallel decrease in the rates of amide and ester hydrolysis following partial inactivation of trypsin by alkali or by the addition of soybean inhibitor, was taken as further evidence that the esterase and amidase activities of trypsin are mediated by the same catalytic centers (232).

Measurements of the rate of hydrolysis of esters of benzoyl-L-arginine in the presence of alcohols indicate that the addition of alcohol causes an initial increase and a subsequent decrease in reaction rate. In 32 volumes per cent ethanol the initial rate is 50 per cent greater than in water. At higher alcohol concentrations the rate again decreased. That the decreasing rates were not the result of mass law effects was shown by the fact that for both the methyl and ethyl esters of benzoyl-L-arginine 16 volumes per cent of methanol causes a 15 per cent increase in initial rate, while ethanol, 1-propanol, and *tert*-butyl alcohol at the same concentration cause increases of 35, 40, and 40 per cent, respectively. Since the combination step between enzyme and substrate must be a very rapid reaction, as evidenced by the adherence of these hydrolyses to zero-order kinetics in aqueous systems, this rate increase must be ascribed to an effect upon the activation step. The addition of benzoyl-L-arginine, ammonium chloride, arginine hydrochloride, and guanidine hydrochloride has no effect upon the hydrolysis of esters in aqueous or alcoholic media (229).

Fruton, Irving, and Bergmann (92, 93, 119) have reported that cathepsin II and papain have the same specificity as does trypsin and, from less complete studies, it appears that bromelin (30) and ficin (118) may fall into the same group.

Grob (98) has found trypsin to be inactivated by reducing agents and to be slightly activated by mild oxidizing agents. These effects were regarded as being due to the influence of the redox potential on enzymatic activity. Peters and Wakelin (212) reported, however, that thiols inactivate trypsin and chymotrypsin and also react with chymotrypsinogen. This inactivation was marked by the appearance of sulfhydryl groups and could not be reversed by disulfide compounds.

Rothen (220, 221) has observed that trypsin inactivates multilayers of bovine albumin deposited on a slide through intervening "blankets" of barium stearate, formvar, polyvinyl chloride polymer, or octadecylamine which may approach 1000 Å. in thickness. The thickness of the blanket necessary to prevent enzymatic action was found to vary with the number and mode of deposition of the underlying layers of bovine albumin. A powerful argument against this demonstration of enzymatic action at a distance has been advanced by Karush and Siegel (130), who found that electron micrographs of replicas of dried multilayers of bovine albumin revealed numerous protrusions which might extend through subsequently deposited blankets (see also reference 233).

Hanig (103), however, has found that when solutions of trypsin and hemoglobin are separated by thin, supported collodion membranes, which were shown to be impermeable to hemoglobin and to trypsin, tyrosine is liberated from the

hemoglobin. The degree of hydrolysis is decreased with increasing membrane thickness up to a critical thickness at which enzymatic action was prevented.

Further arguments against this concept, however, are provided by the difficulties of reconciling enzymatic action at a distance with the two-step reaction kinetics and with the very high substrate specificity exhibited by these enzymes. This high specificity would seem to require that the long-range forces arising at the enzyme surface be nondispersive in character.

The esterase and proteinase activities of trypsin have been found (127) to decrease in a parallel way when trypsin is allowed to react with diisopropyl fluophosphate, but the esterase activity decreases much less rapidly than the proteinase activity when acetylated trypsin is allowed to react with this reagent. This discrepancy was accounted for on the basis that an acetyl group hindered the approach of a protein molecule to the surface, while the small ester substrate molecule was still able to reach the surface.

VI. The Specificity of Chymotrypsins

A. α-CHYMOTRYPSIN

The amino acid derivatives and related compounds which are hydrolyzed by chymotrypsin are shown in table 6. Closely related structures which are resistant to the action of chymotrypsin are also shown in table 6.

A large number of other compounds, dissimilar in structure to those listed in table 6, have been tested as chymotrypsin substrates, with negative results. These are D-leucylglycine, D-leucylglycylglycine, glycylaspartic acid, chloroacetyl-L-leucine, tri-L-alanyl-L-alanine, tetra-DL-alanyl-L-alanine, glycylglycine, pentaglycylglycine, glycyl-L-tryptophan, glycyl-L-alanine, glycyl-L-tyrosine, chloroacetyl-L-tyrosine, which is a substrate for carboxypeptidase (166), and benzoyl-L-leucyl-L-leucylglycine (25). Negative results obtained with compounds bearing a structural similarity to typical substrates will be discussed in detail in subsequent sections (pages 113, 114, and 117).

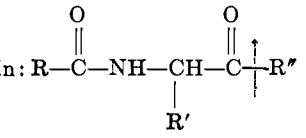
1. The specific amino acid side chain, R′

The second column of table 6 indicates that tyrosine, phenylalanine, tryptophan, methionine, norleucine, and norvaline can supply, to a greater or lesser degree, the necessary structural element in this position. Although benzoyl-L-arginine methyl ester is hydrolyzed by chymotrypsin, *p*-toluenesulfonyl-L-arginine methyl ester is unaffected (232) as are the other typical trypsin substrates, benzoyl-L-argininamide (29) and hippuryl-L-lysinamide (25). This behavior is probably the result of the greater susceptibility of esters to hydrolysis and of the high affinity of benzoyl derivatives for chymotrypsin (*vide infra*).

A similar anomaly which probably also arises from the fact that esters are hydrolyzed much more readily than are amides is seen in the case of the dehydroamino acids, since the ethyl ester of acetyldehydrophenylalanine (table 6, line 46) undergoes slow hydrolysis, while the amides of benzoyldehydrotyrosine

TABLE 6

Specificity of chymotrypsin

A. Amino acid derivatives hydrolyzed by chymotrypsin: 

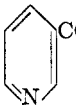
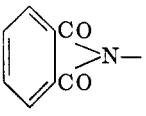
	O ‖ RC—	—NH—CH—CO— R′	—R″	REFERENCES
1.....	Benzoyl	tyrosine	amide	(139)
2.....	Benzoyl	tyrosine	ethyl ester	(139)
3.....	Benzoyl	tyrosine	hydrazide	(182)
4.....	Benzoyl	tyrosyl	glycinamide	(26)
5.....	Benzoyl	phenylalanine	methyl ester	(251)
6.....	Benzoyl	phenylalanine	ethyl ester	(135)
7.....	Benzoyl	phenylalanine	amide	(120)
8.....	Benzoyl	phenylalanine	hydroxamide	(121)
9.....	Benzoyl	arginine	methyl ester	(232)
10.....	Benzoyl	methionine	ethyl ester	(135)
11.....	Benzoyl	norleucine	ethyl ester	(249)*
12.....	Benzoyl	norvaline	ethyl ester	(249)*
13.....	Acetyl	tyrosine	amide	(136)
14.....	Acetyl	tyrosine	ethyl ester	(139)
15.....	Acetyl	tyrosyl	glycinamide	(181)
16.....	Acetyl	phenylalanine	amide	(120)
17.....	Acetyl	phenylalanine	hydroxamide	(121)
18.....	Acetyl	tryptophan	ethyl ester	(135)
19.....	Nicotinyl	tyrosine	amide	(120)
20.....	Nicotinyl	phenylalanine	amide	(120)
21.....	Nicotinyl	methionine	amide	(120)
22.....	Nicotinyl	tryptophan	amide	(120)
23.....	Glycyl	tyrosine	amide	(90)
24.....	Glycyl	tyrosyl	glycinamide	(25)
25.....	Glycyl	phenylalanine	amide	(90)
26.....	Glycyl	glycine	amide	(90)*
27.....	Carbobenzoxy	tyrosyl	glycinamide	(25)
28.....	Carbobenzoxy	tyrosyl	glycylglycinamide	(25)*
29.....	Carbobenzoxy	phenylalanyl	glycinamide	(90)
30.....	Carbobenzoxyglycyl	tyrosine	amide	(90)
31.....	Carbobenzoxyglycyl	tyrosine	ethyl ester	(232)
32.....	Carbobenzoxyglycyl	tyrosyl	glycinamide	(25)
33.....	Carbobenzoxyglycyl	phenylalanine	amide	(90)
34.....	Carbobenzoxyglycyl	phenylalanine	ethyl ester	(232)
35.....	Carbobenzoxyglycyl	phenylalanyl	glycinamide	(25)
36.....	Carbobenzoxyglutamyl	tyrosyl	glycinamide	(27)
37.....	Glutamyl	tyrosyl	glycinamide	(27)*
38.....		Phenylalanine	amide	(90)*
39.....		Phenylalanyl	glycinamide	(90)†

TABLE 6—*Concluded*

	RC(=O)—	—NH—CH(R′)—CO—	—R″	REFERENCES
40.....		Tyrosine	amide	(90)*
41.....		Tyrosyl	glycinamide	(90)†
42.....		Tyrosine	ethyl ester	(135)*

Not shown in this table are compounds such as L-tyrosyl-L-tyrosinamide, L-phenylalanyl-L-phenylalaninamide, L-phenylalanyl-L-tyrosinamide, carbobenzoxy-L-tyrosyl-L-tyrosinamide, carbobenzoxy-L-tyrosyl-L-phenylalaninamide, carbobenzoxy-L-phenylalanyl-L-tyrosinamide, and carbobenzoxy-L-phenylalanyl-L-phenylalaninamide, in which the bond hydrolyzed has not been established. In general, these substrates are hydrolyzed very slowly (90).

B. Related compounds hydrolyzed by chymotrypsin: R—CH(R′)—C(=O)—R″

	R	R′	R″	REFERENCES
43.....	HO—	$C_6H_5CH_2$—	—OCH_3	(251)
44.....	Cl—	$C_6H_5CH_2$—	—OCH_3	(251)
45.....	H—	$C_6H_5CH_2$—	—OCH_3	(251)
46.....	CH_3CONH—	C_6H_5CH= O_2N	—OC_2H_5	(135)*‡
47.....	C_6H_5CONH—	HO-C$_6$H$_4$-CH_2—	—OC_2H_5	(135)
48.....		*p*-$ClC_6H_4CH_2$—	—NH_2	(120)
49.....	CH_3CO—	$C_6H_5CH_2$—	—OC_2H_5	(135)
50.....	C_2H_5OCO—	$C_6H_5CH_2$—	—OC_2H_5	(135)
51.....	HOOC—	$C_6H_5CH_2$—	—OC_2H_5	(135)
52.....	C_6H_5CONH—	C_6H_5—	—OC_2H_5	(135)
53.....	C_6H_5CONH—	*p*-HOC_6H_4CH=	—NH_2	(26)‡
54.....	C_6H_5CONH—	C_6H_5CH=	—NH_2	(26)‡
55.....		$C_6H_5CH_2$—	—OCH_3	(135)
56.....	NH_2CO—	$C_6H_5CH_2$—	—NH_2	(137)

C. Related compounds which are not hydrolyzed by chymotrypsin

* Hydrolyzed very slowly.

† Some hydrolysis probably also occurs at the glycinamide bond (90).

‡ In this structure there is no hydrogen on the α-carbon.

and of benzoyldehydrophenylalanine (lines 53 and 54) are resistant to hydrolysis.

Although glycylglycinamide (line 26) is hydrolyzed by chymotrypsin, and the glycinamide bond is opened in addition to the peptide bond in tyrosyl- and phenylalanyl-glycinamides (lines 39 and 41), these reactions occur at a very low rate and, since no rapidly hydrolyzed compound is known in which glycine contributes the carbonyl group to an amide, ester, or peptide bond, glycine cannot be regarded as meeting the structural requirements for a specific amino acid residue for chymotrypsin substrates. The same arguments apply to norvaline and norleucine (lines 11 and 12). Further, since carbobenzoxyglycyl-L-leucylglycinamide, carbobenzoxyglycyl-L-glutamylglycinamide (25), benzoyl-DL-

TABLE 7

Influence of amino acid residues on chymotryptic hydrolysis at 25°C. and pH 7.8

Unless otherwise indicated, the solvent was 30 volumes per cent methanol

R′	R	R″ = —OC_2H_5				R″ = —NH_2			
		K_m	k_3	C_{max}	References	K_m	k_3	C_{max}	References
		10^{-2} *M*	10^{-2}			10^{-2} *M*	10^{-2}		
o-Nitrotyrosine	Benzoyl	1.1	340	128	(135)				
Tyrosine	Benzoyl	0.4	82	91	(138)	4.2	0.65	0.067	(138)
Tryptophan	Acetyl	0.17	32	83	(230)				
Tyrosine	Acetyl	3.2	260	36	(230)	8.1†	0.29†	0.016†	(136)
Phenylalanine	Benzoyl	0.6	39	30	(135)	0.35*	0.15*	0.2*	
Methionine	Benzoyl	0.08	0.8	4	(135)				
Tryptophan	Nicotinyl					0.4*	0.3*	0.3*	
p-Chlorophenylalanine	Nicotinyl					0.05*	0.03*	0.3*	
Tyrosine	Nicotinyl					1.4*	0.6*	0.2*	
Phenylalanine	Nicotinyl					2.8*	0.4*	0.06*	

* Calculated from the data of Iselin, Huang, MacAllister, and Niemann (120). These determinations, made in aqueous solution, were not intended for quantitative interpretation and are given here merely as a first approximation.

† Measurements made in 21 volumes per cent methanol.

serine ethyl ester, benzoyl-DL-threonine ethyl ester, acetyl-DL-threonine ethyl ester (135), nicotinyl-DL-histidinamide (120), and benzoylalaninamide (26) have been found to be entirely resistant to chymotryptic action, these amino acids, too, must be lacking in the structural elements necessary for specific amino acid side chains.

The quantitative effect of varying the amino acid residue in ester and amide substrates is illustrated in table 7. If C_{max} is used as a rough measure of the overall susceptibility of a substrate to hydrolysis in the presence of chymotrypsin, it appears that the decreasing order of activity of the amino acids is tryptophan > tyrosine > phenylalanine > methionine. The results of Iselin, Huang, MacAllister, and Niemann (120) are in agreement with this conclusion con-

cerning the position of methionine in this series, since nicotinyl-DL-methioninamide is hydrolyzed very slowly by chymotrypsin. From the results shown in table 7 it appears that introduction of a negative substituent into the aromatic ring causes an increase in susceptibility to hydrolysis, since C_{max} is larger for derivatives of nitrotyrosine and *p*-chlorophenylalanine than for the parent compounds, and is also larger for tyrosine substrates than for the phenylalanine analogs.

Kaufman and Neurath have noted (135) that a possible explanation for the ability of methionine to replace an aromatic amino acid in these substrates may be found in the well-known chemical similarity between benzyl and allyl compounds and in the biological and structural similarity of the allyl group and the divalent sulfur atom. This view is supported by the observation that the ethyl esters of benzoylnorleucine and benzoylnorvaline, while sterically similar to the ethyl ester of benzoylmethionine, lack the divalent sulfur atom and are hydrolyzed much more slowly than is the methionine substrate (249).

The distance between the aromatic ring and the α-carbon atom appears to be very critical, since the removal of one methylene group from benzoyl-L-phenylalanine ethyl ester, so as to yield the ethyl ester of *dl*-α-benzaminophenylacetic acid (table 6, line 52), causes total loss of substrate activity. This change in length of the side chain is of the order of 1–2 Å.

2. The "secondary peptide" or equivalent group, R

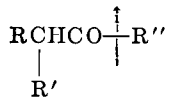
It is clear from table 6 that a very wide range of substitutions may be made in this position without causing the activity of a substrate to disappear. The amino group of an amino acid may be substituted with benzoyl, acetyl, nicotinyl, glycyl, carbobenzoxyl, carbobenzoxyglycyl, or carbobenzoxyglutamyl groups; it may be left as a free amino group, or it may be replaced by a hydroxyl group, by chlorine, or even by hydrogen (table 6, lines 43, 44, and 45). That these various substitutions are not, however, without effect upon substrate activity is shown in table 8, in which the substrates are listed in increasing order of C_{max} for each type of R″ group.

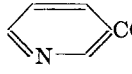
When allowance is made for the effects of the various solvents used in these determinations and for the influence of the R″ groups, it appears that susceptibility diminishes with R in the approximate order: benzoyl > nicotinyl > acetyl > carbobenzoxyglycyl > glycyl for substituted amino acids and continues in the order: *l*-hydroxyl > amino > *dl*-chloro > *d*-hydroxyl > hydrogen for substituents on the α-carbon. From such scanty data as are available for the effect of the carbobenzoxyglutamyl group in this position (27) it can be inferred that it is slightly more active than the carbobenzoxyglycyl group (25).

Although Bergmann and Fruton (26, 90) reported that masking of the amino group of tyrosinamide or of phenylalaninamide with a carbobenzoxyl or benzoyl group causes a loss of substrate activity, as does masking of the amino group of glycyltyrosinamide with a carbobenzoxyl group, they also found the uncharged compounds benzoyl-L-tyrosylglycinamide (26) and carbobenzoxy-L-tyrosylglycinamide (25) to be typical substrates for chymotrypsin. These

TABLE 8

Effect of the substituent R *on the hydrolysis of substrates by chymotrypsin at pH 7.8 and 25°C.*



	R	R′	R″	CONFIGURATION	K_m	k_2	C_{max}	REFERENCES
					$10^{-2}M$	10^{-2}		
1[a]	H—	$C_6H_5CH_2$—	—OCH_3		0.96	0.027	0.012	(253)
2[a]	HO—	$C_6H_5CH_2$—	—OCH_3	*d*	1.65	0.14	0.038	(253)
3[a]	Cl—	$C_6H_5CH_2$—	—OCH_3	*dl*	1.2	0.60	0.14	(253)
4[a]	HO—	$C_6H_5CH_2$—	—OCH_3	*l*	1.1	1.44	0.56	(253)
5[a]	C_6H_5CONH—	$C_6H_5CH_2$—	—OCH_3	L	0.43	53	54	(253)
6[b]	C_6H_5CONH—	$C_6H_5CH_2$—	—OC_2H_5	L	0.57	39	30	(135)
7[b]	CH_3CONH—	*p*-$HOC_6H_4CH_2$—	—OC_2H_5	L	3.1	200	28	(253)
8[b]	C_6H_5CONH—	*p*-$HOC_6H_4CH_2$—	—OC_2H_5	L	0.39	82	91	(138)
9[c]	NH_2—	*p*-$HOC_6H_4CH_2$—	—NH_2	L			0.0003[e]	(90)
10[c]	NH_2CH_2CONH—	*p*-$HOC_6H_4CH_2$—	—NH_2	L	12.2	0.41	0.015	(138)
							0.002[f][b]	(138)
11[b]	$C_6H_5CH_2OCONHCH_2CONH$—	*p*-$HOC_6H_4CH_2$—	—NH_2	L			0.006[f]	(138)
12[d]	CH_3CONH—	*p*-$HOC_6H_4CH_2$—	—NH_2	L	8.1	0.29	0.016	(136)
13[c]	CH_3CONH—	*p*-$HOC_6H_4CH_2$—	—NH_2	L	2.7	0.3	0.048	(230)
14[b]	C_6H_5CONH—	*p*-$HOC_6H_4CH_2$—	—NH_2	L	4.2	0.65	0.067	(138)
15[c]	 CONH—	*p*-$HOC_6H_4CH_2$—	—NH_2	L	1.4	0.6	0.2	[g]

[a] Determinations made in 20 volumes per cent methanol.
[b] Determinations made in 30 volumes per cent methanol.
[c] Determinations made in aqueous system.
[d] Determinations made in 21 volumes per cent methanol.
[e] *C* for a single initial substrate concentration of 0.05 *M*.
[f] *C* for a single initial substrate concentration of 0.025 *M*.
[g] Calculated from data of Iselin, Huang, MacAllister, and Niemann (120).

workers further reported (26) that in the presence of glycinanilide chymotrypsin reacts with benzoyl-L-tyrosine to form benzoyl-L-tyrosylglycinanilide, the synthesis being forced toward completion by the insolubility of the product. The uncertainty regarding the effect of masking of the α-amino group was resolved by Kaufman, Neurath, and Schwert (138), who found that benzoyl-L-tyrosinamide is not only hydrolyzed by chymotrypsin but is hydrolyzed at a higher rate than either L-tyrosinamide or glycyl-L-tyrosinamide. Recently it has been found that acetyl- and benzoyl-L-phenylalaninamides are also hydrolyzed by chymotrypsin (120). From the data given in table 8 for the hydrolysis of L-tyrosinamide, glycyl-L-tyrosinamide, carbobenzoxyglycyl-L-tyrosinamide, and benzoyl-L-tyrosinamide, it seems clear that the presence of a positive charge decreases substrate activity and that the closer this charge is to the α-carbon, the more the activity is decreased. This conclusion is supported by the observation that L-tyrosine ethyl ester is hydrolyzed at a rate too low to be accurately measured (135) and this effect probably accounts, in part, for the low rate of hydrolysis of L-tyrosyl-L-lysyl-L-glutamyl-L-tyrosine by chymotrypsin (213). Although it has been reported (127) that in aqueous solution tyrosine ethyl ester is hydrolyzed at the same rate as benzoyl-L-tyrosine ethyl ester, it seems not unlikely that the very low solubility of the latter substance precludes accurate measurements.

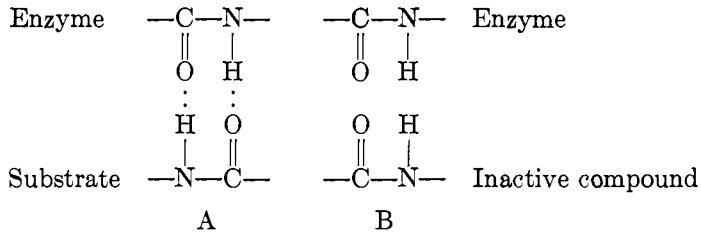
Comparison of the values of k_3 and K_m for the hydrolysis of acetyl-L-tyrosinamide with the values for the hydrolysis of benzoyl-L-tyrosinamide (table 8, lines 12 and 14) or comparison of the values for acetyl-L-tyrosinamide with those for nicotinyl-L-tyrosinamide, both of which were studied in aqueous solution (table 8, lines 13 and 15), indicates that both enzyme–substrate affinity and activation rate are increased when the acetyl group is replaced by an aroyl group. In the cases of acetyl-L-tyrosine ethyl ester and benzoyl-L-tyrosine ethyl ester (lines 7 and 8) no conclusion can be drawn, since k_3 and K_m change in the same direction (page 89). However, in conjunction with information obtained from studies of the inhibition of chymotrypsin by structural analogs of substrates (page 122), it seems clear that the greater activity of the aroyl compounds as compared to their acetyl analogs depends, in large part, upon the greater affinity of the former for the enzyme surface. Recent data on the rates of hydrolysis of acetyl-L-phenylalaninamide and benzoyl-L-phenylalaninamide (120), of benzoyl-L-tyrosylglycinamide and acetyl-L-tyrosylglycinamide (181), of benzoyl-DL-phenylalanine hydroxamide and of acetyl-DL-phenylalanine hydroxamide (121), although not extensive enough for evaluation of the kinetic constants, do confirm the observation that benzoyl derivatives are more active than the corresponding acetyl derivatives.

The importance of the group R in both the formation of the enzyme–substrate complex and the activation of this complex is further illustrated by the first five compounds in table 8. Snoke and Neurath (251) have pointed out that if the nature of R is such that it increases the affinity of the substrate for the enzyme surface, it may also serve to decrease the loss of entropy on activation and may thus increase the rate of activation. For example, if one hydrogen on

the α-carbon of methyl β-phenylpropionate (table 8, line 1) is replaced by chlorine to yield methyl *dl*-α-chloro-β-phenylpropionate (line 3), the activation rate is increased about twenty times. In this case, however, the increase in activation rate cannot be ascribed to an effect on combination with the enzyme surface, since both enantiomorphs of the chloro compound are hydrolyzed at the same rate. An increase in rate is also observed when a hydroxyl group is substituted into methyl β-phenylpropionate to yield methyl *d*-β-phenyllactate (line 2). In view of the high degree of stereospecificity exhibited by chymotrypsin toward all other known substrates, it must be postulated that the increase in rate in these cases is due solely to the effect of the electronegative substituents. This interpretation is confirmed by the fact that the effects of these substituents upon rate of activation parallel their inductive effects as measured by other means. When methyl *l*-β-phenyllactate (line 4) is compared with the *d*-isomer, a further tenfold *increase* in k_3 is found to be accompanied by a *decrease* in K_m. Since the α-hydroxyl of this compound is presumably attached to the same point on the enzyme surface as is the "secondary peptide" bond of benzoyl-L-phenylalanine methyl ester (line 5), the difference in k_3 for the two enantiomorphs must be the result of some preferential orientation of the *l*-isomer at the enzyme surface. When this reasoning is extended to the case of benzoyl-L-phenylalanine methyl ester (line 5), it appears that the amide bond of this substrate must cause a much firmer binding of the substrate to the enzyme than do any of the simpler substituents in this position. From a consideration of the values of k_3 and K_m it appears that the order of affinity of these compounds for the enzyme surface is: benzoyl-L-phenylalanine methyl ester > methyl *l*-phenyllactate > methyl *dl*-chlorophenylpropionate > methyl *d*-phenyllactate. The position of methyl phenylpropionate in this series is indeterminate, since the values of both k_3 and K_m are smaller than the corresponding values for methyl *d*-phenyllactate. It seems probable, however, that it has the lowest affinity of any compound in this series.

From these results it was suggested that the link between enzyme and substrate in this position may be one or more hydrogen bonds, the increased affinity observed with compounds containing a "secondary peptide" bond resulting from the fact that this group can act as both a donor and an acceptor of protons and can thus form two hydrogen bridges. This hypothesis is supported by the observation, cited above, that a positively charged amino group in this position is less effective for substrate activity than is a peptide bond. Kaufman and Neurath (135) found that elimination of the nitrogen atom from the "secondary peptide" bond to yield ethyl *dl*-α-benzyl acetoacetate (table 6, line 49) results in complete loss of substrate activity. Substrate activity is also lost if the secondary peptide group is replaced by an ester group as in diethyl benzylmalonate (table 6, line 50), or by a carboxylate group as in ethyl *dl*-α-benzylmalonate (line 51), or if both hydrogens of the α-amino nitrogen are substituted, as in phthalylphenylalanine methyl ester (line 55). In the latter case loss of substrate activity may also be the result of loss of rotation, owing to the formation of the phthalimide ring. From this evidence it would appear that the hy-

drogen bond formed when the substrate acts as a proton donor is the more important of the two postulated hydrogen bridges between enzyme and substrate. When the elements of the "secondary peptide" bond are reversed, as in the diamide of *dl*-benzylmalonic acid (line 56), substrate activity is lost. Kaufman and Neurath (137) have assumed that in this case no hydrogen bond formation is possible, as illustrated below in diagram B, and that steric interference of the opposing carbonyl oxygens prevents close approach of the enzyme and substrate.



Although Bergmann and Fruton (26) found the amides of benzoyldehydrotyrosine and of benzoyldehydrophenylalanine to be resistant to chymotryptic hydrolysis, the ethyl ester of acetyldehydrophenylalanine (table 6, line 46) is hydrolyzed very slowly (135). It was suggested that only one of the *cis–trans* and keto–enol isomers has a structure appropriate for formation of an activated complex and that the limiting rate is the rate of formation of this isomer. Obviously, in this case no single structural element can be held to account for the resistance of these compounds to hydrolysis.

With the exception of the cases discussed above in which R is a chlorine atom or a hydroxyl group, all known substrates of chymotrypsin belong to the L-series. Although it has been reported (26) that the presence in solution of the D-form of benzoyltyrosylglycinamide prevents the hydrolysis of the L-form of this compound by means of a postulated association of the enantiomorphs in solution, a recent reinvestigation of this topic by MacAllister, Harmon, and Niemann (181) reveals that the hydrolysis of the L-isomer is unaffected by the presence of the D-isomer. Further, no cryoscopic indication of association of the isomers in solution could be found. The problem of competitive inhibition by the D-analogs of substrates will be discussed in a later section (page 124). The specificity of chymotrypsin toward racemic amino acid esters has been used for the resolution of amino acids (48).

3. The hydrolyzable bond, R″

It is shown in table 6 that R″ may be an ester, hydrazide, amide, hydroxamide, glycinamide, or glycylglycinamide group. From the results of Plentl and Page (213) R″ may also be L-lysyl-L-glutamyl-L-tyrosine. If the report is correct that chymotrypsin fails to hydrolyze *N*-carbobenzoxy-L-tyrosylglycine, *N*-carbobenzoxy-L-phenylalanylglycine (25), and carbobenzoxy-L-glutamyl-L-tyrosylglycine, it appears that chymotrypsin has no carboxypeptidase activity

and that the presence of a negative charge near the susceptible bond causes a loss of substrate activity.

Complete data for the effect of R″ upon susceptibility to hydrolysis by chymotrypsin are available only for ester, amide, and glycinamide groups. These data are shown in table 9. From these and such other data as are available (25, 121, 135, 138, 181, 182) the order of susceptibility to hydrolysis may be tentatively listed as: ester > hydroxamide > glycinamide > amide > hydrazide > glycylglycinamide.

It is probable that there is some steric interference with *large* R″ groups since, as noted above, glycinamide is more readily hydrolyzed than is glycylglycinamide from the carbobenzoxy-L-tyrosine derivative, and also since the tetrapeptide of Plentl and Page (213) is very slowly hydrolyzed by chymotrypsin. This steric effect may account for the resistance of the ester of benzoyl-L-phenylalanine with the β-hydroxyl of N-benzoylserine ethyl ester to chymotryptic hydrolysis (135).

TABLE 9

Effect of susceptible group, R″, upon rate of reaction at pH 7.8 and 25°C.

SUBSTRATE	SOLVENT	K_m	k_2	C_{max}	REFERENCES
		10^{-2} M	10^{-2}		
Acetyl-L-tyrosine ethyl ester...	30% methanol	3.07	197	27.9	(253)
Acetyl-L-tyrosylglycinamide...	Water	3.0	0.89	0.13	(181)
Acetyl-L-tyrosinamide.....	Water	3.3	0.27	0.036	(136)

The structural requirements for the more active substrates of chymotrypsin may be summarized as follows:

1. An aromatic ring, or condensed aromatic rings, separated by at least one methylene group from the α-carbon atom. The effect of lengthening the carbon chain or of substitutions on the methylene group has not been established. From the data available it appears that substitutions into the aromatic ring of groups which tend to shift electrons to the methylene bridge increase substrate activity. Clearly, this generalization should be checked by determining whether substitutions which tend to draw electrons away from this position decrease substrate activity.
2. An uncharged polar group, capable of forming hydrogen bonds by proton donation, substituted on the α-carbon in the L-configuration. Maximal activity among known substrates is achieved when this group is the benzoyl or nicotinyl derivative of an α-amino group, in which cases two hydrogen bonds could be formed with the enzyme. The effect of replacement of the remaining hydrogen on the α-carbon has not been uniquely determined, and the effect of electron-directing substituents in the aromatic ring of the benzamido group requires further investigation.

3. The hydrolyzable bond, which may be an amide, hydroxamide, hydrazide, peptide, or ester bond. The group linked by this bond should not be large, since there appears to be a steric factor operative at this position. Although the effect of a positive charge in this region has not been established, present evidence indicates that this group should not bear a negative charge.

B. OTHER CHYMOTRYPSINS

Kunitz (151) has shown that the qualitative specificity of β- and γ-chymotrypsins is the same as that of α-chymotrypsin, since carbobenzoxyglycyl-L-tyrosylglycinamide and benzoyl-L-tyrosylglycinamide are hydrolyzed at the same rate by all three enzymes at equal concentrations of enzyme nitrogen. It has also been found (232) that α- and γ-chymotrypsins hydrolyze benzoyl-L-arginine methyl ester at the same rate, each enzyme having a proteolytic coefficient of about 0.15. These observations indicate that on a molecular basis β- and γ-chymotrypsins are less active than α-chymotrypsin, since the molecular

TABLE 10

Comparison of rates of hydrolysis of substrates by α- and by δ- chymotrypsin at pH 7.8 and 25°C.

SUBSTRATE	α-CHYMOTRYPSIN			δ-CHYMOTRYPSIN		
	K_m	k_3	C_{max}	K_m	k_3	C_{max}
	10^{-2} *M*	10^{-2}		10^{-2} *M*	10^{-2}	
Acetyl-L-tyrosinamide	2.7	0.3	0.048	0.2	0.45	0.96
Acetyl-L-tyrosine ethyl ester	3.2	260	36	1.6	290	77
Acetyl-L-tryptophan ethyl ester	0.17	32	83	0.036	40	490

weights of these enzymes appear to be smaller than that of α-chymotrypsin, while the nitrogen contents are approximately the same.

Fruton (89) has reported that the specificity of B-chymotrypsin is identical with that of α-chymotrypsin. Typical substrates such as carbobenzoxy-L-tyrosylglycinamide and carbobenzoxyglycyl-L-tyrosylglycinamide are hydrolyzed by B-chymotrypsin, while a variety of other amino acid derivatives, including carbobenzoxy-L-glutamyl-L-glutamic acid, benzoyl-L-argininamide, and carbobenzoxyglycyl-L-phenylalanine, are not hydrolyzed by this enzyme.

Similarly, Schwert and Kaufman (230) have shown that δ-chymotrypsin has typical chymotryptic activity. The data shown in table 10 again illustrate the point that an enzyme which has a higher affinity for a substrate, as evidenced by a lower value of K_m, may also activate the substrate more rapidly, presumably because part of the entropy loss upon *activation* by the less active enzyme appears as a loss of entropy of *combination* with the more active enzyme. Comparison of the data obtained for δ-chymotrypsin with that for the α-enzyme also furnishes experimental verification of the point previously made (page 87) that there is no basis for the concept of homospecificity introduced by Irving, Fruton, and Bergmann (118).

C. KINETICS

Most of the kinetic constants in the literature have been cited in tables 6 to 10. Other reported data are for proteolytic coefficients (C) at a single substrate concentration. These include $C = 0.0022$ and 0.0003 for the proteolytic coefficients at 25°C. of glycyl-L-phenylalaninamide and L-phenylalaninamide, respectively (90), $C = 3.3$ for carbobenzoxyglycyl-L-tyrosine ethyl ester in 50 volumes per cent ethanol at 25°C. (232), $C = 0.064$ for the hydrolysis of nicotinyl-L-tyrosine hydrazide in aqueous solution at 25°C. (182), and $C = 3.5$ for benzoyl-L-tyrosylglycinamide in aqueous solution at 40°C. (181).

Although the two-step reaction theory requires that the initial rate of hydrolysis increase with decreasing substrate concentration if the data are plotted according to first-order kinetics (see page 87), and further requires that the apparent first-order reaction constants shall increase with increasing degree of hydrolysis, only the former effect is found with many chymotrypsin substrates. It has been reported (138, 230) that the hydrolysis of benzoyl-L-tyrosinamide, benzoyl-L-tyrosine ethyl ester, acetyl-L-tyrosine ethyl ester, acetyl-DL-tryptophan ethyl ester, benzoyl-DL-methionine ethyl ester, and benzoyl-L-phenylalanine ethyl ester follows first-order kinetics with good agreement throughout the course of the reaction. This anomalous behavior is probably the result of inhibition of the enzyme by the products of hydrolysis (*vide infra*).

1. The effect of alcohol

Since many of the substances studied as substrates are insoluble in water, many of the determinations listed in tables 6 to 10 were made in solutions containing methanol or ethanol. Preliminary measurements (138) of the effect of methanol on the hydrolysis of glycyl-L-tyrosinamide and of benzoyl-L-tyrosine ethyl ester indicated that the proteolytic coefficient, determined at a constant initial substrate concentration, decreases logarithmically with increasing methanol concentration. Kaufman and Neurath (136) have made a more detailed study of the effect of methanol on the values of K_m and k_3 for the chymotryptic hydrolysis of acetyl-L-tyrosinamide. Over the range from 0 to 20.8 volumes per cent of methanol k_3 was found to be constant. On the other hand, the reciprocal of K_m decreased linearly with increasing methanol concentration. Since $1/K_m = k_1/(k_2 + k_3)$, and since k_3 is independent of methanol concentration, it was reasoned that this linear variation could obtain only if k_2 were constant or negligibly small. Since it appeared unlikely that the addition of methanol to the system would influence only one of the reversible reactions whose velocities are represented by k_1 and k_2, it was concluded that in this case k_2 approaches zero and that $K_m = k_3/k_1$. It should be noted that this conclusion cannot be extended to other systems, since the data obtained for the effect of methanol on the hydrolysis of glycyl-L-tyrosinamide and of benzoyl-L-tyrosine ethyl ester (138) do not yield to this simple interpretation.

2. The effect of pH

Although extensive studies have not been made on the effect of pH on the chymotryptic hydrolysis of synthetic substrates, it has been determined that

when activity measurements are made at a single substrate concentration, the pH–activity curve is the same for the hydrolysis of both benzoyl-L-tyrosinamide and benzoyl-L-tyrosine ethyl ester. These measurements were made between pH 6 and pH 9 in 30 volumes per cent methanol (138). Under these conditions a sharp maximum was exhibited at pH 7.8, the activity decreasing to 30 per cent of the maximal value when the pH was increased or decreased by one unit. The pH–activity curve for the hydrolysis of casein by chymotrypsin in aqueous solution (166) shows a much broader maximum than is exhibited with synthetic substrates in alcoholic solution.

D. INHIBITION OF CHYMOTRYPSIN

Kaufman and Neurath (134, 137) have investigated the structural requirements for inhibitors of chymotrypsin. The results of these investigations are shown in table 11.

Although the fact that a substance is a competitive inhibitor is sufficient evidence for assuming that it is attached at the active center of the molecule, there is no *a priori* reason for believing that corresponding structural elements are attached at identical sites on the enzyme surface. In order to demonstrate this point it must be shown that various substituents in both substrate and inhibitor molecules cause parallel changes in the affinity of these molecules for the enzyme.

1. The amino acid side chain

Table 11 indicates that the structural requirements for chymotrypsin inhibitors are fulfilled by the side chains corresponding to phenylalanine, tyrosine, *O*-acetyltyrosine, dehydrophenylalanine, methionine, glycine, and phenylglycine. It will be noted that this list includes, in addition to the groups previously shown to be necessary for substrate activity, several groups which do not contribute to substrate activity, e.g., dehydrophenylalanine, phenylglycine, and glycine.

A comparison of the values of K_i for the benzoyl derivatives of the racemates of the amino acids studied as inhibitors indicates that the order of affinity is: DL-phenylglycine > DL-phenylalanine > glycine > DL-methionine (table 11, lines 5, 10, 11, and 12). Lines 6 and 7 of table 11 indicate that tyrosine and phenylalanine have about the same position in this sequence. Since this order indicates that, as with substrates, aromatic residues are more active than aliphatic groups, there is some basis for the supposition that the side chains of amino acid substrates and inhibitors are oriented in the same way on the enzyme surface. It is clear, however, that it must also be assumed that the enzymatic specificity toward inhibition is much lower, since changes in chain length have much less effect than with substrates.

2. The "secondary peptide" or equivalent group

Cursory examination of the compounds listed in table 11 reveals that this group may be a hydrogen atom, a benzamido group, or an acetamido group,

TABLE 11

Inhibitors of chymotrypsin

	STRUCTURE	CONFIGURATION	K_i
			10^{-2} *M*
1	$C_6H_5CH_2CH_2COOH$		0.45
2	$C_6H_5CH_2CH_2NH_2$		†
3	$C_6H_5CH_2CH(NHCOC_6H_5)COOH$	D	1.5
4	$C_6H_5CH_2CH(NHCOC_6H_5)COOH$	L	2.9
5	$C_6H_5CH_2CH(NHCOC_6H_5)COOH$	DL	2.6*
6	$C_6H_5CH_2CH(NHCOCH_3)COCH_3$	DL	0.79*
7	p-$HOC_6H_4CH_2CH(NHCOCH_3)COCH_3$	DL	0.72
8	p-$CH_3COOC_6H_4CH_2CH(NHCOCH_3)COOH$	L	2.0
9	$C_6H_5CH{=}C(NHCOCH_3)COOH$		1.8
10	$C_6H_5CH(NHCOC_6H_5)COOH$	DL	0.78
11	$CH_2(NHCOC_6H_5)COOH$		4.1
12	$CH_3SCH_2CH_2CH(NHCOC_6H_5)COOH$	DL	4.6
13	$CH_3SCH_2CH_2CH(NHCOCH_3)COOH$	DL	Very large

TABLE 11—*Continued*

Related structures which are not inhibitors

	STRUCTURE	CONFIGURATION	K_i
14	$C_6H_5CH_2CHCOOH$ $\vert$ NH_2	D, L	
15	$C_6H_5CH_2CHCOOH$ $\vert$ $COOH$	*dl*	
16	CH_2COOH $\vert$ $NHCOCH_3$		

* The values given in this table are from Kaufman and Neurath (137). The values marked with an asterisk were established by the equation of Lineweaver and Burk (179) for competitive inhibition. The other values given were calculated from determinations made at a single substrate concentration, assuming competitive inhibition.

† No quantitative data available (137).

but it may not be a carboxyl or amino group if the compound already contains an α-carboxyl group. Closer examination of the data reveals, however, that this group may be an acetamido group *only* if the compound contains an aromatic ring in the amino acid side chain. Thus, acetylglycine is not an inhibitor while benzoylglycine is an inhibitor (lines 11 and 16); also, benzoylmethionine is a much more effective inhibitor than is acetylmethionine (lines 12 and 13), although lines 6 to 9 of table 11 indicate that acetylated aromatic amino acids and amino acid derivatives do act as inhibitors.

From these observations it would appear that benzoylated aromatic amino acids and their derivatives should be very potent inhibitors of chymotrypsin. In general, the data in table 11 confirm this conclusion but, if only free acids are considered, a striking exception to this generalization appears in the case of β-phenylpropionic acid (line 1), which is the most potent inhibitor known at present. The further observation that benzoyl-D-phenylalanine is about twice as active an inhibitor as benzoyl-L-phenylalanine suggests that any substitution on the α-carbon reduces the activity of an inhibitor but that a substitution in the D-configuration causes less loss of activity than does a substitution in the L-configuration. This difference in stereospecificity between inhibitors and substrates (which has also been observed in the case of carboxypeptidase (page 140)) may account for the observation that acetyldehydrophenylalanine is a potent inhibitor, while the ethyl ester of acetyldehydrophenylalanine is a poor substrate. It would be of interest to test this effect further with the antipodes of inhibitors which have been tested only as racemates. As has been pointed out in a preceding section, the observed adherence of the hydrolysis of several substrates to first-order kinetics (135) is probably the result of inhibition by the amino acid derivative formed as one of the products of hydrolysis.

An interesting example of inhibition by the D-form of a substrate has been reported by MacAllister, Harmon, and Niemann (181), who found that the D-form of *N*-acetyltyrosylglycinamide acts as a competitive inhibitor for chymotrypsin.

3. The effect of substituents in the position corresponding to the susceptible bond

If the conclusion is correct that benzoyl derivatives are more active inhibitors than are the corresponding acetyl derivatives, it may be inferred from lines 6 and 7 of table 11 that replacement of the α-carboxyl of an amino acid derivative by a methyl ketone group greatly increases the activity of an inhibitor. This effect was overlooked in the original study of this compound (138), because it was tested against a substrate of high affinity in a solvent of unsuitable composition. It seems possible that the greater activity of the methyl ketone results from the masking of the negative charge of the carboxylate group and that the mutual repulsion of negative charges on the enzyme and carboxylate groups provides the mechanism for clearing the enzyme surface of the products of hydrolysis. If this postulate is correct, it is clear that β-phenethylamine (line 2) may act as an inhibitor by attraction to the negatively charged surface.

Although sufficient data have not been gathered to warrant very specific conclusions concerning the mode of combination of substrates and inhibitors with the enzyme surface, it does appear probable from the preceding discussion of substrate specificity that substrates are attached to the enzyme surface at three points: the aromatic ring of the amino acid, the "secondary peptide" bond, and the hydrolyzable group. It also seems likely that for substrates of high activity all three attachments are essential. By analogy with the classical theory of catalysis of H. S. Taylor (265) it may be reasoned that when the substrate is attached at all three points, or possibly at only two points, it is in a "strained" configuration (95). The attainment of this "strained" configuration, which is the activated state, requires energy. On the other hand, in the case of inhibitors, attachment at any one of the three points would suffice for inhibition. Since attachment of the inhibitor to the enzyme surface in the same configuration as is assumed by the substrate would require energy, it follows that attachment of an inhibitor at more than one point may cause a decrease of inhibitory activity. The structures of specific inhibitors thus may be simpler than those of substrates. In the present case it may be that the "secondary peptide" bond in the L-configuration is essential to the "strained" configuration and that omission of this group or its inversion to the D-form results in an inhibitor which requires a lower energy of activation for attachment to the enzyme surface. The observed lack of substrate activity of the ethyl ester of benzoylphenylglycine and the high inhibitory activity of benzoylphenylglycine also seem to support this view.

E. INHIBITION BY CHEMICAL MODIFICATION OF THE ENZYME

As was mentioned in a preceding section, Jansen and coworkers in A. K. Balls' laboratory (127) have found that very small quantities of diisopropyl

fluophosphate (DFP) completely and irreversibly inhibit chymotrypsin but have no effect upon chymotrypsinogen. The inhibited enzyme can be crystallized by the usual procedure and crystallization is much more complete than with active chymotrypsin. The specific esterase activity of chymotrypsin, measured against tyrosine ethyl ester, and the proteinase activity, measured by the hemoglobin method of Anson, decreased in a parallel manner. The activity of active chymotrypsin is not affected by the addition of inhibited chymotrypsin.

In a second paper (126) these workers reported the results of a study of the stoichiometry of the inhibition reaction. When diisopropyl fluophosphate containing radioactive phosphorus is reacted with chymotrypsin, it is found that 1.1 mole of DFP reacts with 27,000 g. of chymotrypsin, a value which agrees well with the observations previously made on the amount of DFP required to cause 50 per cent inhibition. Jandorf *et al.* (125) report that the inhibition of chymotrypsin by diisopropyl fluophosphate or by tetraethyl phosphate results in a large decrease in the microbiological availability of several amino acids and suggest that this reaction, which appears to be a simple phosphorylation, also involves physicochemical changes in the configuration of the molecule.

Sizer (236) has reported that over the range of redox potential from − 400 mv. to + 500 mv., oxidizing and reducing agents have no effect on the activity of chymotrypsin (page 109) and concludes that disulfide or sulfhydryl groups are not essential for chymotryptic activity. From the kinetics of the reaction of chymotrypsin with ketene and with nitrous acid it was concluded that tyrosine is essential for activity, and from the failure of formaldehyde or phenyl isocyanate to cause an appreciable loss of activity it was deduced that amino groups are not required for the enzymatic action of chymotrypsin.

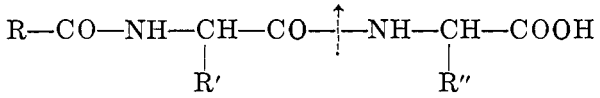
Sizer (237) has reported that tyrosinase oxidizes the phenolic groups of the tyrosyl moeity of many proteins and that chymotrypsin, although oxidized by this enzyme, retains its activity. Edman (76) has replied to this report by stating that tyrosinase does not react with chymotrypsin but only with the split products of the enzyme. Sizer (238) in a further study found that oxidation does occur but to a much smaller extent than was previously found and again found the proteolytic activity to be unchanged. It thus appears that if the observed oxidation is due to oxidation of tyrosyl residues in chymotrypsin, the residues oxidized must be different from the groups essential for proteolytic activity.

VII. The Specificity of Carboxypeptidase

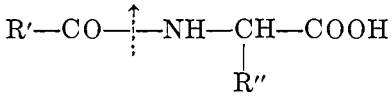
A. SPECIFICITY

The specificity requirements of carboxypeptidase have been the subject of numerous early publications, notably by Abderhalden and coworkers (3, 5, 6) and by Waldschmidt-Leitz (278), which preceded the isolation of the enzyme in crystalline form by Anson (12). These and other publications (22, 32, 35, 36) will receive herein only limited attention, since later work has shown that some of the properties ascribed by these workers to carboxypeptidase were actually those of other enzymes present in the crude pancreatic extract.

Typical substrates for carboxypeptidase may be represented by the structural formula:



This tripeptide is characterized, from right to left, by (*1*) a terminal carboxyl group belonging to the specific amino acid residue R″, (*2*) the hydrolyzable peptide bond, indicated by the broken arrow, (*3*) the amino acid residue R′, whose amino group is substituted by an acyl group containing (*4*) the residue R. The peptide group to the left of R′ will be referred to as the "secondary peptide" group. While the most specific substrates for carboxypeptidase contain this secondary peptide group, it will be shown that this structural element is not requisite and that *N*-acyl amino acids, i.e.,



are likewise hydrolyzed by this enzyme. The hydrolysis of these *N*-acyl amino acids is a genuine attribute of carboxypeptidase and not of an enzymatically distinct "acylase" (1).

The enzymatic activity of carboxypeptidase, as that of other proteolytic enzymes, is restricted to peptides in which the specific amino acid residue, R″, is of the L-isomeric form. This stereoisomeric specificity is adequately demonstrated by the findings that the carbobenzoxyglycyl derivatives of D-phenylalanine (77, 197, 257), D-leucine (257), D-methionine (70), and D-tryptophan (106) are entirely resistant to enzymatic hydrolysis, whereas the corresponding L-isomeric peptides are specific substrates for this enzyme. Although it has been reported that carbobenzoxyglycyl-D-alanine is slowly hydrolyzed by carboxypeptidase (9 and 13 per cent hydrolysis after 7 and 19 hr., respectively) (24), this exceptional finding requires more adequate confirmation before it can be fully accepted. The stereoisomeric specificity of carboxypeptidase has been used to advantage in the enzymatic resolution of phenylalanine, tyrosine, and tryptophan (94).

Limited experimental data indicate that the stereoisomeric specificity of carboxypeptidase applies similarly to the amino acid residue R′ (106, 243).

The requirement of a free carboxyl group in specific substrates is a unique attribute of carboxypeptidase and distinguishes the enzymatic specificity of this enzyme from that of other peptidases (22, 35). In keeping with this requirement, carbobenzoxyglycyl-L-phenylalaninamide (114) and carbobenzoxyglycyl-L-tryptophanamide (241) are entirely resistant to hydrolysis. The specificity of carboxypeptidase also differs uniquely from that of dipeptidases, in that dipeptides with an unsubstituted amino group are either entirely resistant to hydrolysis by carboxypeptidase or else hydrolyzed extremely slowly (22). Included in this group are glycyl-L-tyrosine (114), tyrosyl-L-tyrosine (114), and L-tyrosyl-L-arginine (22). Furthermore, in contrast to dipeptidases, the length of the peptide substrate is not a limiting factor in the specificity requirements

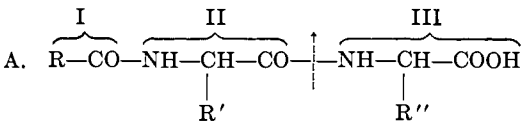
of carboxypeptidase, as evidenced by the hydrolysis of the terminal peptide linkage of the tetrapeptide L-tyrosyl-L-lysyl-L-glutamyl-L-tyrosine (213).

1. The specific amino acid residue, R″

The profound importance of the nature of this residue has been recognized early in specificity studies of this enzyme, and may be evaluated from a comparison of the relative hydrolysis rates of compounds 1 to 10 of table 12. Al-

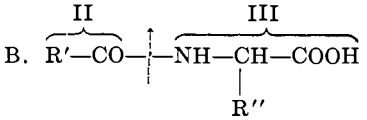
TABLE 12

Substrates for carboxypeptidase



	I	II	III	RATE[a] $C^{25°}_{0.05}$	REFERENCES[b]
1....	Carbobenzoxy	glycyl	phenylalanine	12.0–14.0	(28, *77*, *78*, 91, 93, *114*, *104*, *197*, 218, *241*, 257)
2....	Carbobenzoxy	glycyl	tyrosine	6.2	(28, 93, 114, *257*)
3....	Carbobenzoxy	glycyl	tryptophan	4.7	(106, *241*)
4....	Carbobenzoxy	glycyl	leucine	2.6	(257)
5....	Carbobenzoxy	glycyl	methionine	1.2	(70)
6....	Carbobenzoxy	glycyl	isoleucine	0.54	(257)
7....	Carbobenzoxy	glycyl	alanine	0.04	(24, 114, *257*)
8....	Carbobenzoxy	glycyl	aminoisobutyric acid	0.013	(257)
9....	Carbobenzoxy	glycyl	glycine	<0.001[c]	(114, *218*, 257)
10....	Carbobenzoxy	glycyl	thiazolidine-4-carboxylic acid	+	(241)
11....	Benzoyl	glycyl	phenylalanine	[d]	(252)
12....	Benzoyl	glycyl	phenylglycine	1.0	(252)
13....	Benzoyl	glycyl	lysine	+	(114)
14....	Benzenesulfonyl	glycyl	phenylalanine	[d]	(252)
15....	Carbobenzoxy	alanyl	phenylalanine	11.3[e]	(28)
16....	Carbobenzoxy	alanyl	tyrosine	6.4[e]	(28)
17....	Carbobenzoxy	methionyl	tyrosine	7.8	(70)
18....	Carbobenzoxy	tryptophyl	tyrosine	+	(241)
19....	Carbobenzoxy	tryptophyl	tryptophan	2.3[f]	(241)
20....	Carbobenzoxy	tryptophyl	alanine	0.12	(241)
21....	Carbobenzoxy	tryptophyl	glycine	0.007	(241)
22....	Carbobenzoxy	tryptophyl	proline	0.002	(241)
23....	Carbobenzoxy	glutamyl	phenylalanine	0.8	(28, *70*, 114)
24....	Carbobenzoxy	glutamyl	tyrosine	0.5[e]	(*28*, 114)
25....	Acetyl	dehydrophenylalanyl	phenylalanine	0.003	(257)
26....	Acetyl	dehydrophenylalanyl	leucine	0.002	(257)

TABLE 12—*Concluded*

B. 

	II	III	RATE[a] $C^{25°}_{0.05}$	REFERENCES[b]
27....	Carbobenzoxy	phenylalanine	0.0013[c]	(28)
28....	Carbobenzoxy	tryptophan	0.009[g]	(241)
29....	Carbobenzoxy	tyrosine	0.001[e]	(28)
30....	Benzoyl	phenylalanine	±	(114)
31....	Chloroacetyl	phenylalanine	[d]	(94, 114, 218, *252*)
32....	Chloroacetyl	tyrosine	1.65[h]	(22, 24, 94, *218*)
33....	Chloroacetyl	tryptophan	+	(94, 114)
34....	Formyl	phenylalanine	[d]	(218, *252*)
35....	Acetyl	phenylalanine	[d]	(94, *252*)
36....	Acetyl	tryptophan	0.045[h]	(94, *218*)
37....	Phenylpyruvyl	phenylalanine	±	(91)
38....	Phenylpyruvyl	leucine	±	(91)

[a] $C^{25°}_{0.05}$ denotes the "proteolytic coefficient," calculated from measurements at 25°C. and 0.05 *M* initial substrate concentration, within the pH range of pH 7.3 to pH 7.7. Where the experimental data did not warrant the calculation of rate constants, rates are merely denoted by + (moderate) or ± (slow).

[b] When more than one literature reference is given, that printed in *italics* has been used as reference for the "proteolytic coefficient." This selection was based on the reported value for carbobenzoxyglycylphenylalanine as a standard of reference ($C^{25}_{0.05}$ = 12–14).

[c] The significance of this low value remains questionable (see the text).

[d] See table 13 for complete kinetic data.

[e] The reported values have been corrected to $C^{25°}_{0.05}$ = 13 for the hydrolysis of carbobenzoxyglycylphenylalanine.

[f] Calculated from a single rate value. This substrate has been reported to yield more than 100 per cent hydrolysis per molecule, indicating that hydrolysis of both peptide bonds occurs (241), probably at greatly different rates.

[g] Calculated from the initial slope of the zero-order plot (241).

[h] Calculated from the data of reference 218, at 40°C.

though, as previously discussed (page 87), the proteolytic coefficient calculated from rate measurements at a single initial substrate concentration is not a valid basis of comparison of hydrolysis rates of two or more substrates, lack of more adequate kinetic data for many of the substrates listed in table 12 necessitates an approximate comparison of their rates on this basis. It is evident that phenylalanine is the most specific amino acid residue and that residues containing aromatic structures form more sensitive peptide substrates than those composed of aliphatic chains. The hydrolysis of peptides which contain glycine as the terminal amino acid occurs at a rate so slow that its significance may well be questioned. It has been reported, however, that when the residue R′ is contributed by tryptophan, the resulting peptide, carbobenzoxy-L-tryptophyl*glycine* is hydrolyzed at an appreciable rate. A tryptophyl residue in position R′ also accelerates the rate of hydrolysis of other peptide substrates (241) (table 12).

The spatial separation of the aromatic ring in R″ from the α-carbon atom exerts a marked influence on the hydrolysis rate. Thus, elimination of the β-carbon atom, as in a phenylglycine peptide (compound 12), reduces significantly the sensitivity to hydrolysis. The reported hydrolysis of a lysyl peptide (compound 13) is surprising, since data of the same paper (114) show that, after masking of the ϵ-amino group by a carbobenzoxyl group, the resulting lysyl peptide is inactive toward carboxypeptidase.

Enzymatic studies on substrates for carboxypeptidase which differ from each other in the nature of R″ or R′, respectively, have formed the basis for describing the enzymatic specificity of enzymes in terms of the corresponding "proteolytic quotients" (28). Although subsequent studies appeared to support this interpretation (241, 257), it can, at best, be regarded as a first approximation which, in the present analysis, will be abandoned in favor of the theoretically more adequate comparison of hydrolysis rates in terms of K_m and k_3 (*vide infra*).

2. The amino acid residue, R′

While no significant differences exist between carbobenzoxy*glycyl*, carbobenzoxy*alanyl*, and carbobenzoxy*methionyl* amino acids (compare compounds 1 and 2 with 15 and 16 and with 17 (table 12)), carbobenzoxy*tryptophyl* amino acids (241) appear to be hydrolyzed at an accelerated rate (compounds 18 to 21). In contrast, the introduction of a *glutamyl* residue in position R′ markedly decreases the hydrolysis rate of the resulting peptide (compounds 23 and 24). It is probable that the retarding influence of the latter substituent is caused by the negatively charged γ-carboxyl group, since the other aliphatic residues, even when of comparable chain length, fail to exert an effect. The accelerating influence of the tryptophyl residue in position R′ presumably reflects particularly favorable spatial or electronic relations for enzyme–substrate interaction (241). The effect of other substituents in position R′ on hydrolysis rates of substrates is worthy of further investigation.

The extremely slow hydrolysis rates which result when the unsaturated substituent, dehydrophenylalanine, is introduced in position R′ (compounds 25 and 26) is not necessarily a reflection of the shortened bond distance between the α- and β-carbon atoms of this residue. This effect could also be referred to the potential loss of the hydrogen atom of the secondary peptide bond arising from enolization of this compound, or to the loss of rotation which is caused by the formation of a double bond. (See analogous considerations for chymotrypsin substrates, page 118).

The influence of the nature of the residue R′ in substrates which are devoid of the secondary peptide bond will be considered elsewhere in this discussion (page 133).

3. The acyl group, RCO

The available data limit this analysis to a comparison of the carbobenzoxyl group with the benzoyl group (compounds 1 and 11 of table 12; compounds 1

and 2 of table 13). Since for carbobenzoxyglycylphenylalanine and for benzoylglycylphenylalanine the specific rate constants, k_3, are approximately the same, whereas K_m for the former is higher, it is evident that the affinity of the *N*-benzoyl dipeptide for carboxypeptidase is greater than that of the *N*-carbobenzoxyl dipeptide. Since, moreover, the synthesis of hippurylphenylalanine is experimentally simpler and does not involve the hazardous use of phosgene,

TABLE 13

*Kinetic constants for the hydrolysis of specific substrates by carboxypeptidase at 25°C.**

A. Peptides: R—CO—NH—CH(R″)—COOH

	SUBSTRATE	K_m	k_3	REFERENCES
		M		
1.....	Carbobenzoxyglycylphenylalanine	0.033	2.1	(77)
2.....	Benzoylglycylphenylalanine	0.011	2.0	(252)
3.....	Benzenesulfonylglycylphenylalanine	0.014	0.124	(252)
4.....	Formylphenylalanine	0.036	0.007	(252)
5.....	Acetylphenylalanine	0.155	0.0023	(252)
6.....	Chloroacetylphenylalanine	0.013	0.137	(252)
7.....	Carbobenzoxyglycyltryptophan†		0.147	(241)
8.....	Carbobenzoxytryptophylalanine†		0.012	(241)
9.....	Carbobenzoxytryptophylglycine†		0.0007	(241)

B. Esters: R—CO—O—CH(R″)—COOH

10.....	Benzoylglycyl-β-phenyllactic acid	~0	1.72	(254, 252)
11.....	Acetyl-β-phenyllactic acid	0.013	0.044	(252)
12.....	Chloroacetyl-β-phenyllactic acid	~0	1.30	(252)
13.....	Bromoacetyl-β-phenyllactic acid	0.0016	0.99	(252)

* The dimensions of K_m are moles per liter and of k_3, moles per liter per minute per milligram (enzyme N) per milliliter.

† The hydrolysis of these substrates was reported to follow zero-order kinetics. Since these measurements were limited to a single initial substrate concentration (0.05 *M*), they could not be used for the calculation of K_m. If it is assumed that the zero-order reaction constant is independent of initial substrate concentration, K_m would be too small to be computed from text equation 11 and k_3 would have the value given in this table.

this compound has been proposed as the substrate of choice for carboxypeptidase (252).

4. The hydrolyzable bond

One of the earliest studies of carboxypeptidase by Bergmann and coworkers (22, 36) indicated that the hydrogen atom of the hydrolyzable peptide bond is requisite for enzymatic hydrolysis. Thus chloroacetyl-*N*-methyltyrosine was

resistant to hydrolysis, whereas chloroacetyltyrosine was hydrolyzed at an appreciable rate. However, subsequent studies have failed to substantiate this conclusion. Thus, while crude pancreatic extracts failed to hydrolyze prolyl peptides (22), and carbobenzoxyglycyl-L-proline (257) and carbobenzoxyglycyl-hydroxy-L-proline (241) were not hydrolyzed by the crystalline enzyme (257), carbobenzoxy-L-tryptophyl-L-proline is hydrolyzed, albeit extremely slowly (241). Powerful evidence for the dispensibility of the peptide hydrogen is provided by the enzymatic hydrolysis of specific ester substrates (252, 254), although it should be recognized that such a comparison neglects the differences in electronic configuration between the nitrogen and oxygen atoms. As far as peptides are concerned, the available data suggest that replacement of the peptide hydrogen by another, necessarily larger, substituent greatly decreases hydrolysis rates.

The catalytic activity of carboxypeptidase, like that of trypsin and chymotrypsin (see pages 104 and 119), does not depend on the presence of the peptide nitrogen or the peptide group, since, as already mentioned, specific *esters* are among the most potent substrates for this enzyme (table 13). Indeed, comparison of the specific peptide substrate, benzoylglycyl-L-phenylalanine, with the corresponding ester, benzoylglycyl-β-phenyllactic acid, reveals that their specific rate constants, k_3, are nearly identical, whereas the Michaelis constant, K_m, of the ester is too low to be determined by conventional methods of analysis. Similar comparison of acetylphenylalanine and chloroacetylphenylalanine with their respective ester analogs, i.e., acetyl-β-phenyllactic acid and chloroacetyl-β-phenyllactic acid, demonstrates that in every case the affinity of the ester for the enzyme is greater than that of the corresponding peptide and that in the last two cases mentioned the specific rate of activation of the esters is even higher than that of the peptides (table 13). (See page 116 for analogous considerations of chymotrypsin.)

5. The "secondary peptide" group

For purposes of discussion it is expedient (*1*) to consider substrates in which the elements of this group are modified and (*2*) to compare the hydrolysis rates of substrates which contain the intact secondary peptide group with those of substrates which are entirely devoid of this grouping.

As in the case of the hydrolyzable peptide group, replacement of the hydrogen of the secondary peptide group greatly decreases hydrolysis rates. This is evidenced by a comparison of the hydrolysis rates of benzoylglycylphenylalanine and of benzoylsarcosylphenylalanine (252). Although the hydrolysis rate of benzoylsarcosylphenylalanine was too slow to be interpreted in terms of the kinetic constants, k_3 and K_m, at an initial substrate concentration of 0.04 M the observed velocity was only about 1/2400 of that of benzoylglycylphenylalanine.

Replacement of the secondary peptide group by a sulfonamide group, as in benzenesulfonylglycylphenylalanine (compound 14 of table 12; compound 3 of table 13), causes a sixteenfold decrease in the specific rate of activation (252).

This is in distinct contrast to analogous studies of trypsin, which hydrolyzes α-*p*-toluenesulfonyl-L-argininamide about 1.5 times faster than the corresponding α-benzoyl analog (232).

The distance of separation of the secondary peptide bond from the α-carbon atom of the residue R″ is of critical importance for the susceptibility of the substrates to hydrolysis by carboxypeptidase. This has been convincingly demonstrated by a study of peptides containing β-alanine in place of glycine (104). Thus in 0.05 *M* initial substrate concentration, the hydrolysis rate of carbobenzoxy-*β-alanyl*phenylalanine was approximately one-thousandth that of the carbobenzoxy*glycyl*peptide, and similar comparison of leucyl peptides yielded a ratio of approximately 1:1500. Carbobenzoxyglycyl-*β-alanine*, in contrast to carbobenzoxyglycyl-*α-alanine*, was not hydrolyzed at all by carboxypeptidase.

The effect of the entire omission of the secondary peptide group on hydrolysis rates is most readily evaluated by inspection of table 12 (compounds 27 to 38) and table 13 (compounds 4, 5, 6, 11, 12, and 13).

The *N*-acyl peptides, carbobenzoxyphenylalanine, carbobenzoxytyrosine, and carbobenzoxytryptophan, are evidently hydrolyzed considerably more slowly than the corresponding carbobenzoxyglycyl amino acids, the relative rates being in the proportion of about 1:1000. Qualitatively, a similar comparison holds for benzoylphenylalanine and benzoylglycylphenylalanine. The hydrolysis of carbobenzoxytryptophan occurs, apparently, at a sufficient rate to result in slow, but perceptible, enzymatic hydrolysis of the secondary peptide group of the substrate, carbobenzoxy-L-tryptophyl-L-tryptophan (241).

In the present comparison of *N*-acyl amino acids with *N*-acylglycyl amino acids, the difference in the distance of separation of the acyl group from the hydrolyzable bond has been neglected. However, it appears that the lower susceptibility to hydrolysis of substrates which are devoid of the secondary peptide group has to be ascribed primarily to the absence of this group. This conclusion is supported by the following considerations:

It has been stated elsewhere in this review (page 89) that the Michaelis constant, K_m, is not an explicit measure of the enzyme–substrate affinity but that comparison of K_m and k_3 values for the hydrolysis of structurally related substrate by the same enzyme may yield a relative order of the affinities of the substrates for the enzyme.

If these considerations are applied to the data given in table 13, it follows that in peptide and/or ester substrates the affinity of the substrates for carboxypeptidase decreases in the following order of *N*-substituents: benzoylglycyl > carbobenzoxyglycyl > chloroacetyl > bromoacetyl > formyl > acetyl.

For substrates which are devoid of the secondary peptide group, this sequence bears formal relation to the electronegativity of the *N*-acyl substituent (243). However, substrates which contain the secondary peptide bond are more firmly attached to the enzyme than those which are devoid of this group, despite the fact that the corresponding acyl acids (carbobenzoxyglycine and hippuric acid) are weaker acids than chloro- or bromo-acetic acid (252). The higher affinity

of these compounds apparently lies in their ability to combine with the enzyme through the dual action of the secondary peptide group to form hydrogen bonds by donation and acceptance (135, 251).

The sequence of specific activation rates of the phenylalanyl peptides and phenyllactic acid esters listed in table 13 is similar to that given above for their relative affinities. For substrates devoid of the secondary peptide group, the sequence of enzymatic activation rates parallels the sequence of the *alkaline* hydrolysis of the corresponding esters, whereas the influence of the α-substituent on *acid* hydrolysis is considerably less and yields a different sequence (102). However, as will be shown subsequently (page 136), the specific activation rate of the typical substrate, carbobenzoxyglycylphenylalanine, by carboxypeptidase increases with increasing acidity of the system (between pH 7.8 and 6.5 (197)); hence a correlation between the rate of enzymatic hydrolysis and the electronic properties of the acyl substituent of the substrate (243) is not self-evident (252). One is led to the conclusion that the secondary peptide group in specific substrates for carboxypeptidase, as in those for chymotrypsin (135, 251), is essential for the enzyme to reveal its full catalytic effect.

The present considerations of the substrate specificity of carboxypeptidase converge toward the following optimum structural requirements for specific substrates:

1. An aromatic amino acid, notably phenylalanine, tyrosine, or tryptophan, of the L-isomeric form, containing a free α-carboxyl group. These requirements are equally fulfilled by a structural analog which contains in place of the α-amino group an α-hydroxyl group.
2. The amino or hydroxyl group of group 1 is linked by a peptide or ester bond, respectively, to the carboxyl group of a monoaminomonocarboxylic acid, i.e., tryptophan, glycine, alanine, or methionine, the amino group of which is
3. masked by an acyl group provided by benzoic acid or by monobenzyl carbonate. The secondary peptide group is requisite for the full catalytic activity of the enzyme.

The question of the mutual orientation of enzyme and substrate, either in the Michaelis complex, ES, or in the activated complex, (ES)*, is highly conjectural. As part of a general discussion of dipeptidases, Bergmann (22) advanced the hypothesis that the susceptible peptide group enolizes during complex formation, thus offering points of contact through the free carboxyl group and the enol group. In a later paper, Bergmann and Fruton (24) proposed that the groupings —CONHCH(R″)COOH are arranged in counterclockwise order with the α-hydrogen atom directed toward the enzyme. Presumptive evidence for the activation of carboxypeptidase by magnesium (105, 246) has led Smith (243) to extend Bergmann's "polyaffinity" theory (34) by assuming that the metal combines through two coördinate bonds with the free carboxyl group and the carbonyl oxygen of the susceptible peptide group of the substrate. The resulting chelate ring is said to cause a straining of the hydrolyzable bond.

Despite the sharp differentiation between the substrate specificities of the

three enzymes under consideration herein, it appears more expedient to consider the problem of the mode of enzyme–substrate interaction, and of the nature of the forces involved, jointly for all three enzymes elsewhere in this review (page 144).

B. KINETICS

The rate of hydrolysis of the typical substrate, carbobenzoxyglycylphenylalanine, by carboxypeptidase at pH 7.5 can be quantitatively described by the integrated form of the modified Michaelis–Menten equation (text equation 15). The inadequacy of the interpretation of rate measurements by "proteolytic coefficients" was first demonstrated by this system (77), the calculated "proteolytic coefficients" decreasing with increasing initial substrate concentration. This dependence was equally observed in the presence of 0.04 *M* phosphate buffer (77) as well as in measurements in veronal buffer, pH 7.5 (194). Neither of the reaction products, i.e., carbobenzoxyglycine and L-phenylalanine, exerted an inhibitory effect under the conditions of these measurements (77, 78). Similar adherence to text equation 15 has since been observed for the hydrolysis of benzoylglycylphenylalanine and of benzenesulfonylglycylphenylalanine by carboxypeptidase (252).

The kinetics of the hydrolysis of *N*-acyl amino acids, such as acetyl-, formyl-, and chloroacetyl-phenylalanines (218, 252), is of different nature, since the acid anions which are formed on hydrolysis exert a marked inhibitory activity (114, 218, 252). The inhibition by these ions of the hydrolysis of the corresponding ester substrates is of lower order, since the affinities of the esters toward carboxypeptidase are considerably higher than those of the peptides (252). The hydrolysis of bromoacetyl-β-phenyllactic acid can be described by second-order reaction kinetics, while that of chloroacetyl-β-phenyllactic acid follows zero-order reaction kinetics up to 90 per cent of the reaction course over a fourfold range of initial substrate concentration (252).

The kinetics of hydrolysis of benzoylglycyl-β-phenyllactic acid is unusual (252). At any initial substrate concentration, the hydrolysis rates at 25°C. could be satisfactorily expressed by zero-order reaction kinetics; however, the calculated zero-order reaction constant decreased with increasing substrate concentration.

Since neither the reaction products nor variations in electrolyte concentrations affected the rate of the reaction, it was concluded that the dependence of the apparent zero-order rate constant on initial substrate concentration was the result of the dual action of benzoylglycyl-β-phenyllactic acid as a substrate and an inhibitor (252). Further support for this interpretation was derived from rate measurements at lower temperatures (0°C.) at which the rate increased as hydrolysis proceeded, indicating that the combination of the enzyme with this compound as a substrate is more exothermic than the combination with the same compound as an inhibitor. These considerations are in accord with the preceding discussion of the relative energy requirements for the adsorption of substrates and specific inhibitors of chymotrypsin (page 125).

At a single initial substrate concentration, the hydrolysis of carbobenzoxyglycyltryptophan, -alanine, and -glycine, respectively, likewise follows zero-order reaction kinetics (241).

With the exception of substrates which are hydrolyzed according to zero-order kinetics, the hydrolysis rates of substrates by carboxypeptidase at pH 7.5 follow the general course described by text equation 11, provided the experiments are conducted so as to yield *initial* reaction velocities. This includes also the substrate chloroacetylphenylalanine, since in the initial phase of the reaction the extent of inhibition is negligibly small (252). In all these cases, finite values for K_m and k_3 were obtained. While a plot of the text equation 11 for substrates which are hydrolyzed according to zero-order kinetics extrapolates

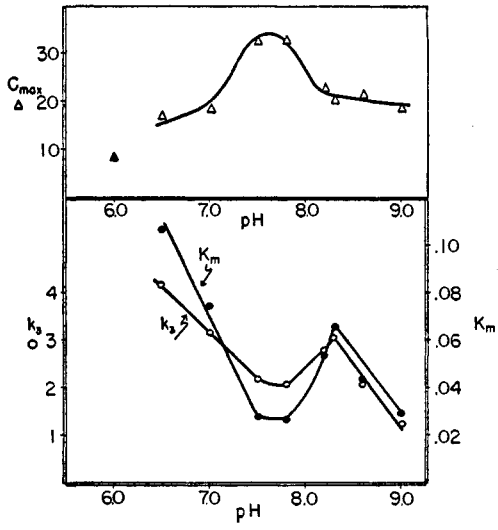


FIG. 1. pH dependence of the hydrolysis rate of carbobenzoxyglycyl-L-phenylalanine by carboxypeptidase. The symbols denote as follows: lower half: ●, K_m, ○, k_3; upper half: △, C_{max}. The point denoted by ▲ is a proteolytic coefficient (C) independent of the substrate concentration. For further details, see the text.

to $K_m \sim 0$, it is evident that also in these cases, K_m must have a finite value although it cannot be experimentally determined.

C. EFFECT OF pH

When the pH dependence of carboxypeptidase activity is expressed in terms of "proteolytic coefficients," determined at an initial concentration of 0.05 M carbobenzoxyglycylphenylalanine, a maximum at about pH 7.5 is obtained (197). A clearer analysis of the effect of pH on enzymatic activity is obtained, however, from the determination of the pH dependence of the kinetic constants, K_m and k_3. The results of such measurements, using carbobenzoxyglycylphenylalanine as substrate, are plotted in figure 1 (195, 197). It will be noted that both constants reach a minimum between pH 7.5 and 7.8. On the acid side of this pH range K_m and k_3 increase, whereas on the alkaline side of this range, a maxi-

mum at pH 8.3 is attained above which the constants decrease again up to pH 9, the upper limit of the stability of carboxypeptidase. At pH 6.0, the initial reaction velocity is practically independent of initial substrate concentration, suggesting that under these conditions K_m is so high that the rate of enzyme–substrate combination becomes the rate-limiting step (see text equation 16). The pH dependence of K_m and k_3 is not entirely the same, with the result that the maximum proteolytic coefficient, $C_{max} = k_3/2.3K_m$ (equation 18), shows a definite maximum between pH 7.5 and 7.8. No specific buffer effect (0.04 M acetate, phosphate, veronal, borate) could be observed in these measurements.

Although the dissociation constant of the carboxyl group of the present substrate is not known, it is probably within the range of that in glycyl- or tyrosyl-peptides (64), i.e., between pH 3.0 and 3.5. Since, within the pH range of the present experiments the carboxyl group of the free substrate is probably completely ionized, the observed pH dependence of K_m and k_3 has to be ascribed to the change in ionization of the enzyme. Since a free carboxyl group is requisite for specific substrates and inhibitors (page 127) of carboxypeptidase, it may be suggested that this group is attracted by a positively charged group of the catalytic centers of the enzyme. If the effect of pH on reaction rates is primarily ascribed to the change of ionization of this group, the ascending portion of the curves shown in the lower half of figure 1, below pH 7.5, could be explained by the increasing ionization of that group within this pH range. From the position of these curves along the pH axis, this group may be tentatively identified with the imidazole nucleus of a histidyl residue (pK in proteins about 5.6–7.0 (64)) or, alternatively, with an α-amino group of a terminal amino acid (pK in proteins 7.6–8.4 (64)). The shape of the curves above pH 7.5, and particularly the peak at pH 8.3, is not readily accounted for. It may be suggested, however, that it is the resultant of the states of ionization of two positively charged groups, the second of which has a pK above 8.0, and which, in the ionized state, has an antagonistic effect on catalysis.

D. INHIBITION

The following types of compounds have been reported to inhibit carboxypeptidase activity: (*1*) reaction products of specific substrates, (*2*) substrates themselves, (*3*) structural analogs of specific substrates, and (*4*) inorganic ions. These will be considered herein in the order given.

1. Reaction products

The inhibition by chloroacetate has already been mentioned. This anion inhibits not only the hydrolysis of the parent substrate, i.e., chloroacetyltyrosine (218), chloroacetylphenylalanine (252), and chloroacetyl-β-phenyllactic acid (254), but also the hydrolysis of the typical substrate carbobenzoxyglycylphenylalanine (78, 114). In the latter system inhibition is of the "indeterminate type" (see page 92), suggesting that, in relatively low concentration, chloroacetate combines with catalytically inactive centers, whereas additional increments of this anion are bound by the active centers as well. Here, as in the case

of the binding of ions and dyes by other proteins (144, 145, 146, 147, 131, 223), the combination is described by more than one parameter.

At pH 9, in the presence of 0.04 *M* buffer salts, L-phenylalanine inhibits competitively the hydrolysis of carbobenzoxyglycyl-L-phenylalanine. K_i, calculated from the anionic concentration of L-phenylalanine at pH 9, is about 6×10^{-3}, as compared to $K_i = 2 \times 10^{-3}$ obtained for D-phenylalanine at pH 7.5 (78). This finding has been interpreted by the assumption that the positively charged amino group of L-phenylalanine, unlike that of the D-isomer, is in sufficient proximity to a positively charged group on the enzyme surface to be repelled electrostatically (78), inhibition by L-phenylalanine occurring only when this isomer is in the *anionic* form.

The inhibition of carboxypeptidase by L-phenylalanine in the presence of relatively high concentrations of phosphate buffer, at pH 7.5, will be considered later in this section (page 141).

2. Substrate

The apparent inhibition of carboxypeptidase (252) by the specific ester substrate, benzoylglycyl-β-phenyllactic acid, has already been described (page 135). It has been reported (257) that relatively resistant substrates of carboxypeptidase inhibit the enzymatic hydrolysis of the highly susceptible substrate, carbobenzoxyglycyl-L-phenylalanine. According to these data, comparable degrees of inhibition were obtained on the addition of carbobenzoxyglycyl-L-alanine, and of the corresponding peptides of glycine, dehydrophenylalanine, D-phenylalanine, and D-leucine. Since, however, subsequent studies (197) failed to corroborate any inhibitory activity of carbobenzoxyglycyl-D-phenylalanine, the reported inhibition by the other peptides requires reinvestigation. The findings (257) of the inhibition of the hydrolysis of a highly susceptible substrate by a less susceptible one, if corroborated, would indicate that the affinities for the enzyme are of comparable magnitude and that the substrates differ from one another primarily in the specific rate of activation, k_3. In general, however, such a situation has not been found to prevail.

3. Structural analogs of specific substrates

Following the observation of a strong inhibitory activity of D-phenylalanine (77), the relative effects of other D-amino acids on the hydrolysis of carbobenzoxyglycylphenylalanine were determined (77, 78). The results are given in table 14. Although these values are only of relative significance, they indicate that the inhibitory activities of D-amino acids parallel the susceptibility to hydrolysis of their corresponding L-peptides (see table 12).

In a subsequent investigation (78), the contribution of the structural components of D-phenylalanine to the inhibition of carboxypeptidase was determined by quantitative rate measurements. The results obtained for competitive inhibitors are given in table 15, in terms of the dissociation constant, K_i, of the enzyme–inhibitor complex. The α-amino group of D-phenylalanine is not required for inhibitory activity since its elimination, as in β-phenylpropionic acid (hydro-

cinnamic acid), greatly enhances the affinity of the resulting compound for the enzyme. The distance of separation of the phenyl ring from the carboxyl group is of critical importance, since an increase or decrease by one methylene group decreases the affinity eighteen- and six-fold, respectively, with respect to β-phenylpropionic acid. If the phenyl group is adjacent to the carboxyl group, as in benzoic acid, "uncompetitive" inhibition of low order results (78). Analogous considerations demonstrated that the phenyl group is not replaceable by a

TABLE 14

Inhibition by D-*amino acids of the hydrolysis of carbobenzoxyglycyl-*L-*phenylalanine by carboxypeptidase (77)**

D-AMINO ACID	$\frac{C}{C_i}$
Phenylalanine	6.0
Histidine	2.1
Alanine	1.6
Isoleucine	1.6
Lysine	1.1

* Inhibition is expressed herein as the ratio of the "proteolytic coefficient" in the absence of added D-amino acid (C), to that obtained for the hydrolysis of carbobenzoxyglycylphenylalanine in the presence of 0.01 M D-amino acid (C_i). Initial substrate concentration = 0.0125 M, T = 25°C., pH = 7.5 (0.04 M phosphate buffer, 0.1 M lithium chloride).

TABLE 15

*Competitive inhibitors of carboxypeptidase (77, 78)**

COMPOUND	K_i
	$10^{-2}M$
D-Phenylalanine	2.0
D-Histidine	20.0
β-Phenylpropionic acid	0.062
γ-Phenylbutyric acid†	1.13
Phenylacetic acid	0.39
p-Nitrophenylacetic acid	2.5

* These measurements were performed at 25°C. and pH 7.5, using carbobenzoxyglycyl-L-phenylalanine as substrate.

† This compound was incorrectly described in the original publication (78) as β-phenylbutyric acid.

methyl group and also that para substitution of a nitro group into the phenyl ring of phenylacetic acid causes a six-fold decrease in the affinity (table 15). The critical importance of a free carboxyl group for specific interaction with carboxypeptidase, previously demonstrated for specific substrates, was likewise reflected by the lack of inhibition by β-phenethylamine.

Under conditions of maximum enzymatic activity, the optical specificity of inhibitors is opposite in sign to that of specific substrates. Since D-phenylalanyl

peptides are neither hydrolyzed (77, 257) nor inhibitory (197), one may conclude that the peptide group is oriented with respect to the enzyme surface in opposite direction to that in L-peptides, the latter probably being in close proximity to the catalytic centers of the enzyme. However, even in D-peptides, the distance of separation between the peptide nitrogen and the enzyme surface must be relatively small, since *N*-substitution of one or two methyl groups into D-phenylalanine (*N*-methylphenylalanine, *N*,*N*-dimethylphenylalanine) causes complete abolition of the inhibitory activity, evidently by steric hindrance, since the positive charge of the amino group remains unaffected by these substitutions. This situation is comparable to that of specific substrates in which replacement of the hydrogen atom of the susceptible or secondary peptide group decreases the susceptibility to hydrolysis (page 132). However, the difference in intermolecular distances between the enzyme and the nitrogens of the two isomeric forms of phenylalanine is sufficiently great to cause electrostatic repulsion of the *zwitterionic* form of L-phenylalanine, which is not inhibitory, in contrast to its D-isomeric form.

4. Inorganic ions

It was recently reported (246, 247) that carboxypeptidase is inhibited by such "metal poisons" as sulfide and cyanide, as well as by the following other inorganic anions: citrate, oxalate, orthophosphate, and pyrophosphate. From the nature of the ions which cause inhibition, it has been concluded that carboxypeptidase is a metal–protein compound and that magnesium is the essential metal ion. The test substrate was carbobenzoxyglycyl-L-leucine and rate measurements were interpreted by "proteolytic coefficients" (initial substrate concentration, 0.05 *M*). The inhibition by 0.2 *M* orthophosphate was reversible by dilution, whereas that caused by cyanide and sulfide required the addition of small amounts of hemoglobin, for which these ions have a high affinity. From the shapes of the curves which were obtained when the remaining activity, in per cent, was plotted against the logarithm of the anion concentration, the number of moles of inhibitor combined per mole of magnesium was estimated to be one for orthophosphate and sulfide, and two for cyanide.

Unlike crystalline enolase (283), which is a typical magnesium-activated enzyme, carboxypeptidase is not inactivated by fluoride, alone or in combination with 0.01 *M* phosphate (247). This has been ascribed to a tighter binding of magnesium in carboxypeptidase, which was also suggested by the failure of magnesium to be removed by prolonged dialysis (247).

In view of the general significance attached to these findings (243), it was deemed of interest to reinvestigate the nature of the inhibition by one of these ions, i.e., orthophosphate (194). In these measurements particular emphasis was laid on the purity of the enzyme since, according to the published values of Smith and Hanson (247), the activity of their enzyme preparation toward carbobenzoxyglycyl-L-leucine was only one-third of that found by other authors (257). The test substrate was carbobenzoxyglycyl-L-phenylalanine and rate measurements, carried out at several different initial substrate concentrations,

were interpreted by the kinetic constants, K_m and k_3. While these measurements will be published in full elsewhere (194), the results obtained to date may be summarized as follows: The initial reaction velocity is unaffected by orthophosphate, in concentrations as high as 0.2 M, which, according to Smith and Hanson (247) causes approximately 75 per cent inhibition. Accordingly, both K_m and k_3, calculated from initial reaction velocities, are independent of the presence of phosphate, as is the inhibition of carboxypeptidase by D-phenylalanine, K_i being 2×10^{-3} in each case. However, the shapes of the hydrolysis curves are dependent on the presence of phosphate, in concentrations of 0.1 M or higher, but not in a concentration of 0.04 M, previously used for quantitative rate measurements (77, 78, 252). The rate of hydrolysis decreases as hydrolysis proceeds, owing to the inhibition by one of the reaction products, i.e., L-phenylalanine. This was also confirmed by measurements in which L-phenylalanine was added to the enzyme–substrate system, in which case also the initial reaction velocity was decreased. It has, therefore, been concluded that combination of carboxypeptidase with orthophosphate does not in itself impair enzymatic activity but does increase the affinity of the enzyme for L-phenylalanine. Inhibition by L-phenylalanine is of considerably lower magnitude than that by D-phenylalanine, even in the presence of 0.2 M phosphate, and greater than that produced by other L-amino acids, such as L-alanine. In the light of these findings, the hypothesis of a specific contribution of magnesium to the catalytic activity of carboxypeptidase (243) requires revision (194).

VIII. Temperature Dependence of Hydrolysis Rates

Table 16 summarizes the values calculated from the temperature dependence of the specific rate of activation, k_3, in terms of the heat, ΔH^*, the entropy, ΔS^*, and free energy, ΔF^*, of the reactions. A cursory inspection of the data reveals that for all enzymatic systems represented in table 16, ΔH^* varies within the limits of approximately 10–16 kcal. per mole, with a median value of approximately 14 kcal. per mole. With one exception, i.e., the hydrolysis of carbobenzoxyglycyl-L-phenylalanine, the activation process is accompanied by a decrease in the entropy of activation, varying between the limits of -6 to -23 E. U., with a median value of -11.6 E. U. The relative magnitudes of these two energetic constants is such as to result in free energies of activation which vary between approximately 13 and 19 kcal. per mole, the median value of the results given in the next to the last column of table 16 being approximately 16 kcal. per mole.

It is to be recognized that the relative and absolute significance of the values given for ΔS^* and for ΔF^* is limited by considerations of both experimental and theoretical nature. The former relate primarily to the precision of the measurements from which these data were calculated. The estimated accuracy in the determination of ΔH^* is about 1 kcal. per mole, and hence that of ΔS^*, approximately 3.5 E.U. Theoretical restrictions to the interpretations of the data are imposed by an inherent ambiguity in the definition of the standard state. While, as previously discussed, k_3 has been referred to unit concentration of the

TABLE 16

Energetic constants for the hydrolysis of specific substrates by crystalline pancreatic proteolytic enzymes

ENZYME	SUBSTRATE	SOLVENT	k_3^0†	ΔH^*	ΔS^*	ΔF^*‡	REFERENCES
			sec.$^{-1}$	*kcal./mole*	E.U.	*kcal./mole*	
Trypsin.....	Benzoyl-L-argininamide	H_2O	0.36	16.9	−6.0	18.0	(229, 232)
	Benzoyl-L-arginine ester§	H_2O	26.7	10.6	−16.5	15.5	(229)
	Sturin¶	H_2O	2140.	11.8	−4.7	13.1	(56, 207)
α-Chymotrypsin.....	Benzoyl-L-tyrosinamide	30% CH_3OH	0.62	14.0	−13.0	17.9	(138)
	Benzoyl-L-phenylalanine methyl ester	30% CH_3OH	51.0	11.9	−11.0	15.2	(253)
	Acetyl-L-tyrosine ethyl ester	30% CH_3OH	193.	10.9	−10.7	14.1	(253)
	Methyl *l*-β-phenyllactate	20% CH_3OH	1.38	10.4	−23.4	17.4	(253)
	Methyl *d*-β-phenyllactate	20% CH_3OH	0.14	14.5	−14.2	18.7	(253)
	Methyl *dl*-α-chloro-β-phenylpropionate	20% CH_3OH	0.14	14.8	−13.2	18.7	(253)
	Methyl β-phenylpropionate	30% CH_3OH	0.026	16.2	−11.8	19.7	(253)
	Pepsin¶	H_2O	218.	11.2	−11.5	17.7	(56, 207)
Carboxypeptidase....	Carbobenzoxyglycyl-L-phenylalanine	H_2O	186.	15.6	+3.6	14.5	(195, 196)
	Chloroacetyl-*l*-β-phenyllactic acid	H_2O	111.	12.1	−8.8	14.7	(252)

* The values given in this table were obtained from measurements carried out at the "pH optimum" of each enzyme, i.e., pH 7.8 for trypsin and α-chymotrypsin and pH 7.5 for carboxypeptidase.

† Except when otherwise indicated, the values for k_3^0 given in this column refer to 25°C. The assumed molecular weights were 36,000 for trypsin and α-chymotrypsin, and 32,000 for carboxypeptidase. In view of more recent sedimentation measurements on trypsin and chymotrypsin (page 80) the values given for systems containing these enzymes are subject to revision.

‡ Except when otherwise indicated, the values given in this column refer to 25°C.

§ Identical values for the methyl, ethyl, isopropyl, cyclohexyl, and benzyl esters of benzoyl-L-arginine.

¶ The values for this substrate refer to a standard temperature of 0°C. k_3^0 was calculated from first-order reaction equation.

enzyme–substrate complex, (ES), other factors which may contribute to the activation process, such as hydrogen ions and solvent molecules, have been neglected in the definition of k_3. The ambiguity inherent in such an approximation has already been demonstrated for one of the present systems, i.e., the large pH dependence of the specific activation rate of the hydrolysis of carbobenzoxyglycyl-L-phenylalanine by carboxypeptidase (page 136), and probably applies also to the other systems represented in table 16. While within a limited range, the specific activation rate of the hydrolysis of acetyl-L-tyrosinamide by chymotrypsin is independent of methanol concentration (136), there is no *a priori* reason to assume that in this, or any other system, ΔH^* and ΔS^* are likewise independent of solvent composition. The problem of the definition of standard states becomes magnified when the changes in free energy and entropy of activation of the enzymatically catalyzed hydrolysis of peptides and esters are to be compared with those which accompany the hydrolysis of these substrates by acids or bases (87, 150, 171, 258, 259, 262). For this reason, such a comparison will not be attempted herein, and further consideration of the results given in table 16 will be limited to tentative interpretations of the variations observed among the different enzymatic systems.

The comparable order of magnitude of the energetic constants obtained with protein, peptide, and ester substrates for the same enzyme adds weight to the assumption that the same active centers of the enzyme surface are involved in all cases. The data given for a large variety of ester substrates for chymotrypsin have been considered (253) in relation to the nature of the α-substituent of the parent ester, i.e., methyl β-phenylpropionate. In these considerations, the influence of the varied substituents on the stability of the substrate, *per se*, has been divorced from the contribution of the same substituent to the catalytic effect of the enzyme through combination. Thus, in the series methyl β-phenylpropionate, methyl *dl*-α-chloro-β-phenylpropionate, methyl *d*-β-phenyllactate, the heat and free energy of activation decrease with increasing electronegativity of the substituent in the α-position, the chlorine and hydroxyl groups occupying similar positions in this respect. These effects have been ascribed to the lowered stability of the substrates resulting from the electronegative constituents. The added contribution which results from combination with the enzyme is evidenced by the even lower heats of activation observed for the substrates which contain either the α-hydroxyl group in the *l*-position, or which contain a secondary peptide bond (benzoyl-L-phenylalanine methyl ester, acetyl-L-tyrosine ethyl ester). With the single exception of the specific ester which contains the α-hydroxyl group in the *l*-position, the heats of activation follow the same sequence as the corresponding free energies of activation, because the entropies of activation are nearly constant. The considerably larger, rate-retarding decrease in the entropy of activation observed for the substrate which contains the α-hydroxyl group in the *l*-configuration (-23 E.U.) has been interpreted by the assumption that the postulated single hydrogen bond, formed between the hydroxyl group and the active center on the enzyme surface (see page 117) permits a larger degree of binding of solvent molecules, an effect which itself would tend to lower the

activation entropy (253). Alternatively, it may be suggested that combination of the hydroxyl group in this configuration (*l*-) with the enzyme forms part of the activation process, whereas in the case of substrates which contain a secondary peptide group such a combination has occurred during the formation of the enzyme–substrate complex, (ES) (253).

Such interpretations have to be considered with caution, as they do not account quantitatively for the differences in entropy changes observed for the tryptic hydrolysis of amide, ester, and protein substrates, respectively, or for the oppositely directed changes in entropy of activation for the hydrolysis of a representative peptide and ester substrate by carboxypeptidase. It is significant, however, that the energetic changes which accompany the tryptic hydrolysis of a series of esters of benzoyl-L-arginine are independent of the nature of the alcohol group.

TABLE 17

Temperature dependence of the Michaelis constant, K_m, for the chymotryptic hydrolysis of specific esters (253)

SUBSTRATE	TEMPERATURE RANGE	ΔE
	°C.	*cal./mole*
Methyl *l*-β-phenyllactate	10–32	6,300
Methyl *d*-β-phenyllactate	10–32	11,100
Methyl *dl*-α-chloro-β-phenylpropionate	9–25	7,600
Methyl hydrocinnamate	15–25	4,400

ΔE is the critical temperature increment, calculated from

$$\frac{\log K_{m_2}}{\log K_{m_1}} = \frac{\Delta E}{2.3R}(1/T_1 - 1/T_2)$$

In view of the preceding discussion of the effect of temperature on K_m (page 99), no interpretation of the calculated values, given in table 17, will be attempted. The limited number of data given in table 17 is presented merely to indicate the magnitude of the changes which has been observed in the study of the hydrolysis of specific esters by chymotrypsin.

IX. General Considerations

In an experimental sense, one of the principal advances in this field has been the demonstration that the range of specificities of the pancreatic proteases is much wider than was inferred from the pioneer work of Bergmann and his collaborators. The chemical reactions which are mediated by these enzymes are the hydrolysis not only of peptides and amides but also of esters, hydrazides, and hydroxamides. The observations made in recent years seem to indicate that the enzyme-catalyzed hydrolysis of synthetic substrates by these pancreatic enzymes follows the same chemical principles as have been established for non-enzymatic hydrolyses.

From the results presented in the preceding sections of this review, a rather generalized picture of these enzymatic reactions can be drawn. Although the conclusions presented here are derived from the views of many investigators, no attempt will be made to establish the antecedents of each viewpoint.

The free energies of formation of peptide, amide, and ester bonds are generally of the order of $\Delta F = -3000$ cal. per mole (110, 116, 188), indicating that the equilibrium in the reversible formation of these bonds is shifted far toward hydrolysis (85). These hydrolytic reactions are generally accelerated by hydrogen or hydroxyl ions, the participation of water *molecules* occupying a subordinate role to the attack of the susceptible bond by these ions (102). A fundamentally similar mechanism must hold for enzyme-catalyzed hydrolysis.

Although in these enzymatic systems the enzyme–substrate complex, (ES), is hypothetical, its existence is a necessary requirement for any interpretation which recognizes the operation of specific short-range forces in the catalytic process. The energetic changes which accompany this reaction cannot be evaluated at the present time since, as has already been discussed (page 98), the kinetics of this step cannot be uniquely determined. The contribution of hydrogen or hydroxyl ions of the solvent to this step likewise remains indeterminate. Limited experimental evidence suggests that the heat of activation in the formation of the enzyme–substrate complex has a small, negative value. This conclusion is derived from the temperature dependence of the interaction between proteins and ions (146) and is in accord with the observed temperature variation of K_m. Accordingly, the changes in free energy are favorable for enzyme–substrate interaction and contribute, in part, to the energetic requirements of the hydrolytic reaction. The major energetic changes, however, probably occur during the activation step.

It appears that the more complete the orientation of the substrate on the enzyme surface in the first step, the less ordering is required for the subsequent activation process. This implies that, in the case of rapidly hydrolyzed substrates, such an orientation brings the molecule into a configuration closely resembling the activated state. Since, for example, the entropy of activation of the hydrolysis of a series of substrates by chymotrypsin has been found to be constant (253), regardless of the presence or absence of the "secondary peptide" group, it has been surmised that for substrates containing this group, part of the entropy change otherwise required for the activation process has been absorbed during the formation of the enzyme–substrate complex. Since trypsin and chymotrypsin have not been shown to contain any elements other than amino acid residues, and since the magnesium of carboxypeptidase does not appear to be directly responsible for the activation process, the forces involved in the adsorption process must be similar to those acting in the specific reactions of antibodies with antigens or haptens (210). The importance of steric factors, which have been shown to be critical to within 1–2 Å., and of hydrogen and electrostatic bonding in the formation of the enzyme–substrate complex have been noted in the preceding text. Van der Waals forces will become important where the appropriate "complementariness" of structures obtains.

Although the activation process is probably much more complex in its details than we can now conceive of, the attack on the susceptible bond may be ascribed to three, not clearly distinguishable, factors: (*1*) Interaction of the enzyme with previously unadsorbed parts of the substrate. By analogy with well-known theories, this interaction may cause sufficient deformation of the hydrolyzable

bond to render it susceptible to attack by the elements of water. (*2*) The activation process may not involve gross changes in configuration of the enzyme–substrate complex but may produce extensive polarization of the hydrolyzable bond. If this polarization approaches ionization of this bond, the approach of hydrogen or hydroxyl ions would be facilitated. (*3*) The enzyme may be capable of concentrating hydrogen or hydroxyl ions of the environment at the site of the hydrolyzable bond so that the cleavage of this bond is the result of proton (or hydroxyl ion) transfer. It is within the realm of possibility that part of the measured energy of activation may be the heat of ionization of a group on the enzyme surface.

Inasmuch as pepsin has been shown to open in egg albumin the same number of bonds, at apparently identical sites, as does acid hydrolysis, pepsin may be conceived of as acting as a proton-transferring enzyme (51). The observation that, in the hydrolysis of carbobenzoxyglycylphenylalanine by carboxypeptidase, the specific rate of activation increases with decreasing pH (page 136), suggests a direct participation of protons in this enzymatic system. However, in the case of substrates for carboxypeptidase which are devoid of the "secondary peptide" group, i.e., *N*-acyl amino acids and their ester analogs (page 134), the rate of enzymatic hydrolysis parallels the rate of alkaline hydrolysis of esters of the acylating groups.

If the views of the activation process advanced in this discussion are correct, the question may be raised why in the case of protein, peptide, or amide substrates, the substrate molecule fails to exert an activating effect on the peptide bonds of the enzyme. The answer to this question must lie in the steric and electronic support given to the active centers of the enzyme by the entire enzyme molecule. This latter effect may also account for the well-established observation that the denatured proteins (including the proteolytic enzymes themselves) are attacked by the proteolytic enzymes much more readily than are native proteins.

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