THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

STUDIES ON THE ACTIVATION AND ACTION OF PORCINE PEPSIN

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

CHARLES G. SANNY

Oklahoma City, Oklahoma

1975

STUDIES ON THE ACTIVATION AND

ACTION OF PORCINE PEPSIN

APPROVED BY

Jan Ann Hartsuck

Jordan J. M. Lang

Albert M. Chandler

Leon Unger

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. Jean A. Hartsuck, Department of Biochemistry and Molecular Biology, for her advice, guidance, and friendship.

I am indebted to Dr. Jordon J. N. Tang, Head of Laboratory of Protein Studies, Oklahoma Medical Research Foundation, for discussion and suggestions.

I wish to thank the members of my dissertation committee, Dr. Jean A. Hartsuck, Dr. Jordon J. N. Tang, Dr. Albert M. Chandler, Dr. Leon Unger, and Dr. Richard Van der Helm for their advice in the preparation of this dissertation.

I am also indebted to Dr. Chadwick A. Cox for his advice.

I also wish to thank Dr. Ronald Coleman for atomic absorbtion analysis, Kenneth Jackson for N-terminal sequence analysis, and Paul Lanier for amino-acid analysis.

I wish to express my gratitude to the Oklahoma Medical Research Foundation for the provision of facilities, equipment, and material and for financial support while a predoctoral fellow.

I also wish to express my gratitude to my wife, my family, and my friends for their continued support during the preparation of this dissertation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

STUDIES ON THE ACTIVATION AND ACTION OF PORCINE PEPSIN

CHAPTER I

INTRODUCTION

The activation of pepsin from pepsinogen and the subsequent action of pepsin in proteolysis have been subjects of study as early as 1882 (1). Methods for preparing crystalline pepsinogen and pepsin inaugurated the modern era of pepsinogen and pepsin investigation (2,3). The following studies confirm and contribute to the current understanding of pepsin activation (Chapter II), and the mechanism of pepsin activity (Chapter III).

Pepsinogen Activation

Herriot, reporting of the properties of swine pepsinogen, observed that pepsinogen was converted to pepsin in solutions more acidic than pH 6.0 (3). Between pH 4.5 and 5.0 the conversion was found to be autocatalytic in which the product of the reaction, pepsin, catalyzed the conversion. Below pH 4.0, the reaction no longer fit simple autocatalytic kinetics. The pH-activation rate profile for the conversion of pepsinogen to pepsin closely resembled the pepsin pH-activity rate profile with a pH optimum at 2.0. This resemblance

supported the autocatalytic activation mechanism (4).

The deviation from simple autocatalytic activation kinetics at pH less than 4.0 was accounted for by the presence of a reversibly dissociable intermediate compound consisting of pepsin and an inhibitor peptide (4).

$$\text{Pepsinogen} \xrightarrow{\text{(Pepsin)}} \text{Pepsin-Inhibitor Complex}$$

$$\text{Pepsin-Inhibitor Complex} \underset{> \text{pH } 5.4}{\overset{< \text{pH } 5.4}{\rightleftharpoons}} \text{Pepsin} + \text{Inhibitor} \longrightarrow \text{Pepsin} + \text{X}$$

The association of the pepsin and inhibitor was pH dependent with little dissociation at pH 6.0 and virtually complete dissociation below pH 5.0 (4). The rate limiting step, at low pH, in the conversion of pepsinogen to pepsin was suggested to be the dissociation of the inhibitor-pepsin complex. This proposal has been supported by Wang and Edelman using fluorescent probes to study the pepsinogen to pepsin conversion (5). Bohak has reported tight binding between chicken pepsin and the activation peptide produced in the conversion of chicken pepsinogen to pepsin (6,7).

The inhibitor peptide has been isolated and found to be a basic, single chain, polypeptide containing 29 amino acids from the amino-terminal portion of the pepsinogen molecule (8-10). The inhibitor peptide plus other low molecular weight products of pepsinogen conversion to pepsin come from the first 44 amino acids of the amino-terminus of pepsinogen (11-13). The amino acid sequence of the amino-terminus of pepsinogen overlapping the amino-terminus of pepsin is shown in Table 1. The presence of basic amino acid residues (9 lysines,

TABLE 1

AMINO ACID SEQUENCE[a] OF THE AMINO-TERMINUS

OF PEPSINOGEN OVERLAPPING THE AMINO-TERMINUS OF PEPSIN

1				5				10						15
Leu	-	Val	-	Lys	-	Val	-	Pro	-	Leu	-	Val	-	Arg
-	Lys	-	Lys	-	Ser	-	Leu	-	Arg	-	Gln	-	Asn	-
Leu	-	Ile	-	Lys	-	Asp	-	Gly	-	Lys	-	Leu	-	Lys
-	Asp	-	Phe	-	Leu	-	Lys	-	Thr	-	His	-	Lys	-
His	-	Asn	-	Pro	-	Ala	-	Ser	-	Lys	-	Tyr	-	Phe
-	Pro	-	Glu	-	Ala	-	Ala	-	Ala	-	Leu	-	Ile	-
Gly	-	Asp	-											

20 25 30 35 40 45

⇣b ↓b

a. Sequence 1 - 39, Ong, E.B. and Perlmann, G.E. (11); sequence 40-47, Pedersen, V.B. and Foltmann, B. (12).

b. ↓, bond normally hydrolysed during acid activation of pepsinogen to pepsin; ⇣, bond of minor hydrolysis during activation (72).

2 histidines, and 2 arginines) contribute to the stabilization of the pepsinogen molecule which is stable between pH 7 and 8, and reversibly denatured above pH 8 (3). Pepsin, on the other hand, is irreversibly denatured above pH 6.0 and denatured at elevated temperatures (3,14). Addition of the activation peptide to pepsin results in a partial stabilization of the pepsin molecule (7,15).

The conversion of pepsinogen to pepsin at acid pH occurs by hydrolysis of the peptide bond between leucine 44 and isoleucine 45 in the pepsinogen molecule (12,13). The mechanism of conversion has not been fully understood.

An acid labile sugar covalently bound to the pepsinogen but not to pepsin was thought to be cleaved by acid prior to activation (16). Later, the carbohydrate content found in a different preparation was negligible, therefore the cleavage of the carbohydrate moiety does not trigger the activation of pepsinogen (17).

Immunochemical studies of the conversion of pepsinogen to pepsin showed pepsinogen and pepsin to have common antigenic determinant groups. These studies suggested that there was "no major conformational alteration" in the conversion process (18). However, optical rotatory properties (15), fluorescent probe studies (5,7,14), and ultraviolet absorbance determinations (19,20), all suggested marked differences between the pepsinogen and pepsin molecules. These observations appeared to be related to the cleavage and dissociation of the activation peptide. Denatured pepsinogen could not be converted to pepsin, suggesting a requirement for secondary and tertiary integrity of the molecule (21).

The quality of the pepsin product formed from pepsinogen is determined by the mechanism of conversion. Rajagopalan, Moore and Stein found commercially available pepsin heterogeneous by several criteria, whereas fresh pepsin, prepared from pepsinogen activated at pH 2.0, yielded a homogeneous pepsin (22). They suggested that during the preparation of commercial pepsin some autolysis had occured. They also noted, in the conversion of pepsinogen to pepsin that if the activation process was carried out between pH 3.0 and 3.9, a heterogeneous pepsin product resulted.

Several mechanisms for the conversion of pepsinogen to pepsin have been proposed. The first mechanism is a bimolecular, autocatalytic (pepsin catalyzed) conversion (as already briefly mentioned above).

$$\text{pepsin} + \text{pepsinogen} \xrightarrow[\text{Acid pH}]{k_2} \text{pepsin} + \text{pepsin} + \text{peptides}$$

The rate of this mechanism is dependent on the pH and on the concentration of both pepsin and pepsinogen (23). This is the mechanism proposed by Herriot (3).

A second mechanism is a unimolecular conversion of pepsinogen to pepsin. In this mechanism, a pepsinogen molecule cleaves its own amino-terminal peptide and forms a pepsin molecule.

$$\text{pepsinogen} \xrightarrow[\text{Acid pH}]{k_1} \text{pepsin} + \text{activation peptide}$$

The rate of this mechanism is dependent only on the pepsinogen concentration and on the pH (23). Bustin and Conway-Jacobs were able to immobilize pepsinogen on Sepharose (resulting in decreased bimolecular interactions) and form active pepsin by exposure to acid (24). Their

experiments suggested pepsinogen was capable of unimolecular activation. The conversion of pepsinogen to pepsin in the presence of excess pepsin substrates (24-26) or competitive inhibitors (25) also suggested a unimolecular activation mechanism.

The third mechanism is a bimolecular, zymogen catalyzed conversion of pepsinogen to pepsin.

pepsinogen + pepsinogen ——→ pepsinogen + pepsin + peptides

This mechanism is postulated on exposure of the active site prior to activation by a conformational change in the pepsinogen molecule (11, 19,26,27).

Kinetic studies, based on the determination of the amount of pepsinogen remaining after various times of activation, showed the rate of disappearance of pepsinogen could be described by a rate equation combining unimolecular and bimolecular rate constants (23). The disappearance of pepsinogen was predominately first-order below pH 3, and predominately second-order in the pH range 3 to 4.5. The kinetic experiments could be interpreted satisfactorily using the first two activation mechanisms described above and did not require assuming the zymogen possessed bimolecular proteolytic activity.

Simulation of the activation process by analog computer has suggested the activation mechanism of pepsinogen to be very complex (20).

Kassell and Kay (27), reviewing the work of others (5,19, 21,23,24,26,28-30), suggested the activation of pepsinogen to pepsin might be better interpreted by assuming that pepsinogen is capable of bimolecular proteolysis. In particular, they cited the experiments of McPhie (19). He observed that the difference spectrum changes,

generated when a pepsinogen solution was adjusted to pH 2.85, could be reversed if the solution was returned to neutrality in less than 2 minutes. This observation was interpreted by Kassell and Kay (27) to imply that in an acidic solution the pepsinogen molecule undergoes a conformational change and then possesses proteolytic activity. However, they believe that if the solution is neutralized in less than 2 minutes the pepsinogen conformational change is reversed. Consequently, they suggested that virtually no pepsinogen activation takes place within 2 minutes at pH 2.85 in a 20 μM pepsinogen solution. This is in contrast to the implications of the kinetic study of pepsinogen activation (23). Calculations from the kinetic constants determined in the study by Al-Janabi, et al (23) show that more than one-half of the pepsinogen would be activated by the first order reaction in 2 minutes at these conditions. I therefore undertook to find out if activation, in the sense of chemical conversion of pepsinogen to pepsin, occurs under these experimental conditions and, if so, how McPhie's results might be interpreted to give a consistent understanding of this system. Chapter II provides chemical and spectral evidence that the conversion of pepsinogen to pepsin takes place at the kinetic rate which was determined previously by Al-Janabi, et al (23).

Mechanism of Pepsin Activity

Once pepsin is formed, it is capable of proteolysis of a large number of natural and synthetic substrates. The pH optimum of peptic hydrolysis of some native and denatured natural substrates is given in Table 2. The activity of pepsin toward natural substrates

TABLE 2

OPTIMUM pH OF PEPTIC HYDROLYSIS OF SOME NATIVE AND DENATURED NATURAL SUBSTRATES

Substrate	pH Optimum		ref.
	Native	Denatured	
Egg albumin	1.0	1.5 - 1.8	31
B-Lactoglobulin	--a	2.0 - 3.0	31
Casein	wide range[b]	--	31
Bovine Hemoglobin	--	≤ 2	31
Bovine Hemoglobin	2.0	2.0[c]	32
"		2-3.5[d]	32
"		3.5[f,g]	32
Bovine Serum Albumin	2.0	2.7[c]	32
"	"	2.0[e]	32
"	"	3.5[g]	32
"	"	3.8[d,f]	32
Bovin Serum Albumin	2.0	2.2; 4.0[h]	33

a. very slow hydrolysis of native substrate
b. constant over entire pH range
c. HCl denatured
d. urea denatured
e. NaOH denatured
f. HCl-Urea denatured
g. NaOH-Urea denatured
h. bimodal: NaCl, I=0.50

appears to be related to the secondary and tertiary structure of the substrate (31-33) and to the nature of the side-chains of the amino acids forming the peptide bond to be hydrolyzed (34).

There have been many reports of synthetic substrate hydrolysis by pepsin (35-67). Factors that influence the specificity of pepsin for synthetic substrates are the nature of the side-chains of the amino acid residues on either side of the peptide bond to be cleaved, the stereo-chemical relationship between the side-chains of the substrate and the enzyme (D or L isomers), and the nature of the groups in the substrate distal to the peptide bond (45,49,59,61-63,67). In general, catalysis is favored between hydrophobic residues in a peptide of L-amino acids with little or no net negative charge at acid pH.

Studies on the pH dependence of hydrolysis of substrates have given insight into the ionization of enzyme functional groups in the free enzyme and in the enzyme-substrate complexes. Table 3 summarizes the substrate and estimated pK's of the functional groups in pepsin. It appears that carboxyl groups at or near the active site are involved in catalysis and/or substrate binding. There seem to be at least two carboxyl groups involved in catalysis, one with an abnormally low pK and one with a normal pK (46,50,51,57,68).

Inactivation of pepsin by compounds which covalently react with functional groups at or near the active site of pepsin have been reported and contribute to the study of the ionization of carboxyl groups in pepsin. Tang (69), studying the specific and irreversible inactivation of pepsin by substrate-like epoxides, inactivated pepsin with 1,2-epoxy-3-(p-nitrophenoxy)propane, (EPNP). He found two moles

TABLE 3

ESTIMATED pK's OF FUNCTIONAL GROUPS IN PEPSIN CALCULATED FROM SYNTHETIC SUBSTRATE HYDROLYSIS

Substrate[a]	pK of Functional Group in		Ref.
	Free Enzyme	Enzyme-Substrate Complex	
Ac-Phe-Diiodotyr	4.25	3.2[b]	42
	--	1.62	46
	--	3.48	"
	--	1.6 (pK_1')[c]	68
	--	4.1 (pK_2')	"
	--	4.7[b]	58
Ac-D-Phe-Tyr	3.0	3.8	48
Ac-D-Phe-Tyr-OMe	3.0	4.2	48
Ac-Phe-3,5 Dibromotyr	0.75 (pK_{e1})	0.89 (pK_{es1})	50
	2.67 (pK_{e2})	3.44 (pK_{es2})	
Ac-Phe-Trp	1.40 (pK_{1E})	1.05 (pK_{1ESH})	51
	4.25 (pK_{2E})	3.70 (pK_{2ESH})	
Ac-Phe-Tyr-NH_2	1.17 (pK_{1E})[c]	1.35 (pK_{1ESH})	51
	4.35 (pK_{2E})	4.15 (pK_{2ESH})	
	--	4.7[b]	58
Ac-Phe-Tyr	1.17 (pK_{1E})	1.12 (pK_{2ESH})	51
	4.35 (pK_{2E})	3.70 (pK_{2ESH})	
	--	4.1[b]	58

TABLE 3 (CONTINUED)

Substrate[a]	pK of Functional Group in Free Enzyme	pK of Functional Group in Enzyme-Substrate Complex	Ref.
Ac-Phe-Phe-OMe	--	1.4 (pK'_1)	68
	--	4.6 (pK'_2)	
Ac-Tyr-Phe-OMe	--	1.8 (pK'_2)	68
	--	4.6 (pK'_2)	
Z-His-Phe-OEt	--	3.8 (pK_a)[b]	61
Ac-Phe-Phe-Gly	--	1.1	57
Ac-Phe-Phe	--	1.1	57
Ac-Phe-Phe-NH_2	--	1.05	57
	--	4.75	
Methyl phenyl sulfite	--	2.6	53
N-TrifluoroAc-Phe	--	3.7[b]	65
	--	4.8	

a. abbreviation: Ac, N-acetyl; His, histidine; Phe, L-phenylalanine; D-Phe, D-phenylalanine; Tyr, tyrosine; Trp, tryptophan; OMe, methyl ester; -OEt, ethyl ester; NH_2, amide; Z, benzoxycarbonyl.

b. involved in binding of substrate to pepsin.

c. pK'_1 and pK'_2 refer to the ionization of two groups. pK_{e_1} and pK_{e_2} or pK_{1E} and pK_{2E} refer to the ionization of the groups in the free enzyme. pK_{es_1} and pK_{es_2} or pK_{1ESH} and pK_{2ESH} refer to the ionization of the groups in the presence of substrate.

of EPNP covalently bound to each mole of pepsin; one aspartic acid residue, and one methionine residue were modified. At lower temperatures, a 1:1 correlation between EPNP incorporation and loss of pepsin activity was observed (70). The residue modified was an aspartic acid, located at position 32 in the pepsin sequence (71,72). The pH-inactivation rate profile for the inactivation of pepsin by EPNP suggested a pK of 2.8 for the active carboxylate ion (70).

A number of diazo compounds have been found that react with pepsin, resulting in essentially complete loss of peptic activity. A list of these compounds is given in Table 4.

The functional group that reacts with the diazo compounds is an aspartic acid residue (84-87) identified in the pepsin sequence as aspartic acid number 215 (72,88). Similar findings have been reported for other acid proteases such as pepsin C (89), bovine pepsins (90) and acid proteases from Aspergillus awomari and Rhodotorula glutinis (91,92).

Copper salts improve the rate and specificity of diazo compounds. A copper-carbene intermediate has been suggested in the mechanism of inactivation (80). From the pH-rate of inactivation profile, the suggested mechanism involves two carboxyl groups, one ionized with a pK near 4 which binds the copper-carbene intermediate, and one protonated with a pK considerably higher which donates a proton in the inactivation reaction (80). The actual mechanism of inactivation is still not known.

Specific but incomplete inactivation by p-bromophenacyl bromide has been attributed to a reaction with an aspartic acid at or near the active site of pepsin (93-95). However, this aspartic acid

TABLE 4

DIAZO INACTIVATORS OF PEPSIN

Compound	Reference
Diphenyldiazomethane (DDM)	73
L-1-diazo-4-phenyl-3-tosylamido butanone (L-DPTB)	74
Diazoacetyl-DL-norleucine methyl ester	75
1-Diazo-4-phenylbutanone (DPB)	76
α-Diazo-p-bromoacetophenone	77
Benzyloxycarbonyl-L-phenylalanyl diazo methane (ZPDM)	78
1-Diazo-3-dinitrophenyl amino propane (IKG)	79
Diazoacetyl-DL-phenylalanine ethyl ester (IGP)	79
Diazoacetylglycine ethyl ester (IGG)	79
α-Diazo-B-p-oxyphenylpropionic ethyl ester	79
Phenylbenzoyldiazomethane, azibenzyl	79
Diazoacetylglycine ethyl ester	80
Diazoacetic acid ethyl ester	80
N-Diazoacetyl-N'-1,2-dinitrophenyl-ethylene diamine (DDE)	81, 82
1-Diazo-2-phenylethane	83
1,1-Bis(diazoacetyl)-2-phenylethane	83
dl-1-Diazoacetyl-1-bromo-2-phenylethene	83

is not the same aspartic acid involved with diazo compounds (77).

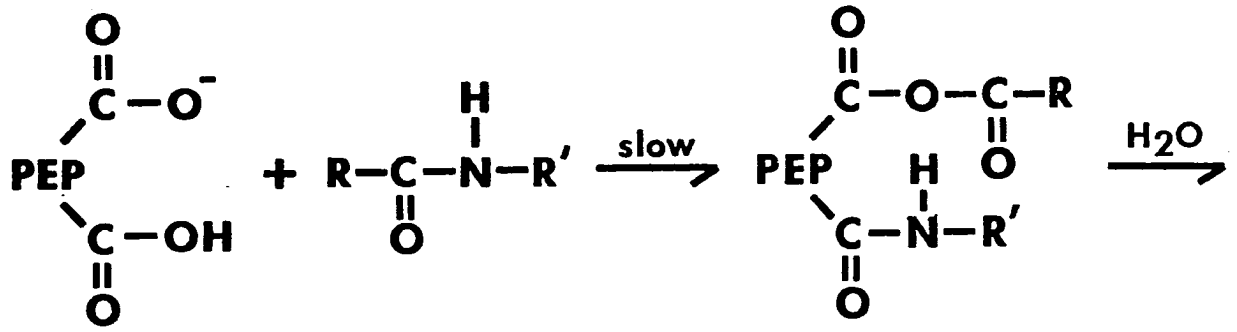
From the above studies on the hydrolysis of synthetic substrates and the inactivation of pepsin, and from other studies, such as O^{18} exchange between pepsin and H_2O^{18} (96-98), and ^{14}C incorporation from ^{14}C trimethyloxonium fluoroborate (99), it has been inferred that at least two carboxyl groups are essential for peptic activity. Current proposals for the mechanism of pepsin action include two carboxyls in the active site.

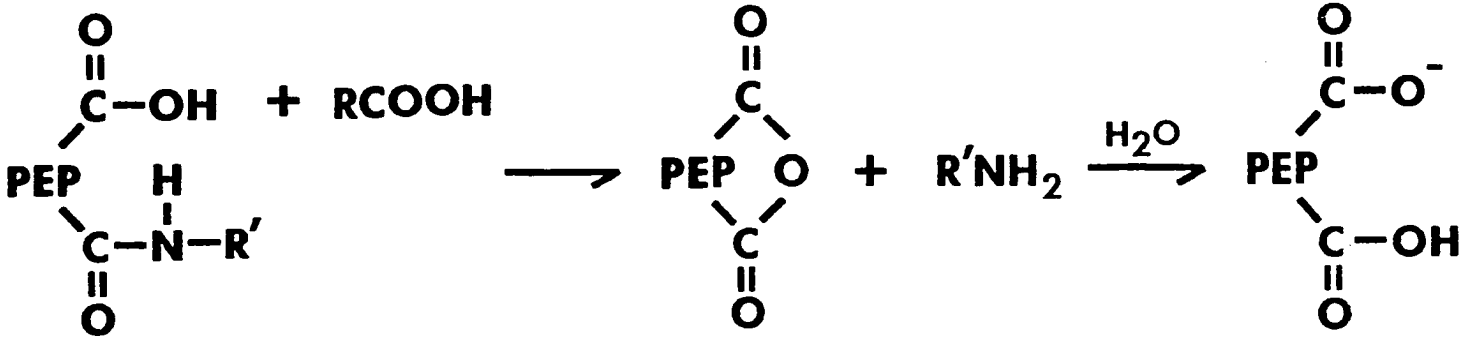
Clement, et al (68) proposed a mechanism for the hydrolysis of neutral dipeptides consistent with two carboxyls kinetically involved in the rate limiting step. Both the acyl and amine half of the dipeptide are covalently bonded to pepsin during hydrolysis, and the rate determining step does not involve proton transfer. The simplified mechanism is described in Figure 1.

The search for an acyl-enzyme intermediate has been inconclusive (100-104), and the search for an amino-enzyme intermediate has met with little success (105,106). Most of the "evidence" for acyl or amide intermediates can be interpreted on the bases of differences in the side-chain binding strengths of the substrates. Studies on the inhibition of pepsin by a variety of competitive and non-competitive inhibitors have suggested an ordered release of products after hydrolysis with the carboxylate product leaving first (107-109).

Knowles, et al (110,111) have proposed mechanisms which account for the catalytic activities of pepsin (hydrolysis, transpeptidation, and ^{18}O-exchange), the involvement of an amino-enzyme intermediate, and the participation of at least two carboxyl groups (one

FIGURE 1: PROPOSED PEPSIN MECHANISM (CLEMENT, et al). This mechanism was proposed by Clement, et al (68) for the hydrolysis of neutral dipeptides.


PEP
C–OH
O
+ R–C–N–R′
H
slow
C–O–C–R
H
H2O


C–OH
PEP
H
C–N–R′
+ RCOOH
C
PEP O
+ R′NH2
H2O
C–O⁻
PEP
C–OH

with pKa 1.0 and one with pKa 4.0) in catalysis. The mechanism for peptide hydrolysis is outlined in Figure 2. The interaction between the substrate and the active site forms a tetrahedral intermediate, which then breaks down into the products. The ^{18}O-exchange is explained by incorporation of ^{18}O into rapidly metabolized carboxyl groups in the active center (96). The ^{18}O-exchange mechanism may involve an anhydride between two carboxyl groups (105).

Wang (112) suggested a mechanism in which pepsin has a slightly distorted peptide linkage at the active center. This mechanism is shown in Figure 3. This mechanism would be consistent with ^{18}O-exchange data if rapid protonation by one carboxyl group would make the other carboxyl carbon a strong electrophile which could react with water. In catalysis, a substrate would react instead of water. The pH dependence of pepsin activity could be explained in part by this mechanism (112). The only lysine in pepsin is changed to glutamine in human pepsin (113), therefore, lysine is not essential in the activity of pepsin and Wang's hypothesis is not valid.

Hartsuck and Tang (70) provided direct evidence for a carboxyl group other than the one modified by diazo compounds. They proposed a mechanism which included an arginine in the active center. This proposal was reinforced by the loss of peptic activity upon modification of argininyl residues near the COOH-terminus of pepsin (114-115). Their mechanism is shown in Figure 4. One of these arginines may be in a position to polarize the carbonyl bond making it susceptible to nucleophilic attack (70). The pKa of the ionized carboxyl would be influenced by a neighboring protonated carboxyl not

FIGURE 2: PROPOSED PEPSIN MECHANISM (KNOWLES). This mechanism was proposed by Knowles, et al (110,111) and accounts for hydrolysis. Other similar mechanisms account for transpeptidation and ^{18}O-exchange.

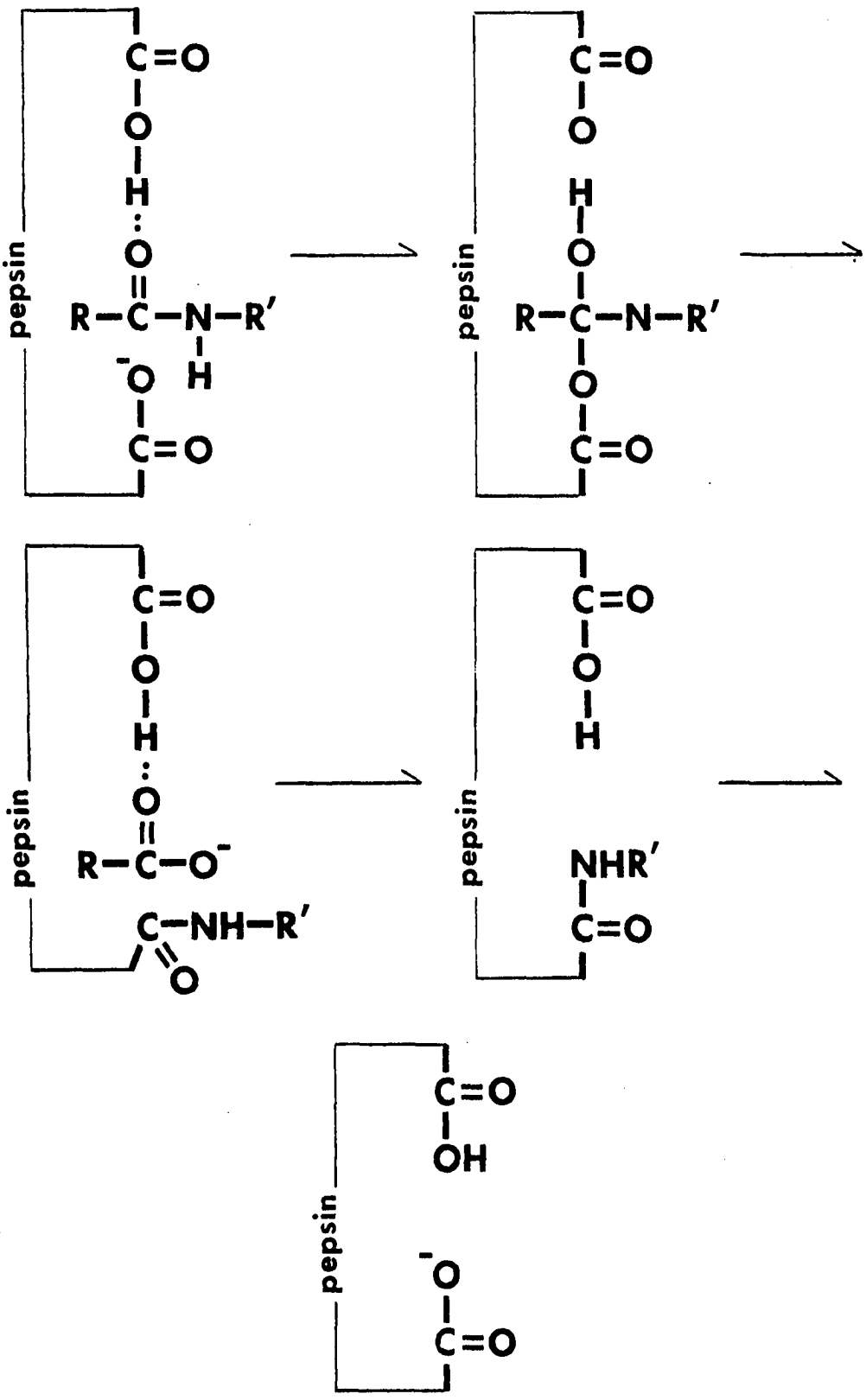

pepsin
C=O
O
H
O
R–C–N–R′
H
O
C=O
pepsin
C=O
O
H
O
R–C–N–R′
O
C=O
pepsin
C=O
O
H
O
R–C–O⁻
C–NH–R′
O
pepsin
C=O
O
H
NHR′
C=O
pepsin
C=O
OH
O⁻
C=O

FIGURE 3: PROPOSED PEPSIN MECHANISM (WANG). This mechanism, proposed by Wang (112), involves a slightly distorted peptide linkage at the active center.

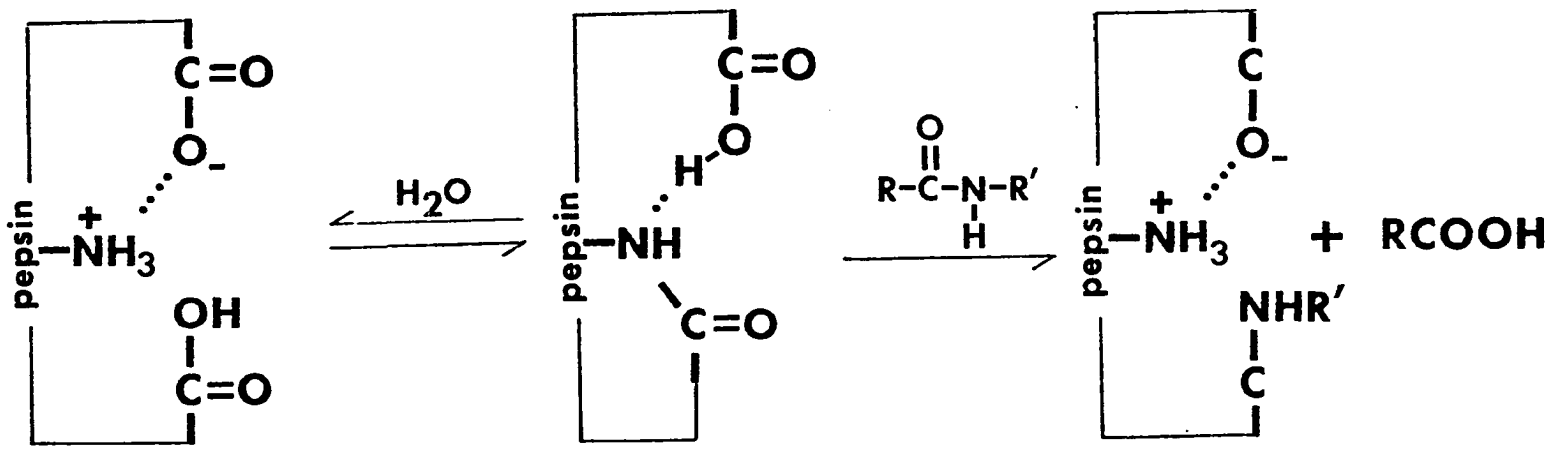
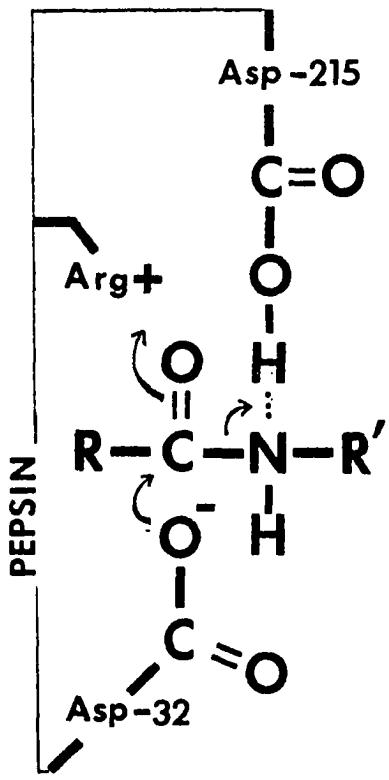

C=O
O
pepsin
NH3
OH
C=O
H2O
H
NH
C=O
O
R–C–N–R′
H
C
O
pepsin
NH3
+ RCOOH
NHR′
C

FIGURE 4: PROPOSED PEPSIN MECHANISM (HARTSUCK AND TANG). This mechanism, proposed by Hartsuck and Tang (70), involves an arginine in the active center polarizing the peptide bond.


Asp-215
C=O
O
H
Arg+
O
R—C—N—R′
H
O⁻
C
O
Asp-32
PEPSIN

directly involved in catalysis. This mechanism is very similar to that proposed independently by Hunkapiller and Richards (65).

Parsons and Raftery (116-119) have been able to study the ionization behavior of the catalytic carboxyls of lysozyme by pH difference titrations of the B-ethyl ester derivative of the aspartic-52 residue of lysozyme relative to native lysozyme. I, therefore, undertook to study the ionization behavior of the carboxyl groups modified by diazoacety-DL-norleucine methyl ester compared to native pepsin by difference titration. Chapter III provides new insight into the ionization of carboxyl groups in the active site of pepsin.

CHAPTER II

CONVERSION OF PEPSINOGEN TO PEPSIN

Materials

Proteolytic Enzymes

Chromatographically pure pepsinogen (Lots PGC1IA and PGC53J413) used in the activation and spectral experiments, crystalline pepsinogen (Lot PG1JA) used for the preparation of pepsin according to the procedure of Rajagopalan, Moore, and Stein (22), and carboxypeptidase A-DFP (COADFP 3AA) were all obtained from Worthington Biochemical Corporation. .

Cation Exchange Resins

SE-Sephadex C-25 and SP-Sephadex C-25 were purchased from Pharmacia.

Ultrafiltration Membranes

Diaflo ultrafiltration membranes were purchased from the Amicon Corporation.

Reagents

Reagents and solvents used in the Protein Sequencer were obtained from Beckman Instruments. All other reagents were analytical

grade.

Methods

Activation of Pepsinogen for Quantitative Analysis of the NH_2 - Terminal Two Residues

Activation mixtures were prepared to quantify the chemical cleavage of pepsinogen when pepsin was formed under various experimental conditions. Pepsinogen, 20 to 25 mg, was dissolved in 2 mM Tris Chloride, pH 7.5, with a final protein concentration of 1 mg per ml (25 μM). An appropriate amount of 0.24 M HCl was added to bring the pH of the solution to the desired value. At the end of the activation period, sufficient 1 M Tris was added to raise the pH to 8.5. The solution was allowed to remain at room temperature for 1 to 2 hours in order to denature any pepsin formed.

For activation at low pH or for short activation times, it was helpful to use a mixture of chloroacetic and hydrochloric acids (22) or 0.02 M sodium citrate buffer to lower the pH. Complete activation to produce homogeneous pepsin was achieved using a 20 minute activation at pH 2.2 (22).

Separation of Pepsinogen-Pepsin Mixture from Activation Peptides

The solutions containing the activation products were concentrated using Amicon ultrafiltration cells (model 12 or model 52) and Diaflo ultrafiltration membranes (UM 05, UM 10 or PM 10). Glass distilled water, followed by the buffer which was to be used in the subsequent column chromatography, was added to the solutions being

concentrated in order to wash out residual salts and to adjust the solution to the required pH. The concentrated pepsinogen-pepsin mixture, protein concentration about 8 mg per ml, was separated from the basic activation peptides on a SE-Sephadex C-25 or SP-Sephadex C-25 column (1.5 cm x 30 cm), equalibrated and eluted with 0.4M sodium acetate, pH 6.0 or pH 4.4 (22). Under these conditions the basic activation peptides are bound to the column (22). Initially pH 6.0 was used to guard against further pepsinogen activation during chromatography. However, while these experiments were underway, it was found that pepsinogen, which is free of pepsin, does not activate at pH 4.4 during a 7 hour incubation period. Therefore, subsequent columns were eluted at pH 4.4. The flow rate was 1 ml per minute and 2 to 3 ml fractions were collected at 13^0C. The protein peak was pooled and dialyzed for 12 hours against 2 changes of glass distilled water which had been adjusted to pH 10 with concentrated ammonium hydroxide. This step assured denaturation of all remaining pepsinogen. The dialyzed protein was lyophilized.

Quantitative Analysis of the NH_2-Terminal Two Residues

The NH_2-terminal two residues of the peptide free activation products were analyzed using a Beckman model 890C Protein Sequencer with a Fast Protein-Quadrol Program No. 072172C. This is similar to the single coupling, double cleavage method of Edman and Begg (120). The conversion of thiazolinines to phenylthiohydantoin (PTH)[1]-amino

---

[1]Abbreviation: PTH, phenylthiohydantoin.

acid derivatives and their subsequent extraction has been described (120). The PTH-amino acids were identified using a Beckman GC-65 gas-liquid chromatograph equipped with two columns of SP-400 packing (121).

For quantitative measurement of PTH-amino acids from a mixture of pepsinogen and pepsin, two steps of Edman degradation were carried out in the sequencer. The NH_2-terminal leucine and isoleucine, derived from pepsinogen and pepsin, respectively, were determined together because their PTH derivatives did not separate during the gas-liquid chromatography. The PTH-valine and PTH-glycine, derived from the second residues of pepsinogen and pepsin, respectively, were determined from separate peaks in the gas-liquid chromatography. Other PTH-amino acids were determined in gas-liquid chromatography with and without silylation (121). Quantitative yields were calculated from PTH-amino acid standards run under the same conditions.

Control mixtures containing known ratios of pepsinogen and pepsin were prepared from the pure proteins and the second residues were quantitatively determined to establish the accuracy of this method of quantification. The ratio of PTH-valine to PTH-glycine was taken as the ratio of pepsinogen to pepsin in the sample. The determinations were accurate to within 3%.

COOH-Terminal Analysis

One milligram samples of the activation products, which had been isolated for quantitative amino-terminal analysis, were subjected to carboxypeptidase A digestion at 37^0C for 5 hours (122). After lyophilization, the free amino acids were analyzed in a Beckman 120B analyzer.

Difference Spectra

Initially, the procedure of McPhie (19) was followed. At room temperature a cuvette containing 2 ml of 20 μM pepsinogen in 2mM Tris chloride, pH 7.5, was adjusted to pH 2.85 by the addition of a previously determined quantity of 1 N perchloric acid. After an elapsed time of less than 2 minutes, the solution was raised to pH 7.3 by the addition of 1 M Tris. The difference spectra, with 20 μM pepsinogen in 2 mM Tris chloride, pH 7.5, as a reference, were recorded on a Cary model 15 spectrophotometer using the 0 to 0.1 absorbance scale.

In later experiments, 0.24 M HCl acid instead of 1 N perchloric acid was used to lower the pH in the reaction cuvette. Also, 0.24 M HCl acid and 1 M Tris were added to the reference cuvette in the same amounts as added to the reaction cuvette but in reverse order. For studies at pH 8.5 an appropriate amount of 1 M Tris was added to the reaction and reference cuvettes to raise the pH to 8.5.

All pH measurements were made with a Radiometer pH meter 26 equipped with a GK2311C combination electrode. In the above experiments, after raising the pH of the reaction cuvette, the difference spectra were recorded at various times. The length of time required to record a difference spectrum from 260 nm to 320 nm was about 30 seconds.

In a control experiment, 0.5 mg/ml and 1.0 mg/ml pepsinogen solutions were activated at pH 2.85 for 1.5 minutes. The change in absorbance at 287 nm during incubation at pH 8.5 of the 1.0 mg/ml solution was twice the change in absorbance of the 0.5 mg/ml solution at all incubation times up to one hour. Apparently, the pepsin concentration after activation was directly proportional to the change

in absorbance at 287 nm during incubation at pH 8.5.

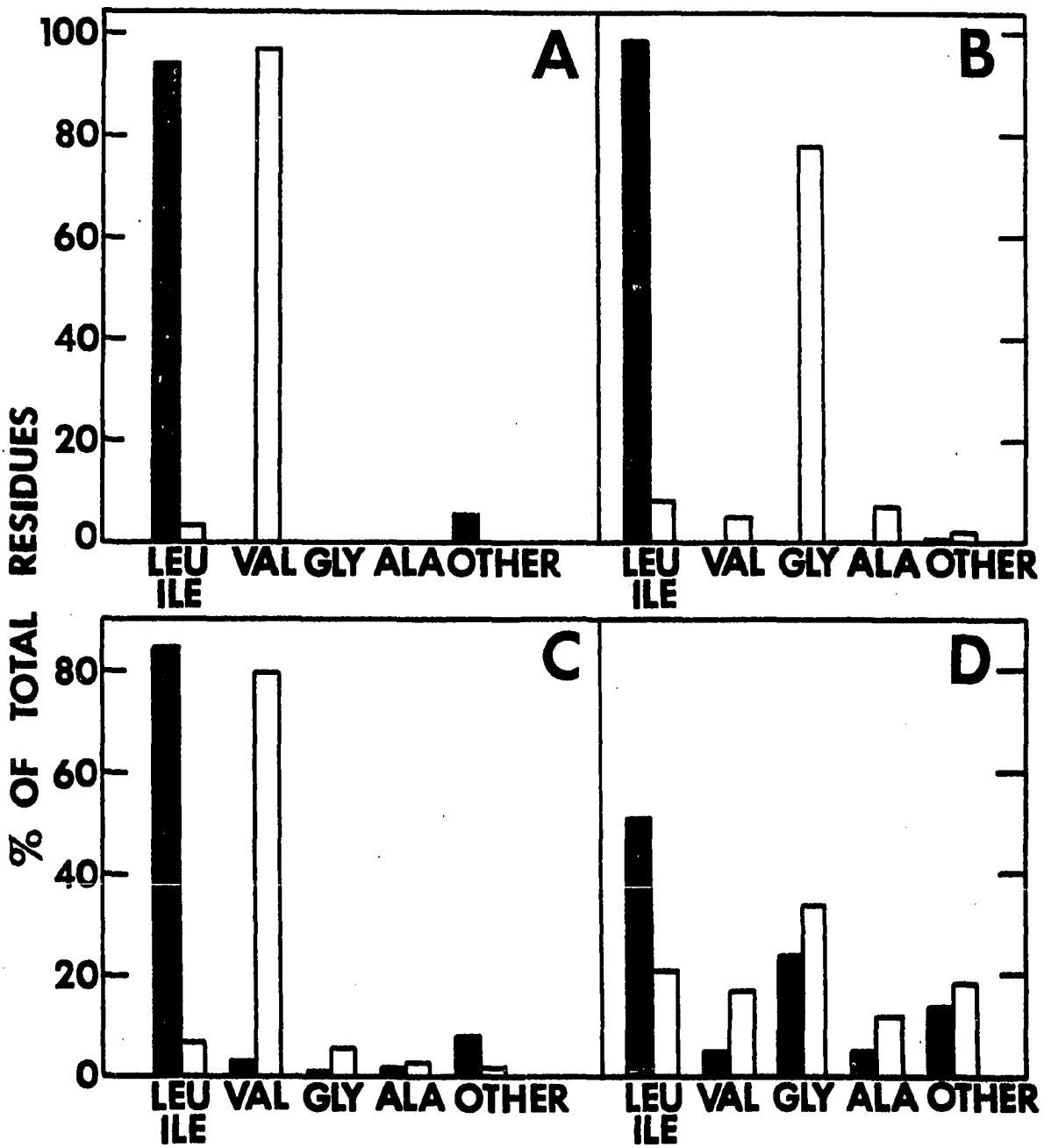
Results

Quantitative measurement of the NH_2-Terminal Two Residues in the Peptide-free Activation Products

Figure 5 summarizes the quantitative measurement of the first two residues of pepsinogen (Worthington, PGC), of pepsin prepared by the method of Rajagopalan et al. (22), and of the peptide-free reaction products after pepsinogen activation for 30 seconds and 1.5 minutes at pH 3.2. The first two residues of pepsinogen, leucine and valine (11), were determined without significant contamination (Fig. 5,a). Complete activation of pepsinogen for 20 minutes at pH 2.2 (22) produced pepsin with the predominant NH_2-terminal sequence of Ile-Gly- (Fig. 5,b) as expected (72, 123, 124). The completeness of activation under these conditions is indicated by the small amount of PTH-valine found in the second residue. A separate experiment, not shown in Fig. 5, in which activation at pH 2.2 was carried out for only 1.5 minutes produced relative amounts of the NH_2-terminal residues similar to those shown in Fig. 5,b (the 1.5 minute activation data are shown in Table 5). Activation at pH 3.2 (Fig. 5,c and 5,d) produced much more heterogeneity in the NH_2-terminal two residues of pepsin than was observed in the pH 2.2 activation experiments.

Table 5 lists the percentage of PTH-amino acids attributable to pepsinogen, pepsin with NH_2-terminal sequence Ile-Gly-, and other activation products in pH 2.2 and pH 3.2 activation mixtures. Presumably the other activation products are pepsin molecules with other

FIGURE 5: NH_2-TERMINAL TWO RESIDUES OF PEPSINOGEN ACTIVATION PRODUCTS AFTER PEPTIDES HAVE BEEN REMOVED. Solid bars, first residue; open bars, second residue. A, Pepsinogen Worthington PGC; B, Pepsin from pepsinogen activated for 20 minutes, pH 2.2, 14°C (22); C, Pepsinogen activated for 30 seconds at pH 3.2; D, Pepsinogen activated for 1.5 minutes at pH 3.2. Leucine and isoleucine were not distinguishable (see Methods).


A
B
C
D
% OF TOTAL RESIDUES
100
80
60
40
20
0
LEU ILE
VAL
GLY
ALA
OTHER

NH_2-termini. The peptide products had been removed as described above. The expected percentages of pepsinogen, Ile-Gly- pepsin[2] and other products are tabulated for comparison. These calculated values were derived from the rate constants[3] k_1 = 0.3 min^{-1} and k_2 = 2.5 mg^{-1} min^{-1} for activation at pH 3.2 and k_1 = 2.1 min^{-1} and k_2 = 1.6 mg^{-1} min^{-1} for activation at pH 2.2 (23). It was assumed in these calculations that first order activation yields a specific pepsin product with the NH_2-terminal sequence Ile-Gly-, whereas second order activation yields a variety of pepsin species. The assumption is confirmed by the fact that the observed yields of second glycine shown in Table 5 agree with the amount expected from first order activation.

COOH-Terminal Analysis

Carboxyl-terminal analysis of reaction products from 1.5 minute activation at pH 2.2, 30 second activation at pH 3.2, and 15 minute activation at pH 4.0 suggests increasing heterogeneity of the COOH-terminal residues as the reaction is shifted from a predominantly first order activation mechanism at pH 2.2 to a predominantly second order activation mechanism at pH 4.0. The ratios for the COOH-terminal amino acids relative to alanine as 1.0 are: valine, 0.34, leucine, 0.28, for 1.5 minute activation at pH 2.2; valine 0.52, leucine, 0.38, tyrosine, 0.37, phenylalanine, 0.29, for 30 second activation at pH 3.2; and valine, 1.0, isoleucine, 0.20, leucine, 1.51, tyrosine, 0.76,

---

[2]Abbreviation: Ile-Gly-pepsin, a pepsin molecule with NH_2-terminal sequence Ile-Gly-.

[3]k_1 is the rate constant for unimolecular activation, and k_2 is the rate constant for bimolecular, pepsin catalyzed, activation.

TABLE 5

PERCENTAGES OF ACTIVATION PRODUCTS

Activation Conditions	30 sec at pH 3.2		1.5 min at pH 3.2		1.5 min at pH 2.2	
Quantification of Products	Obs %	Calc %	Obs %	Calc %	Obs %	Calc %
Pepsinogen	80.0	75.3	17.3	12.4	7.7	7.2
Ile-Gly- pepsin	6.4	13.4	34.4	25.3	79.7	76.3
Other pepsins	13.6	11.3	48.4	62.3	12.6	16.5

Observed percents, obs. %, were derived from the sequence analysis of the first two NH_2-terminal residues of the activation products assuming Leu-Val- to be the first two residues of pepsinogen, Ile-Gly- to be the first two residues of pepsin from a first order activation mechanism, and other NH_2-terminal residues to be from pepsin formed by a second order activation mechanism. Calculated percents, calc. %, were derived from first and second order rate constants previously reported (23).

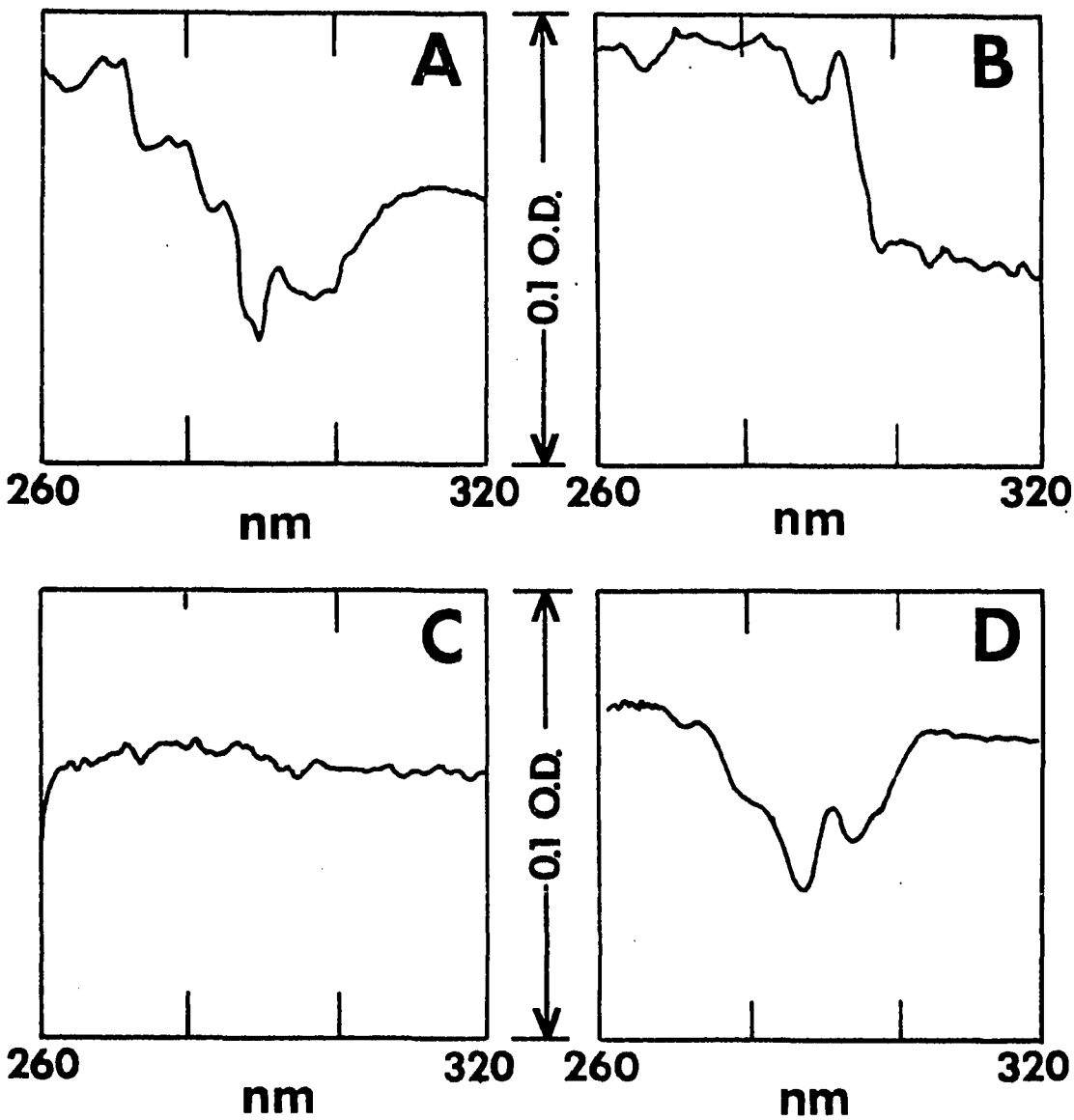
phenylalanine, 0.90, for 15 minute activation at pH 4.0. The expected COOH-terminal sequence of both pepsin and pepsinogen is -Pro-Val-Ala (125-128). Carboxypeptidase A digestion would release only alanine and valine. The unexpected carboxy-termini must result from internal cleavage due to peptic autodigestion which appears to be more prominent under conditions which favor second order activation. Autodigestion is supported by the fact that hydrophobic residues, susceptible to peptic cleavage, appear conspicuously in the COOH-terminal analysis.

Although the existence of internal cleavage points may complicate the quantitative evaluation of the NH_2-terminal sequences (Table 5), several facts support the conclusion that the NH_2-terminal data can be used as a measure of pepsin formation. First, the pH 2.2 activation products contain only a very small amount of the unexpected NH_2- and COOH-terminal residues. Second, in the pH 3.2 activation products, the amount of pepsinogen plus pepsin with an NH_2-terminal Ile-Gly- sequence should be represented in the yields of NH_2-terminal PTH-leucine plus PTH-isoleucine and of second residue PTH-valine plus PTH-glycine. In the 1.5 minute activation experiment at pH 3.2 the combined yields, 51.2% for the first NH_2-terminal residues and 51.6% for the second residues, agree well. Third, COOH-terminal data are a collection of free amino acids from continuous carboxypeptidase A digestion; so it is not possible to quantify their origin.

Difference Spectra

Figure 6 shows four difference spectra which were observed during 1.5 minute pepsinogen activation at pH 2.85 and during the

FIGURE 6: DIFFERENCE SPECTRA OF PEPSINOGEN SOLUTIONS. A, Sample, 20 μM pepsinogen during 1.5 minute activation at pH 2.85; reference, 20 μM pepsinogen, pH 7.5. B, Sample, 20 μM pepsinogen immediately following neutralization to pH 7.3 after 1.5 minute activation at pH 2.85; reference, 20 μM pepsinogen, pH 7.5. C, Same conditions as B but 1 minute after neutralization of sample to pH 7.3. D, Sample, 20 μM pepsinogen incubated 12 minutes at pH 8.5 after 1.5 minute activation at pH 2.85; reference, 20 μM pepsinogen, pH 8.5.


A
B
C
D
0.1 O.D.
0.1 O.D.
260
320
nm
260
320
nm
260
320
nm
260
320
nm

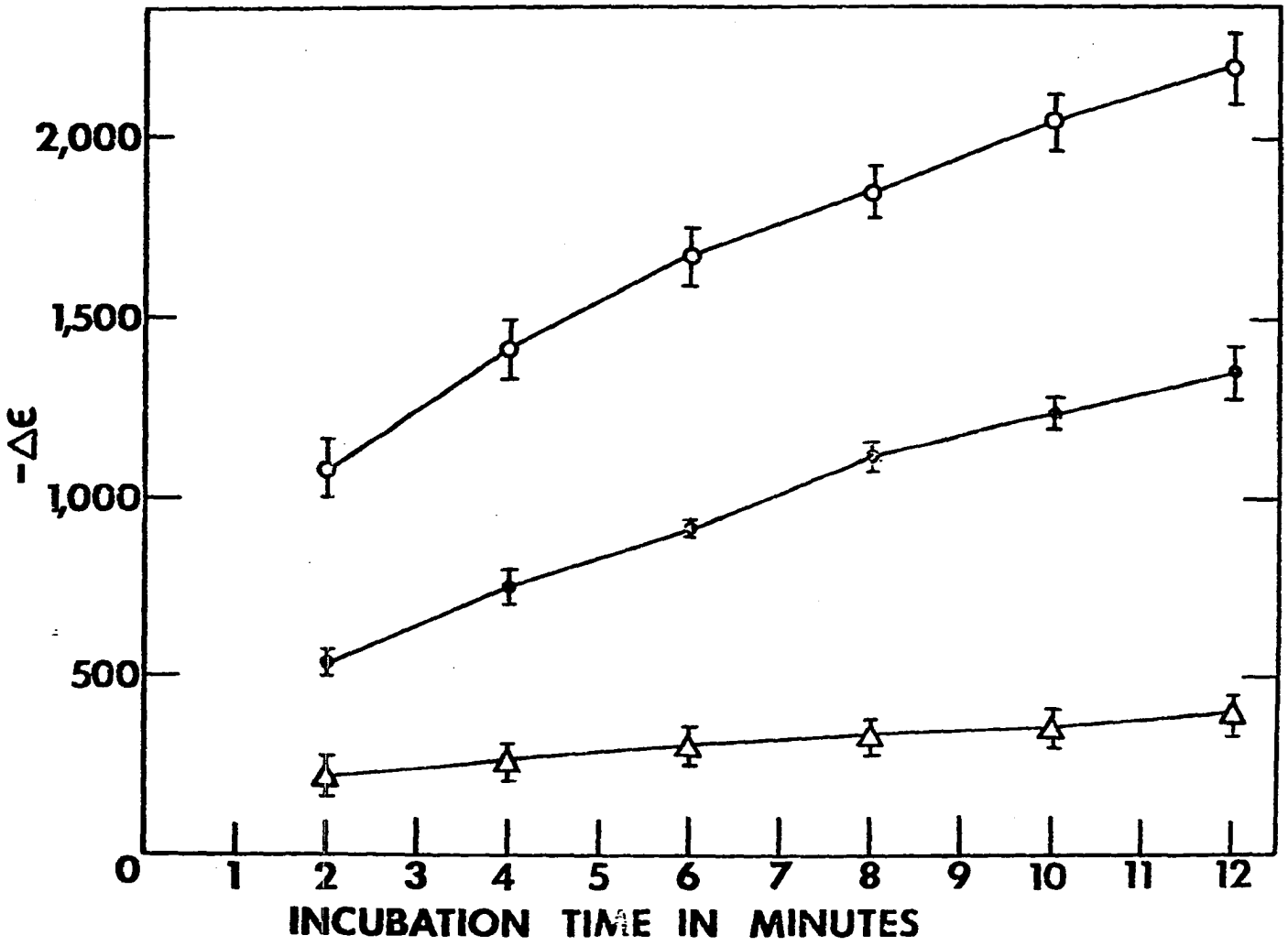
subsequent incubation in neutral and then alkaline solutions. The difference spectrum at pH 2.85 (Fig. 6,a) was very similar to that of pepsin versus pepsinogen. An interpretation of this spectrum as it relates to the absorbance of aromatic residues in pepsinogen has been discussed (19). After neutralization of the acidic pepsinogen solution, an intermediate phase (Fig. 6,b) and then subsequent disappearance of the difference spectrum (Fig. 6,c) were observed. This confirms in general the results of McPhie (19). If the 1.5 minute-activated pepsinogen solution was taken to pH 8.5, the disappearance of the difference spectrum was only temporary. After incubation at pH 8.5 a difference spectrum (Fig. 6,d), similar to that of the acidified pepsinogen solution, reappeared. Moreover, it was found that the regeneration of the difference spectrum also took place when the activated pepsinogen solution was maintained at pH 7.3, but the rate of regeneration was about ten times slower than at pH 8.5.

Rate of Reappearance of the Difference Spectra

Pepsinogen solutions were acidified to pH 2.85 for 0.5, 1 and 1.5 minutes and the pH of each solution was then raised to 8.5. The rate of reappearance of the difference spectrum was measured by observing the change in abosrbance ($\Delta\varepsilon$) at 287 nm. The results in Fig. 7 show that both the rate of appearance of the difference spectra and the maximum attained difference are dependent on the activation time.

Since the values of $\Delta\varepsilon$ reflected the amount of pepsin formed during activation, the pepsin concentration and the rate of pepsinogen activation could be calculated from the data in Fig. 7. This calcula-

FIGURE 7: ABSORBANCE CHANGES AT 287 nm of 20 μM PEPSINOGEN INCUBATED AT pH 8.5 AFTER ACTIVATION AT pH 2.85. Δ, 30 second activation; ●, 1 minute activation; 0, 1.5 minute activation; $\Delta\varepsilon$ is the absorbance difference (units $M^{-1}cm^{-1}$); reference, 20 μM pepsinogen, pH 8.5. Each point represents three independent experiments ± SEM.


2,000
1,500
1,000
500
0
-Δε
1
2
3
4
5
6
7
8
9
10
11
12
INCUBATION TIME IN MINUTES

tion, for the first order activation rate constant at pH 2.85, is described in the following section. Earlier, inactivation of pepsin at pH 8.5 was shown to reach completion within 12 minutes (23); this time course is consistent with the development of the difference spectra.

Calculation of the First Order Pepsinogen Activation Rate Constant

The integrated first order activation equation is

$$\ln \frac{\{Pgn\}}{\{Pgn_0\}} = k_1 t$$

where {Pgn} is the pepsinogen concentration at time t, $\{Pgn_0\}$ is the initial pepsinogen concentration, and k_1 is the first order rate constant.

If the magnitude of the difference spectrum at 287 nm, which develops during incubation at pH 8.5, is a direct measure of the amount of pepsin in a partially activated pepsinogen solution then

$$\{Pgn_0\} \propto \Delta\varepsilon_{max}$$

$$\text{and } \{Pgn\} \propto \Delta\varepsilon_{max} - \Delta\varepsilon_t$$

where $\Delta\varepsilon_{max}$ is the maximum observable difference at 287 nm after 100% pepsinogen activation and $\Delta\varepsilon_t$ is the maximum observable difference after activation for time t. If $\Delta\varepsilon_{t1}/\Delta\varepsilon_{t2}$ is constant for any pH 8.5 incubation time, then the spectral change may be observed after any period of incubation time as well as after a maximal spectral change has occurred. The ratios among $\Delta\varepsilon_t$'s for the different activation experiments at six different times of incubation are tabulated in Table 6. The data in this table support the hypothesis that these ratios are constant. The

TABLE 6

RATIOS OF THE ABSORBANCE CHANGES AT 287 nm
OF PEPSINOGEN INCUBATED AT pH 8.5 FOR
VARIOUS TIMES AFTER ACTIVATION AT
pH 2.85

Incubation time at pH 8.5 (minutes)	ΔεRatios					
	B/A		C/A		C/B	
2	2.48	(1.81-3.62)	5.00	(3.60-7.34)	2.01	(1.76-2.31)
4	2.90	(2.23-3.92)	5.46	(4.24-7.33)	1.88	(1.66-2.14)
6	3.06	(2.56-3.77)	5.58	(4.52-7.09)	1.83	(1.70-1.95)
8	3.44	(2.87-4.22)	5.69	(4.72-7.04)	1.66	(1.55-1.77)
10	3.52	(2.96-4.29)	5.86	(4.90-7.14)	1.66	(1.54-1.79)
12	3.45	(2.87-4.18)	5.62	(4.75-6.73)	1.63	(1.47-1.80)
ave.	3.14		5.54		1.78	

A, Δε due to 30 second activation. B, Δε due to 1 minute activation. C, Δε due to 1.5 minute activation. Reference 20 μM pepsinogen, pH 8.5. These ratios are calculated from the data displayed in Figure 7. The range is enclosed in parentheses.

amount of pepsin formed was therefore calculated from the $\Delta\varepsilon_t$ after 10 minute incubation at pH 8.5. The average change in absorbance at 287 nm after 8, 10 and 12 minute activation and 10 minute incubation at pH 8.5 was taken as $\Delta\varepsilon_{max}$ (4334±261 M^{-1} cm^{-1}).

The integrated first order rate equation which is shown above can be rewritten as

$$\ln\left(\frac{\Delta\varepsilon_{max}-\Delta\varepsilon_t}{\Delta\varepsilon_{max}}\right) = k_1 t$$

Fig. 8 shows a plot of $\ln\left(\frac{\Delta\varepsilon_{max}-\Delta\varepsilon_t}{\Delta\varepsilon_{max}}\right)$ versus t. The k_1 derived from the slope of the least squares line through these points is 0.44 min^{-1} and that line has an intercept which corresponds to 10% error in the initial value of $\frac{\Delta\varepsilon_{max}-\Delta\varepsilon_t}{\Delta\varepsilon_{max}}$. Earlier studies had determined a first order rate constant of 0.5 min^{-} at pH 2.85 (23).

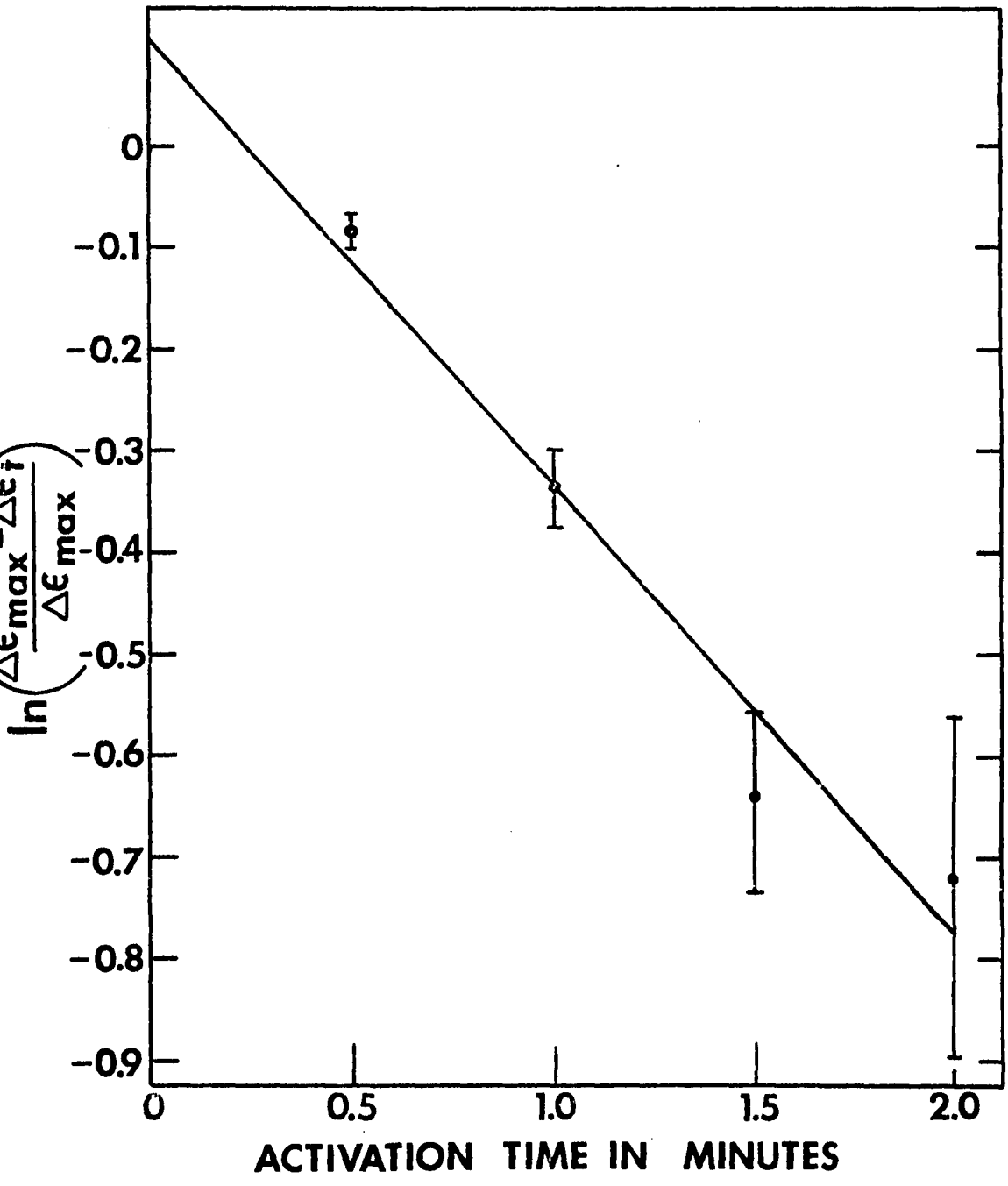
Discussion

The activation of pepsinogen is apparently achieved by the initial cleavage of a peptide bond followed by a very rapid development of proteolytic activity. This conclusion is supported by the agreement between the reported activation rate constant (0.5 min^{-1}) (23) and the rate constant determined spectrally in this study (0.44 min^{-1}) as well as by the agreement between the calculated per cent of pepsinogen activation and the per cent of pepsinogen activation observed in the NH_2-terminal analysis. The data does not support the hypothesis of intermolecular proteolytic activity of the zymogen after a conformational change (27).

The intramolecular cleavage of porcine pepsinogen apparently produced pepsin with the NH_2-terminal sequence Ile-Gly-. The quantifi-

FIGURE 8: PLOT OF $\ln\left(\frac{\Delta\varepsilon max-\Delta\varepsilon_t}{\Delta\varepsilon max}\right)$ VERSUS ACTIVATION TIME, t.

$\Delta\varepsilon_{max}$ is average absorbance difference measured at 287 nm of 20 μM pepsinogen activated at pH 2.85 for 8, 10 and 12 minutes measured 10 minutes after the pH was raised to 8.5. $\Delta\varepsilon_{max}$=4334±261 $M^{-1}cm^{-1}$. $\Delta\varepsilon_t$ is the average absorbance difference of 20 μM pepsinogen activated for time t, measured 10 minutes after the pH was raised to 8.5. Reference, 20 μM pepsinogen, pH 8.5. The solid line is a least squares fit of the data. The slope of this line implies a k_1 of 0.44 min^{-1}. The intercept corresponds to a 10% error in the initial value of $\frac{\Delta\varepsilon_{max}-\Delta\varepsilon_t}{\Delta\varepsilon_{max}}$.


0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
$\ln\left(\frac{\Delta\epsilon_{max}-\Delta\epsilon_i}{\Delta\epsilon_{max}}\right)$
0
0.5
1.0
1.5
2.0
ACTIVATION TIME IN MINUTES

cation of the yield of these two residues, under activation conditions which favor the unimolecular reaction, agrees well with the amounts predicted from the preciously determined activation rate constants (23). This tramolecular activation site must be located between residues 44 and 45 in the pepsinogen molecule (12,13). Intuitively, it seems that a unimolecular activation mechanism, if it is due to a single reaction and kinetically fits a first order treatment, would produce a homogeneous reaction product. An activation system which favors the first order reaction does produce a homogeneous pepsin with a single amino terminus (22,24). However, in the bimolecular activation reaction, particularly in view of the wide specificity of pepsin (34,129,) there is no mechanistic reason why the pepsin product must have a homogeneous amino terminus. The results shown in Table 5 were calculated assuming that unimolecular activation produced a single NH_2-terminal sequence Ile-Gly- and that bimolecular activation produced a heterogeneous NH_2-terminal sequence but not Ile-Gly-. Ile-Gly- could result from bimolecular activation, but a peptide bond involving isoleucine is not favored by pepsin (34, 129) and thus bimolecular cleavage at this site (between residues 44 and 45 of pepsinogen) is probably insignificant.

Autodigestion could play a role in producing heterogeneous amino and carboxyl-termini (22). It has been argued in the Results section that the amount of COOH-terminal heterogeneity which was observed was not sufficient to invalidate the conclusion that activation produces a homogeneous Ile-Gly-pepsin.

Considering the results of McPhie (19), the temporary disappearance of the difference spectrum between an activated pepsinogen solution and a native pepsinogen solution, as shown in Fig. 7, is best explained by the association of the basic activation peptide with pepsin. Strong binding of this peptide to pepsin, as demonstrated by its ability to inhibit peptic activity has been shown to occur (4,130). The following scheme summarizes an interpretation of the processes which take place when a pepsinogen solution is made acidic and then quickly returned to neutral pH.

Native Pepsinogen

↓ pH 2.85

Pepsinogen + pepsin + peptides

↓ pH 7.3

Pepsinogen + (pepsin · peptides)[4]

↓ pH 8.5

Pepsinogen + denatured pepsin + peptides

Pepsinogen is activated either intramolecularly or intermolecularly (pepsin catalyzed). If the activation mixture is neutralized quickly, the activation products, pepsin and peptides, associate to form a complex with a conformation similar to that of pepsinogen. However, during alkaline incubation, a pH dependent destruction of this complex occurs so that a difference spectrum is generated.

---

[4]A pepsin-peptide complex that resembles native pepsinogen spectrophotometrically.

CHAPTER III

PEPSIN ACTIVITY: IONIZATION BEHAVIOR OF CARBOXYL GROUPS MODIFIED BY DIAZO ACETYL-DL-NORLEUCINE METHYL ESTER

Materials

Pepsinogen

Pepsinogen (lots 1IA and 1JA) used in the preparation of pepsin according to the procedure of Rajagopalan, et al (22) was obtained from Worthington Biochemical Corporation.

Glycyl-DL-Norleucine

Glycyl-DL-norleucine (lots ZZ1055 and ZZ1200) used in the preparation of diazoacetyl-DL-norleucine methyl ester[5] by the procedure of Rajagopalan, et al (75) was obtained from Schwarz-Mann. Glycyl-DL-norleucine (lot 7410) used in the preparation of diazoacetyl-DL-norleucine ^{14}C-methyl ester was obtained from Nutritional Biochemicals Corporation.

---

[5]For preparation of glycyl-DL-norleucine methyl ester, see appendix.

^{14}C-Methanol

^{14}C-methanol, used in the preparation of diazoacetyl-DL-norleucine ^{14}C-methyl ester by the general procedure of Rajagapolan, et al (75), was purchased from New England Nuclear Corporation.

Chromatography Resins

SP-Sephadex C-25 and Sephadex G-25 were from Pharmacia. Chelex 100 was obtained from Bio-rad Laboratories.

Carbonate-Free KOH

Carbonate-free KOH (Dilut-it) was purchased from J. T. Baker Chemical Company.

Filtration Membranes

Diaflo ultrafiltration membranes were purchased from the Amicon Corporation. Filters of 0.45μ pore size were obtained from Millipore Filter Corporation.

Reagents

Other reagents were obtained from commercial sources and were of the highest purity available.

Methods

Preparation of Homogeneous Pepsin

Crystalline pepsinogen was converted to pepsin by the procedure of Rajagopalan, Moore and Stein (22). The pepsin produced was essentially homogeneous as determined by analysis of the first two amino-terminal residues (see Methods and Results in Chapter II).

Proteolytic Activity

The proteolytic activity was measured with acid denatured hemoglobin as substrate. The procedure was essentially that of Anson and Mirsky (131) modified by Tang (132).

Preparation of Pepsin Inactivated by Diazoacetyl-DL-Norleucine Methyl Ester

Homogeneous pepsin was inactivated with diazoacetyl-DL-norleucine methyl ester[6] according to the procedure of Rajagapolan, Stein and Moore (75), with cupric chloride instead of cupric acetate in the reaction mixture. The reaction mixture was passed through a Sephadex G-25 column (equilibrated with H_2O adjusted to pH 5.0 with HCl) to remove residual reagents and salt. Since Cu++ binds to pepsin (79), a suspension of Chelex 100 (which had been converted to the potassium form, washed with glass distilled water, and adjusted to pH 5.0 with HCl) was added to the pooled, desalted protein peak from the Sephadex G-25 elution to remove cupric ions still bound to the protein. The pH of the protein solution, in the presence of Chelex 100, gradually increased, therefore, 0.1 M HCl was added at intervals during a 5 minute incubation period to maintain the pH between 4.5 and 5.5. It was necessary to maintain the pH below 6.0 to avoid any alkaline denaturation of the protein. The mixture was then filtered through a 0.45μ millipore filter and the filtrate was dialyzed against H_2O, pH 5.0.

---

[6]Pepsin inactivated by diazoacetyl-DL-norleucine methyl ester will be abbreviated norleu-pepsin.

In other experiments, the pepsin and reagents were passed through a Chelex 100 column equilibrated with 0.05 M potassium acetate, pH 5.0, to remove the Cu++ before desalting on Sephadex G-25.

Atomic absorption analysis for Cu++ in the final product showed less than one Cu++ per 50 protein molecules. Amino acid analysis gave 1.2 norleucines incorporated per pepsin molecule, and proteolytic activity measured with hemoglobin as substrate was essentially zero.

Inactivation of Pepsin by Diazoacetyl-DL-Norleucine ^{14}C-Methyl Ester

Pepsin was reacted with diazoacetyl-DL-norleucine ^{14}C-methyl ester by the procedure of Rajagapolan, Stein and Moore (75).

The protein solution was dialyzed with 0.05 M potassium acetate, pH 5.0, by diafiltration on PM 10 ultrafiltration membrane until the radioactivity of the dialysate was constant for successive measurements and close to background. The extent of incorporation of ^{14}C into the protein was determined from the specific radio activity of the inactivator and from the concentration of the inactivated pepsin.

Test for the Reversibility of Pepsin Titrations

To test if pepsin could be reversibly titrated, the activity of pepsin was measured with hemoglobin as a substrate before and after titration with HCl or KOH. One sample, after titration with HCl, was dialyzed against H_2O. A small amount of KOH was added to the dialysate

to raise the pH to 5.0. After dialysis, the activity was measured and the solution was retitrated with HCl.

Amino-Terminal Analysis after Titration

Quantitative analysis of the first two amino-terminal residues of pepsin and norleu-pepsin[7] after titration was carried out using automated Edman degradation and gas-liquid chromatography according to the procedure described in Chapter II.

Preparation of Proteins for pH Titrations

Homogeneous pepsin collected from SP-Sephadex C-25 column chromatography was desalted on Sephadex G-25 (equilibrated with H_2O, pH 5.0), and the pooled protein peak was dialyzed against H_2O (pH 5.0). Desalted pepsin and desalted pepsin inhibited with diazoacetyl-DL-norleucine methyl ester (norleu-pepsin) were concentrated to approximately 5 mg/ml using a ultrafiltration membrane PM 10 or UM 10. The concentrate was then dialyzed further against H_2O (pH 5.0) and finally against KCl (pH 5.0).

After dialysis, the two protein solutions were adjusted to the same concentration by matching the optical densities at 280 nm on a Zeiss spectrophotometer utilizing 1/2 cm cuvettes. Determination of the relative protein concentrations using the method of Lowry, et al (133) confirmed the reliability of the optical density measurements.

---

[7]Abbreviation: norleu-pepsin, pepsin inactivated with diazoacetyl-DL-norleucine methyl ester.

pH Titrations

A protein sample, 1.5 or 2 ml, was pipetted into a titration vessel (Radiometer, Type V521). The titration vessel was fitted on a titration assembly (Type TTA31) equipped with a thermostatted jacket (Type V526), a nitrogen gas inlet, a polyethylene titrant delivery tube, and a combined electrode (Type GK 2320 C). Nitrogen gas was bubbled through H_2O in a thermostatted vessel (Type V520) before delivery into the titration vessel. The titration assembly was equipped with a magnetic stirrer to ensure rapid mixing of the titrant with the protein solution. The temperature was maintained by a Hetotherm water circulator (Model 01 T 623) coupled with a Cold Finger portable immersion cooler.

The pH of the protein solutions was adjusted with 2 M carbonate-free KOH to slightly higher than the starting pH of the titration, and then the pH was lowered to the starting pH with HCl titrant. The solutions were allowed to equilibrate for 15 minutes; then titrant was added to the solutions by means of an autoburette (Type ABU 11b) equipped with a 0.25 ml burette assembly (Type B260). The volume of titrant could be measured to 0.1 µl. After a volume of titrant was added, the solutions were allowed to equilibrate before the addition of more titrant. The volume of titrant added to the solutions was measured as a function of pH. The pH was measured on the expanded scale of a Radiometer pH meter (Type PHM 26c) standardized with pH 7.0 and pH 2.0 buffers.

The volume of titrant added was measured at each 0.100 pH unit. The volume of titrant added was corrected for the titration of

the solvent by subtraction of solvent titration data from protein titration data. The corrected volume of titrant added to the protein solution was converted to moles H^+ added per mole pepsin.

Difference Titration Curves

A difference titration curve was determined by subtracting for each 0.100 pH unit the titration curves for the norleu-pepsin from the titration curves for the native pepsin.

Theoretical Difference Titration Curves

The average number of H^+ associated with each protein molecule can be theoretically calculated by

1. $$\bar{\nu} = n - \bar{r}$$

where $\bar{\nu}$ is the average number of H^+ associated with each protein molecule, n is the total number of binding sites, and $\bar{r}$ is the average number of H^+ dissociated from each protein molecule.

The average number of H^+ dissociated from each protein molecule is defined as

2. $$\bar{r} = \frac{\{H^+\}_d}{\{P\}_t}$$

where $\{H^+\}_d$ is the concentration of dissociated H^+ and $\{P\}_t$ is the total concentration of protein, P.

The dissociation constants for the dissociation of H^+ from the protein are defined as

$$K_1 = \frac{\{H^+\}\ \{PH_{n-1}\}}{\{PH_n\}}$$

3. $$K_2 = \frac{\{H^+\}\ \{PH_{n-2}\}}{\{PH_{n-1}\}}$$

$$K_n = \frac{\{H^+\} \quad \{P\}}{\{PH\}}$$

where n is the total number of H^+ binding sites.

The concentration of H^+ dissociated is described as follows

4. $\{H^+\}_d = \{PH_{n-1}\} + 2\{PH_{n-2}\} + \ldots + n\{P\}$

The total concentration of protein is given by

5. $\{P\}_t = \{PH_n\} + \{PH_{n-1}\} + \{PH_{n-2}\} + \ldots + \{P\}$

From rearrangement of equation 3,

6. $$\{PH_{n-1}\} = \frac{K_1\{PH_n\}}{\{H^+\}}$$

$$\{PH_{n-2}\} = \frac{K_2\{PH_{n-1}\}}{\{H^+\}}$$

$$\{P\} = \frac{K_n\{PH\}}{\{H^+\}}$$

where K_1, K_2, ... , K_n are the dissociation constants for the polyvalent protein.

From equations 2, 4, 5 and 6, one can obtain a general expression for $\bar{r}$.

7. $$\bar{r} = \frac{K_1/\{H^+\} + 2K_1K_2/\{H^+\}^2 + \ldots + n\ K_1K_2 \ldots K_n/\{H^+\}^n}{1 + K_1/\{H^+\} + K_1K_2/\{H^+\}^2 + \ldots + K_1K_2 \ldots K_n/\{H^+\}^n}$$

After multiplying both the numerator and denominator of equation 7 by $\{H^+\}^n$, equation 7 can be transformed into

8. $$\bar{r} = \frac{G_1}{G_1 + \{H^+\}} + \frac{G_2}{G + \{H^+\}} + \ldots + \frac{G_n}{G_n + \{H^+\}}$$

where G_1, G_2, ..., G_n are titration constants related to K_1, K_2, ..., K_n by the following equations (134).

$$K_1 = G_1 + G_2 + \ldots + G_n$$

$$K_1K_2 = G_1G_2 + G_1G_3 + \ldots + G_1G_n + \ldots G_{n-1}G_n$$

9. $K_1K_2K_3 = G_1G_2G_3 + G_1G_2G_4 + \ldots G_{n-1}G_{n-2}G_n$

$$\text{---}\ K_1K_2 \ldots K_n = \text{---}\ G_1G_2 \ldots G_n$$

Equations 8 and 9 were derived by equating the denominator of equation 7 (after multiplication by $\{H^+\}^n$) with the expansion of the polynomial

10. $(G_1 + \{H^+\})\ (G_2 + \{H^+\}) \ldots (G_n + \{H^+\})$

The above transformation has been described by von Muralt (134).

Equation 8 can also be derived by describing the polyvalent protein as a mixture of monovalent acids in equal quantities. The dissociation constants are defined as

$$G_1 = \frac{\{H^+\}\ \{A_1\}}{\{A_1\ H\}}$$

$$G_2 = \frac{\{H^+\}\ \{A_2\}}{\{A_2H\}}$$

$$G_n = \frac{\{H^+\}\ \{A_n\}}{\{A_nH\}}$$

where G_1, G_2, ..., G_n are the dissociation constants for the monovalent acids A_1, A_2, ..., and respectively. AH is the protonated form and A is the deprotonated form.

The concentration of H^+ dissociated is given by

12. $\{H^+\}_d = \{A_1\} + \{A_2\} + \ldots + \{A_n\}$

Also, as described above,

$$\{P\}_t = \{A_1\} + \{A_1H\} = \{A_2\} + \{A_2H\}$$

$$= \ldots \{A_n\} + \{A_nH\}$$

Therefore, from equations 2, 12 and 13,

$$\bar{r} = \frac{\{A_1\} + \{A_2\} + \ldots + \{A_n\}}{\{A_1\} + \{A_1H\}}$$

$$14. \quad = \frac{\{A_1\}}{\{A_1\} + \{A_1H\}} + \frac{\{A_2\}}{\{A_2\} + \{A_2H\}} + \ldots + \frac{\{A_n\}}{\{A_n\} + \{A_nH\}}$$

Rearrangement of equation 11 gives

$$\{A_1\} = \frac{G_1\{A_1H\}}{\{H^+\}}$$

$$\{A_2\} = \frac{G_2\{A_2H\}}{\{H^+\}}$$

$$\{A_n\} = \frac{G_n\{A_nH\}}{\{H^+\}}$$

Substitution of equations 15 in equation 14, and multiplication of both numerator and denominator by H^+ gives

$$16. \quad \bar{r} = \frac{G_1}{G_1 + \{H^+\}} + \frac{G_2}{G_2 + \{H^+\}} + \ldots + \frac{G_n}{G_n + \{H^+\}}$$

which is identical to equation 8.

The relationships between K_1, K_2, ..., K_n and G_1, G_2, ..., G_n from equation 9 have been derived for a trivalent acid calculated as if there were three monovalent acids present in equivalent quantities by Simms (135). A discussion of the relationship between dissociation constants and titration constants for polyvalent acids can also be found in Edsall and Wyman (136).

The average number of H^+ associated with each protein molecule by equation 1 can be calculated by substituting equation 16 in equation 1, giving

$$17. \quad \bar{\nu} = n - \left(\frac{G_1}{G_1 + \{H^+\}} + \frac{G_2}{G_2 + \{H^+\}} + \ldots + \frac{G_n}{G_n + \{H^+\}} \right)$$

If one titratable group is modified in such a way that it can no longer be titrated, and if all other groups remain unaltered and their titration constants do not change, then the average difference between the association of H^+ with the unmodified protein and the

association of H^+ with the modified protein could be expressed as

$$18. \qquad \Delta\bar{\nu} = (n_n - n_d) - \left(\frac{G}{G + \{H^+\}}\right)$$

where n_n is the total number of binding sites in the native protein, n_d is the total number of binding sites in the modified protein, and G is the titration constant in the native protein of the group modified in the derivative. In this case, $(n_n - n_d)$ would equal 1 and G would equal the dissociation constant, K.

If the modification only shifts the titration constant of one group, then

$$19. \qquad \Delta\bar{\nu} = (n_n - n_d) - \left(\frac{G}{G + \{H^+\}} - \frac{G'}{G' + \{H^+\}}\right)$$

where G' is the titration constant of the shifted group in the derivative. In this case, $(n_n - n_d)$ would equal zero, and G would equal K in the native protein and G' would equal K' (the dissociation constant of the shifted group in the derivative).

If a modification of the protein removes one group and shifts the dissociation constant of another, then a combination of equations 18 and 19 gives

$$20. \qquad \Delta\bar{\nu} = (n_n - n_d) - \left(\frac{G_1}{G_1 + \{H^+\}} + \frac{G_2}{G_2 + \{H^+\}} - \frac{G'}{G' + \{H^+\}}\right)$$

where G_1 and G_2 are the titration constants of the groups in the native protein. A modification of this type has been suggested in the esterification of Asp-52 with a shift in the dissociation constant of Glu-35 in lysozyme (116-119).

The average difference between the association of H^+ with native pepsin and the association of H^+ with pepsin inhibited by

diazoacetyl-DL-norleucine methyl ester was fitted to an equation of the form

$$\Delta\bar{\nu} = 1 - \left(\frac{G_1}{G_1 + \{H^+\}} + \frac{G_2}{G_2 + \{H^+\}} + \frac{G_3}{G_3 + \{H^+\}} + \frac{G_4}{G_4 + \{H^+\}} - \frac{G_1'}{G_1' + \{H^+\}} - \frac{G_2'}{G_2' + \{H^+\}} - \frac{G_3'}{G_3' + \{H^+\}}\right) + 0.2 - 0.2\left(\frac{G_5}{G_5 + \{H^+\}} + \frac{G_6}{G_6 + \{H^+\}} - \frac{G_4'}{G_4' + \{H^+\}}\right) \quad 21.$$

Where G_1, G_2, ... G_6 are titration constants of native pepsin, and G_1', G_2', G_3' and G_4' are shifted titration constants in norleu-pepsin.

A theoretical difference titration curve was fitted to the experimental difference titration curve by guessing values for the titration constants until a close fit was achieved.

Calculation of Dissociation Constants

Dissociation constants for the ionization of groups modified by diazoacetyl-DL-norleucine methyl ester were calculated from titration constants which fit equation 21 to the experimental difference titration data. The relationship between the titration constants and the dissociation constants has been given in equation 9. The dissociation constants are defined in equation 3.

The dissociation constants for the groups in native pepsin were calculated from G_1, G_2, G_3 by equation 9. Since $G_1' << G_2' << G_3'$, then $K_1' = G_1'$, $K_2' = G_2'$ and $K_3' = G_3'$ where K_1', K_2', and K_3' are the shifted dissociation constants in norleu-pepsin. Both the titration constants and dissociation constants are not "intrinsic" constants, but may have been raised or lowered by electrostatic, hydrogen bonding, or hydrophobic effects (117).

Results

Incorporation of Inactivator into Pepsin

The incorpcration of inactivator was stoichiometric with respect to both norleucine incorporation as analyzed by amino-acid analysis (1.2/pepsin), and methyl ester incorporation as analyzed by ^{14}C radioactivity (0.95/pepsin).

Reversibility of Pepsin Titration

The titration of the pepsin with HCl did not significantly affect the activity of the pepsin. Reverse titration with KOH to pH 5.9 resulted in 50% loss of activity possibly due to alkaline denaturation in the immediate vicinity of the KOH delivery. Pepsin dialyzed against H_2O after titration with HCl did not lose any proteolytic activity. Retitration of the dialyzed protein with HCl resulted in the consumption of more HCl than in the first titration. The difference in HCl consumption could result from autodigestion during titration and/or dialysis. As described below, some autodigestion did occur during titration, but not enough to invalidate the results.

Analysis of Amino-Terminal Residues after Titration

Analysis of the first two amino-terminal residues of pepsin after titration showed approximately 2-5% phenylalanine as compared to glycine as the second amino-terminal residue. The 2-5% phenylalanine could result from autodigestion during titration. The auto-

digestion does not significantly effect the titration data. Norleu-pepsin had only glycine as the second amino-terminal residue.

Difference Titration Data

Table 7 lists the conditions under which native pepsin and norleu-pepsin were titrated.

The average difference titration between the moles of H^+ added to native pepsin minus the moles of H^+ added to norleu-pepsin, in units of moles H^+/mole pepsin, is shown in Figure 9. The theoretical volume of acid required to titrate one carboxyl group was calculated from the molarity of the enzyme solution obtained from its absorbancy at 280 nm (69) (molar extinction coefficient 44,000) and the normality of the titrant. Figure 9 shows a difference of greater than one carboxyl group between native and norleu-pepsin. The data suggest at least two carboxyl groups are not available for titration in norleu-pepsin compared to native pepsin in the pH range 5.9-2.4. The effects of temperature do not contribute significantly, within experimental error, to the difference titrations.

Theoretical Difference Titration Curves

Figure 10 shows the fit of equation 21 to the experimental difference titration data. The titration constants which gave a good fit are listed in Table 8.

In equation 21, two sites of inactivator binding are described; one is the active site and the other is a partially reactive site not necessarily involved in catalysis. Since 1.2 norleucines per pepsin are incorporated upon inactivation, apparently a second

TABLE 7

TITRATION CONDITIONS

Experiment	Protein Concentration mg/ml	Temperature	KCl, M	Titrant HCl, M	pH Range
1	3.5	23°C	0.40	0.01	5.9 - 3.2
2	3.4	23°C	0.40	0.01	5.9 - 3.2
3	4.9	14°C	0.50	0.04	5.4 - 2.4
4	4.9	14°C	0.50	0.04	5.4 - 2.4

FIGURE 9: DIFFERENCE TITRATION. The average difference titration between moles of H^+ added to native pepsin minus the moles of H^+ added to norleu-pepsin in units of Δ moles H^+ per mole pepsin; ●, difference titration, 14°C; O, difference titration, 23°C, ± range.

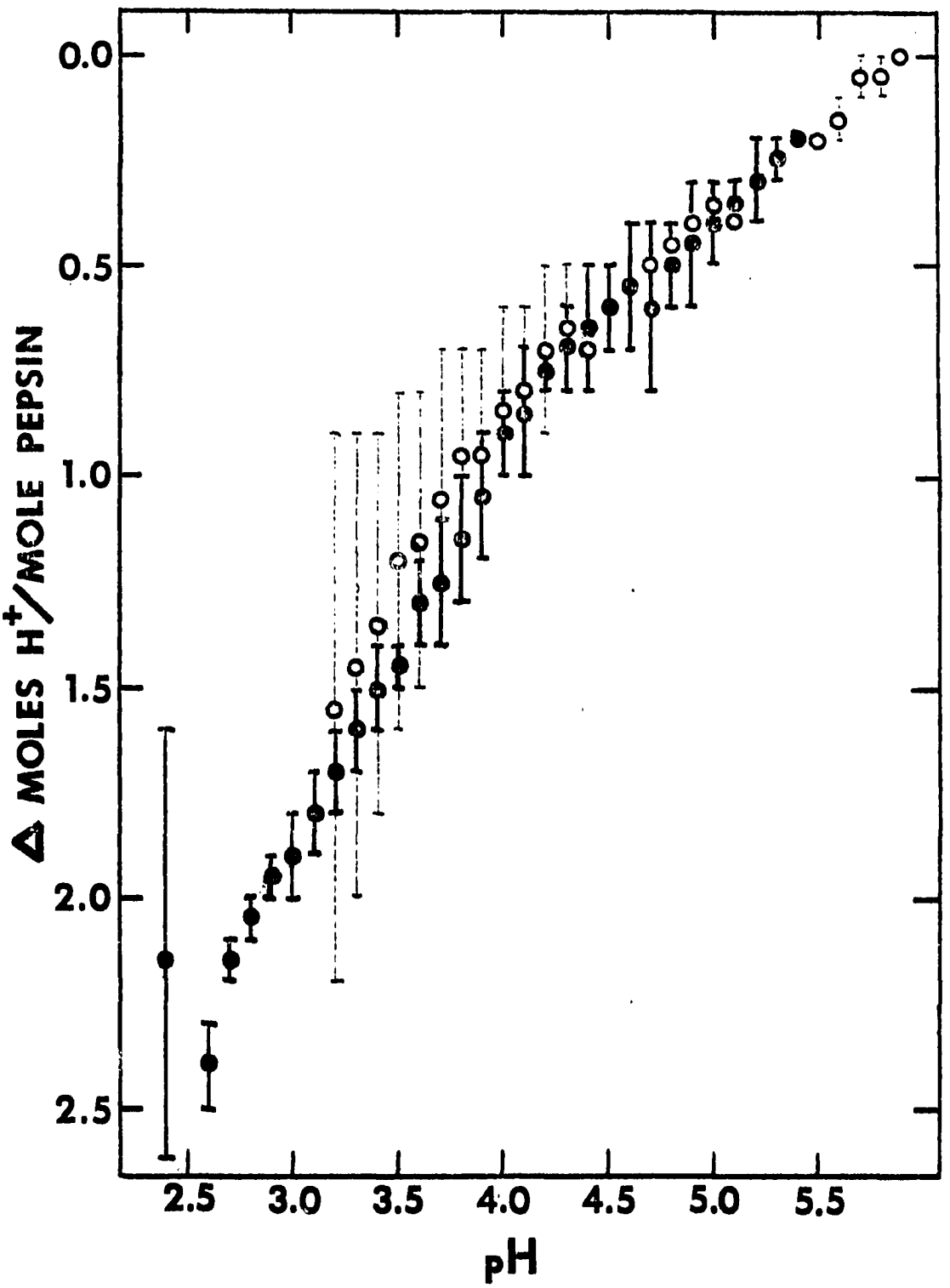

0.0
0.5
1.0
1.5
2.0
2.5
Δ MOLES H+/MOLE PEPSIN
2.5
3.0
3.5
4.0
4.5
5.0
5.5
pH

FIGURE 10: FIT OF EQUATION 21 TO DIFFERENCE TITRATION. Equation 21 was fitted to the difference titration data with titration constants listed in Table 8. The dashed line is the fit of equation 21 with G_4, G_3' , G_6, and G_4' equal to zero.

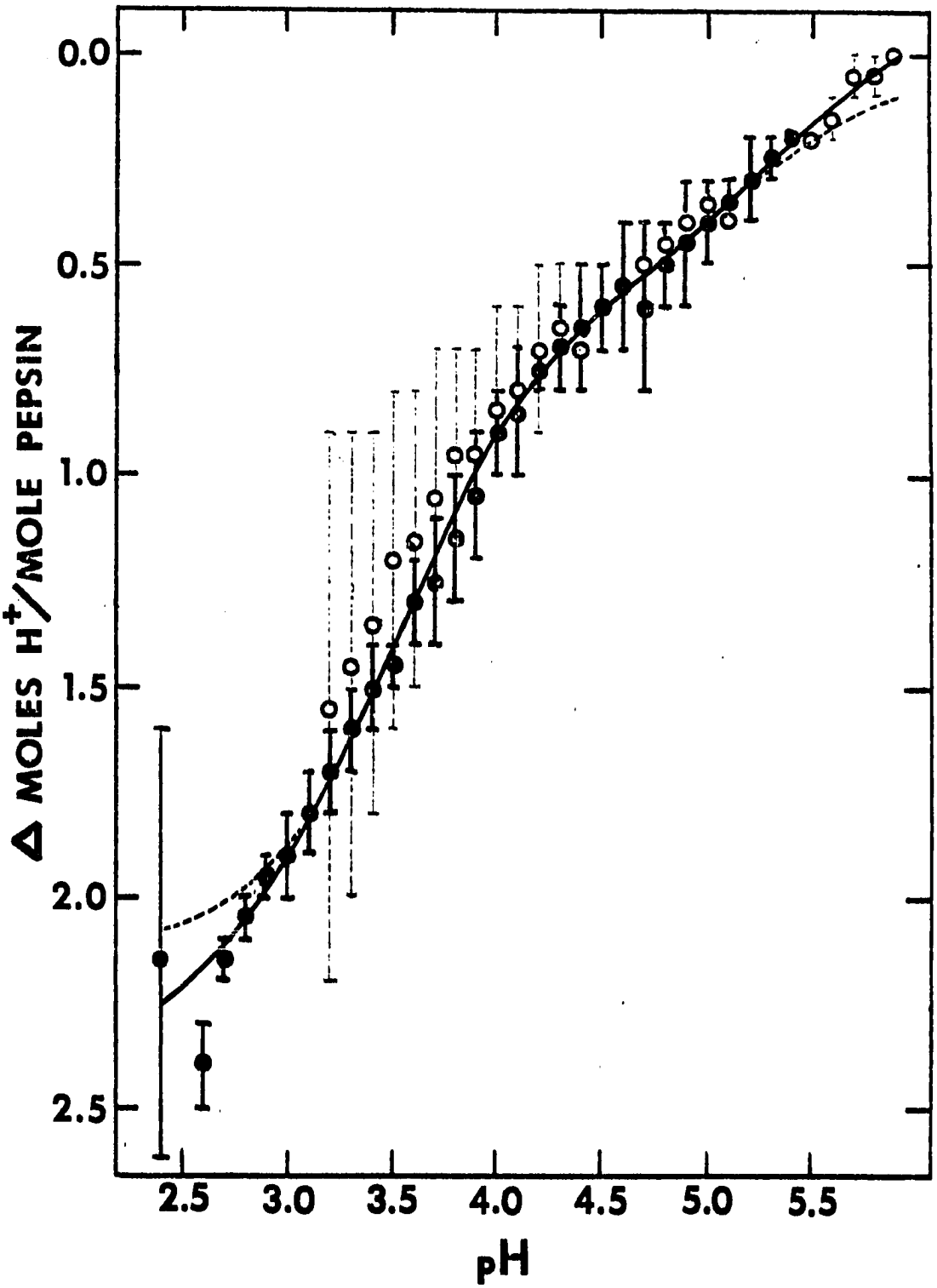

0.0
0.5
1.0
1.5
2.0
2.5
Δ MOLES H+/MOLE PEPSIN
2.5
3.0
3.5
4.0
4.5
5.0
5.5
pH

TABLE 8

TITRATION CONSTANTS DETERMINED

FOR THEORETICAL DIFFERENCE

TITRATION CURVE

		pG	Range
Active Site	pG_1	4.97	4.8 - 5.3
	pG_2	3.34	3.2 - 3.6
	pG_3	3.85	3.6 - 4.2
	pG_4	$\geq$6.5	----
	pG_1'	4.6	4.1 - 4.8
	pG_2'	$\leq$1.5	----
	pG_3'	$\geq$8.1	----
Second Site	pG_5	3.0	2.1 - 5.2
	pG_6	5.0	3.6 - 5.8
	pG_4'	$\leq$2.5	----

carboxyl is 20% esterified. The total difference in the consumption of acid during titration (approximately 2.4 equivalents) also suggests a second partially reactive carboxyl group.

If the secondary modification site does not involve a carboxyl group which shifts in norleu-pepsin in addition to the esterified group, then in equation 21, G_6 and G_4' are 0. In this case the theoretical curve does not fit the titration data below pH 3 as shown in Figure 10. Titration constants G_4 and G_3' were included in equation 21 to fit at the high pH range. The theoretical curve calculated with G_4 and G_4' equal to 0 is also shown in Figure 10.

The error limits in the titration constants used in the curve fitting were estimated by varying each titration constant derived from the best fit of equation 21 until the curve exceeded the limits of the 14^0C titration data. The range of these constants is given in Table 8.

Dissociation Constants

The dissociation constants for the ionization of groups at the active site of pepsin calculated (using equation 9) from the titration constants in Table 8 are listed in Table 9. The range was estimated by using the lower and upper limits of the titration constants in the calculations. As mentioned in the Methods section, the dissociation constants in the modified enzyme were equated to the titration constants. The secondary site was not included in these calculations.

Microconstants for the ionization of groups in pepsin were not calculated because the calculation required ab initio knowledge of

TABLE 9

DISSOCIATION CONSTANTS[a] OF GROUPS AT THE ACTIVE SITE OF PEPSIN CALCULATED FROM TITRATION CONSTANTS

	pK	Range
pK_1	3.22	3.05 - 3.43
pK_2	3.94	3.72 - 4.26
pK_3	5.01	4.84 - 5.34
pK'_1	4.6	4.1 - 4.8
pK'_2	≤ 1.5	----

a. derived from equation 9, and from $pK'_1 = pG'_1$, $pK'_2 = pG'_2$.

three microconstants. It might be reasonable to assign the microconstant for the ionization of the acid of highest pK, when the other acids are ionized, to be equal to K_3. The ionization of the shifted carboxyl in the derivative (K_1') could be assigned to one of the microionizations of the acid of intermediate pK in the presence of a protonated species for the acid of highest pK. These two relations are not sufficient to determine the other microconstants.

Discussion

In the titration of pepsin and pepsin inactivated with diazoacetyl-DL-norleucine methyl ester, two carboxyl groups are not titratable and one carboxyl group has a pK shift in the inactivated pepsin in the pH range 2.4 to 6.0. One carboxyl group not titratable is esterified by the inactivator. The origin of the other group not titratable is not certain; however, four possible explanations have been considered. First, autodigestion during titration might expose new carboxyl groups; however, analysis of the first two amino-terminal residues of pepsin and norleu-pepsin after titration suggests no significant autodigestion. Second, the additional carboxyl group could be attributed to Cu++ binding to carboxyl groups in norleu-pepsin, but atomic absorption analysis for Cu++ revealed an insignificant amount of bound Cu++. Third, another carboxyl group could be covalently bound to the inactivator. A covalent bond formed at the methyl ester bond of the inactivator would result in the loss of the methyl group, however, the incorporation of ^{14}C into pepsin upon inactivation rules out this possibility. It is possible that in the framework of the

three-dimensional structure of the pepsin active center, the acetyl α-carbon might form a diester with the second carboxyl group of the enzyme as well as with Asp-215. This diester would normally be very unstable, however, unless stabilized in some way by the tertiary structure of the protein. Attack by a second carboxyl group at other sites on the inactivator would probably result in the loss of the norleucine moiety. This loss is inconsistent with the amino acid analysis. Finally, the pK of the secord group may be shifted out of the titration range due to strong hydrogen bonding which would lower the pK, or due to burying of the carboxyl group which would make it inaccessable to solvent and raise its pK. The best curve fit includes a shift to a lower pK for this carboxyl group.

A slightly better fit of a theoretical titration curve to the data is achieved when a fourth group with pK $\geq$ 6.5 in pepsin and a pK $\geq$ 8.1 in norleu-pepsin is added to the difference titration equation (equation 21). The extra constants do not significantly alter the other constants in the equation. The pK of the fourth group is suggestive of either an imidazole group of a histidine side chain or an α-NH_3+ group (137,138). The amino-terminus of pepsin could be in close proximity to the active site if intramolecular activation of pepsinogen to pepsin is catalyzed by the pepsin active site (23). Upon binding of substrate or inactivator to pepsin, the environment and/or the conformation of the amino-terminus could change and cause the pK to shift. The amino-terminus does not participate directly in catalysis (3,139) but certainly might be near enough to affect the titration in the active site.

The pK_2 and pK_3 are consistent with the mechanism proposed by Lundblad and Stein (80) for the participation of carboxyl groups in the inactivation of pepsin by diazoacetyl-DL-norleucine methyl ester. The pK_2 can be identified with the ionization of the carboxyl that binds the copper carbene intermediate. They proposed that this carboxyl group has a pK near 4 as supported by synthetic substrate hydrolysis (43,46,51). The pK_3 can be identified with the ionization of the protonated carboxyl which forms the ester bond in the proposed inactivation mechanism. This carboxyl group does not necessarily participate directly in catalysis.

From the titration data, at least one carboxyl group has a shift in its pK. If pK_3 is identified with the esterified Asp-215 in norleu-pepsin, and if one assumes the smallest available shift, then pK_1' would be the shifted pK in norleu-pepsin of the carboxyl group identified with pK_2 in pepsin. The pK shift would be from 3.94 in pepsin to 4.6 in norleu-pepsin suggesting either a change in hydrogen bonding or a change to a somewhat more hydrophobic environment of this carboxyl group in norleu-pepsin. The pK_1 may be related to the ionization of a carboxyl group implicated in the reaction of pepsin with EPNP (70). The pK of this carboxyl has been determined to be 2.85 $\pm$ 0.5 (70) compared to $pK_1 = 3.22$ from difference titration data. If this carboxyl group is shifted in norleu-pepsin to the pK_2' of ≤ 1.5, then the titration data would be consistent with the pK's of functional groups calculated from the kinetics of synthetic substrate hydrolysis (Table 3, Chapter I). These kinetic studies conclude that at least two carboxyl groups are required for catalysis, one with a pK around 1 and

the other with a pK between 3 and 4 (43,46,51). Hollands and Fruton (55) have shown that the pH optimum of substrate hydrolysis can vary widely depending on the structure and charge of the substrate. The pH optimum for Z-His-Phe-Phe-OMe is about 4 (55). This pH optimum would suggest involvement in catalysis of a carboxyl group with a pK higher than 4. This could be identified with K_3 from the titration data. A carboxyl group implicated in the binding and hydrolysis of cationic substrates has an estimated pK of 3.8 (55). This pK is similar to pK_2 = 3.94 deduced from difference titration data. Studies with N-methyl-2-anilinonaphthalene-6- sulfonyl (mansyl) peptides as fluorescent probes for pepsin-substrate interaction have suggested the active site of pepsin has considerable conformational flexibility (66). The inactivation of pepsin by tosyl-L-phenylalanyldiazomethane also lowers the polarity of the mansylamide-binding site (66). The shift in pK's, as seen in the difference titration studies, could be due to a change in the environment of carboxyls in the active site of pepsin by inactivation with diazoacetyl-DL-norleucine methyl ester and is consistent with the above pepsin-substrate interaction studies (66).

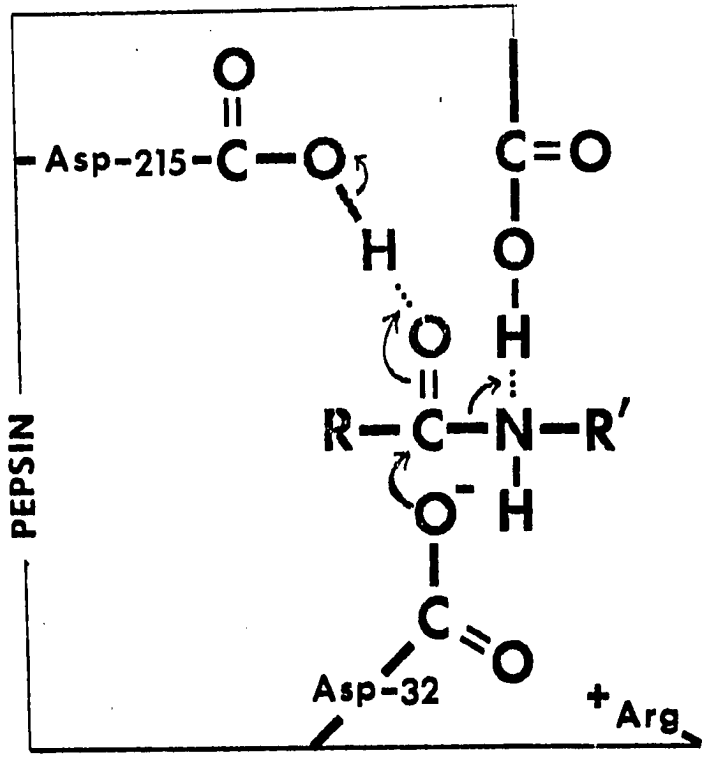
The ionization of a carboxyl group in the active site of pepsin identified with K_2 or K_3 from titration data is consistent with the proposed mechanisms of pepsin action deduced from synthetic substrate hydrolysis (68,110,111) as shown in Figures 1 and 2 of Chapter I. A pK of 4.6 for a catalytic group in the presence of substrate has been proposed by Clement, et al (68) which is similar to pK_i from titration data. Cornish-Bowden and Knowles (57) have also proposed a pK of 4.75 for a catalytic group in the presence of substrate.

A mechanism of pepsin action proposed by Hartsuck and Tang (70) is consistent with the pK's from titration data. In their mechanism, as shown in Figure 4 of Chapter I, the ionized carboxyl, presumably identified with a pK of about 3 is a nucleophile which attacks a polarized carbonyl bond. Proton donation from another carboxyl, presumably Asp-215, completes the scheme. Recently, however, Hunkapiller and Richards (65) proposed a mechanism where the carbonyl bond was polarized by a protonated carboxyl group rather than an arginine as proposed by Hartsuck and Tang (70). In this mechanism, three carboxyl groups are involved in catalysis. This mechanism is supported by the titration data.

Figure 11 shows a proposed mechanism of pepsin action. This proposal is essentially that of Hartsuck and Tang (70) with a carboxyl group instead of an arginine polarizing the peptide bond as proposed by Hunkapiller and Richards (65). The nucleophile is the ionized Asp-32 as proposed by Hartsuck and Tang (70). The presence of an arginine near this group could explain its abnormally low pK. The assignment of Asp-215 to the polarizing carboxyl was made on the assumptions that the carboxyl group with a pK of 3.94 in the native enzyme and 4.6 in the inactivated enzyme is involved in proton donation. This assumption is supported by synthetic substrate hydrolysis (57,68,110,111).

In conclusion, three carboxyl groups and possibly one α-amino group are implicated in the difference titration of pepsin compared to pepsin inhibited with diazoacetyl-DL-norleucine methyl ester. One carboxyl, presumably Asp-215 esterified by diazoacetyl-DL-norleucine methyl ester can be tentatively assigned a pK of 5. Another carboxyl,

FIGURE 11: PROPOSED MECHANISM OF PEPSIN ACTION. This mechanism is essentially that of Hartsuck and Tang (70) with a carboxyl group polarizing the peptide bond as proposed by Hunkapiller and Richards (65) instead of an arginine. Asp-215 has been assigned to the polarizing carboxyl and a different carboxyl is involved in proton donation at the peptide bond. The pK of the ionized carboxyl is lowered by the presence of an arginine.


PEPSIN
Asp-215
Asp-32
+Arg
R
R'

possibly Asp-32, with a pK of about 3.2 in native pepsin, changes upon inactivation. It's pK is probably shifted to below 1.5. A shift in pK occurs in a third carboxyl with a pK of 4.0 in pepsin and a pK of 4.6 in the modified pepsin. The pK's derived from difference titration data are consistent with pK's estimated from substrate hydrolysis (see Table 3, Chapter I) and from inactivation studies with substrate-like inactivators (70,80).

CHAPTER IV

SUMMARY

Exposure of pepsinogen to acid for less than 2 minutes yields a product with proteolytic activity. This activity is due to intramolecular and intermolecular formation of pepsin from pepsinogen. No evidence is found for intermolecular proteolytic activity in the zymogen. These conclusions are based upon two sets of experiments. First, chemical cleavage of pepsinogen during short activation is demonstrated by quantitative analysis of the NH_2-terminal two residues of the pepsin and pepsinogen in an activation mixture. In addition, quantitative NH_2-terminal analyses after activation under different conditions confirm a previous inference that the product of unimolecular pepsinogen activation is homogeneous whereas biomolecular activation produces a pepsin product with a variety of NH_2-termini. Second, spectral changes which occur upon acidification of pepsinogen solutions are shown to be consistent with the chemical cleavage of pepsinogen during acidification. The first order rate constant for pepsinogen activation, calculated from these spectral experiments, agrees well with the value previously determined.

Three carboxyl groups are implicated in the active site of pepsin by difference titration of pepsin compared to pepsin inactivated

with diazoacetyl-DL-norleucine methyl ester. One carboxyl, presumably Asp-215 in the pepsin sequence, can be tentatively assigned a pK of 5. Another carboxyl modified by inactivation, possibly Asp-32, can be assigned a pK of about 3.2 in native pepsin with a shift in pK to $\leq$ 1.5 upon inactivation. A shift in pK also occurs in a third carboxyl with a pK of 4.0 in pepsin and a pK of 4.6 in the modified pepsin. The dissociation constants derived from difference titration data are consistent with estimated pK's of functional groups in the active site of pepsin from substrate hydrolysis and from inactivation by substrate-like inactivators. The difference titration data supports a mechanism of pepsin action involving three carboxyl groups in the active site.

BIBLIOGRAPHY

1. Langley, J. N. 1882, J. Physiol. 3:246.

2. Northrop, J. H. 1930 Crystalline Pepsin, J. Gen. Physiol. 13:739.

3. Herriot, R. H. 1938 Isolation, Crystallization, and Properties of Swine Pepsinogen, J. Gen. Physiol. 21:501.

4. Herriot, R. M. 1939 Kinetics of the Formation of Pepsin from Swine Pepsinogen and Identification of an Intermediate Compound, J. Gen. Physiol. 22:65.

5. Wang, J. L. and Edelman, G. M. 1971 Fluorescent Probes for Conformational States of Proteins IV. The Pepsinogen-pepsin Conversion, J. Biol. Chem. 246:1185.

6. Bohak, Z. 1969 Purification and Characterization of Chicken Pepsinogen and Chicken Pepsin, J. Biol. Chem. 244:4638.

7. Bohak, Z. 1973 The Kinetics of the Conversion of Chicken Pepsinogen to Chicken Pepsin, Eur. J. Biochem. 32:547.

8. Herriot, R. M. 1941 Isolation, Crystallization, and Properties of Pepsin Inhibitor, J. Gen. Physiol. 24:325.

9. Vunakis, H. V. and Herriot, R. M. 1956 Structural Changes Associated with the Conversion of Pepsinogen to Pepsin I. The N-terminal Amino Acid Residue and Amino Acid Composition of the Pepsin Inhibitor, Biochim. Biophys. Acta. 22:537.

10. Vunakis, H. V. and Herriot, R. M. 1957 Structural Changes Associated with the Conversion of Pepsinogen to Pepsin II. The N-terminal Amino Acid Residues of Pepsin and Pepsinogen; the Amino Acid Composition of Pepsinogen, Biochim. Biophys. Acta. 23:600.

11. Ong, E. B. and Perlmann, G. E. 1968 The Amino-terminal Sequence of Porcine Pepsinogen, J. Biol. Chem. 243:6104.

12. Pedersen, V. B. and Foltmann, B. 1873 The Amino Acid Sequence of a Hitherto Unobserved Segment From Porcine Pepsinogen Preceeding The N-Terminus of Pepsin, FEBS Letters 35:255.

13. Stepanov, V. M., Baratova, L. A., Pugacheva, I. B., Belyanova, L. P., Revina, L. P. and Timokhina, E. A. 1973 N-Terminal Sequence of Swine Pepsinogen and Pepsin. The Site of Pepsinogen Activation, Biochem. Biophys. Res. Comm. 54:1134.

14. Perlmann, G. E. 1964 Comments on Correlation Between Optical Rotation, Fluorescence, and Biological Activity of Pepsinogen, Biopolymers Symp. No. 1, p. 383.

15. Perlmann, G. E. 1961 The Optical Rotatory Properties of Pepsinogen, J. Mol. Biol. 6:452.

16. Neumann, H. and Zehavi, U. 1969 Acid Labile Sugar in Pepsinogen, Biochem. Biophys. Res. Comm. 36:152.

17. Stepanov, V. M., Timokhina, E. A., Baratova, L. A., Belyanova, L. P., Korzhenko, V. P. and Zhukofa, I. G. 1971 On The Activation of Pepsinogen, Biochem. Biophys. Res. Comm. 45:1482.

18. Schalamowitz, M., Shaw, A. and Jackson, W. T. 1964 Pepsinogen and Pepsin: Further Immunachemical Studies of the Conformational Changes Involved in the Formation of Porcine and Human Pepsins from Their Zymogens, Biochemistry 3:636.

19. McPhie, P. 1972 A Spectrophotometeric Investigation of the Pepsinogen-Pepsin Conversion, J. Bio. Chem. 247:4277.

20. Hayashi, K., Koga, D., Aso, Y., Hayashida, E., Kaneda, T., Funatsu, M. and Matsui, T. 1973 On the Activation Process of Pepsinogen, J. Fac. Agr., Kyushu Univ. 18:21.

21. Funatsu, M., Harada, Y., Hayashi, K. and Jirgensons B. 1971 Studies on the Mechanism of Activation of Pepsinogen Part I. Conformational Change of Pepsinogen in the Process of Activation in Acid Solution, Agr. Biol. Chem. 35:566.

22. Rajagopalan, T. G., Moore, S. and Stein, W. H. 1966 Pepsin from Pepsinogen. Preparation and Properties, J. Biol. Chem. 244:4940.

23. Al-Janabi, J., Hartsuck, J. A. and Tang, J. 1972 Kinetics and Mechanism of Pepsinogen Activation, J. Biol. Chem. 247:4628.

24. Bustin, M. and Conway-Jacobs, A. 1971 Intramolecular Activation of Porcine Pepsinogen, J. Biol. Chem. 246:615.

25. Funatsu, M., Harada, Y., Hayashi, K. and Kaneda T. 1972 Studies on the Mechanism of Activation of Pepsinogen Part II. Effects of Substrate and Competitive Inhibitor on the Activation of Pepsinogen, Agr. Biol. Chem. 36:305.

26. McPhie, P. 1974 Pepsinogen: Activation by a Unimolecular Mechanism, Biochem. Biophys. Res. Comm. 56:789.

27. Kassell, B., and Kay, J. 1973 Zymogens of Proteolytic Enzymes, Science. 180:1022.

28. Foltmann, B. 1966 A review on Prorennin and Rennin, Compt. Rend. Trav. Lab. Carlesberg. 35:143.

29. Foltmann, B. 1971 in McKenzie, H. A. (Editor). Milk Proteins, p. 217, Academic Press, New York.

30. Wong, R. C. Nakagawa, Y. and Perlmann, G. E. 1972 Studies on the Nature of the Inhibition by Gossypol of the Transformation of Pepsinogen to Pepsin, J. Biol. Chem. 247:1625.

31. Christensen, L. K. 1954 Concerning the pH Optimum of Peptic Hydrolysis, Arch. Biochem. Biophys. 57:163.

32. Schlamowitz, M. and Peterson, L. U. 1959 Studies on the Optimum pH for the Action of Pepsin on "Native" and Denatured Bovine Serum Albumen and Bovine Hemoglobin, J. Biol. Chem. 234:3137.

33. Schlamowitz, M. and Peterson, L. U. 1961 The Effect of Sodium Chloride on Peptic Digestion of Bovine Serum Albumin, Biochim. Biophys. Acta. 46:381.

34. Tang, J. 1963 Specificity of Pepsin and its Dependence on a Possible "Hydrophobic Binding Site", Nature 199:1094.

35. Fruton, J. S. and Bergmann, M. 1938 The Specificity of Pepsin Action, Science 87:557.

36. Fruton, J. S. and Bergmann, M. 1939 The Specificity of Pepsin, J. Biol. Chem. 127:627.

37. Bergmann, M. and Fruton, J. S. 1941 The Specificity of Proteinases, Adv. Enzymol. 1:63.

38. Harington, C. R. and Pitt Rivers, R. V. 1944 The Synthesis of Crysteine-(Cystine-) Tyrosine Peptides and the Action Thereon of Crystalline Pepsin, Biochem. J. 38:417.

39. Dekker, C. A., Taylor, Jr., S. P. and Fruton, J. S. 1949 Synthesis of Peptides of Methionine and Their Cleavage by Proteolytic Enzymes, J. Biol. Chem. 180:155.

40. Baker, L. E. 1951 New Synthetic Substrates For Pepsin, J. Biol. Chem. 193:809.

41. Baker, L. E. 1954 The Kinetics of the Action of Pepsin on Synthetic Substrates, J. Biol. Chem. 211:701.

42. Kozlov, L. V., Ginodman, L. M., Orekhovich, V. N. and Valueva, T. A., 1966 Free Energy of Hydrolysis of the Peptide Bond and Enzymatic Synthesis of Esters of N-Acetyldipeptides, Biokhimiya 31:315.

43. Jackson, W. T., Schlamowitz, M. and Shaw, A. 1965 Kinetics of the Pepsin-catalyzed Hydrolysis of N-Acetyl-L-phenylalanyl-L-diiodo-tyrosine, Biochemistry 4:1537.

44. Zeffren, E. and Kaiser, E. T. 1966 The Pepsin-Catalyzed Hydrolysis of N-Acetyl-L-phenylalanyl-L-3-5-dibromotyrosine at pH2, J. Amer. Chem. Soc. 88:3129.

45. Inouve, K., Voynick, I. M., Delpierre, G. R. and Fruton, J. S. 1966 New Synthetic Substrates for Pepsin, Biochemistry 5:2473.

46. Clement, G. E. and Snyder, S. L. 1966 The Kinetics of the Pepsin-Catalyzed Hydrolysis of N-Acetyl-L-tyrosine Methyl Ester, J. Amer. Chem. Soc. 88:5338.

47. Jackson, W. T. Schlamowitz, M. and Shaw, A. 1966 Kinetics of the Pepsin-Catalyzed Hydrolysis of N-Acetyl Dipeptides, Biochemistry 5:4105.

48. Lutsenko, N. G., Ginodman, L. M. and Orekhovich, V. N. 1967 Determination of the Ionization Constants of the Functional Groups of the Active Center of Pepsin, Biokhimiya 32:223.

49. Inouye, K. and Fruton, J. S. 1967 Studies on the Specificity of Pepsin, Biochemistry 6:1765.

50. Zeffren, E. and Kaiser, E. T. 1967 The pH Dependence of the Pepsin-Catalyzed Hydrolysis of N-Acetyl-L-phenylalanyl-L-3,5-dibromotyrosine, J. Amer. Chem. Soc. 89:4204.

51. Denburg, J. L., Nelson, R. and Silver, M. S. 1968 The Effect of pH on the Rates of Hydrolysis of Three Acylated Dipeptides by Pepsin, J. Amer. Chem. Soc. 90:479.

52. Reid, T. W. and Fahrney, D. 1967 The Pepsin-Catalyzed Hydrolysis of Sulfite Esters, J. Amer. Chem. Soc. 89:3941.

53. Reid, T. W., Stein, T. P. and Fahrney, D. 1967 The Pepsin-Catalyzed Hydrolysis of Sulfite Esters. Resolution of Alkyl Phenyl Sulfites, J. Amer. Chem. Soc. 89:7125.

54. Doherty, D. G. and James, J. 1968 Pepsin-Catalized Hydrolysis of Picolinoyl-, Nicotinoyl- and Isonicotinoyl-L-Phenylalanyl-L-Phenylalanine Ethyl Ester, Fed. Proc. 27:784.

55. Hollands, R. and Fruton, J. S. 1968 Kinetics of the Hydrolysis of Synthetic Substrates by Pepsin and by Acetyl-Pepsin, Biochemistry 7:2045.

56. Schlamowitz, M. and Trujillo, R. 1968 A New Solubilizing Group for Synthetic Pepsin Substrates, Biochem. Biophys. Res. Comm. 33:156.

57. Cornish-Bowden, A. J. and Knowles, J. R. 1969 The pH-Dependence of Pepsin-Catalysed Reactions, Biochem. J. 113:12.

58. Jackson, W. T., Schlamowitz, M., Shaw, A. and Trujillo, R. 1969 The Effect of pH on the Kinetic Constants of Peptic Substrates and Inhibitors, Arch. Biochem. Biophys. 131:374.

59. Sachdev. G. P. and Fruton, J. S. 1969 Pyridyl Esters of Peptides As Synthetic Substrates of Pepsin, Biochemistry 8:4231.

60. May, S. W. and Kaiser, E. T. 1969 The Pepsin-Catalyzed Hydrolysis of Bis-p-nitrophenyl Sulfite and Its Inhibition by Diphenyl Sulfite at pH 2, J. Amer. Chem. Soc. 91:6491.

61. Hollands, T. R., Voynick, I. M. and Fruton, J. S. 1969 Action of Pepsin on Cationic Synthetic Substrates, Biochemistry 8:575.

62. Trout, G. E. and Fruton, J. S. 1969 The Side-Chain Specificity of Pepsin, Biochemistry 8:4183.

63. Medzihradszky, K., Voynick, I. M., Medzihradszky-Schweiger, H. and Fruton, J. S. 1970 Effect of Secondary Enzyme-Substrate Interactions on the Cleavage of Synthetic Peptides by Pepsin, Biochemistry 9:1154.

64. Hubbard, C. D. and Stein, T. P. 1971 The Pepsin Catalysed Hydrolysis of Bis-P-Nitrophenyl Sulfite, Biochem. Biophys. Res. Comm. 45:293.

65. Hunkapiller, M. W. and Richards, J. H. 1972 Studies on the Catalytic Mechanism of Pepsin Using a New Synthetic Substrate, Biochemistry 11:2829.

66. Sachdev, G. P., Brownstein, A. C. and Fruton, J. S. 1973 N-Methyl-2-anilinonaphthalene-6-sulfonyl Peptides as Fluorescent Probes for Pepsin-Substrate Interaction, J. Biol. Chem. 248:6292.

67. Sampath-Kumar, P. S. and Fruton, J. S. 1974 Studies on the Extended Active Sites of Acid Proteinases, Proc. Nat. Acad. Sci. 71:1070.

68. Clement, G. E., Snyder, S. L., Price, H. and Cartmell, R. 1968 The pH Dependence of the Pepsin-Catalyzed Hydrolysis of Neutral Dipeptides, J. Amer. Chem. Soc. 90:5603.

69. Tang, J. 1971 Specific and Irreversible Inactivation of Pepsin by Substrate-like Epoxides, J. Biol. Chem. 246:4510.

70. Hartsuck, J. A. and Tang, J. 1971 The Carboxylate Ion in the Active Center of Pepsin, J. Biol. Chem. 247:2575.

71. Chen, K. S. C. and Tang, J. 1972 Amino Acid Sequence Around the Epoxide-reactive Residues in Pepsin, J. Biol. Chem. 247:2566.

72. Tang, J., Sepulveda, P., Marciniszyn, Jr., J. Chen, K. C. S., Huang, W-Y, Tao, N., Liu, D. and Lanier, J. P. 1973 Amino-Acid Sequence of Porcine Pepsin, Proc. Nat. Acad. Sci. 70:3437.

73. Delpierre, G. R. and Fruton, J. S. 1965 Inactivation of Pepsin by Diphenyldiazomethane, Proc. Nat. Acad. Sci. 54:1161.

74. Delpierre, G. R. and Fruton, J. S. 1966 Specific Inactivation of Pepsin by a Diazo Keytone, Proc. Nat. Acad. Sci. 56:1817.

75. Rajagopalan, T. G., Stein, W. H. and Moore, S. 1966 The Inactivation of Pepsin by Diazoacetyl-norleucine Methyl Ester, J. Biol. Chem. 241:4295.

76. Hamilton, G. A., Spona, J. and Crowell, L. D. 1967 The Inactivation of Pepsin by an Equimolar Amount of 1-Diazo-4-Phenylbutanone-2, Biochem. Biophys. Res. Comm. 26:193.

77. Erlanger, B. R., Vratsanos, S. M., Wassermann, N. and Cooper, A. G. 1967 Sterochemical Investigation of the Active Center of Pepsin Using a New Inactivator, Biochem. Biophys. Res. Comm. 28:203.

78. Ong, E. B. and Perlmann, G. E. 1967 Specific Inactivation of Pepsin by Benzyl-oxycarbonyl-L-Phenylalanyldiazomethane, Nature 215:1492.

79. Kozlov, L. V., Ginodman, L. M. and Orekhovich, V. N. 1967 Inactivation of Pepsin with Aliphatic Diazocarbonyl Compounds, Biokhimiya 32:1011.

80. Lundblad, R. L. and Stein, W. H. 1969 On the Reaction of Diazoacetyl Compounds with Pepsin, J. Biol. Chem. 244:154.

81. Stepanov, V. M., Lobareva, L. S. and Mal'tsev, N. I. 1968 Coloured Inhibitors of Pepsin, Biochim. Biophys. Acta. 151:719.

82. Valueva, T. A. and Ginodman, L. M. 1970 Investigation of Hog Pepsin Incubated with Diazocarbonyl Reagents, Biokhimiya 35:837.

83. Husain, S., Ferguson, J. B. and Fruton, J. S. 1971 Bifunctional Inhibitors of Pepsin, Proc. Nat. Acad. Sci. 68:2765.

84. Fry, K. T. 1968 A reactive Aspartyl Residue of Pepsin, Biochem. Biophys. Res. Comm. 30:489.

85. Stepanov, V. M. and Vaganova, T. I. 1968 Identification of the Carboxyl Group of Pepsin Reacting with Diazoacetamide Derivatives, Biochem. Biophys. Res. Comm. 31:825.

86. Bayliss, R. S., Knowles, J. R. and Wybrandt, G. B. 1969 An Aspartic Acid Residue at the Active Site of Pepsin, Biochem. J. 113:377.

87. Fry, K. T., Kim, O-K, Spona, J. and Hamilton, G. A. 1970 Site of Reaction of a Specific Diazo Inactivator of Pepsin, Biochemistry 9:4624.

88. Moravek, L. and Kostka, V. 1974 Complete Amino Acid Sequence of Hog Pepsin, FEBS Letters 43:207.

89. Kay, J. and Ryle, A. P. 1971 An Active Site Peptide from Pepsin C, Biochem. J. 123:75.

90. Meitner, P. A. 1971 Bovine Pepsinogens and Pepsins. The Sequence Around a Reactive Aspartyl Residue, Biochem. J. 124:673.

91. Kovaleva, G. G., Shimanskaya, M. P. and Stepanov, V. M. 1972 The Site of Diazoacetyl Inhibitor Attachment of Acid Proteinase of Aspergillus Awamori - An Analog of Penicillopepsin and Pepsin, Biochem. Biophys. Res. Comm. 49:1075.

92. Liu, C. L. and Hatano, H. 1974 An Aspartic Acid Residue at the Active Site of Rhodotorula Glutinis Acid Protease, FEBS Letters 42:352.

93. Erlanger, B. F., Vratsanos, S. M., Wassermann, N. and Cooper, A. G. 1962 Specific and Reversible Inactivation of Pepsin, J. Biol. Chem. 240:3447.

94. Gross, E. and Morell, J. L. 1966 Evidence for an Active Carboxyl Group in Pepsin, J. Biol. Chem. 341:3638.

95. Erlanger, B. F., Vratsanos, S. M., Wassermann, N. and Cooper, A. G. 1966 A. Chemical Investigation of the Active Center of Pepsin, Biochem. Biophys. Res. Comm. 23:243.

96. Shkarenkova, L. S., Ginodman, L. M., Kozlov, L. V. and Orekhovich, V. N. 1967 Incorporation of O^{18} From H_2O^{18} into Carboxyl Groups in the Active Center of Pepsin, Biokhimiya. 33:154.

97. Hollands, T. R. and Fruton, J. S. 1969 On the Mechanism of Pepsin Action, Proc. Nat. Acad. Sci. 62:1116.

98. Silver, M. S., Stoddard, M. and Stein, T. P. 1969 On the Mechanism of the Pepsin-Catalyzed Exchange of Carboxylic Acids with Water-^{18}O, J. Amer. Chem. Soc. 92:2883.

99. Paterson, A. K. and Knowles, J. R. 1972 The Number of Catalytically Essential Carboxyl Groups in Pepsin. Modification of the Enzyme by Trimethyloxonium Fluoroborate, Eur. J. Biochem. 31:510.

100. Akhtar, M. and Al-Janabi, J. M. 1962 Studies on the Mechanism of Action of Pepsin, Chem. Comm. p. 859.

101. Akhtar, M. and Al-Janabi, J. M. 1962 The Labelling of a Catalytic Group of Pepsin; Evidence for an Acyl Intermediate, Chem. Comm. p. 1002.

102. Cornish-Bowden, A. J., Greenwell, P. and Knowles, J. R. 1969 The Rate-Determining Step in Pepsin-Catalysed Reactions, and Evidence against an Acyl-Enzyme Intermediate, Biochem. J. 113:365.

103. Akhtar, M. 1970 Studies on the Mechanism of Action of Pepsin; a Caution, J. Chem. Soc. D6:361.

104. Takahashi, M., Wang, T. T. and Hofmann, T. 1974 Acyl Intermediates in Pepsin and Penicillopepsin Catalyzed Reactions, Biochem. Biophys. Res. Comm. 57:39.

105. Silver, M. S., Stoddard, M., 1972 Amino-Enzyme Intermediates in Pepsin-Catalyzed Reactions, Biochemistry 11:191.

106. Antonov, V. K., Rumsh, L. D. and Tikhodeeva, A. G. 1974 Kinetics of Pepsin-Catalysed Transpeptidation: Evidence for the "Amino-Enzyme" Intermediate, FEBS Letters, 46:29.

107. Knowles, J. R., Sharp, H. and Greenwell, P. 1968 The pH-Dependence of the Binding of Competitive Inhibitors to Pepsin, Biochem. J. 113:343.

108. Greenwell, P., Knowles, J. R. and Sharp, H. 1969 The Inhibition of Pepsin-Catalysed Reactions by Products and Product Analogues. Kinetic Evidence for Ordered Release of Products, Biochem. J. 113:363.

109. Kitson, T. M. and Knowles, J. R. 1971 The Inhibition of Pepsin-Catalysed Reactions by Structural and Sterochemical Product Analogues, Biochem. J. 122:241.

110. Knowles, J. R., Bayliss, R. S., Cornish-Bowden, A. J., Greenwell, P., Kitson, T. M., Sharp, H. C. and Wybrant, G. B. 1970 in P. Desnuelle, H. Neurath, and M. Ottensen (Editors). Structure-Function Relationships of Proteolytic Enzymes, p. 237, Munksgaard, Copenhagen, Denmark.

111. Knowles, J. R. 1970 On the Mechanism of Action of Pepsin, Phil. Trans. Roy. Soc. Lond. B257:135.

112. Wang, J. H. 1970 in P. Desnuelle, H. Neurath, and M. Ottensen (Editors). Structure-function of Proteolytic Enzymes, p. 251, Munksgaard, Copenhagen, Denmark.

113. Huang, W-Y. and Tang, J. 1970 Carboxyl-Terminal Sequence of Human Gastricsin and Pepsin, J. Biol. Chem. 245:2189.

114. Huang, W. Y. and Tang, J. 1971 Modification of Arginine in Pepsin by 2,3-Butanedione, Fed. Proc. 30:1183.

115. Kitson, T. M. and Knowles, J. R. 1971 The Effect of Arginine Modification on the pH Dependence of Pepsin Activity, FEBS Letters 5:377.

116. Parsons, S. M. and Raftery, M. A., 1970 Ionization Behavior of the Catalytic Carboxyls of Lysozyme, Biochem. Biophys. Res. Comm. 41:45.

117. Parsons, S. M. and Raftery, M. A. 1972 Ionization Behavior of the Catalytic Carboxyls of Lysozyme. Effects of Ionic Strength, Biochemistry 11:1623.

118. Parsons, S. M. and Raftery, M. A. 1972 Ionization Behavior of the Catalytic Carboxyls of Lysozyme. Effects of Temperature, Biochemistry 11:1630.

119. Parsons, S. M. and Raftery, M. A. 1972 Ionization Behavior of the Cleft Carboxyls in Lysozme-Substrate Complexes, Biochemistry 11:1633.

120. Edman, P. and Begg, G. 1967 A Protein Sequenator, Eur. J. Biochem. 1:80.

121. Pisano, J. J. and Bronzert, T. J. 1969 Analysis of Amino Acid Phenythiohydantoins by Gas Chromatography, J. Biol. Chem. 244:5597.

122. Ambler, R. P. 1967 in C. H. W. Hirs (Editor). Methods in Enzymology, Vo. XI, p. 155, Academic Press, New York.

123. Tang, J. 1970 Amino Acid Sequence Near the Amino Terminus of Porcine Pepsin. Biochem. Biophys. Res. Comm. 41:697.

124. Stepanov, V. M., Timokhina, E. A., Baratora, L. A., Belyanove, L. P., Korzhenko, V. P. and Zhukova, I. G. 1971, On the Activation of Pepsin, Biochem. Biophys. Res. Comm. 45:1482.

125. Dopheide, T. A. A., Moore, S. and Stein, W. H. 1967 The Carboxyl-terminal Sequence of Porcine Pepsin, J. Biol. Chem. 242:1833.

126. Perham, R. N. and Jones, G. M. T. 1967 The Determination of the Order of Lysine-containing Tryptic Peptides of Proteins by Diagonal Paper Electrophoresis. A Carboxyl-terminal Sequence for Pepsin, Eur. J. Biochem. 2:84.

127. Matveeva, R. A., Krivtsov, V. E. and Stepanov, V. M. 1968 Structure of Peptides Obtained from Tryptic Digest of Hog Pepsin, Biokhimiya 33:167.

128. Kostaka, V., Moravek, L. and Sorm, F. 1970 Amino Acid Sequence of C Terminal Fragment of Hog Pepsin, Eur. J. Biochem. 13:447.

129. Fruton, J. S. 1971 in P. D. Boyer (Editor). The Enzymes, Vol. III, p. 119 Academic Press, New York.

130. Anderson, W. and Harthill, J. E. 1973 Pepsin Inhibitory Activity Amongst Activation Peptides of Pepsinogen, Nature 243:417.

131. Anson, M. L. and Mirsky, A. E. 1932 The Estimation of Pepsin with Hemoglobin, J. Gen. Physiol. 16:59.

132. Tang, J. and Tang, K. I. 1963 Purification and Properties of a Zymogen from Human Gastric Mucosu J. Biol. Chem. 238:606.

133. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. 1951 Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193:265.

134. von Muralt, A. L. 1930 The Titration Constants of Multivalent Substances, J. Amer. Chem. Soc. 52:3518.

135. Simms, H. S. 1926 Dissociation of Polyvalent Substances I. Relation of Constants to Titration Data, J. Amer. Chem. Soc. 48:1239.

136. Edsall, J. T. and Wyman J. 1958 Biophysical Chemistry, Vol. I, p. 477, Academic Press, New York.

137. Tanford, C. 1962 in C. B. Anfinsen, Jr., M. L. Anson, K. Bailey and J. T. Edsall (Editors). Advances in Protein Chemistry, Vol. XVII, p. 69, Academic Press, New York.

138. Nozaki, Y. and Tanford, C. 1967 in C. H. W. Hirs (Editor). Methods in Enzymology, Vol. XI, p. 715, Academic Press, New York.

139. Herriott, R. M. and Northrop, J. H. 1934 Crystalline Acetyl Derivatives of Pepsin, J. Gen. Physiol. 18:35.

APPENDIX

PREPARATION OF GLYCYL-DL-NORLEUCINE METHYL ESTER

Glycyl-DL-norleucine methyl ester was prepared by mixing glycyl-DL-norleucine with methanol (99.9% pure) in a molar ratio of 1:8. The mixture was cooled in a ice-salt bath (-4°C) after which a slight excess of thionyl chloride to methanol (molar ratio 1.1:1) was added to the mixture. The solution was allowed to warm to 40°C and was maintained at 40°C in a thermostatted vessel for 2 hours. The excess thionyl chloride and methanol were removed by distillation, and the residue (thick solution) was "dried" at 100°C under vacuum. The "dried" residue, (which had a thick, sticky, consistency) was dissolved in a small amount of methanol. Ether was added until the solution turned slightly turbid. This required a fairly large volume of ether compared to methanol. Crystals of glycyl-DL-norleucine methyl ester began forming in one hour. Yield was 70-80%, M. P. 118-120°C.