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COMPARATIVE STUDIES ON THE SPECIFICITY

OF HUMAN GASTRICSIN AND PEPSIN

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS--Continued

COMPARATIVE STUDIES ON THE SPECIFICITY OF HUMAN GASTRICSIN AND PEPSIN

CHAPTER I

INTRODUCTION

Broadly speaking, the word "specificity" expresses the general idea that enzymes catalyze some reactions but not others, and in many cases, catalyze the reactions of some compounds of a class much more efficiently than other members of the class.

Although all proteolytic enzymes catalyze the hydrolysis of peptide linkages, the specificity is often a complex problem. Some proteolytic enzymes are the exopeptidases which require either a terminal amino or carboxyl group for catalysis. These enzymes usually hydrolyze residues from the ends of polypeptide chains in a sequential manner. The endopeptidases, on the other hand, do not require free terminal groups for their activity. This group of proteolytic enzymes, though capable of hydrolyzing an amino acid residue from the end of a polypeptide chain, generally prefer to hydrolyze a long polypeptide into shorter peptides. The specificity of endopeptidases can also be considered with respect to the type of amino acid residues which are preferentially selected by the enzymes for the site of hydrolysis. For some endopeptidases, the specificity is very narrow. Trypsin,

for example, attacks only the peptide bonds on the carboxyl side of either lysine or arginine residues. It also attacks the aminoethylated cysteine, which is analogous to lysine. However, most of the endopeptidases have a much wider specificity. They are capable of hydrolyzing peptide linkages involving various amino acids. The study of the specificity of this type of endopeptidase, such as pepsin, is a much more complex problem.

Although pepsin has been known and studied extensively over a period of more than thirty years, the isolation and study of a second protease present in the stomach of higher mammals was initiated only ten years ago (1). In the study of human gastric proteolytic enzymes, Richmond et al. (2) succeeded in separating two proteolytic enzymes, pepsin and gastricsin, from human gastric juice. Gastricsin was later crystallized and partially characterized by Tang et al. (3). It was shown to differ from pepsin in pH optimum, electrophoretic mobility on paper and starch, and in heat inactivation. In 1962, Tang and Tang (4) reported the purification and properties of a zymogen from human gastric mucosa which upon activation produced both gastricsin and pepsin. In 1964, Mills (5) studied several physicochemical properties of human pepsin, gastricsin and their zymogen. This study included the determination of the sedimentation coefficients, diffusion coefficients, intrinsic viscosities, amino acid compositions, molecular weights by various methods and carboxyl terminal residue for each of these proteins. Gastricsin has also been isolated from porcine mucosal extracts. The properties of porcine gastricsin were found to be similar to those of human enzyme (6). Several properties of these two enzymes

are compared in Tables 1 through 4 (7).

Gastricsin and pepsin are endopeptidases which hydrolyze proteins at various rates depending on the substrate used. For example, gastricsin hydrolyzed bovine hemoglobin about 30% faster than pepsin (Table 2). However, when egg albumin was used as the substrate, the rate of peptic hydrolysis was twice that of gastricsin. In order to explain why gastricsin hydrolyzed hemoglobin faster than pepsin, one may assume that there are more bonds in hemoglobin which are susceptible to hydrolysis by gastricsin than by pepsin. Various synthetic dipeptides have been tested as substrates of gastricsin and pepsin. In all cases, pepsin hydrolyzes the synthetic substrates at a higher rate (Table 2). It is interesting to note that N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT), which is the best synthetic substrate yet found for peptic hydrolysis, was resistant to cleavage by gastricsin.

The study of the specificity of pepsin has a long and rewarding history. Since the discovery of the first synthetic substrates, CBZ-Glu-Tyr (8), by Fruton and Bergmann in 1939, several investigators, notably Baker (1951), have prepared peptide derivatives that have proved to be valuable. Bergmann and Fruton (9) reviewed the results of work with derivatives of glutamic acid, glutamine, tyrosine and glycine, and found that N-protected derivatives of glutamyl tyrosine were digested by pepsin most rapidly with a pH optimum near 4.0. Peptides bearing a free amino group were hydrolyzed much more slowly than those in which the amino group was masked. Harrington and Pitt-Rivers (10) studied peptides of tyrosine with cystine or cysteine, and found that the derivatives of cysteine were hydrolyzed more rapidly than the

TABLE 1

COMPARISON OF SOME PROPERTIES OF HUMAN GASTRICSIN AND PEPSIN (7)

Enzyme	Optimal pH	Relative Proteolytic Activity[a]	Relative Milk-Clotting Activity	Relative Electrophoretic Mobility[b]	Effluent pH[c]	% Inactivation at 70°, 10 min
Gastricsin	3.2	100	100	60	4.4	80
Pepsin	1.5-2.0	74	103	100	4.0	33

[a]Bovine hemoglobin was used as substrate.

[b]Electrophoresis was carried out in starch gel at pH 6.5 (sodium phosphate buffer, ionic strength 0.1).

[c]Amberlite IRC-50 column eluted with 0.2 M sodium citrate buffer.

TABLE 2

MAXIMUM VELOCITY IN THE HYDROLYSIS OF SYNTHETIC PEPTIDE AND NATURAL PROTEIN SUBSTRATES BY HUMAN GASTRICSIN AND PEPSIN (7)

Substrate	Gastricsin	Pepsin
Synthetic	μmoles/mg/hr	μmoles/mg/hr
Carbobenzoxy-L-glutamyl-L-tyrosine[a]	0.017	0.034
Carbobenzoxy-glycyl-L-phenylalanine[a]	.002	.006
Carbobenzoxy-L-glutamyl-L-phenylalanine[a]	.001	.024
N-acetyl-L-phenylalanyl-L-tyrosine[b]	.230	.770
N-acetyl-L-phenylalanyl-L-diiodotyrosine[b]	0	3.920
Protein[c]		
Bovine Hemoglobin	3850	2813
Egg Albumin	103	262

[a]The reaction mixture contained 8.3 μmoles of substrate and 1.6 mg of enzyme in 1 ml of 0.1 M sodium citrate-HCl buffer, pH 2.5. The incubation was carried out at 38° for 24 hours (36).

[b]The reaction mixture contained 0.5 μmole of substrate and 50 μg of enzyme in 1 ml of 0.005 M sodium citrate buffer, pH 3.5. The incubation was carried out at 37° for 60 minutes (6).

[c]The hydrolysis of protein substrates is expressed as μmoles of tyrosine released by the method of Anson and Mirsky (38).

TABLE 3

PHYSICAL CONSTANTS OF HUMAN PEPSIN, GASTRICSIN AND ZYMOGEN (7)

Protein	Molecular Weight[a]	Intrinsic Viscosity, $[\eta]$	Diffusion Coefficient, $D_{20,w} \times 10^7$	Axial Ratio, a/b
		dl/g		
Gastricsin	31,000	0.100	9.6	160
Pepsin	34,100	0.045	8.7	6
Zymogen[b]	38,900	0.100	7.7	50

[a]Average value from three methods of determination:
1. Sedimentation equilibrium
2. Sedimentation-Diffusion
3. Calculated from amino acid composition

[b]"Zymogen" was designated as the common inactive precursor of gastricsin and pepsin.

TABLE 4

AMINO- AND CARBOXYL-TERMINAL RESIDUES OF HUMAN PEPSIN, GASTRICSIN AND ZYMOGEN (7)

Protein	Amino-terminal[a]	Carboxyl-terminal[a]
Pepsin	Valine	- Leu - Phe - Ala
Gastricsin	Serine	- Thr - Phe - Ala
Zymogen	Leucine	- Val - Ser - Ala

[a]Determined by Dinitrophenyl and Carboxypeptidase methods according to Frankel-Conrat et al. (40).

corresponding cystine peptides. The carbobenzoxy derivatives were hydrolyzed faster than the free dipeptides and hydrolysis in each case was faster at pH 4.0 than at pH 1.8. Dekker, Taylor and Fruton (11) examined the action of pepsin on carbobenzoxy-L-methionyl-L-tyrosine and on the corresponding free dipeptide at pH 4 and found that both were hydrolyzed, but the free dipeptide was hydrolyzed more slowly than the N-substituted derivative. From these studies it appears that a free carboxyl terminal residue is not essential in a synthetic substrate for pepsin, and that the proximity of an α-amino group is inhibitory to varying degrees.

Baker (12) tested a variety of substrates in which two aromatic residues were linked together and in which the amino group was blocked by an acetyl or carbobenzoxy group. She found that these substrates were much more rapidly hydrolyzed than those previously studied with a pH optimum near 2.0. Compounds in which the amino acid residue provided the carboxyl moiety of the peptide bond in the D-configuration were not hydrolyzed. Baker (12) also observed that one of Bergmann's substrates, carbobenzoxy-L-glutamyl-L-tyrosine, was hydrolyzed optimally at pH 2.0, when the substrate concentration was as low as that which she employed with her other substrates (0.002 M). The most rapidly hydrolyzed of these substrates, N-acetyl-L-phenylalanyl-L-diiodotyrosine was suitable for assaying pepsin.

In 1960, Bovey and Yanari (13) reviewed the works on synthetic substrates which showed that pepsin could hydrolyze bonds formed by the amino or carboxyl groups of phenylalanine, tyrosine, glutamic acid, cystine and cysteine. Other types of synthetic substrates have not been

examined in detail, and the best picture of the specificity of pepsin has come from recent studies with protein substrates. R. L. Hill (14) tabulated a list of the bonds split by pepsin in seven proteins or polypeptides. This list includes tobacco mosaic virus, egg-white lysozyme, ribonuclease, human hemoglobin (α-chain), insulin, human hemoglobin (β-chain), and β-melanocyte stimulating hormone. The amino acid sequences of each of these substances has been established (15-21).

In 1955, Bailey, Moore and Stein (22) reported on the peptide products obtained by the peptic hydrolysis of ribonuclease A which had been oxidized by performic acid. The products were examined by means of Dowex 50-X2-column chromatography. Eight peptides were isolated and the amino acid composition of each was determined.

In 1965, Scheraga (23) reported the results of an investigation of the peptic digestion products of ribonuclease A. The peptic digest was fractionated using gel filtration column chromatography (Bio-Rex 70) and Dowex 50-X2-column chromatography. The sites of peptic cleavage have been partially identified as phe-asp [120-121], met-ser [79-80], thr-phe [45-46], gln-ala [55-56] and phe-glu [8-9].

In 1963, Tang (24) proposed that the specificity of pepsin can be accounted for if it is assumed that pepsin requires a "hydrophobic binding site" near susceptible bond. This suggestion was based on the calculation of the frequency of appearance of amino acids with hydrophobic side chains at positions on either side of the bond which was hydrolyzed. This proposal was supported by a study of the kinetics of the competitive inhibition of pepsin by aliphatic alcohols (25). Aliphatic alcohols, from methanol to amyl alcohol, were shown to inhibit

the hydrolytic activity of pepsin with both protein and synthetic dipeptide substrates. The inhibition appeared to increase with the size of the alcohol molecules. The ΔF^{o}_{H-C} for the formation of a complex between the enzyme and the hydrocarbon chain of alcohols increased linearly with an increasing number of carbon atoms in the hydrocarbon chain of the inhibitor. This was interpreted as supporting evidence that pepsin forms a hydrophobic bond with the side chain of the amino acid in the substrate as the first step of its enzymic mechanism.

In 1966, Fruton (33) reported the synthesis of several new peptide substrates for crystalline swine pepsin. They include carbobenzoxy-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester, and related peptide derivatives, in which one or both L-phenylalanyl residues have been replaced by L-tyrosyl or L-tryptophanyl residues. These compounds, as well as glycyl-glycyl-L-phenylalanyl-L-phenylalanine ethyl ester, were cleaved rapidly at the peptide bond between the two aromatic amino acid residues, the pH optimum being near 4. Carbobenzoxy-L-histidyl-L-phenylalanyl-L-phenylalanine was hydrolyzed by pepsin more slowly than the corresponding ethyl ester, with a pH optimum near 3. The α-carboxylate group adjacent to the sensitive peptide bond is inhibitory to pepsin action.

In 1963, Lockshina et al. (26) reported that pepsin has esterase activity. They showed that in the presence of 0.027 mM pepsin, acetyl-L-phenylalanyl-β-phenyl-L-lactic acid (2.7 mM) was 30% cleaved in 48 hours at 37° and pH 2.0. The rate of hydrolysis was followed by measuring the disappearance of ester group by the hydroxamic acid

reaction. In 1966, Fruton et al. (27) confirmed the finding of Lokshina and found that carbobenzoxy-L-histidyl-p-nitro-L-phenylalanyl-β-phenyl-L-lactic acid methyl ester was also hydrolyzed by pepsin. The ester bond cleaved was the one linking the carboxyl group of p-nitro-L-phenylalanine and the hydroxyl group of β-phenyl-L-lactic acid methyl ester.

The kinetics of the pepsin-catalyzed hydrolysis of several synthetic dipeptide substrates have been studied by several investigators (28-33). The Michaelis constants of those substrates are shown in Table 5.

In the present investigation, the specificities of pepsin and gastricsin were studied by using oxidized bovine ribonuclease A, glucagon and several synthetic dipeptides as substrates. The experimental procedure included the incubation of each of these substrates with each enzyme and the subsequent isolation and identification of amino acids or peptides resulting from the enzymic digestion. The latter along with the knowledge of the primary structure of glucagon and oxidized ribonuclease A (34-35) made it possible to identify those peptide bonds which were split by each enzyme. The bonds susceptible to both enzymes or to either one alone are listed in Table 6.

The kinetics of the gastricsin catalyzed hydrolysis of three synthetic dipeptide substrates, CBZ-L-tyr-L-ala, CBZ-L-tyr-L-ser and CBZ-L-tyr-L-thr, were also investigated.

TABLE 5

THE MICHAELIS CONSTANTS OF HOG PEPSIN ACTION ON SYNTHETIC SUBSTRATES

Substrate	pH Optimum	Km (M)	Reference
CBZ-L-Tyr-L-Tyr	4.0	1.89×10^{-3}	Laidler (1950) (28)
CBZ-L-Tyr-L-TyrOEt	4.0	1.78×10^{-3}	
CBZ-L-Phe-L-Tyr	2.0	2.1×10^{-4}	Silver (1965) (29)
Ac-L-Phe-L-Tyr	2.0	1.95×10^{-3}	
Ac-L-Phe-L-Tyr	2.0	2.4×10^{-3}	Baker (1954) (30)
Ac-L-Tyr-L-Tyr	2.0	6.3×10^{-3}	
Ac-L-Tyr-L-Tyr	2.0	6.1×10^{-3}	Schlamowitz (1965) (31)
Ac-L-Tyr-L-Phe	2.0	2.0×10^{-3}	
Ac-L-Phe-L-Tyr	2.0	2.2×10^{-3}	
Ac-L-Phe-L-Phe	2.0	0.16×10^{-3}	
Ac-L-Phe-L-Diiodotyr	2.0	7.5×10^{-5}	
Ac-L-Phe-L-Dibromotyr	2.0	9.3×10^{-5}	Kaiser (1966) (32)
CBZ-L-His-L-Phe-L-PheOEt	4.0	1.8×10^{-4}	Fruton (1966) (33)
CBZ-L-His-L-Phe-L-TyrOEt	4.0	2.3×10^{-4}	
CBZ-L-His-L-Phe-L-TrpOEt	4.0	2.3×10^{-4}	
CBZ-L-His-L-Tyr-L-TyrOEt	4.0	2.4×10^{-4}	
Gly-Gly-L-Phe-L-TyrOEt	4.0	2.8×10^{-4}	

TABLE 6

THE SPECIFICITY OF PEPSIN AND GASTRICSIN FOR HYDROLYSIS OF PEPTIDE BONDS IN GLUCAGON AND OXIDIZED RIBONUCLEASE A

Bonds Susceptible to Both Enzymes			Bonds Susceptible to Either Enzyme	
			Gastricsin	Pepsin
Glucagon				
Phe-Thr [6-7]	Asp-Tyr [9-10]	Phe-Val [22-23]	Tyr-Ser [10-11]	
Leu-Met [26-27]			Tyr-Leu [13-14]	
Oxidized Ribonuclease A				
Ala-Ala [4-5]	Phe-Glu [8-9]	Ser-Arg [32-33]	Tyr-Ser [76-77]	Val-Ala [108-109]
Asp-Leu [34-35]	Val-Asp [43-44]	Thr-Phe [45-46]		
Glu-Ser [49-50]	Leu-Ala [51-52]	Phe-Val [46-47]		
His-Glu [48-49]	Ala-Asp [52-53]	Glu-Ala [55-56]		
Arg-Glu [85-86]	Ser-Ser [89-90]	Met-Ser [79-80]		
Glu-Ser [74-75]	Tyr-Pro [92-93]	Val-His [118-119]		
Phe-Asp [120-121]				

Note: The numbers inside brackets indicate the position of amino acid residues in the protein molecule.

CHAPTER II

MATERIALS AND METHODS

Materials

Human Gastric Juice

Samples of human gastric juice were obtained from fasting patients at the University Hospital and the Veterans Administration Hospital in Oklahoma City, Oklahoma. The gastric juice was maintained at 4°. Samples from several patients were pooled, dialyzed against distilled water, and lyophilized.

Carboxypeptidase A

Carboxypeptidase A (diisopropylphosphofluoridate treated 5 x crystallized water suspension) was obtained from Mann Research Laboratories, New York, New York.

Bovine Hemoglobin

Bovine hemoglobin was obtained from Pentex Incorporated, Kankakee, Illinois.

Bovine Ribonuclease A

Bovine ribonuclease A was obtained from Mann Research Laboratories, New York, New York.

Crystalline Porcine Glucagon

Crystalline Porcine Glucagon was a gift of Eli Lilly & Co., Indianapolis (Lot No. 258-234B-167-1).

Dansyl Chloride

5-Dimethyl amino-1-naphthalene sulfonyl chloride (Lot 52654) (dansyl chloride) was obtained from K & K Laboratories, Inc., Plainview, New York.

Ninhydrin

Ninhydrin was obtained from Pierce Chemical Company, Rockford, Illinois.

Phenylisothiocyanate

Phenylisothiocyanate was obtained from Fisher Scientific Company, Fair Lawn, New Jersey, and was distilled before use.

Synthetic Peptides

Chromatographically pure samples, as tested by the company, were obtained from Cyclo Chemical Corporation, Los Angeles, California. A list of N-carbobenzoxy-dipeptides (N-CBZ-dipeptides) is shown as follows.

N-CBZ-L-tyrosyl-L-serine (Lot M-2585)

N-CBZ-L-tyrosyl-L-tyrosine (Lot M-2601)

N-CBZ-L-tyrosyl-L-alanine (Lot M-2840)

N-CBZ-L-tyrosyl-L-leucine (Lot M-2603)

N-CBZ-L-tyrosyl-L-threonine (Lot M-2682)

N-CBZ-L-tyrosyl-L-glycine (Lot G-1367)

N-CBZ-L-tyrosyl-L-valine (Lot G-1883)

N-CBZ-L-seryl-L-tyrosine (Lot G-1400)

N-CBZ-L-alanyl-L-tyrosine (Lot M-2254)

N-CBZ-L-phenylalanyl-L-serine (Lot G-1124)

1-Fluoro-2,4-Dinitro Benzene

1-Fluoro-2,4-dinitro benzene was obtained from Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York.

Dye Markers

The dye used as markers in high voltage electrophoresis were Orange G and Xylene Cyanol blue FF which were obtained from K & K Laboratories, Inc., Plainview, New York.

Methods

Preparation of Human Pepsin and Gastricsin

Human pepsin and gastricsin were prepared by fractionation of human gastric juice with an ion-exchange column of Amberlite IRC-50 (XE-64) using the procedure of Richmond *et al.* (2) as modified by Tang *et al.* (3, 36).

Preparation of Oxidized Ribonuclease A

Oxidized ribonuclease A was prepared according to the procedure of Hirs (37). Performic acid was prepared by adding 0.5 ml of 30% hydrogen peroxide to 9.5 ml of 99% formic acid and the resulting solution was allowed to stand at room temperature for 2 hours in a stoppered test tube. In another glass stoppered test tube, 200 mg of ribonuclease A

were dissolved in 5.0 ml of 99% formic acid, and 1.0 ml of anhydrous methanol was added to prevent freezing of the solution when it was cooled as described below. The quantity of performic acid used was 12 times that required to transform 3 cystine residues in the protein to 6 cysteic acid residues, and at the same time converted 3 methionine residues to the methionine sulfone. The protein solution and performic acid were placed in a bath at -10°, and then mixed together. After 2.5 hours the contents of the tube were rinsed, using 50 ml of ice cold water, into a flask containing 350 ml of water at 0°. The aqueous solution was then freeze-dried. Remaining traces of performic acid were removed by dissolving the dry protein in 8.0 ml of water, followed by repetition of the lyophilization step.

Protein Concentration

Protein concentration was determined spectrophotometrically at 280 mμ in a Hitachi-Perkin-Elmer Model 139 spectrophotometer. The molar extinction coefficients used were 52,104 and 47,952 for human pepsin and gastricsin respectively (6).

Proteolytic Activity

The procedure of Anson and Mirsky (38) was used to determine proteolytic activity. The incubation mixture contained 1.25% bovine hemoglobin and 0.01 ml enzyme solution in 2 ml of 0.2 M sodium citrate buffer, pH 2.5. At the end of 10 minutes of incubation at 37°, 1 ml of 10% trichloroacetic acid was added and the mixture was filtered. The absorbance of the filtrate at 280 mμ was then determined in a Hitachi-Perkin-Elmer Model 130 spectrophotometer. The optical density

of the filtrate after subtracting the value of the blank, was used as a measure of proteolysis. The specific activities (change in optical density at 280 mμ per mg of enzyme per 10 minutes at 37°) of gastricsin and pepsin were 168 and 140 respectively (6).

Amino Acid Analyses

The purified peptides (0.02--0.2 μmole) were hydrolyzed with 0.1 ml of 5.7 N glass distilled HCl in an evacuated tube (9.8 cm x 5 cm) at 110° ± 1° for 24 hours. The hydrolysates were evaporated in a vacuum desiccator at room temperature, redissolved with a small amount of distilled water, and the evaporation repeated. The final residues were dissolved by the addition of 50 μl of water. Aliquots which represented about one tenth of the dissolved residue were spotted on Whatman No. 1 paper. High voltage paper electrophoresis was carried out at pH 2.0 and 6000 volts (120 volts/cm) for twenty minutes. Known quantities of several amino acids were applied as standards. After the electrophoresis, the paper was stained with ninhydrin collidine reagent. The spots obtained were compared visually with the colors produced by the standards to estimate the amounts of amino acids in the samples. This also permitted an estimation of the volume of the remaining aliquots which should be used for quantitative amino acid determination in the amino acid analyzer. The proper volume of the remaining solution was then mixed with 0.2 N sodium citrate buffer, pH 2.2 to make a final volume of 1.0 ml.

The amino acid composition of each hydrolysate was determined according to the procedure of Spackman, Stein and Moore (39) with a

Beckman Spinco Amino Acid Analyzer Model 120B, equipped with a rapid-flow system (flow rate 67 ml/hour) and a high sensitivity range card (5 x amplification). The sensitivity of the instrument is about 0.001 μmole. Identical amounts of the hydrolysates were applied to the 40 cm column and to the 5 cm column.

The neutral and acidic amino acids were chromatographed on the 40 cm column containing Amberlite IRC-120C resin (a very fine pulverized 8% cross-linked sulfonated polystyrene resin), and eluted initially with pH 3.25 buffer for 48 minutes, and finally with a pH 4.25 buffer for 1 hour and 40 minutes. The basic amino acids were chromatographed on the 5 cm column containing the same resin but were eluted with a pH 5.28 buffer for 1 hour.

Standard amino acid mixtures were chromatographed at the beginning and the end of each batch of ninhydrin reagent used for color development. Because of the variability introduced by different preparations of ninhydrin color reagent, it was necessary to chromatograph standard amino acid mixtures at the beginning and the end of each batch of reagent to standardize the color constants. The average of the results of the color yield for each amino acid was used to calculate its constant by the usual height-width method. One of the standard amino acids analyses is shown in Figure 1.

Amino-terminal Amino Acid Determination

Dinitrophenyl method (40, 41). The peptide was dissolved in 0.1 ml of 1% trimethylamine, and to this was added a solution of 10 μl of 1-fluoro-2,4-dinitrobenzene (FDNB) dissolved in 0.2 ml of ethanol.

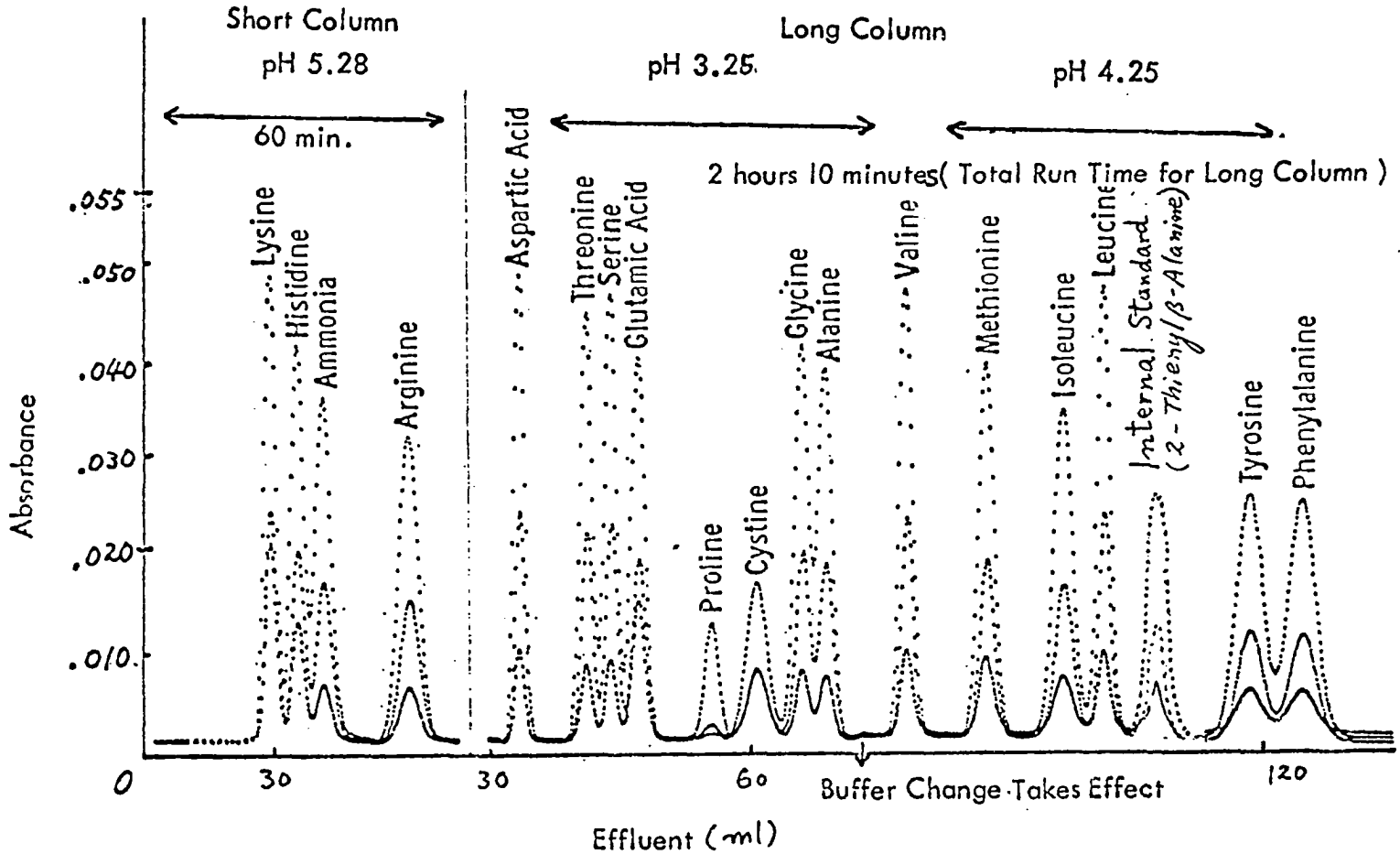


Figure 1. The Chromatogram from Standard Amino Acids Analysis

After shaking at room temperature for overnight in the dark room, a few drops of water and trimethylamine solution were added and the excess FDNB was removed by three times extraction with ether. The residue was evaporated to dryness under vacuum, and 0.1 ml of 5.7 N HCl was added. The tube was then sealed under vacuum and heated for 16 hours at 110°C. The hydrolysate was evaporated to dryness. Aliquots of the hydrolysates were subjected to quantitative amino acid analysis, and the rest of the samples were analyzed for DNP amino acids by two dimensional thin layer chromatography. Silica gel G was used as the supporting phase. The following two solvent systems were used: (1) Toluene / pyridine / ethylene chlorohydrin / 0.8 N ammonium hydroxide (100 : 30 : 60 : 60); (2) Chloroform / benzyl alcohol / acetic acid (70 : 30 : 3).

Dansyl method (42). DNS-derivatives of the peptides were used to characterize the amino-terminal residues according to Gray and Hartley (43). Aliquots of the peptides containing from 1 to 3 μmoles were evaporated to dryness in a vacuum desiccator at room temperature, and dissolved in 20 μl of 0.1 M sodium bicarbonate in ammonia free water. They were then evaporated to dryness in a vacuum desiccator. Ten μl each of ammonia free water and DNS-chloride solution (2.5 mg/ml in acetone) were added and the mixture was incubated for 2 hours at 37°. The reaction mixture was dried in a vacuum desiccator and the residue was redissolved in 20 μl of 6 N HCl and hydrolyzed in a sealed tube (0.5 cm x 3 cm) at 110° ± 5° for 16 hours. The product was evaporated in a vacuum desiccator over NaOH pellets, and 10 μl of 1N ammonium hydroxide solution was added. The sample was spotted on Whatman No. 1

paper (98 cm long with the origin 30 cm from the cathode end), and subjected to electrophoresis on a water cooled flat plate in a buffer solution containing 8 ml of pyridine and 10 ml of glacial acetic acid per one liter of solution, to make the pH exactly 4.55. The electrophoresis was carried out at 6 kilovolts (61 volts/cm) for three hours at a current of 50 milliamperes. The circulating water was at room temperature. The standard DNS-derivatives of amino acids served as markers and a dye mixture was also spotted on both edges of the paper. The amino acid derivatives were detected by their fluorescence when illuminated under ultraviolet light. All the DNS-amino acids give a yellow fluorescence. Most of the DNS-amino acids could be identified readily by electrophoretic separation at pH 4.55, except for DNS-glycine, DNS-alanine, and DNS-serine which were often masked by a strong blue fluorescence of the DNS-sulfonic acid. Hence the position corresponding to DNS-sulfonic acid and the three DNS-amino acid was cut out and was stitched to Whatman No. 1 paper. Further electrophoretic separation was carried out at pH 2 and 6 kilovolts (120 volts/cm) for 20 minutes in a Savant high-voltage electrophoresis tank. DNS-serine, DNS-alanine and DNS-glycine could be identified by this procedure.

Carboxyl-terminal Amino Acid Determinations

A 25 µl aliquot of DFP-treated carboxypeptidase A suspension was removed from its container using a syringe. One ml ice-cold distilled water was added, and the suspension was centrifuged. One hundred µl of 1% sodium bicarbonate solution were added to the precipitate. Three drops of freshly prepared 0.1 N NaOH solution were added to dissolve the enzyme. An equal number of drops of 0.1 N HCl

solution was then added to neutralize the enzyme solution. This was followed by diluting the solution to 1.25 ml with 0.2 M N-ethyl morpholine acetate buffer, pH 8.0. The resulting enzyme solution had the concentration of about 1 mg/ml. Thirty µl of this enzyme solution were added to 100 µl of the 0.2 M N-ethyl-morpholine acetate solution, pH 8.0, containing a suitable amount of peptide (0.02 µmole to 0.1 µmole). The mixture was incubated at 37° for 5 hours. When the kinetics of amino acid release were studied, aliquots were taken for quantitative amino acid analysis at various time intervals ranging from 2 to 6 hours (44).

Edman Degradation

Edman degradation (45) was carried out by the phenylisothiocyanate procedure described by Sjoquist (46). The dried peptide was added to 0.2 ml of 50% redistilled pyridine and 0.1 ml of 5% PITC (phenylisothiocyanate) in pyridine. The mixture reacted at 37° for 2.5 hours. Then the product was extracted 3 times with 2 ml each of benzene in order to remove the unreacted reagent. The solution was dried in a vacuum desiccator. To the residue o.1 ml of trifluoroacetic acid was added and the mixture was allowed to react for 1 hour at room temperature. The reaction product was dried over NaOH pellets in the desicator.

Ninhydrin Method

The quantitative determination of free amino groups was carried out according to the procedure of Rosen (47). The reaction mixture consisted of 1.0 ml sample, containing 0.02 to 0.4 µmole of peptide;

0.5 ml of 4 M sodium acetate buffer (pH 5.3) containing 2 x 10^{-4} M NaCN; and 0.5 ml of 3% ninhydrin solution in methyl cellosolve. The reaction mixture was heated 15 minutes in a boiling water bath, and 5 ml of isopropyl alcohol-water solution (1 : 1) was added at the end of this period. After the solution cooled to room temperature, the color was read in a Hitachi-Perkin-Elmer spectrophotometer at 570 mμ.

High-Voltage Paper Electrophoresis

For the high voltage electrophoretic separation of peptides and amino acids, an apparatus similar to that designed by Michl (48) (1951) was used. The buffers were: pH 6.5 pyridine-acetic acid-water (25 : 1 : 225 by volume); pH 3.5 pyridine-acetic acid-water (1 : 10 : 90 by volume); pH 2.0 formic acid-acetic acid-water (1 : 4 : 45 by volume).

Ninhydrin-Cadmium Acetate Reagent

The cadmium acetate reagent (49) for staining peptides was prepared by mixing 6 g of cadmium acetate, 300 ml of glacial acetic acid and 600 ml of water. Fifteen ml of cadmium acetate reagent was mixed with 100 ml of 1% ninhydrin in acetone.

Ninhydrin-Collidine Reagent

The spray reagent of ninhydrin-collidine (50) for staining amino acids was prepared by mixing 10 ml of 1% ninhydrin in acetone, 40 ml of distilled acetone and 2 ml of collidine (2,4,6-trimethyl-pyridine).

Pauly Reagent

The Pauly reagent (51) for staining tyrosine and histidine was

prepared by mixing 3 volumes of 1% sulfanilic acid in 1 N HCl, 2 volumes of 5% sodium nitrite solution, and 5 volumes of 15% sodium carbonate solution. This was prepared just before use.

CHAPTER III

RESULTS

Hydrolysis of Glucagon

Digestion of Porcine Crystalline Glucagon

The digestion of glucagon was carried out at the respective optimal pH of each enzyme: in pH 2.1 buffer [formic acid / acetic acid / water (1 : 4 : 45)] for pepsin; and pH 3.1 buffer [pyridine / acetic acid / water (1 : 14 : 85)] for gastricsin. The ratio of substrate to enzyme was 50 to 1 (w/w). Aliquots of digest (0.1 ml) were removed at intervals and the increase in free amino groups due to enzymic hydrolysis was determined by the ninhydrin reaction. The rate of the hydrolysis of glucagon by pepsin and gastricsin is shown in Figure 2. It is apparent that the hydrolysis was completed after 10 hours of incubation.

Peptide Isolation

The glucagon peptides in the digestion mixtures were separated using high voltage paper electrophoresis. The results on Whatman No. 1 paper at pH 6.5 are shown in Figure 3. After the paper was stained with ninhydrin cadmium acetate reagent (49), the peptides appeared as pink, red, or orange spots. A separate run was made and treated with

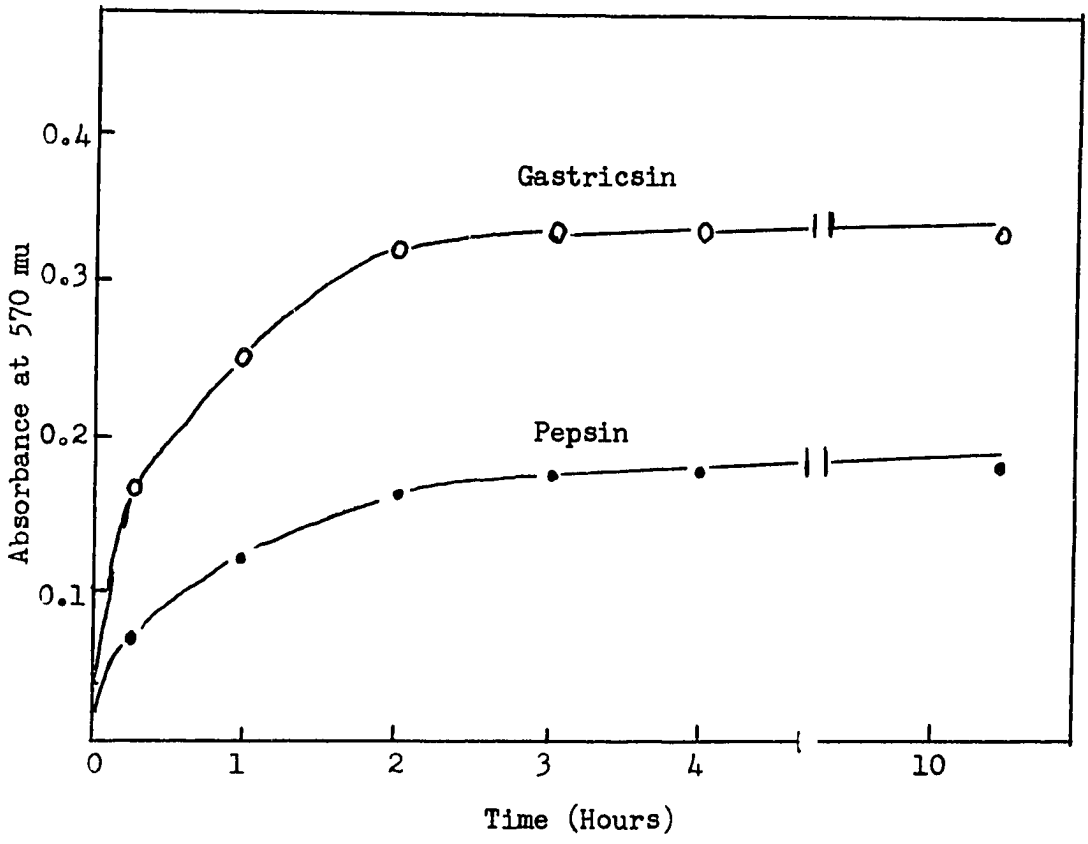


Figure 2. Time course of digestion of glucagon by pepsin and gastricsin. The incubation mixture (3 ml), containing 30 mg glucagon and 0.6 mg of enzyme, was incubated at 37° for 10 hours. Aliquots (0.1 ml) were removed for ninhydrin color reaction.

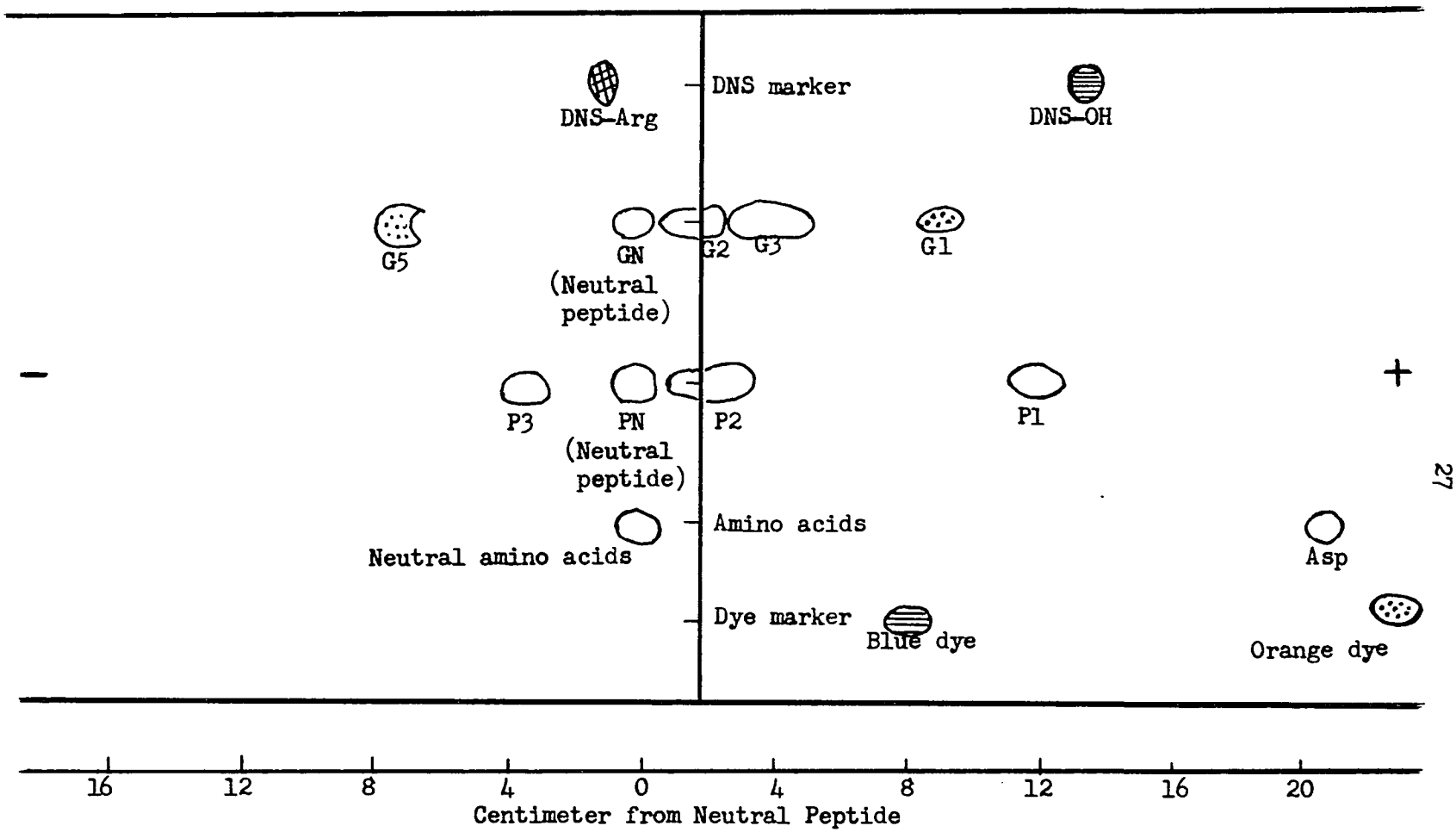


Figure 3. High-voltage electrophoretic separation of peptides derived from the hydrolysis of glucagon by pepsin and gastricsin. The electrophoresis was carried out at pH 6.5 and 3 kilovolts (60 volts/cm) for 45 minutes. P: stand for peptides derived from pepsin digest G: stand for peptides derived from gastricsin digest DNS-Arg: dansyl arginine DNS-OH: dansyl sulfonic acid ⬭ red color spot ⬭ blue color spot or blue fluorescence ⬭ orange color ⬭ yellow fluorescence

Pauly reagent in order to locate tyrosine and histidine containing peptides. With each electrophoresis, the dye marker and fluorescent markers were included with each electrophoretic run. These markers were used as references for electrophoretic mobility.

GN and PN mark the positions of neutral peptides. The pattern of acidic and basic peptides appeared to be different in the two digests. Peptides P1, G1 and G5 were relatively pure as judged from their mobility and amino acid compositions. The neutral peptides (PN, GN) and the slow moving peptides (P2, G2, G3) were further separated by high voltage electrophoresis at pH 2.0 and pH 3.5 respectively. The electrophoretic patterns of these peptides are shown in Figure 4. Both PN and GN were further separated into 3 spots (PN1, PN2, PN3, and GN1, GN2, GN3) by this procedure. The peptides which moved slowly at pH 6.5 (G2, G3, and P2) were also separated from minor impurities. Preparative runs were carried out in which the digestion mixture was applied on Whatman No. 3 MM paper as a band, and the purification carried out as described above. The peptides were eluted from the paper, dried in the desiccator, and saved for further analyses.

Structure of Glucagon Peptides

Each purified peptide was subjected to amino acid analysis, and then to N-terminal amino acid analysis by dansyl method. The dansyl amino acids were identified using a water cooled flat plate high-voltage electrophoretic apparatus. The results on Whatman No. 3 MM paper are shown in Figures 5 and 6.

Amino acid composition of glucagon peptides. The amino acid compositions of the purified peptides from the glucagon hydrolysate are

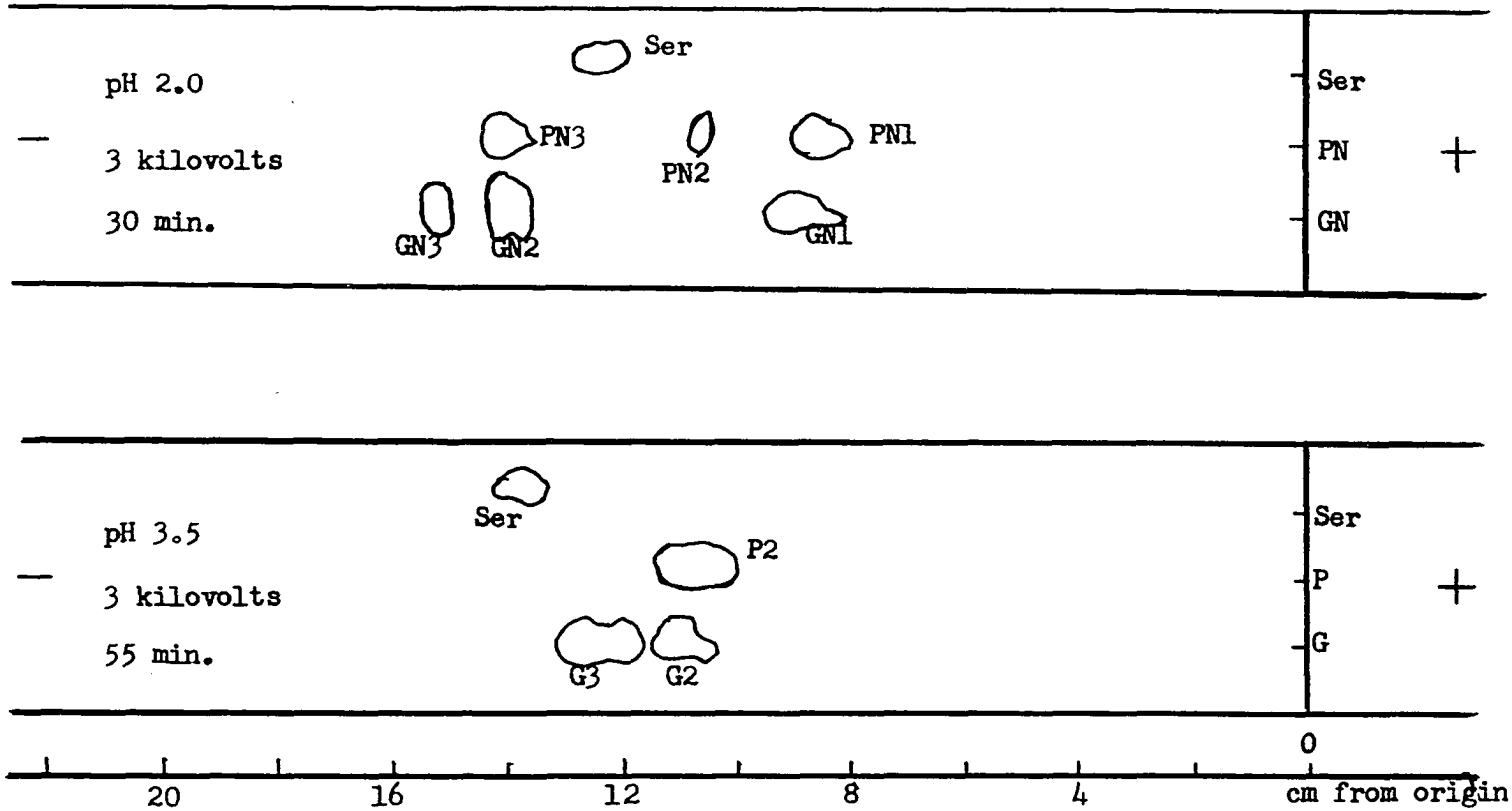


Figure 4. High voltage paper electrophoretic separation of neutral peptides derived from the hydrolysis of glucagon by pepsin and gastricsin. ⬭ red color spot
PN: stand for neutral peptides from the hydrolysis glucagon by pepsin
GN: stand for neutral peptides from the hydrolysis glucagon by gastricsin

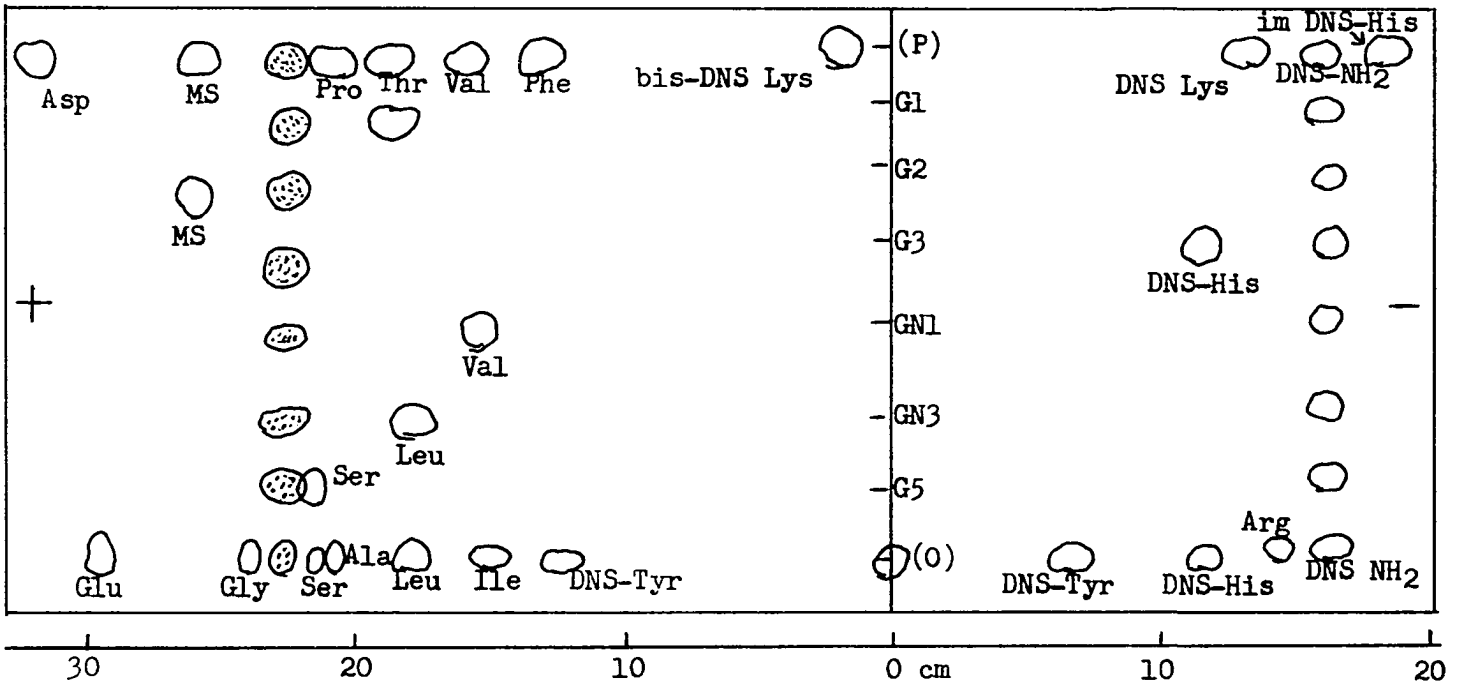


Figure 5. Electrophoresis of DNS-amino acids from the hydrolysis of DNS-derivatives of gastricsin peptides (G1, G2, G3, GN1, GN3, G5) on Whatman No. 3 MM paper at pH 4.55 on a flat plate. ⊛ : blue fluorescence of dansyl sulfonic acid, ○ : yellowish blue fluorescence of DNS-amino acids. (P) and (O) are standard DNS-amino acid mixtures.

G2 peptide was oxidized with performic acid and N-terminal amino acid was determined as dansylmethionine sulfone (MS).

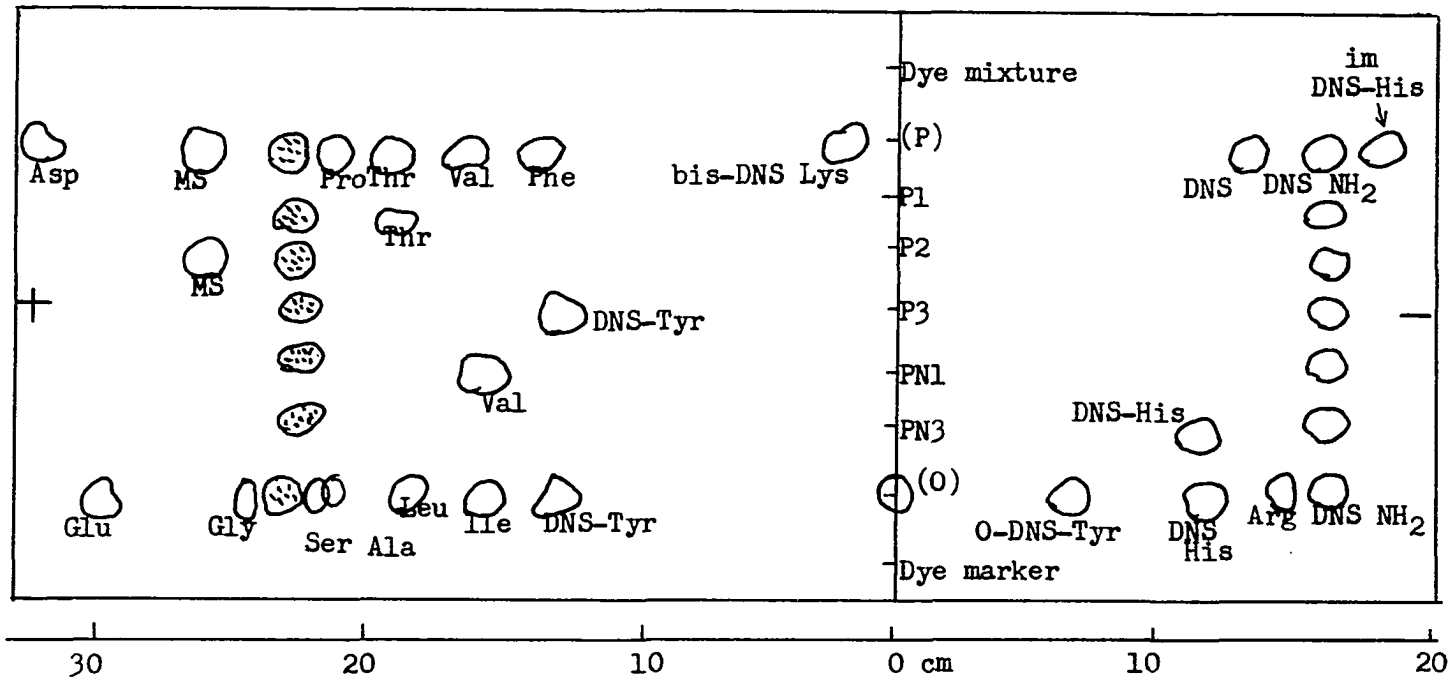


Figure 6. Electrophoresis of DNS-amino acids from the hydrolysis of DNS-derivates of peptic peptides (P1, P2, P3, PN1, PN3) on Whatman No. 3 MM paper at pH 4.55 on a flat plate.

◎ : blue fluorescence of dansyl sulfonic acid
○ : yellowish blue fluorescence of DNS-amino acids
(P) and (O) are standard DNS-amino acid mixtures.

P2 peptide was oxidized with performic and N-terminal amino acid was determined as dansylmethionine sulfone (MS).

shown in Table 7. The data are expressed as number of amino acid residues per peptide. The analytical results show the number of amino acid residues close to integral numbers. Most peptides appeared to be reasonably free from contamination, as judged by the fact that most of the values are near an integral number, and contain a single amino-terminal amino acid.

Sequence of glucagon peptides. From the amino acid composition and the N-terminal amino acids of the peptides, the sequences of these purified peptides could be established from the known sequence of glucagon, as shown in Table 8. Five pure peptides were identified in the pepsin digest and six peptides were identified in the gastricsin digests.

The Site of Bond Cleavage

From the data described above, it was thus possible to reconstruct the sites of bond cleavage by the two enzymes, as shown in Figure 7. The sequence of glucagon is shown in the two lines of Figure 7. The solid arrows show the peptides resulting from pepsin digestion, and the dotted arrows indicate the peptides resulting from gastricsin digestion. Four peptide bonds were cleaved by pepsin. They are: phe-thr [6-7], asp-tyr [9-10], phe-val [22-23], leu-met [26-27]. Six peptide bonds were cleaved by gastricsin. They are: phe-thr [6-7], asp-tyr [9-10], tyr-ser [10-11], tyr-leu [13-14], phe-val [22-23], leu-met [26-27]. Particularly interesting is the finding that tyr-ser [10-11] and tyr-leu [13-14] were only cleaved by gastricsin and not by pepsin.

TABLE 7

AMINO ACID COMPOSITIONS OF PEPTIDES DERIVED FROM HYDROLYSIS OF GLUCAGON BY PEPSIN AND GASTRICSIN

	Peptides												
	Pepsin						Gastricsin						
Amino Acids	P1	P2	P3	PN1	PN2	PN3	G1	G2	G3	GN1	GN2	GN3	G5
Aspartic Acid	1.20	1.14	2.29				1.39	1.00	1.31	1.15	2.40	2.14	
Glutamic Acid			1.19	1.03	1.03	0.98			1.26	1.03	1.75	1.14	
Threonine	1.00	0.80			1.00	0.92	1.00	1.00	2.32	1.02	6.67		
Serine	0.84		1.74		1.41	1.00	0.74		1.87		1.36	1.00	1.01
Glycine					0.68	0.88			1.34		0.67		
Alanine			1.15								1.00	1.08	
Valine				0.87						1.00			
Methionine		1.00						1.01		1.29			
Leucine			0.78	1.00	0.93					1.18	0.95	1.00	
Tyrosine			2.05		0.70		0.95						0.91
Phenylalanine			0.89		1.00	0.92			1.30		1.48	0.60	
Tryptophane				1.00						1.00			
Histidine					0.32	1.34			1.00		0.35		
Lysine			1.00		0.52								
Arginine			2.30								2.03	1.68	1.00
Total Residues	3	3	13	4		6	4	3	9	7		9	3

TABLE 8

SEQUENCE OF PEPTIDES DERIVED FROM THE HYDROLYSIS OF GLUCAGON BY PEPSIN AND GASTRICSIN

Peptide No.	Sequences
P1	Thr-Ser-Asp ⌈7-9⌋
P2	Met-Asn-Thr ⌈27-29⌋
P3	Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe ⌈10-22⌋
PN1	Val-Gln-Trp-Leu ⌈23-26⌋
PN2	⌈1-6⌋ + Leu + ⌈10-13⌋
PN3	His-Ser-Gln-Gly-Thr-Phe ⌈1-6⌋
G1	Thr-Ser-Asp-Tyr ⌈7-10⌋
G2	Met-Asn-Thr ⌈27-29⌋
G3	His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp ⌈1-9⌋
GN1	Val-Gln-Trp-Leu-Met-Asp-Thr ⌈23-29⌋
GN2	GN3 + His -------- Phe ⌈1-6⌋
GN3	Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe ⌈14-22⌋
G5	Ser-Lys-Tyr ⌈11-13⌋

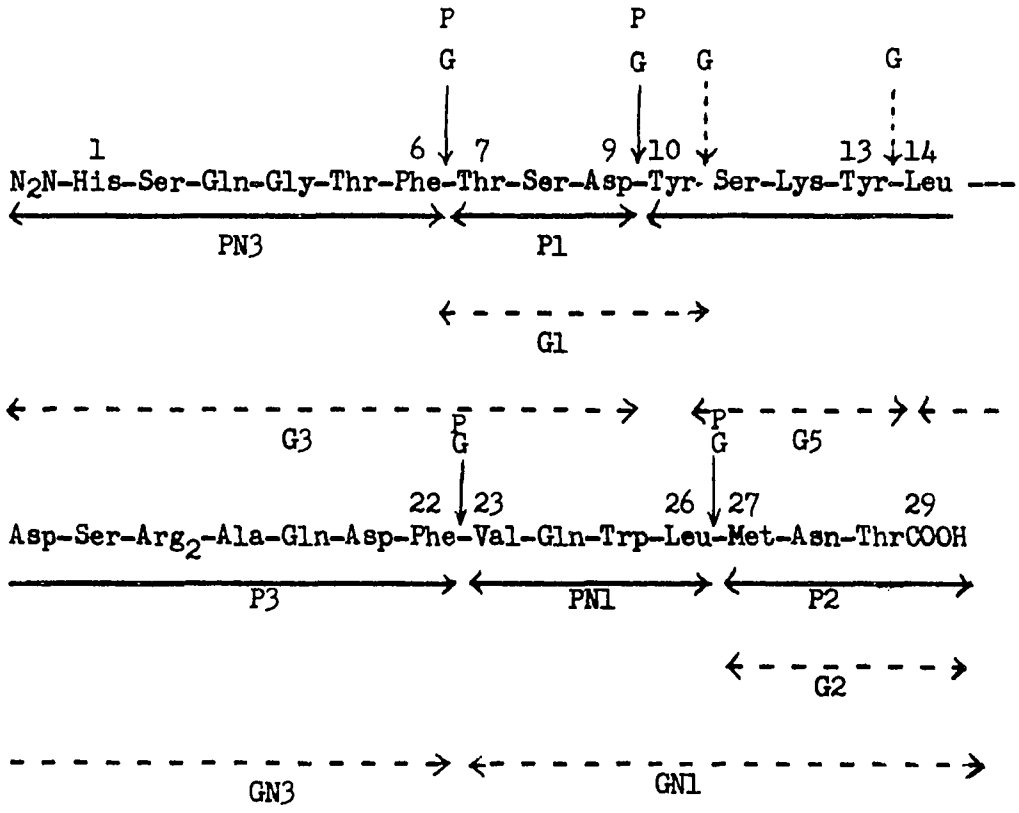


Figure 7. Primary Structure of Glucagon (34) and the Sites of Bond Cleavage by Pepsin and Gastricsin.

Hydrolysis of Oxidized Ribonuclease A

Enzymic Digestions

Digestion of oxidized ribonuclease A by human pepsin and gastricsin were carried out at pH 2.0 (0.2 N Na-citrate-HCl buffer) and 37° for pepsin; at pH 3.1 (0.2 N sodium citrate buffer) and 37° for gastricsin. The incubation mixture contained 10 mg oxidized ribonuclease A and 0.2 mg of enzyme in a volume of 1 ml buffer solution. At various stages of the digestion, 0.1 ml aliquots of the solutions were removed, and the increase of free amino groups in the solution was determined by ninhydrin reaction. The digestion was completed after 24 hours incubation, as judged by the course of hydrolysis shown in Figure 8.

Peptide Separation and Isolation

Chromatography on a column of Amberlite IR-120B was selected as the method to separate peptides from the digest of oxidized ribonuclease A. A column of 0.9 x 50 cm was used and the resin was prepared according to the procedure of Spackman, Moore and Stein (52). The column was first eluted with pH 3.25, 0.2 N citrate buffer for 3 hours, then with pH 4.25, 0.2 N citrate buffer for another 3 hours. Prolonged eluting with pH 4.25 buffer or pH 5.28 buffer did not produce further ninhydrin positive substances.

The separation patterns of the two enzymic digests of oxdized ribonuclease A were first investigated, using the automatic recording system of a Beckman Spinco Model 120B amino acid analyzer with a flow rate of 30 ml per hour. As shown in Figure 9, the chromatographic

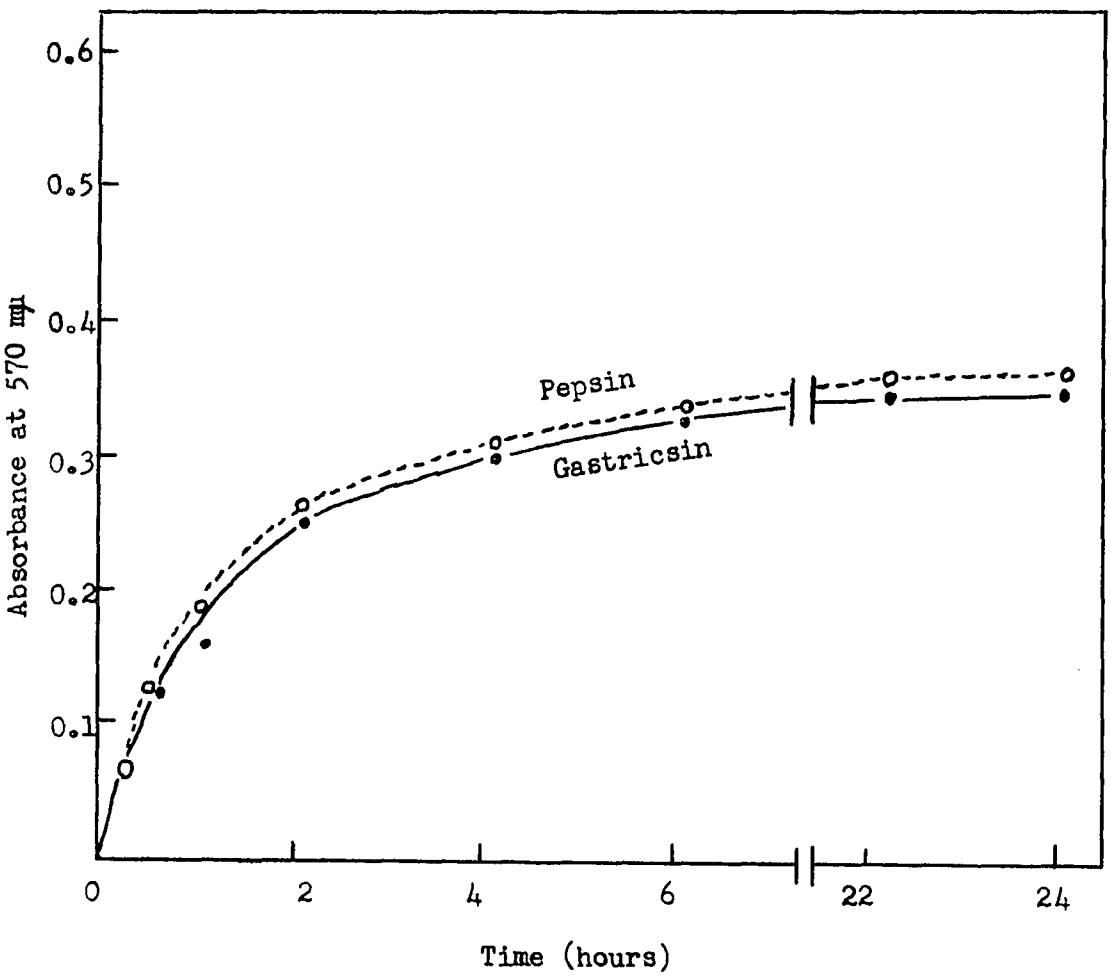
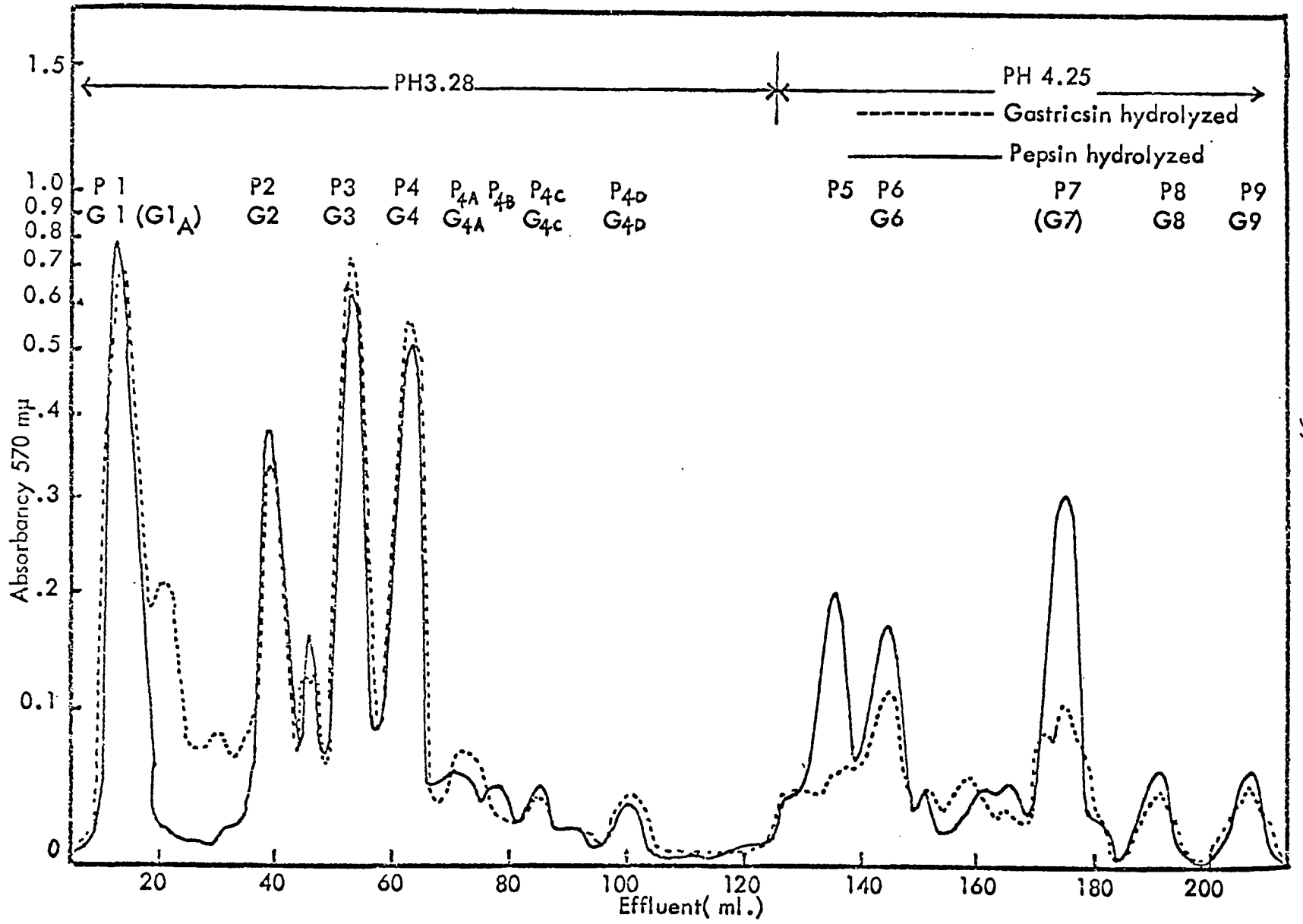


Figure 8. Time course of digestion of oxidized ribonuclease A by pepsin and gastricsin. Digestions of oxidized ribonuclease A were carried out with pepsin at pH 2.0 and 37° for 24 hours, and with gastricsin at pH 3.1 and 37° for 24 hours. Aliquots (0.1 ml) were removed for ninhydrin color reaction.

Figure 9. Elution pattern of the peptides derived from the hydrolysis of oxidized ribonuclease A by gastricsin and pepsin. The enzymic digests were chromatographed at 50° on a column of IR-120B (0.9 x 50 cm), 200 to 400 mesh, with 0.2 N sodium citrate buffers, at a flow rate of 30 ml per hour.


PH3.28
PH 4.25
Gastricsin hydrolyzed
Pepsin hydrolyzed
P 1
G 1 (G1A)
P2
G2
P3
G3
P4
G4
P4A P4B
G4A
P4C
G4C
P4D
G4D
P5
P6
G6
P7
(G7)
P8
G8
P9
G9
Absorbancy 570 mμ
Effluent(ml.)
0
0.1
.2
.3
.4
0.5
0.6
0.7
0.8
0.9
1.0
1.5
20
40
60
80
100
120
140
160
180
200

patterns of the peptides resulting from digestions by the two enzymes are similar. Several major peaks from pepsin digestion (P1, P2, P3, P4, P8 and P9) coincide well with those resulting from gastric digestion (G1, G2, G3, G4, G8 and G9). Minor differences could be observed between the two patterns. Among them were G1A, P5 and P7. The two chromatographic patterns were highly reproducible. In order to identify the major peptides, preparative runs were made using 50 mg of oxidized ribonuclease A in each enzyme digest under the same fractionation conditions. The fractions were pooled, desalted with Dowex-50 resin, and further purified by means of high voltage paper electrophoresis. Before application of the samples to the paper, the peptides were desalted by passing through a Dowex 50 x 2 (H^+ form) column. This was done by adjusting the pool of peptide peaks to pH 2.2 with 6 N HCl, and introducing this into a column (0.9 cm x 15 cm) of Dowex 50 x 2 resin (H^+ form). Citric acid or mono sodium citrate were washed out thoroughly with distilled water. This was followed by elution with 0.8 N ammonium hydroxide solution to obtain the purified peptides. The pattern of high voltage electrophoresis is shown in Figure 10. The solid circles represent major spots and the dotted dircles represent minor spots. Electrophoresis was carried out in a solution of pH 3.5 pyridine acetate buffer (pyridine / acetic acid / water 1 : 10 : 90) at 4000 volts (80 volts/cm) for 100 minutes. For preparative purposes, the samples were applied on the middle line of the papers. After electrophoresis, the separated bands were eluted with distilled water and saved for further analyses.

In order to have an independent separation method for this

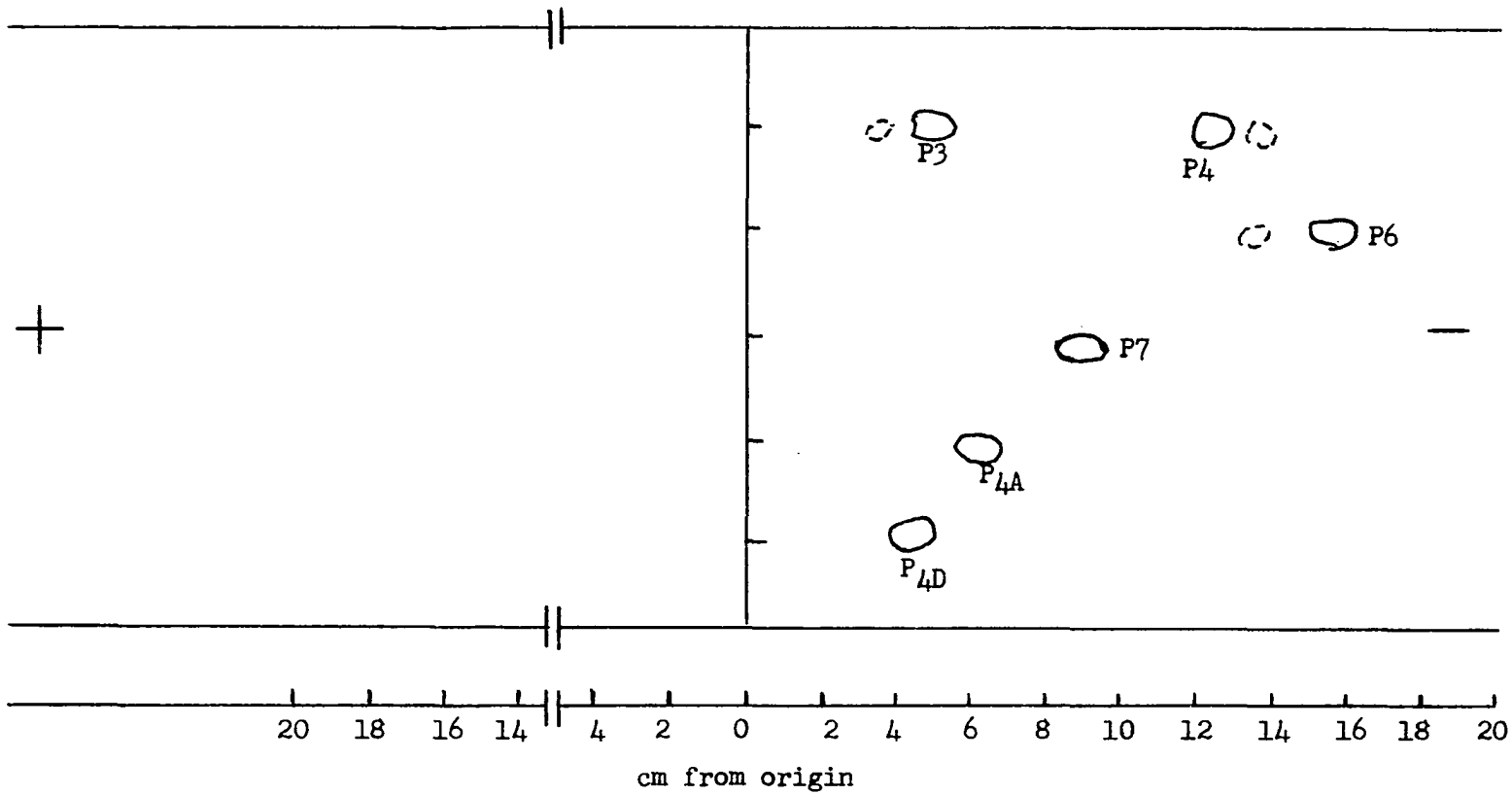


Figure 10. High voltage paper electrophoretic separation of peptides derived from the hydrolysis of oxidized ribonuclease A by pepsin. The electrophoresis was carried out at 4000 volts (80 volts/cm) at pH 3.5 for 100 minutes. The solid circles indicate the major spots, and the dotted circle indicate trace peptides.

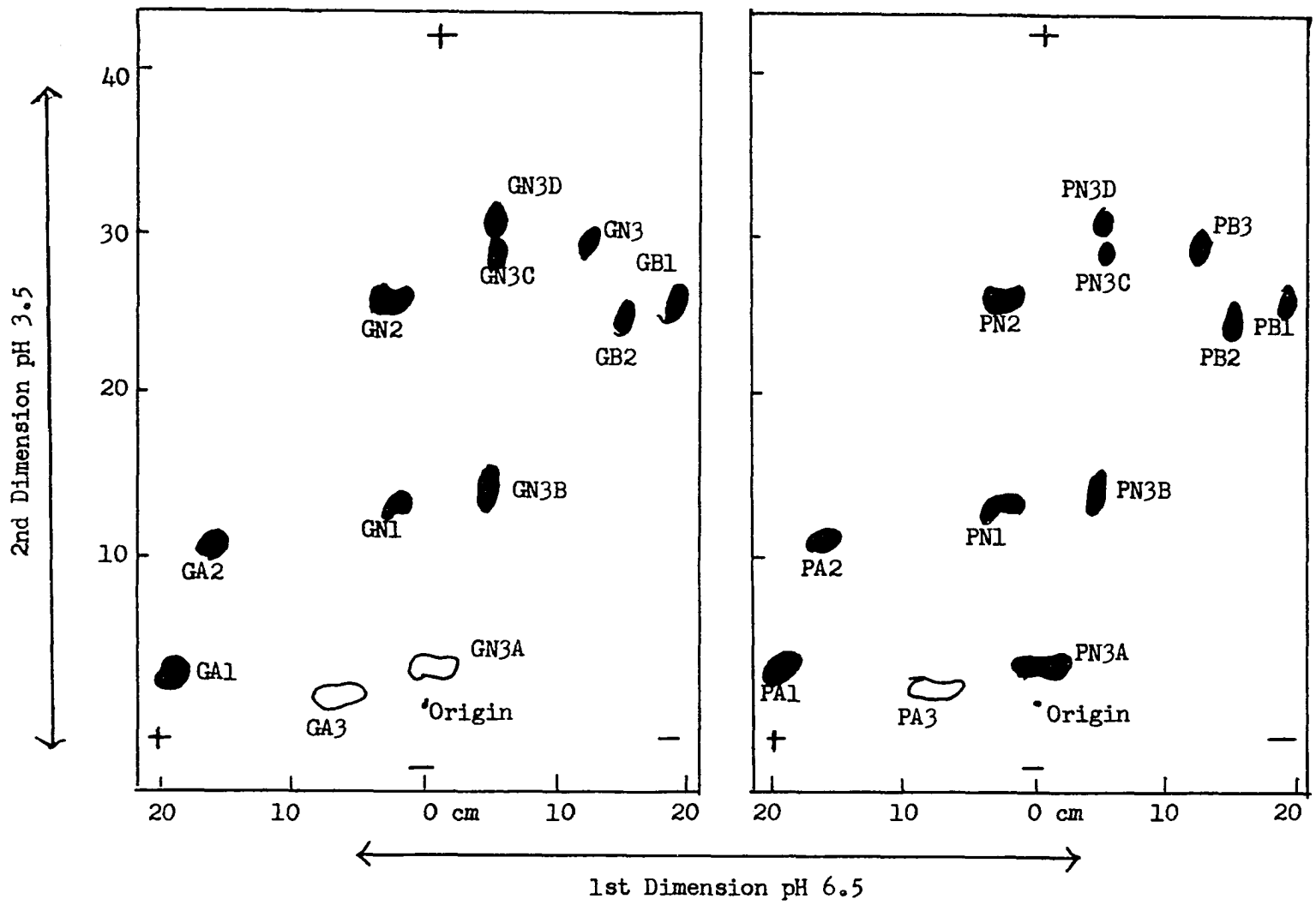
study, two dimensional high voltage paper electrophoresis was carried out on both enzymic digests. The conditions of incubation were much the same as previously described except for a switch to volatile buffers: pH 2.1 [formic acid / acetic acid / water (1 : 4 : 45)] for pepsin; and pH 3.1 [pyridine / acetic acid / water (1 : 14 : 85)] for gastricsin. The fingerprint patterns are shown in Figure 11. The first dimensional high voltage paper electrophoresis was carried out at pH 6.5. The second dimension was run at pH 3.5. The shaded spots are those found at identical positions in the two patterns. The light spots are those found to be at different positions.

For preparative purposes, the samples were applied as a band near the middle of a full sheet of Whatman No. 3 MM paper. After electrophoresis at pH 6.5, a narrow guide strip was cut out and stained with ninhydrin-cadmium reagent to locate peptide bands. Each band was cut out separatly and stitched to another sheet of Whatman No. 3 MM paper. The second electrophoresis was carried out at pH 3.5. Each purified peptide band was eluted with water, and dried in a desiccator over concentrated sulfuric acid. The purified peptides were saved for further analyses.

Analyses of Digestion Products

The purified peptides, obtained from column chromatography and two dimensional high voltage paper electrophoretic procedure, were analyzed for their amino acid compositions and for their N-terminal amino acids using either dinitrophenyl or dansyl method. From this information, the amino acid sequence of peptides could be inferred from

Figure 11. Two dimensional high voltage paper electrophoresis of the peptides derived from the hydrolysis of oxidized ribonuclease A by pepsin and gastricsin. Left side: the pattern of gastricsin digests. Right side: the pattern of peptic digests. The shaded spots are those found at identical positions in the two patterns. The light spots are those found to be at different positions.


2nd Dimension pH 3.5
40
30
20
10
GN3D
GN3
GB1
GN3C
GN2
GB2
GN3B
GN1
GA2
GA1
GN3A
GA3
Origin
PN3D
PB3
PN3C
PN2
PB1
PB2
PN3B
PN1
PA2
PA1
PN3A
PA3
Origin
20
10
0 cm
10
20
1st Dimension pH 6.5

the known sequence of ribonuclease A. The amino acid composition as well as the sequence of the peptides are shown in Tables 9, 10, 11 and 12.

The Site of Bond Cleavage

From the data obtained above, it was possible to indicate the site of peptide bond cleavage by these two enzymes (Figure 12). The solid arrows show the points of cleavage by both enzymes, and the dotted arrows indicate the bonds cleaved by either enzyme alone.

A number of different peptide bonds were split by both enzymes (Table 6). The tyr-ser bond [76-77] was cleaved only by gastricsin, not by pepsin, while val-ala [108-109] was cleaved only by pepsin, not by gastricsin.

Hydrolysis of Synthetic Dipeptides

The Hydrolysis of CBZ-dipeptides

It has been shown in the above experiments that two peptide linkages in glucagon were hydrolyzed only by gastricsin. They are tyr-ser and tyr-leu bonds. In the case of oxidized ribonuclease A, the bond, tyr-ser, was cleaved only by gastricsin. The result seem to indicate that gastricsin has certain degree of specificity for tyr-x bond.

It was decided then to try a series of tyrosyl dipeptides as substrates for the enzymes. If the observed differences in the hydrolysis of glucagon and ribonuclease A by the two enzymes could be demonstrated in synthetic dipeptide, some of the tyrosyl-dipeptides

TABLE 9

AMINO ACID COMPOSITIONS OF PEPTIDES FROM THE OXIDIZED RIBONUCLEASE A HYDROLYSATES

Amino Acid	Peptides					
	P3	P4	P_{4A}	P_{4D}	P6	P7
Aspartic Acid	1.01	1.12				
Glutamic Acid		1.13			0.98	
Alanine	1.04	1.01			1.27	
Valine	1.02	0.92				
Leucine				0.95		
Serine	0.95		2.02	1.10		
Threonine			1.04		0.91	
Tyrosine			0.85			
Phenylalanine						0.95
Methionyl Sulfone			1.00			
Lysine					0.95	
Total No. of Residue	4	4	5	2	4	1

TABLE 10

PROBABLE STRUCTURES OF PRODUCTS FORMED BY THE ACTION OF PEPSIN ON OXIDIZED RIBONUCLEASE A

Peptide or Amino Acid	Sequence	Position in Ribonuclease A
P6	Lys-Glu-Thr-Ala	1-4
P7	Phe	46
P_{4D}	Ser-Leu	50-51
P4	Ala-Asp-Val-Gln	52-55
P_{4A}	Ser-Tyr-Ser-Thr-Met SO_2	75-79
P3	Asp-Ala-Ser-Val	121-125

Note:

N-terminal amino acid of each peptide was determined by dinitrophenyl method.

TABLE 11

AMINO ACID COMPOSITION OF PEPTIDES DERIVED FROM THE HYDROLYSIS OF OXIDIZED RIBONUCLEASE A BY PEPSIN AND GASTRICSIN

Amino Acid	GN1	GN2	GN3B	GN3C	GN3D	GB1	GB2	GB3	GA1	GA2	GA3	PA3
Aspartic Acid	1.10	1.08		1.36					1.00	1.11	2.84	3.20
Glutamic Acid			1.06	1.00	1.00		1.03	1.06		1.34	2.61	2.20[b]
Threonine	0.936	1.00			0.99			1.12			1.00	1.25
Serine				0.98	0.83	0.84	1.09		0.78		1.36	1.34
Glycine					1.13						1.07	1.38
Alanine				1.00				1.00			2.09	2.26
Valine			1.00						1.01	1.00	2.09	2.30
Methionine							0.94		1.02	0.83		
Leucine	1.00			0.77			1.00					
Tyrosine						1.25					0.80[a]	0.76
Phenylalanine				0.78	0.92		0.99					
Histidine					0.92		0.95					
Lysine						1.00		0.97			1.64	1.70
Arginine				1.00								
Cysteic Acid											2.61	2.90
Total Residue	1 + 2	2	2	2 + 4 + 1	4 + 2	3	6	4	4	4	19	18

[a]Tyrosine was determined as a C-terminal amino acid followed by serine.

[b]Glutamine was determined as a C-terminal amino acid followed by tyrosine.

TABLE 12

PROBABLE STRUCTURE OF PRODUCTS FORMED BY THE ACTION OF GASTRICSIN ON OXIDIZED **RIBONUCLEASE A**

Peptide No.	Sequence
GN1	Leu + Asn-Thr [44-45]
GN2	Asn-Thr [44-45]
GN3B	Val-Gln [54-55]
GN3C	Arg-Asp [33-34] + Phe + Ala-Leu-Ser-Glu [49-52]
GN3D	Phe-His [120-121] + Glu-Thr-Gly-Ser [86-89]
GB1	Ser-Lys-Thr [90-92]
GB2	Phe-Val-His-Glu-Ser-Leu [46-51]
GN3	Lys-Glu-Thr-Ala [1-4]
GA1	Asp-Ala-Ser-Val [121-124]
GA2	Ala-Asp-Val-Gln [52-55]
GA3	Ala-Val-Cys-Ser-Gln-Lys-Asn-Val-Ala-Cys-Lys-Asp-Gly-Gln-Thr-Asn-Cys-Thr-Gln-Ser-Tyr [56-76]

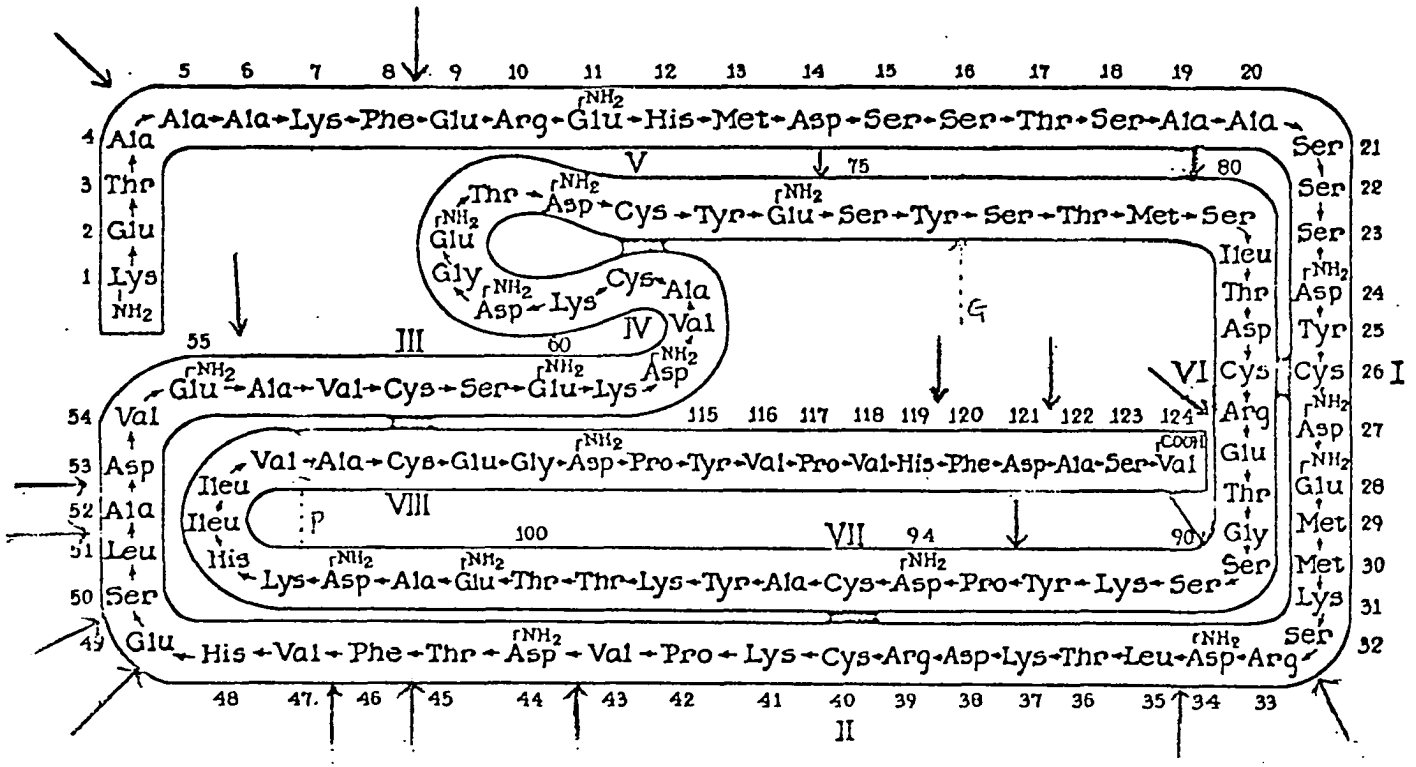


Figure 12. The amino acid sequence of bovine pancreatic ribonuclease A (35), and the position of cleavage by pepsin and gastricsin.

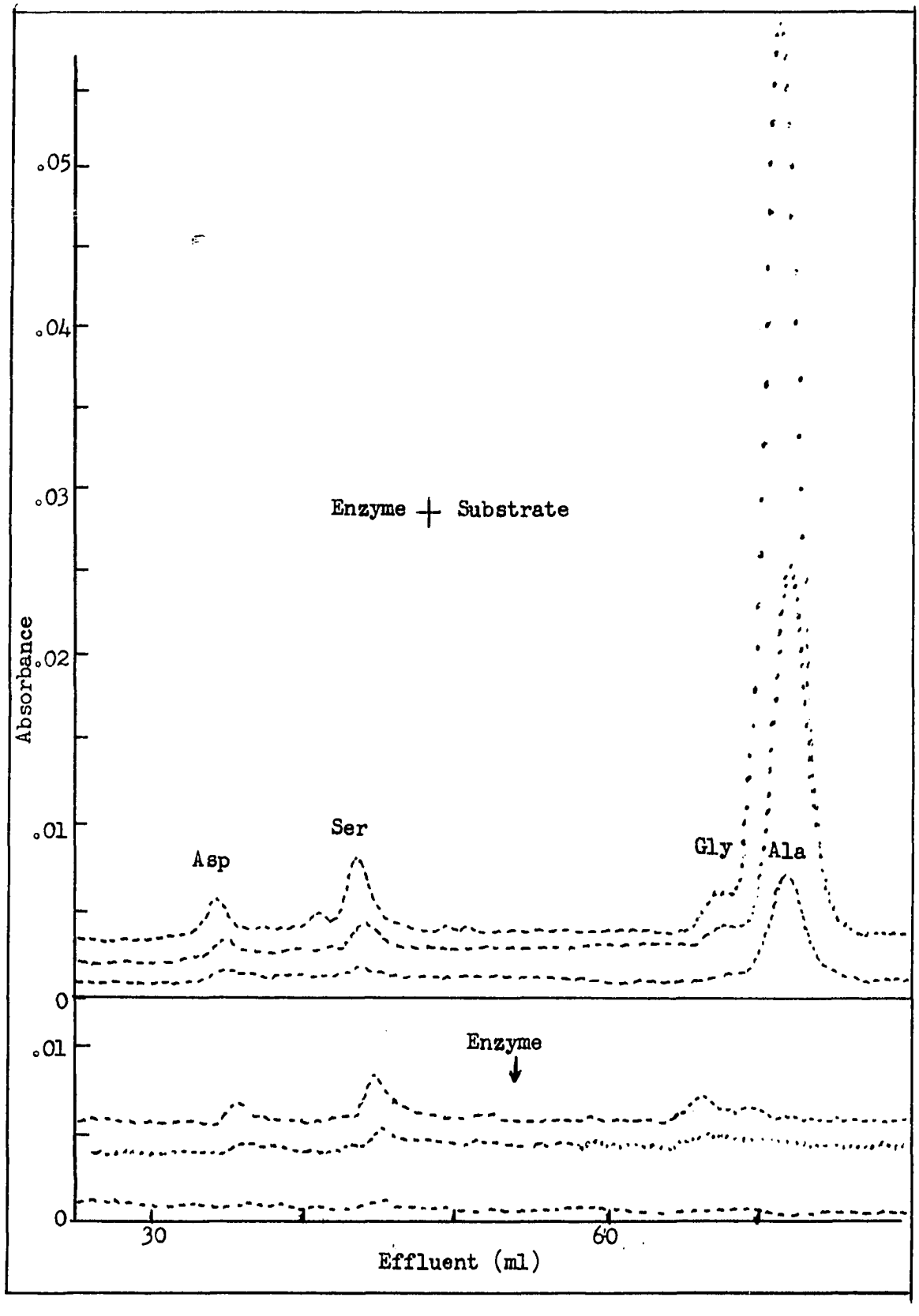
——→ The sites of bond attacked by both enzymes.

- - - → The sites of bond attacked by either enzyme alone.

such as CBZ-tyr-ser or CBZ-tyr-leu would be hydrolyzed by gastricsin but not by pepsin. In this series of experiments , 0.2 mg to 1 mg of the peptide was dissolved in 0.5 ml of 0.1 N citrate-HCl buffer, pH 2.0. Due to low solubility of most of the peptides, it was necessary to heat this peptide solution to 60°C for 5 minutes with vigorous shaking. An highest amount of each peptide that would dissolved completely by this procedure was used as substrate. Enough of each peptide was used to produce near saturated solution. Fifty µl of enzyme solution (4 mg/ml aqueous solution) was added to each tube containing 0.5 ml of 0.1 N citrate-HCl buffer and a substrate. After incubation at 37° for 5 to 8 hours, the reaction was stopped by heating the mixture in a boiling water bath for 5 minutes. The precipitate of inactive enzyme was filtered off and 0.2 ml aliquots of the filtrate were analyzed with the amino acid analyzer.

In a preliminary experiment, it was found that during the incubation at 37° for 7 hours, there was essentially no hydrolysis of the peptide in the absence of the enzyme. However, the incubation of either human pepsin or gastricsin, in the absence of the substrate peptide, resulted in a slight increase of ninhydrin positive substance, indicating the presence of some autodigestion of the enzyme. It was thus necessary to distinguish between the true hydrolysis of the peptide, and the ninhydrin positive substances formed by autodigestion of the enzymes. This was accomplished by means of a Beckmann Spinco Model 120B amino acid analyzer. The chromatograms from amino acid analyses are shown in Figure 13. Small ninhydrin-positive peaks were always observed in the record from the amino acid analyzer, perhaps

Figure 13. The chromatograms from amino acid analyses of the hydrolysis of CBZ-L-tyrosyl-L-alanine by gastricsin and of the control experiment of enzyme incubation alone.


.05
.04
.03
.02
.01
0
Absorbance
Enzyme + Substrate
Asp
Ser
Gly
Ala
.01
0
Enzyme
30
60
Effluent (ml)

due to the autodigestion of the enzyme. Since the rate of hydrolysis of synthetic peptides was measured after only 2 to 8 hours of incubation, the effect of autodigestion was assumed to be insignificant. The chromatogram from amino acid analysis of incubation with enzyme alone indicate several low peaks at the position of aspartic acid, serine, threonine, glycine and alanine. The amino acids analysis chromatogram of the CBZ-tyr-ala hydrolysate showed a high peak of alanine compared with small peaks of autodigestion products. It is apparent that this high peak of alanine was the product of the hydrolysis of CBZ-tyr-ala.

The analytical results obtained for CBZ-dipeptides are shown in Table 13. The first five synthetic dipeptides were the results of work previously reported by Tang (7, 36). Other CBZ-dipeptides were shown in the decreasing order of the specific activity of gastricsin.

The Hydrolysis of Iodinated Dipeptides

Iodination of peptides. In order to study the steric effect of iodine atoms incorporated into the peptide substrates on the specificity of the two enzymes, three dipeptides, CBZ-L-tyr-L-ala, CBZ-L-tyr-L-ser, CBZ-L-tyr-L-thr, were iodinated according to the procedure of Hughes and Straessle (53) with slight modification. The dipeptides (5 mg) were dissolved in a ml of 0.1 M sodium bicarbonate solution. To this solution, iodine solution (100 mg KI + 100 mg I_2 + 5 ml H_2O) was added dropwise with vigorous shaking at room temperature until the slightly yellow color persisted. Next several drops of 6 N HCl was added to make the solution acidic and the white precipitate that appeared was washed twice with several drops of water to remove potassium iodide

TABLE 13

THE HYDROLYSIS OF SYNTHETIC DIPEPTIDES BY GASTRICSIN AND PEPSIN

Substrate	Gastricsin	Pepsin
	Specific Activity[a]	
N-acetyl-L-phe-L-tyr	.23	.77
N-acetyl-L-phe-L-diiotyr	0	3.92
CBZ-L-glu-L-tyr	.0168	.0342
CBZ-gly-L-phe	.0018	.0060
CBZ-L-glu-L-phe	.0012	.0240
CBZ-L-tyr-L-ala	.0910	.002
CBZ-L-tyr-L-thr	.0521	.002
CBZ-L-tyr-L-leu	.0272	.002
CBZ-L-tyr-L-ser	.0193	.002
CBZ-L-tyr-L-val	.0084	.0042
CBZ-L-tyr-L-tyr	.0076	.0337
CBZ-L-tyr-L-phe	.0021	.0212
CBZ-L-tyr-gly	.0002	0
CBZ-L-ser-L-tyr	0	0
CBZ-L-ala-L-tyr	0	0
CBZ-L-phe-L-ser	0	0

[a] μmole amino acid released per mg enzyme per hour.

and hydrochloric acid. The washed precipitate was then dissolved in pH 2.0 at 60°C to make a saturated solution.

Enzymic digestion of iodinated peptides. Fifty µl of either pepsin or gastricsin solution containing 0.2 mg of enzyme was added to each 0.5 ml of saturated solution of iodinated dipeptide. The mixture was incubated at 37° for 5 hours. The reaction was stopped by heating in a boiling water bath for 10 minutes to inactivate the enzyme. Aliquots (0.2 ml) were taken for amino acid analysis. The specific activity of gastricsin toward the iodinated dipeptides was 30% lower than with the CBZ-tyrosyl dipeptides as substrates. Although pepsin did not hydrolyze CBZ-L-tyrosyl-L-alanine, CBZ-L-tyrosyl-L-serine and CBZ-L-tyrosyl-L-threonine, pepsin could hydrolyze the iodinated CBZ-tyrosyl-dipeptides to the extent of 50% of the specific activity of gastricsin toward CBZ-tyrosyl dipeptides.

Kinetic Measurements

From the results shown above, it is clear that pepsin does not hydrolyze the dipeptides, CBZ-tyr-ala, CBZ-tyr-ser and CBZ-tyr-thr. It was thus decided to study the kinetic of the hydrolysis of these particular substrates by gastricsin.

Determinations of enzymic hydrolysis were carried out in the same manner as described previously. The incubation mixture contained 0.2 mg of purified gastricsin, and substrate at the desired concentration, in a total of 0.55 ml of 0.1 N sodium citrate buffer, pH 2.0. This is the optimal pH for the hydrolysis of synthetic substrates by gastricsin. The samples were incubated at 37° for 2 to 7 hours depending on the

rate of hydrolysis. After incubation, the enzyme was inactivated by placing the tubes in a boiling water bath for 10 minutes. The samples were then filtered, and aliquots of the filtrates (0.2 ml) were adsorbed on the column (0.9 x 40 cm) of the amino acid analyzer and eluted with 0.2 M sodium citrate pH 3.25. Two control samples were used. One contained the enzyme without substrate, and the other contained the substrate alone. The enzyme control sample showed the presence of several small ninhydrin positive peaks appearing at the positions of aspartic acid, threonine, serine, glycine and alanine. Since the peaks in this blank resulted from autodigestion, they were substracted from the free amino acid values determined in the digestion mixture. The substrate control showed no peaks of ninhydrin positive substances.

The Time Course of Hydrolysis of A Synthetic Dipeptide with Gastricsin

When CBZ-L-tyrosyl-L-serine (1.8 umole) was incubated with gastricsin (0.4 mg) in 1 ml of 0.1 M sodium citrate-HCl buffer at pH 2.0 and 37°, an apparent linear increase of ninhydrin color reaction with time was observed (Figure 14). Under these experimental conditions only 2.7% of substrate was hydrolyzed after 6 hours of incubation.

pH-Activity Curve

The pH-activity curve for the hydrolysis of CBZ-L-tyr-L-ser by gastricsin was obtained by measuring quantitatively the ninhydrin color reaction after incubating enzyme and substrate at pH values ranging from 1.5 to 4.5. The incubation mixture (0.5 ml) contained 0.2 mg of

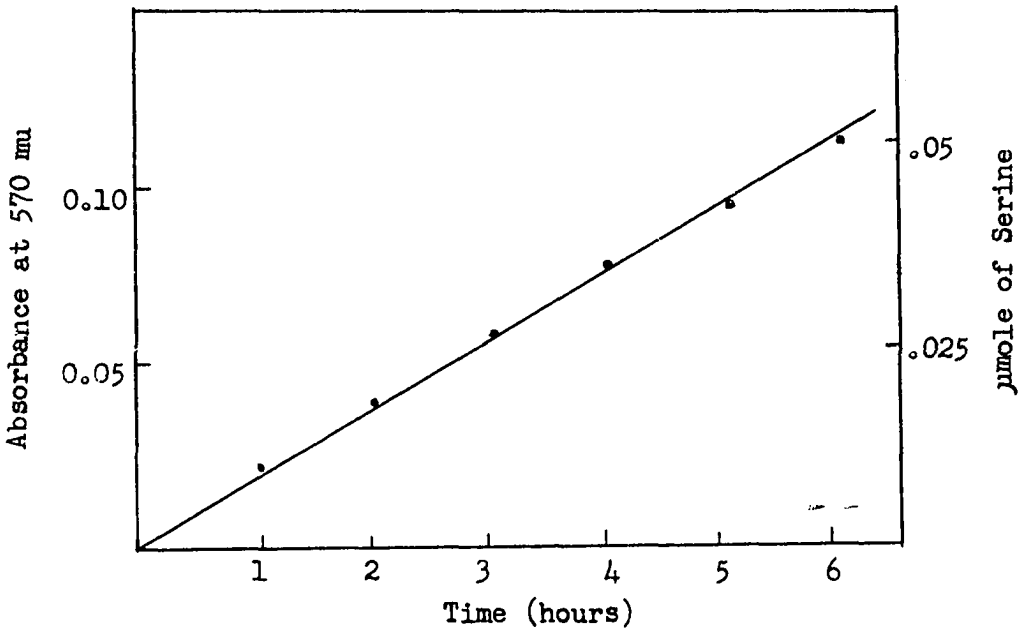


Figure 14. Hydrolysis of N-CBZ-L-tyrosyl-L-serine by gastricsin (0.4 mg) at pH 2 and 37°. The extent of hydrolysis was determined by the ninhydrin method. Aliquots (0.1 ml) were analyzed at one hour intervals.

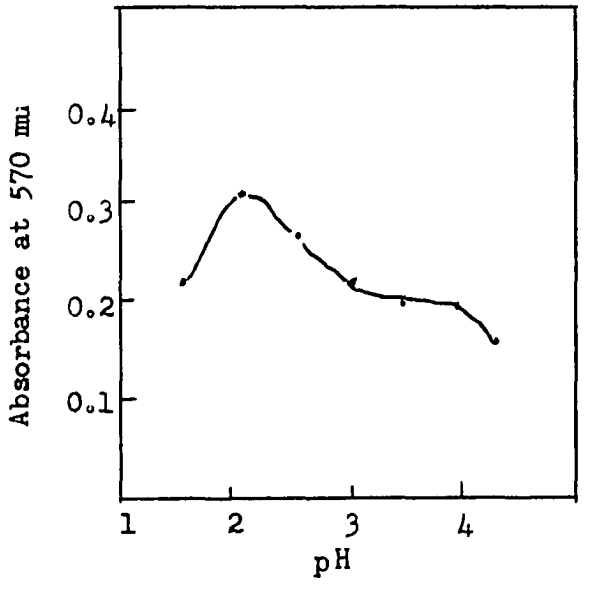


Figure 15. The pH-activity curve of gastricsin toward CBZ-L-tyrosyl-L-serine.

gastricsin and a saturated solution of CBZ-L-tyrosyl-L-serine (1.8 mM) in 0.1 M sodium citrate-HCl buffer. The maximum activity was observed at pH 2.0 (Figure 15), which differed from the optimum pH 3.0 of gastricsin when hemoglobin was used as substrate.

The Rate of Hydrolysis Versus Enzyme Concentration

Five-tenths ml each of 0.5 mM CBZ-L-tyr-L-ala was incubated with five different concentrations of enzyme at pH 2.0 and 37° for two hours. The rate of hydrolysis was measured by quantitative amino acid analysis. Figure 16 shows that the rate of hydrolysis was strictly proportional to enzyme concentration.

The Graphical Determination of K_m and V_m

For each substrate, the rate of hydrolysis was measured at five different substrate concentrations. In all experiments, the initial rate of the reaction was measured by limiting the hydrolysis below 15% of the total substrate concentration. All kinetic experiments were plotted according to the method of Lineweaver and Burk (54) (Figures 17, 18 and 19). The values of K_m and V_m were graphically determined from these plots, and a list of the values of K_m, K_3 and $-\Delta F^\circ$ are shown in Table 14.

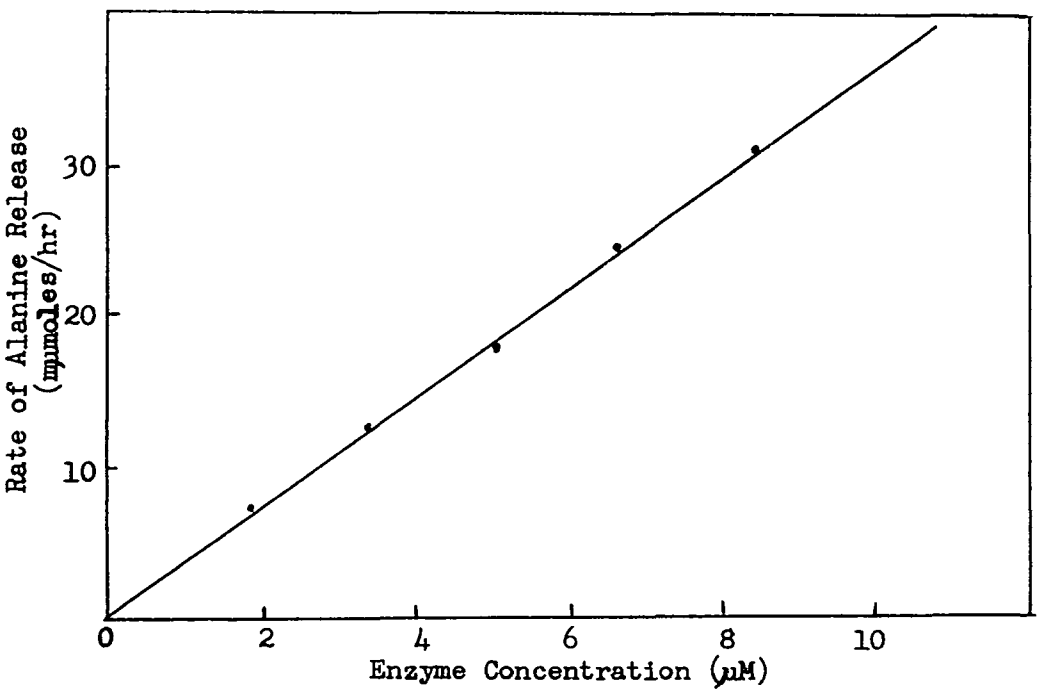


Figure 16. Proportionality of velocity to enzyme concentration in the gastricsin assay. The extent of peptide hydrolysis was determined by amino acid analysis. The substrate was 0.5 mM CBZ-L-tyr-L-ala. Hydrolyses were performed in 0.1 M sodium citrate-HCl buffer, pH 2.0, and 37°.

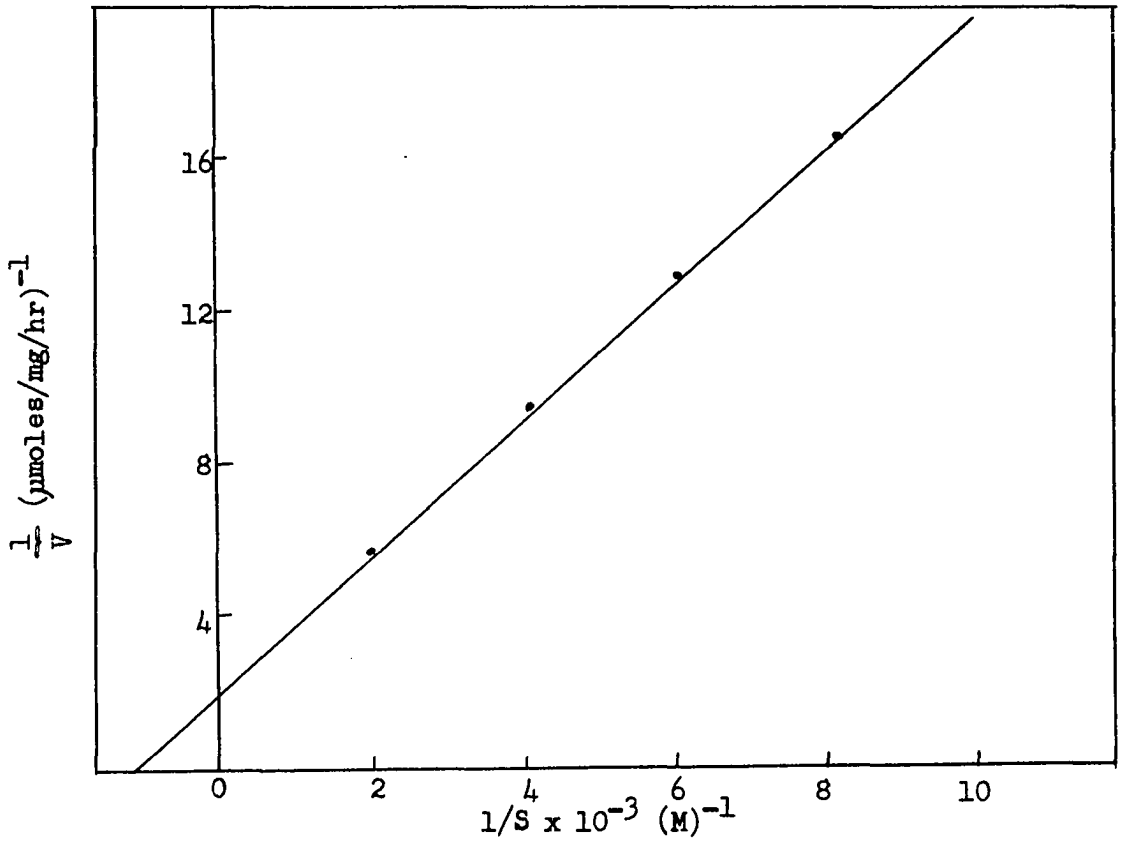


Figure 17. Graphical determination of K_m and V_m for the action of gastricsin on CBZ-L-tyr-L-ala. Enzyme concentration was 0.4 mg/ml. Hydrolyses were performed at 37°, in 0.1 M sodium citrate HCl buffer, pH 2.0. The alanine released from hydrolysis was determined by amino acid analysis.

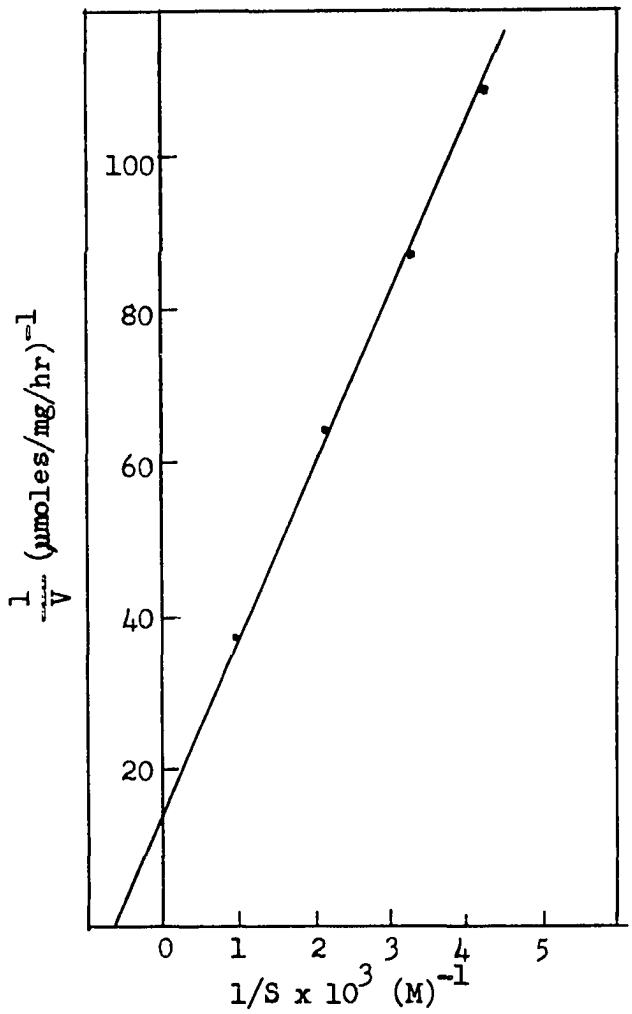


Figure 18. Graphical determination of K_m and V_m for the action of gastricsin on CBZ-L-tyr-L-ser. Enzyme concentration was 0.4 mg/ml. Hydrolyses were performed at 37°, in 0.1 M sodium citrate-HCl buffer, pH 2.0. The serine released from dipeptide was determined by amino acid analysis.

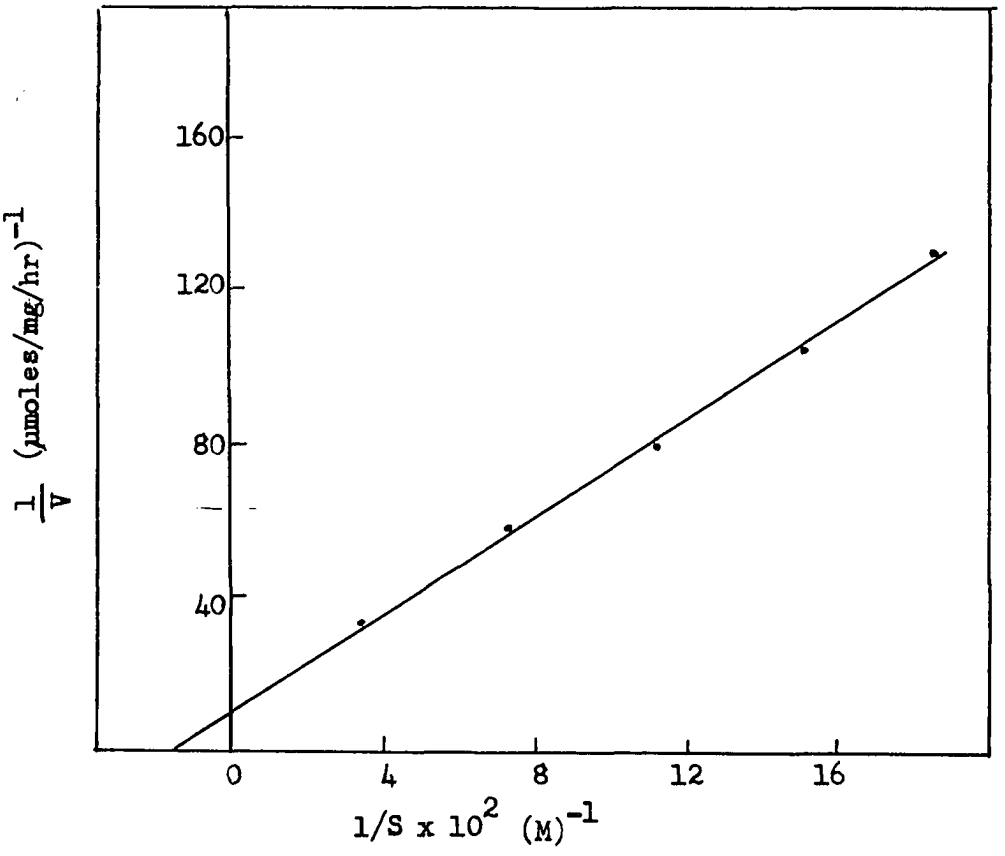


Figure 19. Graphical determination of K_m and V_m for the action of gastricsin on CBZ-L-tyr-L-thr. Enzyme concentration was 0.4 mg/ml. Hydrolyses were performed at 37°, in 0.1 M sodium citrate-HCl buffer, pH 2.0. The threonine released from dipeptide was determined by amino acid analysis.

TABLE 14

KINETICS OF PEPSIN AND GASTRICSIN ACTION
ON SYNTHETIC DIPEPTIDES AT pH 2.0

Dipeptide Substrates of Pepsin	K_m (M)	K_3 sec^{-1}	$-\Delta F^o$ Kcal/mole
N-acetyl-L-phenylalanyl -L-diiodotyrosine(31)	7.5×10^{-5}	20×10^{-2} sec^{-1}	5.85
N-acetyl-L-phenylalanyl -L-dibromotyrosine(32)	9.3×10^{-5}	1.97×10^{-2} sec^{-1}	- -
N-acetyl-L-tyrosyl-L-phenylalanine (31)	2.0×10^{-3}	0.3 min^{-1}	3.8
N-acetyl-L-tyrosyl-L -tyrosine (31)	6.1×10^{-3}	0.88 min^{-1}	3.8
Dipeptide Substrates of Gastricsin	**K_m (M)**	**K_3 hr^{-1}**	**$-\Delta F^o$ Kcal/mole[a]**
CBZ-L-tyrosyl-L-alanine	7.7×10^{-4}	15.40	4.41
CBZ-L-tyrosyl-L-ser	2.0×10^{-3}	2.38	3.83
CBZ-L-tyrosyl-L-threonine	8.3×10^{-3}	3.09	2.95

[a]The standard free energies of combination of gastricsin with each substrate ($-\Delta F^o$) were calculated on the assumption that K_m (Michaelis constant) is equal to K_s (Dissociation constant) and using the expression $-\Delta F^o = 2.303\ RT \log K$ where $R = 1.986$ cal x deg^{-1} x $mole^{-1}$, $T = 310^o$, $K = \frac{1}{K_s}$.

CHAPTER IV

DISCUSSION

The present study was undertaken to obtain detailed information on the specificity of gastricsin and pepsin. As reviewed in the introduction, it has been established that these two enzymes differ from each other in many properties, such as optimal pH, electrophoretic mobility, molecular weight, N-terminal amino acid, the sequence of the C-terminal first 3 amino acid residues, amino acid composition, and even the overall conformation of the molecules.

Several questions seem to be relevant to this current study.
(a) How does the specificity of gastricsin differ from pepsin and through which specific amino acid side chains is this reflected?
(b) Are there bonds that are susceptible to attack by both enzymes?
(c) Can the specificity of pepsin and gastricsin observed with proteins also be demonstrated with synthetic dipeptide substrates?

In this investigation, to these questions the answer are provided by the experimental results obtained from the hydrolysis of oxidized ribonuclease A, glucagon, and from the kinetic studies with a series of synthetic dipeptides, CBZ-L-tyrosyl-L-alanine, CBZ-L-tyrosyl-L-serine, and CBZ-L-tyrosyl-L-threonine.

Hydrolysis of Protein Substrates

On the basis of the results obtained from the identification of the peptides from glucagon and oxidized ribonuclease A hydrolysates, common sites of bond cleavage by both enzymes could be inferred. Those susceptible bonds are shown in Table 6.

Pepsin and gastricsin cleave similar bonds. There are only a few bonds cleaved by gastricsin but not by pepsin and vice versa (Table 6). The tyrosyl-serine bond was cleaved in both proteins by gastricsin; tyrosyl-leucine was cleaved only by gastricsin when glucagon was used as the substrate molecule.

Although there are six tyrosine residues in the oxidized ribonuclease A molecule, five of them are linked with unfavorable amino acid residues, such as cysteic acid or a basic amino acid. Only the tyrosine residue at position 76 has two serine residues on either side. Therefore tyr-ser [76-77] is susceptible to gastricsin hydrolysis.

The amino acids on either side of the common susceptible bonds in both protein hydrolysates contain hydrophobic side chains. This suggests that hydrophobic interactions may be required for hydrolysis to occur in either case.

The peptide bonds tyr-ser and tyr-leu were cleaved only by gastricsin but not by pepsin. This interesting finding led us to test a series of CBZ-tyrosyl dipeptides and other related dipeptides. The reason that pepsin does not hydrolyze tyr-ser or tyr-leu bond may be that the binding of this part of side chain of amino acid to pepsin is not as strong as to gastricsin.

Hydrolysis of Synthetic Dipeptides

The results obtained in the study of the hydrolysis of dipeptides by gastricsin and pepsin agree with the observation made when glucagon and oxidized ribonuclease A were used as substrates. It may be recalled that there were three linkages in glucagon and oxidized ribonuclease A cleaved by gastricsin but not by pepsin. They are all tyrosyl-x peptide linkages. It is clear from the results that some of the tyrosyl dipeptides, i.e., CBZ-tyr-leu, CBZ-tyr-thr, CBZ-tyr-ser, and CBZ-tyr-ala are specific synthetic substrates for gastricsin but not for pepsin. Since the hydrolysis of peptide bonds by pepsin is known to be dependent on the hydrophobic interaction of the enzyme and the hydrocarbon side chain of the substrate (24), it is interesting to see the effect of the size of the hydrophobic side chain of the substrate on the hydrolysis by gastricsin. From the results given in Table 13, it was found that variations in the nature of the amino acid on either side of the susceptible peptide bond could affect the susceptibility of the bond to hydrolysis by gastricsin. CBZ-ser-tyr and CBZ-ala-tyr were resistant to hydrolysis by gastricsin; while CBZ-tyr-ser and CBZ-tyr-ala were readily hydrolyzed by gastricsin. When phenylalanine was substituted for tyrosine in a CBZ-tyrosyl dipeptide, there was a large decrease in the rate of hydrolysis. Although a side chain of phenylalanine is a good hydrophobic group, CBZ-phe-ser was resistant to gastricsin attack. This observation may be explained by stating that hydrogen bonding may play some role in the binding of dipeptide substrates with gastricsin; or some residue of tyrosine in the gastricsin molecule may exist in the active site of the molecule.

The fact that N-acetyl-L-phenylalanyl-L-diiodotyrosine was hydrolyzed by pepsin but not by gastricsin may be accounted for by the steric hindrance of the L-diiotyrosine side chain of the substrate to the enzyme-substrate binding of gastricsin. From this difference in specificity, it might be explained that pepsin probably requires hydrophobic regions on the second amino acid in the peptide. This is also supported by the evidence that CBZ-L-tyr-L-tyr and CBZ-L-tyr-L-phe were readily cleaved by pepsin.

When CBZ-diiodotyrosyl dipeptides were used as substrates for pepsin, pepsin hydrolyzed them 50-70% as well as gastricsin could hydrolyze the same non-iodinated substrates. An explanation is that iodination of tyrosine increased the pepsin-substrate binding. On the other hand, gastricsin hydrolyzed CBZ-diiodotyrosyl dipeptides at 70% of the hydrolysis rate of the CBZ-tyrosyl dipeptides. This observation may be explained by stating that steric hindrance overcomes hydrophobic effect in case of gastricsin.

The Kinetic Data

From the data in Table 13, CBZ-tyr-ala, CBZ-tyr-ser, CBZ-tyr-thr were found to be good synthetic substrates for kinetic investigation. The primary purpose of these studies was to assess the influence of alanine, serine and threonine on the kinetic parameters of the hydrolysis of dipeptides by gastricsin.

Since the hydrolysis rate of dipeptides was relatively slow, we assumed that the observation made on the gastricsin-catalyzed hydrolysis of CBZ-L-tyrosyl dipeptides fits the following kinetic

scheme:

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} E + P_1 + P_2$$

E represents gastricsin; S represents the CBZ-dipeptides; P_1 is CBZ-tyrosine, and P_2 represents either serine, alanine or threonine. Kinetic data have been plotted throughout the present study according to the equation. K_m and V_m were obtained from the plot according to the method of Lineweaver and Burk, and K_3 was calculated from V_{max} as shown in Figures 19, 20 and 21.

$$\frac{[S_o]}{V} = \frac{1}{V_{max}}[S_o] + \frac{K_m}{V_{max}}$$

$$K_m = \frac{K_3 + K_2}{K_1} \qquad K_3 = \frac{V_{max}}{[E]}$$

In order to calculate standard free energies of association, it was assumed that $K_2 \gg K_3$ and, therefore, K_m is numerically equal to K_s. Values for K_1 for different enzyme-substrate systems have been reported to vary from 2.1×10^7 to 6×10^{10} M^{-1} min^{-1} (54) (Eigen and Hammes 1963). If it is assumed that K_1 in this gastricsin substrate system lies within this range, then K_2 should be between 16×10^4 and 48×10^7 min^{-1}. The observed value of K_3 is less than 1% of the calculated K_2. Then standard free energies of association were calculated using the expression $-\Delta F^\circ = 2.303\ RT \log K$, where $R = 1.986$ cal x deg^{-1} x $mole^{-1}$, $T = 310^\circ$ and K, association constant, $= \frac{1}{K_m}$. The calculated $-\Delta F^\circ$ are shown in Table 14. With reference to influences on binding, it is observed that the binding energy of dipeptides ($-\Delta F^\circ$) increases in the following order: CBZ-tyr-thr <

CBZ-tyr-ser < CBZ-tyr-ala.

With reference to the influence on the catalytic hydrolysis of CBZ-dipeptides, the values of K_3 in Table 14 indicate that the nature of amino acids at the C-terminal end is important. The K_3 values for the three dipeptides increase in the following order: CBZ-tyr-ser < CBZ-tyr-thr < CBZ-tyr-ala.

The compactness of the hog pepsin molecule has been attributed to hydrophobic bonding due to the high percentage of amino acids with nonpolar side chains (55). Hydrogen bonds are relatively unimportant in maintaining the conformational structure of active pepsin (55). The similarity in amino acid composition and physical properties between hog pepsin and human pepsin suggests a similar importance of hydrophobic bonding in maintaining the structure of human pepsin. The relatively lower percentage of leucine, isoleucine and valine residues in gastricsin may account for the more relaxed, elongated structure of gastricsin (56). It is highly probable that these structural differences are responsible for the specificity differences found in this study of pepsin and gastricsin. In other words, the tertiary structure as well as the probable amino acid sequence differences are also reflected in the specificity of these two enzymes as expressed in strength of binding and hydrolytic rate.

CHAPTER V

SUMMARY

Studies were made on the specificity of human gastric proteolytic enzymes, pepsin and gastricsin using oxidized ribonuclease A and glucagon. The two enzymes show a broad specificity toward these substrates in catalyzing the hydrolysis of tyrosyl, phenylalanyl, leucyl, glutaminyl and aspartyl peptide bonds etc. The bonds cleaved by both enzymes were similar in most case. However, three peptide linkages were found to be hydrolyzed only by gastricsin. They are tyrosyl-serine and tyrosyl-leucine in glucagon and tyrosyl-serine in oxidized ribonuclease A. Since all three bonds were tyrosyl-x peptide linkages, they may reflect a difference in the specificity of the two enzymes. A series of synthetic tyrosyl dipeptides were then used as the substrates of gastricsin and pepsin in order to see whether the specificity difference observed in the hydrolysis of these protein substrates by the two enzymes was a general phenomenon.

Three synthetic dipeptides, CBZ-L-tyr-L-ala, CBZ-L-tyr-L-ser, CBZ-L-tyr-L-thr, were found to be hydrolyzed by gastricsin but not pepsin. This observation thus agrees with the results obtained when glucagon and oxidized ribonuclease A were used as substrates.

Kinetic measurements were carried out using these three

synthetic substrates. The pH optimum of gastricsin toward the synthetic dipeptides was found to be pH 2.0. Values of K_m and K_3 were determined. These values were $K_m = 7.7 \times 10^{-4}$ M, $K_3 = 15.40$ hr^{-1} for CBZ-L-tyr-L-ala, $K_m = 2.0 \times 10^{-3}$ M, $K_3 = 2.38$ hr^{-1} for CBZ-L-tyr-L-ser, and $K_m = 8.3 \times 10^{-3}$ M, $K_3 = 3.09$ hr^{-1} for CBZ-L-tyr-L-thr respectively. The nature and the position of the amino acids in dipeptide was found to affect the K_m and K_3 values. The significance of the differences as well as the general similarity in specificity between the enzymes was discussed. Most observations on the specificity and kinetic parameters of the two enzymes can be explained, at least in part, on the hydrophobic nature of the binding of the substrate and enzyme, and on the steric effect of the side chains of amino acid residues in the substrates.

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