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COMPARATIVE STRUCTURAL STUDIES ON HUMAN GASTRIC PROTEOLYTIC ENZYMES AND THEIR ZYMOGEN

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CHAPTER I

INTRODUCTION

An elucidation of the structural differences between zymogens and their activation products should contribute to our understanding of the relationship between the chemical structure of proteins and their biological activity. A comparison of structural differences before and after activation is now possible for several zymogens of the digestive tract including pepsinogen, trypsinogen, chymotrypsinogen and procarboxypeptidase largely as a result of the work of Northrop, Kunitz, Herriott, Desnuelle and Neurath (1-4). These comparisons provide an insight into the nature of the activation process. The activation is in each case catalyzed by proteolytic enzymes which hydrolyze a limited number of peptide bonds. Most of the potentially susceptible bonds are not attacked which suggests that they are protected as a result of the configuration of the protein. A major aspect of the problem under consideration is whether this configuration and the enzymatically active configuration are present prior to activation.

Structural changes resulting from the activation of zymogens can be studied by employing physico-chemical techniques such as methods

of sedimentation, diffusion, viscometry, electrophoresis and optical rotatory dispersion. Other techniques used to detect conformational changes include immunological studies and chemical modifications of susceptible groups. Alterations in the primary structures can be detected by end group analysis methods and by amino acid composition studies. If relatively large fragments are split off in the activation process, their size, shape, end group character and chemical composition can also be studied by the above methods. The detection of free amino acids and those contained in small peptides should confirm changes in the primary structure of an activated zymogen which are not accounted for by the composition of other activation products.

The above techniques have been applied to studies on the activation of several pancreatic zymogens including trypsinogen, chymotrypsinogen and procarboxypeptidase (2-4). The primary event in the activation of both porcine and bovine trypsinogen is the hydrolysis of a lysyl-isoleucine bond resulting in the liberation of an amino terminal hexa-or octapeptide. A corresponding event in the activation of bovine chymotrypsinogen A and B is the splitting of an arginyl-isoleucine bond, but in this case an amino terminal cystine residue prevents the liberation of a peptide. In all four of these cases the enzyme which is formed has an amino terminal isoleucyl-valyl-glycine sequence. The significance of this sequence is uncertain but Labouesse et al. (5) have recently reported data suggesting that the ionization state of the amino terminal isoleucine of δ-chymotrypsin controls both the conformation of the enzyme and its catalytic properties. The above changes in chemical structure appar-

ently induce conformational changes which have been detected by measurements of optical rotation and hydrodynamic properties. Neurath and Dixon (6) suggest that the changes observed in optical rotation result from the formation of a more nearly helical configuration in the enzyme which permits the interaction of histidine and serine side chains forming the active site.

The activation of bovine pancreatic procarboxypeptidase A is even more complex due to the occurrence of the zymogen in a peculiar state of aggregation (3). Activation of the zymogen with trypsin may produce an endopeptidase, an exopeptidase (carboxypeptidase) or an enzyme with both types of activity, depending on the activation conditions. The formation of the exopeptidase alone occurs with extensive degradation of the zymogen.

Hog pepsinogen and pepsin have been studied extensively over a period of more than thirty years. Herriott pioneered the study of the activation of hog pepsinogen in 1938 (7,8). Since then several physico-chemical properties of pepsin including the sedimentation coefficient, the diffusion coefficient, the molecular weight, the intrinsic viscosity and the optical rotation have been reported by a number of workers (9). The amino acid composition, number of chains and the N- and C-terminal sequences have been determined for both pepsin and its precursor (9). These results show that each consists of only one chain and that pepsin apparently constitutes the C-terminal part of the pepsinogen molecule. Knowledge of conformational differences is limited to that obtained from studies of optical rotatory properties (8), effect of iodination of pepsinogen

on enzyme activity and reactions of pepsinogen and pepsin with anti-pepsinogen antibodies (10). These studies indicate that conformational alterations involve only limited portions of the molecule.

The study of homogeneous preparations of human gastric proteolytic enzymes was not initiated until 1957 when Richmond et al. (11) succeeded in completely separating two proteolytic enzymes, pepsin and gastricsin from human gastric juice. Gastricsin was crystallized and partially characterized by Tang et al. (12). It was shown to differ from pepsin in pH optimum, electrophoresis migration on paper and heat inactivation. In 1962 Tang and Tang (13) reported the purification and properties of a zymogen from human gastric mucosa which when activated produced both gastricsin and human pepsin. It is the purpose of this study to determine and compare the structural properties of human pepsin, gastricsin and their zymogen. The results of this study, presented in the following pages, include the determination of the sedimentation coefficient; diffusion coefficient; intrinsic viscosity; amino acid composition; molecular weight by various methods; and the carboxyl terminal sequence of amino acids for each of these three proteins, the zymogen, gastricsin and pepsin.

CHAPTER II

MATERIALS AND METHODS

Materials

Human Gastric Juice

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

Human Gastric Mucosa

Human stomachs were supplied by hospitals in Oklahoma City, Oklahoma. The principal source of human gastric mucosa was the normal portion of stomach specimens removed during the gastrectomy of ulcer patients at the Veterans' Administration Hospital. Post mortem examinations at the University Hospital provided another source. Stomachs, from ulcer patients at St. Anthony's Hospital, which had been preserved in formaldehyde, served as a third source of gastric mucosa.

Carboxypeptidase

Carboxypeptidase A (5x cryst.H_2O susp.) was obtained from Mann Research Laboratories, New York 6, N. Y.

Bovine Hemoglobin

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

N,N-diethylaminoethylcellulose (DEAE-cellulose)

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

Methods

Preparation of Human Pepsin and Gastricsin

Human pepsin and gastricsin were prepared by the fractionation of human gastric juice with an ion-exchange column of Amberlite IRC-50 (XE-64) according to a modified procedure of Richmond et al. (11) as described by Tang et al. (12). An attempt was made to further purify the human pepsin by passing it through a DEAE-cellulose column, which had been equilibrated with acetate buffer pH 5.3, and eluting it with a NaCl gradient. No further purification was achieved.

Preparation of Zymogen

The zymogen was prepared from human gastric mucosal tissue by extraction with 0.1M $NaHCO_3$ solution, precipitation in 80% saturated $(NH_4)_2SO_4$ solution, and chromatographic separation on a DEAE-cellulose column according to the procedure described by Tang and Tang (13). Some of the DEAE-chromatographed zymogen was further purified on a Sephadex G-75 column.

Proteolytic Activity

The proteolytic activity of gastricsin and human pepsin was determined by a modified procedure of Anson and Mirsky (14) as described by Tang and Tang (13). The zymogen was activated by incubation in a pH 2.5, 0.2M sodium citrate buffer for 10 minutes prior to measuring proteolytic activity as described above.

Nitrogen Determination

Nitrogen was determined by the method developed by Lang (15). The digestion mixture contains 40 g K_2SO_4, 2 ml selenium oxychloride, 250 ml H_2O and 250 ml H_2SO_4. Samples containing 1 to 10 µg of nitrogen are added to 0.2 ml of the digestion mixture. The tubes are heated in a sand bath at a temperature between 100° and 200° until all of the water has evaporated. Marbles are placed on the mouths of the digestion tubes and the temperature is increased to about 300°. After a 1-2 hour digestion period at this temperature the tubes are cooled to room temperature. To the acid digest is added 1.4 ml of water followed by the rapid addition of 5.0 ml of Nessler's reagent. The tubes are protected from light for thirty minutes and then read at 420 mµ.

Protein Concentration

Protein concentration was determined spectrophotometrically at 280 mµ in a Hitachi-Perkin-Elmer Model 139 spectrophotometer applying the extinction coefficient determined for each protein from nitrogen determinations.

Density Measurements

The densities of solutions were measured at 15° and 25° with 2 and 5 ml pycnometers. Extrapolations were made using the results of the above determinations for the values used at other temperatures.

Viscosity Measurements

Viscosity measurements were made with an Ostwald viscometer having a flow time for 3 ml of water of 207.0 sec at 6.9°. The average deviation was about 0.25 sec for five readings. All solutions and buffers were filtered through sintered glass before being pipetted into the viscometer. A volume of 3 ml of solution or distilled water was used for each determination. The viscometer was cleaned with chromic acid cleaning solution and rinsed several times with distilled water and finally acetone before the introduction of each solution. The experiments were either carried out in a constant cold room at $6.9° \pm 0.1°$ or in a water bath at room temperature or at 0.0°.

The viscosities of buffer solutions were calculated according to the equation:

$$\eta/\eta_0 = (t/t_0)\ (\rho/\rho_0)$$

where η_0, t_0 and ρ_0 are the viscosity, outflow time and density of distilled water, respectively, and η, t and ρ are corresponding values for the buffer solution.

The viscosities of pepsin, gastricsin and zymogen were studied in water and in buffer solutions. The buffer solutions used were Na^+,

CH_3COO^-, Cl^-, pH 5.0 with an ionic strength of 0.1 for pepsin and gastricsin and 0.2M K^+, $HPO_4^=$, pH 7.5 for zymogen. The data were plotted as $\eta sp/C$ against C, where ηsp is the specific viscosity and C is the concentration in grams per ml of solution. ηsp is determined as $t-t_o/t_o$ where t and t_o are solution and solvent flow times, respectively. The intrinsic viscosity, $[\eta]$ is equal to the value of this plot at zero concentration plus a correction factor, $(1-\bar{V}\rho_o)/\rho_o$, which arises because the solution and solvent have different densities. In this factor ρ_o is the solvent density and $\bar{V}$ is the partial specific volume of the protein, which is 0.73 ml per g, based on amino acid compositions, for each of the proteins studied. The correction factor in each instance was equal to 0.003 dl/g. The axial ratio of each protein was determined from the parameter β which is calculated from the relation (16)

$$\beta \equiv \frac{Ns^o_{20,w}[\eta]1/3\eta_o}{M^{2/3}(1-\bar{v}_2\rho_o)\ (100)^{1/3}}$$

where η_o and ρ_o are the viscosity and density of the solvent respectively, N is Avogadro's number, $s^o_{20,w}$ is the value of the sedimentation coefficient in water at 20° and M is the molecular weight.

Diffusion

Diffusion is the transfer of material which occurs whenever a concentration gradient exists in a solution and which tends to equalize the concentration everywhere. The diffusion coefficient, D, is a measure of the mass of solute transported across a plane of known cross section in a given period of time under the influence of a known

driving force. At a given temperature, the driving force for the diffusion of a protein solution is essentially equal to the concentration gradient. The free diffusion method, which is used in these experiments, is based on Fick's second law of diffusion

$$\frac{dc}{dt} = D \frac{d^2c}{dx^2}$$

where the rate of change of concentration with time is related to the rate at which the concentration gradient is changing with position, x, in the cell. These experiments involve both schlieren and Rayleigh optical systems. The diffusion experiments were performed with either an ultracentrifuge (with a synthetic boundary cell) or a Tiselius electrophoresis cell. In the former case, the apparent diffusion coefficient, D app, was calculated from schlieren patterns with the equation

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

where A is the area under the boundary, $H_{max.}$ is the maximum ordinate of the gradient curve, ω is the angular velocity of the ultracentrifuge, and t is the time in seconds. In the latter studies, using the Spinco Model H electrophoresis-diffusion instrument, the factor $(1-\omega^2 st)$, which is an adjustment for the effect of sedimentation on diffusion, is ignored. The D app calculated according to the equation above was first corrected for zero time imperfection. This was done by plotting D app against 1/t to determine the true t_o which was then used to recalculate D app. The D app. values were then further corrected to diffusion coefficients corresponding to a temperature of 20^o in a solvent with the viscosity of water, according to the following

equation (17)

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

where t is temperature, ($\eta_{solv.}/\eta_w$) 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°.

Diffusion coefficients from Rayleigh patterns were calculated by the method of Longsworth (18) as described by Schachman (17). Computations by this method involve the assumption that the concentration distribution in the boundary is Caussian and therefore Tables of Probability Functions (19) were used.

Most of these studies were performed with the Model H electrophoresis-diffusion instrument. Either the standard cell or the micro cell were used depending on the amount of material available. The experimental procedures used were essentially those described by Schachman (17). Artificial sharpening of the boundary was performed for each experiment. About 15 ml were used in the standard cell and about 3 ml in the micro cell. All experiments were performed at 0.1° after dialysis against the same buffer for at least 24 hours. The buffers used were the same as those used for sedimentation studies. Eight or more photographs were taken during a 2-3 day period. A few photographs were made soon after the final sharpening of the boundary was completed and the diffusion cell isolated. These photographs provided a means for calculating the fractional number of fringes (17) and for obtaining sharp schlieren peaks. Later photographs permitted a measure of the distance between pairs of fringes.

A few runs were made with the Spinco Model E ultracentrifuge using essentially the same conditions as those described below for sedimentation velocity studies except that the instrument was operated at about 10,000 rpm with the synthetic boundary cell.

Ultracentrifugation

The Spinco Model E ultracentrifuge equipped with the RTIC unit for indicating and regulating temperature was used for sedimentation velocity and sedimentation equilibrium studies. The experimental procedures used were essentially those described by Schachman (17). The rotor was precooled to a temperature of about 20° and maintained at a constant temperature, in most cases, between 17° and 25°. Photographs of the schlieren pattern were taken by the automatic mechanism of the ultracentrifuge. The positions of the sedimenting boundaries on the photographic plates were determined with a Nippon Kogaku K K Shadowgraph Model 6 which gave an accuracy of 0.0001 cm.

Studies with human pepsin and gastricsin were carried out in a Na^+, CH_3COO^-, Cl^- buffer at pH 5.0 with an ionic strength of 0.1. Studies with the zymogen were carried out in a 0.2M K^+, $HPO_4^=$ buffer at pH 7.5. The protein solutions were equilibrated with their respective buffers by dialysis at least 15 hours at 6° before use.

Sedimentation coefficient determination. The sedimentation coefficient is defined as the velocity of the sedimenting molecules per unit field

$$s = \frac{1}{\omega^2 x} \frac{dx}{dt}$$

where $\omega^2 x$ is the centrifugal field strength and dx/dt is the sedimentation velocity. ω is the angular velocity in radians per second, x is the distance in cm from the axis of rotation to the boundary (maximum ordinate in refractive index gradient curve) and t is the time in sec. Integrating the above expression between t_o and t gives

$$s = \frac{1}{2(t-t_o)} \ln \frac{x}{x_o}$$

where x and x_o are positions of the boundary at time t and t_o respectively. A plot of the logarithm of the distance from the center of rotation to the boundary as a function of time gives a straight line. The apparent sedimentation coefficients were calculated from the slope of this line according to the following equation

$$s_{obs.} = \frac{2.303(\text{slope})}{\omega^2}$$

The value of $s_{obs.}$ was corrected to $s_{20,w}$ which is its value in a solvent with the density and viscosity of water at 20° according to the equation

$$s_{20,w} = s_{obs.} \left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta}{\eta_o}\right)\left(\frac{1-\bar{V}\rho_{20,w}}{1-\bar{V}\rho,t}\right)$$

where $\eta t/\eta_{20}$ corresponds to the viscosity of water at the experimental temperature, t, relative to that at 20°, (η/η_o) is the relative viscosity of the solvent to that of water. $\rho_{20,w}$ and ρ_t are the densities of water at 20°, and the solvent at t°, respectively, and $\bar{V}$ is the partial specific volume of the solute.

The ultracentrifuge was operated at its top speed of 59,780 rpm. Photographs were taken at 8 minute intervals after constant velocity of the rotor had been attained. Usually 10 photographs were

taken in each experiment. A conventional 12-mm ultracentrifuge cell with a 4° sector centerpiece was used for concentrations of protein above 0.5 g/100 ml. For lower concentrations a synthetic boundary cell, also with a centerpiece thickness of 12-mm and a 4° sector angle, was used. The concentration range studied was from about 0.2 g/100 ml to 1.5 g/100 ml.

Molecular weights by sedimentation velocity method. Molecular weights were determined with the use of sedimentation velocity and diffusion data according to the Svedberg equation

$$M = \frac{RTs}{D(1-\bar{V}\rho)}$$

where R is the gas constant, 8.314 X 10^{-7} erg/mole/degree, T is the absolute temperature, s and D are as previously defined when corrected to 20° in water and $\bar{V}$ and ρ are also as previously defined.

Molecular weights from sedimentation equilibrium studies. Sedimentation equilibrium is obtained when the transport of solute by sedimentation equals its transport by diffusion. Analysis of sedimentation data is based on the equation of Svedberg and Peterson (20,21) which, when ideality is assumed, takes the form

$$M = \frac{2RT}{(1-\bar{V}\rho)\omega^2} \frac{d \ln c}{dx^2}$$

where c is concentration evaluated at the radial distance x. The above equation is converted to the following form

$$M = \frac{2RT}{(1-\bar{V}\rho)\omega^2(x_b^2-x_m^2)} \cdot \frac{c_b-c_m}{c_o}$$

where the subscripts b and m refer to the bottom of the cell and the meniscus respectively and c_o refers to the initial concentration. The

quantities x_o and x_m are determined directly from measurements on the photographic plates with the microcomparator. The quantity c_b-c_m is obtained by integrating across the whole cell using the equation

$$c_b-c_m = \int_{x_m}^{x_b} \left(\frac{dc}{dx}\right) dx$$

where dc/dx values are directly determined (in arbitary units) as the ordinates of schlieren patterns and dx is the value of the interval between the readings. A factor must be applied which corrects for the magnification of the camera lens. The value for c_o is obtained by measuring the area under the boundary in a synthetic boundary cell. Ginsberg et al. (22) have pointed out that if homogeneity of the material is assumed the determination of c_o is not necessary thereby eliminating the experiment with the synthetic cell boundary. The equation then used is

$$M = \frac{RT}{(1-\bar{V}\rho)\omega^2} \frac{\frac{1}{x_b}\left(\frac{dc}{dx}\right)_b - \frac{1}{x_m}\left(\frac{dc}{dx}\right)_m}{c_b-c_m}$$

Archibald (23) has suggested that the conditions of sedimentation equilibrium are met both at the meniscus and at the bottom of the cell at all times during the run. The equation describing these conditions is

$$M = \frac{RT}{(1-\bar{V}\rho)\omega^2} \frac{(dc/dx)_m}{x_m c_m} = \frac{RT}{(1-\bar{V}\rho)\omega^2} \frac{(dc/dx)_b}{x_b c_b}$$

The values of c_m and c_b are determined by employing an equation derived by Klainer and Kegeles (24)

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

where X refers to a position in the plateau region (where dc/dx is equal to zero). The equation for determining c_b is analagous except that the value of the last term is added to c_o.

The ultracentrifuge was operated at a speed of 8,766 rpm. Pictures were taken at 2 minute intervals, after a constant velocity had been attained, for the approach to equilibrium studies. Equilibrium through the entire cell appeared to be attained after about 25 hours at which time photographs were taken for this study. Other photographs were taken at earlier times in order to have a record of the rate of approach to equilibrium. A conventional 12-mm ultracentrifuge cell with a 4^{o} sector centerpiece was used. The concentration range studied was from 1.0 g/100 ml to 1.5 g/100 ml.

Amino Acid Analyses

Duplicate samples of 3.5 mg of thoroughly dialyzed and lyophilized protein were hydrolyzed with 1 ml of 5.7N glass distilled HCl in an evacuated tube at $110^{o} \pm 1^{o}$ for 20, 40 and 70 hours. The hydrolysates were evaporated in a rotating evaporator at 37^{o}, redissolved with distilled water, and the evaporation repeated. The residue was then dissolved in 0.2N citrate buffer of pH 2.2 and the volume adjusted to 5 ml. The concentration of protein in each hydrolysate was determined from nitrogen analyses using a nitrogen factor for conversion to dry weight based on 16% nitrogen.

The amino acid composition of each hydrolysate was determined according to the procedure of Spackman, Stein and Moore (25) with a Spinco Amino Acid Analyzer Model 120 B. Identical 2 ml aliquots of

the hydrolysates were applied to the 150-cm column and the 15-cm column. The neutral and acidic amino acids were chromatographed on the 150-cm column containing Amberlite IR-120 resin (a very finely pulverized 8% cross-linked sulfonated polystyrene resin), and eluted initially with a pH 3.25 buffer and finally with a pH 4.25 buffer. The basic amino acids were chromatographed on the 15-cm column with the same resin but were eluted with a pH 5.28 buffer. Ninhydrin reagent was added to the column effluents and the absorption of the ninhydrin reaction products was measured at 570 mμ (two degrees of amplification) for all amino acids except proline which was measured at 440 mμ.

Standard amino acid samples were run at the beginning and end of each ninhydrin batch. The average of the results of the color yield for each amino acid was used to compute its constant. The area under each peak was determined by multiplying the height of the peak by the width at half the height except for small or broad peaks in which case the area was determined by integrating the area under the peak.

Tryptophan was determined by a modification of the method of Spies and Chambers (26) developed by Harrison and Hofmann (27). This modification involves denaturation by heating and partial digestion of the protein with a mixture of chymotrypsin and trypsin prior to the color forming reaction with the p-dimethylaminobenzaldehyde-H_2SO_4 reagent of Spies and Chambers. The spectrum of the final colored solution was determined for each determination to make sure that a shift in maximum absorption had not taken place.

Carboxyl-terminal Amino Acid Sequence

The carboxypeptidase method was used for C-terminal group analysis and stepwise degradation from the carboxyl end (28). About 0.7 μmole of protein, which had been dialyzed against 0.005M pH 7.5 phosphate buffer for at least 24 hours, were incubated with about 0.1 mg of diisopropylfluorophosphate-treated carboxypeptidase in 2 ml of 0.005M phosphate buffer at pH 7.5. The incubation was carried out at room temperature for as long as 24 hours, and samples of 0.5 ml were removed at several incubation times. Each sample was placed in a boiling water bath for 3 minutes after which 1 ml of 0.4M citrate buffer pH 2.2 was added. The sample was further acidified to pH 2.2 with 0.1N HCl, filtered through sintered glass, diluted to 2 ml with H_2O and stored in the refrigerator. The samples were subjected to amino acid analysis using the 50-cm column of the Model 120-B Amino Acid Analyzer.

Analyses were made of samples of carboxypeptidase, gastricsin, pepsin and zymogen incubated separately and treated as described above as a test for contamination or autodigestion.

CHAPTER III

RESULTS

Physical Measurements

Sedimentation Coefficients

The preparations of human pepsin, gastricsin and zymogen each showed a single symmetrical boundary in sedimentation velocity studies. The symmetry was not altered during 72 minutes of sedimentation at 59,780 rpm. A typical sedimentation pattern is shown in Figure 1.

The sedimentation coefficients have been determined for each of the proteins at various concentrations and corrected to standard conditions using the procedures described in Methods. The relative viscosity of the solvent to that of water, which is essentially temperature independent, was found to be 1.016 for the acetate buffer and 1.008 for the phosphate buffer. Density measurements showed that the acetate and phosphate buffers were respectively 1.0035 and 1.0056 g per ml at 4.0°. The value used for $\bar{V}$ was that determined from the amino acid composition which is 0.73 ml/g for each of the proteins (see the section on "Partial Specific Volume").

The $S_{20,w}$ values obtained for the three proteins at different concentrations are shown in Figure 2. An effect of concentration on the sedimentation velocity of gastricsin is apparent. Each value

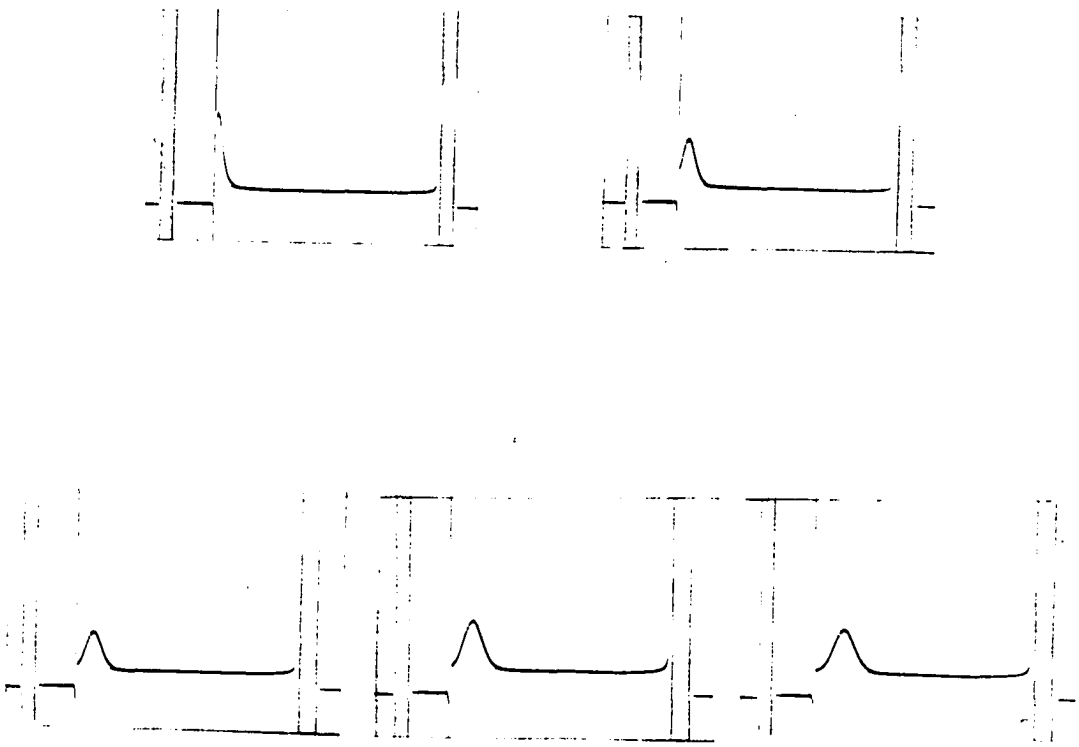


Figure 1. Sedimentation of 1.3% zymogen in K^+, $HPO_4^=$ buffer, 0.2M, pH 7.5. Photographs were made at 8 minute intervals. The exposure time was 15 seconds and the phaseplate angle was 80° for the first three pictures and 75° for the last two. The temperature of the rotor during the experiment was 18.0°.

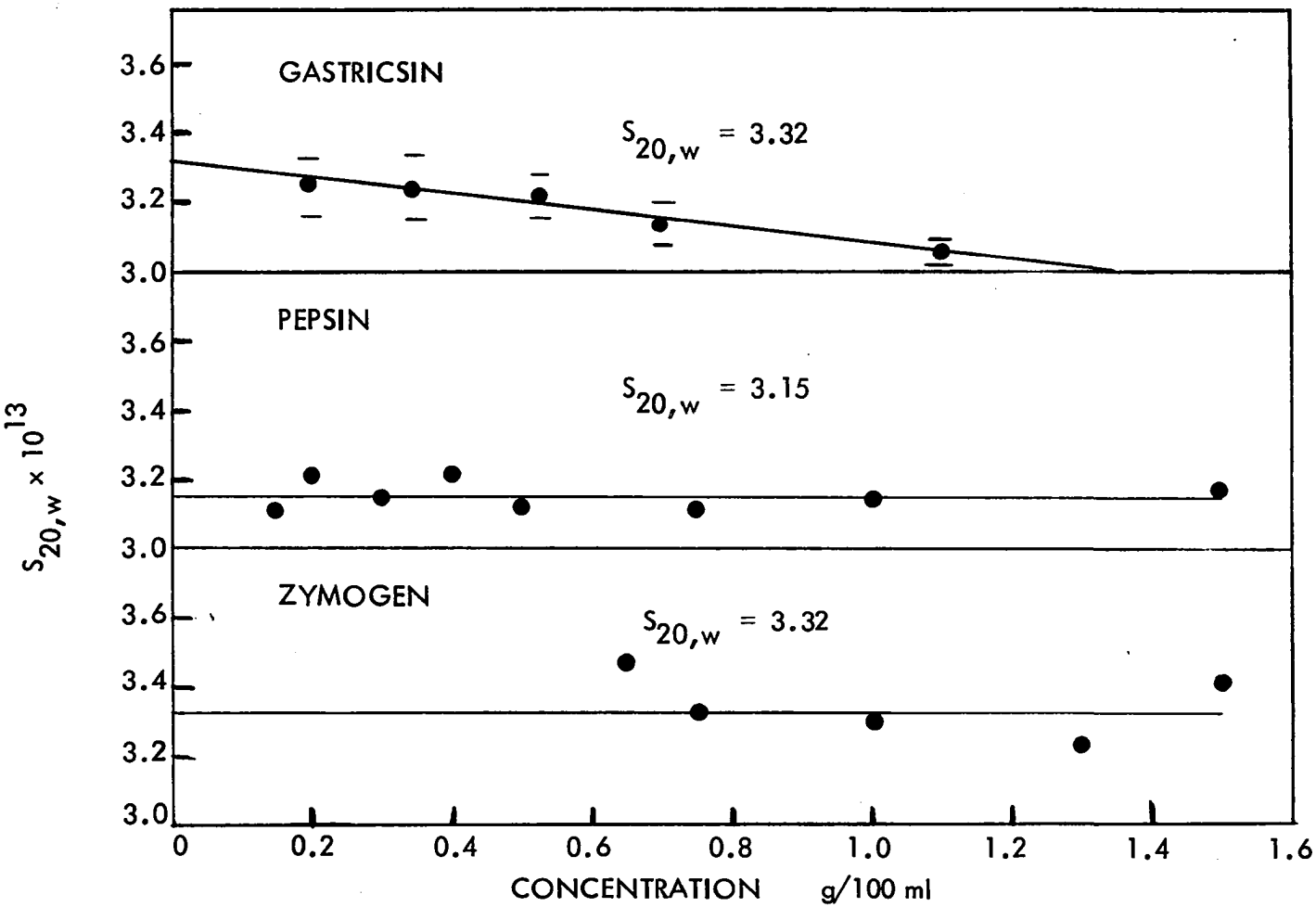


Figure 2. Sedimentation coefficients of gastricsin, pepsin and zymogen as a function of protein concentration. A concentration effect on the sedimentation coefficient is apparent for gastricsin. The bars above and below the experimental values, in the case of gastricsin, represent the average deviation. Each value represents a mean of from 3 to 5 determinations.

shown for gastricsin represents an average of from three to five determinations and thus the concentration dependence and extrapolated value seem to be well established. An extrapolation to zero concentration gives the accepted value of 3.32S. Sedimentation coefficients for the human pepsin and zymogen do not appear to be concentration dependent. The average value for the zymogen is 3.32S $\pm$ 0.08S with six determinations and that for pepsin is 3.14S $\pm$ 0.04S with eight determinations. The $S_{20,w}$ value for the HCHO modified zymogen was found to be 2.9 $\pm$ 0.1 as a result of two experiments with a concentration of about 0.7 g/100 ml of zymogen.

The concentration dependence of the $S_{20,w}$ of gastricsin suggests either excess hydration or an elongated asymmetrical gastricsin molecule as opposed to more spherical, symmetrical pepsin and zymogen molecules.

Diffusion Coefficients

Typical schlieren and Rayleigh patterns from diffusion experiments performed as previously described are seen in Figure 3. This is a reproduction of a plate containing a photographic record of an experiment in which the diffusion of a 0.75 g/100 ml solution of gastricsin in the micro cell was studied using the Model H electrophoresis-diffusion instrument. Shown are 2 of 12 photographs taken over a span of two days. These early pictures show a sharp symmetrical schlieren pattern and Rayleigh fringes which are sufficiently close together to allow the measurement of fractional numbers of fringes. Readings from 4 photographs of this experiment at 121, 204, 296 and

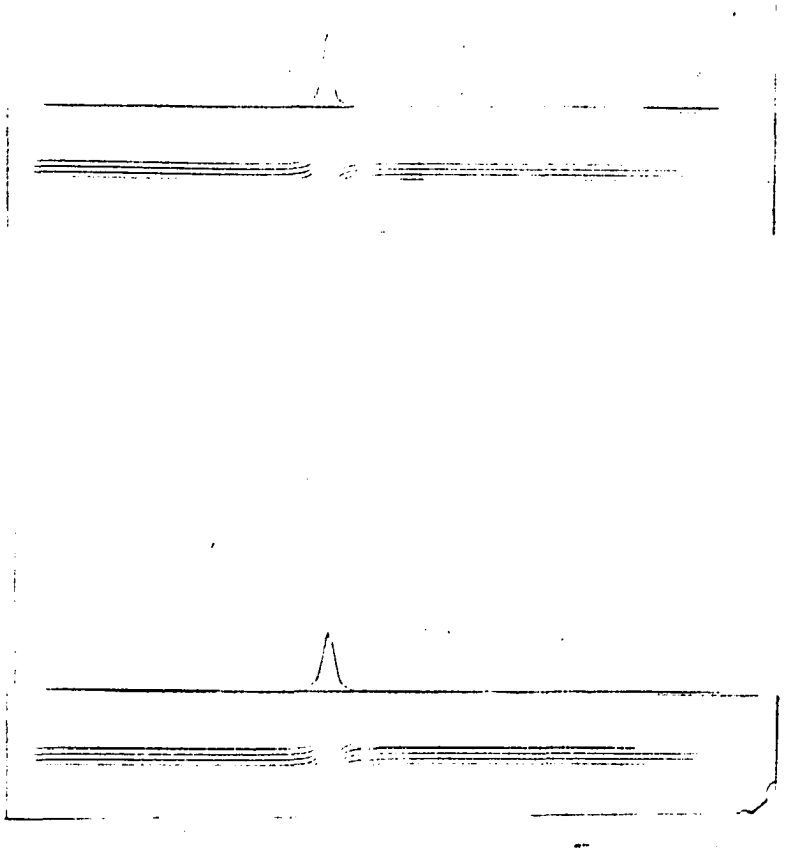


Figure 3. Photograph of schlieren pattern and Rayleigh fringe record of diffusion experiment. Gastricsin, 0.75%, in acetate buffer, pH 5.0, μ 0.1. Diffusion occurred in micro cell of Model H electrophoresis-diffusion instrument at 0.1°. Photographs were made on metallographic plates with an exposure time of 5.7 seconds. The lower photograph was taken 45 minutes and the upper 14 minutes, after boundary sharpening was completed.

402 minutes after the formation of the sharpened boundary gave a D app. in cm^2/sec of $4.85 \times 10^{-7} \pm 0.02 \times 10^{-7}$ from the schlieren pattern and a D app. of $4.90 \times 10^{-7} \pm 0.14 \times 10^{-7}$ from the Rayleigh fringes at 0.1°. These values corrected to $D_{20,w}$ are 9.40×10^{-7} and 9.49×10^{-7} respectively.

A composite of the results from diffusion experiments is presented in Table 1. The diffusion behavior of pepsin, gastricsin, zymogen and the formaldehyde modified zymogen was studied using the Model H electrophoresis-diffusion instrument at 0.1°. The diffusion of pepsin and gastricsin was also studied using the Model E ultracentrifuge operated at about 10,000 rpm at 20.0°. These values for $D_{20,w}$ will be used with sedimentation velocity data for calculating molecular weights by the well known Svedberg equation. The striking effect of temperature on the diffusion behavior of gastricsin can not be explained at this time but this may be related to the relative unstability of gastricsin observed during storage. The formaldehyde affected zymogen diffuses more rapidly than even gastricsin which is in agreement with its relatively low sedimentation coefficient reported earlier.

Molecular Weights By Sedimentation Velocity Method

The molecular weights of pepsin, gastricsin and zymogen were determined using the sedimentation velocity method. The calculated sedimentation coefficients, diffusion coefficients, partial specific volumes and resulting molecular weights are summarized in Table 2. The molecular weights calculated by this method are 32,900 for pepsin

TABLE I

COMPARISON OF $D_{20,w}$ VALUES OF PEPSIN' GASTRICSIN' ZYMOGEN AND ZYMOGEN-HCHO**

Protein	Concentration Range g/100 ml	Temperature	Number of Determinations	$D_{20,w}$ X 10^7 cm^2/sec
Pepsin	0.13 - 1.0	0.1°	6	8.7 ± 0.4
Pepsin	0.5	20.0°*	2	8.3 ± 1.1
Gastricsin	0.40 - 0.75	0.1°	2	9.6 ± 0.2
Gastricsin	0.7 - 1.9	20.0°*	3	5.2 ± 0.8
Zymogen	0.1 - 1.3	0.1°	3	7.7 ± 0.6
Zymogen-HCHO**	0.1 - 1.0	0.1°	2	11.5 ± 0.1

*Determined using Model E Ultracentrifuge.
**Zymogen isolated from formaldehyde treated gastric mucosa.

and 30,900 for gastricsin. The molecular weight of zymogen determined by the sedimentation velocity method is 38,800 which is significantly higher than those of the enzymes resulting from its activation. All of these molecular weights will be subsequently compared with those obtained by other methods. The value of the molecular weight of the formaldehyde modified zymogen obtained by this method is 23,000.

TABLE 2

MOLECULAR WEIGHT DETERMINATION OF PEPSIN, GASTRICSIN AND ZYMOGEN BY SEDIMENTATION VELOCITY METHOD

	$S_{20,w}$ X 10^{-13}	$D_{20,w}$ X 10^{-7}	$\bar{V}$ ml/g	Molecular Weight
Pepsin	3.15	8.7	0.732*	32,900
Gastricsin	3.32	9.6	0.727*	30,900
Zymogen	3.32	7.7	0.729*	38,800
Zymogen-HCHO	2.9	11.5	0.73†	23,000

*Calculated from amino acid composition (see Table 8).
†Assumed.

Molecular Weights from Equilibrium Studies

Approach to equilibrium. Typical schlieren patterns of homogeneous protein preparations studied during the approach to equilibrium were obtained for pepsin and gastricsin. Figure 4 is a reproduction of the photographic record of an experiment with a pepsin solution containing 1.0 g/100 ml. The values for the molecular weight of pepsin according to the results of this particular experiment calculated from the meniscus and the bottom of the cell are 31,350 ± 530 and 33,080 ± 700 respectively. Two other experiments gave similar results. The

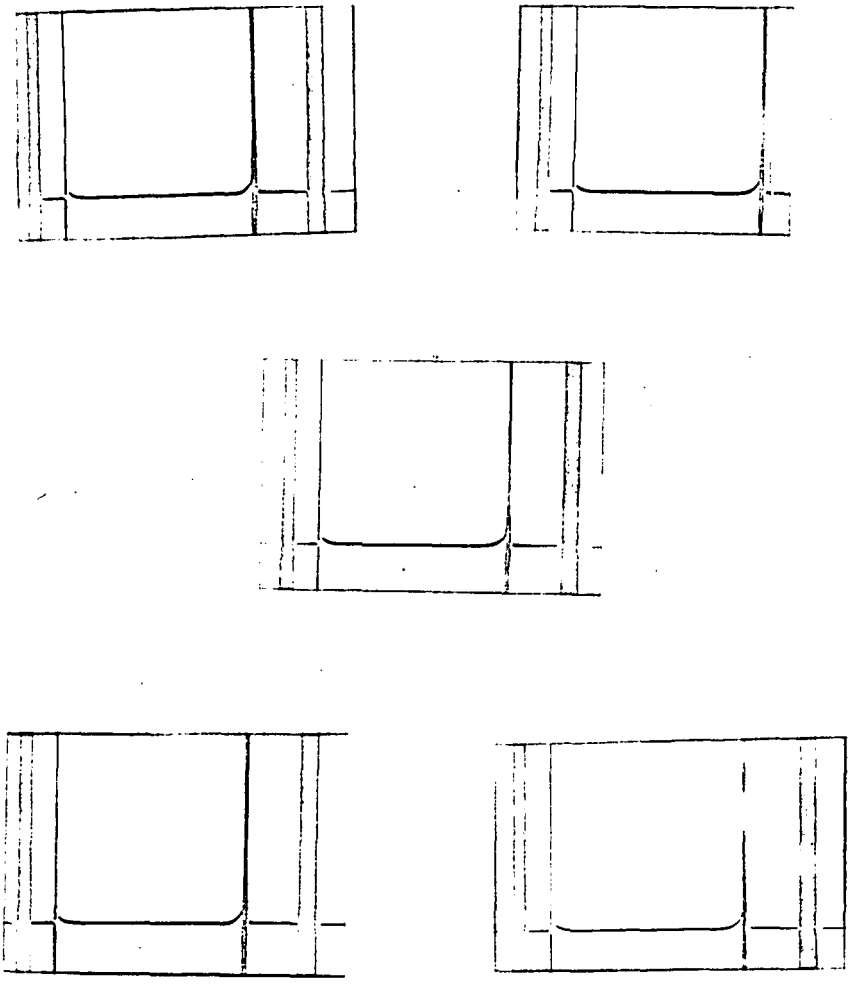


Figure 4. Schlieren patterns obtained in a study of pepsin during the approach to sedimentation equilibrium. Ultracentrifuge speed is 8,766 rpm, and all pictures are taken at an angle of 80° for the schlieren diaphragm. About 0.4 ml of 1.0% pepsin solution is placed in the cell on top of 0.1 ml of silicone oil. The direction of sedimentation is from left to right. These photographs were made 10, 12, 14, 16 and 18 minutes after the attainment of constant velocity. The exposure time was 15 seconds and the temperature of the rotor was 27.4°.

result obtained for the molecular weight of gastricsin according to the results of one experiment calculated from the bottom of the cell is 34,000 ± 1,000. However, calculations from the meniscus gave a value of 56,000 ± 4,000. Two other experiments with gastricsin gave similar high values. Tang (28) reported large variations in similar studies with gastricsin. These variations suggest the existence of denatured or aggregated material which is not inconsistent with some relatively low diffusion coefficients reported earlier for this protein.

Sedimentation equilibrium. Figure 5 is a photographic reproduction of the schlieren patterns obtained during a sedimentation equilibrium study of pepsin. A progressive increase in the curvature representing the concentration gradient is noted as the time of ultracentrifugation increases. After 120 minutes (middle picture) the plateau region has disappeared. It appears that sedimentation equilibrium is attained after 25.5 hours of centrifugation at 8,766 rpm as shown in the fourth picture from the left. The last picture is a reproduction of the schlieren pattern obtained with the synthetic boundary cell and for the purpose of determining a measure of the original concentration.

The value of the molecular weight for pepsin calculated from the pattern shown in picture 4 is 34,000. This value may not be as reliable as those determined by other methods because it was evident from the pattern that a thickening of the boundary existed at the interface between the protein solution and the silicone oil. This necessitates a somewhat hazardous extrapolation to the bottom of the cell. A similar phenomenon was observed in experiments with gastricsin.

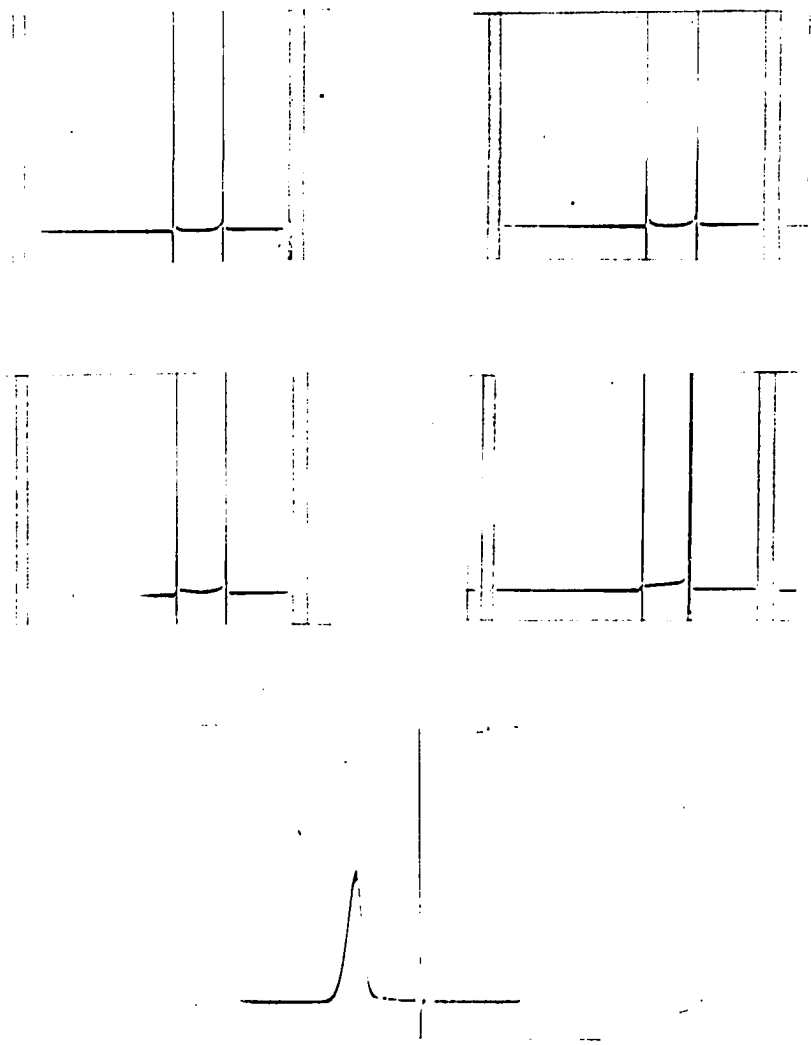


Figure 5. Schlieren patterns obtained in a study of pepsin before and after sedimentation equilibrium had been attained. Ultracentrifuge speed is 8,766 rpm, and all pictures are taken at an angle of 80° for the schlieren diaphragm. About 0.1 ml of a 1.5% pepsin solution is placed in the cell on top of 0.1 ml of silicone oil. The direction of sedimentation is from left to right. The first, second and third photographs from left to right were made 16, 24 and 120 minutes respectively and the fourth 25.5 hours after the attainment of constant velocity. The last picture is a reproduction of the photographic record of a synthetic boundary cell run.

The value of the molecular weight of gastricsin determined from a photograph representing apparent equilibrium throughout the cell is 31,000.

Viscosity

Viscosity data for gastricsin and pepsin in water are shown in Figure 6, where the $\eta sp/C$ is plotted against concentration. The slopes and intercepts were determined by the method of least squares. The intrinsic viscosity, $[\eta]$, values were calculated as described in the Methods. Temperature effects on viscosity are not apparent in these studies. A striking difference is observed in the data for these two proteins. A definite dependence of viscosity on concentration is observed for gastricsin in contrast to a slight negative slope for the pepsin concentration vs. $\eta sp/C$ curve. Furthermore, the calculated $[\eta]$ of 0.124 dl/g for gastricsin is significantly higher than that of 0.079 dl/g for pepsin. The non-Neutonian effect and relatively high $[\eta]$ observed for gastricsin suggest interactions between non-spherical rod like molecules. The negative slope observed for the pepsin data is similar to that observed by Edelhoch (29) for hog pepsin in 0.00Γ/2 phosphate buffer resulting in a value of 0.063 dl/g. The values for $[\eta]$ obtained in water cannot be compared directly with those obtained in salts solutions in which electroviscous effects are normally obviated. However, this does not detract from the significance of viscosity differences observed between these proteins in water.

Viscosity data for pepsin and gastricsin in acetate buffer and for zymogen in phosphate buffer at 6.5° are presented in Table 3. These data do not show a dependence of viscosity on concentration for

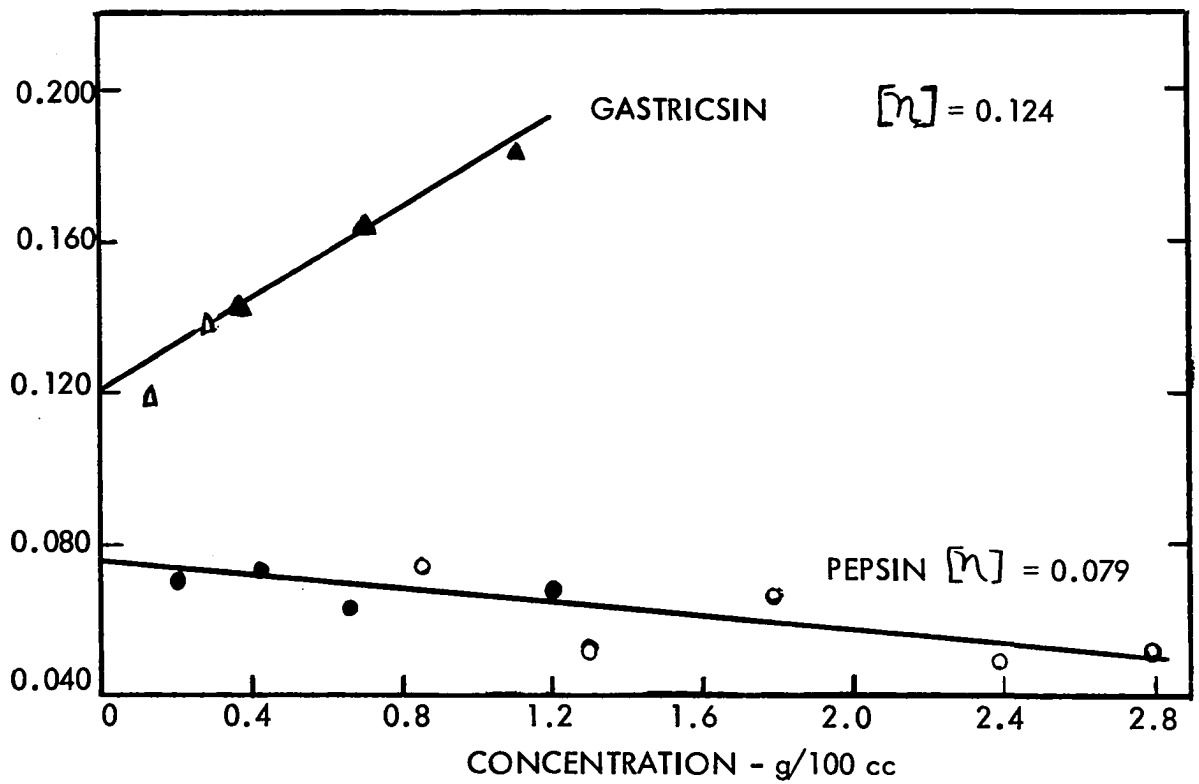


Figure 6. Viscosity data for gastricsin and pepsin obtained in water. ▲ and ● at 6.9°. ◬ at 27.7° and o at 0.0°.

TABLE III

INTRINSIC VISCOSITY

Protein	Concentration Range in g/100 ml	Number Of Determinations	$[\eta]$ dl/g (a)	β (10^6) (d)	Axial Ratio a/b (d)
Pepsin	0.9-1.5	4	.045± .008 (b)	229	6
Gastricsin	0.3-0.5	3	.100±.008 (b)	338	160
Zymogen	0.6-1.5	4	.100±.008 (c)	298	50

(a) Average of values of η sp/C values.
(b) Measured in acetate buffer pH 5.0, μ=0.1.
(c) Measured in phosphate buffer 0.2M, pH 7.5.
(d) See Methods.

any of these proteins. However, the concentration range of gastricsin solutions is narrow and does not preclude concentration effects over a wider range. Nevertheless, the value of $\eta sp/C$ at the low concentration studied would be expected to be near the true intrinsic viscosity. The value of $[\eta]$ presented for each protein represents an average of the values of $\eta sp/C$ plus the density correction factor. The value for human pepsin of 0.045 dl/g is well within the range of values of $[\eta]$ reported for hog pepsin (29-31). Its lack of dependence on concentration is in accord with the expected behavior of non-deformable globular proteins (32). The values of $[\eta]$ for gastricsin and zymogen of 0.100 dl/g suggest that these molecules are more asymmetrical or flexible.

The values for the parameter β (see Methods) are 2.29×10^6, 3.38×10^6 and 2.98×10^6 for pepsin, gastricsin and zymogen respectively. The axial ratios determined from β for prolate ellipsoids are 6, 160 and 50 for pepsin, gastricsin and zymogen respectively. These values also indicate that pepsin is a globular type protein and that gastricsin and zymogen are shaped more like long rods.

Amino Acid Composition

The amino acid compositions of the zymogen, human pepsin and gastricsin are given in Tables 4-7. All values presented in Tables 4-6, except those for serine, ammonia and tryptophan, are the averages of duplicate determinations at hydrolysis times of 20, 40 and 70 hours. Two methods of calculations were used to determine the approximate number of residues of each amino acid. One method is based on the molecular weight determined from sedimentation and diffusion data. The

other method is based on corrections which are needed to give an integral number of histidine, proline and phenylalanine residues. It is first assumed that the number of histidine residues per mole of protein is 3 for the zymogen and 1 for both pepsin and gastricsin. This assumption is based on approximate molecular weights determined by physical methods. The corrections based on the number of residues of histidine do not necessarily correct for experimental errors occurring in the analysis of neutral and acidic amino acids. Therefore, further corrections were based on the closest intergral numbers of proline and phenylalanine residues. This choice was made because proline and phenylalanine are present in relatively low amounts. Furthermore, they have been shown, in other proteins, to be completely hydrolyzed after 20 hours and not degraded within a period of at least 140 hours (33) and they are eluted from the column at relatively different times. The final correction factor for each hydrolysate was an average of corrections needed to bring the number of each of these three residues to the closest integral number.

Tables 4-6 show that the standard deviation is $\geq$ one residue for aspartic acid, glutamic acid, glycine, valine, isoleucine and threonine for at least one of the proteins. Aspartic acid, glutamic acid and glycine are present in the highest amounts and the percentage deviation is not inconsistent with that for other residues. For some other proteins leucine and isoleucine have been shown to require approximately 70 hours for complete hydrolysis and threonine to be progressively degraded with time (34). More analyses are required to obtain more precise values for these amino acids. On a percentage

basis the standard deviation for half cystine is very high for the zymogen and pepsin. This is perhaps due to the fact that the peaks obtained for this residue were broad and thus it was difficult to measure the area accurately.

The method of determining the number of serine and amide residues is illustrated in figure (7). The number of amide residues for the zymogen is not shown because it was larger than the theoretical value obtained if all the aspartic and glutamic acid residues were in the amide form. This discrepancy is apparently due to protein bound ammonia resulting from the dialysis of the zymogen preparation against ammonia water at a pH above 7.5. Analysis of a single sample of another preparation gave a value of 59 amide residues per mole which has been incorporated into these data. The degradation of serine in zymogen and gastricsin does not appear to be linear during the hydrolysis time studied. Noltmann et al. (33) suggested that the degradation kinetics for each amino acid depends upon the particular composition of the individual protein. Therefore, the apparently higher value of serine in pepsin compared to the zymogen is probably the result of serine being bonded to different residues in the protein.

Table 7 shows the amino acid composition of the three proteins when the number of residues based on the two methods of calculation are expressed as closest integral numbers. The agreement between the two methods of calculation appears to be satisfactory and allows a comparison of the composition of the three proteins from which emerge significant differences. It is noted that the numbers of residues of lysine, histidine and arginine are much lower in the enzymes

than in the zymogen. This disproportionate loss of basic amino acids is similar to that observed in the activation of hog pepsinogen (35). The numbers of aspartic acid (includes asparagine) residues, isoleucine residues and serine residues found in gastricsin are significantly less than the numbers of the corresponding residues found in pepsin and zymogen. Similarily the number of glutamic acid (includes glutamine) residues and the number of leucine residues are significantly less in pepsin than the numbers of the corresponding residues in the other two proteins. Other differences are by comparison minor but they will need to be reexamined when N- and C-terminal sequence studies are carried out.

The zymogen obtained from mucosa which had been exposed to formaldehyde contains about one half as many of each of the amino acids as the unmodified zymogen according to the analysis of one sample hydrolyzed for a period of 40 hours.

Partial Specific Volume

Table 8 shows the partial specific volume, $\bar{V}$, of zymogen, pepsin and gastricsin calculated from the weight percentages of the amino acids. The $\bar{V}$ values are 0.729, 0.732 and 0.727 ml per g for zymogen, pepsin and gastricsin respectively.

Molecular Weights Calculated From Amino Acid Compositions

The molecular weights calculated from the amino acid compositions are shown in Table 7. The calculation of the amino acid composition by two methods results in two values for the molecular weight of each protein. It should be recognized that the molecular weights

resulting from the amino acid composition based on molecular weights determined by physical methods restricts the resulting molecular weight. On the other hand, molecular weights resulting from the amino acid composition based on corrections needed for intergral numbers of selected amino acids represent values determined independently of physical methods. Values calculated by this latter method are 38,183, 34,439 and 32,250 for zymogen, pepsin and gastricsin respectively. These values compare favorably with those determined by physical methods as will be emphasized subsequently.

Comparison Of Molecular Weights

A composite of the values of the molecular weights of zymogen, pepsin and gastricsin, determined by various methods, is presented in Table 9. The values determined by the different methods are in good agreement for each protein. The poorest agreement is found between the value of the molecular weight for gastricsin determined by the Archibald method and those values determined by other methods. This was not unexpected in view of the large variations observed among values obtained for gastricsin by the Archibald method. This value for gastricsin was not used in determing an average of the molecular weight values. The averages of the molecular weight values are 38,500, 33,400 and 31,300 for zymogen, pepsin and gastricsin respectively. Therefore, the activation of the zymogen to pepsin results in a decrease in the molecular weight of about 5,000 and the activation to gastricsin of about 7,000.

Carboxyl-terminal Amino Acid Sequence

Figures 8 and 9 show the moles of amino acids released by carboxypeptidase A treatment of pepsin and gastricsin respectively as a function of time. As reported earlier by Tang and Tang (36) alanine is the C-terminal amino acid for each of these proteins. The second amino acid from the C-terminal end in both instances is shown to be phenylalanine. An earlier report (36), which stated that it was leucine, was based on a two demensional chromatographic separation with which it is not easy to distinquish spots for these two amino acids. The third amino acid released is leucine from pepsin and threonine from gastricsin. At 4.5 hours of incubation a second residue of alanine has apparently been released from gastricsin but not from pepsin. The data shown in these figures do not include values of amounts of amino acids which did not exceed 0.4 residues per mole of gastricsin or 0.2 residues per mole of pepsin at 5 minutes of incubation. At 4.5 hours of incubation these values were 2.0, 1.3 and 1.2 residues of serine, tyrosine and isoleucine respectively from gastricsin and 0.9, 0.3 and 0.2 residues of serine, tyrosine, and isoleucine respectively from pepsin. An analysis of samples after an incubation time of 24 hours showed more differences.

Limited studies with zymogen chromatographed on DEAE-cellulose and then Sephadex G-75 showed a release of 0.58, 0.48, 0.42 and 0.38 residues per mole of protein of alanine, serine, valine and phenylalanine respectively after 30 seconds of incubation with carboxypeptidase A.

Therefore, these results indicate that the amino acid sequence at the C-terminal end is--leucyl, phenylalanyl, alanine for

pepsin,--threonyl, phenylalanyl, alanine for gastricsin and--valyl, seryl, alanine for zymogen. Other differences in the C-terminal portions of the pepsin and gastricsin molecules are revealed when the release of other amino acids is compared.

TABLE 4

AMINO ACID COMPOSITION OF ZYMOGEN

Amino Acid Residue	No. of Residues for MW 38,000	No. of Residues for Closest Integral No. of His, Pro, Phe
Lysine	7.96 ± 0.47	7.26 ± 0.19
Histidine	3.18 ± 0.19	3.04 ± 0.10
Ammonia*	59	59
Arginine	6.60 ± 0.37	6.02 ± 0.17
Aspartic Acid	37.6 ± 0.9	36.9 ± 1.4
Threonine	25.0 ± 0.3	28 **
Serine**	39	41
Glutamic Acid	39.0 ± 1.0	40.1 ± 0.5
Proline	19.1 ± 0.7	18.8 ± 0.3
Glycine	35.5 ± 1.5	35.0 ± 1.0
Alanine	21.6 ± 0.9	21.1 ± 0.8
Half Cystine	5.17 ± 0.7	5.1 ± 0.7
Valine	24.2 ± 1.4	25.1 ± 1.1
Methionine	6.35 ± 0.31	6.2 ± 0.2
Isoleucine	20.5 ± 1.4	20.2 ± 0.6
Leucine	26.5 ± 0.5	27.4 ± 0.2
Tyrosine	14.9 ± 0.9	14.8 ± 0.2
Phenylalanine	15.6 ± 0.4	16.0 ± 0.4
Tryptophan***	5	5

* based on one determination, hydrolyzed 40 hours.
** determined by extrapolation (see Figure 7).
*** determined by modified method of Spies and Chambers (26,27).

TABLE 5

AMINO ACID COMPOSITION OF HUMAN PEPSIN

Amino Acid Residue	No. of Residues for MW 32,000	No. of Residues for Closest Integral No. of His, Pro, Phe
Lysine	0.26 ± 0.08	0.29 ± 0.08
Histidine	0.97 ± 0.11	1.01 ± 0.02
Ammonia*	35	42
Arginine	2.92 ± 0.25	3.11 ± 0.21
Aspartic Acid	34.6 ± 2.5	37.8 ± 2.2
Threonine	22.5 ± 0.4	24.7 ± 0.1
Serine*	37	41
Glutamic Acid	28.4 ± 0.7	31.4 ± 1.3
Proline	17.5 ± 0.5	18.8 ± 0.1
Glycine	32.3 ± 1.1	35.7 ± 0.3
Alanine	15.9 ± 1.0	17.3 ± 0.7
Half Cystine	3.49 ± 1.0	3.8 ± 1.0
Valine	21.8 ± 2.8	25.4 ± 2.2
Methionine	3.73 ± 0.50	4.1 ± 0.5
Isoleucine	22.5 ± 0.4	24.5 ± 0.7
Leucine	19.6 ± 1.0	21.8 ± 0.6
Tyrosine	13.3 ± 0.3	14.7 ± 0.1
Phenylalanine	13.8 ± 0.3	15.0 ± 0.3
Tryptophan**	6	6

* determined by extrapolation (see Figure 7).
** determined by modified method of Spies and Chambers (26,27).

TABLE 6

AMINO ACID COMPOSITION OF GASTRICSIN

Amino Acid Residue	No. of Residues for MW 31,000	No. of Residues for Closest Integral No. of His, Pro, Phe
Lysine	0.50 ± 0.08	0.52 ± 0.10
Histidine	1.20 ± 0.30	1.25 ± 0.34
Ammonia*	48.8	49
Arginine	2.51 ± 0.16	2.61 ± 0.16
Aspartic Acid	25.1 ± 0.6	26.1 ± 0.8
Threonine	19.9 ± 0.4	21.9 ± 1.4
Serine*	31.8	31
Glutamic Acid	37.6 ± 0.2	39.2 ± 0.5
Proline	17.3 ± 0.6	18.2 ± 0.4
Glycine	31.4 ± 0.9	33.0 ± 1.1
Alanine	17.7 ± 0.4	18.3 ± 0.5
Half Cystine	4.74 ± 0.05	5.0 ± 0.2
Valine	21.6 ± 0.4	22.5 ± 0.3
Methionine	4.65 ± 0.09	5.0 ± 0.2
Isoleucine	12.0 ± 0.3	13.0 ± 0.3
Leucine	23.3 ± 0.3	23.9 ± 0.5
Tyrosine	16.1 ± 0.3	16.9 ± 0.3
Phenylalanine	15.1 ± 0.1	15.8 ± 0.2
Tryptophan**	5	5

* determined by extrapolation (see Figure 7).
** determined by modified method of Spies and Chambers (26,27).

TABLE 7

AMINO ACID COMPOSITIONS AND MOLECULAR WEIGHTS
OF ZYMOGEN, PEPSIN AND GASTRICSIN

	ZYMOGEN		PEPSIN		GASTRICSIN	
Amino Acid	(1)	(2)	(1)	(2)	(1)	(2)
Lysine	8	7	0	0	1	1
Histidine	3	3	1	1	1	1
Ammonia	59	59	35	42	47	49
Arginine	7	6	3	3	3	3
Aspartic Acid	38	37	35	38	24	26
Threonine	25	28	23	24	19	22
Serine	39	41	37	43	31	31
Glutamic Acid	39	40	28	31	36	39
Proline	19	19	18	19	17	18
Glycine	36	35	32	35	30	33
Alanine	22	21	16	17	17	18
Half Cystine	5	5	4	4	5	5
Valine	24	25	22	25	21	23
Methionine	6	6	4	4	5	5
Isoleucine	21	20	23	24	12	13
Leucine	27	27	20	21	23	24
Tyrosine	15	15	13	15	16	17
Phenylalanine	16	16	14	15	15	16
Tryptophan	5	5	6	6	5	5
Molecular Weight	38,112	38,183	31,787	34,439	30,166	32,250

(1) closest integral no. of residues based on molecular weights determined by physical methods.

(2) closest integral no. of residues based on corrections needed for nearest integral number of histidine, proline and phenylalanine residues.

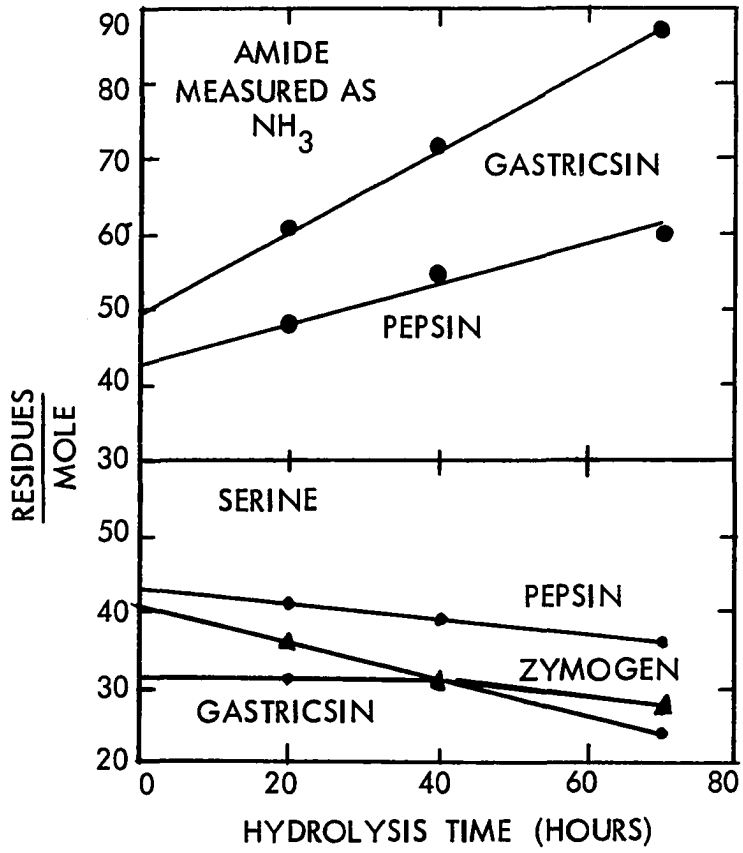


Figure 7. Determination of amide and serine residues by extrapolation to zero time hydrolysis.

TABLE 8

PARTIAL SPECIFIC VOLUMES OF ZYMOGEN, PEPSIN AND GASTRICSIN

	ZYMOGEN			PEPSIN		GASTRICSIN	
Amino Acid	$\bar{v}$	wt. %*	$\bar{v}$ x wt. %	wt. %*	$\bar{v}$ x wt. %	wt. %*	$\bar{v}$ x wt. %
Lysine	0.82	2.36	1.94	0.00	0.00	0.00	0.00
Histidine	0.67	1.08	0.71	0.41	0.27	0.43	0.29
Arginine	0.70	2.24	1.73	1.39	0.97	0.99	0.69
Aspartic Acid	0.60	2.42	1.45	5.75	3.45	0.72	0.43
Asparagine**	0.62	8.68	5.38	7.04	4.36	8.55	5.30
Threonine	0.70	10.91	5.22	7.20	5.04	6.72	4.70
Serine	0.63	7.46	5.92	10.59	6.67	8.55	5.39
Glutamic Acid	0.66	3.40	2.24	3.80	2.51	5.63	3.71
Glutamine**	0.67	10.15	6.80	7.91	5.30	10.00	6.70
Proline	0.76	4.86	3.69	5.47	4.16	5.53	4.20
Glycine	0.64	5.26	3.37	5.92	3.79	5.78	3.70
Alanine	0.74	3.93	2.91	3.58	2.65	4.05	3.00
Half Cystine	0.63	1.36	0.86	0.92	0.58	1.63	1.03
Valine	0.86	6.27	5.39	7.06	6.07	6.90	5.93
Methionine	0.75	2.07	1.55	1.56	1.17	2.08	1.56
Isoleucine	0.90	5.96	5.36	8.06	7.25	4.66	4.19
Leucine	0.90	8.05	7.25	7.05	6.35	8.60	7.74
Tyrosine	0.71	6.45	4.58	7.26	5.15	8.78	6.23
Phenylalanine	0.77	6.20	4.77	6.55	5.04	7.46	5.74
Tryptophan	0.74	2.45	1.81	3.31	2.45	2.95	2.18
$\bar{V}$			0.729 ml/g		0.732 ml/g		0.727 ml/g

* based on molecular weight of 38,000, 34,000 and 32,000 for zymogen, pepsin and gastricsin, respectively.

** to take into account the amide groups it was assumed that they were approximately equally distributed between the glutamic and aspartic residues.

TABLE 9

MOLECULAR WEIGHTS

	ZYMOGEN	PEPSIN	GASTRICSIN
Sedimentation Velocity Method	38,800	32,900	30,900
Amino Acid Composition	38,200	34,400	31,900
Archibald Method		32,200	34,000*
Sedimentation Equilibrium		34,000	31,000
Average	38,500 ± 300	33,400 ± 800	31,300 ± 400

* not included in average.

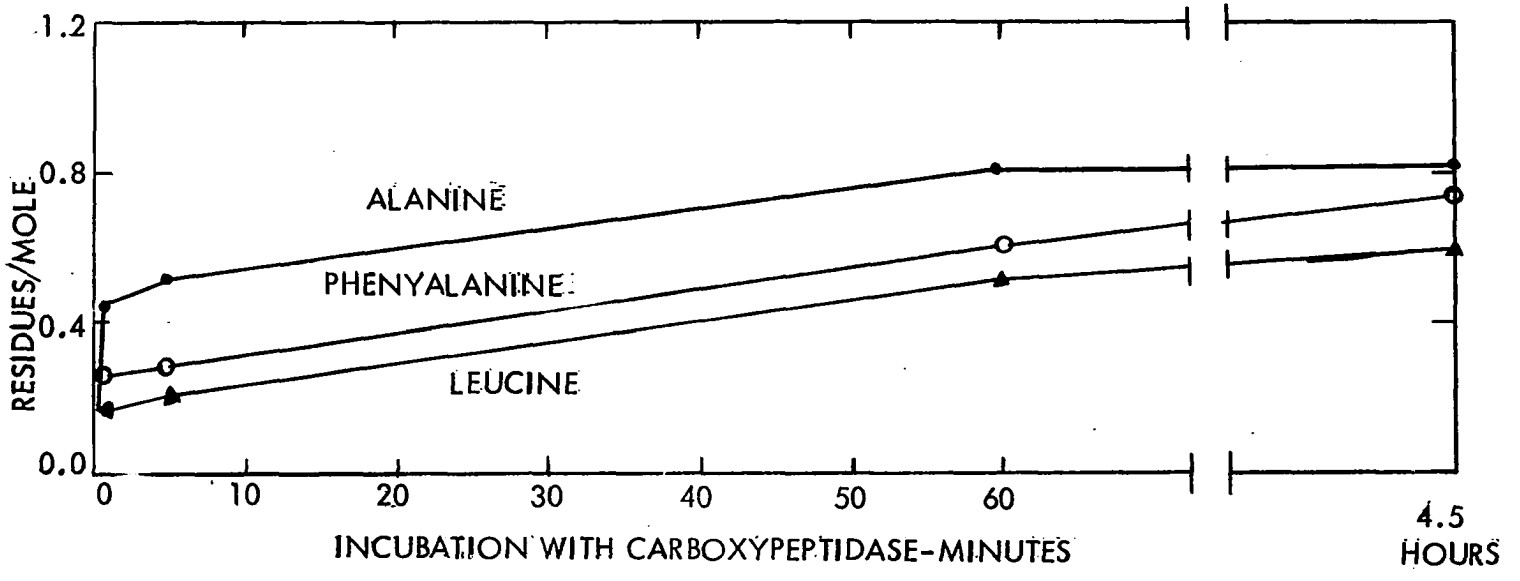


Figure 8. Release of amino acids from pepsin by carboxypeptidase at room temperature.

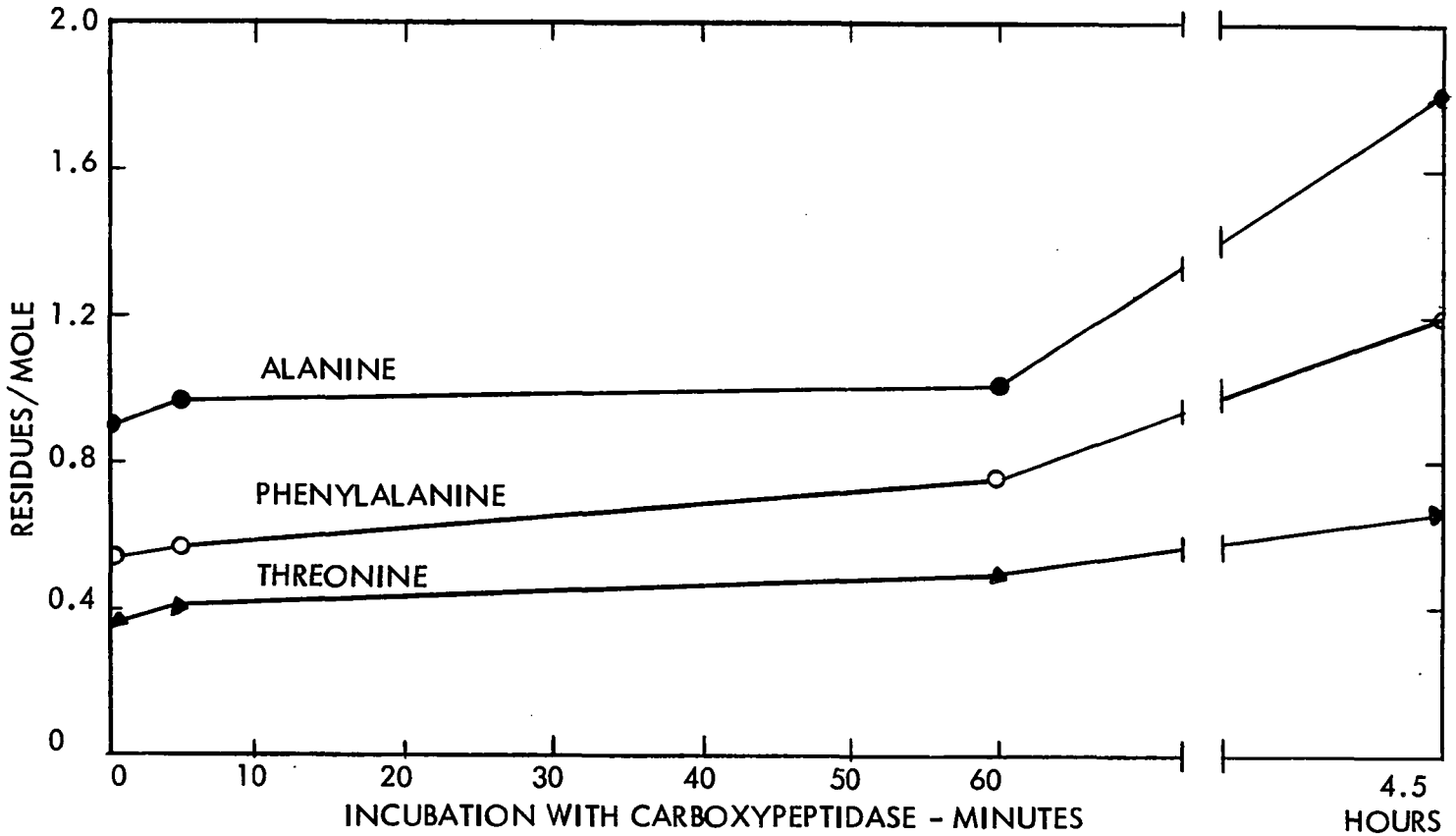


Figure 9. Release of amino acids from gastricsin by carboxypeptidase at room temperature.

CHAPTER IV

DISCUSSION

The formation of human pepsin and gastricsin by the activation of a zymogen was reported by Tang and Tang (13). Although the zymogen preparation appeared to be homogeneous using the criteria of ultracentrifugation and starch-gel electrophoresis, it might be argued that enzymes as similar as gastricsin and pepsin could be formed from similar zymogens which would be difficult to separate. However, there are other pieces of evidence against this possibility. Firstly, the ratio of the amount of gastricsin to pepsin formed varies with the pH at which the zymogen is activated yet the sum of the amounts of the enzymes formed remains constant (28). Secondly, only one N-terminal amino acid residue was found (Tang, unpublished observation). Finally, immunodiffusion experiments performed by Schlamowitz et al. (37) showed that the human zymogen formed only a single reaction band when the preparation was cross reacted with antibodies of hog pepsinogen. All of this evidence in support of the proposal that one zymogen is the precursor of both gastricsin and pepsin along with the lack of any evidence to the contrary should be highly convincing.

The proposed scheme that both enzymes are formed from one zymogen suggests several possibilities. One is that each enzyme could be formed from an entirely different part of the zymogen which exists

as an aggregate of enzyme precursors. An example of this is found in the formation of two enzymes by the activation of bovine procarboxypeptidase A as mentioned in the Introduction (3). The results of the molecular weight studies indicate that only about one-fifth of the zymogen molecule is lost in either the formation of pepsin or gastricsin. This eliminates the possibility mentioned above. Another possibility is that one enzyme is formed by a modification of the other which is an intermediate in the zymogen activation. This type of sequential formation is found in the conversion of porcine carboxypeptidase A_1 to carboxypeptidase A_2 which seems to result simply from the release of the carboxypeptidase A_1 amino-terminal dipeptide, alanyl arginine (38). A higher number of glutamic acid residues in gastricsin, which has a smaller molecular weight than pepsin, rules out a sequential formation of one of these enzymes from the other. Furthermore, no interconversion of gastricsin and pepsin could be observed when either enzyme was incubated alone at various conditions including its optimal pH (28). The difference in amino acid composition, mentioned above, also eliminates autocatylsis or partial denaturation as explanations for the formation of two enzymes from one zymogen. An explanation which is consistent with presently known structural differences between these enzymes is that they are formed at about the same time as a result of different types of changes in the primary structure of the zymogen.

The possibility that the zymogen or the enzymes could consist of more than one chain needs to be considered. The C-terminal sequence, determined with carboxypeptidase A, is different for each protein and

there is no indication of the release of more than one C-terminal amino acid. The N-terminal amino acid, determined by the DNP method, is valine, serine and one of the leucines for human pepsin, gastricsin and zymogen, respectively, and the amount of each does not exceed one residue per mole of protein (28, unpublished data). Therefore, end group analyses show that human pepsin, gastricsin and zymogen each consists of only one chain.

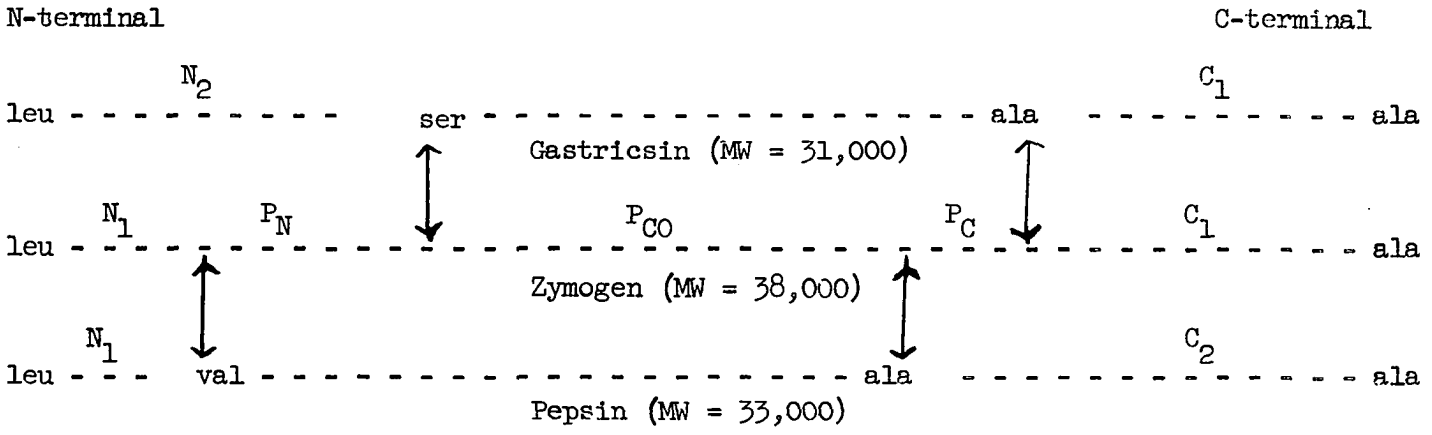
The proposal of a scheme in which only one zymogen is involved brings out certain discrepancies in the amino acid compositions. There appear to be more aspartic acid, serine, isoleucine, tyrosine and tryptophan residues in either gastricsin or pepsin than in the zymogen. The discrepancy of only one aspartic acid residue is not considered significant because of the high number of these residues per molecule and because the average deviation is from 1-2 residues. Discrepancies in the number of serine residues may be due to an error in extrapolation, which is a common difficulty. Isoleucine has been shown to require approximately 70 hours for complete hydrolysis and tyrosine is progressively degraded with time after 20 hours of hydrolysis (34). Therefore, the limited number of runs made in these studies may account for discrepancies in the case of these two amino acids. The method used for tryptophan determination may result in a relatively large error because of differences in the response of each protein to the partial denaturation and enzymatic hydrolysis prior to the colorimetric determination (see Methods).

As mentioned earlier, the number of glutamic acid residues in gastricsin precludes the formation of the entire smaller gastricsin

molecule from the larger pepsin molecule. Therefore, the gastricsin molecule must contain some portion of the zymogen protein which is not contained in pepsin. The facts that, according to amino acid analysis data, gastricsin contains all of the phenylalanine residues found in the zymogen and that the C-terminal end of pepsin has one of these phenylalanine residues indicates that the gastricsin molecule contains the C-terminal portion of pepsin. (The C-terminal alanine of pepsin must be included because the C-terminal of gastricsin is not phenylalanine). It follows that, pepsin (the larger of the two enzymes) contains the N-terminal portion of gastricsin. This is supported by the fact that pepsin seems to contain as many serine residues as the zymogen and one of these, as mentioned earlier, forms the N-terminal of gastricsin. A schematic representation of the formation of gastricsin and pepsin from their zymogen is presented on the following page.

According to this scheme the zymogen molecule is hydrolyzed in a minimum of four places to yield two enzymes. The enzymes contain a relatively large common portion, P_{CO}, of the zymogen molecule. The gastricsin molecule is shown to contain the C-terminal portion of the pepsin molecule and the pepsin molecule contains the N-terminal portion of the gastricsin molecule. As shown, there are at least four peptides, C_1, C_2, N_1 and N_2 released in the formation of the two enzymes. Two of these peptides, N_1 and N_2, have N-terminal leucine residues and the other two, C_1 and C_2, have C-terminal alanine residues. According to the above scheme and the amino acid composition differences, the most significant of which are shown in Table 10, it can be con-

SCHEME FOR ZYMOGEN ACTIVATION



(see text for explanation of above scheme)

TABLE 10

AMINO ACID CONTENT DIFFERENCES BETWEEN HUMAN ZYMOGEN, GASTRICSIN AND HUMAN PEPSIN (a)

Amino Acid	N_2+C_2(b)	N_2-N_1(c) or P_N	C_2-C_1(d) or P_C	N_1+C_1(e)
Lysine	7	0	1	6
Histidine	2	0	0	2
Arginine	3	0	0	3
Aspartic Acid (includes asparagine)	11	11	0	0
Threonine	6	2	0	4
Serine	11	11	0	0
Glutamic Acid	9	0	9	0
Alanine	4	0	1	3
Isoleucine	9	9	0	0
Leucine	6	0	3	3
Phenylalanine	1	0	1	0
Total	69	33	15	21

(a) used numbers of residues in column (2) Table 7; used average value when number in pepsin exceeds that in zymogen.

(b) number of residues found in zymogen which are not found in both pepsin and gastricsin, obtained by subtracting the number of each residue in pepsin or gastricsin (whichever is smaller) from the number in zymogen.

(c) number of residues found in zymogen portion, P_N, obtained by determining residues found in pepsin which are not found in gastricsin.

(d) number of residues found in zymogen portion, C_N, obtained by determining residues found in gastricsin which are not found in pepsin.

(e) number of residues found in peptides C_1 and N_1 obtained by subtracting the sum of the number of residues in P_N and P_C from the sum of the number of residues in N_2 and C_2.

cluded that peptides C_1 and N_1 contain about 21 amino acid residues of which about one-half are basic amino acids, lysine, histidine and arginine. Furthermore, it can be stated that P_C, the zymogen portion which represents the difference in composition between peptides C_1 and C_2, contains 9 glutamic acid (including glutamine) residues which make up about three-fifths of its composition and P_N, the zymogen portion which represents the difference between peptides P_1 and P_2, contains 11 aspartic acid (including asparagine), 11 serine, 9 isoleucine and 2 threonine residues. The amino acid analysis data show that the portion of the zymogen from which gastricsin is formed contains all of the glutamic acid residues found in the zymogen whereas that portion from which pepsin is formed contains all of the aspartic acid, serine and isoleucine residues which are found in the zymogen molecule. The exact significance of this distribution of these amino acids is uncertain at this time. Nevertheless, the difference in the primary structures of pepsin and gastricsin must be responsible for differences in their conformation.

Several similarities are revealed when the human gastric proteolytic enzyme system is compared with the porcine gastric proteolytic enzyme system. Activation occurs autocatalytically in each system with the hydrolysis of several peptide bonds resulting in a disproportionate loss of basic amino acids. Similarities in physical and chemical properties between human pepsin and zymogen and the corresponding porcine proteins are seen in Table 11. Only limited studies have been carried out on hog pepsinogen, but the closeness of the values for the molecular weight, sedimentation coefficient

TABLE II

PHYSICAL AND CHEMICAL PROPERTIES OF HUMAN AND HOG GASTRIC PROTEOLYTIC ENZYMES AND ZYMOGENS

	Human Pepsin	Gastricsin	Zymogen	Hog Pepsinogen (d,e)	Hog Pepsin (d,f,g)
$S_{20,w}(10^{-13})$	3.15	3.32[a]	3.32	3.2 - 3.3	3.20
$D_{20,w}(10^{-7})$	8.7	9.6	7.7		8.71
V (ml/g)	0.732	0.727	0.727		
M W	33,400	31,300	38,500	41,000	35,700
cc/g	4.5[b]	10.0[b]	10.0[b]		3.1
	7.9[c]	12.4[c]			
a/b	6	160	50		3
C-terminal (⟶)	Leu-Phe-Ala	Thr-Phe-Ala	Val-Ser-Ala	Val-Leu-Ala	Val-Leu-Ala
N-terminal[f]	Valine	Serine	Leucine or Isoleucine	Isoleucine	Leucine
Lys, Arg. and Hist.	4	5	16	18	4
Aspartic Acid-NH_2	38	26	37	46	44
	69[h]	65[h]	77[h]	76[h]	71[h]
Glutamic Acid-NH_2	31	39	40	30	27
Ser. and Thr.	67	53	69	75	72
Val., Leu. and Isoleu.	70	60	72	83	76

a. Concentration dependent.
b. Measured at 20° C in buffer of 0.1 ionic strength.
c. Measured in H_2O
d. C - and N-terminal data obtained from review by Bovey, F. A. and Yanari (9).
e. Data obtained from Arnon, R. and Perlmann, G. (39).
f. Data obtained from Edelhoch, H. (29).
g. Amino Acid composition data obtained from Blumenfeld, O. and Perlmann, G. (40).
h. Sum of aspartic acid, asparagine, glutamic acid and glutamine residues.

and amino acid composition with those for the corresponding human zymogen suggests very similar structures. Likewise the same properties of hog pepsin plus the diffusion coefficient, intrinsic viscosity and axial ratio, are essentially the same as those for human pepsin. It follows that gastricsin is as dissimilar to hog pepsin as it is to human pepsin.

The activation of human zymogen results in a change in the C-terminal sequence. This is markedly different from the activation scheme of hog pepsinogen. According to a reference to the preliminary data of Van Vanakis and Herriott (41) carboxypeptidase A released the same three amino acids, in the same order, from both hog pepsinogen and pepsin. However, the validity of this species difference in gastric zymogen activation needs to be further substantiated by a more critical study on the C-terminal of hog pepsinogen and pepsin.

The existence of groups of enzymes which have the same enzymatic action but which differ in their physical, chemical or immunological properties is well established. One of these groups is represented by the lactic acid dehydrogenases which have been extensively studied by Kaplan et al. (42). These enzymes are called isozymes or isoenzymes and the phenomenon of their existence has been referred to as 'molecular heterogeneity'. The implication associated with isozymes seems to be that they have identical specificities. In reality, it seems unlikely that any two enzymes which differ significantly in their molecular structures can have identical specificities under all conditions. It seems inevitable that the enzymic properties will be affected by the overall conformation of the molecule, even though

the precise structure of the active center may be identical for the two enzymes. Human pepsin and gastricsin have been shown to have similar but not identical specificities. They were shown to attack certain proteins and synthetic peptides at different relative rates (28). The question of whether gastricsin and human pepsin are isozymes seems to be purely academic. Tang has introduced the term 'heterogeneous enzyme system' to include systems in which enzymes have similar but not identical specificities (28).

The reasons for the differences in specificities of enzymes must be found in the structural differences. It is now possible to evaluate several structural differences between gastricsin and pepsin. Table 11 represents, in part, a composite of values which have thus far been obtained for such an evaluation.

Differences in the gross conformation between human pepsin and gastricsin are apparent, in particular, from viscosity measurements. It is known that solutions of globular proteins are only slightly more viscous than the pure solvent whereas solutions of proteins having rod-like or randomly coiled structures may have a much higher viscosity than the pure solvent. The value of 4.5 cc/g obtained for the intrinsic viscosity of human pepsin in buffer is well within the range of values (2.4 cc/g - 5.0 cc/g) assembled by Schachman (43) for 12 compact globular proteins. The value of 10.0 cc/g for the intrinsic viscosity of gastricsin is significantly higher than those values obtained for globular proteins and approaches those values reported for randomly coiled chains. Effects of macromolecules on the viscosity of a liquid are higher in water than in buffer solutions. This is due to the elec-

troviscous effect and to a tendency of the molecule to stretch because of the presence of charged groups which are not damped by the ionic strength effect of the solvent. The correspondingly higher values of $[\eta]$ for both pepsin and gastricsin in water are therefore expected. The concentration dependence of the value of $[\eta]$ for gastricsin is apparently associated with the high axial ratio of its molecule. These elongated molecules apparently interfere with the movements of each other at higher concentrations. This same explanation may be applied to the concentration dependence of the sedimentation coefficient value of gastricsin. Axial ratios derived from viscosity measurements are shown in Table 11. The values indicate that the human pepsin molecule is only slightly elongated with an axial ratio, a/b, of 6. On the other hand, both gastricsin and zymogen molecules are considerably more elongated with a/b values of 160 and 50 respectively. It is well known, however, that the intrinsic viscosity is dependent on both the axial ratio of the protein and the hydration effect. These two factors are separated on the assumption that the elongation of protein molecules follows theoretical elipsoid evolution. A deviation from ideality, therefore, could contribute to the value of the calculated axial ratio. It is for these reasons that perhaps the axial ratios calculated for human pepsin, gastricsin and zymogen should be considered only in a relative manner. However, in view of the large differences in the calculated axial ratios for these three proteins, it seems safe to conclude that gastricsin has a much elongated molecule while the pepsin molecule deviates only slightly from being spherical.

Although the axial ratios of pepsin and gastricsin are ex-

tremely different the enzymatic properties are similar. This can only be explained on the basis that the active center of the enzymes are almost identical whereas the overall conformations are different. This would require a fairly rigid structural support around the active center. Possibly disulfide bonds provide this structural rigidity.

The relative unstability of gastricsin noted in its decline in activity, its lower diffusion coefficient at 20° (compared to that at 0.1°) and its decreased solubility with aging has been repeatedly observed in our laboratory. The unstability is perhaps associated with the elongation of the gastricsin molecule. The internal stabilizing bonds in such conformation are perhaps less effective.

The compactness of the hog pepsin molecule has been attributed to hydrophobic bonding due to the high percentage of amino acids with non polar side chains (44). This is substantiated by the fact that on short exposure of hog pepsin to urea or guanidine in a pH range of 3.0 to 5.0 at 25° the optical rotatory properties remain unchanged and the enzyme is fully active. This indicates hydrogen bonds are relatively unimportant in maintaining the conformational structure of active pepsin. The similarity in amino acid composition and physical properties between hog pepsin and human pepsin suggests a similar importance of hydrophobic bonding in maintaining the structure of human pepsin. The relatively fewer numbers of leucine, isoleucine and valine residues in gastricsin may account for the more relaxed structure of gastricsin.

CHAPTER V

SUMMARY

Studies were made on the physical and chemical properties of human pepsin, gastricsin and their zymogen.

Their sedimentation coefficients were found to be 3.15 S for human pepsin and 3.32 S for both gastricsin and zymogen. The sedimentation coefficient of gastricsin was found to be concentration dependent. The diffusion coefficients were found to be 8.7×10^{-7} cm^2/sec, 9.6×10^{-7} cm^2/sec and 7.7×10^{-7} cm^2/sec for human pepsin, gastricsin and zymogen respectively when determined at 0.1° with the Spinco Model H electrophoresis-diffusion instrument. A value of 5.2×10^{-7} cm^2/sec was obtained for gastricsin when measured at 20° with the Model E ultracentrifuge. The intrinsic viscosities measured in buffer were found to be 4.5 cc/g for human pepsin and 10.0 cc/g for both gastricsin and zymogen. The values determined in water were 7.9 cc/g and 12.4 cc/g for human pepsin and gastricsin respectively. The intrinsic viscosity of gastricsin in water was found to be concentration dependent.

The amino acid composition has been determined for each of these proteins. The zymogen contains about four times as many basic amino acids as either of the enzymes. Human pepsin contains a significantly higher number of aspartic acid, isoleucine and serine residues per molecule than gastricsin which contains a significantly

higher number of glutamic acid residues. Carboxy terminal sequence studies, using carboxypeptidase A, showed that the C-terminal sequences are -leu-phe-ala COOH, thr-phe-ala COOH and val-ser-ala COOH for human pepsin, gastricsin and zymogen respectively. The differences in amino acid content and in C-terminal sequences have been used in explaining a proposed mechanism for the activation of the zymogen.

Molecular weights determined by the sedimentation velocity method, the Archibald method, sedimentation equilibrium studies and from the amino acid analyses data gave average molecular weights of 33,400, 31,300 and 38,500 for the human pepsin, gastricsin and zymogen respectively. The values for the partial specific volume used in these determinations were calculated from the amino acid compositions.

Evidence for a more relaxed elongated gastricsin molecule compared to the pepsin molecule has been discussed. The mechanism by which two enzymes are formed from one zymogen has also been discussed in terms of structural differences found in this study. A comparison has been made showing that porcine pepsin and pepsinogen are very similiar in physical and chemical properties to human pepsin and its zymogen, respectively, but that gastricsin, by the same criteria, is different from any of these other proteins.

BIBLIOGRAPHY

1. Northrop, J.H., Kunitz, M. and Herriott, R.M., Crystalline Enzymes. second edition, New York: Columbia University Press, (1948).

2. Neurath, H., Advances Protein Chem. 12, 319 (1957).

3. Neurath, H., Federation Proceedings, 23, 1 (1964).

4. Desnuelle, P. and Rovery, M., Advances Protein Chem. 16, (1961).

5. Labouesse, B., Carlsson, K., Oppenheimer, H.L. and Hess, G.P., in Goodwin, T.W., Harris, J.I., and Hartley, B.S. (Eds.) Structure And Activity Of Enzymes, London and New York; Academic Press, (1964).

6. Neurath, H. and Dixon, G., Federation Proceedings, 16, 791 (1957).

7. Herriott, R.M., J. Gen. Physiol., 22, 65 (1938).

8. Herriott, R.M., J. Gen. Physiol., 21, 501 (1938).

9. Bovey, F.A. and Yanari, S.S., in Boyer, P.D., Lardy, H. and Myrback, H. (Eds.), The Enzymes, IV, New York and London: Academic Press (1960).

10. Schlamowitz, M., Varandani, P. and Wissler, F., Biochem. 2, 238 (1963).

11. Richmond, V., Caputto, R. and Wolf, S., Arch. Biochem. Biophys., 66, 155 (1957).

12. Tang, J., Wolf, S., Caputto, R. and Trucco, R.E., J. Biol. Chem. 234, 1174 (1959).

13. Tang, J. and Tang, K.I., J. Biol. Chem. 238, 606 (1963).

14. Anson, M.L. and Mirsky, A.E., J. Gen. Physiol., 16, 59 (1932).

15. Lang, C.A., Anal. Chem., 30, 1692 (1958).

16. Scheraga, H.A. and Mandelkern, L., J. Am. Chem. Soc., 75, 179 (1953).

17. Schachman, H.K., in Colowick and Kaplan (Eds.), Methods in Enzymology, Vol. 4, Academic Press, Inc., New York, pp. 32 (1957).

18. Longsworth, L.G., J. Am. Chem. Soc., 74, 4155 (1952).

19. Tables of Probability Functions (Federal Work Agency, Works Project Administration, Superintendent of Documents, Washington, D.C., 1941.

20. Svedberg, T. and Pedersen, K.O., The Ultracentrifuge, Oxford University Press, London and New York, Johnson Reprint Corporation, New York, pp 8-9 (1940).

21. Schachman, H.K., Ultracentrifugation in Biochemistry, Academic Press, Inc., New York, pp 201-208 (1959).

22. Ginsberg, A., Appel, P. and Schachman, H.K., Arch. Biochem. and Biophys., 61, 545 (1956).

23. Archibald, W.J., J. Phys. and Colloid Chem., 51, 1204 (1957).

24. Klainer, S.M. and Kegeles, J., J. Phys. Chem., 59, 952 (1955).

25. Spackman, D.H., Stein, W.H. and Moore, S., Anal. Chem., 30, 1190 (1958).

26. Spies, J.R. and Chambers, D.C., Industr. Engng. Chem. (Anal. Ed.), 21 (1949).

27. Harrison, P.M. and Hofmann, T., Biochem. J., 80, 38P (1961).

28. Tang, J., Ph.D dissertation, U. of Okla. (1961).

29. Edelhoch, H., J. Am. Chem. Soc., 79, 6100 (1957).

30. Polson, A., Kolloid Z., 88, 51 (1939).

31. Jirgensens, B., Arch. Biochem. Biophys., 39, 261 (1952).

32. Buzzell, J.G. and Tanford, C., J. Phys. Chem., 60, 1204 (1956).

33. Noltmann, E.A., Mahowald, T.A. and Kuby, S.A., J. Biol. Chem., 237, 1146 (1962).

34. Mahowald, T.A., Noltmann, E.A. and Kuby, S.A., J. Biol. Chem., 237, 1138 (1962).

35. Van Vanakis, H. and Herriott, R.M., Biochim. et Biophys. Acta., 23, 600 (1957).

36. Tang, K. and Tang, J., Federation Proceedings, 20, 239 (1961).

37. Schlamowitz, M., Shaw, A. and Jackson, W., Biochem. 3, 639 (1964).

38. Folk, J., J. Biol. Chem., 238, 3895 (1963).

39. Arnon, R. and Perlmann, G., J. Biol. Chem., 238, 963 (1963).

40. Blumenfeld, O.O. and Perlmann, G.E., J. Gen. Physiol., 42, 553 (1959).

41. Herriott, R., J. Gen. Physiol., 45, 57 (1962).

42. Kaplan, N.O., Ciotti, M.M., Hamolsky, M. and Bieber, R.E., Science, 131, 392 (1960).

43. Schachman, H.K., Cold Spring Harbor Symposia on Quantitative Biology, XXVIII, 409 (1963).

44. Perlmann, G., Proc. Nat. Acad. Sci. U.S., 45, 915 (1959).