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GRADUATE COLLEGE

GASTRIC PROTEOLYTIC ENZYMES

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JORDAN JIEN-NAN TANG

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GASTRIC PROTEOLYTIC ENZYMES

APPROVED BY

DISSERTATION COMMITTEE

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GASTRIC PROTEOLYTIC ENZYMES

CHAPTER I

INTRODUCTION

The gastric juice of all vertebrates contains proteolytic activity. In 1836, Schwann (1) for the first time described the presence of pepsin in gastric juice. Northrop (2) in 1930 obtained crystalline pepsin from hog gastric mucosa and further showed the crystalline enzyme to be a homogeneous preparation (3).

Pepsin is perhaps one of the enzymes which has been most intensively studied. The enzyme catalyzes the hydrolysis of most proteins, with the exception of some keratins and the protamines. It possesses an optimal activity at a pH range of 1.5 to 2.0. The molecular weight of this enzyme is about 34,000 as originally determined by Northrop (4) using osmotic pressure and diffusion methods, but it has been repeatedly corrected in recent years by use of various modern molecular weight determination methods (5). Pepsin has long been known to lose enzymatic activity irreversibly at neutral and alkaline pH values. As a protein, it showed high acidity, and was found to migrate toward the anode in the electrophoretic field at pH 1.0 (6,7). This may be explained by the results of amino acid analysis which showed that of a total of 400 amino acid residues of

the pepsin molecule, 46 were aspartic acid and 32 were glutamic acid, while there were only 12 residues of lysine and 3 residues of arginine (5,8). Pepsin also contains one phosphate group for each molecule of enzyme (9).

The precursor of pepsin, pepsinogen, was first discovered in 1882 by Langley (10) who observed that the proteolytic activity in gastric mucosa was stable in alkaline solution. However, the stability in alkali was lost once the solution had been acidified. This result was correctly interpreted as the conversion of pepsinogen into pepsin upon acidification. Herriott (11) later succeeded in preparing crystalline pepsinogen, which was found to be devoid of proteolytic activity and had no milk clotting activity. In further studies on the properties of pepsinogen, Seastone and Herriott (12) found that pepsinogen has a molecular weight of 42,000 and an isoelectric point at pH 3.7. Pepsin and pepsinogen were also found to be immunologically distinct from each other.

The conversion of pepsinogen to pepsin takes place in an acid solution at a pH lower than 6.0 (13). The mechanism of activation of pepsinogen by hydrogen ion was first studied by Herriott et al (14) and later by Van Vunakis and Herriott (5, 15, 16). It was found that during the activation six small peptides were cleaved from the amino end of the pepsinogen molecule. One of the peptides was named pepsin-inhibitor which reversibly inhibits the milk clotting activity of pepsin at pH 5. This inhibitor was purified and crystallized by Herriott (17) and was found to have a molecular weight of about 5,000.

The study on the specificity of pepsin toward chemically

synthesized dipeptides was first performed by Fruton and Bergmann (18). It was observed that pepsin attacks several of these synthetic substrates containing tyrosine or phenylalanine. Among them, carbobenzoxy-L-glutamyl-L-tyrosine, carbobenzoxy-L-glutamyl-L-phenylalanine and carbobenzoxy-glycyl-L-phenylalanine were hydrolyzed most readily. More recently several other peptides were synthesized and found to be substrates for pepsin, they are: carbobenzoxy-L-cysteyl-L-tyrosine and carbobenzoxy-L-tyrosyl-L-cysteine(19), carbobenzoxy-L-methionyl-L-tyrosine (20), and carbobenzoxy-L-tyrosyl-L-tyrosine and carbobenzoxy-L-phenylalanyl-L-tyrosine (21); the latter was found to be hydrolyzed at a much higher rate than all the other synthetic peptides.

The studies of specificity of pepsin on synthetic peptides has certain disadvantages. Chemical synthesis of new peptide substrates is an elaborate and time-consuming task. Further, it is doubtful that the results obtained from these experiments represent the true specificity of the enzyme toward protein substrates, which are usually hydrolyzed at a much higher rate. This prompted the use of certain proteins or polypeptides of known sequential structures as the substrates for specificity studies. Insulin was used by Sanger and Tuppy (22, 23), alpha-corticotropin by Cole and co-workers (24), and several polypeptides from lysozyme by Acher et al (25). It was found that pepsin can also attack many peptide bonds which do not contain an aromatic amino acid. It is not possible yet to interpret these results on a generalized basis of the specificity of pepsin.

The transpeptidation activity of pepsin has been recently reported by Neumann et al (26). However, this same group of workers

has also reported that pepsin without transpeptidation activity can be prepared from pepsinogen (27). It seems that transpeptidation is not an intermediate step of the enzymatic action of pepsin.

The presence of a second proteolytic enzyme beside pepsin in the stomach of the human and other species of animals has been a subject of extensive controversy in the past two decades. Freudenberg (28) in 1940 studied the pH optimum curve of human gastric juice. It was found that in addition to the well known pH optimum of pepsin there was another optimum at pH 3.5. He interpreted this observation as the existence of a second proteolytic enzyme in the human gastric juice, and he named this new enzyme gastric 'cathepsin'. The observation of Freudenberg was confirmed by Buchs (29) and Merten and Ratzer (30).

In the studies of hog pepsin, Herriott et al. obtained two pepsin fractions from a preparation of crude pepsin. One of the fractions, which he called pepsin A, was found to have higher enzymatic activity as well as higher solubility (31). However, both fractions were found to have the same pH optimum. On the contrary, Pope and Stevens (32) in studies of the effect of pH on the activity of amorphous pepsin on hemoglobin and diphtheria antitoxin observed a pH curve with seven optima ranging from pH 1.5 to 6.0.

In 1940 Norris and Elam (33) found that the pH dependent curve of crystalline salmon pepsin showed two distinct optima at pH 1.4 and pH 3.3 respectively. A similar result was observed for the pH dependent curve of the gastric mucosa extract of the shark (Carcharinus milberti) by Sprissler (34).

The attempts at the purification of a second proteolytic enzyme

from the gastric juice or the gastric mucosa of different sources have not been successful until quite recently. Merten et al. (35) used ammonium sulfate precipitation and electrophoresis in the Tiselius apparatus and on paper strips. They obtained from hog mucosa extract a 'cathepsin'-rich fraction which was said to have the pepsin:'cathepsin' ratio of 0.88 in the best preparation. The criterion used to determine quantitatively the amount of pepsin and 'cathepsin' was to measure the proteolytic activities at pH 1.8 and 3.5 respectively. However, it is well known from the pH dependent curve of Northrop's crystalline pepsin that pepsin itself still possesses about 50% of its maximum activity at pH 3.5, therefore, the true ratio of pepsin:'cathepsin' of Merten's preparation should be much higher. Also, none of his preparations showed a single pH optimum at 3.5. The pH curve is always accompanied by a distinct pepsin optimum peak. Taylor and O'Brien (36), after trying many different methods, reported in 1955 that they were unable to separate this second proteolytic enzyme from human gastric juice. The first complete separation of this two enzymes from human gastric juice was reported by Richmond et al. (37) who used ion-exchange chromatography with Amberlite IRC-50 resin column. Two proteolytic enzymes was reported to have the pH optima at the pH range of 1.5-2.0 and 3.0-3.5 respectively. However, as recent as 1958, Masch (apparently unaware of the work of Richmond et al.) denied the presence of gastric 'cathepsin' on the basis that the second pH optimum observed in the gastric juice may be the property of pepsin itself (38).

The problem concerning the origin of an additional proteolytic enzyme in the gastric juice was further complicated by the study of

Perlmann (39) who obtained an active fragment from the dialysate of a pepsin solution which had undergone autodigestion. Funatsu and Tokuyasu (40, 41) have recently been able to confirm this experiment. Further fractionation of the active fragments revealed several components, one of which had a molecular weight lower than 10,000. It then seems conceivable that the other proteolytic enzymes present in the gastric juice may be the products of the autodigestion of pepsin, even though a change of the pH optimum was never observed for the autodigestion of pepsin.

In a recent study, Ryle and Porter (42) obtained from a crude porcine pepsin preparation two proteolytically active minor components which they called 'parapepsin I' and 'parapepsin II'. Both enzymes have the identical pH optimum of pepsin at pH 2.0 and account for less than 5 per cent of total peptic activity in the procine pepsin preparation. It is interesting that 'parapepsin I' was unable to attack hemoglobin but hydrolyzed the snythetic substrate acetylphenylalanyl-diiodotyrosine. On the contrary, 'parapepsin II' was inactive toward the synthetic peptide and hydrolyzed hemoglobin rapidly. These two 'parapepsins' apparently are not the gastric 'cathepsin' proposed by Freudenberg, since their pH optima are at pH 2.0 instead of pH 3.5 for gastric 'cathepsin'.

Rennin, the milk-clotting enzyme, is known to be present in the fourth stomach of the young calf. This enzyme has been purified in crystalline form independently by Berridge (43) and by Hankinson (44). The proteolytic activity of rennin is markedly lower than that of pepsin. A recent study by Fish (45) indicates that the enzyme has an optimal pH at 4.0 and attacks the B-chain of insulin at the following

peptide bonds: -leucyl-valyl-, -leucyl-tyrosyl-leucyl-, and -phenylalanyl-phenylalanyl-tyrosyl-. It is to be noted that some of these linkages are also cleaved by pepsin (22, 23). The milk-clotting activity of rennin requires the presence of calcium ion, however, little is known about the mechanism of its action. The enzyme is secreted into the fourth stomach of the calf as the zymogen, prorennin. The purified prorennin has no milk-clotting activity unless activated with hydrogen ion (46). The presence of rennin in other species of animals has not been demonstrated.

It is the purpose of this study to elucidate the presence of proteolytic enzymes in the gastric content of animal species. During the progress of this investigation, it was found that among the several different species studied, human gastric juice is the only material containing a proteolytic enzyme with an optimum at pH 3.0. Therefore, the proteolytic enzymes in human gastric juice were studied the most extensively. The results of this study, which are to be presented in the following pages, include the separation and isolation of various proteolytic enzymes from human gastric juice; the comparison of the physicochemical and enzymatic properties; the origin of these enzymes, and finally the comparison of proteolytic enzymes from different animal species. Part of the results in this study have been communicated (47, 48, 49, 50).

A special remark should be made here concerning the name 'gastric cathepsin', which designated the gastric proteolytic enzyme with a pH optimum at 3.5 (28). The only reason for the proposal of this name by Freudenberg was due to the resemblance of its pH optimum to cathepsin c. However, the name cathepsin is understood to be used only for intra-

cellular enzymes which carry out the process of autolysis of the tissue, and should not be used for a proteolytic enzyme which carries out the digestion of foodstuff in the stomach. This prompted us to use the term 'gastricsin', which is in agreement with the unsystematic names used for many digestive enzymes.

CHAPTER II

MATERIALS AND METHODS

Materials

Human Gastric Juice

Samples of human gastric juice were collected from patients at the University Hospital and the Veterans' Administration Hospital in Oklahoma City, Oklahoma. The secretion of gastric juice was stimulated by injecting intravenously 10 units of insulin immediately preceding each collection. The gastric juice was collected in jars, cooled to 4°, and maintained at that temperature. Samples from several patients were pooled, dialyzed against distilled water, and lyophilized in batches of about 250 ml.

Human Gastric Mucosa

The samples of human gastric mucosa were obtained from three hospitals in Oklahoma City, Oklahoma: University Hospital, Veterans' Administration Hospital, and Wesley Hospital. The normal portion of the stomachs obtained at gastrectomy of ulcer patients was cooled in an ice bath and the mucosa was removed. The freshly removed mucosa was immediately processed.

Rat Gastric Juice

The following procedures were used in obtaining the rat gastric juice. The rats were fasted for 12 hours in order to empty their stomachs. The animals were anesthetized with ether, and ligation of the pyloric end of the stomachs was made by cutting open the abdomen of the animals. The abdomen of the rats was closed by sutures. Four hours after the ligation of the stomach, the animals were anesthetized again and the stomachs were removed. The contents of the stomachs were pooled, centrifuged to remove insoluble residues, dialyzed against several changes of distilled water and lyophilized.

Dog Gastric Juice

The dog gastric juice was collected from Heidenhain pouches of dogs during a period of several hours. The collected juice was dialyzed and lyophilized.

Porcine Gastric Mucosa

Porcine stomach was obtained from Wilson and Company packing house, Oklahoma City, Oklahoma. The stomach was cooled in an ice bath immediately after slaughtering and the gastric mucosa was removed.

Porcine Pepsin, Twice-crystallized

The enzyme was purchased from General Biochemicals, Chagrin Falls, Ohio.

Porcine Pepsin, 1:10,000

The preparation was purchased from Mann Research Laboratories, Inc., New York 6, N.Y.

Rennin, Crystalline

The enzyme was purchased from Nutritional Biochemicals Corporation, Cleveland.

Carboxypeptidase

Five times-crystallized carboxypeptidase was purchased from Mann Research Laboratory, New York 6, N. Y.

Bovine Hemoglobin

Bovine hemoglobin powder was obtained from Pentex Incorporated, Kankakee, Ill.

Synthetic Peptides

Chromatographically pure samples were obtained from Mann Research Laboratory, New York 6, N.Y. The synthetic peptides are carbobenzoxy-L-glutamyl-L-tyrosine, carbobenzoxy-L-glutamyl-L-phenylalanine and carbobenzoxy-glycyl-L-phenylalanine.

N,N-diethylaminoethylcellulose (DEAE-cellulose)

DEAE-cellulose was purchased from Eastman Organic Chemicals, Rochester 3, N. Y.

Methods

Proteolytic Activity

A modified procedure of Anson and Mirsky was used (51) with bovine hemoglobin as substrate to determine the proteolytic activity. To 4 volumes of 2.5 per cent substrate solution one volume of a 0.6 N HCl solution was added. The incubation mixture contained 1.0 ml. of acidified

substrate solution, 1.0 ml. of enzyme solution in 0.04 N HCl. After 10 minutes of incubation at room temperature, 1.0 ml. of 10% trichloroacetic acid was added. After 5 minutes standing at room temperature, the precipitate was filtered off with Whatman No. 50 filter paper. The optical density at 280 mu of the filtrate was measured in a Bechman DU spectrophotometer. Since the filtrate of non-digested bovine hemoglobin solution always had appreciable amounts of absorption in the ultraviolet region, it was necessary to run a blank with every determination. The value obtained from the blank was deducted from all other values of enzyme determination. The method was found to be linear up to optical density 1.8.

Rennin Activity

The milk-clotting activity was determined according to the procedures described by Berridge (52). Skim milk powder (Pet Milk Company) was used as substrate. A solution which contained 12 gm. of milk powder and 0.01 M $CaCl_2$ in a volume of 100 ml. was dispensed into test tubes in 10 ml. quantities. The test tubes were placed in a boiling water bath for 2 hours. The enzyme solution was diluted with distilled water to a concentration so that when 1 ml. was added to 10 ml. of milk solution, clotting would take place in about 5 minutes. Time was recorded with a stop watch at the first appearance of the clotting.

Nitrogen Determination

The modified microdiffusion technique of Seligson and Seligson (53) was used. The digestion mixture contained 3 M H_2SO_4, 0.6 M K_2SO_4 and 0.017 M H_gSO_4. The washing solution contained 0.05 M $Na_2S_2O_3$ and

1.0 M NaOH. A sample of about 0.2 ml. containing 10-25 micro-gm. of nitrogen (approximately 0.1 mg. of protein) were put into a 25 ml. Erlenmeyer flask and 0.5 ml. of digestion-mixture solution was added. The flask was placed on a hot plate until all water had evaporated. The flask was then covered with a glass marble, the temperature of the hot plate was raised so that a continuous reflux of sulfuric acid could take place in the flask. After four hours of digestion, the flask was removed from the hot plate and allowed to cool slowly. The marble was rinsed with 2 ml. of washing solution into the flask. Three pellets of NaOH were placed in the flask and the flask was immediately closed with a stopper which contained a drop (about 0.05 ml.) of concentrated sulfuric acid in the inner well of the stopper. The flask was then placed in an electric shaker and agitated for 12 hours. After this diffusion period, the stopper was removed from the flask and placed on a test tube containing 10 ml. of Nessler's solution. The tube was then shaken to mix the sulfuric acid drop with Nessler's solution. After 5 minutes of standing, the developed color was read in a Beckman DU spectrophotometer at 420 mu. The standards were run with ammonium sulfate and carried through the same procedures.

Protein Concentration

The protein concentration was determined spectrophotometrically at 280 mu in a Beckman DU spectrophotometer. The extinction coefficients of several purified enzymes were determined which allowed the calculation of the protein concentration with this method. The method was found to be in good agreement with the determination of nitrogen.

Ninhydrin Method

The quantitative determination of amino acid was carried out according to the procedures of Rosin (54). The reaction mixture consisted of 1.0 ml. sample containing 0.02-0.4 u mole of amino acid, 0.5 ml. of 4 M sodium acetate buffer (pH 5.3) containing 2×10^{-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. After the solution cooled to room temperature, the color was read in a Beckman DU spectrophotometer at 570 mu. The standards were carried out at the same time with amino acids.

Paper Chromatography of Amino Acids

Descending paper chromatography was run on Whatman No. 1 filter paper with butanol-acetic acid-water as solvent, which was prepared by mixing n-butanol, glacial acetic acid and distilled water in a ratio of 4:1:5 in a separatory funnel. After complete separation, the upper layer (organic phase) is used as solvent and an aliquot of the lower layer is placed in the chromatographic chamber (55). The chromatography was allowed to run for 12 to 14 hours. The paper was dried at room temperature and then placed in an oven of 70°C for 15 minutes. The paper was dipped quickly in 5% ninhydrin in acetone and allowed to drain to dryness. The amino acid spots appeared after the paper was placed in an oven at 60°C for 15 minutes.

Amino-terminal (N-terminal) Amino Acid Determination

The fluorodinitrobenzene (FDNB) method was used with slight

modification as described by Fraenkel-Conrat *et al.* (55) About 7 mg. of enzyme (0.2 u mole) was suspended in 3 ml. of 0.1 N KCl at 40° and the pH was adjusted to 8.0 with 0.05 N KOH. 0.1 ml. of FDNB was added, and the solution was stirred vigorously in order to maintain saturation with the reagent. The pH was maintained at 8.0 by intermittent addition of the standard alkali. The process was continued until the plot of alkali uptake as a function of time had flattened to the constant background value (2 hours was found to be adequate). The reaction mixture was transferred to a centrifuge tube, and extracted three times with peroxide-free ether to remove excess FDNB. The DNP-protein was then precipitated by acidification, and the suspension was again extracted with ether. The DNP-protein was washed successively in water, acetone and ether, and then placed over P_2O_5 *in vacuo* to dry.

The DNP-protein was hydrolyzed as a 1% solution in a sealed evacuated tube with 7.5 N glass-distilled HCl. Hydrolysis for 4 hours at 105° was found to give the highest yield of N-terminal amino acid. After hydrolysis, the solution was diluted until the HCl concentration was 1 N and extracted three times with peroxide-free ether. The combined ether extract was evaporated to dryness and the dinitrophenol present in the residue was sublimated at 55° in an evacuated flask fitted with a cold finger loaded with crushed solid carbon dioxide.

The identification of DNP-amino acid was carried out with two dimensional paper chromatography (58). The solvents were prepared as follows:

'Toluene' solvent. A mixed solution of 30 ml. of toluene, 9 ml. of pyridine, and 18 ml. of 2-chloroethanol was placed in a separatory

funnel, and 18 ml. of 0.8 N ammonia was added down the wall of the funnel, avoiding undue mixing of the layers. The mixture was allowed to stand thus, without shaking, for the duration of the equilibration period, after which period of time the lower aqueous layer was withdrawn and discarded. The organic layer was used as solvent.

Phosphate solvent. 1.5 M phosphate buffer of pH 6.0 was prepared by dissolving 138 gm. of $NaH_2PO_4 \cdot H_2O$ and 71 gm. of Na_2HPO_4 in 1 liter of distilled water.

The quantitative determination of the amount of N-terminal DNP-amino acid obtained from the chromatography was done by quantitative elution of spots from the paper with 4 ml. of 1% sodium bicarbonate solution and the optical density of the eluents were determined in a Beckman DU spectrophotometer at 360 mu. The loss of the DNP-amino acid during this process was calculated by treating a known amount of chemically pure DNP-amino acid (the same DNP-amino acid was used as standard as that found in the sample after its identity was known from the preliminary experiment) with the same procedures. The adsorption of this standard DNP-amino acid at 360 mu before and after treatment was determined, and the loss of DNP-amino acid was calculated from the difference in the adsorption. The quantitative value of N-terminal DNP-amino acid was corrected for the loss during the process and further calculated as the per cent of yield.

$$\% \text{ yield} = \frac{\text{Amount of N-terminal DNP-amino acid recovered (corrected)}}{\text{Theoretical amount of N-terminal amino acid present in the protein}} \times 100$$

Carboxyl-terminal (C-terminal) Amino Acid Determination

The carboxypeptidase method was used for C-terminal amino acid residue analysis (55). Protein (1 u mole) was incubated with diisopropylfluorophosphate-treated carboxypeptidase (about 0.15 mg.) in a 2 ml. solution buffered with 0.1 M ammonium acetate buffer at pH 7.5. The incubation was carried out at room temperature for 12 hours, and several samples of 0.25 ml. each were taken at different incubation times. Two methods were used to determine the amino acids in the incubation mixture.

(1) FDNB method (58) was used. The procedure was similar to that described for N-terminal amino acid determination except that no acid hydrolysis was necessary.

(2) The procedures of White et al. (59) were also used. To 0.25 ml. incubation mixture 1 ml. of absolute ethyl alcohol was added. After standing for approximately 60 minutes, the precipitate was removed by centrifugation. The residue was washed twice with 0.5 ml. of alcohol, and the supernatants were combined. This alcohol solution was evaporated in a boiling water bath to dryness and the residue was heated in an oven of 70° for several hours to remove any ammonium acetate which might still have been present. The residue was then subjected to paper chromatography for amino acids (see a separate section above for procedures). The ninhydrin positive spots were identified and cut out. The pieces of paper which contained the spots were eluted separately with 3 ml. of a solution containing 75% acetone and 25% water. The absorbency of the eluents at 570 mu were determined in a spectrophotometer. The amino acid standards were also run with the same procedures, and from this the quantity of amino acids in the samples

were calculated. The method was found to be linear up to 0.2 u mole of amino acid.

Electrophoresis on Starch Gel

The procedure of Smithies (60) was followed with the use of a Reco model E-800-2 electrophoresis cell to which was adapted a frame of Simplex-glass to contain the potato starch gel. Gel strips of 3 x 33 cm. with the thickness of 0.8 cm. were prepared and left standing over night before use. A slit of 0.2-cm. width was cut in the gel at a distance of one third of the length from the cathode. Approximately 2 mg. of a crystalline enzyme sample were blended with adequate amounts of supporting potato starch and applied in the slit. The electrophoresis was performed at a constant gradient with a field strength of 4.55 volts per cm. at room temperature with tap water running in the cooling system. After the electrophoresis was completed, the gel was cut parallel to the bottom surface and one half was stained with Amidoblack 10B. The remaining half of the gel was cut perpendicularly to the long axis of the strip at 0.5-cm. intervals and the pieces were put into test tubes, and then frozen (-20°C) and thawed twice. The enzyme was extracted from the gel with 3 ml. of distilled water, and the proteolytic activities of the extracts measured.

Electrophoresis on Raw Starch

The procedures and equipment used in raw starch electrophoresis were similar to that of starch gel electrophoresis. After electrophoresis, the starch block was cut and eluted with buffer solution. The supernatants were measured for protein concentration and enzymatic

activity.

Electrophoresis on Paper

The Spinco Durrum type cell was used for paper electrophoresis with pH 5.0 acetate buffer, (ionic strength = 0.1), prepared as described by Miller and Golder (61). Spinco electrophoresis paper strips (part 300-028 of Beckman Instruments, Spinco Division) were used. The experiments were run with eight 3-cm. strips at a constant field strength of 140 volts during 24 hours at 4°C. The proteins were dissolved in the buffer and applied in quantities of 3 mg. per strip. After electrophoresis, the strips were dried, and the proteins were stained with bromophenol blue.

Molecular Weight Determination

The molecular weight determinations were made with two methods:

Sedimentation velocity method. The experimental procedures for sedimentation velocity measurements were carried out according to the procedures described by Schachman (62).

(A) The determination of sedimentation coefficients. The apparent sedimentation coefficient ($s_{app.}$) was determined according to the equation of Kegeles and Gutter (59):

$$s_{app.} = \frac{2.303 \ (d \log x / dt)}{60 \ (\omega^2)} \qquad (1)$$

where x is the distance of boundary from center of the rotation at the time t (in seconds) and ω is the angular velocity of the centrifugation.

The photographs of the schlieren pattern were taken by the automatic mechanism of the Spinco Model E ultracentrifuge at 4 or 8 minute intervals. The position of the sedimenting boundaries were

determined with a micro-comparator which gave an accuracy of 0.0001 cm. The values of d log x were plotted against t. The slope of this straight line represents d log x / dt which can be further calculated to $s_{app.}$ as shown in equation (1). The value of $s_{app.}$ was changed to $s_{20,w}$ (sedimentation coefficient under standard conditions of 20°C and water as solvent) according to the equation of Svedberg and Pedersen (64).

$$s_{20,w} = s_{app.} \times \frac{\eta}{\eta_{20,w}} \frac{(1 - \bar{V}\rho)_{20,w}}{(1 - \bar{V}\rho)} \qquad (2)$$

where η and $\eta_{20,w}$ are the viscosity of the solvent at the temperature of the experiment and the viscosity of water at 20°C respectively, $\bar{V}$ is the partial specific volume of the protein and ρ is the density of the solvent.

(B) The determination of diffusion coefficients. The diffusion experiments were performed with a synthetic boundary cell in a Spinco Model E Ultracentrifuge. The diffusion time was determined with a stop watch which was started at the observation of the boundary. The apparent diffusion coefficient, $D_{app.}$, was calculated with the equation:

$$D_{app.} = \frac{1}{4\pi t} \left(\frac{A}{H_{max.}}\right)^2 (1 - \omega^2 st) \qquad (3)$$

where A is the area under the boundary of the schlieren pattern, $H_{max.}$ is the maximum ordinate of the gradient curve, ω is the angular velocity of the ultracentrifuge, s is the sedimentation coefficient, and t is the time in seconds. The calculated $D_{app.}$ were plotted against 1/t, and the value of $D_{app.}$ at 1/t = 0 was used as the true apparent diffusion coefficient.

The $D_{app.}$ was also calculated according to another equation:

$$\sigma^2 = \frac{1}{2\pi} \left(\frac{A}{H_{max.}}\right)^2 \qquad (4)$$

The second moments, σ^2, obtained from the above equation at different times were plotted against time in seconds. The slope of this plot is equal to 2D. The diffusion coefficient corresponding to a temperature of 20°C in a solvent with the viscosity of water, $D_{20,w}$ was calculated (62) according to the following equation:

$$D_{20,w} = D_{app.} \left(\frac{293}{273 + t}\right) \left(\frac{\eta_{solv.}}{\eta_w}\right) \left(\frac{\eta_{t,w}}{\eta_{20,w}}\right) \qquad (5)$$

where t is temperature in °Centigrade, $(\eta_{solv.}/\eta_w)$ is the relative viscosity of solvent and water and $(\eta_{t,w}/\eta_{20,w})$ is the relative viscosity of water at temperature t and at 20°C.

(C) The determination of the density of the buffer solution. The density of the buffer solution was measured with a 5 ml. pycnometer. The density was determined at two temperatures, 27°C and 37°C. Extrapolation was made from the results of the above determinations for the values to be used at other temperatures.

(D) The determination of the viscosity of the buffer solution. The viscosity of the buffer solution was determined in an Ostwald viscometer, which was equilibrated in a constant temperature water bath of 37°C. A volume of 2 ml. of buffer or distilled water was pipetted into the viscometer. After the temperature was equilibrated, the flow time was recorded by a timer having an accuracy of 0.01 second. Triple readings were made with good agreement. The equation used for calculation was taken from Schachman (62):

$$\eta/\eta_o = (t/t_o)\ (\rho/\rho_o) \qquad (6)$$

where η_o, t_o and ρ_o are the viscosity, outflow time, and density of the distilled water, respectively, and η, t and ρ are those of buffer solution.

(E) Calculation of the molecular weight. The Svedberg's equation was used in the calculation of molecular weight (64):

$$\text{M. Wt.} = \frac{R\ T\ s}{D\ (1 - \bar{V}\rho)} \tag{7}$$

where R is the gas constant, 8.314×10^7 erg/mole/degree, T is the absolute temperature S and D are the value calculated for standard condition of 20°C in water, and other quantities are as defined previously.

Archibald method for molecular weight determination. The equation of Archibald was used in the calculation (65):

$$\text{M. Wt.} = \frac{R\ T}{(1 - \bar{V}\rho)\omega^2} \quad \frac{(dc/dx)_m}{x_m \quad c_m} \tag{8}$$

where $(dc/dx)_m$ is the concentration gradient at the meniscus, x_m is the position of the meniscus and c_m is the concentration at the meniscus. In cases involving the bottom of the solution, $(dc/dx)_b$, c_b and x_b are used.

The value of (dc/dx) and x were determined directly by reading the photographic plate in the micro-comparator. The value c was determined according to the equation derived by Klainer and Kegeles (66):

$$c_m = c_o - \frac{1}{x_m^2} \int_{x_m}^{X} x^2 \ (dc/dx) \ dx \tag{9}$$

where X refers to a position in the plateau region (where dc/dx is equal to zero), and c_o is the initial concentration which is determined by measuring the area under the boundary in a synthetic boundary cell.

CHAPTER III

RESULTS

Separation of Gastricsin from Pepsin

The human gastric juice was fractionated with an ion-exchange column of Amberlite IRC-50 (XE-64) by a modified procedure of Richmond et al. (67). Two gm. of dialyzed and lyophilized human gastric juice powder were dissolved in 30 ml. of 0.2 M sodium citrate buffer (pH 3.0). The insoluble residue was removed by centrifugation in the cold at 10,000 x g. A column of resin (4.4 x 15 cm.) was equilibrated with 0.2 M sodium citrate buffer of pH 3.0 and the surface of the buffer solution was adjusted to the same level as the top of the resin column. The centrifuged gastric juice solution was then pipetted into the column on top of the resin. The solution was permitted to flow slowly into the resin by opening the valve under the column. The slow flow rate was used until all the gastric juice solution had flowed into the resin. The valve was closed and more pH 3.0 sodium citrate buffer was carefully layered at the top of the resin to form a buffer layer of about 15-20 cm. in height. The column was then connected by means of polyethylene tubing to a reservoir which contained the same buffer, thus making the system a closed one so that any outflow of the effluent from the column would be replaced by the same volume of buffer from the reservoir. The outflow

of the effluent was then connected to an automatic fraction collector and the valve was opened and adjusted to a flow rate of 10 ml. per 8 minutes. The effluent was collected in 10 ml. fractions, and each fraction was read in a Beckman DU spectrophotometer for its optical density at 280 mu. The elution with 0.2 M sodium citrate buffer, pH 3.0, was continued until the absorption at 280 mu in the effluent reached a base line value which is usually less than O.D 0.1 at 280 mu. The eluent buffer (both at the top of the resin column and in the buffer reservoir) was then changed to 0.2 M sodium citrate buffer of pH 3.8. The procedure was repeated using successively 0.2 M sodium citrate of pH 3.0, 3.8, 4.2, and 4.6. The further change of buffer was done only after the pH of the effluent had reached the pH of influent buffer.

The resulting chromatographic pattern revealed three protein peaks which were eluted at pH 3.2, 4.0 and 4.4 of the effluent respectively (Figure 1). The first peak is thought to be the "break-through peak" (i.e., the materials which are not retained by the column). This peak is usually asymmetrical and its shape is not reproducible. The second and third peaks are usually sharp and symmetrical, and the pH values, at which the two peaks are eluted, are found to be highly reproducible in the various column fractionations done. The relative size of these two peaks were evaluated by integration of the area under the two peaks. It was found that the ratio was about 4 to 1. This chromatographic procedure was found to give constant results in thirty to forty different runs. A larger scale fractionation column for preparative purposes was also found to give successful results. The

size of the folumn was 8 x 20 cm. and was able to separate the components of 10 gm. of dialyzed and lyophilized gastric juice. By means of the proteolytic activity measurements of the fractions, it was found that both the peaks eluted at pH 4.0 and 4.4 correspond to the proteolytic activity. The first peak was found to be devoid of proteolytic activity (Figure 1).

The pH optimum was determined for the two proteolytically active peaks. It was found that the fraction which eluted at effluent pH 4.0 showed an optimum of pH 2.0 (Figure 2), while the fraction eluted at pH 4.4 of the effluent showed an optimum of pH 3.2. The former, apparently, corresponds to the known pH optimum of pepsin, while for the latter, which corresponds to the pH optimum of the enzyme postulated by Freudenberg, we have proposed the designation, gastricsin.

The effluent fractions under both the pepsin and gastricsin peaks were pooled separately, dialyzed thoroughly against several changes of distilled water, and lyophilized. When each fraction was rechromatographed on an Amberlite IRC-50 resin column, it did not lead to further fractionation, and the pH values of the effluent at which the peaks appeared were again consistent.

Isolation and Crystallization of Gastricsin

In order to further study this new enzyme, gastricsin, it was necessary to attempt its further purification. After several attempts, fractionation and crystallization procedures were then developed for this purpose, as follows:

About 20 to 30 mg. of the gastricsin powder, which was obtained from the ion-exchange chromatography, was dissolved with 2.0 ml. of

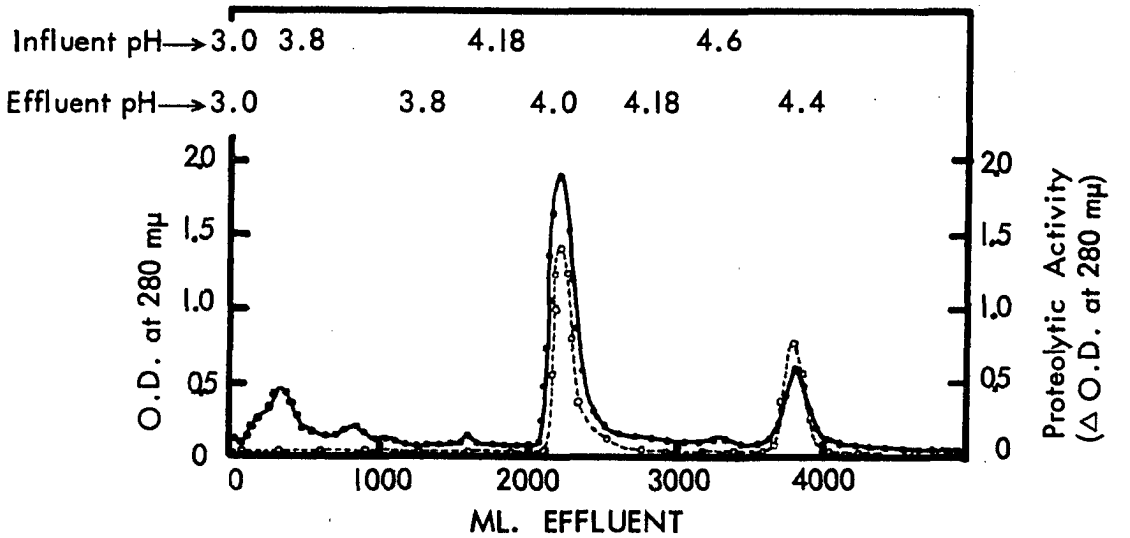

Figure 1.-Chromatography of dialyzed human gastric juice on a column of Amberlite IRC-50 (XE-64).

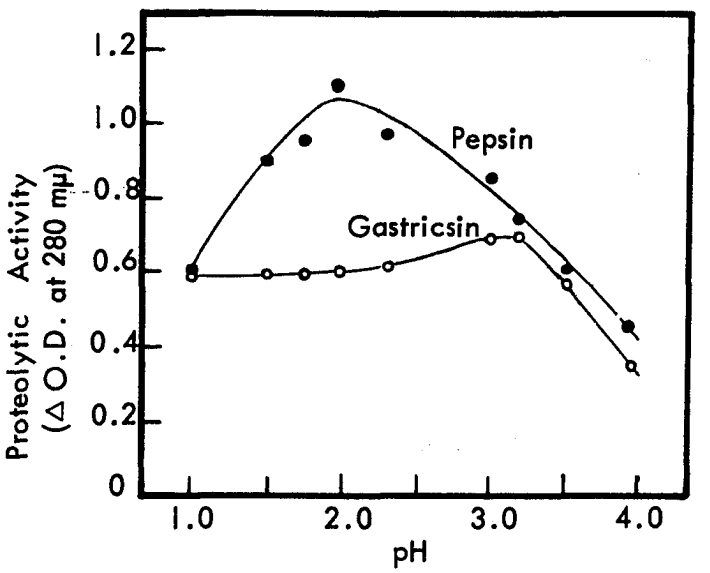

Figure 2.-The proteolytic activity of gastricsin and pepsin at different pHs. The enzyme preparations were obtained from ion-exchange chromatography, and bovine hemoglobin was used as substrate.

distilled water at 4° and centrifuged at 2000 r.p.m. for 10 minutes, and the supernatant solution discarded. The precipitate was dissolved with 2.0 ml. of water and the previous precipitation was repeated. The precipitate was dissolved with 2 ml. of cold sodium acetate buffer (pH 5.0). A clear solution was obtained to which crystalline ammonium sulfate was added in small portions until the first indication of protein precipitation was observed (approximately 0.2 gm. of ammonium sulfate was required). The tube was then placed in a water bath at 20° and left for 5 minutes. If the turbidity disappeared, drops of saturated ammonium sulfate solution were added until it reappeared. The tube was then moved to a 40°-water bath, in which, after 5 minutes, the turbidity usually disappeared. When it did not, however, the insoluble material was removed by centrifugation. The tube was placed in a beaker containing 2 liters of water at 30° and moved into the 4°-cold room where, after 6 to 8 hours, a white precipitate was formed. The first precipitate, however, was mostly noncrystalline and was removed by centrifugation in the cold room and at a low speed. The supernatant solution was left standing at 4° for a period of 20 to 30 hours during which crystals were formed. The crystals of gastricsin, shown in Figure 3, were transparent, and the microscopic observations were best made in dim light. Seeding of crystalline gastricsin reduced the time required for the crystals to appear. After 2 to 3 days, the first collection of crystals was made by centrifugation at low speed. The supernatant solution still contained enzyme activity, and the formation of more crystals could be obtained by addition of a few drops of saturated ammonium sulfate solution.

Table 1 shows a protocol of the purification procedure. The

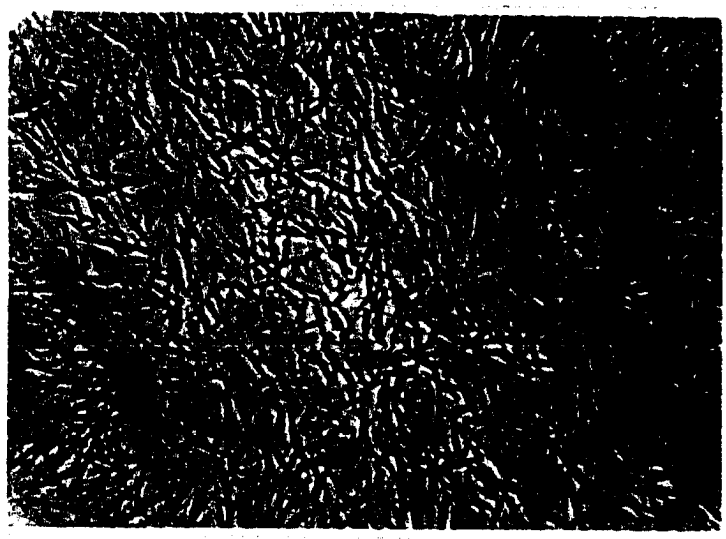

Figure 3.-Crystalline gastricsin at 480x magnification.



Figure 4.-Recrystallized gastricsin at 480x magnification.

TABLE 1

PURIFICATION AND CRYSTALLIZATION OF GASTRICSIN

Purification Procedures	Dry Weight	Specific activity	Total activity
	mg.	$O.D._{280}$/ mg. protein	%
Freeze-dried gastric juice	2000	4.6*	100
Chromatographic fraction	36	153.6	60
First crystals	7.2	227.5	17.8
Second crystals	6.7	242.5	17.6

*There is no accurate determination of this value, because the activities of pepsin and gastricsin overlap in any measurement of these enzymes in total gastric juice. The value for gastricsin given in this table was calculated from the total proteolytic activity recovered from the column after adding the values of both peaks and then assuming that the per cent recoveries for pepsin and gastricsin were the same.

specific activities of the crystalline gastricsin were 25 to 30 per cent higher than that of the lyophilized materials obtained by chromatography, and the yields, related to the same starting material, varied from 20 to 30 per cent. The crystallization procedure has been repeated more than 30 times in this study.

The shape and the activity of the crystals remained unaltered in the mother liquor after 1 to 2 months at 4°, but at room temperature they tended to dissolve.

The recrystallization can be performed without the necessity for refractionation with ammonium sulfate, and the crystals obtained had the same characteristics as those of the first crystallization. However, gastricsin was also recrystallized using a different procedure and the resulting crystals had a different shape. In this procedure, about 10 mg. of crystalline gastricsin was dissolved in 1 ml. of distilled water. To this solution, a saturated ammonium sulfate solution was added at a rate of a few drops per day. The solution was kept in the cold during this process. After four or five additions of ammonium sulfate, crystalline gastricsin appeared in square-shaped plates (Figure 4). This method of recrystallization was less reproducible and often resulted in the precipitation of noncrystalline material, therefore, it was not used in the preparation of crystalline for further studies.

Homogeneity of Crystalline Gastricsin

Several methods were used for the analysis of the homogeneity of crystalline gastricsin:

Ultracentrifugation

The sedimentation analysis of crystalline gastricsin was performed with a Spinco model E ultracentrifuge. The crystalline enzyme was dissolved in 0.1 M sodium acetate buffer at pH 5.0 (ionic strength 0.1) to a concentration of 0.1 per cent. After this solution was dialyzed against several changes of the same buffer, it was used in the ultracentrifugation studies. When the enzyme solution was centrifuged at 59,780 r.p.m., with the temperature control regulating at 20°C, a single symmetrical sedimenting boundary was observed in five different runs. A typical sedimentation pattern is shown in Figure 5. Change of enzyme concentration and centrifugal speed did not alter the appearance of the boundary.

The homogeneity of human pepsin, purified by chromatography, was also analyzed by means of the sedimentation method. Under the same conditions as described above, only one sedimenting boundary was observed for the human pepsin preparation.

The homogeneity of crystalline gastricsin was also studied using the method of the transient states during the approach to sedimentation equilibrium (Archibald method). The criteria of the homogeneity based on the agreement between the molecular weight value determinations calculated from the meniscus and from the bottom of the solution phase at the schlieren optical pattern. The closeness of the values obtained in the case of gastricsin (see a separate section for molecular determination) indicating a homogeneous preparation.

Diffusion

The diffusion experiments were performed in an ultracentrifuge

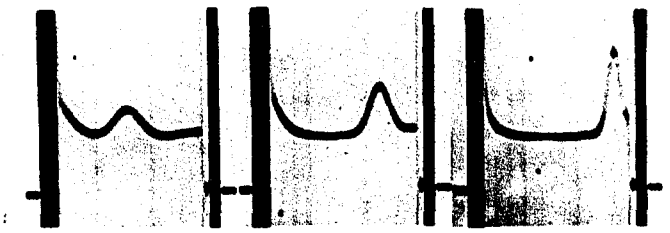

Figure 5.-Ultracentrifugation of crystalline gastricsin. The pictures from right to left were taken at 32, 80 and 160 minutes.

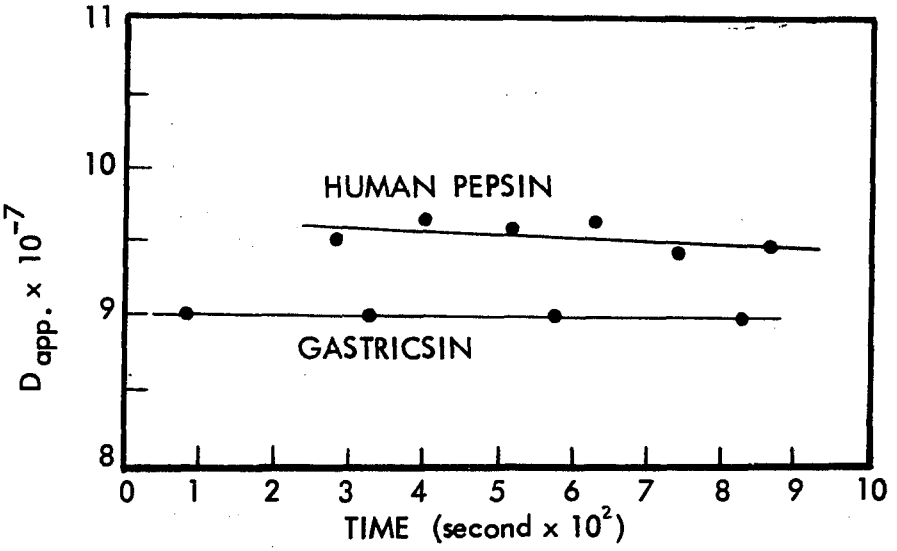

Figure 6.-The plot of apparent diffusion coefficient, $D_{app.}$, at different time, t, for crystalline gastricsin and chromatographically purified human pepsin.

as described in the method for molecular weight determination. The zero time correction was made by plotting $D_{app.}$ against 1/t as suggested by Schachman (62). The $D_{app.}$, after the zero time correction, were plotted against t. For both crystalline gastricsin and purified human pepsin, the plots resulted in horizontal lines indicating that they were homogeneous preparations (Figure 6). The plots of second moments, σ^2, against 1/t resulted in straight lines (Figure 7) for both preparations. This also indicates that the preparations were homogeneous.

Zone Electrophoresis on Starch Gel

Only a single protein band was found in the electrophoresis of crystalline gastricsin on starch gel. The enzymatic activity was present in the extracts from the zone corresponding to the dyed area (Figure 8).

From the results of above experiments it was concluded that crystalline gastricsin was a homogeneous preparation. Human pepsin, which was purified with ion-exchange chromatography, also appears to be homogeneous using the criteria of sedimentation and diffusion. However, critical analysis on starch gel electrophoresis was not carried out for human pepsin preparation.

Molecular Weight of Gastricsin and Human Pepsin

The molecular weights were determined for gastricsin and pepsin using the sedimentation velocity method. The calculated sedimentation coefficients and diffusion coefficients are summarized in Table 2. The molecular weight of gastricsin was found to be 36,027 and that of human pepsin to be 31,390. In the calculation of molecular weights, a value

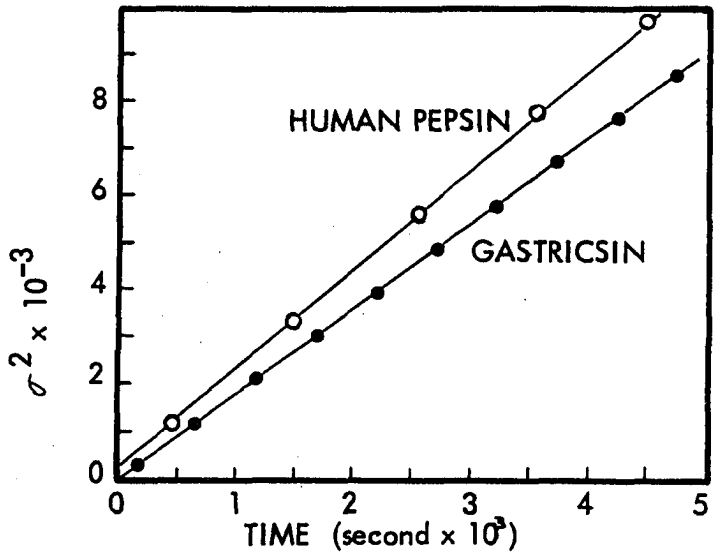

Figure 7.-The plot of second moments, σ^2, against time, t, for gastricsin and human pepsin.

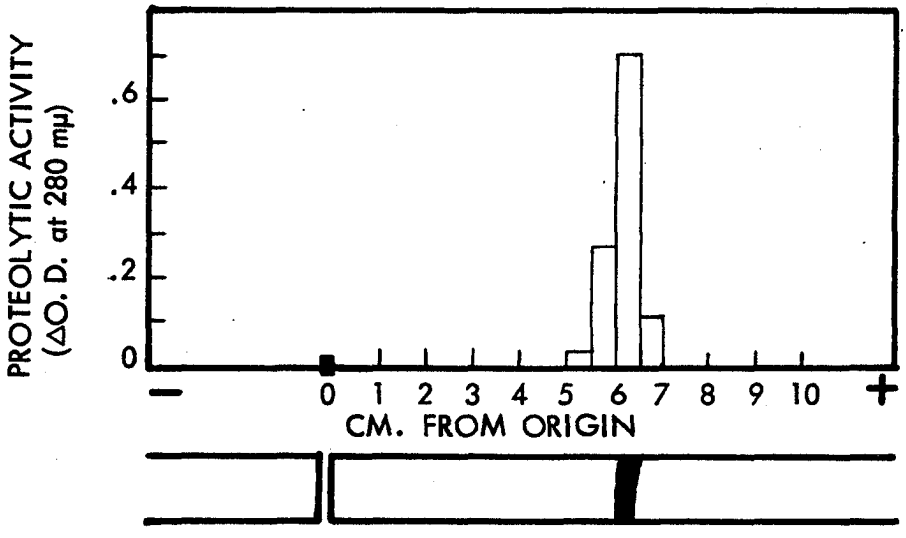

Figure 8.-Zone electrophoresis of gastricsin. The lower part of the graph shows the zone electrophoretic pattern on starch gel. The upper graph shows the location of activity in the starch gel.

of 0.725 was used for partial specific volume.

The molecular weight of gastricsin was also determined by the Archibald method. The values calculated from the meniscus and the bottom of the solution phase were 31,285 and 32,523 respectively. The molecular weight of human pepsin was not determined by this method.

TABLE 2

MOLECULAR WEIGHT DETERMINATION OF GASTRICSIN AND PEPSIN BY SEDIMENTATION VELOCITY METHOD

	$S_{20,w}$ $x10^{-13}$	$D_{20,w}$ $x10^{-7}$	Molecular Weight
Gastricsin	3.53	8.90	36,027
Human Pepsin	3.33	9.65	31,390

The values for the molecular weight of gastricsin as determined by the two methods are slightly different. Several determinations were made with the sedimentation velocity method and the results were highly reproducible. However, large variations were observed among the values obtained from the different runs by the Archibald method. This suggests that more confidence should be placed on the molecular weight obtained from the sedimentation velocity method.

Comparisons of the Physico-chemical Properties of Gastricsin and Human Pepsin

Ultraviolet Absorption Spectrum

The ultraviolet absorption spectra (Figure 9) were determined in a Cary recording spectrophotometer. Crystalline gastricsin

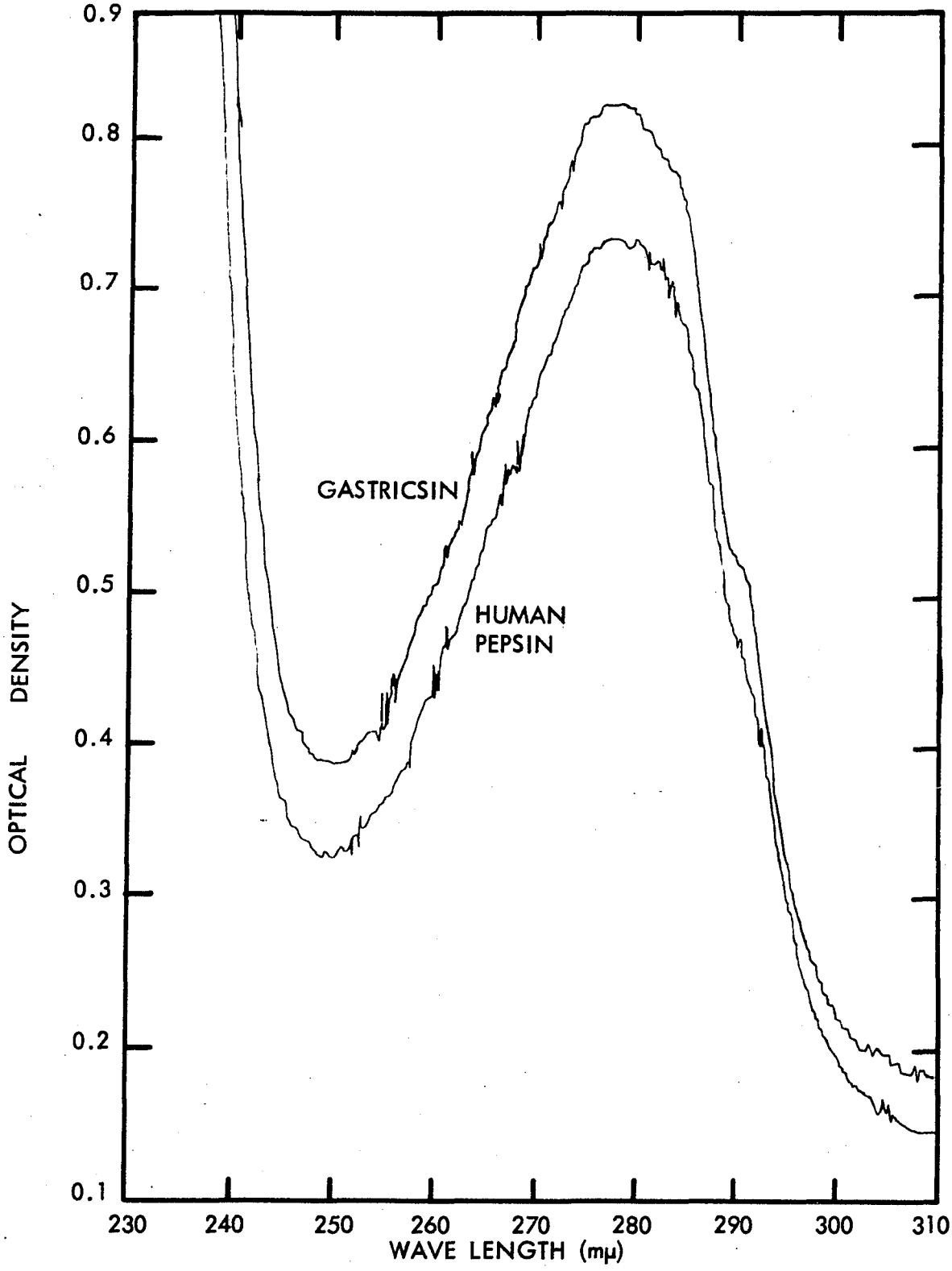

Figure 9.-The absorption spectrum of gastricsin and human pepsin in the ultraviolet region.

in distilled water shows a maximum absorption at 278 mu ($E^{1\%}_{1cm}$ = 12.83) and a minimum absorption at 247 mu ($E^{1\%}_{1cm}$ = 4.56). The curve is typical of a simple protein and indicates that gastricsin has no ultraviolet-absorbing cofactor. A similar spectrum was observed for human pepsin. The adsorption peak of both enzymes has a shoulder at 290 mu.

Electrophoretic Migration

In the case of paper electrophoresis, human pepsin was observed to migrate 9.0 cm. toward the anode (Figure 10) and crystalline hog pepsin migrated 9.7 cm. Gastricsin did not show a significant migration from the origin. The electrophoresis of a mixture of pepsin and gastricsin resulted in the separation of the two enzymes, but when human pepsin was mixed with hog pepsin only one band was observed in the paper strip after electrophoresis.

It should be pointed out that when the electrophoresis of gastricsin was performed on paper or on starch gel (see the section on the homogeneity of crystalline gastricsin) under otherwise similar conditions, the enzyme migrated on starch gel but remained at the origin on paper. Furthermore, neither protein nor gastricsin activity was eluted with distilled water or acetate buffer. Both observations were interpreted to mean that gastricsin is either denatured or strongly adsorbed by paper.

Comparisons of the Enzymatic Properties of Gastricsin and Human Pepsin

Optimal pH

The pH optimum curves were obtained by measuring the proteolytic

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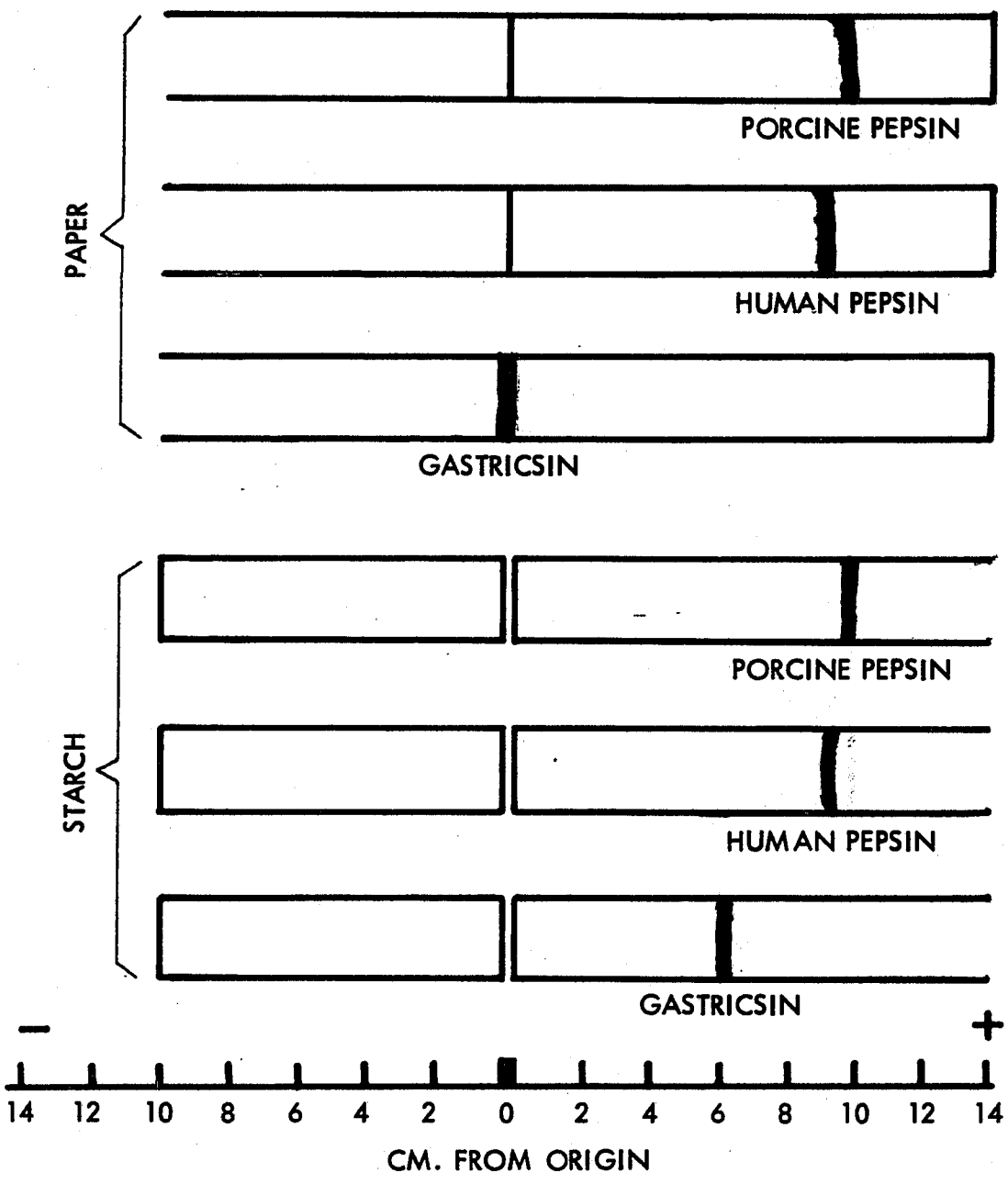

Figure 10.-Electrophoretic migration of gastricsin and human and porcine pepsin on filter paper and starch gel.

activities at pH values ranging from 1.1 to 4.0. The digestion mixture contained 2 per cent hemoglobin in 0.1 M citrate-HCl buffer. Optimal activity of human pepsin was observed at pH 2.0 (Figure 2), which corresponds to the known optimal pH of hog pepsin. The pH curve for cyrstalline gastricsin showed activity from pH 1.1 to 3.5, with a well defined optimum at pH 3.0 (Figure 11). A slight but consistent difference was found between the previously observed pH curve of gastricsin prepared by chromatography and the one shown in Figure 11. The difference would appear to indicate that the preparations from the column were contaminated with small amounts of pepsin.

Heat Inactivation

Solutions of human gastricsin and pepsin at both pH 3.2 were prepared, so that the activities of both enzymes would not differ more than 10 per cent when measured according to the method of Anson and Mirsky (51). One milliliter aliquots of each solution were incubated separately for 10 minutes in a constant temperature water bath at 45°, 50°, 55°, 60°, 65°, 70°, and 75°, and the proteolytic activities of the same solutions were determined again. The results were calculated as percentage of inactivation (Figure 12), with the activity of enzymes incubated at 45° taken as zero per cent inactivation. The enzyme solutions incubated at 75° were 100 per cent inactivated. It was found that the relative heat stabilities for both enzymes at different pH's were the reverse of the relative activities at the same pH's. For example, Figure 11 shows that at pH 2.0 and 65°, gastricsin was 44.8 per cent inactivated, whereas pepsin as 69.0 per cent inactivated. The

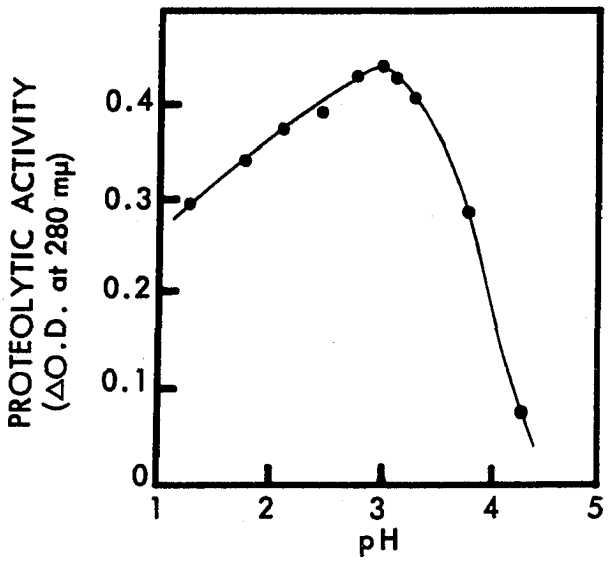

Figure 11.-The pH curve of crystalline gastricsin.

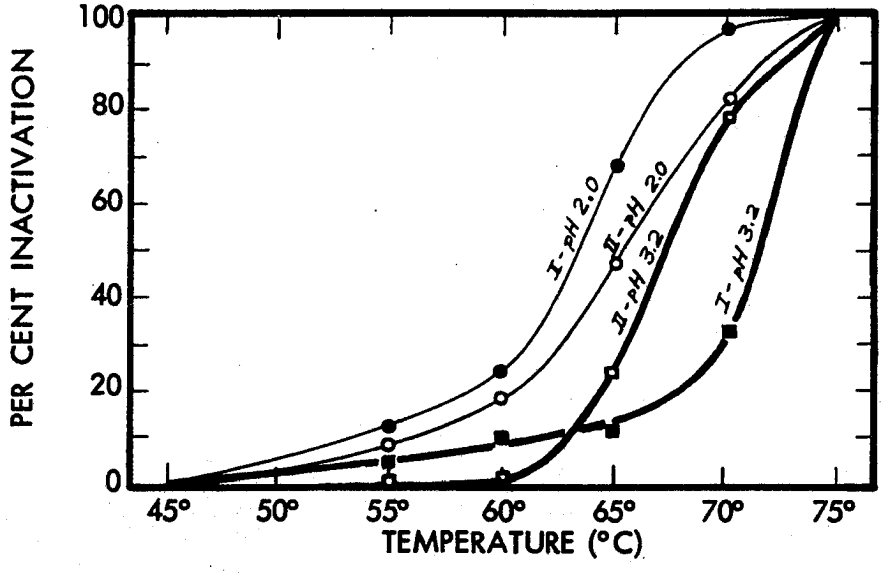

Figure 12.-Heat inactivation of human pepsin and gastricsin. I-Human pepsin and II-Gastricsin

results for relative stabilities of the two enzymes at pH 3.2 were reversed. After incubation at 65° gastricsin was 22.3 per cent inactivated and pepsin was only 11.2 per cent inactivated. These results have been interpreted to mean that at least part of the inactivations were due to autodigestion.

Milk-Clotting and Proteolytic Activities

Solutions of crystalline hog pepsin (0.25 mg. per 100 ml.), crystalline rennin (0.245 mg. per 100 ml.), human pepsin (0.26 mg. per 100 ml.), and crystalline gastricsin (0.284 mg. per 100 ml.), were compared for milk-clotting and proteolytic activities. The milk-clotting activities of gastricsin and pepsin, when calculated as per cent of the activity of rennin, were: gastricsin, 43.3 per cent; porcine pepsin, 65.6 per cent; and human pepsin, 44.6 per cent (Table 3). On the other hand, gastricsin was found to have a higher proteolytic activity than pepsin and rennin when measured with the method of Anson and Mirsky (51). Porcine pepsin was found to have 66.6 per cent of the proteolytic activity of gastricsin, and human pepsin and rennin had 80.0 and 8.15 per cent, respectively.

The Effect of Urea on the Optimal pH of Gastricsin and Pepsin

The proteolytic activity of gastricsin and human pepsin was measured in the presence of 3.6 M urea in buffers with pH values ranging from 1.1 to 5.0. Bovine hemoglobin was used as substrate. The assay of proteolytic activity was essentially the same as the procedure of Anson and Mirsky (51) as described in the section of experimental methods.

It was found that the optimal pH of gastricsin shifted from pH

3.0 to pH 4.1 in the presence of 3.6 M urea. Under identical conditions, the optimal pH of human pepsin was found to be shifted from pH 2.0 to pH 3.0 (Figure 13). The effect of urea on the specific activities of both enzymes was studied quantitatively. In the presence of urea, the specific activity of gastricsin at its optimal pH (4.1) decreased about 60 per cent compared to the specific activity of gastricsin in the absence of urea (at pH 3.0). Similarly, human pepsin showed a 77 per cent decrease of specific activity in the presence of urea.

TABLE 3

COMPARISON OF MILK-CLOTTING AND PROTEOLYTIC ACTIVITIES OF RENNIN, PEPSIN AND GASTRICSIN

	Concentration	Clotting Time*	Relative milk-clotting activity	Proteolytic activity†	Relative proteolytic activity
	μg./ml.	sec.	$\frac{Cr \times Tr}{C \times T} \times 100$‡	ΔO.D.280	$\frac{(PA) \times Cg}{(PA)g \times C} \times 100$§
Rennin	2.45	248	100	0.081	8.15
Gastricsin	2.84	492	43.3	1.151	100
Porcine Pepsin	2.50	370	65.6	0.675	66.6
Human Pepsin	2.60	511	44.6	0.843	80.0

*Measured according to the method of Berridge.

†Method of Anson and Mirsky.

‡Cr is the concentration of rennin, Tr is the clotting time for rennin. C and T are the concentration and clotting times for the respective enzymes.

§(PA)g is the O.D. at 280 mμ of the deproteinized solution from gastricsin assay, Cg is the concentration of gastricsin, and C and (PA) are the concentration and the O.D. at 280 mμ from the deproteinized solutions from the assays of the respective enzymes.

In order to determine whether the enzymes were inactivated by urea

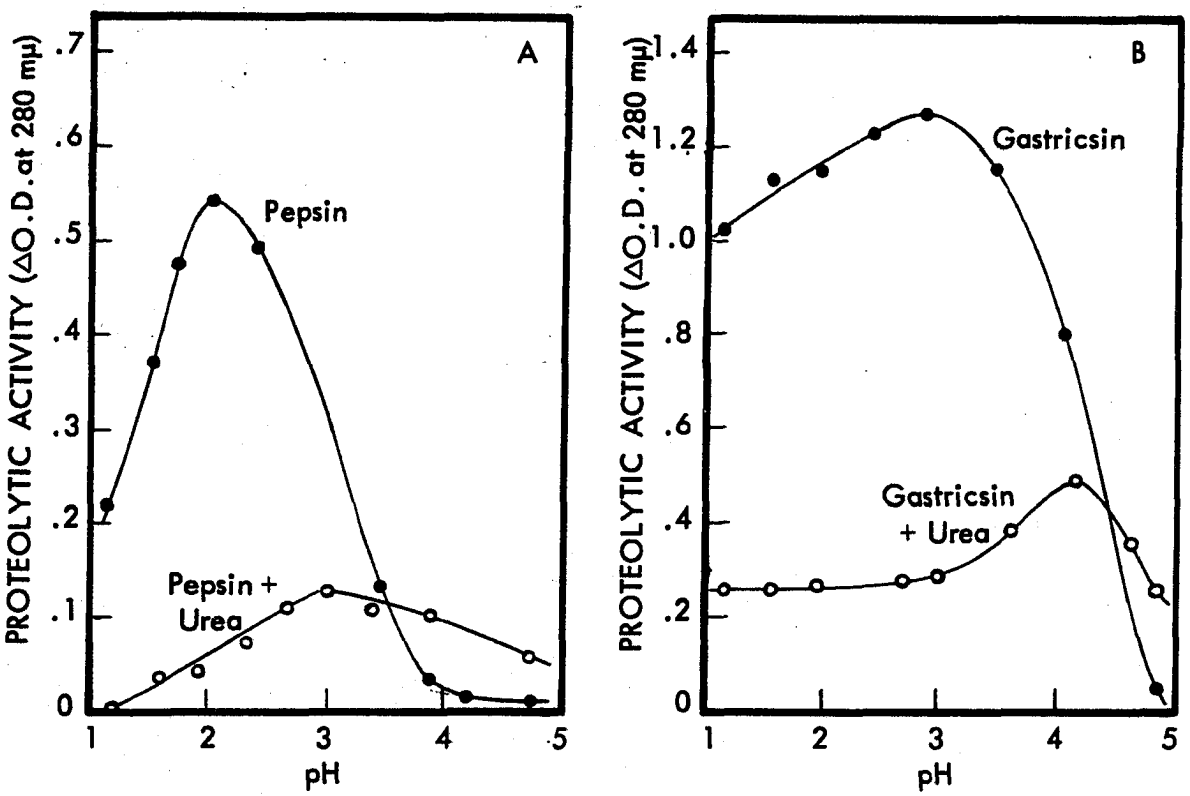

Figure 13.-The effect of urea on the pH optima of gastricsin and human pepsin. A - Pepsin and pepsin with urea. B - Gastricsin and gastricsin with urea.

during the incubation, the enzymes were incubated with substrate both in the presence and absence of urea. Samples were taken at two minutes time intervals over a period of 20 minutes. The proteolysis was stopped by immediate mixing with trichloroacetic acid. The optical density at 280 mu of the filtrate was determined for each sample. It was found that the progress of proteolysis was linear with time in all cases (Figure 14). This indicates that no inactivation of the enzymes had taken place during the incubation.

Activators and Inhibitors

Cysteine at the concentration of 1×10^{-3}M slightly activates gastricsin. No substantial activation or inactivation was obtained with any of the following salts tested at a concentration of 1×10^{-3}M: KCl, $CuCl_2$, KI, sodium citrate, $CaCl_2$, KCN, $FeCl_3$, sodium acetate, NaF, M_gSO_4, $SnSO_4$, $SrBr_2$, $MnSO_4$ and $LiSO_4$. Ascorbic acid at the same concentration also failed to show an effect.

The Terminal Amino Acid Residues of Gastricsin and Human Pepsin

N-terminal Amino Acid

The N-terminal amino acid residue of gastricsin was found to be serine, and that of human pepsin was found to be valine. The quantitative recovery is shown in Table 4. The recovery of N-terminal valine from human pepsin was 97.5%. However, 56% of serine was recovered from N-terminal position of gastricsin. An increase of hydrolyzing time did not improve the recovery.

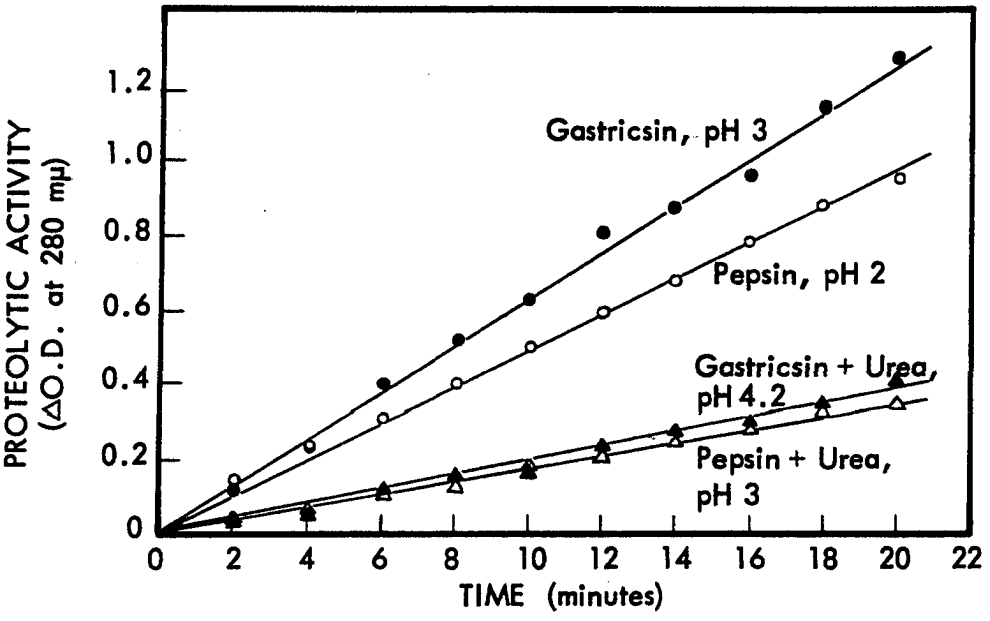

Figure 14.-The proteolytic activity of gastricsin and human pepsin at the presence and absence of urea.

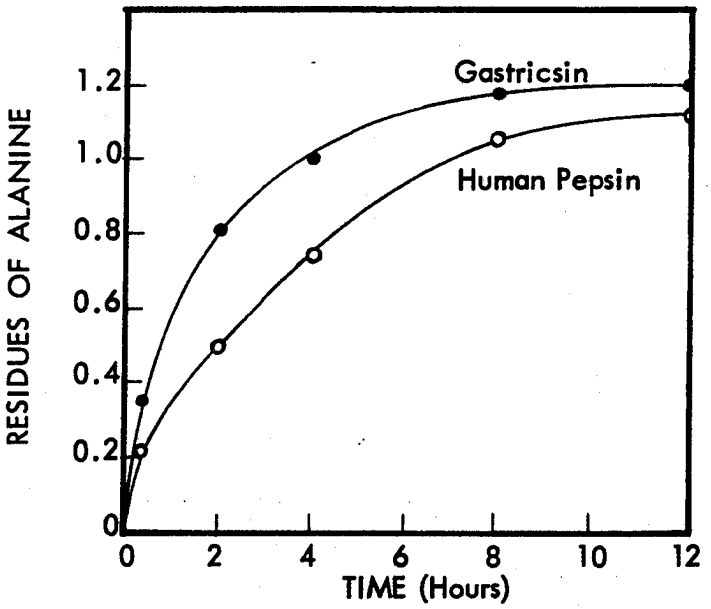

Figure 15.-The release of C-terminal alanine from gastricsin and human pepsin by carboxypeptidase.

TABLE 4

THE RECOVERY OF DNP-AMINO ACIDS FROM THE N-TERMINAL OF GASTRICSIN AND HUMAN PEPSIN

	DNP-Amino Acid	u mole DNP-Amino Acid per u mole of Protein
Gastricsin	Serine	0.560
Human Pepsin	Valine	0.975

C-Terminal Amino Acid Sequence

In the preliminary experiments, it was found that after a very short incubation of either gastricsin or human pepsin with carboxypeptidase, alanine was always the amino acid appearing in a significant amount on paper chromatography. This suggests that alanine is the C-terminal amino acid for both enzymes. The amount of alanine released during the incubation was followed by using the procedures of White et al. (Figure 15). It was found that after 12 hours of incubation, alanine approached one residue for each molecule of either gastricsin or human pepsin.

Duplicate samples of four hours incubation were taken for gastricsin and human pepsin. The free amino acids in the samples were determined with the FDNB method. In the incubation of either gastricsin or human pepsin, alanine was present in the highest quantity, then followed leucine and valine (Table 5).

The above results suggest that both gastricsin and human pepsin are molecules containing a single peptide chain. The two enzymes appear to possess different N-terminal amino acid residues, but the same three

amino acid sequence at the C-terminal of the molecules, which is -valyl-leucyl-alanine. It should be pointed out that two dimensional paper chromatography did not allow the separation of DNP-isoleucine. This may alter the conclusion concerning the C-terminal amino acid sequence. However, the fact that this C-terminal sequence is shared also by porcine pepsin (16) seems to be more than a coincidence.

TABLE 5

AMINO ACIDS RELEASED FROM GASTRICSIN AND HUMAN PEPSIN BY THE ACTION OF CARBOXYPEPTIDASE

	Alanine	Leucine	Valine
Gastricsin	0.97*	0.32	0.20
Human Pepsin	0.75	0.58	0.17

*All values given in u mole amino acid per u mole protein.

The results on C- and N-terminal amino acids are compared for gastricsin, human pepsin and porcine pepsin in the following diagram:

Enzyme	Amino Acid Sequence
Gastricsin	H_2N-Ser.....Val-Leu-Ala-COOH
Human Pepsin	H_2N-Val.....Val-Leu-Ala-COOH
Porcine Pepsin	H_2N-Isoleu.....Val-Leu-Ala-COOH

Specificity of Gastricsin

The affinity of gastricsin and human pepsin toward different protein substrates was found to be different. Egg albumin and bovine hemoglobin were used as substrates and were incubated with two enzymes

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Specificity of Gastricsin

The affinity of gastricsin and human pepsin toward different protein substrates was found to be different. Egg albumin and bovine hemoglobin were used as substrates and were incubated with two enzymes

at pH 1.5 with all other conditions identical to those described in the methods section. It was found that gastricsin hydrolyzed bovine hemoglobin at a higher rate than did pepsin (Table 6). On the contrary, pepsin shows higher specific activity than gastricsin when egg albumin is used as the substrate.

TABLE 6

THE SPECIFIC ACTIVITIES OF HUMAN PEPSIN GASTRICSIN TOWARD DIFFERENT PROTEIN SUBSTRATES

Substrate	Pepsin	Gastricsin
	specific activity*	specific activity*
Bovine Hemoglobin	468.9	641.4
Egg albumin	43.6	17.2

*Specific activity = u mole tyrosine released per mg. enzyme per 10 minutes.

Three different synthetic peptides, which are known to be the substrates of crystalline hog pepsin (18), were tested on human pepsin and crystalline gastricsin. The peptides tested were carbobenzoxy-L-glutamyl-L-tyrosine, carbobenzoxy-glycyl-L-phenylalanine and carbobenzoxy-L-glutamyl-L-phenylalanine. Twenty mg. of the peptide was dissolved in a small amount of water and 1 N NaOH was added dropwise until it dissolved completely. A solution containing 10 mg. of enzyme in citrate-HCl buffer, pH 2.5, was added to peptide solution and the volume was brought to 6 ml. with the same buffer. The pH of the solution was checked with a pH meter and readjusted to pH 2.5 when necessary. Two

controls of the experiment were made, one was without substrate, and the other was without enzyme. In the preliminary experiments, it was found that during the incubation of these solutions at 38°C for 24 hours there was essentially no hydrolysis of peptide in the absence of enzyme. However, the incubation of either human pepsin or crystalline gastricsin, in the absence of substrate peptide, resulted in a significant increase of ninhydrin positive substance. This indicated the presence of an autolysis of the enzymes. It was necessary to distinguish between the measurement of the true hydrolysis of the peptide and the autolysis, which may even differ from the enzyme control due to the presence of competition between substrate and autolysis.

A procedure was then developed to isolate and measure quantitatively the amino acid hydrolyzed from the substrate peptide. Two aliquots of samples were taken at certain time intervals of the incubation, and were quantitatively transferred on a sheet of Whatman No. 1 filter paper along with the amino acid standards. Descending paper chromatography for amino acid was run. After the samples had been chromatographed, the path of one of the two samples on the paper was cut apart from the other and sprayed with ninhydrin solution to locate the amino acid spot. The amino acid spot was compared to the standards to make certain its identity. The paper strip of the other sample, which was not developed with ninhydrin, was cut out at the same position where the amino acid spot was located. The amino acid on the paper was eluted by capillary action with water. In order to eliminate some other ninhydrin positive materials, presumably ammonium salts, the sample was treated with 0.5 ml. of 0.1 N $NaHCO_3$ and dried in a turning evaporator. The excess alkali was

neutralized with acetic acid, and the excess acid was also eliminated in vacuum. The sample was then analyzed quantitatively with the ninhydrin method of Rosen (54). The procedure was first applied to different amounts of a standard amino acid, and the recovery was found to be quantitative.

Figure 16 shows the comparison of the direct measurement of ninhydrin color and the measurement after the application of this procedure on the incubation of carbobenzoxy-glutamyl-tyrosine with both gastricsin and pepsin along with the controls with enzyme alone. In the direct measurement, the ninhydrin color produced by incubating gastricsin or pepsin alone exceeded that of the substrate incubated with gastricsin. However, from the purified amino acid system (i.e., tyrosine in this case), it is clear that the ninhydrin positive substances produced in the autodigestion of gastricsin and pepsin were not the amino acid in question. From the quantitative comparison of the ninhydrin color of the substrate incubated with enzymes in both direct and purified systems, it is also clear that the autodigestion did not take place to the extent as the enzymes incubated alone (Figure 16). Similar results were observed for other two synthetic substrates.

Crystalline gastricsin was found to be capable of hydrolyzing all three synthetic peptides. The quantitative results of the hydrolysis, which were obtained from the purification procedures of the amino acid, showed that crystalline gastricsin has less affinity for all three peptide substrates than pepsin (Table 7). The ratio between the activity of gastricsin and pepsin varies from substrate to substrate. For carbobenzoxy-glutamyl-tyrosine, gastricsin showed about 50% of the activity of pepsin, in the case of carbobenzoxy-glycyl-phenylalanine, gastricsin

had 30% of the specific activity of pepsin, however, gastricsin hydrolyzed carbobenzoxy-glutamyl-phenylalanine at a rate only 5% that of pepsin.

TABLE 7

THE HYDROLYSIS OF SYNTHETIC PEPTIDES BY CRYSTALLINE GASTRICSIN AND PEPSIN

Substrate	Gastricsin	Pepsin
	specific activity*	specific activity*
CBZ-glu-tyr.**	0.0028	0.0057
CBZ-gly-phen.	0.0003	0.0010
CBZ-glu-phen.	0.0002	0.0040

*Specific activity = u mole amino acid released per mg. enzyme per 10 minutes.
**CBZ=carbobenzoxy; glu = glutamyl; tyr = tyrosine; gly = glycyl and phen. = phenylalanine.

The 'Zymogens' in Human Gastric Mucosa and Their Activation

In order to study the origin of gastric proteolytic enzymes, attempts were made to extract human gastric mucosa with an alkaline solution and to study the proteolytic activity of this extract. This is based on the possibility that human gastric proteolytic enzymes, like the porcine pepsin, originate from an inactive precursor present in the gastric mucosa.

Human gastric mucosa was separated from other tissues, minced, ground and extracted with 0.1 M $NaHCO_3$ solution as described by Herriott (11). The extract was centrifuged at 2°C, and the supernatant was dialyzed in a 4°C cold room against several changes of ammonium hydroxide

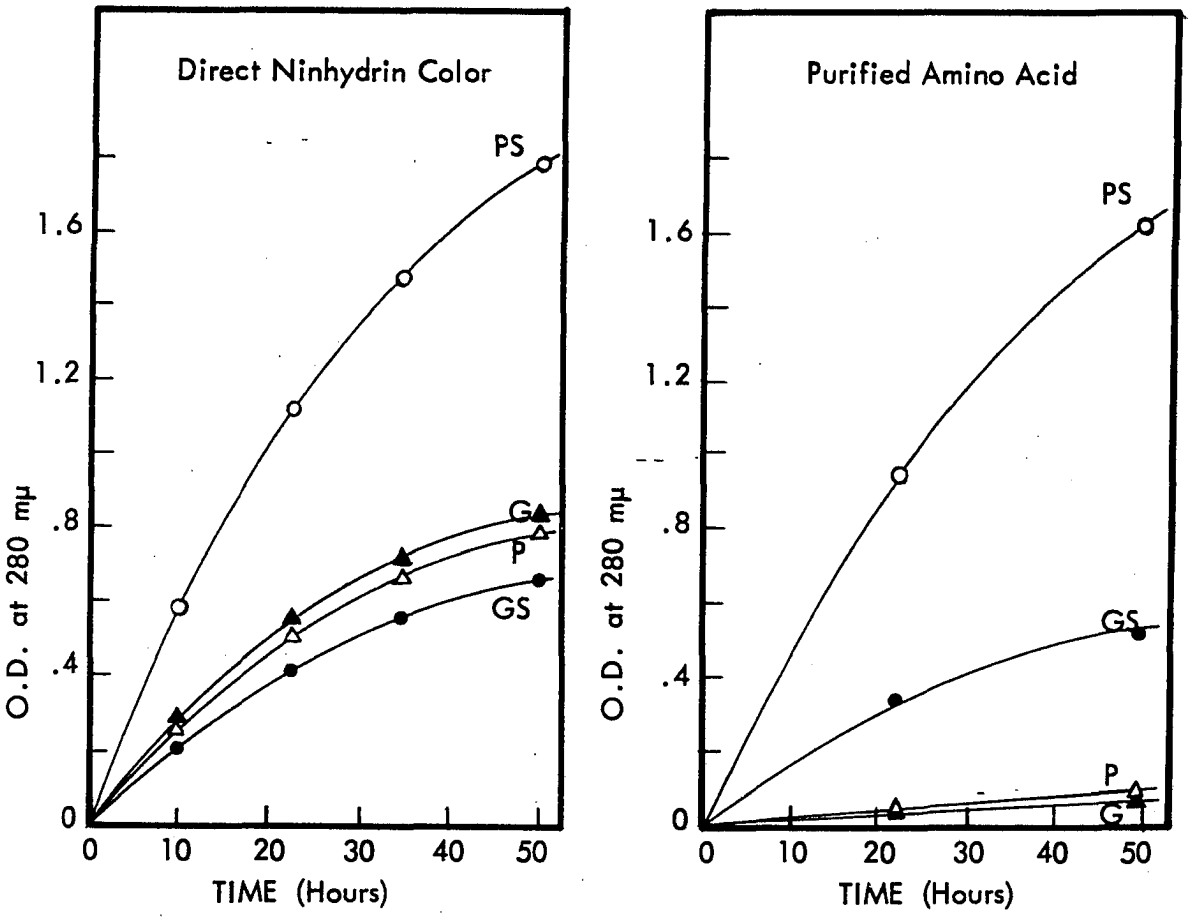


Figure 16.-The hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosin by human pepsin and gastricsin. The left part of the figure shows the results made with direct ninhydrin measurement. The right part of the figure shows the measurement with ninhydrin of the purified tyrosine hydrolyzed from the peptide. The abbreviations: G-gastricsin; S-substrate; P-human pepsin.

solution of pH 8.0-8.5. The dialyzed extract was then lyophilized.

The Presence of Gastricsin and Pepsin in the Acidified Human Gastric Mucosa Extract

The pH dependence of the proteolytic activity of the extract of human gastric mucosa was studied. The extract was dissolved in the buffer of pH 2.5, incubated for three hours at room temperature and the proteolytic activity was determined at different pH values. The results show the presence of two optimal pH's (2.0 and 3.0) corresponding to those of pepsin and gastricsin (Figure 17).

In order to have further proof of the presence of gastricsin and pepsin in the gastric mucosa, the extract was dissolved in sodium citrate buffer of pH 3.0 and was fractionated in a small column (1.2 x 10 cm.) of Amberlite IRC-50 (XE-64) resin according to the procedure previously described. Two protein peaks were eluted at pH 4.02 and pH 4.43 of the effluent respectively, which corresponds to the pH values where pepsin and gastricsin are normally eluted. The proteolytic activity measurement of the fractions showed that both of these two peaks correspond to enzyme activity (Figure 18).

Fractionation of Extract of Human Gastric Mucosa on DEAE-cellulose Column

The lyophilized extract of human gastric mucosa (1.665 gm.) was dissolved in 100 ml. of 0.005 M potassium phosphate buffer, pH 7.5. The solution was centrifuged and the supernatant was passed slowly through a DEAE-cellulose column (3.0 x 22 cm.) which had been previously equilibrated with the same buffer. A concentration gradient from 0.005 M to 0.33 M

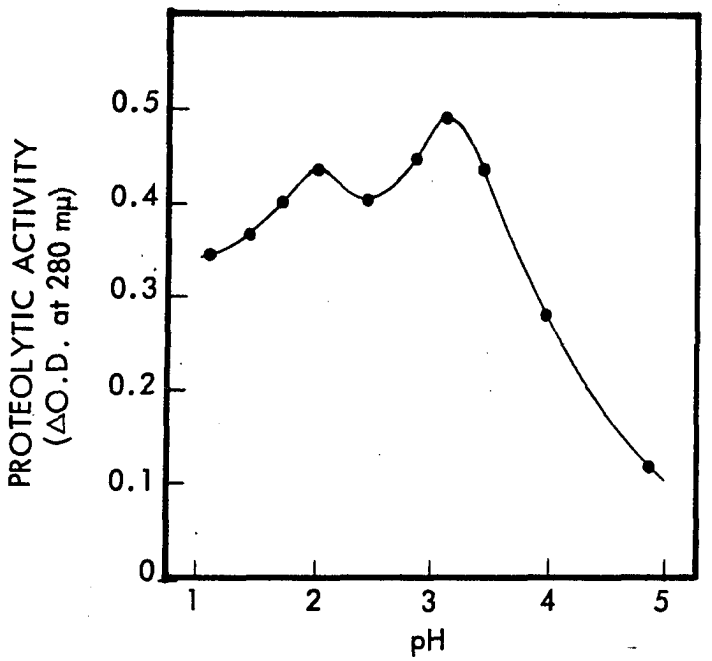


Figure 17.-The pH dependent curve for the proteolytic activity of human gastric mucosa extract.

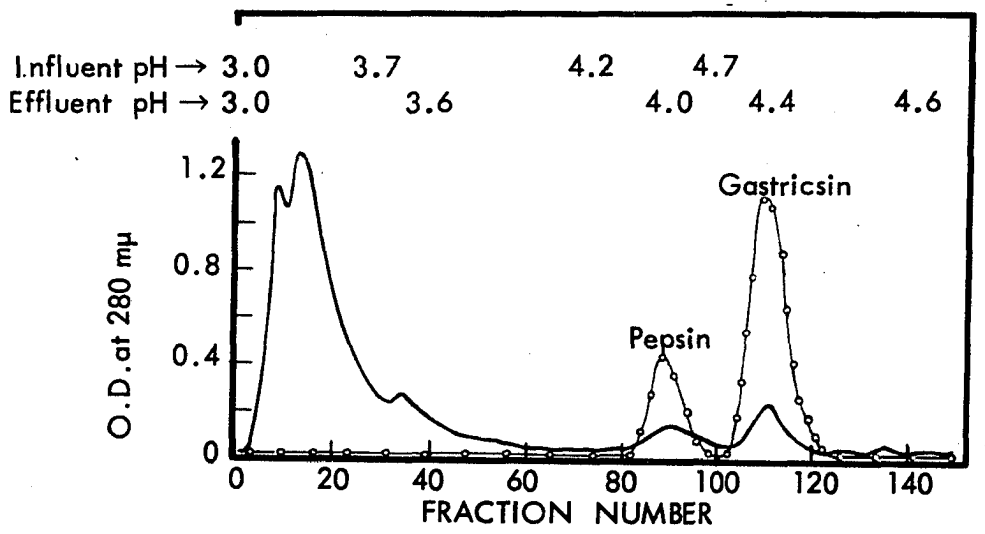


Figure 18.-Fractionation of acidified extract of human gastric mucosa in an Amberlite IRC-50 column. The solid line shows the protein concentration. The line with open circles represents the proteolytic activity.

of phosphate was applied during the elution, while the pH of the buffer was maintained at 7.5. The effluent was collected in a fraction collector with 5.0 ml. of solution in each test tube. The fractions were analyzed for protein content and for proteolytic activity. Two proteolytically active peaks were observed (Figure 19), which were eluted at 0.12 M and 0.2 M of phosphate concentration respectively. Both activity peaks are quite symmetrical and well separated from each other, however, neither corresponded well to a particular protein peak. For the purposes of convenience in further discussion, the larger proteolytic peak, which was eluted second in the chromatography, is called 'zymogen I', while the smaller proteolytic peak is called 'zymogen II'.

Fractionation of Extract of Human Gastric Mucosa in Starch Electrophoresis

Soluble starch of reagent grade was blended in a buffer of sodium phosphate, pH 7.0 and ionic strength 0.1, to form the supporting material for electrophoresis. A constant voltage was applied with the field strength of 6 volts per cm. About 15-20 mg. of lyophilized human gastric mucosa extract was applied with the soluble starch at the origin, which was a slit 0.5 in width. After the voltage had been applied for 17 hours, the starch block was cut perpendicular to the long axis of the strip at 0.5-cm. intervals and the pieces were put into separate test tubes and each eluted with 2 ml. of sodium phosphate buffer (pH 7.0). The test tubes were stirred thoroughly and allowed to stand in a cold room (4°C) for a few hours until the starch sedimented. The clear supernatant fractions were analyzed for protein content and proteolytic activity.

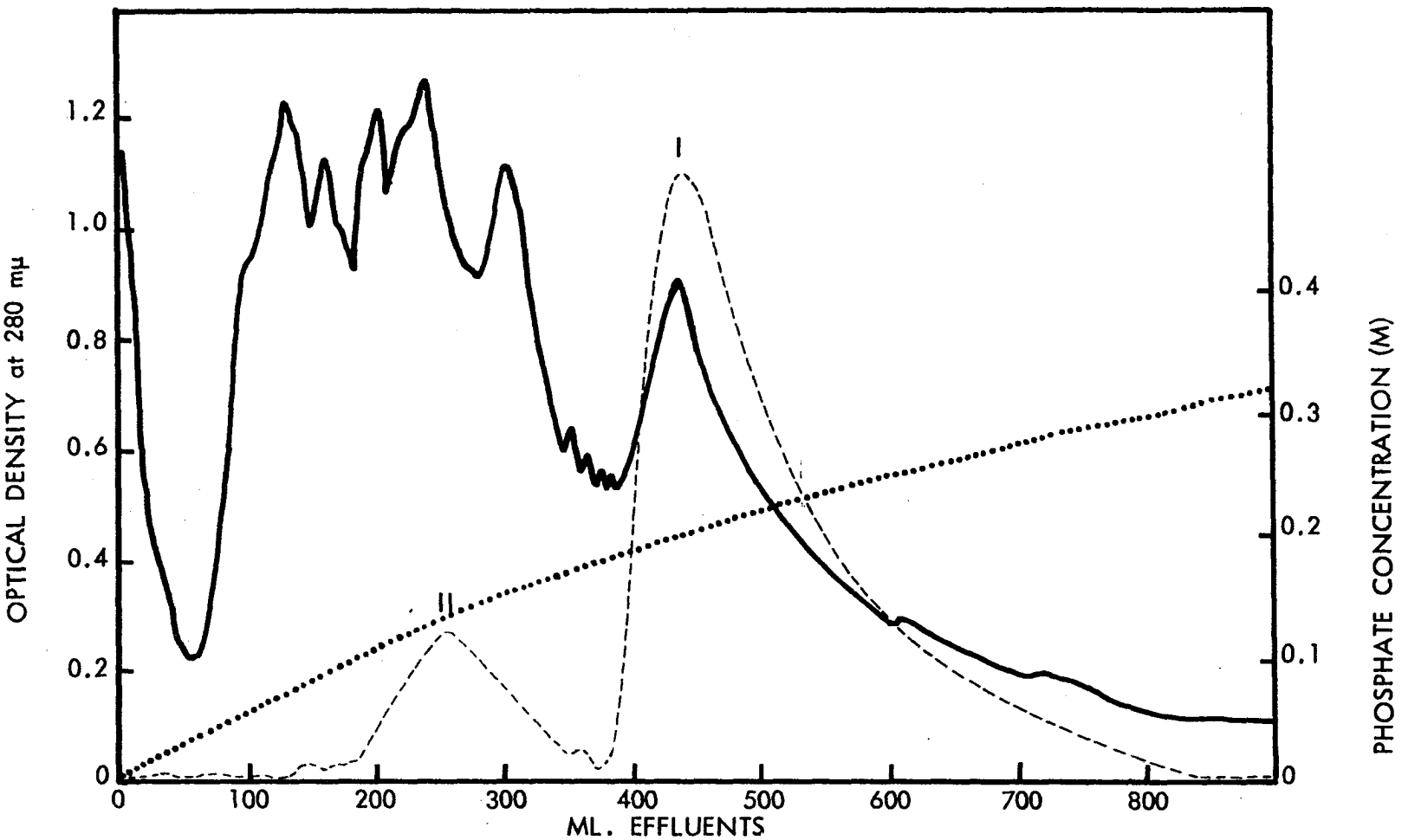


Figure 19.-The fractionation of extract of human gastric mucosa in a DEAE-cellulose column. The solid line represents the protein concentration, the broken line represents the proteolytic activity and the dotted line represents the phosphate concentration.

Two proteolytic peaks were observed in the starch electrophoresis (Figure 20). The relative sizes of the two peaks were identical with those observed when the separations were accomplished chromatographically using a DEAE-cellulose column (Figure 19). (This comparison is valid, since the measurements of proteolytic activity were quantitative). The identity of the two proteolytically active peaks was further studied by comparing their electrophoretic mobility with those of the 'zymogen I' and 'zymogen II' fractions from the column chromatography. It was found that 'zymogen I' as well as the fast moving activity peak migrated 9 cm. from the origin toward the anode, while the 'zymogen II' and the slow moving activity peak migrated 6.5 cm. toward the anode. The protein pattern, again, did not correspond well with the proteolytic activity.

The Zymogen Nature of 'Zymogen I' and 'Zymogen II'

It is known from the work of Northrop et al. (3) that pepsinogen is stable in alkaline media and possesses no milk clotting activity. However, the acidification of pepsinogen causes the loss of alkaline stability and the appearance of milk clotting activity. Similar experiments were carried out for 'zymogen I' and 'zymogen II'. The pH of each 'zymogen' solution, which was in a phosphate buffer of pH 7, was adjusted to pH 2 with 3 N HCl. After 2 minutes, the pH of the solution was readjusted to pH 8 with 1 N NaOH solution, and 0.1 ml. aliquots of each sample were taken at 10 minute intervals and mixed immediately with 1.0 ml. of pH 2 citrate buffer. The samples were analyzed for proteolytic activity, and compared to the non-acidified 'zymogens' as well as gastricsin, which were run simultaneously under identical conditions. It was found that both 'zymogens' were stable at pH 8.0, while

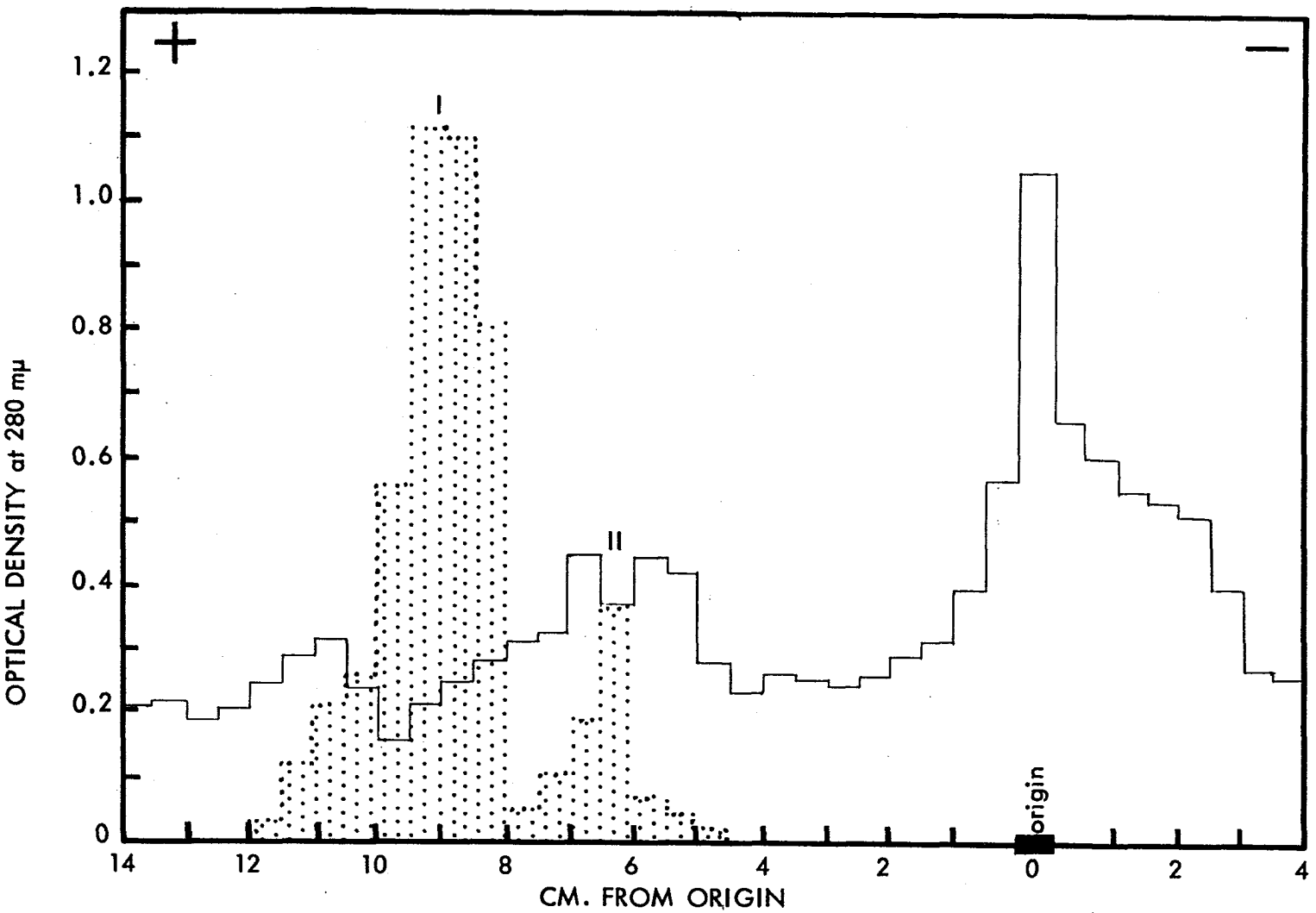


Figure 20.-Starch electrophoresis of extract of human gastric mucosa. The solid line indicates the protein concentration, the dotted area indicates the proteolytic activity.

acidification caused rapid loss of activity at pH 8.0. Gastricsin and pepsin also lost activity at pH 8. A typical experiment is shown in Figure 21.

Milk clotting activity was not observed for either 'zymogen' until after it was acidified. It was not possible to measure milk clotting activity quantitatively for either 'zymogen', since the activity was very low, it required 20 to 30 minutes to clot the milk solution. However, in the case of non-acidified 'zymogen', no milk clotting was found after 5 hours of incubation.

From the above results it was concluded that both 'zymogen I' and 'zymogen II' have the characteristics of the precursors for acid-active proteolytic enzymes (3, 46). The 'zymogens' are activated at an acidic pH as in the case of pepsinogen and prorennin.

The Activation Products of the 'Zymogens'

The pH optimum curves of the proteolytic activity from the activation of 'zymogen I' and 'zymogen II' were determined by measuring the proteolytic activity at pH's from 1.1 to 4.9. 'Zymogen II' was found to have a wide activity range from pH 1.5 to 4.9 with a well-defined optimum at pH 3.0. 'Zymogen I' showed two optimal pH's (1.8 and 3.2) and a rapid decrease in activity when the pH approached 4.0 (Figure 22). From this result, it seems possible that 'zymogen I' produces both pepsin and gastricsin upon activation. It was desirable to fractionate the activation products of the 'zymogen I' preparation.

Due to the fact that only a limited amount of material for 'zymogen I' preparation was available, a technique was developed to enable the use of an Amberlite IRC-50 (XE-64) ion-exchange column

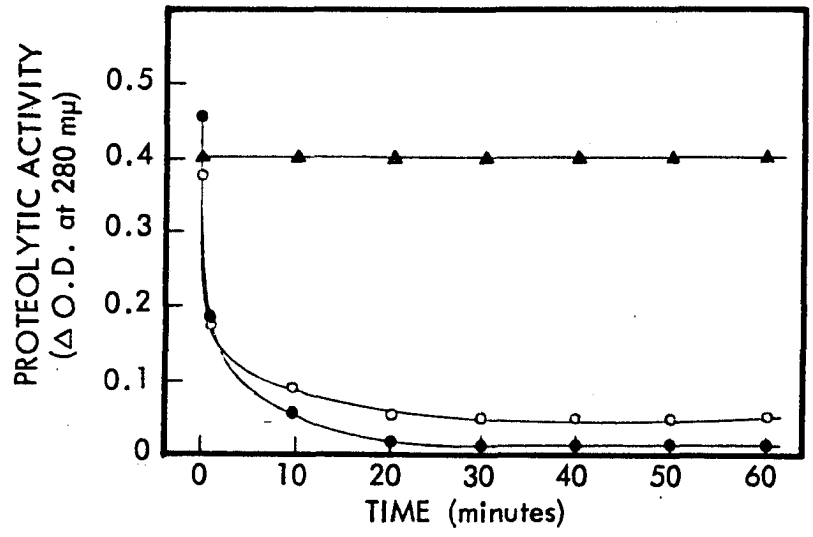


Figure 21.-The stability of 'zymogen I', acid activated 'zymogen I' and gastricsin at pH 8. ▲—▲ 'zymogen I', ○—○ acidified 'zymogen I' and ●—● gastricsin.

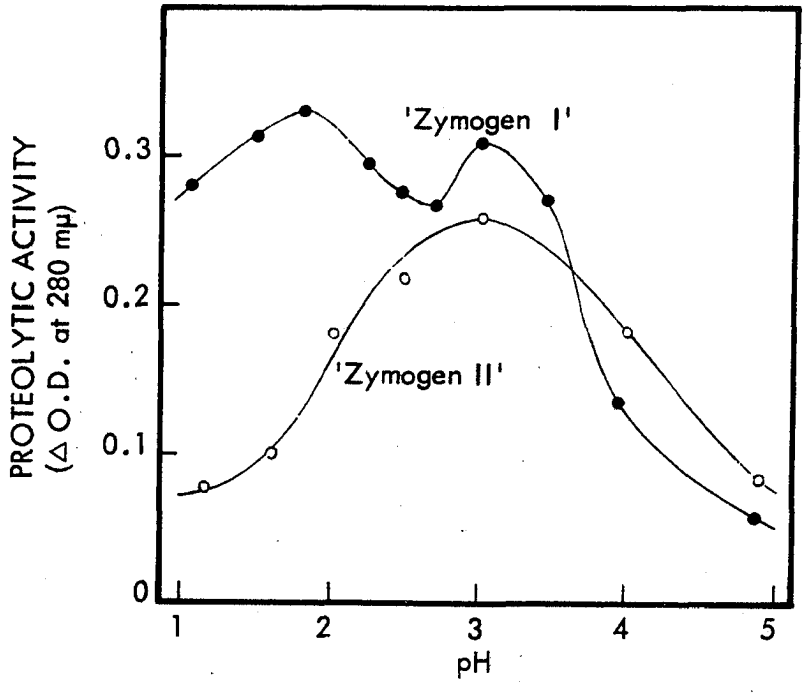


Figure 22.-The pH optimum curves of 'zymogen I' and 'zymogen II'.

fractionation on a microscale. A column of 0.4 x 5.0 cm. of Amberlite IRC-50 resin was equilibrated with sodium citrate buffer, pH 3.0. About 1 ml. of 'zymogen I' solution (pH 3.0) was passed through the column slowly and the column was washed with 10 ml. of pH 3.0 sodium citrate buffer. From the experiments on the separation of pepsin and gastricsin it was known that each enzyme is eluted only at a certain pH of the effluent, namely pH 4.0 for pepsin and pH 4.4 for gastricsin. The column was first eluted with pH 4.2 buffer then with pH 4.4 buffer. The eluent solution was collected in a drop counting fraction collector with five drops in each tube, and the proteolytic activity was measured for each of the tubes. The amount of each buffer required to elute gastricsin and pepsin was decided from a preliminary experiment in which purified gastricsin and pepsin were mixed and adsorbed on an identical micro-column to the maximum capacity of the resin and eluted with the same buffer. It was found that the enzyme peaks eluted at 15 tubes after the change of the buffer; no more enzyme activity could be detected after 30 or 40 tubes. In this preliminary experiment, 0.1 mg. each of gastricsin and pepsin was found to be separated completely using this technique.

A 'zymogen I' solution was acidified to pH 2.5 and fractionation was made in a micro-column on the samples taken at 1 minute, 2½ hours and 5 hours after the activation. Both gastricsin and pepsin peaks were observed in all three samples (Figure 23). After 1 minute of acid activation, the size of gastricsin peak was larger than that of pepsin, however, the relative size of the two peaks was about the same and did not change from 2½ to 5 hours of acid activation. 'Zymogen II' did not

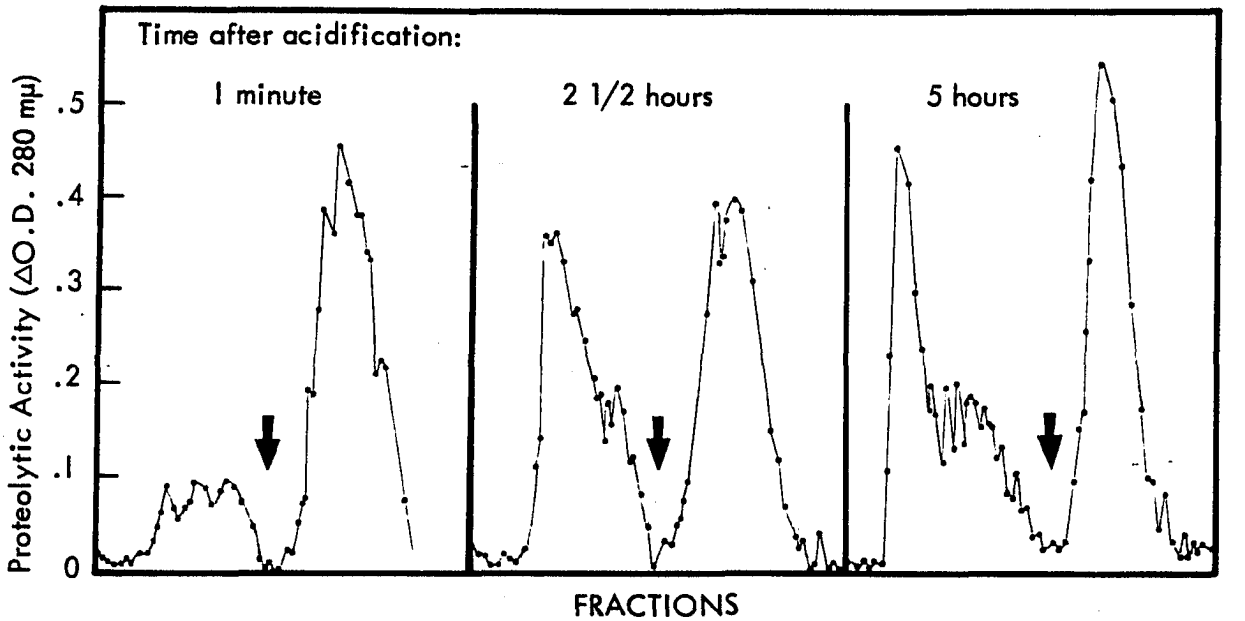


Figure 23.-The fractionation of activation products of 'zymogen I' in a micro-column of Amberlite IRC-50 resin. The position of the arrow indicates the change of buffer of pH 4.2 to 4.4.

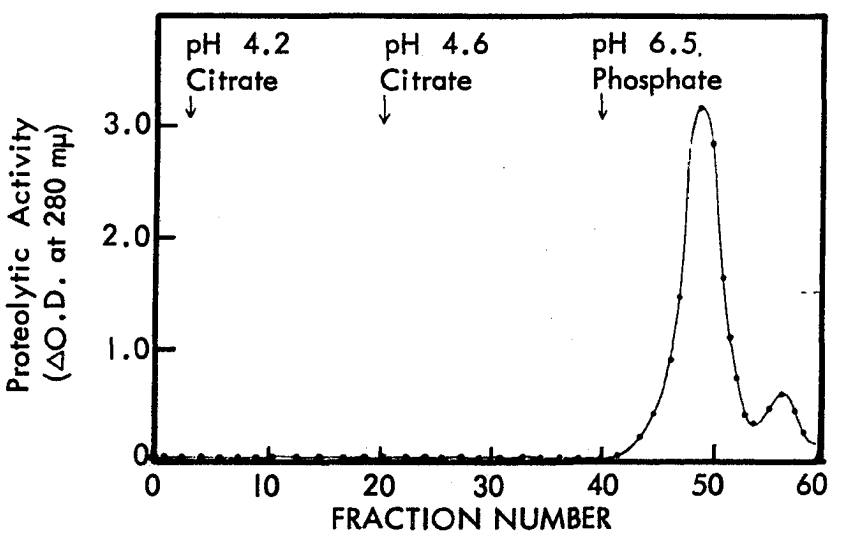


Figure 24.-The fractionation of activation products of 'zymogen II' in a micro-column of Amberlite IRC-50 resin.

give gastricsin or pepsin after acid activation, however, a proteolytically active fraction was eluted from the micro-column with pH 6.5 sodium phosphate buffer (Figure 24). This proteolytically active peak was never observed in the fractionation of human gastric juice.

Experiments on the Interconvertibility of Gastricsin and Pepsin

Gastricsin and pepsin were incubated separately at their optimal pH for 5½ hours at room temperature. The incubated solutions were fractionated in an Amberlite IRC 50 column (1.7 x 20 cm.). The non-incubated pepsin and gastricsin solution were also fractionated separately in resin columns as controls. It was found that no conversion between pepsin and gastricsin had occurred. No significant change in the chromatographic behavior of either enzyme was observed when it was incubated.

Comparison of the Proteolytic Enzyme of Different Species of Animals

Fractionation of Gastric Contents of Different Species of Animals

In order to compare the proteolytic enzymes present in different species of animals, rat and dog gastric juice as well as extract of porcine gastric mucosa were fractionated in the ion-exchange column, Amberlite IRC-50. The fractionation procedures were the same as used in the separation of gastricsin and pepsin. Rat and dog gastric juice were dialyzed against several changes of distilled water at 4°C and lyophilized. The extract of porcine mucosa was made on the minced and ground mucosa with 0.1 M sodium bicarbonate, using the procedures of Herriott (11). The extract was centrifuged at 2°C, and the supernatant was dialyzed in a 4°C cold room against several changes of ammonium

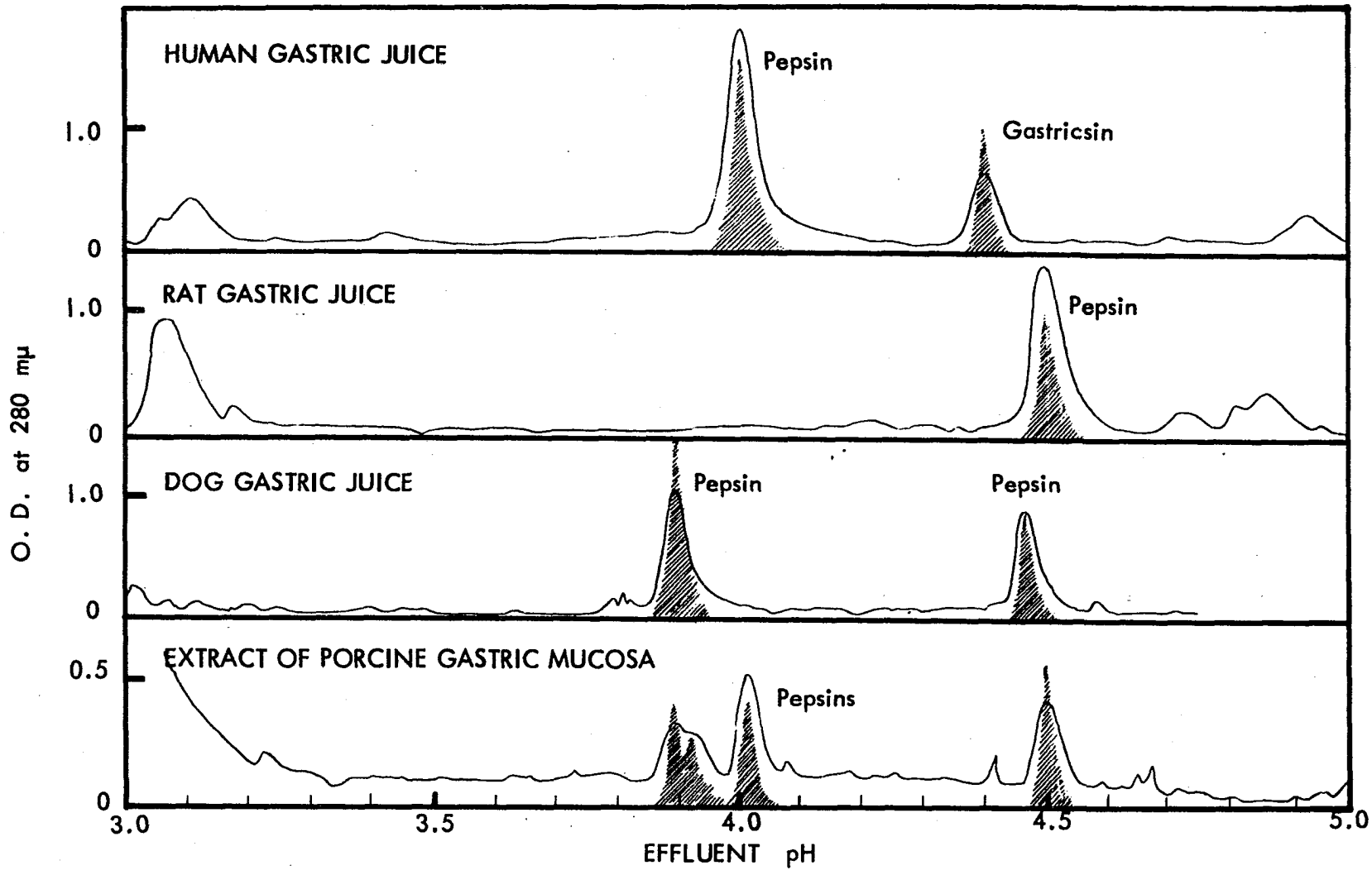


Figure 25.-The fractionation of gastric proteolytic enzymes from different species of animals and man.

hydroxide solution of pH 8.0-8.5. The dialyzed extract was then lyophilized.

The results of the fractionation are summarized in Figuer 25, in which the protein content (solid line) and proteolytic activity (shade area) are plotted against pH of the effluent. In the fractionation of rat gastric juice only one proteolytic peak was found to be eluted at pH 4.5 of the effluent. In the case of fractionation of dog gastric juice, there were two proteolytic peaks eluted at pH 3.91 and 4.48 of the effluent respectively. The fractionation of extract of porcine gastric mucosa is shown in the last chromatographic pattern in Figure 22. The lyophilized extract was incubated for 10 minutes in the starting sodium citrate buffer of pH 2.5, which presumably activated the zymogen. The fractionation otherwise was carried out in the same manner as the others. Four proteolytically active peaks were eluted at pH 3.88, 3.93, 4.04 and 4.5 of the effluent. Two other commercially available pepsin preparations, crude porcine pepsin (1 : 10,000) and crystalline porcine-pepsin, were also fractionated by the same procedures. Similar results were observed in both cases, however, the relative size of the proteolytically active peaks did show some variations. In crystalline porcine pepsin, the peak at pH 4.5 of the effluent was distinctly smaller than that in the extract of porcine gastric mucosa and crude porcine pepsin (Figure 26). A new protein peak with low proteolytic activity (shown in broken line) was found at pH 4.2 of the eluent, this peak was not observed in crude preparations. It is interesting that in crystalline porcine pepsin a proteolytically inactive peak was found at pH 2.5 of the effluent. The fractionation of crude porcine pepsin showed essentially

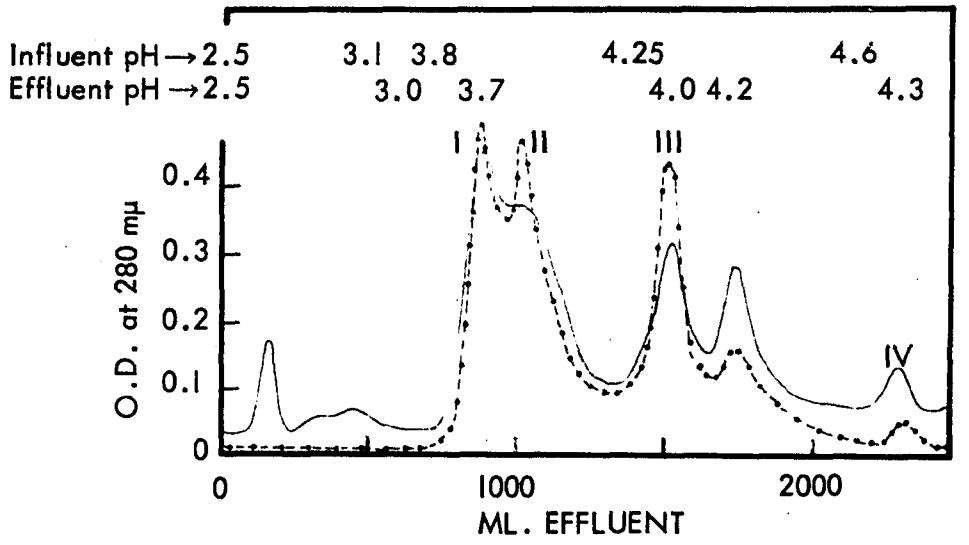


Figure 26.-Fractionation of crystalline, porcine pepsin in an Amberlite IRC-50 resin column. The solid line represents the protein concentration and the broken line represents the proteolytic activity.

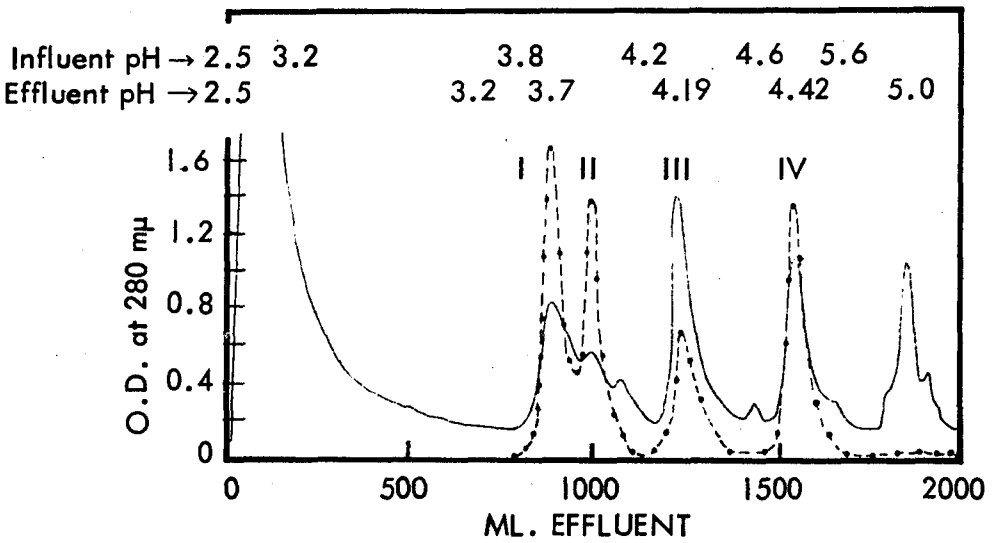


Figure 27.-Fractionation of crude porcine pepsin in an Amberlite IRC-50 resin column. The solid line represents the protein concentration and the broken line represents the proteolytic activity.

the same pattern as in extract of porcine gastric mucosa. The first two proteolytic enzymes seem to be better separated (Figure 27).

Optimal pH Values of Different Proteolytically Active Fractions

None of the proteolytically active fractions from hog, dog and rat show the pH optimum characteristics of gastricsin. The proteolytically active fraction from rat gastric juice has a broad range of pH in which it is active (approximately from pH 1.5 to pH 4.0). However, a distinct maximum activity is shown at pH 2.0 which is characteristic of a gastric pepsin. In dog gastric juice, both proteolytically active fractions have the same optimal pH at 1.5 (Figure 28). The first fraction, eluted at pH 3.9, seems to have a broader range of active pH than the second fraction, eluted at pH 4.5 of the effluent. The two proteolytic enzymes from dog gastric juice also have the characteristics of the pH optimum of pepsin. The optimal pH of proteolytically active fractions from crude porcine pepsin is shown in Figure 29. The first three fractions have the same optimal pH at 1.5. However, the fourth proteolytic enzyme fraction, which was eluted at pH 4.5 of the effluent, has an optimal pH lower than pH 1.0. These porcine gastric proteolytic enzymes also seem to be similar pepsins.

Starch Electrophoresis of Extract of Porcine Gastric Mucosa

The fractionation of extract of porcine gastric mucosa in starch electrophoresis was carried out in the same manner as the fractionation of extract of human gastric mucosa. The electrophoresis was run at room temperature for 17 hours with tap water flowing in the cooling system. The buffer used was sodium phosphate, pH 7.0, ionic strength 0.1 (61).

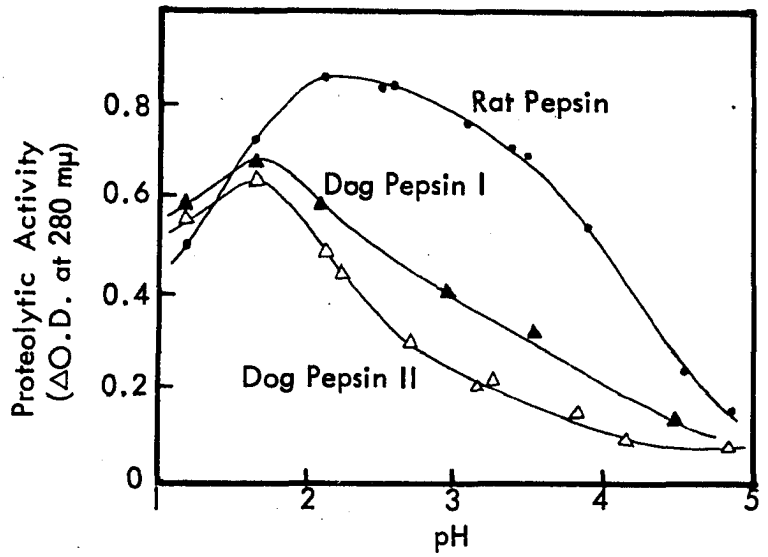


Figure 28.-The pH optima of rat pepsin and dog pepsins. (Dog pepsin-I: eluted at pH 3.9, -II: eluted at pH 4.5. cf. Figure 25).

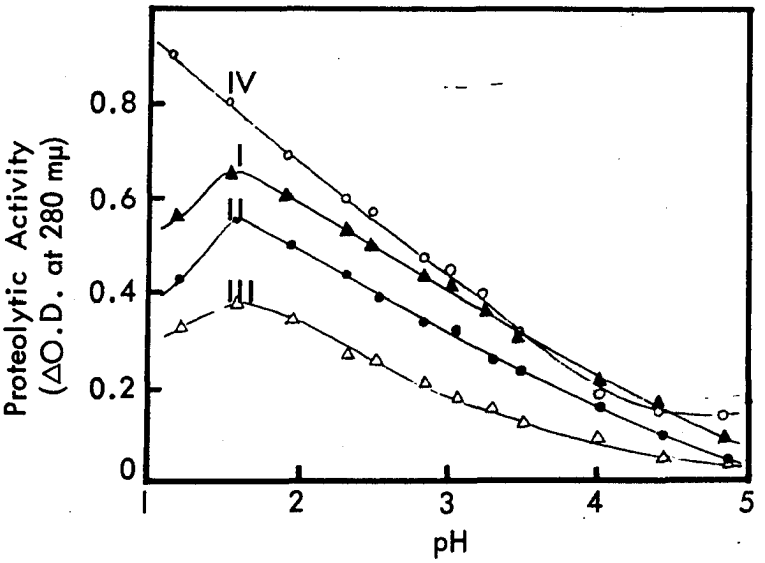


Figure 29.-The pH optima of porcine pepsins. The numbers in the graph correspond with the numbers marked in the chromatographic pattern (cf. Figure 27).

The constant potential of 150 volts was applied across a length of 33 cm. of the starch block. Two parallel blocks of starch strips were prepared. To one of the strips, approximately 15 mg. of extract of porcine gastric mucosa was applied with buffer wet starch into a 0.3 cm. slit, which was located midway along the starch strip. Approximately 2 mg. of crystalline hog pepsin were applied in the same manner to the other starch block. The analysis of protein and proteolytic activity was carried out in the same way as described previously.

Two zymogen fractions (Figure 30, under shaded area) were found to be present in extract of porcine gastric mucosa. The electrophoretic mobility of these two zymogens are markedly lower than crystalline porcine pepsin. The two zymogens were found to migrate 8 and 5 cm. toward the anode, compared to 13 cm. for crystalline pepsin. None of the zymogen fractions corresponded to the major protein peaks. The zymogens were found to be stable in alkaline solution and both were found to have an optimal pH of 1.5.

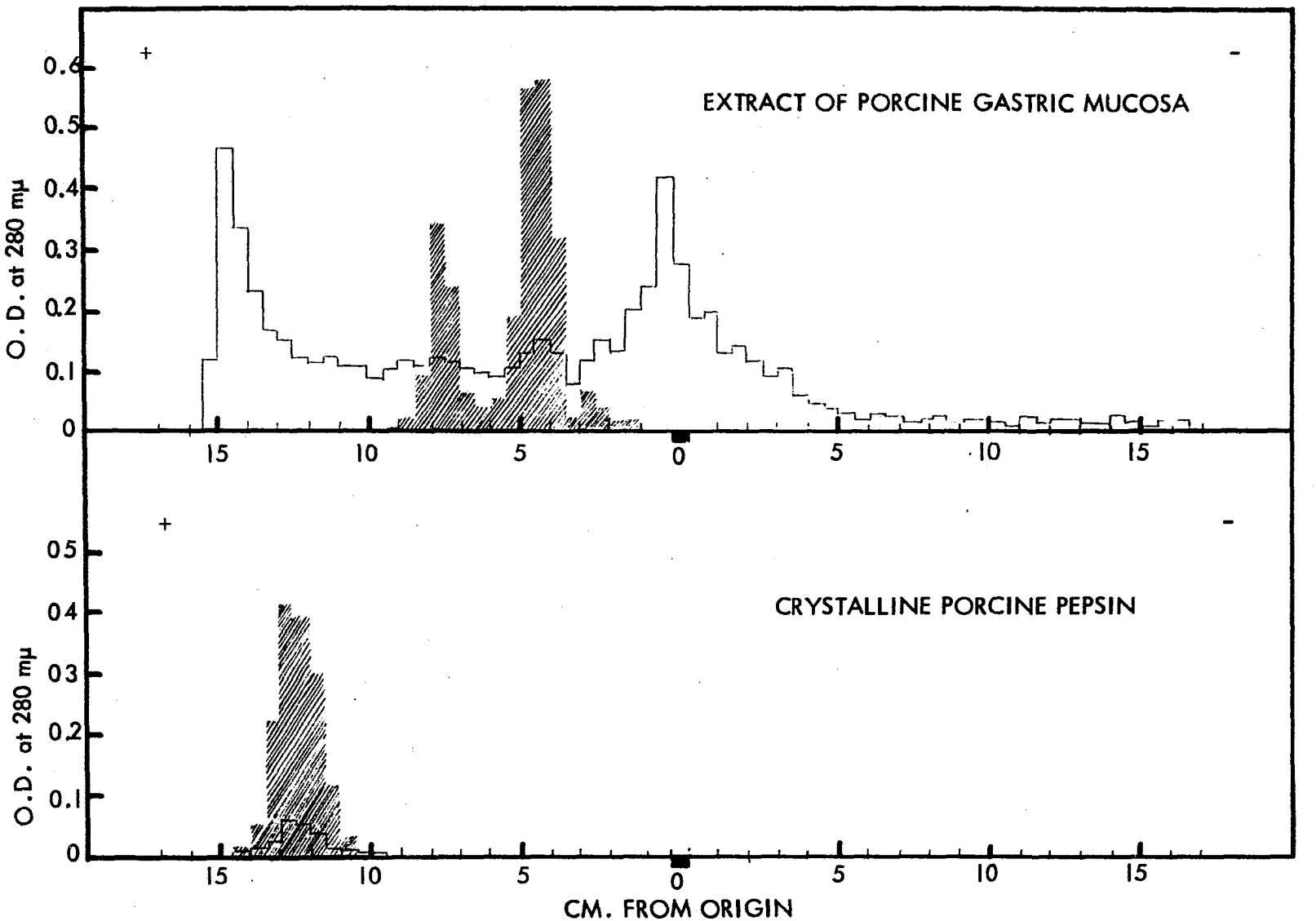


Figure 30.-Starch electrophoresis of extract of porcine gastric mucosa and crystalline porcine pepsin. The solid line shows the protein concentration, while the shaded area shows the proteolytic activity.

CHAPTER IV

DISCUSSION

The 'Heterogeneous System' of Gastric Proteolytic Enzymes

In most of the species studied, there are more than one proteolytic enzyme in the stomach content. In dog gastric juice, there are two enzymes similar to pepsin. In different porcine gastric enzyme preparations, at least four pepsins were found. In the gastric juice of human beings, it seems to have developed into two proteolytic enzymes of different pH optima, gastricsin and pepsin. However, there is only one pepsin in the gastric juice of the rat. The studies on gastric mucosa have revealed that there are two zymogens present in both the human and the porcine gastric mucosa.

It has been known for some time that there may exist a group of enzymes which apparently have the same enzymatic action, but differ in their physical, chemical, or immunological properties. Kaplan *et al.* (68) called this 'molecular heterogeneity' of the enzymes. The efforts in this field were first concentrated on the demonstration of the differences between certain enzymes of different organs. Immunological differences have been shown for muscle and liver phosphorylases (69), and alkaline phosphatases from different organs (70). Differences between the lactic dehydrogenases of the different organs have been

extensively studied by Kaplan et al. (68) in several species of organisms. More recently, the "molecular heterogeneity" of the enzymes in a single organ have been observed for crystalline ribonuclease (71), and lactic dehydrogenase (72).

The molecular heterogeneity of porcine pepsin was first reported by Herriott et al. by obtaining pepsin A from porcine gastric mucosa (31). Two minor proteolytic enzymes, 'parapepsins', have recently been reported to be present in porcine gastric mucosa (42).

The term 'molecular heterogeneity' of an enzyme seems to indicate that identical specificity exists among these heterogeneous species of molecules of a specific enzyme. This is, indeed, not the case found in this study for gastric proteolytic enzymes in the human stomach. Therefore, the term 'heterogeneous enzyme system' seems to be a less rigid term and will be used in further discussion.

In considering the origin of the 'heterogeneous system' of gastric proteolytic enzymes one might argue that it could be the product of partial alteration of the secondary and tertiary structure of the molecule of a single enzyme. This possibility seems to be unjustified in view of the different N-terminal amino acids in gastricsin and pepsin in human gastric juice. Furthermore, the 'heterogeneous system' was observed from freshly activated preparations of gastric mucosa from both human beings and the hog (Figures 18, 22 and 24). The activation periods were one and three minutes at room temperature. No great extent of denaturation should be expected under such conditions.

The problem of autolysis has to be considered in discussing the origin of 'heterogeneous systems' of gastric proteolytic enzymes. In the

specificity studies (Figure 16), significant amount of ninhydrin positive substances were liberated during the incubation of human gastricsin or pepsin over a 24 hour period. Perlmann has reported that the dialyzable fragments of autolyzed porcine pepsin retain some proteolytic activity (39). One of the active fragments of pepsin, which has recently been isolated by Funatsu and Tokuyasu (40), has a sedimentation coefficient of 0.7 S compared to 2.9 S of pepsin (73). However, it is not likely that the active fragment of hog pepsin is responsible for the 'heterogeneous system' observed in this study. According to Funatsu and Tokuyasu (41) the active fragments only formed in the presence of a certain preservatory substance and upon continuous dialysis of the fragments during the autolysis. The 'heterogeneous system' observed after short periods of activation again serve as strong evidence against the 'autolysis theory'. In the case of human gastric proteolytic enzymes, direct evidence has been presented previously that there is no interconversion of gastricsin and pepsin when the isolated enzyme is incubated alone at its optimal pH.

It is quite possible that the 'heterogeneous system' of enzymes is derived from the same zymogen either by a series of modifying steps or by several different means of activation. The fact that there were four porcine pepsins and only two zymogens found in the porcine gastric mucosa tends to support this possibility. In the case of human gastric proteolytic enzymes, a single zymogen fraction has been shown to yield both gastricsin and pepsin upon activation. The possible mechanisms in this system will be further discussed.

The biological significance of the 'heterogeneous system' of

gastric proteolytic enzymes seems to be best explained in the case of the human being. The difference in the optimal pH of gastricsin and pepsin tend to suggest that they carry out the digestive process at different degrees of acidity of the human stomach. The pH optimum of gastricsin and pepsin (pH 3.0 and 1.5) are, indeed, well within the pH range of human stomach contents known to physiologists. The fact that there is only one pepsin in the gastric juice of rats seems justified also, since the pH dependent curve of rat pepsin shows a much broader range of activity (Figure 27). The pepsins in dog gastric juice, though have the same optimal pH at 1.5, show the difference in activity at higher pH range (Figure 27). It is interesting to see that three of the four pepsins in porcine gastric mucosa have an optimal pH 1.5 while the fourth one has an optimal pH lower than pH 1. It would be interesting to correlate the acidity of the porcine stomach with the optimal pH observed in this study.

Due to the fact that there are two zymogens present in either human or hog gastric mucosa, it is interesting to consider the possible genetic control of their synthesis. It is reasonable to assume that these two zymogens, as two different protein molecules, should be controlled by different genes in their synthesis. During recent years, a concept has been formed mainly from the studies of amino acid synthesis, namely that organisms tend to carry the minimum genes possible for their survival (74). It is known that the essential amino acids, which are not synthesized in animal organisms, are those amino acids which require the greatest number of enzymatic steps in their synthesis from the intermediates of the main metabolic pathways. Due to the stabilization of

food intake in animals, it is likely, according to this concept, to eliminate first the more elaborate process in amino acid synthesis for the benefit of the economy of the organism. It is obvious then, the 'heterogeneous system' of enzymes, which should be controlled by multiple genes, is at variance with the concept of 'minimum genes'. However, one can argue that due to the stabilization of environment and food, most mutations might be expected to be lethal. The carrying of extra genes would be of great biological significance in maintaining the species characteristics.

The Gastric Proteolytic Enzymes of Man

The Physiological Significance of Gastricsin

The physiological significance of gastricsin and pepsin in the human stomach has been briefly discussed in the preceeding section. The quantitative study in fractionation of human gastric juice tends to indicate that the role of gastricsin in digestion is an important one. By measuring the area occupied by both enzymes in the chromatographic pattern of somewhat more than 40 ion-exchange resin chromatograms, it appears that the ratio of pepsin to gastricsin in human gastric juice is fairly constant at 4:1. However, when the calculation is made relative to their proteolytic activities at the respective optimal pH's of both enzymes, the ratio is usually between 2:1 and 3:1 . It is interesting to note that freshly-activated extract of human gastric mucosa shows twice as much proteolytic activity of gastricsin as of pepsin (Figures 18 and 22). It is possible that gastricsin actually plays a more important role in the initial phase after the activation

of zymogen. The total activity ratio of 2:1 observed for pepsin and gastricsin may be due to the instability of gastricsin in the gastric juice or in the processing.

The specificity studies on protein substrates of both enzymes show that gastricsin attacks certain protein substrates faster than pepsin (Table 6). This indicates that gastricsin and pepsin perhaps digest somewhat different species of proteins according to the structural preferences.

The Identity and Presence of Gastricsin

Gastricsin is undoubtedly the same enzyme which was designated as 'gastric cathepsin' by Freudenberg (28). The pH optimum of this enzyme obtained from total human gastric juice in earlier works was approximately pH 3.5 (28, 29, 30). The purified gastricsin from the ion-exchange column has an optimal pH of 3.2. However, after final crystallization, the optimal pH is consistently at pH 3.0. Merten and Ratzer (30) reported that the slow migrating fraction in the electrophoresis of human gastric juice is rich in 'gastric catheptic activity'. In this study, it has been shown that gastricsin migrates at a slower rate than pepsin in electrophoresis on either paper or starch gel.

The idea that gastricsin could be identified with the milk-clotting enzyme, rennin, was discarded in the light of the finding that striking differences exist in the ratio of proteolytic to milk-clotting activities for crystalline gastricsin and rennin (see Table 2).

It is interesting that gastricsin has not been found in the gastric juice or gastric mucosa of species other than man. The presence of two pH optima (pH 1.5 and 3.5) in the extract of porcine gastric

mucosa was reported in earlier works (29). However, none of the active fractions obtained in the fractionation (which constitutes more than 95% of the total proteolytic activity) could account for the optimal activity at pH 3.5 (see Figure 25). Kaplan et al. have suggested that the development of the 'molecular heterogeneity' of certain enzymes could be a reflection on the history of evolutional development of the species (68). It will be of interest to correlate the pattern of gastric proteolytic enzymes in the different species of animals and seek the presence of gastricsin in the species of the primates.

The Differences and Similarities Between Gastricsin and Pepsin

Human pepsin is a more acidic protein than gastricsin. It migrates faster toward the anode in the electrophoretic field, and is eluted at lower pH of the effluent in the cation exchange resin column (see Figures 1 and 10). The two enzymes show differences also in heat stability. The preincubation of gastricsin or pepsin at different temperatures indicates that pepsin is more stable at pH 3.2 and gastricsin is more stable at pH 2.0 (Figure 12).

In the enzymatic activity, besides the difference in optimal pH of the two enzymes, the ratio between milk-clotting activity and proteolytic activity are also somewhat different. The specificity studies show that the two enzymes have different affinities toward protein and synthetic substrates (Tables 6 and 7).

However, the two enzymes are not without similarities. The fact that the pH optimum shifted about 1 pH scale toward the alkaline side in the presence of urea, for both enzymes, tends to indicate a similarity

on the basic mechanism of the two enzymes. The shift of the pH optimum of porcine pepsin by urea was first observed by Schlamowitz and Peterson (75). It was interpreted as meaning that the hydrolysis of a protein substrate consists of two steps. The first step is the breaking of secondary and tertiary bonds in the substrate molecule, this step was assumed to have a pH optimum of 1.5 and is, in the ordinary condition, the rate-limiting step. The second step, which was assumed to have a different pH optimum of 3.5, is the hydrolysis of peptide bonds of the substrate. It was believed that in the presence of urea the first step is eliminated, and the pH optimum of the second step appears in the measurement with an increase in the specific activity. In the experiment on the urea effect on the pH optimum of gastricsin and human pepsin, the quantitative results show a decrease of specific activity accompanied by a shift of the pH optimum. This would indicate that the second step, if existing, must be a slow one. If this is true, then no shift in the pH optimum should be observed when the first step is eliminated. Therefore, it would tend to suggest that the mechanism proposed by Schlamowitz and Peterson is invalid. A more reasonable hypothesis seems to be that urea actually affects the pK of certain dissociable groups of the enzyme which are involved in the enzymatic action, thus shifting the pH optimum.

The Origin of Gastricsin and Pepsin in the Human Stomach

The possible mechanisms by which gastricsin and pepsin are generated from zymogen I of human gastric mucosa is a problem to be discussed with special interest. Zymogen I, which was obtained from a DEAE-cellulose column, produces both gastricsin and pepsin after acid activation (Figure 22). If one assumes that this zymogen I preparation did not

contain more than one zymogen, then it requires that gastricsin and pepsin each possesses a portion of the zymogen I molecule. Due to the fact that the C-terminal amino acid sequences are identical for gastricsin, human pepsin, hog pepsin and hog pepsinogen, and also that the activation of hog pepsinogen occurs by cleaving certain peptides only from the N-terminal, the following relationship between the molecules of gastricsin, pepsin and zymogen I seems to be more preferable:

Protein Molecule

N-terminal		C-terminal	
----------	----------	(val-leu-ala.)	Zymogen I
Serine----------	----------	val-leu-ala.	Gastricsin
Valine----------	----------	val-leu-ala.	Pepsin

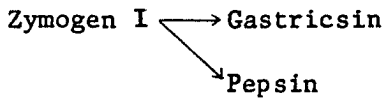
In this scheme, different lengths of the peptide chain are split off from the N-terminal of the zymogen I chain to produce gastricsin or pepsin. The sedimentation coefficient of the two enzymes tends to suggest that gastricsin is slightly larger in molecular weight than pepsin.

There are two different mechanisms by which gastricsin and pepsin can most likely be derived from zymogen I:

Mechanism 1: Stepwise conversion:

Zymogen I $\longrightarrow$ Gastricsin $\longrightarrow$ Pepsin

Mechanism 2: Direct conversion:



The fact that there was no interconversion observed for gastricsin and pepsin (after incubation of two enzymes separately at their respective optimal pH) tends to argue in favor of Mechanism 2. However, it is

apparent that autodigestion may not be the physiological means of interconversion of the two enzymes. In studying the activation product of zymogen I with micro-column fractionation, gastricsin and pepsin were found to be produced at a ratio of two to one after 1 minute of activation (Figure 22). After two and a half hours of activation, the ratio was one to one for the two enzymes. This seems to be in favor of the idea that gastricsin is produced first in the activation then is converted to pepsin (Mechanism 1). However, the possibility cannot be ruled out that the two different means of activation in Mechanism 2 have two different types of kinetics for the reaction.

There are several factors which seem more likely to be involved in the production of gastricsin and pepsin under physiological conditions. The influence of pH on the activation of gastric proteolytic enzyme was not known until the recent report of Neumann and Sharon (27) who observed that hog pepsins obtained from the activation of pepsinogen at different pH's are different in transpeptidation activities. It is possible that acidity of the human stomach, which may be influenced by the food and the hydrochloric acid secretion of the stomach, may control the activation mechanism in producing a proteolytic enzyme of proper pH optimum. Other factors, such as other enzyme or non-enzyme components in the stomach, have never been demonstrated to influence the activation process of gastric zymogen. However, their participation is not entirely impossible.

The Physiological Fate of 'Zymogen II'

It is interesting that 'zymogen II' obtained from DEAE-cellulose column (Figure 19) and electrophoresis (Figure 20) has an optimal pH of

3.0 (Figure 21). However, after activation at pH 2.5 and fractionation on an Amberlite IRC-50 column, the activity was found to be eluted in the region of pH 6.5 (Figure 24). This proteolytically active fraction has never been observed to occur in the fractionation of human gastric juice. This indicates that 'zymogen II' is not the precursor of either gastricsin or pepsin. Two other possibilities remain: (a) 'Zymogen II' is activated into an intracellular enzyme and it is not secreted into the stomach. However, the pH optimum of activated 'zymogen II' is about 3.0 which is a rather low pH for physiological environment other than stomach contents. Moreover, 'zymogen II' is activated by an acid pH which is undoubtedly not present intracellularly. (b) 'Zymogen II' is an intermediate in the activation of 'zymogen I' to gastricsin, but the acidification is not the physiological means of activation. There has been no evidence in favor of or against either of the possibilities. Whether or not 'zymogen II' plays a role in gastric digestion can still not be concluded with certainty.

The Porcine Pepsins

It is interesting that crystalline porcine pepsin was resolved into several active fractions in the fractionation with an ion-exchange column (Figure 25). An inactive peak was also observed in the chromatogram. It appears that the twice crystallized porcine pepsin is not a homogeneous preparation.

The proteolytically active fraction eluted at pH 4.5 of the effluent is of special interest. It shows a pH optimum lower than 1.0, such a low pH optimum has never been reported previously.

None of the active fractions correspond to the para-pepsins of

Ryle (42). It could be that para-pepsins are present in such small amounts that they are covered by the other active peaks in the chromatogram. It is not known whether one of the active fractions of the ion-exchange chromatogram actually corresponds to the pepsin A of Herriott (31). The comparison of the properties of these fractions will be of great interest in clarifying these questions.

CHAPTER V

SUMMARY

Studies were made on the gastric proteolytic enzymes of man and of several other species of animals. Two proteolytic enzymes were isolated from human gastric juice. One of the enzymes was human pepsin, the other was a new enzyme which was named gastricsin. Further purification resulted in the crystallization of gastricsin. This crystalline enzyme was found to be a homogeneous preparation.

The chemical, physical and enzymatic properties of gastricsin and human pepsin were compared. Gastricsin was found to have a molecular weight of 36,027 compared to 31,390 for human pepsin. The optimal pH of gastricsin was 3.0 and that of human pepsin was 2.0. The two enzymes were also found to differ in electrophoretic mobility, heat stability, milk-clotting activity, proteolytic activity with synthetic peptide and protein substrates, and the effect of urea on their optimal pH's. The terminal amino acid determinations revealed that the two enzymes had different N-terminal amino acid (serine for gastricsin and valine for human pepsin), however, the sequence of the last three amino acids at the C-terminal appeared to be the same (-valyl-leucyl-alanine).

The origin of gastricsin and human pepsin was also studied. Two zymogens were found in the alkaline extract of human gastric mucosa. One

of the zymogens, which was a single fraction in both ion-exchange chromatography and electrophoresis, was shown to be activated to both gastricsin and pepsin. The second zymogen was also activated to a proteolytic enzyme which apparently is not present in human gastric juice.

It was found that gastricsin was absent in the gastric preparations of several species of animals. Instead, several pepsins were found in extract of porcine gastric mucosa and dog gastric juice. Two possible mechanisms were proposed for the production of these 'heterogeneous systems' of gastric proteolytic enzymes. The biological significance of these 'heterogeneous enzyme systems' is also discussed.

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