Pepsinogen B: the Zymogen of Pepsin B

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1. Pepsinogen B, the precursor of pepsin B, has been isolated by ion-exchange chromatography and gel filtration from neutral extracts of pig gastric mucosa. The material possesses potential activity against acetyl-L-phenylalanyl-L-di-iodotyrosine and against gelatin but has little, if any, potential activity against haemoglobin. 2. The material appears homogeneous in the ultracentrifuge, but on gel filtration and on electrophoresis in starch gel it is shown to be contaminated with a small amount of material having potential activity against haemoglobin. On electrophoresis in starch gel also the material is shown to contain about equal amounts of two major components, both of which have potential activity against the synthetic substrate. Pepsin B has also been shown to contain two active components by electrophoresis under the same conditions. 3. The zymogen is similar to pepsinogen and pepsinogen C in its molecular weight and general physico-chemical properties, but differs from these zymogens in the nature of its *N*-terminal residues. It is possible that one of the components contains 1 mole of bound phosphate/mole. 4. The material is activated rapidly at pH 2 and more slowly at pH 4. At both pH values the kinetics of the activation reaction are complex.

The Report of the Commission on Enzymes of the International Union of Biochemistry (1961; London: Pergamon Press Ltd.) recommends (p. 114) that the enzymes formerly known as parapepsins (EC 3.4.4.2) (Ryle & Porter, 1959) should be renamed pepsin B. Since there are two separate parapepsins it is intended to rename parapepsin I pepsin B and parapepsin II pepsin C. The name pepsin A has already been applied by Herriott, Desreux & Northrop (1941) to a highly active enzyme they obtained by fractional crystallization procedures. The term pepsin B has been used by Baker (1954) to refer to a fraction of a pepsin preparation that differed from the starting material and from pepsin A in some of its properties.

This material has not been further characterized and it seems simplest to follow, as far as possible, the published recommendations of the Commission and use pepsin B for parapepsin I. Some properties of the enzymes and zymogens are listed in Table 1.

The isolation of the zymogen of pepsin C, one of the minor proteolytic components of crude porcine pepsin, has already been reported (Ryle, 1960). This zymogen was obtained from the fraction of the gastric mucosa that could be extracted with 0.45 saturated ammonium sulphate but precipitated by 0.68 saturation. No evidence was found during the chromatographic purification of pepsinogen C for the presence of a zymogen corresponding to pepsin

Table 1. *Some properties of the gastric proteases and zymogens isolated by chromatography*

Abbreviations: Hb, haemoglobin; ALPLD, acetyl-L-phenylalanyl-L-di-iodotyrosine.

Enzyme ...	Pepsin B (parapepsin I)	Pepsin C (parapepsin II)	Pepsin D	Pepsin
Order of elution from DEAE-cellulose at pH 4.0	First	Second	?	Third
Substrate for assay	ALPLD	Hb	Hb and ALPLD	Hb and ALPLD
Zymogen ...	Pepsinogen B	Pepsinogen C	Pepsinogen D	Pepsinogen
Order of elution from DEAE-cellulose at pH 6.9	First	Fourth	Second	Third
Substrate for assay	ALPLD	Hb	Hb and ALPLD	Hb and ALPLD

B, although it seemed likely that such a protein was produced by the mucosa. Chromatographic examination of the material precipitated by 0.68 saturated ammonium sulphate showed that only pepsinogen and pepsinogen C were present, but extracts made with dilute buffer of the tissue residue that was insoluble in 0.45 saturated ammonium sulphate contained not only these zymogens but also what appeared to be the zymogen of pepsin B. The present paper describes the purification and characterization of pepsinogen B, which is more simply prepared from extracts of the mucosa made with dilute buffer in the first place.

Preliminary accounts of part of this work have appeared (Ryle, 1961, 1963).

EXPERIMENTAL

Buffer solutions. Except where otherwise stated buffer solutions were made from sodium salts and their concentrations are given with respect to the total concentration of the buffering component.

Materials. Haemoglobin (bovine haemoglobin enzyme substrate powder) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

Gelatin (lot 141) was obtained through the generosity of Mr A. G. Ward of the British Gelatine and Glue Research Association and had an isoelectric point of pH 5.0.

Acetyl-DL-phenylalanyl-L-di-iodotyrosine was in part a gift from Mrs R. V. Pitt-Rivers, National Institute for Medical Research, Mill Hill, London, N.W.7, and in part a preparation made in this Laboratory.

Acetyl-L-phenylalanyl-L-di-iodotyrosine was prepared by the method described by Baker (1951) and had m.p. 231° (decomp.), $[\alpha]_D^{25} + 30.5^\circ$ (c 2 in pyridine).

DEAE-cellulose was prepared as described by Peterson & Sober (1956) and had about 1 m-equiv. of basic groups/g.

Hydrolysed starch for use in electrophoresis experiments was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

Acid phosphatase (potato) was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. (lot H 1748). The soluble part of this material was subjected to chromatography on CM-cellulose at pH 4.5 and two fractions with activity against *p*-nitrophenyl phosphate were found: phosphatase A (unretarded) and phosphatase B (eluted with a gradient of increasing ionic strength). These two accounted for most of the soluble protein of the preparation.

Methods

Activity determination with haemoglobin. Assays of activity against haemoglobin were made in duplicate at pH 1.7 by the method, based on that of Northrop, Kunitz & Herriott (1948), that was used in previous work (Ryle, 1960). Since no detectable difference was found in the extent of digestion of the haemoglobin whether the pepsinogen or pepsinogen C solutions were activated by preincubation in acid solution before the addition of the haemoglobin or whether the acid haemoglobin solution was added directly to the solution of the zymogen, this latter simpler procedure was used.

The increases in extinction of the deproteinized solutions at 280 m μ were converted into proteolytic units (haemoglobin substrate), designated [P.U.]^{Hb}, by reference to standard curves prepared for purified pepsin or pepsin C.

Activity determinations with acetylphenylalanyldi-iodotyrosine. In the earlier stages of this work acetyl-DL-phenylalanyl-L-di-iodotyrosine was used as substrate as described previously (Ryle, 1960), but later, when it became necessary to use a new preparation of this compound, it was found that the enzymes digested the new preparation less readily than the old. The diastereoisomeric substrate is not crystalline and apparently contains some inhibitory impurities. The extent of digestion found with this substrate was converted into proteolytic units (acetyl-DL-phenylalanyl-L-di-iodotyrosine substrate), designated [P.U.]^{APD}, by reference to standard curves prepared for each enzyme and zymogen. In the later stages of the work crystalline acetyl-L-phenylalanyl-L-di-iodotyrosine was used as substrate and the method of assay was simplified by including a 10 min. period for activation of the zymogen before the substrate was added. This preincubation did not affect the activity determined. Both activation and assay were performed at 35.5°. To 0.5 ml. of solution containing zymogen or enzyme was added 0.25 ml. of HCl of such concentration as to give a final pH of 2.0 in the digestion mixture (0.088 N-HCl when the zymogen was dissolved in 0.02 M-phosphate buffer, pH 6.9). After 10 min. 0.25 ml. of 0.001 M-acetyl-L-phenylalanyl-L-di-iodotyrosine in 0.01 N-NaOH was added, followed 20 min. later by 1.0 ml. of the modified ninhydrin reagent of Moore & Stein (1954). All assays were carried out in duplicate, together with duplicate blank reactions in which the ninhydrin reagent was added to the acidified solution at the end of the 10 min. preincubation period and the substrate later. The colour was then developed in the usual way and the mean increment in E_{570} determined. These values were found to be proportional to the amount of enzyme or zymogen taken, up to the amount that liberates 0.1 μ mole of di-iodotyrosine, and the rate of digestion is constant over the first 20 min. for the highest amounts of enzyme or zymogen used in the assays. Activities are therefore expressed as μ moles of di-iodotyrosine liberated/min., and for pepsinogen B 1.0 milli-[P.U.]^{APD} corresponds to a liberation of 6.8 μ moles/min. with acetyl-L-phenylalanyl-L-di-iodotyrosine as substrate. An experimentally determined value of 22.8 was taken for the millimolar extinction coefficient (related to di-iodotyrosine) for the colour formed by reaction of di-iodotyrosine with the ninhydrin reagent.

Extraction of zymogens from gastric mucosa. The zymogens were extracted by a modification of the method of Herriott (1948b) in which the extract was made with dilute buffer solution. All operations were performed at 2–5°. Pig stomachs obtained fresh from the slaughter-house were transported to the laboratory on ice, and the mucosa from the dark body region (sometimes called fundus) was separated, minced and stirred for 2 hr. or more with 4 vol. of 0.02 M-phosphate buffer, pH 6.9, in the presence of toluene or of penicillin (5 units/ml.) and streptomycin (50 μ g./ml.) to inhibit bacterial growth. Filter-Cel (100 g./l. of buffer) and Hyflo Super-Cel (50 g./l. of buffer) were then stirred in and the liquid was separated by decantation, centrifugation and filtration through a bed of Hyflo Super-Cel to yield a reddish, slightly cloudy solution. The potential activity of this solution against haemoglobin was determined; it was not possible to determine the potential activity against

acetyl-DL-phenylalanyl-L-di-iodotyrosine because of the very high blank values.

Chromatography of the mucosal extract. The slimy solution obtained as above ran very slowly into columns of DEAE-cellulose, and it was expedient to adsorb the zymogens on to the ion-exchanger before packing the column. A measured volume of DEAE-cellulose, equilibrated with and suspended in 0.02M-phosphate buffer, pH 6.9, was added to the mucosal extract, stirred for 30 min. and centrifuged off. The residual haemoglobin-digesting activity of the supernatant was then determined, and the supernatant was treated with a calculated quantity of the DEAE-cellulose suspension sufficient to adsorb 120% of the residual zymogen. In this way the potential activity of the solution was decreased from an initial value of about 60 milli-[P.U.]^{ab}/ml. to less than 0.1 milli-[P.U.]^{ab}/ml. The DEAE-cellulose with the zymogens adsorbed was then washed by centrifugation with 0.02M-phosphate buffer, pH 6.9, and poured, suspended in the same buffer, on to the top of a column of DEAE-cellulose (10 cm. $\times$ 5 cm.) equilibrated with the same buffer. The total height of the column was then usually about 20 cm. The column was eluted with an exponential gradient obtained by passing a solution 0.02M with respect to phosphate buffer, pH 6.9, and 0.45M with respect to NaCl into a 2 l. mixing vessel containing 0.02M-phosphate buffer, pH 6.9. The buffers were kept saturated with toluene. The extinction of the fractions (50 ml.) at 280 m μ was determined as a measure of protein concentration, and the potential enzymic activities of suitable fractions were determined.

Rechromatography of fractions containing pepsinogen B. The fractions from the leading edge of the main pepsinogen peak in which the activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine was considerably greater than that against haemoglobin were pooled and concentrated. The concentrated solution was then dialysed against 0.02M-phosphate buffer, pH 6.9, to remove NaCl and run on to the top of a column (2.4 cm. $\times$ about 20 cm.) of DEAE-cellulose prepared in the same buffer. The column was then eluted as before, but since the column was narrower the gradient along the column was correspondingly less steep. The fractions containing the pepsinogen B were pooled, concentrated and dialysed as before, and chromatographed for a third time under the same conditions as for the second chromatogram. Appropriate fractions from these chromatograms were pooled and concentrated.

Gel filtration of zymogen. Material obtained by the chromatographic procedure described above was further purified by passage through columns (about 2.4 cm. $\times$ 40 cm.) of Sephadex G-100 or G-200, medium grade (Pharmacia, Uppsala, Sweden), in 0.02M-phosphate buffer, pH 6.9, containing NaCl (0.45M) to decrease electrostatic interaction between the zymogen and the material from which it was to be separated. Suitable fractions from these columns were pooled, dialysed against distilled water to remove salts and freeze-dried.

Concentration of zymogen solutions. Solutions of the zymogen were concentrated when necessary either by dialysis in Visking tubing (24/32 in.) against polyethylene glycol, mol.wt. 20000 or 15000 (L. Light and Co. Ltd., Colnbrook, Bucks.), or by ultrafiltration under 60 cm. Hg pressure through Visking tubing (24/32 in.) supported by a sleeve of silk net. Since some material from the polyethylene glycol was found to enter the dialysis sac, and since this material does not completely separate from the proteins on

Sephadex columns although it is without observable effect on the potential activity of the zymogen, the concentration steps after the last chromatography of a preparation were always performed by the rather slower ultrafiltration method.

Determination of total nitrogen. Nitrogen was determined by the micro-Kjeldahl procedure on samples containing about 0.2 mg. of N.

Determination of phosphate. Phosphate was estimated by a modification (T. S. Work, personal communication) of the method of Allen (1940), in which the protein is digested with 2.0 ml. of 2N-H₂SO₄ and 2 drops of 60% (w/w) perchloric acid and after hydrolysis of pyrophosphate is made to 5 ml. with 1 ml. of the amidol-bisulphite reagent [1% (w/v) amidol in 20% (w/v) sodium metabisulphite], 1 ml. of 3.3% (w/v) ammonium molybdate and water. Because the amounts of protein available in these studies were limited the method was scaled down to a final volume of 3 ml.

It was hoped that bivalent sulphate ions would successfully displace phosphate ions, which appeared to be rather strongly bound to the protein, and this exchange was attempted by gel filtration. Samples of pepsinogen B (10 and 42 mg. in two experiments), dissolved in 3 ml. of 0.1M-phosphate buffer, pH 6.9, were applied to columns (1.2 cm. $\times$ 40 cm.) of Sephadex G-25 packed in 0.1M-Na₂SO₄ adjusted to pH 6.9. The columns were eluted with the same solution and the protein emerged with a maximum at 22 ml. and the phosphate at 74 ml. There was no overlap of the peaks of protein and phosphate.

Determination of N-terminal amino acid residues. The techniques used were largely those described by Porter (1957). Weighed samples of the protein were allowed to react with 2,4-dinitrofluorobenzene at pH 8 or 9 in aqueous solution at 40°, the pH being held constant by the addition of dilute NaOH controlled by a Radiometer (Copenhagen, Denmark) titrator type TTT 1a. Correction for breakdown of the DNP-amino acids was made by control experiments in which the DNP-amino acid was hydrolysed in the presence of DNP-protein. The ether-soluble DNP-amino acids were separated by chromatography on paper with 2-methylbutan-2-ol (Blackburn & Lowther, 1951) and estimated spectrophotometrically. Where necessary the identification was confirmed by chromatography on paper in phosphate buffer (Levy, 1954). The DNP-amino acids that were not extracted with ether were extracted with ethyl acetate and separated by chromatography on Whatman no. 4 paper after 2 hr. equilibration with the solvent system 'isoamyl alcohol'-pyridine-water (7:7:6, by vol.), which was described by Baglioni (1961) for the chromatography of peptides. (The alcohol used here was British Drug Houses laboratory reagent 'isoamyl alcohol' and was described as 2-methylbutan-1-ol with 15-30% of 3-methylbutan-1-ol.) The chromatograms were allowed to develop for about 5 hr., and good separations of standard DNP-amino acids were obtained with less tailing than is found with several other systems.

Electrophoresis in starch gel. This was performed in horizontal gels by the method of Smithies (1955) in 0.02M-phosphate buffer, pH 6.9. The protein sample (0.25-0.5 mg.) was applied in 0.01 ml. buffer to a segment (0.5 cm. $\times$ 0.9 cm.) of Whatman no. 3MM filter paper that was inserted into a cut in the gel. The potential gradient was measured at the start of the run by inserting probe electrodes, attached to a voltmeter, into the gel. The power was supplied by a Vokam

2541 unit (Shandon Scientific Co. Ltd., London, W.10), run at constant voltage. After the electrophoresis the gel was sliced and part was stained for protein or peptides by the modification of the method of Reindel & Hoppe (1954) described by Ryle & Porter (1959). Since in this method as originally applied the citrate buffer was essential to reduce the excess of chlorine and prevent the formation of a dark background, sliced gels that contained phosphate buffer were soaked for 30 min. in 0.05 M-citrate buffer, pH 5.6, and blotted lightly before the chlorine treatment. The rest of the gel was available for detection of potential enzymic activity by three methods. Activity against gelatin was detected by soaking the gel for 30 min. in 0.05 N-HCl and then laying it on an exposed and developed photographic plate that had been similarly soaked for 5 min. After incubation for about 30 min. at room temperature, the zones of digestion of the gelatin became apparent on gently washing the plate in cold water. Activity against acetyl-L-phenylalanyl-L-di-iodotyrosine was detected by laying on a similarly acidified gel a piece of Whatman no. 1 filter paper wetted with 0.01 M-acetyl-L-phenylalanyl-L-di-iodotyrosine in 0.02 N-NaOH. After incubation for 1 hr. at 30° the paper was removed, dried in air and stained by dipping in 0.25% ninhydrin in ethanol containing 1% (v/v) of pyridine, and the colour was allowed to develop at room temperature. Activity against haemoglobin was detected by a similar technique suggested by Dr I. Kushner; the slice of gel was not acidified, but covered with a strip of Whatman no. 3MM filter paper wetted with 2% haemoglobin-0.14 N-HCl. After 45 min. at room temperature the paper was removed and the gel was stained with Naphthalene Black 10B (G. T. Gurr Ltd., London, S.W.6) in the usual fashion. The enzymes or zymogens are detected as white areas on the blue ground, and the method permits the detection of less than 5 μ g. of pepsinogen.

Treatment of zymogens with potato phosphatases. Two phosphatases (phosphatases A and B) were separated from commercially obtained potato phosphatase (see the Materials section). One unit of phosphatase is defined as the amount of enzyme that caused a rate of liberation of 1 μ mole of *p*-nitrophenol/min. from 1 mm-*p*-nitrophenyl phosphate in acetate buffer, pH 5.0 and 10.1, containing MgCl₂ (5 mM) at 37°. The zymogen (25 mg./ml.) was incubated with phosphatase A (0.2 unit/ml.) and phosphatase B (0.12 unit/ml.) in 0.1 M-acetate buffer, pH 5.6, containing MgCl₂ (0.01 M), at 35° for 28 hr. Samples of the incubation mixture were applied directly to segments of Whatman no. 3MM paper for electrophoresis in starch gel beside samples of the zymogen that had been similarly incubated in the absence of phosphatase.

Mixed chromatogram of pepsin B and activated pepsinogen B. A portion (12.8 mg.) of pepsinogen B was dissolved in 5 ml. of water and adjusted at 35.5° to pH 2.0 by the addition of 0.068 ml. of 1.14 N-HCl. After 5 min. the pH was raised by the addition of 0.25 ml. of 2.0 M-acetate buffer, pH 5.6. This solution was then mixed with 2 ml. of 0.1 M-acetate buffer, pH 5.6, containing 5.5 mg. of pepsin B, and the whole was chromatographed on DEAE-cellulose at pH 5.6.

Progress of activation of pepsinogen B at pH 2.0 and 4.0. The progress of the conversion of the zymogen into the enzyme was followed by making use of the fact that the enzyme can be assayed by its activity in lowering the viscosity of gelatin solutions at pH 5.0, whereas the zymogen has no activity and is only very slowly converted into the

enzyme at this pH. For the studies at both pH values the zymogen was dissolved in NaCl solution calculated to give a final ionic strength of 0.09 and final zymogen concentration of 0.05 mg./ml. The pH was then adjusted by the addition of acid (0.011 vol. of 1.0 N-HCl for pH 2.0 and 0.00105 vol. of 0.1 N-HCl for pH 4.0). There was no change of pH of the reaction at pH 2 in 1080 min. The reaction at pH 4 was maintained at that pH by the occasional addition of 0.05 N-HCl: 0.043 ml. was required over the first 5 hr., after which no further additions were made. At 11.5 hr. the pH had fallen to 3.92.

Samples were removed from the activation mixtures at suitable times and adjusted to pH 5.6 to stop the activation, so that determinations of the gelatin-liquefying activity could be made later. The samples from the pH 2 solution were added to 4 vol. of acetate buffer whose composition was such that the final mixture contained 0.1 M-acetate buffer, pH 5.6; the samples from the pH 4 solution were added to 1 vol. of 0.2 M-acetate buffer, pH 5.6.

Activity determination with gelatin. Since it was necessary in some experiments to use quite large volumes of partly activated zymogen the method used by Ryle & Porter (1959) was slightly modified. To 7.5 ml. of 5% (w/w) gelatin and 0.6 ml. of 0.29 N-HCl a total of 7.5 ml. of 0.1 M-acetate buffer, pH 5.6, containing the enzyme or zymogen was added. The viscosity of the mixture, of pH 5.0, was then determined at 2 min. intervals over a period from 4 to 12 min. after mixing in an Ostwald viscometer (t_{H_2O} 26.5 sec.) at 35.5°. One unit of gelatin-liquefying activity, designated [P.U.]^{Gel.}, is the amount of enzyme that causes a 1% fall in the reduced viscosity of 5.2 ml. of the final mixture/min. The assay differs from that used before in that the final mixture contains a higher concentration of salt.

RESULTS

Extraction and chromatography of zymogens. The crude mucosal extracts usually contained about 10 [P.U.]^{MB} of potential activity/stomach taken, although occasionally the amount was half or twice this. These variations in amounts could not be correlated with variations in the time of extraction or the method of separation of the extract from the insoluble material, and they may represent variations in the zymogen contents of the mucosae of the pigs.

Fig. 1 shows the chromatography of the zymogens from 12 mucosae. The peak at 4.5 l. effluent volume, which has potential activity against haemoglobin but not against acetyl-DL-phenylalanyl-L-di-iodotyrosine, contains pepsinogen C. The main peak at 2.9 l., with potential activity against both substrates, is largely pepsinogen, for which the activity measured with the two substrates should be numerically the same. However, at the leading edge of this peak it is apparent that the ratio of the potential activities against the two substrates is not unity; there is an excess of potential activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine. The fractions from this region of the chromatogram were pooled,

Fig. 1. Chromatography of gastric mucosal extract. Crude zymogens from 12 body mucosae were applied to a column (20 cm. $\times$ 5 cm.) of DEAE-cellulose in 0.02 M-phosphate buffer, pH 6.9. The gradient was 0–0.45 M-NaCl, the mixing-vessel volume being 21. The flow rate was approx. 50 ml./hr. $\circ$ — $\circ$, Potential activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine; $\bullet$ — $\bullet$, potential activity against haemoglobin; —, E_{280} ; ----, calculated concn. of chloride in the effluent. The arrow shows the point of application of the gradient, and the bar the fractions pooled for further treatment.

Fig. 2. Rechromatography of crude pepsinogen B. Material from the first chromatogram (Fig. 1) was applied to a column (21 cm. $\times$ 2.4 cm.) of DEAE-cellulose in 0.02 M-phosphate buffer, pH 6.9. The gradient was 0–0.45 M-NaCl, the mixing-vessel volume being 21. The flow rate was approx. 22 ml./hr. $\circ$ — $\circ$, Potential activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine; $\bullet$ — $\bullet$, potential activity against haemoglobin; —, E_{280} ; ----, calculated concn. of chloride in the effluent. The bar shows the fractions pooled for further treatment.

concentrated and dialysed, and subjected to rechromatography as shown in Fig. 2.

On this chromatogram with a less-steep gradient of sodium chloride concentration the third peak showing potential activity, emerging at 2.2 l., contains material that does not separate on mixed chromatograms from material taken from the trailing edge of the pepsinogen peak on chromatograms like that shown in Fig. 1, and is therefore pepsinogen. (The high ratio of potential activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine to that against haemoglobin seen in this peak was not usually observed in such chromatograms and may be spurious.) The peak emerging at about 1.6 l. shows potential activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine but none against haemoglobin, and contains the pepsinogen B. The middle peak, which contains material that separates from pepsinogen on mixed chromatograms, represents a new zymogen, pepsinogen D. Pepsinogens C (Fig. 1), B and D (both Fig. 2) account for about 12, 4 and 6% respectively of the total zymogen. as estimated from the sizes of the chromatographic peaks.

Fig. 3 shows the fractionation by gel filtration on Sephadex G-100 of pepsinogen B obtained from chromatograms such as that shown in Fig. 2. It is apparent that some material of high molecular size

Fig. 3. Gel filtration of pepsinogen B on Sephadex G-100. The pepsinogen B (16 [P.U.]^{APD}) was applied to a column (43.5 cm. $\times$ 2.2 cm.) in 0.02 M-phosphate buffer, pH 6.9, containing NaCl (0.45 M). The flow rate was approx. 20 ml./hr. $\circ$ — $\circ$, Potential activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine; $\bullet$ — $\bullet$, potential activity against haemoglobin; —, E_{280} . The bars ('1st cut' and '2nd cut') show the fractions pooled for further treatment.

is separating from the zymogen, but the skewed shape of the curve suggests that there is some interaction between the zymogen and the inactive material in spite of the high sodium chloride concentration in the eluting buffer. Although the presence of

toluene in the eluting buffer has made it impossible to determine accurate E_{260}/E_{280} ratios, the inactive material on these Sephadex columns does show a high extinction at $260m\mu$ and may be partly nucleic acid. Fig. 3 shows that the peak of potential activity against haemoglobin does not coincide with the

Fig. 4. Final chromatography of pepsinogen B. The pepsinogen B (238 mg.) was applied to a column (25 cm. $\times$ 2.4 cm.) of DEAE-cellulose in 0.02 M-phosphate buffer, pH 6.9. The gradient was 0.1–0.45 M-NaCl, the mixing-vessel volume being 2 l. $\circ$, Potential activity against acetyl-L-phenylalanyl-L-di-iodotyrosine (the curve has not been drawn in); $\bullet$, potential activity against haemoglobin; $\triangle$ — $\triangle$, ninhydrin reaction; —, E_{280} ; ----, calculated concn. of chloride in the effluent. The bar shows the fractions pooled for further treatment.

major peak of extinction, suggesting that the low potential activity present is not, or is not all, an intrinsic property of pepsinogen B but may be attributed to some other protein.

The material from the first cut shown in Fig. 3 (90–110 ml.) was refractionated on a column (49 cm. $\times$ 2.2 cm.) of Sephadex G-200, medium grade, with the same eluent as used above, and separation of zymogen from inactive material about as useful as that on Sephadex G-100 was obtained.

The material from the second cut from the Sephadex G-100 column was examined in the ultracentrifuge in phosphate buffer, pH 6.9 and 7.0, and the sedimentation pattern showed the presence of about 10% of heterogeneous material sedimenting more slowly than the major component. The material from the Sephadex G-100 and G-200 columns was combined and resubjected to chromatography in the hope of separating the slowly sedimenting material, and particularly the potential activity against haemoglobin, from the pepsinogen B. This chromatogram is shown in Fig. 4. Although some ninhydrin-positive material with little extinction at $280m\mu$ was removed, no separation of the potential activities against haemoglobin and acetyl-L-phenylalanyl-L-di-iodotyrosine was achieved. The pooled material from this chromatogram was concentrated by ultra-filtration and finally fractionated on a column of Sephadex G-200 (Fig. 5) on which a single almost symmetrical peak of extinction at $280m\mu$ was

Fig. 5. Final gel filtration of pepsinogen B. The pepsinogen B (potential activity 35.4 μ mole of di-iodotyrosine/min.) was applied to a column (49 cm. $\times$ 2.2 cm.) of Sephadex G-200 in 0.1 M-phosphate buffer, pH 6.9. The 10 ml. fractions were collected at a flow rate of approx. 5 ml./hr. $\blacktriangle$, E_{280} ; $\circ$, potential activity against acetyl-L-phenylalanyl-L-di-iodotyrosine; $\bullet$, potential activity against haemoglobin; $\triangle$, ninhydrin reaction; $\square$, concn. of chloride. The bar shows the fractions pooled for further investigation.

obtained, showing nearly constant ratios of potential activity against acetyl-L-phenylalanyl-L-di-iodotyrosine to E_{280} and to ninhydrin-reactivity across the whole peak; although, as on the Sephadex G-100 column (Fig. 3), the peak of potential activity against haemoglobin does not coincide with that against acetyl-L-phenylalanyl-L-di-iodotyrosine so that the activity against haemoglobin must be due, at least in part, to a contaminant. The material of the peak was pooled, dialysed against distilled water and freeze-dried (105 mg.) and has been used for all the final studies reported in the present paper, except the mixed chromatography with pepsin B.

Throughout this fractionation the pH was maintained at 6.9 to minimize the risk of inactivation or partial activation of the zymogen, which is stable in solution at this pH as judged by its potential activity. The recovery of activity from the chromatographic columns was generally about 80% and from Sephadex 100%.

Sedimentation and diffusion studies. In the preliminary communication (Ryle, 1961) data were given for a sample of pepsinogen B that had not been purified by gel filtration: S_{20} 3.17s and D_{20} 5.40×10^{-7} cm.²/sec., leading to mol. wt. 57 200. The more highly purified preparation obtained in the present work has been re-examined by Dr H. J. Cruft. A single sedimentation run was performed with a 1.3% solution in phosphate buffer, pH 6.9 and 1.0. The material gave a single symmetrical peak with sedimentation coefficient 2.88s. Correction of this value for the concentration-dependence previously determined gave $S_{20,w}^0$ 2.99s.

The diffusion coefficient was determined in a Tiselius electrophoresis apparatus at 20° over a period of 25 hr. in the same buffer as was used for the ultracentrifuge experiments. The distribution curve at the end of this time was normal and the determined value for $D_{20,w}$ was 7.40×10^{-7} cm.²/sec. Taking an assumed value for the partial specific volume of 0.75 the molecular weight obtained is 39 000. This value is very much less than that previously reported, largely because of the much greater value for the diffusion coefficient now obtained. The difference in the diffusion behaviour of the two samples of the zymogen suggests that under the conditions of the experiment there is some interaction between the zymogen and some other material that was removed by gel filtration.

Electrophoresis in starch gel. Despite the near-homogeneity of pepsinogen B as judged by its behaviour on chromatography, gel filtration and ultracentrifugation, electrophoresis in starch gel at pH 6.9 (Fig. 7) or 5.6 showed the presence of two major components detectable by the chlorine method. Both components showed potential activity against acetyl-L-phenylalanyl-L-di-iodotyrosine and against gelatin, and it thus appears that pep-

Fig. 6. Rechromatography of pepsin C and some pepsin B. The material (10 [P.U.]^{Hb} and 4.6 [P.U.]^{APD}) was applied to a column (43 cm. $\times$ 2.4 cm.) of DEAE-cellulose in 0.1 M-acetate buffer, pH 4.0. The gradient was 0.0–0.2 M-NaCl, the mixing-vessel volume being 1.5 l. ○, Activity against acetyl-L-phenylalanyl-L-di-iodotyrosine; ●, activity against haemoglobin; —, E_{280} .

sinogen B occurs in two forms. The haemoglobin-digesting activity of the preparation was found as a diffuse zone ahead of the major components, but behind control samples of pepsinogen (which appeared as two barely separated bands) or pepsinogen D. The amount of this activity was judged to be the equivalent of pepsinogen equal to more than 1% but less than 10% of the weight of the pepsinogen B.

Heterogeneity of pepsin B. Ryle & Porter (1959) found that pepsin B migrated as a single component in starch gel at pH 5.6 and that it appeared homogeneous on chromatography and in the ultracentrifuge. Some evidence that pepsin B contains more than one component has, however, been obtained. Fig. 6 shows the result of an experiment in which some pepsin C that was known to be contaminated with pepsin B was chromatographed at pH 4.0, but with a less-steep salt gradient than that which had been formerly used. There is clear evidence of partial separation of the pepsin B into two active components, the peaks at 1.15 and 1.27 l.

Two components were also demonstrated by electrophoresis in starch gel in 0.02 M-phosphate buffer, pH 6.9. After electrophoresis at 3.3 v/cm. for 18 hr., staining by the chlorine method revealed two bands for pepsin B, at 18.2 and 18.8 cm. from the origin in the direction of the anode. Both bands were also detected by their activity against acetyl-L-phenylalanyl-L-di-iodotyrosine and against gelatin.

Specific activity of pepsinogen B. Pepsinogen B has a specific potential activity of 1.84μ moles of di-iodotyrosine liberated/min./mg. of N at 35.5° with acetyl-L-phenylalanyl-L-di-iodotyrosine as substrate. This corresponds to 0.27 [P.U.]^{APD}/mg. of N, which is higher than that (0.22 [P.U.]^{APD}/mg. of

N) previously reported (Ryle, 1961) for material that had not been purified by gel filtration. The specific activity of pepsin B measured with the two substrates on the same preparation of enzyme was 0.33 [P.U.]^{APD}/mg. of N and 1.8₆ μ moles of di-iodotyrosine liberated/min./mg. of N at 35.5°. The ratio [P.U.]^{APD}/ μ mole of di-iodotyrosine liberated/min. is thus 0.15 for the zymogen and 0.18 for the enzyme. It is probable that the inhibitory compounds present in the preparation of acetyl-DL-phenylalanyl-L-di-iodotyrosine used (see the Experimental section) inhibit not only the activity of the enzyme but also the process of activation itself, resulting in a lower specific activity for the zymogen when acting on the diastereoisomeric substrate. Since the molecular weight of the enzyme is 38 600 (Ryle & Porter, 1959), close to that of the zymogen, the similarity of the specific activity of the enzyme and specific potential activity of the zymogen indicates the same molecular (potential) activity for the two, unless there should prove to be a large difference in their nitrogen contents.

N-Terminal analysis. Analyses for the N-terminal amino acid residues in the zymogen were carried out by the fluorodinitrobenzene method on two samples of the purified material. One sample (10 mg.) was allowed to react at pH 8.0 and 40° for 6 hr. after heating at 100° for 15 min. to denature the protein and render its terminal groups more accessible to the reagent. A second sample (20 mg.) was allowed to react with fluorodinitrobenzene at pH 9.0 for 4 hr. and in this case the DNP-protein was isolated and washed with ethanol before hydrolysis. The results of these N-terminal analyses are shown in Table 2. Although, as with the related enzymes and zymogens, it is difficult to obtain protein free from adventitious amino groups, probably due to adsorbed peptides, it seems likely that the preparation contains two proteins in about 3:1 molar ratio. This result is not incompatible with the finding of two active components on electrophoresis in starch gel.

Phosphorus content of pepsinogen B. Since pepsin (Northrop, 1948) and pepsinogen (Herriott, 1948a) each contain 1 mole of bound phosphate/mole,

whereas pepsin B contains none (Ryle & Porter, 1959), it was decided to determine the phosphate content of pepsinogen B. Analysis of the freeze-dried material obtained as above gave an improbably high value, presumably owing to contamination with phosphate from buffers. Samples of the protein were therefore passed through Sephadex G-25 columns packed with 0.1 M-sodium sulphate and good separation of the protein from inorganic phosphate was found. Tests of the gelatin-liquefying activity showed that the zymogen had undergone no activation during this treatment. The first analysis with relatively small amounts of material showed the presence of a fraction of a mole of phosphate/mole of protein. More material was then separately treated on a similar column of Sephadex and used in the second and third analyses. In the second analysis the material was also used for N-terminal amino acid analysis, and the phosphate estimation was performed on the aqueous solution remaining after extraction of DNP-amino acids with ether and with ethyl acetate.

The results of these analyses are shown in Table 3. The various treatments failed to remove the phosphate from the protein, although some of that remaining after passage through the Sephadex column was removed by the precipitation and washing of the DNP-protein. Since the amounts of phosphate in each analysis are very small (about 0.05 μ mole), the accuracy of the estimations cannot be good.

Treatment of pepsinogen B with potato phosphatases
The above results, together with those of the N-terminal amino acid analysis, made it seem possible

Table 3. *Phosphate content of pepsinogen B*

Expt. no.	Wt. of sample (mg.)	Phosphate content (mole/mole of protein)
1	4.8	0.29
	5.2	0.31
2	7.0 (DNP-protein)	0.38
	10.7 (DNP-protein)	0.30
3	11.7	0.47
	12.7	0.49

Table 2. *N-Terminal residues of pepsinogen B*

The values at pH 9.0 are calculated for an assumed molecular weight of the DNP-protein of 43 000. N.D., Not determined.

pH of reaction	DNP-amino acids found (mole/mole of protein)							
	Met	His	Asp*	Glu*	Ser*	Thr*	Ala*	Leu*
8.0	0.74	0.38	0.02	0.04	0.05	0.0	0.05	N.D.
9.0	0.86	0.28	0.09	0.07	0.05	0.03	0.07	0.13

* Values not corrected for loss of DNP-amino acid during hydrolysis.

that one of the two components of pepsinogen B detectable in starch-gel electrophoresis experiments has *N*-terminal histidine and 1 mole of phosphate/mole. Perlmann (1958) has shown that the phosphate of pepsin and pepsinogen can be removed by treatment with an acid phosphatase from potatoes. A mixture of two enzymes (phosphatases A and B) separated by chromatography from the commercially available material was allowed to attack pepsinogen B and, in a separate control experiment, pepsinogen. Starch-gel electrophoresis of samples at 4 hr. and at 28 hr. showed the same result (Fig. 7). Although a decrease in the mobility of both the components of pepsinogen is detectable, no such change is visible with either of the components of pepsinogen B. In separate experiments it was shown that incubation of the zymogens at pH 5.6 did not affect their mobility.

Activation of pepsinogen B. Although the routine assay of pepsinogen B included a period of 10 min.

Fig. 7. Electrophoresis of pepsinogen B and pepsinogen in starch gel and the effect of phosphatases. Diagrammatic drawings of bands detected by chlorine after electrophoresis in 0.02M-phosphate buffer, pH 6.9, at 3 v/cm. are shown. B, Pepsinogen B incubated (1) with and (2) without phosphatases; electrophoresis for 15 hr.; P, pepsinogen incubated (1) with and (2) without phosphatases; electrophoresis for 12 hr. Full details are given in the Experimental section.

Fig. 8. Mixed chromatogram of pepsin B and activated pepsinogen B. The activated pepsinogen B (12.8 mg., activated at pH 2 and 35.5° for 5 min.) and pepsin B (5.5 mg.) were applied to a column (22 cm. $\times$ 1.2 cm.) of DEAE-cellulose in 0.1M-acetate buffer, pH 5.6. The gradient was 0.05M-NaCl, the mixing-vessel volume being 250 ml. ○, Activity or potential activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine (the whole curve has not been drawn in); △, activity against gelatin at pH 5.0; —, E_{280} ; ----, calculated concn. of chloride in the effluent.

during which the zymogen was incubated in acid solution before the addition of the substrate acetyl-L-phenylalanyl-L-di-iodotyrosine, such a period of preactivation in fact makes no difference to the measured activity, suggesting that the process of activation is very rapid at pH 2. Fig. 8 shows the result of an experiment in which pepsinogen B that had been activated at pH 2 and 35.5° for 5 min. was readjusted to pH 5.6, mixed with half its weight of pepsin B and chromatographed. The total activity against acetyl-DL-phenylalanyl-L-di-iodotyrosine recovered from the column was 85% of that applied, and the major peak at 310 ml. contains 84% of the eluted activity or activity equal to 1.8 times that of the pepsin B applied to the column, and must therefore contain activity derived from the zymogen. That this peak is in fact enzyme is shown by its high gelatin-liquefying activity, measured at pH 5.0, at which the activation of the zymogen is very slow. The smaller peak at 200 ml., emerging somewhat later than is expected for pepsinogen B by comparison with another experiment in which pepsinogen B was eluted at pH 5.6, apparently contains zymogen, for it has a low gelatin-liquefying activity. The very small intermediate peak was not investigated. A second such mixed chromatogram gave a similar result.

Fig. 9 shows the progress of activation of the zymogen at pH 2.0 and 35.5° as measured by development of gelatin-liquefying activity. About 40% of the final activity is formed in the first 2 min. and the rest more slowly in a process following first-order kinetics. At pH 4.0 a very different behaviour is found (Fig. 10). The activation is very much slower and shows a distinctly sigmoid course, but is not truly autocatalytic. Presumably an auto-

Fig. 9. Activation of pepsinogen B at pH 2.0. Pepsinogen B solution (0.05 mg./ml. in 0.08M-NaCl) was adjusted at 35.5° pH 2.0 with *N*-HCl. (a) Course of the activation. (b) First-order plot for the early stages of the reaction, taking the initial concentration, a , as the mean final activity liberated, i.e. 25.8 [P.U.]^{gel} V-/ml.

Fig. 10. Activation of pepsinogen B at pH 4.0. ●—●, Pepsinogen B solution (0.05 mg./ml. in 0.09 M-NaCl) was adjusted at 35.5° to pH 4.0 with 0.1 N-HCl. —, Calculated curve for an autocatalytic reaction with a final value of 20.4 [P.U.]^{Gel. V-/ml.} and a time for half-reaction of 345 min.

catalytic process is involved together with a second process following different kinetics. The final ratios of gelatin-liquefying activity to activity against acetyl-L-phenylalanyl-L-di-iodotyrosine attained at pH 2.0 in 400 min. or at pH 4.0 at 9 days are close (2370 and 2230 [P.U.]^{Gel. V-/μmole} of di-iodotyrosine/min.), but not yet equal to that of pepsin B (2760 [P.U.]^{Gel. V-/μmole} of di-iodotyrosine/min.).

DISCUSSION

When the precursor of pepsin C was isolated (Ryle, 1960) no evidence was found in the chromatograms for the existence of a zymogen for the related enzyme pepsin B. It is now clear that the reason for this is that pepsinogen B is not extracted from the pig gastric mucosa by the mildly alkaline solution of 0.45 saturated ammonium sulphate used to extract pepsinogen and pepsinogen C. The material whose isolation is reported above has no activity against gelatin at pH 5.0, but is converted at low pH values into an enzyme that does digest gelatin and acetyl-L-phenylalanyl-L-di-iodotyrosine and has little activity against haemoglobin, as measured by the formation of products soluble in trichloroacetic acid solution and absorbing light at 280 mμ. This specificity is a characteristic of pepsin B. The bulk of the product of acid-activation of pepsinogen B does not separate on ion-exchange chromatography from pepsin B, and the potential specific activity of the zymogen is very close to the specific activity of pepsin B; the molecular weights of the two are also very similar. On these grounds it can therefore be

concluded that the material isolated is in fact the zymogen of pepsin B.

The purest preparation so far obtained, which has been used for almost all the studies reported above, shows heterogeneity of two different orders. Both gel-filtration and starch-gel electrophoresis experiments indicate the presence of some contaminating material having proteolytic activity against haemoglobin but which represents less than 10% of the total material. This contaminant has a different electrophoretic mobility in starch gel from pepsinogen or pepsinogen D; it may be yet another zymogen or zymogens or it may be a degraded form of one of the known zymogens. Although the preparation appeared otherwise homogeneous on chromatography and gel filtration and in the ultracentrifuge, two active components are detected by electrophoresis in starch gel. The finding of this heterogeneity prompted a re-examination of pepsin B previously thought to be homogeneous, and chromatographic and electrophoretic evidence for the presence of two components in the enzyme, also in about equal amounts, was obtained. It is suggested that there are two zymogens, each convertible into one of the two enzymes. These findings pose some problems in nomenclature, but it seems unnecessary to attempt to solve them until the two components can be separated and individually characterized. For the present pepsinogen B will be used to refer to the mixture of the two components.

The presence of the two components in pepsinogen B can be correlated with the finding of about 0.8 *N*-terminal methionine and 0.3 *N*-terminal histidine residues/molecule and about 0.3 g. atom of phosphorus/mole. The simplest possibility is that one of the components has *N*-terminal histidine and one phosphate residue and the other *N*-terminal methionine and no phosphate. Pepsin B has been shown (Ryle & Porter, 1959) to have *N*-terminal alanine and no phosphate, so that if the simplest hypothesis is correct activation of the zymogens must involve removal of the *N*-terminal part of the molecules and the loss of the phosphate. If there is indeed bound phosphate in one of the components of pepsinogen B it must be bound in a fashion different from that in pepsinogen, for treatment with potato phosphatases produces no change in electrophoretic mobility. This could be due to failure of the phosphatases to remove the phosphate residue, or less probably to some special feature of the structure such that the phosphate was not ionized at pH 6.9, or such that dissociation of a proton from some other group occurred at pH 6.9 when the phosphate was removed. The solution of this question must probably await the separation of the two components.

The preliminary kinetic studies reported above show that the activation of pepsinogen B, like that of pepsinogen C, is complex. At pH 4.0 the process

is partly autocatalytic, and at pH 2.0, although all the potential activity against acetyl-L-phenylalanyl-L-di-iodotyrosine, measured at pH 2, is quickly liberated, there is rapid formation of only part of the activity against gelatin, measured at pH 5, the rest of the activity being liberated in a process showing first-order kinetics. This behaviour might be explained by the liberation of a peptide that inhibits the enzyme at pH 5 but does not do so at pH 2, and is slowly digested at pH 2; pepsin and the pepsin inhibitor behave in a similar fashion (Herriott, 1941).

This evidence of the incompleteness of the activation after a short time at pH 2 may account for the finding that not all the activity on a mixed chromatogram of pepsin B and activated pepsinogen B (Fig. 8) behaved as pepsin B; the chromatogram in fact suggests that there may be more than one partial activation product. The problem of the activation of pepsinogen B is further confused by the presence of two components, and will not be easily solved until they can be separated.

In the course of this work it has been incidentally demonstrated that chromatographically purified pepsinogen, obtained from the third peak of chromatograms such as that shown in Fig. 2, contains two components separable by electrophoresis in starch gel. Pepsinogen B contains two similarly separable components and pig gastric mucosa yields pepsinogens C and D as well. All these zymogens, except the two components of pepsinogen, are known to give rise to different enzymes, some of which are known to differ in their specificity, but the function of this multiplicity of enzymes, most of which account for only a minor part of the total, remains obscure. The mucosae of some individual pigs contain all four characterized zymogens, but in others one or other of the minor components is apparently missing (Ryle, 1963). It is not known whether the nature of the zymogens formed or secreted by one individual is constant or varies. A similar complexity of the proteolytic enzymes (Tang, Wolf, Caputto & Trucco, 1959; Seijffers, Segal & Miller, 1963*b*) and zymogens (Tang & Tang, 1963; Seijffers, Segal & Miller, 1963*a*) is found in man, though here the relative amounts of the various components seem to be more nearly equal. The investigators of the human enzymes and zymogens have not used acetylphenylalanyldi-iodotyrosine as a substrate, and it is not really possible to recognize

the enzymes or zymogens from one species as the equivalents of those from the other.

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