Studies on the Heterogeneity of Human Gastric Zymogens

By MICHAEL D. TURNER, JAGDISH C. MANGLA, I. MICHAEL SAMLOFF,
LEON L. MILLER AND HARRY L. SEGAL

*The Gastroenterology Unit, Department of Medicine, University of Rochester School of Medicine and
Dentistry, Rochester, N.Y. 14620, U.S.A.*

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The peptic zymogens of human gastric mucosa have been studied in the past by three different techniques: phosphate-gradient chromatography on a column of DEAE-cellulose, chloride-gradient chromatography on DEAE-cellulose and agar-gel electrophoresis. These techniques have given different results, each suggesting the presence of a different number of pepsin zymogens in human gastric mucosa. In the present experiments gastric mucosal homogenates were subjected to analysis by all three techniques. The two major zymogen peaks obtained from phosphate-gradient chromatography were found to be composed of the three fractions found on chloride-gradient chromatography; these fractions in turn were shown to be heterogeneous on agar-gel electrophoresis. The latter technique demonstrated the presence of seven separable enzymically active components in human gastric mucosal homogenates. The relationships of the components separated by the three techniques are discussed.

Since the work of Ryle & Porter (1959) and Richmond, Tang, Wolf, Trucco & Caputto (1958) it has become clear that many non-ruminant mammals have more than one proteinase in their gastric juice. The precursors of these proteinases in the human gastric mucosa have been studied by so many different analytical methods, however, that there is no agreement at present on the number of pepsin zymogens present in man.

Tang & Tang (1963) obtained two zymogen fractions from an extract of human gastric mucosa by column chromatography: the major one they termed IA and the minor one IB. They found that on acid activation zymogen IA gave two proteolytic enzymes corresponding in chromatographic behaviour to the 'pepsin' and 'gastricsin' that they had previously described in human gastric juice (Richmond *et al.* 1958). Zymogen IB apparently behaved similarly (Tang, Mills, Chiang & de Chiang, 1967). Despite finding two partially separable zymogens, Tang & Tang (1963) concluded that human gastric mucosa contains a single zymogen that on activation gives rise to two distinct enzymes. Seijffers, Segal & Miller (1963), employing chloride-gradient elution chromatography on DEAE-cellulose, found three distinct zymogen fractions in fundic mucosal extracts that they termed pepsinogens I, II and III. On activation with acid, pepsinogen I gave a single pepsin, pepsin I, and pepsinogen III a single pepsin, pepsin III. Pepsinogen II gave two chromatographically

distinguishable pepsins, termed IIA and IIB. These investigators concluded that human gastric mucosa contains four distinct pepsinogens, each of which on activation gives a single pepsin. Kushner, Rapp & Burtin (1964) separated the proteolytic zymogens of human gastric mucosal extracts into four bands by electrophoresis in agar gel. These they termed I, II, III and IV in order of decreasing electrophoretic mobility. The relationship of the zymogens detected by these three different techniques is obscure.

In the present investigation zymogen IA was prepared by the method of Tang & Tang (1963), and examined by chloride-gradient chromatography and electrophoresis in an attempt to reconcile the results of the different methods of analysis and thus to clarify the question of the number of human gastric pepsinogens.

MATERIALS AND METHODS

Gastric mucosa was obtained from the resected stomachs of patients undergoing subtotal gastrectomy for treatment of duodenal ulcer. Immediately after removal the tissue was cooled in ice and washed with 0.1M-NaHCO₃ at 4°C. The mucosa was removed by blunt dissection and homogenized first in a Waring blender and then in a Duall homogenizer at 4°C in 0.1M-NaHCO₃, 3 ml of solution being used/g of mucosa. It was then processed by the method described by Tang & Tang (1963).

DEAE-cellulose (lot no. 1410) was obtained from the Brown Chemical Co., Berlin, N.H., U.S.A. Before use it

was washed with 0.5M-NaOH containing 0.5M-NaCl and with the buffer used for chromatography. This DEAE-cellulose was used for chromatography with the phosphate gradient described by Tang & Tang (1963) and for chromatography with a chloride gradient by a minor modification of the method of Seijffers *et al.* (1963). Difco Noble agar was used for agar-gel electrophoresis. Bovine haemoglobin type II, lot no. 107B-1340, was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Buffers. 5mm-Phosphate buffer, pH 7.50, was prepared by mixing 5mm-K₂HPO₄ with 5mm-KH₂PO₄ in a proportion of approx. 4:1 and adjusting the pH by the addition of the appropriate 5mm-potassium phosphate solution. 0.2M-Potassium phosphate buffer, pH 7.50, was prepared in a similar manner. 0.1M-Acetate buffer, pH 5.30, was made from 0.1M-sodium acetate and 0.1M-acetic acid: appropriate amounts of NaCl were added for chloride-gradient elution chromatography. Citrate buffers were prepared as follows: pH 3.0 buffer by mixing 1750 ml of 0.2M-citric acid, 390 ml of 0.2M-trisodium citrate and 28.79 g of NaCl; pH 4.18 buffer by mixing 500 ml of 0.2M-citric acid, 450 ml of 0.2M-trisodium citrate and 3.10 g of NaCl; pH 4.60 buffer by mixing 500 ml of 0.2M-citric acid and 700 ml of 0.2M-trisodium citrate. 0.1M-Tris-glycine buffer, pH 8.3, for electrophoresis was made by mixing 260 ml of 0.1M-tris with 3000 ml of 0.1M-glycine solution and adjusting the pH with appropriate amounts of either constituent.

Proteolytic activity. The proteolytic activity of the activated zymogen fractions was determined by a modification (Seijffers *et al.* 1963) of the procedure of Anson & Mirsky (1932), with Folin-Ciocalteu reagent being used for the development of the colour, which was compared with standards containing known amounts of added L-tyrosine. All enzyme solutions tested were diluted sufficiently to give results in the near-linear range of the method. One unit of enzyme activity was defined as that amount of enzyme in 1 ml of solution that released an average of 1 μ mol (181 μ g) of 'tyrosine-like substance'/min from 5 ml of 2% acid haemoglobin substrate during a 1 h incubation at 37°C.

Milk-coagulating activity. The milk-coagulating activity of the zymogen fractions before and after activation with acid was measured by the technique described by Seijffers, Miller & Segal (1964).

Phosphate concentration. The concentration of phosphate in fractions obtained from the phosphate-gradient column was measured by a technique [U.S. Air Force *Manual of Clinical Chemistry* (1967) no. 160-49. Washington: Department of the Air Force], modified from that of Fiske & Subbarow (1925).

Preparation of zymogen IA. The homogenate of gastric mucosa in 0.1M-NaHCO₃ was centrifuged at 13000 rev./min at 4°C for 1 h in a Sorvall centrifuge with an angle-head rotor of radius 11.0 cm. The precipitate was discarded and enough saturated (NH₄)₂SO₄ in 0.1M-NaHCO₃ was added to the supernatant to bring the concentration to 25% saturation. The mixture was kept for 15 min at 4°C and the precipitate was removed by centrifugation at 4°C and discarded. Further saturated (NH₄)₂SO₄ in 0.1M-NaHCO₃ was then added to the supernatant to bring the concentration to 80% saturation. The mixture was kept at 4°C for 4 h and the precipitate removed by centrifugation. The precipitate thus obtained was dissolved in 50 ml of

5mm-phosphate buffer, pH 7.50, and dialysed for 24 h at 4°C against three changes of the same buffer. After removal of a sample for measurement of enzymic activity the dialysis residue was applied to a 3 cm $\times$ 30 cm column of DEAE-cellulose that had been packed under gravity and equilibrated with 5mm-phosphate buffer as described by Tang & Tang (1963). The column was eluted with the 5mm-phosphate buffer until the E_{280} value of the eluate was less than 0.1 when measured in a 1 cm cuvette in a Hitachi-Perkin-Elmer spectrophotometer. This required some 500 ml of buffer. The column was then connected to a gradient-elution apparatus composed of two cylinders of similar diameter each containing 3500 ml of buffer. The two chambers were connected by a siphon so that the level of fluid in each chamber fell equally throughout the elution. The mixing cylinder initially contained 3500 ml of 5mm-phosphate buffer, pH 7.50, and the second cylinder 0.2M-phosphate buffer, pH 7.50. Throughout the chromatography the temperature of the column was maintained at 2-3°C by circulation of thermostatically controlled coolant through a jacket surrounding the column. Fractions of volume 10 ml were collected. The volume of buffer passed through the column from the time it was connected to the gradient-elution apparatus until the appearance of the first fraction containing zymogen was termed the elution volume, V_e , of the zymogen: for the purposes of this calculation the volume of the initial buffer required to lower the extinction of the eluate to less than 0.1 was ignored. The fractions containing the peak of zymogen IA were pooled and concentrated by dialysis through Visking tubing under a pressure of 600 mmHg at 4°C. The fractions corresponding to the peak of zymogen IB were concentrated similarly. After concentration some 50-fold, the material was stored at -20°C until used for further study.

Rechromatography of zymogen IA by phosphate-gradient elution. To determine whether the zymogen IA retained its characteristics on rechromatography, the concentrated zymogen IA from the previous experiments was dialysed overnight against three changes of 5mm-phosphate buffer at 4°C and applied to a second 3 cm $\times$ 30 cm column of DEAE-cellulose equilibrated with 5mm-phosphate buffer, pH 7.50. This was eluted with a phosphate gradient in a manner similar to that used for the initial chromatography, with the exception that the preliminary washing of the column with 5mm-phosphate buffer was omitted on rechromatography. Eluate fractions were collected as before and samples of each assayed for proteolytic activity and phosphate in the standard manner. The fractions containing the peak of zymogen activity were again concentrated by dialysis.

Chromatography of acid-activated zymogen IA. To determine whether the zymogen IA prepared as described above gave the same products on acid activation as the material prepared by Tang & Tang (1963), a portion of the concentrated zymogen IA from the previous column containing 12.02 units of activity was activated by acidification to pH 2.0 with m-HCl. After standing at room temperature for 20 min the acidified material was dialysed against 0.2M-citrate buffer, pH 3.0, for 24 h at 4°C. The proteolytic activity of the activated zymogen was measured and an amount containing 1.56 units of activity applied to a column (5.0 cm $\times$ 0.5 cm) of Amberlite IRC-50 in 0.2M-citrate buffer, pH 3.0. After the activated zymogen

solution had run into the column the resin was washed with 5.0 ml of 0.2 M-citrate buffer, pH 3.0. The column was eluted first with 20 ml of 0.2 M-citrate buffer, pH 4.18, and then with 20 ml of 0.2 M-citrate buffer, pH 4.6. Fractions of volume 1.7 ml were collected and analysed for proteolytic activity.

Chloride-gradient chromatography of zymogen IA on DEAE-cellulose. The zymogen IA was next studied by chloride-gradient chromatography. A column (2 cm × 49 cm) of DEAE-cellulose equilibrated with 0.1 M-acetate buffer, pH 5.30, was prepared in a manner similar to that described by Seijffers *et al.* (1963). A portion of the concentrated zymogen IA obtained from rechromatography on the phosphate-gradient column was dialysed overnight at 4°C against three changes of 0.1 M-acetate buffer, pH 5.30. After dialysis the activity of the solution was measured by the standard technique and a volume containing 78.16 units of proteolytic activity applied to the DEAE-cellulose column. The zymogen was allowed to run into the DEAE-cellulose and followed by a wash with a small amount of 0.1 M-acetate buffer, pH 5.3. Further buffer was then applied to the column to a height of some 5–8 cm above the level of the DEAE-cellulose and the whole column connected to a Varigrad composed of nine cylinders each of which contained 350 ml of buffer and NaCl at concentrations of 0.11 M, 0.11 M, 0.12 M, 0.12 M, 0.13 M, 0.13 M, 0.14 M, 0.14 M, 0.16 M respectively. The column was eluted at a rate of 45 ml/h and the eluate collected in 15 ml fractions. Throughout the chromatography the temperature of the column was maintained at 7°C by circulation of a coolant through the jacket of the column. Samples of the fractions were examined for proteolytic activity. The chloride concentration of the fractions was measured by titration with AgNO₃, with Na₂CrO₄ as indicator.

Phosphate-gradient rechromatography of fractions from the chloride-gradient column. To determine whether the zymogen IA had been altered in some way by the chloride-gradient chromatography, the fractions containing each of the three peaks of pepsinogen activity from the chloride-gradient column were concentrated by dialysis, combined and rechromatographed as before on a phosphate-gradient column.

After they had been combined and concentrated, the peaks from the chloride-gradient column were dialysed against 5 mm-phosphate buffer, pH 7.50. The activity of a sample of this material was measured and the remainder applied to a column (3 cm × 30 cm) of DEAE-cellulose previously equilibrated with 5 mm-phosphate buffer, pH 7.50. Elution with a phosphate gradient was then carried out as described above. Fractions of volume 10 ml were collected and were assayed for proteolytic activity and phosphate concentration.

Agar-gel electrophoresis. Agar-gel electrophoresis of gastric mucosal homogenate and zymogen preparations was carried out by a modification of the technique of Kushner *et al.* (1964). A glass plate (20 cm × 20 cm) was coated with 50 ml of 1.5% Difco Noble agar in 0.1 M-tris-glycine buffer, pH 8.3. After cooling, the plate was kept overnight in a covered dish saturated with water vapour at 4°C. The next morning a line of slots each 1 mm thick and 1 cm wide were cut in the agar about 2 cm from one side of the plate. Each zymogen preparation subjected to electrophoresis was first diluted with 0.1 M-tris-glycine

buffer, pH 8.3, to give a solution with a proteolytic activity of 10 units/ml. This was mixed with an equal volume of tris-glycine buffer containing 3% of agar at 56°C and placed in a slot in the electrophoresis plate. The plate was then put in a Brinkmann thin-layer electrophoresis chamber, the metal cooling plate of which was cooled by circulating water through it at 1°C. As markers for the progress of electrophoresis 2–4 µl of Brinkmann RBY dye were placed on the surface of the agar at either end of the line of slots containing the samples. The current was switched on and the potential across the agar plate was measured with a voltmeter: electrophoresis was then carried out at 700 V and 40 mA for 4 h, by which time the red component of the RBY dye mixture had migrated off the anodal end of the plate and the blue component was some 10 cm from the origin. During the electrophoresis any globules of liquid that appeared on the surface of the agar were removed with filter paper. After electrophoresis the plate was placed in an acid solution of haemoglobin made from 5 parts of aq. 0.8% denatured bovine haemoglobin and 1.25 parts of 0.2 M-HCl. After 15 min the excess of liquid was drained off the plate, which was then incubated at 37°C for 2 h in a sealed plastic dish, the atmosphere of which was saturated with water vapour. After incubation the plate was removed and washed in 5% (v/v) acetic acid in 50% (v/v) ethanol. The plate was then kept in fresh acetic acid-ethanol mixture overnight. The following morning the plate was removed and dried in a current of air. The undigested haemoglobin remaining in the agar was stained by placing the plate in aq. 2% (v/v) acetic acid for 7 min followed by immersion in 0.5% Buffalo Black NBR (Amido-Schwarz B) in acetic acid-methanol (1:9, v/v) for 5–6 min. The bands of zymogen appeared as clear areas in a blue background of undigested haemoglobin (Fig. 1).

Ultracentrifugation. Rechromatographed zymogen IA and peaks I and III from the chloride-gradient column were centrifuged in a sucrose density gradient in a preparative Beckman ultracentrifuge (model L2-65).

The gradient was prepared with a Buchler gradient generator. This consisted of two cylindrical chambers of identical diameter connected at their lower ends through a 2 mm stopcock. One cylinder, the mixing chamber, was equipped with a vibrating mixer and a further capillary tube from which the fluid could be drained into the centrifuge tube. After the stopcock had been closed, 2.2 ml of 20% (w/v) sucrose in 0.1 M-phosphate buffer, pH 7.4, was placed in the mixing chamber, and 2.0 ml of 5% (w/v) sucrose in the same buffer was placed in the other cylinder. The vibrator was started, the stopcock opened and the sucrose solution drained slowly into the plastic centrifuge tube. Care was taken to ensure that as little mixing as possible occurred in the centrifuge tube during the preparation of the gradient, the end of the capillary used for filling the tube being kept about 3–4 mm above the surface of the liquid in the centrifuge tube throughout. After the gradient had been prepared, 0.5 ml of zymogen in 0.1 M-phosphate buffer, pH 7.4, was gently layered on to its surface.

After ultracentrifugation in a rotor of 8.9 cm radius for 22 h at 36000 rev./min the contents of the tubes were fractionated by piercing the bottom of each with a Buchler tube fractionator. In all, 21 fractions, each of 16 drops, were obtained from each tube. The proteolytic activity

Fig. 1. Tracing of actual agar-gel electrophoresis plate of fractions from preparation II of fundic gastric mucosa. Upper and lower wells contained whole mucosal extract (M): seven bands showing enzymic activity were demonstrable in this extract. P I, P II and P III represent the first, second and third peaks of zymogen obtained from chloride-gradient chromatography of the same extract. Zymogens IA and IB and pre-IA were prepared by phosphate-gradient chromatography of the same fundic mucosal extract. It should be noted that zymogen IA (Z IA) is composed of a mixture of the components of P I, P II and P III. Zymogen IB (Z IB) is composed of a mixture of the more mobile zymogens. Pre-IA appears to be homogeneous and is probably a cathepsin.

of a sample of each fraction was then estimated by the modified Anson & Mirsky (1932) technique.

RESULTS

Preparation of zymogen IA. Zymogen IA was prepared on two occasions from two different pooled preparations of human gastric mucosa. The first preparation (preparation I) was made from 99.6g of mucosa obtained from four stomachs. After ammonium sulphate precipitation and dialysis against 5mM-phosphate buffer 208ml of material was obtained with an enzymic activity of 80.46 units/ml. The second preparation (preparation II) was made from 95.7g of mucosa obtained from three stomachs. Its final volume after dialysis against 5mM-phosphate buffer was 180ml; this preparation had an enzymic activity of 80.54 units/ml.

A 62ml portion of preparation I was used for phosphate-gradient elution chromatography on DEAE-cellulose. Two peaks of enzymic activity were detected (Fig. 2) in the column eluate. The first, and larger, one appeared at an elution volume of 3100ml and a phosphate concentration of 0.11M in the eluate: this was termed 'zymogen IA'

Fig. 2. Phosphate-gradient column chromatography of an extract of fundic gastric mucosa (preparation I) on DEAE-cellulose. $\circ$, Enzymic activity of the fractions; $\bullet$, phosphate concentration. It should be noted that the zymogens are eluted in two major fractions. The first (zymogen IA) appears at a phosphate concentration of about 0.11M and the second (zymogen IB) at 0.16M.

after the nomenclature of Tang & Tang (1963). The second peak, termed 'zymogen IB', appeared at 4800ml and a phosphate concentration of 0.16M.

Preparation II was also chromatographed on the phosphate column. This preparation showed three peaks of proteolytic activity in the column eluate (Fig. 3). The first peak, termed 'pre-IA', appeared at an elution volume of 1910ml and a phosphate molarity of 61mM. This material was not detected in preparation I. The other two peaks of enzymic activity corresponded to zymogens IA and IB of preparation I, appearing at 3200ml with a phosphate concentration of 0.11M and at 4900ml with a phosphate concentration of 0.16M respectively.

Rechromatography of zymogen IA by phosphate elution. The fractions constituting zymogen IA were used for rechromatography on a similar column with the same conditions for elution. The zymogen was eluted as a single peak with an elution volume of 4400ml at a phosphate concentration of 0.116M. Almost all of the enzymic activity was eluted at a phosphate concentration below 0.142M, although a small amount of activity above this concentration was observed (Fig. 4).

Chromatography of acid-activated zymogen IA. On chromatography on Amberlite IRC-50, the activated zymogen IA showed two peaks of proteolytic

Fig. 3. Phosphate-gradient column chromatography of an extract of fundic gastric mucosa (preparation II) on DEAE-cellulose. Three major peaks of zymogen activity are visible, Z IA and Z IB, appearing at the same phosphate concentrations as IA and IB of preparation I, and a third peak, pre-IA, appearing at a phosphate concentration of 61 mM. O, Enzymic activity of the fractions; ●, phosphate concentration.

Fig. 4. Rechromatography of zymogen IA peak from previous chromatography (Fig. 3) of preparation II (fraction volume 10ml). O, Enzymic activity of samples of the fractions; ●, phosphate concentration. The material appears predominantly as a single peak, the elution of which commences at a phosphate concentration of 0.116 M.

activity corresponding to the 'pepsin' and 'gastric-sin' obtained by Tang & Tang (1963) from their zymogen IA preparation (Fig. 5). Of the enzymic activity of the material applied to the column, 89.3% was recovered in the eluate.

Rechromatography of zymogen IB with phosphate-gradient elution. Zymogen IB from preparation I was concentrated and rechromatographed on DEAE-cellulose with phosphate-gradient elution. The enzymic activity appeared as a single peak with

Fig. 5. Amberlite IRC-50 column chromatography of acid-activated sample of zymogen IA from rechromatographed material from preparation II (Fig. 4). ●, Enzymic activity of samples of the fractions. Two peaks of enzymic activity are observed, corresponding to the 'pepsin' and 'gastric-sin' described by Richmond *et al.* (1958).

Fig. 6. Phosphate-gradient rechromatography on DEAE-cellulose of zymogen IB from preparation I (Fig. 2). O, Enzymic activity of the fractions; ●, phosphate concentration. The material first appears at 5300 ml as a single peak of zymogen activity at a phosphate concentration of 0.16 M (cf. zymogen IA in Fig. 4).

an elution volume of 5300 ml at a phosphate concentration of 0.16 M (Fig. 6).

Chloride-gradient chromatography of zymogen IA on DEAE-cellulose. A concentrate of zymogen IA from preparation I after dialysis against 0.1 M-acetate buffer, pH 5.30, contained 22.48 units/ml. A 35 ml portion of this material was placed on the chloride-gradient column and eluted as described above. The proteolytic activity appeared in three major peaks with elution volumes of 323, 872 and 1340 ml. The chloride concentrations at which the peaks appeared were 0.118, 0.124 and 0.131 M respectively. These three peaks of enzymic activity corresponded to the pepsinogen peaks I, II and III found by Seijffers *et al.* (1963) on chloride-gradient chromatography of human gastric mucosal extracts.

A 4 ml portion of concentrated zymogen IA

obtained from the phosphate-gradient rechromatography of preparation II having a proteolytic activity of 19.50 units/ml was equilibrated with 0.1M-acetate buffer, pH 5.3, placed on a DEAE-cellulose column and eluted with a chloride gradient. The proteolytic activity again appeared in three major peaks, with elution volumes of 397, 1135 and 1716.9 ml at chloride concentrations of 0.114, 0.125 and 0.136M respectively (Fig. 7). Samples of the peak material were pooled and assayed for enzymic activity: 91.3% of the enzymic activity applied to the column was recovered in the three peaks.

Rechromatography of zymogens from the chloride-gradient column by phosphate-gradient elution. After concentration and dialysis the combined three peaks of zymogen activity from the chloride-gradient column were found to have an activity of 68.96 units/ml. All this material was rechromatographed on the DEAE-cellulose phosphate-gradient column. The enzymic activity appeared in the eluate as a single peak with an elution volume of 4400 ml at a phosphate concentration of 0.118M (Fig. 8). After concentration by dialysis and correction for the activity removed for preliminary analysis, 83% of the enzymic activity originally applied to the column was recovered in the eluate.

Agar-gel electrophoresis. On electrophoresis in agar gel, fundic gastric mucosal extracts from a number of subjects showed seven clearly distinguishable bands of enzymic activity; these were labelled A, B, C, D, E, F and G in order of increasing mobility (Fig. 1). In some mucosae band A appeared double and may be heterogeneous.

When the zymogen IA preparations were sub-

jected to electrophoresis and compared with the original mucosal homogenates from which they were derived, they were found to contain bands B-G (Fig. 1). Zymogen IB also appeared to be heterogeneous on electrophoresis: it contained predominantly band G contaminated with band F but with only small amounts of the less mobile bands (Fig. 1).

The enzymic activity of all bands except band A was destroyed by neutralization after acidification,

Fig. 8. Phosphate-gradient chromatography of recombinant pepsinogen peaks I, II and III from the chloride-gradient column of Fig. 7. O, Enzymic activity of the fractions; ●, phosphate concentration. It should be noted that the three zymogens now appear as a single peak closely corresponding in elution behaviour to the original rechromatographed zymogen IA (cf. Fig. 4).

Fig. 7. Chloride-gradient chromatography of zymogen IA from preparation II previously rechromatographed on the phosphate-gradient column (Fig. 4). O, Enzymic activity of the fractions (each 15 ml); ●, chloride concentration. It should be noted that the material shows three major peaks of zymogen corresponding to pepsinogens I, II and III described by Seijffers *et al.* (1963).

Fig. 9. Sucrose-density-gradient ultracentrifugation of rechromatographed zymogen IA (▲), pepsinogen peaks I (■) and III (●) from the chloride-gradient column. It should be noted that the three materials show similar distributions with maximum concentrations in fraction 12. A mixture of these components could not be separated into its components by this technique. Fraction 1 was taken from the bottom of the centrifuge tube and fraction 21 from the top.

indicating that these bands represented pepsinogens. The activity of band *A* was retained after acidification and neutralization. Electrophoresis of the material from the pre-IA peak obtained from phosphate-gradient chromatography of zymogen preparation II gave only band *A* (Fig. 1).

Pepsinogen peaks I, II and III from chloride-gradient chromatography on DEAE-cellulose were also examined by agar-gel electrophoresis. Peak I corresponded to bands *B* and *C*. Peak II gave bands *E* and *F*, and peak III bands *F* and *G* (Fig. 1).

Sucrose-density-gradient ultracentrifugation. The zymogen IA appeared to be homogeneous after centrifuging for 22h in a sucrose density gradient. It showed essentially the same distribution of enzymic activity as that seen with peaks I and III from the chloride-gradient column (Fig. 9).

DISCUSSION

Of the three methods of analysis of human gastric mucosal zymogens used in this study, agar-gel electrophoresis appeared to be the most discriminating. The electrophoretic method employed was different from the original technique of Kushner *et al.* (1964). Tris-glycine buffer was used in place of barbital buffer and acid haemoglobin was employed as the substrate for detection of enzymic activity. Of all the numerous modifications in the techniques tried, this was found to give the greatest resolution of the different proteolytically active bands. It resulted in the finding of seven electrophoretically distinct components in most homogenates of human gastric mucosa as compared with the four originally reported by Kushner *et al.* (1964). The proportions of the different detectable bands of proteolytic activity varied in different mucosae, and in the electrophoreses of some mucosal homogenates band *A* appeared double so that eight enzymically active components could then be seen. The relationship of the four electrophoretically separable zymogens described by Kushner *et al.* (1964) to those reported here is not entirely clear. It does seem, however, that band 4 described by Kushner *et al.* (1964) corresponds to band *A* of the present study, since it is the least mobile of all the zymogens detected by this method and is stable to alkali after preliminary acidification. Electrophoresis also showed that the material designated 'pre-IA' (Fig. 1) from the phosphate-gradient column corresponded to band *A* of the whole mucosal homogenates. Its alkali-stability suggests that this material is a cathepsin.

The zymogen IA prepared in these studies appeared very similar to that obtained by Tang & Tang (1963), since it showed the following properties of the material that they prepared: it was prepared in an identical manner; it was the first,

and larger, component of two partially separable zymogen fractions obtained by phosphate-gradient chromatography; it appeared to be homogeneous on ultracentrifugation, although a sucrose density gradient was used in these studies rather than conditions for the measurement of sedimentation velocity; and finally it gave, on acid activation, as did their zymogen, two chromatographically separable enzyme fractions that corresponded to the 'pepsin' and 'gastricsin' described by Richmond *et al.* (1958). The zymogen IA obtained in these studies differed from that described by Tang & Tang (1963) only in that it was eluted from the DEAE-cellulose column at a phosphate concentration of 0.11 instead of 0.07M. However, this difference is readily explicable on the basis of differences in the batches of DEAE-cellulose used in these experiments and those of Tang & Tang (1963): it is well known that different batches of DEAE-cellulose do not behave reproducibly and show very marked differences in behaviour when used for chromatography of proteins in general (Himmelhoach & Peterson, 1966) and gastric zymogens in particular (Seijffers *et al.* 1963).

When the zymogen IA preparations were rechromatographed on DEAE-cellulose with phosphate-gradient elution they appeared to be homogeneous, but when subjected to chloride-gradient chromatography by a minor modification of the method of Seijffers *et al.* (1963) the three pepsinogen fractions described by them were obtained with 91% recovery of the enzymic activity applied to the column. When these three fractions were then pooled, concentrated and reappplied to a phosphate column a single zymogen peak corresponding in behaviour to the original rechromatographed zymogen IA was again obtained with an 83% recovery of the enzymic activity. This indicated that the detection of three separate fractions in the zymogen IA on chloride-gradient chromatography did not result from damage to a pure single zymogen on the chloride-gradient column.

This conclusion was confirmed by the finding that the fractions from both the phosphate-gradient column and the chloride-gradient column did not coagulate milk without preliminary acidification, although they were very active after acidification, indicating that the fractions had not been activated by chromatography. The zymogen IA preparations were also found to be heterogeneous on agar-gel electrophoresis, as were the pepsinogen fractions from the chloride-gradient column. Moreover, the components of these fractions and of the zymogen IA preparations were clearly identifiable on electrophoresis in the original mucosal homogenate, so that there was no suggestion of degradation of the zymogens as a result of either chromatographic technique.

The concept of zymogen IA as a pure substance

Fig. 10. Diagrammatic representation of the correspondence of the three methods used to study human gastric zymogens. In addition to bands *F* and *G* some preparations of zymogen IB contain small amounts of band *E*.

and as the single zymogen precursor of a number of gastric enzymes is no longer tenable. It is clear that zymogen IA is a mixture of the pepsinogen fractions I, II and III studied by Seijffers *et al.* (1963), and that these are also impure since on electrophoresis they are separable into a number of distinct components.

Pepsinogen fraction I described by Seijffers *et al.* (1963) appears to contain predominantly the zymogen of gastricsin, since on acid activation it shows little or no hydrolytic activity against *N*-acetylphenylalanyldi-iodotyrosine (G. Guarasci & M. D. Turner, unpublished work), a property of gastricsin (Chiang, Sanchez-Chiang, Wolf & Tang, 1966), whereas it readily hydrolyses haemoglobin and cytochrome *c* with the production of peptides very different from those produced by acid-activated pepsinogen fractions II and III. After activation by acid, pepsinogen fraction I shows much greater resistance to neutralization than do fractions II and III (Turner, Tuxill, Miller & Segal, 1967), as well as different pH optima for proteolysis. These observations suggest that there are considerable differences between some of the zymogens present in pepsinogen fraction I and those of fractions II and III. The heterogeneity observed on electrophoresis within these latter zymogen fractions may represent minor differences in amino acid composition or in the degree of phosphorylation, similar to the differences observed in some of the hog pepsinogens and pepsins (Perlmann, 1958; Ryle & Hamilton, 1966).

It is not yet completely certain that the individual electrophoretic bands do not represent partial degradation products of a smaller number of zymogens produced during the process of extraction or electrophoresis. Indeed, the detection of what is probably a cathepsin in the gastric mucosa raises this possibility anew. At the moment, therefore, the significance of the finding of a number of separable zymogen components on electrophoresis is not entirely clear. It is, however, possible from these studies to establish the relationship between the human gastric zymogens detected by the different analytical methods (Fig. 10) and to conclude that the human gastric mucosa is like that of the pig (Ryle, 1960, 1965; Ryle & Hamilton, 1966; Lee & Ryle, 1967) in containing a number of proteinase proenzymes. This demonstrated multiplicity of human gastric zymogens suggests that many of the past studies of human pepsinogens and pepsins have been made on mixtures, and the conclusions drawn from these investigations may require some re-evaluation and revision.

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