Pepsin from Pepsinogen

PREPARATION AND PROPERTIES

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SUMMARY

Commercial samples of crystalline swine pepsin have been found to be heterogeneous by a number of criteria. Several active fractions can be obtained by chromatography on hydroxylapatite with phosphate buffers, pH 5.7, of increasing molarity as eluents. This has been found to be an effective procedure for evaluating the homogeneity of pepsin. End group analysis by the cyanate method yields 1 eq of isoleucine but as many as 9 other amino-terminal residues in fractional molar amounts. Treatment with carboxypeptidase A liberates 1 eq of alanine and fractional molar amounts of five other amino acids. These results suggest that commercial pepsin is a mixture of autodigested products, cleavage having occurred at various points in the pepsin chain. Evidence of fragmentation is also observed when reduced and carboxymethylated pepsin is passed through columns of Sephadex. It seems probable that autodigestion occurs during the industrial preparation of 1:10,000 pepsin, the starting material from which crystalline pepsin has traditionally been prepared.

In order to obtain pepsin more suitable for structural studies and for investigations of the active site of the enzyme, it is necessary to begin with pepsinogen. In agreement with the results of others, we find that commercial samples of the zymogen are essentially homogeneous. Chromatography on diethylaminoethyl Sephadex A-25 and on hydroxylapatite does not reveal heterogeneity. Leucine is the sole amino-terminal residue found by the cyanate method. Treatment with carboxypeptidase A liberates 1 eq of alanine and fractional molar amounts of five other amino acids. These results suggest that commercial pepsin is a mixture of autodigested products, cleavage having occurred at various points in the pepsin chain. Evidence of fragmentation is also observed when reduced and carboxymethylated pepsin is passed through columns of Sephadex. It seems probable that autodigestion occurs during the industrial preparation of 1:10,000 pepsin, the starting material from which crystalline pepsin has traditionally been prepared.

A relatively homogeneous pepsin may be readily obtained by the activation of pepsinogen at 14°C and pH 2 for 20 min. Separation of the enzyme from the peptides formed during the activation process is accomplished by passage through a column of sulfoethyl Sephadex C-25 at pH 4.4 and 0°C. The acidic pepsin passes through unretarded, whereas the basic peptides are tenaciously held and may be eluted with 0.1 M NH₂OH. Pepsin prepared in this manner in 95% yield is homogeneous on DEAE-Sephadex A-25 and on hydroxylapatite. It shows no evidence of heterogeneity upon reduction, carboxymethylation, and passage through Sephadex. It yields isoleucine as the sole amino-terminal and alanine as the sole carboxyl-terminal residue. Carboxypeptidase B does not liberate basic amino acid residues. Failure of the carboxypeptidases to liberate any amino acid other than alanine suggests that proline is the third residue from the carboxyl terminus of both pepsinogen and pepsin. A proline residue in this position would block further action by either carboxypeptidase A or B.

The homogeneity of the pepsin formed depends critically upon the pH of the activation process. If activation is carried out at pH 3 or pH 3.9 instead of at pH 2, additional active species are obtained.

Amino acid analyses have been carried out on pepsinogen and on the pepsin derived from it. The zymogen has been found to have 363 amino acid residues; the enzyme, 321. In the activation process, 42 residues, including 9 of lysine, 2 of histidine, and 2 of arginine, are cleaved off. These are almost exactly accounted for by the amino acid composition of the total peptide fraction obtained from the activation mixture.

Pepsin freshly prepared from pepsinogen has about 1.3 times the activity of the commercial product against hemoglobin as a substrate, but somewhat less activity against acetylatedlanylidiiodotyrosine.

Preliminary evidence is mentioned which suggests that freshly prepared pepsin may differ in several properties from the commercial material. The investigation of these and other aspects of the behavior of pepsin should be facilitated by the availability of homogeneous, reproducible preparations of the enzyme.

The present work grew out of attempts to label the active site of pepsin and thereby to learn the nature of the amino acid residues that participate most directly in the catalytic process. Preliminary results suggested that the commercial, crystalline pepsin being used was heterogeneous, a supposition supported by considerable evidence to be found in the literature. Steinhardt (1) pointed out in 1939 that pepsin preparations do not exhibit constant solubility. Electrophoretic heterogeneity was

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find that columns of the adsorbent can be used to fractionate minor proteolytically active components. Recently, Stepanov, Vaganova, and Kuznetsov (6), and Vaganova, Levin, and Stepanov (7) have demonstrated that pepsin has multiple end groups when tested by the usual procts but that commercial crystalline pepsin is heterogeneous in the course of the present work. From commercial pepsinogen, samples obtained from Worthington, and the acetyl-n-phenylalanyl-n-phenylalanine employed as substrate was purchased from Cycle Chemical Corporation. The acyl dipeptide gave within 0.3% of the theoretical values upon elemental analysis for carbon, hydrogen, and nitrogen, and liberated 1.0 ± 0.05 residue of phenylalanine upon treatment with carboxypeptidase A. Acetyl-l-phenylalanyl-l-DL-diiodotyrosine was a sample prepared by Dr. R. Pitt-Rivers and kindly supplied by Dr. Gertrude E. Perlmann.

Chromatography of Pepsin on Hydroxylapatite (cf. References 8 and 14)—Columns of two sizes were used: for the most part, 0.9 × 30 cm in a chromatograph tube 0.9 × 40 cm for analytical purposes, and 2 × 30 cm in a tube 2 × 40 cm for preparative scale work. The chromatograph tubes were provided with sintered glass plates of medium porosity or Grade 50-55 porous Teflon discs. All columns were prepared and operated in the cold room at 4°. Hydroxylapatite was prepared as a suspension in 0.001 M sodium phosphate buffer, pH 6.8. The solid, which settles fairly rapidly, was brought into suspension by shaking and vigorous stirring, and the suspension was poured into the chromatograph tube. The delivery tip of the tube was closed off and the tube was filled to the top with the suspension. The hydroxylapatite was allowed to settle to a height of 10 cm before the delivery tip was opened. When the adsorbent had settled, the clear supernatant was removed, the top 1 to 2 cm of the settled bed were stirred, and a second portion of the gel suspension was added. This procedure was repeated until the height of the bed settled under gravity was 5 to 10 cm more than that ultimately desired. Packing was then continued under pressure with the aid of a Milton Roy Minipump. A rate of 20 ml per hour against a pressure of 0 to 15 p.s.i. was used to pack the analytical column; a rate of 40 to 50 ml per hour against a pressure of 10 to 25 p.s.i. was used to pack the preparative scale column. The pressure varied with different batches of adsorbent. Columns were washed with 5 to 8 column volumes of 0.03 M sodium phosphate, pH 5.7 ± 0.05, until the influent and effluent were of the same pH, ±0.02 pH unit (Radiometer, type PHM 4 pH meter).

The buffers used were all prepared by suitable dilution of a 0.3 M stock phosphate buffer, pH 5.7, which was prepared by diluting 275 ml of m NaH₂PO₄ and 24 ml of m Na₂HPO₄ to a volume of 1 liter. In an analytical experiment, 25 mg of pepsin in 2 ml of 0.03 M phosphate buffer were applied to the column. Elution was begun at a rate of 15 to 20 ml per hour (maintained by a pump). Fractions of 2 ml were collected. After 50 ml had been eluted, the buffer in the reservoir, on top of the resin surface, in the pump, and in all the input tubing was changed to 0.05 M phosphate buffer, pH 5.7. Subsequently, at 50-ml intervals, the eluent was changed successively in the same manner to 0.10, 0.15, and 0.20 M phosphate buffer, pH 5.7. Since satisfactory resolution can be obtained with a simpler elution schedule, in later analytical chromatograms 0.1 M phosphate buffer was used as eluent immediately after the sample had been applied to the column. Subsequent changes to 0.15 and 0.20 M phosphate buffer were made after about 25 and 20 ml, respectively.

The column effluent was monitored continuously at 254 m by an ISCO ultraviolet recording analyzer (model UA, Instrumenta Specialties Company, Inc.). Fractions found to contain material absorbing at 254 m were subjected to ninhydrin analysis after alkaline hydrolysis (cf. Fruchter and Crestfield (15)). Samples of 100 ml were always used unless otherwise stated. The protein concentration in selected fractions was also determined at 280 m with the aid of a Zeiss PMQ II spectrophotometer. Portions of these same fractions were assayed for pepsin activity by one of the procedures described in a later section.

In preparative work with columns 2 × 30 cm, 100 mg of pepsin were added to the column. The flow rate was about 35 ml per hour, and 4-ml fractions were collected. Buffers changes to 0.15 and 0.20 M buffer were made after about 40 and 115 ml, respectively.

After a run, columns were re-equilibrated with starting buffer and could be used for three to five successive chromatograms. When a discolored zone appeared at the top of the column, the upper 1 to 2 cm of adsorbent were removed and replaced with fresh adsorbent. Occasionally the column was washed after use

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with 2 to 4 column volumes of 0.6 M phosphate buffer, pH 5.7 (250 ml of 0.2 M NaH2PO4 plus 52 ml of 0.1 M Na2HPO4 diluted to a volume of 500 ml). Columns were stored at 4°C when not in use.

When materials emerging from the hydroxylapatite columns were to be rechromatographed on the same columns, the pooled effluent fractions were first passed through a column, 2 X 30 cm, of Sephadex G-25 that was equilibrated and operated with 0.1 M phosphate buffer, pH 7.3.

**Chromatography of Pepsinogen on Hydroxylapatite**—The procedure of packing and operating the columns was the same as that described for pepsin, except that the column was equilibrated initially with 0.05 M phosphate buffer, pH 7.3. The protein was applied to the column in the same buffer and elution was continued with this buffer for 55 ml, after which the eluent was changed to a 0.075 M phosphate buffer at pH 7.3 to elute thezymogen. Both buffers were appropriate dilutions of 0.1 M stock phosphate buffer (19.314 g of Na2HPO4·7H2O plus 4.98 g of NaH2PO4·H2O, diluted to 1 liter with water).

**Chromatography of Pepsinogen and Pepsin on DEAE-Sephadex**—Before use, the fines were removed from the DEAE-Sephadex A-25; for this purpose, 10 g of resin were suspended in 300 ml of 0.05 M Tris-HCl buffer, pH 7.25, 0.2 M in NaCl (45 ml of 0.1 M NaCl plus 25 ml of 0.2 M Tris, made to a volume of 100 ml, and 1.17 g of solid NaCl were added). After 15 min of settling, the supernatant was removed by gentle suction and the settled resin was suspended as before and allowed to settle for 10 min. The process was repeated three times more with settling times of 10, 5, and 5 min. The settled resin was suspended in about 3 volumes of buffer and poured into a chromatograph tube, 0.9 X 30 cm, the delivery tip of which was closed off. After the resin had settled to a height of 5 cm, the delivery tip was opened. Column packing was continued with the aid of a Minipump set to deliver 25 ml per hour. After the resin had settled, the supernatant fluid was removed and more resin suspension was added. This operation was repeated until the column height had reached 27 cm. The column was washed with 300 ml of buffer. It was operated at a pressure of 10 p.s.i. A 40-ml sample of pepsinogen dissolved in 2 ml of the equilibrating buffer was applied to the column. Gradient elution was begun at once. A 75-ml reservoir was filled with the equilibrating buffer and into it was allowed to flow a buffer 0.05 M in Tris-HCl and 0.4 M in NaCl (the equilibrating buffer with twice the concentration of NaCl). The column was operated at 4°C at a rate of 20 ml per hour, and 2-ml fractions were collected. The effluent was monitored with the aid of the ISCO ultraviolet recording analyzer.

For the chromatography of pepsin, DEAE Sephadex A-50 was used. The column was prepared and operated as described above except that 0.05 M acetate buffer, pH 4.3 ± 0.03, 0.2 M in NaCl, was the initial eluent. A gradient toward 0.5 M NaCl was begun immediately after addition of the sample.

**Preparation of Pepsin from Pepsinogen**—On the basis of the earlier work of Herrtrot (16), a pH of 2 was chosen for the activation of pepsinogen. A 75-ml sample of pepsinogen was dissolved in 8 ml of glass-distilled water to yield a slightly turbid solution, pH 5 to 6. The solution was transferred to a jacketed vessel equipped with a magnetic stirrer and fitted with electrodes from a radiometer TTTT autotitrator. Chilled water from a thermostated bath was circulated through the jacket of the vessel so as to maintain the solution at 14°C ± 1°C. Temperature equilibrium was attained within 2 to 3 min. In a typical experiment, the solution was brought to pH 2.0 by the addition of 0.75 ml of a chloroacetic acid-HCl mixture (9 ml of 2 N chloroacetic acid + 1 ml of 0.1 N HCl). The acid must be added rapidly, in 20 to 25 sec. The amount of acid needed was determined in a trial experiment with a small sample. With some preparations which contain a large amount of morganite salt, more acid was needed; when this was the case, the concentration of HCl was increased, rather than that of monochloroacetic acid. The solution was maintained at pH 2.0 and 14°C for exactly 20 min, at which time 0.75 ml of 4 N acetate buffer, pH 5.0 (cf. Reference 17), was added to bring the mixture to pH 4.40. The activation mixture was applied promptly (within 5 min) to a column, 2.5 X 30 cm, of sulfoethyl Sephadex C-25 equilibrated with a 0.4 M sodium acetate buffer, pH 4.38 ± 0.03 (40.29 g of sodium acetate·3H2O plus 29 ml of glacial acetic acid, made to a volume of 2 liters). The column was operated at 4°C under gravity at a flow rate of about 60 ml per hour. Fractions of 4 ml were collected. The effluent fractions which contained the material emerging at the void volume, as shown by the recorder, were pooled within a few minutes after elution. The solution was brought to pH 5 by the addition of a few drops of 2 N sodium acetate. The pepsin solution was then frozen in 10-ml lots and stored at -20°C.

The frozen samples were thawed immediately before use and converted to a solution of the desired pH and ionic composition by passage at 4°C through a column, 2 X 30 cm, of Sephadex G-25. Desalting could be accomplished if the Sephadex column had been equilibrated with glass-distilled water. For chromatography on hydroxylapatite, the pepsin solution was converted to 0.1 M phosphate, pH 5.7, by gel filtration. To elute the basic peptides formed during activation from the sulfoethyl Sephadex column used above, the eluent was changed to 0.1 N Na,HPO4 after 100 ml had passed through the column.

In studies on the effect of pH on the activation process, a pepsinogen solution (15 mg of pepsinogen in 2 ml of glass-distilled water) was first brought to pH 1.9 with a few drops of 6 N HCl and activation was allowed to proceed for 20 min at 14°C. In a parallel experiment, the solution was brought to pH 6.5 with 0.3 ml of 4 N acetate buffer, pH 5, and the mixture was applied to a column, 2 X 9 cm, of sulfoethyl Sephadex equilibrated with 0.03 M NaH2PO4, pH 4.4. The effluent fractions containing pepsin were pooled and applied to a hydroxylapatite column, 0.9 X 14 cm, operated as described previously. In activation experiments at pH 3 and pH 4, 3 N acetic acid was used to lower the pH, rather than 6 N HCI.

**Amino-terminal Analyses of Pepsin and Pepsinogen**—Amino-terminal analyses of samples of pepsin and pepsinogen were carried out by the cyanate method of Stark and Smyth (18). Neither amino-terminal cystine nor cysteine was sought. The commercial protein preparations were freed of low molecular weight contaminants by passage through a column, 2 X 65 cm, of Sephadex G-25 in water prior to carboxymyiation. In the end group method itself, there is a second gel filtration step which separates the denatured protein fraction from any autocatalytically formed small peptides. Samples of pepsin were also analyzed for end groups after reduction and carboxymethylation by the procedure of Crestfield, Moore, and Stein (19).

**Carboxyl-terminal Analyses of Pepsin and Pepsinogen**—Native, urea-denatured, or reduced and carboxymethylated proteins (19) were employed. For urea denaturation, 60 mg of pepsin plus 2.4 g of urea were dissolved in 3 ml of 0.1 M phosphate buffer, pH 8.0. The volume was adjusted to 5 ml and the solution was
placed in a jacketed vessel of the pH-stat. A few drops of NaOH were added to bring the solution to pH 8.6, whereupon the mixture was maintained at 52°C for 45 min. The solution was desalted on Sephadex as described above.

For enzymatic digestion, approximately 0.4 µmole of freshly gel-filtered pepsin in 0.6 ml of water was added to 1.2 ml of 0.1 M phosphate buffer, pH 8.0, and to this mixture was added 0.2 ml of a carboxypeptidase A solution prepared as described by Hiro, Moore, and Stein (20). Digestion was allowed to proceed at 25°C ± 2°C. Aliquots (0.5-ml) were pipetted into centrifuge tubes and acidified with 0.1 ml of HCl to precipitate the protein. The mixture was cooled at 0°C for 15 min and then centrifuged at room temperature. The supernatant solution was rotary-evaporated to dryness, and the residue was taken up in 1.5 to 2 ml of 0.2 M citrate buffer, pH 2.2, and submitted to amino acid analysis on the amino acid analyzer (see below).

When pepsinogen was treated with carboxypeptidase A for 6 hours as outlined above, no free amino acids were found on analysis, but a small peptide peak was noted near the phenylalanine position. This was a result of pepsin activity generated in the acidification step, since it was present in an equal amount in the zero time fraction. In order to determine the carboxy-terminal group, reduced carboxymethylated pepsinogen prepared by the method of Crestfield et al. (19) was employed. After 8 and 16 hours of digestion by carboxypeptidase A, 1-ml portions were removed and 5 ml of 1% picric acid solution were added. The mixture was cooled at 0°C for 15 min and centrifuged; 5 ml of the supernatant solution were applied to a small bed of a carboxypeptidase A solution prepared as described by Hirs, 25°C ± 2°C. Aliquots (0.5-ml) were pipetted into centrifuge tubes and acidified with 0.1 ml of HCl to precipitate the protein. The mixture was cooled at 0°C for 15 min and then centrifuged at room temperature. The supernatant solution was rotary-evaporated to dryness, and the residue was taken up in 1.5 to 2 ml of 0.2 M citrate buffer, pH 2.2, and subjected to amino acid analysis on the amino acid analyzer (see below).

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Amino Acid Analyses—For the amino acid analyses given in Table II, pepsinogen and freshly prepared, purified pepsin were desalted on a column, 2.5 x 40 cm, of Sephadex G-50 equilibrated with 0.05 M N-ethylmorpholine acetate, pH 8. The alkaline pH employed was in order to inactivate pepsin. Morpholine acetate is volatile and was largely removed by lyophilization. A weighed sample of protein (about 1 mg) of known moisture, ash, and nitrogen content was hydrolyzed in 1 ml of 6 N HCl (21). The dry hydrolysate was taken up in 3 ml of pH 2.2 buffer and 1-ml aliquots were analyzed by ion exchange chromatography according to the accelerated procedure of Spackman (22) and as modified by the use of a range card in the recorder so as to permit quantitative analysis in the range of 0.01 to 0.10 µmole of amino acid (cf. Reference 24 for references). The increased sensitivity was particularly useful in the determination of the basic amino acids, which are present in pepsin in very small amounts.

Pepsin Assay—Pepsin activity was routinely determined with the use of hemoglobin or acetyl-l-phenylalanyl-l-phenylalanine as substrates. The hemoglobin assay was that of Anson (25), modified slightly. Hemoglobin substrate powder (2.5 g) was dissolved in 100 ml of glass-distilled water and filtered through Pyrex glass wool. To 25 ml of the filtrate were added 4 to 4.2 ml of 0.3 N HCl to bring the solution to pH 1.8 ± 0.05. A 2-ml portion of this solution was pipetted into a test tube, 16 x 150 mm, immersed in a water bath at 37°C ± 0.1°C. After 2 to 3 minutes, to permit temperature equilibration, 100 µl of a pepsin solution containing 5 to 12 µg of pepsin were added. After 4 min, the reaction was terminated by the addition of 4 ml of 5% trichloracetic acid solution. The mixture was allowed to remain at 37°C for 3 to 4 min and was then filtered through a double layer of filter paper, Whatman No. 3 on top and Schleicher and Schuell No. 576 underneath. Blank determinations were carried out in the same way, except that trichloracetic acid was added to the substrate solution before the enzyme was added. The absorbance was read at 590 nm in a 1-cm cell against water as a blank with the aid of a Zeiss PM II spectrophotometer. Activity is expressed as change in absorbance at 590 nm per hour per 100-µl sample. Specific activity is defined as the change in absorbance at 280 nm per hour per liter equivalent. The concentration of pepsin was determined by the ninhydrin method after alkaline hydrolysis (15).

Activity against acetyl-l-phenylalanyl-l-phenylalanine was determined by the procedure of Baker (26). Hydrolysis was followed by the ninhydrin method. Activity against acetyl-l-phenylalanyl-nL-didiotyrosine was determined by the method of Jackson, Schlamowitz, and Shaw (27). The concentration of pepsin was determined as above.

For comparison of the specific activities of purified and commercial pepsin, assays against hemoglobin and acetyl-l-phenylalanyl-l-phenylalanine were carried out as described above. The purified pepsin employed was freshly prepared by activation of pepsinogen; the enzyme was recovered from the sulfoethyl Sephadex C-25 column and desalted on a column of Sephadex G-25 operated with water as the eluent. The commercial pepsin was passed through sulfoethyl Sephadex C-25 and Sephadex G-25 in exactly the same manner. The protein concentrations of the solutions used for assay were determined by amino acid analysis. In the calculation of enzyme content, pepsin was assumed to possess 1 lysine, 1 histidine, and 2 arginine residues per molecule (cf. Table II).

Results

Heterogeneity of Commercial Pepsin—Several different samples of crystalline pepsin from two sources were all found to be markedly heterogeneous either by chromatography under conditions that do not permit significant autodigestion, or by end group analysis, or by both. The presence of a mixture of enzymatically active proteins is seen in Fig. 1. The effluent curve was obtained when crystalline, commercial pepsin was chromatographed by stepwise elution with phosphate buffer, pH 5.7, on a column of hydroxylapatite. Two clearly separated peaks of active material are visible (Peak I and Peak III), with a more diffuse active zone (Peak II) between. The first peak emerges with the 0.15 M buffer, as does the diffuse zone. The second peak does not emerge until the concentration of phosphate buffer is raised to 0.2 M. Between 50 and 90% of the protein applied to the column, as measured by ninhydrin after alkaline hydrolysis, can be accounted for in the peaks shown in Fig. 1, and no further material can be eluted from the column by an increase in the molarity of the eluent to 0.25 or 0.30 M phosphate, pH 5.7. The material which appears at the breakthrough volume is inactive and presumably represents breakdown products. Since nothing further emerges until the concentration of the eluent reaches 0.15 M, columns are now routinely started with a 0.1 M phosphate buffer as eluent, followed by the introduction of 0.15 and 0.20 M buffers (see "Experimental Procedure"). Although the relative sizes of Peaks I and III may vary, all commercial samples of pepsin examined so far yield...
Chromatography at 4°C of commercial pepsin on a column, 0.9 × 40 cm, of hydroxylapatite. A sample of 25 mg was applied to the column. Phosphate buffers, pH 5.7, of increasing molarity were employed as eluents. The molarities and points of change (arrows) of the buffers are indicated at the top of the figure. Effluent fractions of 2 ml were collected at a rate of 15 to 20 ml per hour. Protein concentration was determined on 0.1-ml samples by ninhydrin analysis after alkaline hydrolysis. The assay procedures are described under “Experimental Procedure.”

Fig. 2. Chromatography of reduced, carboxymethylated commercial pepsin on a column, 2 × 65 cm, of Sephadex G-25. Water was the eluent; the rate was 56 ml per hour; the effluent was collected in fractions of 2 ml; and the protein concentration was determined on 0.1-ml samples by the ninhydrin method after alkaline hydrolysis. Further evidence of heterogeneity was afforded by results obtained when commercial pepsin was reduced and carboxymethylated by the procedure of Crestfield et al. (19), the resulting mixture was passed through a column of Sephadex G-25 equilibrated with 0.1 M phosphate buffer to lower the phosphate concentration from 0.15 M. The protein was then applied to a column, 0.9 × 35 cm, of hydroxylapatite equilibrated with 0.1 M phosphate buffer, pH 5.7. Nothing emerged until the eluent was changed to 0.15 M, whereupon a single sharp peak appeared which had 80 ± 5% of the protein and 100 ± 5% of the activity applied to the column. No further protein emerged on continued elution with a 0.2 M buffer. When Peak III was rechromatographed similarly, except that a column 16 cm in height was employed, no protein emerged in 0.10 M or 0.15 M buffer, but 85 ± 5% of the protein was recovered as a single sharp peak at the breakthrough volume of the 0.20 M buffer. It thus appears that the effluent curve shown in Fig. 1 is a true reflection of heterogeneity and not an artifact of chromatography.

Fig. 1. Chromatography of reduced, carboxymethylated commercial pepsin on a column, 2 × 65 cm, of Sephadex G-25. Water was the eluent; the rate was 56 ml per hour; the effluent was collected in fractions of 2 ml; and the protein concentration was determined on 0.1-ml samples by the ninhydrin method after alkaline hydrolysis.
occupy the amino terminus in pepsin. Reduction and carboxymethylation, followed by gel filtration on Sephadex G-25, yielded a preparation of Lot 710 that still possessed a number of extraneous amino-terminal residues. The results for amino-terminal threonine and serine are least certain. In the first place, serine and threonine hydantoins decompose during the alkaline hydrolysis used in the cyanate method and, consequently, the values for these end groups incorporate large correction factors. In the second place, pepsin contains a residue of phosphoserine flanked on either side by threonine and glutamic acid (30). Stark and Smyth (18) have emphasized the problems involved in determining the end groups of phosphoproteins with the aid of the cyanate method; in the experiments summarized in Table I, abnormally large amounts of threonine and glutamic acid were found in the blank analyses that are always required in the cyanate method.

Treatment of Lot 707 or of urea-denatured Lot 710 with carboxypeptidase A liberated a number of amino acids in addition to alanine, the residue reported to be carboxyl-terminal in pepsin (7, 32). In the past, results of this general character have been employed in attempts to deduce the carboxyl-terminal sequence in pepsin, on the assumption that the preparation being examined consisted of one unbroken peptide chain (34). The data in Table I render this assumption extremely unlikely. If carboxypeptidase were cleaving amino acids stepwise from the carboxyl terminus of a single chain, the relative amounts of the different residues liberated from Lots 707 and 710 should be the same. They are not, however. From the data in Table I, the carboxyl-terminal sequence for Lot 707 would seem to be -Phe-Tyr-Ile-Val-Leu-Ala, whereas the sequence for Lot 710 would seem to be -Ile-Phe-Leu-Tyr-Val-Ala. Since it will be shown later that terminal groups. In the cyanate amino-terminal procedure, carbamylation is carried out in 8 M urea. This may explain why RCM- pepsin, Lot 710, appears to have more amino- than carboxyl-terminal groups. In the cyanate amino-terminal procedure, carbamylation is carried out in 8 M urea, which apparently disperses aggregates.

Further evidence that the crystalline pepsin preparations here studied have been fragmented by proteolysis is provided by an experiment in which the proteins in Peak I and Peak III from a hydroxylapatite column (pepsin, Lot 707) were separately treated with carboxypeptidase A. Digestion was allowed to proceed for 12 hours in each case, by which time a full residue of alanine had not been liberated. However, the quantities of the other amino acids, relative to alanine, liberated from the two protein preparations were markedly different. The ratios relative to alanine were: leucine, 0.85; phenylalanine, 0.72; tyrosine, 0.53; valine, 0.40; isoleucine, 0.16, for the protein from Peak I; and leucine, 0.69; phenylalanine, 0.56; tyrosine, 0.18; valine, 0.16; isoleucine, 0.06, for the protein from Peak III. These results suggest that Peak I represents enzymes more highly fragmented than those in Peak III, which is consistent with the observation that on standing or aging, the amount of Peak I material in pepsin preparations tends to increase at the expense of Peak III.

Homogeneity of Pepsinogen—Previous studies (10, 11) have suggested that commercial samples of pepsinogen are essentially homogeneous. We have confirmed and extended these observations. As may be seen in Fig. 3, A and B, pepsinogen, Lot 9163, yielded a single peak upon chromatography on either DEAE-Sephadex A-25 or hydroxylapatite. Similar results were obtained with two other commercial lots. There is a suggestion of inhomogeneity on the trailing edge of the curve shown in Fig. 3A, which is manifested by a decrease in the specific activity. Evidence was found for the presence of a small percentage of low molecular weight material, which could readily be removed by gel filtration on a column, 2 × 65 cm, of Sephadex G-25 equilibrated with water. When pepsinogen so treated was analyzed by the cyanate procedure, leucine (0.76 eq) was found as the sole COOH- and NH₂-terminal residues, respectively, in freshly prepared pepsin.

### Table I

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<th>Amino acid</th>
<th>COOH-terminal residues in</th>
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<tr>
<td>Alanine</td>
<td>0.90</td>
<td>1.09</td>
</tr>
<tr>
<td>Valine</td>
<td>0.32</td>
<td>0.18</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.29</td>
<td>0.10</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.55</td>
<td>0.13</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.20</td>
<td>0.15</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.10</td>
<td>0.11</td>
</tr>
</tbody>
</table>

1 These residues might be expected to arise from a peptide such as Thr-Ser-P-Glu that could easily form during the cyclization procedure, which involves treatment with hot, strong mineral acid. Such an acidic peptide would appear in the hydantoin fraction, as Stark and Smyth (18) have pointed out. During the subsequent hydrolyses of the hydantoin fraction with hot 0.2 M NaOH, the peptide would be hydrolyzed and some threonine and glutamic acid would be lost, but phosphoserine would probably be almost completely destroyed owing to its elimination (31).

2 The abbreviation used is: RCM, reduced, carboxymethylated.

3 I. A. A. Doplo, unpublished experiments.

4 T. A. A. Dopheide, unpublished experiments.

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9 The abbreviation used is: RCM, reduced, carboxymethylated.
Insofar as we are aware, a carboxyl-terminal analysis of pepsinogen has never been published. It has been stated (34) that the carboxyl-terminal residue is alanine, but the citation has always been to unpublished work. It has been our experience that native pepsinogen is resistant to the action of carboxypeptidase A. If a 12-hour digest is freed of protein by acidification in the usual way, some activation of pepsinogen occurs and peptide fragments may appear as a result of peptic action, but no alanine (less than 0.05 residue) or other free amino acids can be detected upon amino acid analysis. If, however, reduced, carboxymethylated pepsinogen is treated with carboxypeptidase A, alanine is the only free amino acid found (0.73 and 1.12 eq after 8 and 16 hours of digestion, respectively). This reinforces the conclusion drawn earlier that the other amino acids liberated from commercial pepsin by carboxypeptidase A derive from other portions of the molecule and are an evidence of autodigestion. Addition of purified carboxypeptidase B to the RCM-pepsinogen from which carboxyl-terminal alanine has been removed does not liberate any lysine or arginine.

The failure of carboxypeptidase A to liberate any amino acids other than alanine from RCM-pepsinogen, suggests that the residue immediately preceding alanine at the carboxyl terminus is not sensitive to carboxypeptidase. Such would be the case if the third residue from the carboxyl terminus were proline, as is the case in the tobacco mosaic virus protein (35). Carboxypeptidase A liberates only the carboxyl-terminal threonine from that protein.

The demonstrable heterogeneity of commercial crystalline pepsin prompted us to investigate pepsin freshly prepared by the activation of pepsinogen according to the general procedure of Herriott (16, 36). Since Herriott had shown that activation proceeded optimally at pH 2, this condition was chosen for test. It was found to be important to lower the pH rapidly and to maintain it at a constant level in order to get reproducible results. For this purpose a mixture of 2 M monochloroacetic acid and HCl proved to be satisfactory. The acid should be added in a period no greater than 20 to 25 sec to the chilled, well-stirred solution of pepsinogen. After 20 min at 14°C, tests showed that pepsin activity had reached a maximum, that there was no residual pepsinogen, and that the pepsin formed was homogeneous and had not yet begun to autodigest.

Several procedures were tried in order to separate rapidly and efficiently the newly formed pepsin from the low molecular weight cleavage products that arise during activation. The work of Herriott (16, 36) has shown that these cleavage products, which are basic, form complexes with pepsin at low pH, but that these complexes tend to dissociate between pH 4 and pH 5. Passage through Sephadex G-25 at pH 4.4 did not effect complete separation, as could be shown by amino acid analysis. The protein component often had slightly more than the theoretical quantity of 1 lysine residue per molecule. When an acidic column packing was employed, however, namely sulfoethyl Sephadex C-25, the basic, low molecular weight cleavage products were tenaciously held by the resin, whereas the acidic pepsin passed rapidly through the column unretarded, as may be seen in Fig. 4. To minimize autodigestion, all operations were carried out at 4°C after the initial 20-min activation at 14°C. The basic peptides may be eluted from the column with 0.1 M NH_4OH. The yield of pepsin is over 90% of theoretical. From here on, we will refer to the enzyme obtained in this way as “freshly prepared pepsin,” to distinguish it from the commercial product. The specific activity of freshly prepared pepsin against hemoglobin is 22,500 A absorbance units at 280 μM per hour per μMole of pepsin, which is higher than the value of 17,600 found for one sample of commercial pepsin (Worthington, Lot 710). By contrast, the specific activity against acetyl-L-phenylalanyl-DL-diiodotyrosine is slightly higher (approximately 20%) for the commercial pepsin preparation. The method of determining these specific activities is described under “Experimental Procedure.”

The enzyme can be stored at -20°C in pH 4.4 buffer for at least 2 to 3 months without change in activity. No amino acid other than alanine was liberated by carboxypeptidase A from pepsin.

One commercial sample of carboxypeptidase B was tested and found to liberate substantial quantities of both arginine and lysine. It apparently was contaminated with proteinases.
stored at 20° for 1 month. Longer periods of storage have not yet been checked.

Homogeneity of Pepsin Freshly Prepared from Pepsinogen—Freshly prepared pepsin is homogeneous on both DEAE-Sephadex A-50 and hydroxylapatite, as may be seen in Figs. 5 and 6A. Moreover, the specific activity is constant across the peak. The position of the single peak on hydroxylapatite (Fig. 6A) is nearly but not quite identical with that of Peak III obtained from commercial pepsin (Fig. 1). Freshly prepared pepsin is also essentially homogeneous on end group analysis. The cyanate method applied to freshly prepared RCM-pepsin yielded 0.75 residue of isoleucine as an amino-terminal residue, and a trace (0.03) of leucine. Occasionally traces (0.01 to 0.06 residue) of glycine and alanine were also found. A sample that had not been reduced and carboxymethylated gave the same result. Freshly prepared pepsin yielded 0.94 residue of alanine as the sole amino acid on treatment with carboxypeptidase A for 15 hours. After reduction and carboxymethylation, a similar result was obtained: 0.96 residue of alanine plus a trace (0.04 residue) of valine. The freshly prepared enzyme showed no evidence of heterogeneity after reduction, carboxymethylation, and passage through Sephadex. This is in contrast to the result obtained with commercial pepsin and shown in Fig. 2.

It is important to note that the homogeneity of the product formed is critically dependent upon the pH at which activation is carried out. In Fig. 6 are shown effluent curves obtained when samples of pepsin formed by activation at pH 2, 3, and 4 are chromatographed on hydroxylapatite. Activation at pH 2 yields a single product (Fig. 6A), as has been noted before. After activation at pH 3 for 20 min, heterogeneity is beginning to be apparent (Fig. 6B), whereas the product obtained after activation at pH 4 for 30 min appears to be markedly heterogeneous (Fig. 6C). Activation slows down as the pH is raised, so that the total yield of activity after 30 min at pH 4 is 48% of theoretical. At pH 3, activation is 97% complete. The heterogeneity shown in Fig. 6C does not seem to be a result of autodigestion, because 1 eq of alanine is the only amino acid residue

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**Fig. 4.** Separation of pepsin from the peptides formed during the activation of pepsinogen. A column, 2.5 X 30 cm, of sulfoethyl Sephadex C-25 was operated at 4° with 0.4 M acetate buffer, pH 4.4, as eluent. The rate was 60 ml per hour, and fractions of 4 ml were collected. The mixture obtained by activation of 75 mg of pepsinogen was applied to the column. To elute the peptides, 0.1 M NH₄OH was employed as eluent (see top of figure).

**Fig. 5.** Chromatography of pepsin freshly prepared from pepsinogen on a column, 0.9 X 22 cm, of DEAE-Sephadex A-50. Sample, 5 mg; eluent, 0.05 M acetate buffer, pH 4.5 ± 0.05, with a gradient of NaCl concentrations from 0.2 M toward 0.5 M; rate, 8 ml per hour; 2-ml effluent fractions. The ninhydrin color values were obtained on 0.1-ml samples after alkaline hydrolysis.

**Fig. 6.** The enzymatically active components formed upon activation of pepsinogen (25 mg) at pH 2.0 (A), pH 3.0 (B), and pH 3.9 (C). The activation mixture was in each case passed first through a column of sulfoethyl Sephadex C-25 (cf. Fig. 4), and the protein fraction was placed upon a column, 0.9 X 14 cm, of hydroxylapatite operated in the manner described in the legend to Fig. 1. Phosphate buffers, pH 5.7, of the indicated molarity were introduced as eluents at the points indicated by the arrows at the top of each figure.
Duplicate analyses by ion exchange chromatography (22) were performed on 22-hour and on 48-hour hydrolysates. Unless otherwise indicated, the averages of all of these values were employed to derive the figures in Columns 2 and 3. Replicate analyses always agreed to ±3%. For the calculation of the molar ratios the average micromoles of histidine, lysine, and arginine found were taken to represent 3, 10, and 4 residues per molecule for pepsinogen and 1, 1, and 2 residues per molecule for purified pepsin.

The calculated molecular weight includes the contribution of liberated from either of the fractions seen in the figure on treatment with carboxypeptidase A.

Amino Acid Analyses—In Table II are given the results of amino acid analyses of pepsinogen, the pepsin prepared from it, and the peptide fraction liberated (second peak, Fig. 4) in the process. The protein preparations analyzed probably contained some residual N-ethylmorpholine from the gel filtration step, since the weight and nitrogen recoveries were 91% for pepsinogen and 81% for pepsin. The results do not exclude the possibility that pepsinogen and pepsin contain some ninhydrin-negative constituents in addition to 1 residue of phosphate (40); no chromatographic evidence was obtained for amino sugars.

The molar ratios of amino acids present in amounts less than 10 residues per molecule are close to integral values. The calculated difference between pepsinogen and pepsin is 42 residues; the composition of the unpurified peptide material cleaved off in the conversion is within ±1 residue of the calculated amounts of each amino acid, which is about as close agreement as can be expected when an analysis of the crude peptide fraction is compared with the difference between two large analytical values.

Commercial pepsin has recently been analyzed chromatographically by Blumenfeld and Perlmann (39), and pepsinogen, by Arnon and Perlmann (10). The present results are similar, but define with greater precision the composition of the peptides removed by cleavage in the transformation process and the compositions of purified preparations of the two proteins.

The amino acid composition in the last column of the table is also compatible with the sequence of the first 18 residues at the amino terminus of pepsinogen reported by Lokshina and Orekhovich (41). From their data and from the results in Table II, it can be expected that the next 24 amino acid residues in the pepsinogen sequence will prove to be very rich in basic amino acids.

### DISCUSSION

**Heterogeneity of Commercial, Crystalline Pepsin—**As was pointed out in the introductory section, there have been numerous scattered indications in the literature that the commercial pepsin preparations so widely used at present are inhomogeneous. The results discussed in the previous sections of this paper leave little doubt that this is the case. Since all of the protein components appear to possess pepsin activity, the simplest interpretation of all of the results is that these preparations contain a mixture of autodigestion products. It seems probable that cleavage has taken place after the first and before the last half-cystine residue in the linear sequence, so that the fragments thus produced are still held together by disulfide bonds. Since pepsin is not highly specific in its action on other proteins, it is not surprising that autodigestion occurs in several different parts of the molecule and gives rise to a complex mixture of products. It is not possible to state at present whether small peptide fragments are cleaved away as in the autodigestion of pepsinogen (42), or whether the single original chain is simply cleaved in several different places.

* Duplicate analyses of 22-hour hydrolysates were made on the peptide fraction.
* The average 22- and 48-hour values for threonine, serine, and tyrosine have been extrapolated to zero time to correct for destruction during hydrolysis (37).
* Half-cystine and methionine were determined as cysteic acid and methionine sulfoxone on the performic acid-oxidized protein (38). A 150-cm ion exchange column was used for the complete resolution of methionine sulfoxone and aspartic acid (27).
* The average 48-hour values were taken for valine and isoleucine.
* The tryptophan value is taken from the data of Arnon and Perlmann (10) and Blumenfeld and Perlmann (39).
* An approximate value for the amide NH3 of pepsin was derived with carboxypeptidase A.
* The calculated molecular weight includes the contribution of 1 phosphate residue on serine (40). The values in this table are to be compared with the ultracentrifugally determined values of similarly prepared samples, as described in the accompanying paper (13): pepsinogen, 40,000 ± 1,200; pepsin, 32,700 ± 1,200.
* The nitrogen contents found by Kjeldahl analysis were 15.0% for pepsinogen and 14.7% for pepsin.
It is instructive to inquire into the sources of the heterogeneity of commercial samples of the enzyme. Although pepsin is derived from pepsinogen in vivo, this is not true of the commercially available preparations. Commercially, the enzyme is made by the procedure of Northrop (43), which starts from 1:10,000 pepsin. The 1:10,000 material is made from autolyzed hog stomachs. The gastric mucosa is exposed for many hours, possibly days, to mild hydrochloric acid and incubated at about 50°C for several hours. The digestion mixture is allowed to settle, and the clear supernatant liquid is chilled to about 4°C and concentrated under reduced pressure. The mucin is precipitated with alcohol and removed, after which the pepsin is precipitated by the addition of more alcohol. The pepsin is filtered off and vacuum-dried.

It is clear that during this procedure the pepsinogen in the hog gastric mucosa is exposed for many hours, possibly days, to mild acidity, a condition known to promote autodigestion of the pepsin formed. Thus, there seems little doubt that the starting material used in the Northrop procedure contains a mixture of active enzymatic species of similar properties, and it is not surprising that a single homogeneous product is not readily obtained from it. Similar problems were encountered in earlier years in the preparation of other proteolytic enzymes, such as trypsin and chymotrypsin, and perhaps one of the major items of novelty in the present findings is that they have not been reported before.

It should be pointed out that Stepanov et al. (5-7) have found multiple end groups in pepsin, but they have interpreted their findings as an indication that autodigestion was proceeding at pH 8 during the course of the end group determinations. In view of the results presented in this communication, it is difficult to escape the conclusion that the autodigestion, which Stepanov assumed had occurred in the course of the experimental manipulations, had actually taken place during the preparation of the enzyme, as has been suggested above. Thus, while we agree that autodigestion is responsible for the multiplicity of end groups, we disagree as to when the autodigestion occurred.

It will be of interest to learn to what extent the properties of pepsin freshly prepared from pepsinogen differ from those of the commercial product. For example, Dopheideg has found that the rate of inactivation at pH 6.2 of freshly prepared pepsin differs from that of commercial pepsin or of the Peak I and Peak II fractions (Fig. 1) derived from it. Early in the present investigation it was noted that commercial pepsin could be about 50% inactivated by treatment at pH 5.9 with 0.4 M cyanate. Freshly prepared pepsin is virtually unaffected by cyanate, however.

One of the practical consequences of the finding that commercial pepsin is a mixture of autodigested species is that this material is not suitable for structural studies. An unbroken peptide chain is needed, and this can most easily be obtained by working with pepsinogen or with pepsin freshly derived from it. This too is reminiscent of the experience with trypsin and chymotrypsin. In the work which led to the elucidation of the structure of these enzymes, the zymogens were studied, not the commercially available active enzymes.

If the supposition is correct that, in commercial pepsin, peptide bonds in the more central portions of the peptide chain have been cleaved, this indicates that a single intact polypeptide chain is not essential for activity. It suggests, in addition, that a more controlled proteolytic cleavage of an intact peptide chain might yield useful information as to the relationship between structure and function, just as has been true in the study of other enzymes, notably ribonuclease (cf. Reference 44 for references).

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