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Extraction, Gel Filtration Pattern, and Receptor Binding of Porcine Gastrointestinal Glucagon-Like Immunoreactivity

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Summary. Different techniques for the extraction and initial purification of porcine gastrointestinal glucagon-like immunoreactivity (GLI) were compared with reference to yield, and preservation of number and pattern of GLI components. The conventional acid-ethanol technique combined with ethanol-ether purification gave high yields and a reproducible pattern of components. Large amounts of tissue were more easily extracted using another technique, based on extraction by boiling, extraction and precipitation with acetone, and – if necessary – salting out. – By means of the latter two techniques mucosal tissue from all of the porcine gastrointestinal tract was extracted and subjected to gel filtration. Glucagon-like peptides were searched for using: – 1. a radioimmunoassay which quantifies gut type glucagon (GTG), as well as pancreatic type glucagon (PTG), 2. a radioimmunoassay highly specific for pancreatic type glucagon (PTG), and 3. a radioreceptor assay based on binding of glucagon to porcine liver cell membranes. – The oesophageal, the fundic, and the antro-pyloric parts of the gastric mucosa contained very small amounts of GLI. The cardiac gland region contained small amounts of a peptide indistinguishable from "true" glucagon. The duodenal mucosa contained small amounts of "true" glucagon and may be a smaller, glucagon-like peptide. The mucosa of the small intestine contained large amounts of both high and low molecular weight GTG and, in addition, PTG of high molecular weight and "true" glucagon. The colon also contained these components with "true" glucagon in high concentrations. Only small GTG and "true" glucagon were receptor-active, the former with less than its immunometric potency.

Key words: Glucagon, chromatography, gastrointestinal hormones, gut glucagon, enteroglucagon, radioimmunoassay, hormone receptors, diabetes.

The interest in the glucagon-like gut peptides [1, 2] was nourished greatly by the recent discovery of small amounts of presumably true glucagon in extracts of porcine duodenal mucosa [3]; large amounts were also identified in gastrointestinal mucosa of dogs [4]. Furthermore, true A-cells or glucagon-cells were identified in the canine gastric mucosa [3–6]. The high content of glucagon in the canine gastric mucosa (which equals the content of the pancreas [4]) may be a characteristic of this species, since others found only small amounts in the gastric mucosa of primates [7]. Likewise only few glucagon cells were identified in the gastric mucosa of pigs and rats [4].

The pig is being used extensively for research in gastrointestinal endocrinology because of ready availability and because of the similarity between porcine and human physiology, especially of the gastrointestinal tract [8]. The present study was therefore undertaken to map the occurrence of the various forms of glucagon-like peptides in the porcine gastrointestinal mucosa. Also, some technical notes on the extraction and initial purification of these peptides are presented.

In this report the following definitions and abbreviations are employed: Glucagon is the well-known hyperglycaemic 29-amino-acid peptide (MW 3485), originally extracted from the pancreas. Glucagon-Like Immunoreactivity (GLI) covers peptides of gastrointestinal (including pancreatic) origin, which will bind to certain so-called cross-reacting, antiglucagon antisera. Pancreatic Type Glucagon (PTG) indicates peptides of pancreatic and gastrointestinal origin, which behave like true glucagon in their reactions with the various glucagon antisera. Gut Type Glucagon (GTG) (synonymous with gut glucagon, gut GLI, and enteroglucagon) covers peptides, primarily of gastrointestinal origin, which do not bind to so-called specific antisera, but only to

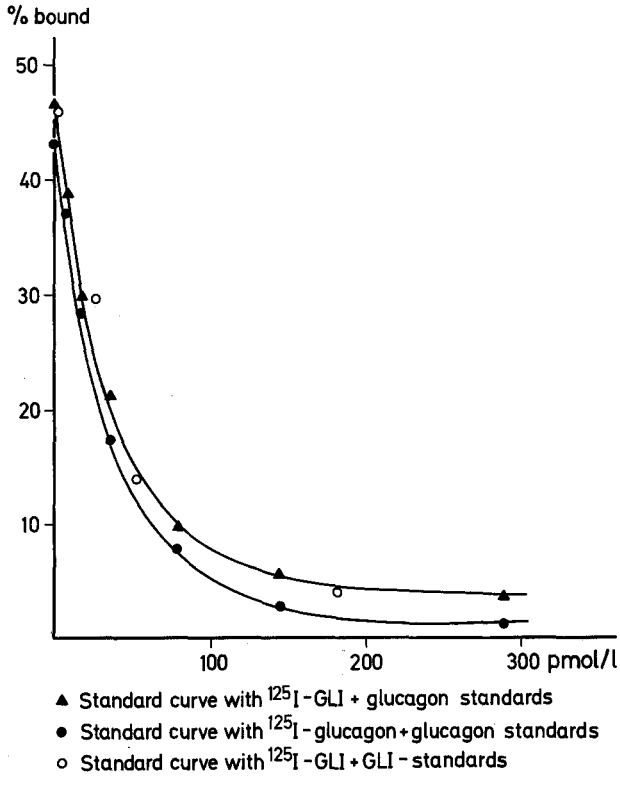

Fig. 1. Standard curves resulting from incubations of antiserum 4304 (12) with ^{125}I-labelled glucagon and glucagon (●), ^{125}I-labelled GLI I (see text), and glucagon (▲), and ^{125}I-labelled GLI I and GLI I (○). Ordinate: binding of the labelled peptide, expressed as percentage of total. Abscissa: concentration of unlabelled peptide. Each point represents the mean of triplicate determinations

so-called cross-reacting antisera. GTG, therefore, has to be calculated by subtracting PTG from GLI.

Methods and Materials

Assays

Radioimmunoassays of pancreatic type glucagon (PTG) and GLI were performed as described earlier [9]. GTG concentrations may then be calculated by subtracting the PTG-concentrations obtained with the specific antiserum from the total GLI-concentrations obtained with the cross-reacting antiserum. Separation of bound and free peptide was carried out using ethanol [10] or activated charcoal [11]. The results obtained with the two techniques were similar. The antiserum used for determination of PTG showed no cross-reaction with vasoactive intestinal polypeptide, secretin, cholecystokinin, insulin, and gastrin. Its specificity towards GTG is best appreciated by inspection of Figures 4 to 8, from which it appears that virtually no GTG-cross-reaction can be demonstrated, in spite of GTG-concentrations exceeding 5000 pmol-equivalents/1. Furthermore, it cross-reacts less than 1% with GLI I (see below) in concentrations up to 10^{-7} mol/l. The antiserum used for the determination of GLI (4304) does not crossreact with any of the above mentioned non-glucagon gut peptides. Although raised against glucagon [12] it cross-reacts strongly with GTG; with this antiserum dilution curves of glucagon and a partly purified extract of porcine gut (MUC 101, a gift from A. Moody, NOVO Research Institute, Copenhagen, Denmark) are completely superimposable. Furthermore (Fig. 1), standard curves resulting from incubations with ^{125}I-labelled glucagon (monoiodinated [13], purchased from the NOVO Research Institute) and glucagon (highly purified, NOVO) are virtually identical to curves resulting from incubations with ^{125}I-labelled gut GLI I (= porcine large molecular weight GTG, a gift from A. Moody and F. Sundby, NOVO Research Institute) and glucagon, and to curves from incubations with ^{125}I-labelled GLI I and highly purified GLI I (a gift from F. Sundby, NOVO [14]). As GLI I is a highly purified peptide of known molecular size and amino acid composition [14], the results of the GLI measurements are expressed in molar units. The unit "pmol-equivalents/l", however, has been preserved owing to the less well-characterized components.

Receptor-interaction was investigated using a previously described radioreceptor assay [15], which is based on the binding of labelled glucagon to purified porcine liver cell membranes. The assay was modified slightly, incubations being performed at 4 °C overnight, instead of 45 min at 22 °C. The results obtained with the two techniques were similar.

Gel Filtration

For gel filtration Sephadex G-50 superfine (Pharmacia, Uppsala, Sweden) was used. Columns were either K 50/100 (Pharmacia), 50 × 1000 mm, bed volume 1600 ml, or siliconized glass columns, 22 × 2000 mm, bed volume 650 ml. The columns were equilibrated and eluted with 0.5 mol/l acetic acid (Merck no. 63) at 4 °C if not otherwise stated. A constant flow of 42 ml/h (K 50/100) and 12 ml/h (22 × 2000 mm) was maintained by means of peristaltic pumps (P-3, Pharmacia), and fractions were collected every 20 min by means of an automatic fraction collector. Columns were calibrated with ^{125}I-labelled human albumin, unlabelled glucagon, GLI I, and ^{22}NaCl. Labelled albumin and ^{22}NaCl were added to all samples as internal standards. Sample volume never exceeded 1% of bed volume. Proper packing was controlled with Blue Dextran (Pharmacia). Elution positions are referred to by

means of the coefficient of distribution $K_{av} = (V_e - V_o)/(V_t - V_o)$. V_o is the elution volume of a substance completely excluded from the gel, V_e is the elution volume of the substance studied, and V_t is the total volume of the gel bed. GLI I and glucagon eluted with $K_{av} = 0.22$ and $K_{av} = 0.65$ respectively.

Extraction

Gastrointestinal mucosal tissue was obtained from anaesthetized 20–30 kg pigs of Danish landrace and frozen immediately between blocks of dry ice. The frozen tissue was stored at −20 °C.

Acid Ethanol. Most workers have used acidified ethanol for extraction, a procedure originally devised for peptide extraction from the pancreas [16]. The initial steps in acid ethanol extraction were considered important, as pilot experiments indicated that neglect of some precautions led to impaired yield and recovery of components. The following procedure was adopted: Frozen mucosa (the volume of which in ml was estimated to be equal to its weight in grams + 10%) was crushed and plunged into 4 volumes of an extraction solution, which consisted of absolute ethanol, 635 ml; 37% HCl, 15 ml; distilled water 150 ml. This solution was precooled to −20 °C. The mixture was then homogenized for 1 min in a Waring type blender, passed through a fine meshed sieve to remove connective tissue, homogenized briefly in a Potter-Elvehjelm homogenizer and left for four hours in the cold. The homogenate was then centrifuged for 30 min at 2000 x G. The supernatant was decanted for further purification.

The Boiling Method. Mutt used boiling of the tissue in water as the initial step in the extraction of secretin [17]. It was therefore felt that the standard extraction method of Kenny [18] was not necessarily the most expedient for both yield and preservation of the heterogenous pattern of intestinal glucagons.

First it was established that boiling in water for 20 min did not affect the immunoreactivity of glucagon (not shown). Extraction of intestinal mucosa by boiling was accomplished in the following manner: Frozen mucosa was crushed finely and plunged into 4 volumes of vigorously boiling distilled water. After 20 min boiling the suspension was cooled in an ice bath to 0 °C, acidified with glacial acetic acid to 1 mol/l and homogenized in a Waring type blender for 1 min. After 4 h in the cold the suspension was centrifuged at 2000 × G for 30 min and the supernatant deep frozen or freezedried.

Further Purification

The different approaches are summarized in Tables 1 and 2. First the standard procedure of Kenny [18], slightly modified [3] as shown in part A, Table 1. Second (part B, Table 1), extraction of dried acid ethanol extracts with either 0.4 mol/l sodium phosphate, pH 7.5, or 50% acetone [19–20], and precipitation with acidified 97% acetone [20]. Acetone was UVASOL®, No. 22, Merck, Darmstadt. Third (part C, Table 1), salting out the acid extract after evaporation of ethanol with 15% (W/W) sodium chloride [21] or 20% sodium chloride [22]. Fourth (Table 2), salting out boiled extracts with 20% sodium chloride or ammonium sulphate [23]. The same extract of intestinal mucosa was used for all studies on acid ethanol extracts, and the whole experiment was performed twice. All precipitates that formed with the different procedures were, sometimes after further extraction with 0.4 mol/l sodium phosphate buffer, pH 7.5, solubilized with 8 mol/l urea in 1 mol/l acetic acid, and dialyzed in 8/32 Visking tubes for 48 h against several changes of 1 mol/l acetic acid at 4 °C. All fractions dissolved in 0.4 mol/l phosphate buffer were first subjected to radioimmunoassay, appropriately diluted, and then dialyzed against 1 mol/l acetic acid prior to gel filtration. Sodium phosphate buffer, 0.4 mol/l, pH 7.5, was chosen because glucagon, which is poorly soluble in dilute salt solutions of this pH, will dissolve in this buffer up to a concentration of 0.3 mmol/l; on the other hand this buffer cannot be used at 4 °C.

From the results in Tables 1 and 2 and Figures 2 and 3 a number of conclusions can be drawn: 1. The overall yield of glucagon-like immunoreactivity is probably not affected by the initial extraction procedure; 2. There are very small losses of total immunoreactivity and no losses of immunoreactive components in the various precipitates of the Kenny procedure; 3. Acetone extraction with subsequent precipitation is as effective as ethanol-ether as a means of purification, whereas evaporation of the acid-ethanol to dryness leads to serious losses of immunoreactivity; 4. 15% NaCl will not, at these protein concentrations, salt out the immunoreactivity, whereas 20% is fully effective. Thus, the combination of acid-ethanol extraction and salting out represents a useful alternative to the Kenny procedure; 5. Ammonium sulphate added to 40% saturation (at 4 °C (24)) will, at the present concentration of protein, precipitate 87% of the immunoreactivity present.

The gel filtration pattern of immunoreactivity appeared unaffected by the various purification procedures. Consistently only one peak with K_{av}

Table 1. Purification of acid ethanol extracts

	Procedure	GLI[a]	Gel filtration
Acid ethanol extract of porcine intestinal mucosa	A add NH_3 (25 %) to pH 8.0 centrifuge → supernatant + 1.8 vol abs ethanol + 3 vol ether at −20°C → precipitate dried in vacuum, extracted with 0.4 mol/l sodium phosphate pH 7.5 centrifuge → supernatant	0.14–0.37	1 peak (Fig. 2 A)
	→ precipitate dissolved in 8 mol/l urea, 1 mol/l acetic acid. Dialysed against 1 mol/l acetic acid	0.023–0.052	no peaks
	→ precipitate extracted with 0.4 mol/l sodium phosphate pH 7.5 centrifuge → supernatant	0.002–0.012	no peaks
	→ precipitate dissolved in 8 mol/l urea, 1 mol/l acetic acid. Dialysed against 1 mol/l acetic acid.	0.012	no peaks
	B evaporated to dryness extracted with 50% acetone in water. centrifuge → dry matter extracted with 0.4 mol/l sodiumphosphate, pH 7.5	0.066	not tested
	→ precipitate extracted with 75% acetone in water. centrifuge → precipitate dissolved in 8 mol/l urea 1 mol/l acetic acid. Dialysed against 1 mol/l acetic acid.	0.0006	not tested
	→ Supernatants combined acetone added to 97%. 4 N HCl added dropwise till precipitate is formed. centrifuge → supernatant discarded		
	→ precipitate extracted with 0.4 mol/l sodium phosphate pH 7.5 centrifuge → supernatant	0.040	1 peak Kav = 0.23
	→ precipitate dissolved in 8 mol/l urea, 1 mol/l acetic acid. dialysed against 1 mol/l acetic acid	0.034	1 peak (Fig. 2 B)
	C Ethanol evaporated off, residue diluted with 0.001 N HCl. centrifuge → supernatant + NaCl 15% (w/w) for 4 h at 4°C centrifuge → precipitate dissolved in 2 mol/l urea 1 mol/l acetic acid, dialysed against 1 mol/l acetic acid	0.040	1 peak (Fig. 2 C)
	→ supernatant	0.16	not tested
	→ supernatant + NaCl 20% (w/w) for 4 h at 4°C centrifuge → supernatant	0.017	not tested
	→ precipitate dissolved in 2 mol/l urea, 1 mol/l acetic acid, dialysed against 1 mol/l acetic acid	0.29	1 peak Kav = 0.25
	→ precipitate dissolved in 8 mol/l urea, 1 mol/l acetic acid, dialysed against 1 mol/l acetic acid	0.0057–0.0085	not tested

[a] Amount of immunoreactive material in the fraction expressed as nmol-equivalents/g mucosa (wet weight) as assayed against standards of glucagon. Where several fractions were analysed, the range is shown

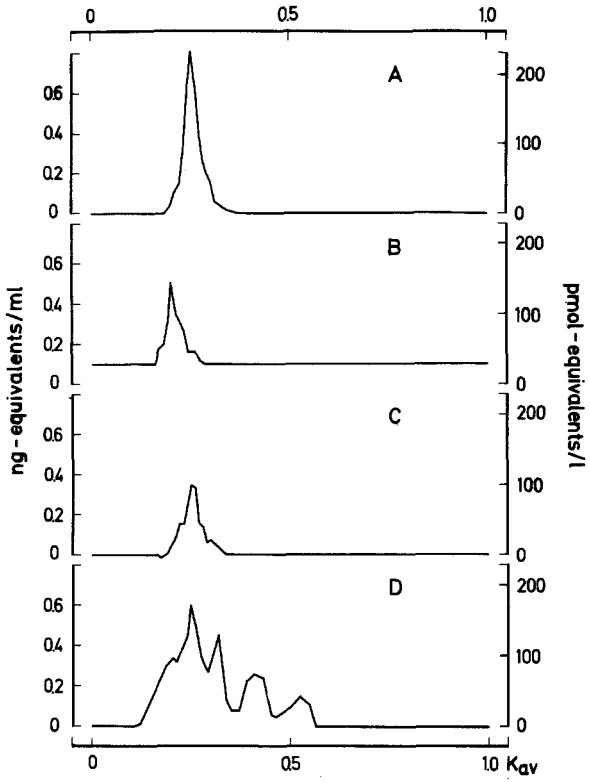

Fig. 2. Elution pattern of glucagon-like immunoreactivity in extracts of porcine small intestine after various purification procedures, and gel filtration on 2000 × 22 mm Sephadex G-50 SF columns eluted with 0.5 mol/l acetic acid. The capital letters A-D refer to the different purification procedures, as indicated in the outer right columns of Tables I and II. Ordinates: concentration of immunoreactivity in the column effluent, as measured with the assay system for total GLI against standards of glucagon. Abscissa: coefficient of distribution (K_{av})

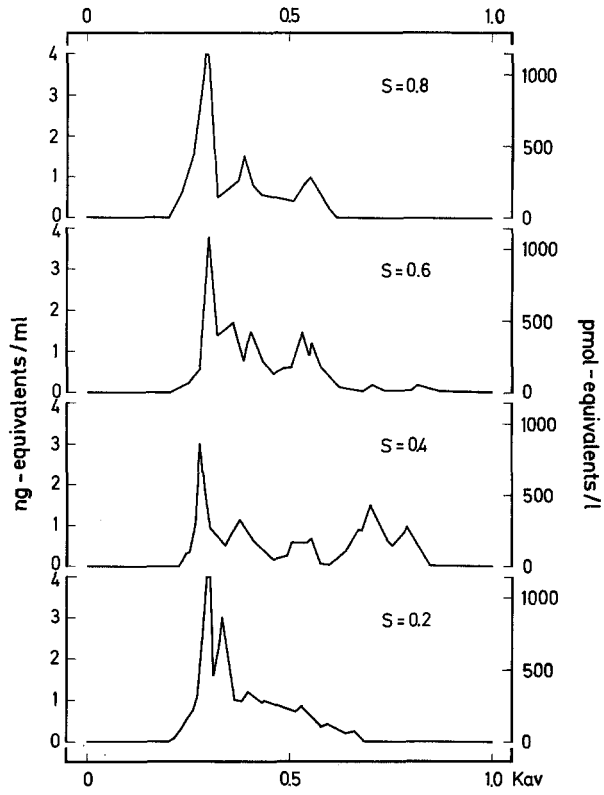

Fig. 3. Elution pattern of glucagon-like immunoreactivity in extracts of porcine small intestine, prepared by the boiling method, and purified further by salting out with ammonium sulphate at various degrees of saturation as indicated in Table II. Finally filtered on 2000 × 22 mm Sephadex G-50 SF columns eluted with 0.2 mol/l glycine buffer, pH 8.8, containing in addition 0.1% albumin. Ordinates: concentration of immunoreactivity in the column effluent, as measured with the assay system for total GLI against standards of glucagon. Abscissa: coefficient of distribution (K_{av})

0.24–0.31 was found in acid ethanol extracts. The extracts obtained by boiling appeared more heterogenous (Fig. 2, D). As at least two molecular forms of GTG were expected [1, 2], the dialysis step, common to all purifications and regularly used by other workers [18, 23] was examined in further experiments.

It was found that iodinated glucagon does not pass Visking dialysis tubing but adheres firmly to the wall. Unlabelled glucagon on the other hand will pass through, depending on the time of dialysis. 50% of unlabelled glucagon in a concentration of 6.5 nmol/l can be recovered from 50 volumes of dialysis buffer after 24 hours at 4 °C. Dialysis in Visking tubes was therefore abandoned. Undamaged membrane filters (Diaflo, UM 2, Amicon) retain glucagon fully. This probably explains partly the preservation of lower molecular size components in the boiling-ammonium-sulphate experiments (Fig. 3).

As a result of the above investigations, two procedures were defined, one for analytical investigations of small samples, the other for convenient handling of large samples for preparative purposes. The first is Kenny's acid-ethanol technique, slightly modified [4]. The other is a combination of extraction by boiling, freeze-drying of the acidified supernatant, repeated extraction (3 times) with icecold 50% acetone, precipitation with acidified 97% acetone at −20 °C, drying of precipitate in a stream of N_2, and final dissolution of the precipitate in dilute HCl added to a pH of 2.5. When processing very large samples (more than 1 kg of mucosa) salting out with 20% (W/W) NaCl and subsequent pressure dialysis is included at this stage. The final solutions are then subjected to gel filtration. Recovery of labelled glucagon added to the extraction medium was 59.3% and 67.6% respectively, the latter not including salting out. During the subsequent study of porcine gastrointestinal tissue, these two techniques were used in parallel.

Table 2. Purification of Extracts obtained by boiling

	Procedure		GLI[a]	Gel filtration
extract of porcine intestinal mucosa obtained by boiling	NaCl added to 20% (W/W) 4 h at 4°C centrifuge →	Supernatant	0.02	not tested
		precipitate dissolved in 2 mol/l urea, 1 mol/l acetic acid dialysed against 1 mol/l acetic acid	0,25–0,26	4 peaks (Fig. 2 D)
	+ ammonium sulphate to sat. 0.2 centrifuge → supernatant	precipitate diluted with and pressuredialysed with 0.5 mol/l acetic acid. Freeze-dried and dissolved in glycine buffer	0.15	see Fig. 3
	+ ammonium sulphate to sat. 0.4 centrifuge → supernatant		0.057	
	+ ammonium sulphate to sat. 0.6 centrifuge → supernatant		0.012	
	+ ammonium sulphate to sat. 0.8 centrifuge → supernatant discarded →		0.014	

[a] Amount of immunoreactive material in the fraction expressed as nmol-equivalents/g mucosa (wet weight). Where several fractions were analysed the range is show

Results

The gel filtration results are shown in Figures 4–8 and summarized in Table 3.

The porcine gastric mucosa is divided into four easily identified parts: the oesophageal region, the cardiac gland region, the fundus gland region, and the pyloric region [25], each of which was extracted and analysed separately. In pigs of 25 kg the total weight of the gastric mucosa (including the muscularis mucosae layer) is distributed approximately as follows: oesophageal region: 5 g; cardiac gland region: 13–16 g; fundic gland area: 40–50 g; and the pyloric gland region: 12–15 g (range of 2–3 determinations).

1. The Oesophageal Region. 2.5 g (wet weight) was extracted with acid-ethanol and 5 g by boiling. The concentration of GLI was less than 0.003 nmol-eq/g (i. e. the detection limit for assay on crude tissue extracts, which are routinely diluted at least 100 times before assay), and no peaks could be identified on gel filtration.

2. The Cardiac Gland Region. GLI-concentrations ranged from 0.01 to 0.04 nmol-eq/g (3 determinations) of which approximately 80% was PTG. Results were identical with either technique of extraction. The gel filtration pattern is seen in Figure 4. A large peak with a distribution coefficient (K_{av}) of 0.66 was identified equally well by all three assays, and co-eluted with glucagon marker ($K_{av} = 0.65$). A smaller GTG peak (i. e. a peak, which is detected with the GLI-assay, but not the PTG-assay) eluted with a K_{av} of 0.49.

3. The fundus and pyloric gland regions. In spite of extractions with either technique of more than 40 g and 15 g respectively, the extracted amounts of GLI were insignificant (less than 0.003 nmol-eq/g – 5 experiments) and no peaks were identifiable upon gel filtration (3 experiments, not shown).

4. The duodenum. The concentration of GLI ranged from 0.009 nmol-eq/g to 0.015 nmol-eq/g of which approximately 20% was PTG. The gel filtration results of both extractions are shown (Figs. 5 and 6). Both showed a significant PTG peak at K_{av} 0.66–0.65, corresponding to glucagon, and both peaks were registered with the receptor assay. The acid-ethanol extract, however, contained another PTG-peak with K_{av} 0.90, also detected with the re-

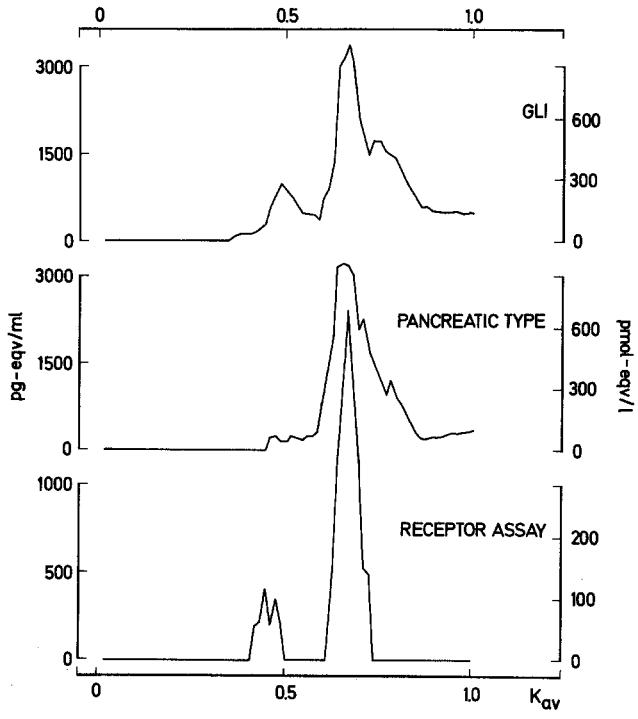

Fig. 4. Elution pattern of glucagon-like components in an extract of the cardiac gland region of the porcine gastric mucosa, extracted and purified by the boiling method and filtered on 50 × 1000 mm columns of Sephadex G-50 SF, eluted with 0.5 mol/l acetic acid. Extract of 16 g mucosa (wet weight) was applied to the columns, and the effluent lyophilized and reconstituted in assay buffer (12) or a Krebs-Ringer-bicarbonate solution (15) before assay. Each eluted fraction was assayed in duplicate using assay systems for total GLI (upper diagram), pancreatic type glucagon (central diagram), and using radioreceptor-assay (lower diagram). Standards were highly purified glucagon (NOVO Research Institute, Copenhagen) which necessitates the use of the unit pg or pmol equivalents of glucagon per ml or 1. Concentrations in the effluent as indicated on the ordinates. Note the different scales. Abscissa: coefficient of distribution

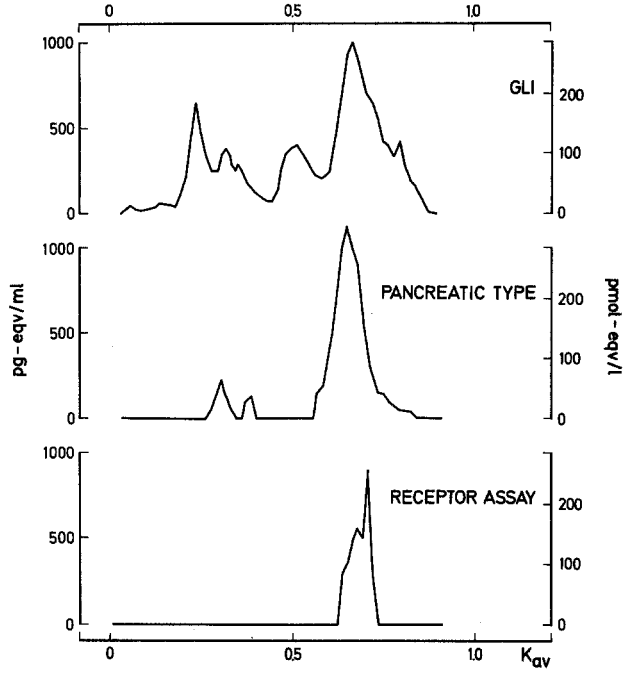

Fig. 5. Elution pattern of glucagon-like components in an extract of 15 g of porcine duodenal mucosa, obtained by the boiling method and separated by gel filtration. For explanations see legend to Fig. 4

ceptor-assay. Furthermore, a PTG peak ($K_{av} = 0.30$) and two GTG peaks were found (Fig. 5). This was the only discrepancy noted between the component patterns of the two extraction methods.

5. Small intestine. The whole of the porcine small intestinal mucosa weighs 600–800 g (range of 8 determinations in 20–30 kg pigs). The concentration of GLI is high with a maximum of 0.42 nmol-eq/g in the ileum, of which approximately 5% is PTG. The elution pattern is shown in Figure 7. This pattern has been reproduced 6 times irrespective of extraction method. It shows the following characteristic features: Two major peaks of GTG, one with $K_{av} = 0.22$ and one with $K_{av} = 0.49$. Refiltration of these components does not change their elution pos-

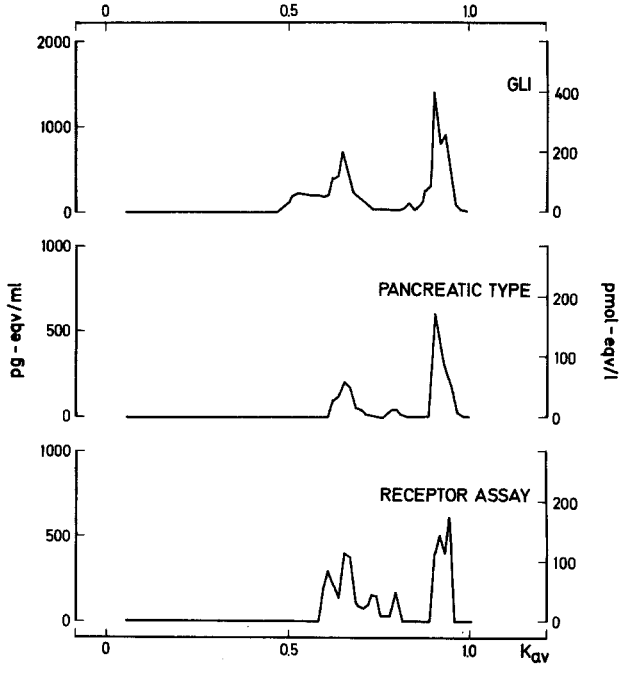

Fig. 6. Elution pattern of glucagon-like components in an acid-ethanol extract of 3.9 g of porcine duodenal mucosa, separated by gel filtration. For explanations see legend to Fig. 4

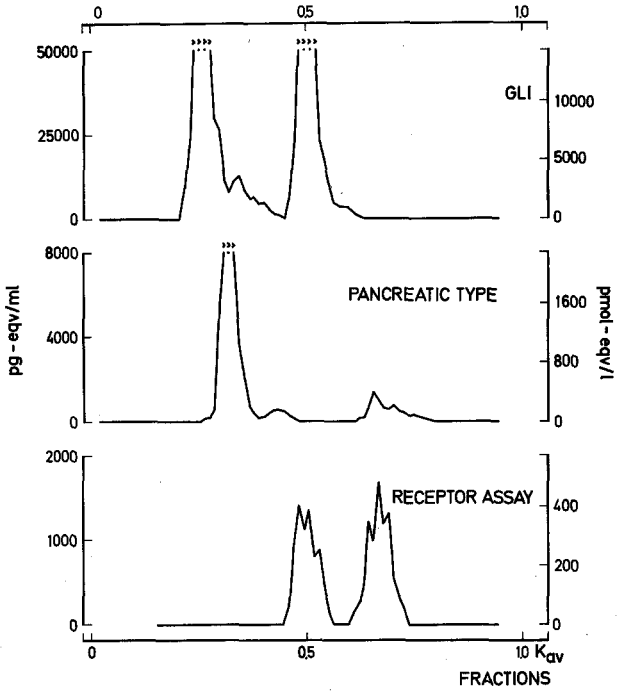

Fig. 7. Elution pattern of glucagon-like components in an acid-ethanol extract of 21.4 g of porcine ileum-mucosa, separated by gel filtration. For explanations see legend to Fig. 4

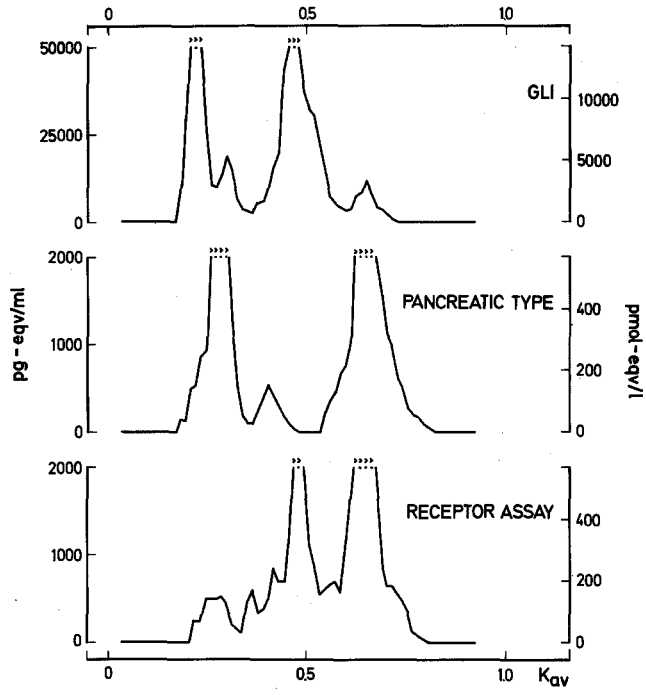

Fig. 8. Elution pattern of glucagon-like components in an acid-ethanol extract of 21.2 g of porcine colon-mucosa, separated by gel filtration. For explanations see legend to Fig. 4

Table 3. Concentration and components of glucagon-like immunoreactivity in porcine gastrointestinal mucosa

Region of gastro-intestinal mucosa	Concentration of GLI in nmol-equivalents/g tissue	Percentage PTG of GLI	PTG-components K_{av} (approximate) 0.30	0.65	0.90	GTG-components K_{av} (approximate) 0.22	0.49
oesophageal	<0.003	–					
cardiac	0.01–0.04	80%		+§			+§
fundus	<0.003	–					
pyloric	<0.003	–					
duodenum	0.009–0.015	20%	+	+§	+§	+	+§
small intestine	0.42	5%	+	+§		+	+§
colon (caecum)	0.3	12%	+	+§		+	+§

+ indicates the presence of that particular component
§ indicates that the component binds to hepatic glucagon receptors

ition (but the large GTG may be resolved into further components, see discussion). Further, a prominent peak of large PTG ($K_{av} = 0.30$) is consistently found and variable amounts of PTG ($K_{av} = 0.65$). Only small GTG ($K_{av} = 0.49$) and small PTG ($K_{av} = 0.65$) are receptor-active, the former with only one tenth of its immunometric concentration.

6. The colon. The colon is a huge viscus in the pig and only data for the caecum and the proximal part of the spiral coil are presented here. The concentration of GLI is high, 0.3 nmol-eq/g, with approximately 12% PTG. The pattern of components (Fig. 8) is similar to that of the ileum, but the PTG peaks are much more prominent. Again small GTG and small PTG are receptor-active.

Discussion

In the present report the various forms of glucagon-like immunoreactivity and glucagon-like receptor-activity in the porcine gastrointestinal mucosa are described. The major findings were:

1. The gastric mucosa of the pig contains a pep-

tide which, from its immunological behaviour, its molecular size, and its ability to compete with labelled glucagon for binding to porcine liver cell membranes, is indistinguishable from true glucagon. But this "true glucagon" is only found in the cardiac gland region, and only by 1/50–1/100 of its concentration in the canine oxyntic mucosa [4]. By contrast with dogs and cats [4] the pig seems to resemble primates in this respect. Bloom et al. [7] found approximately 1.1 μg-equivalent of PTG in the gastric fundus of the baboon, corresponding to 0.31 nmol-equivalents. The total amount of PTG in the porcine cardiac gland region may range from 0.15 to 0.60 nmol.

2. This "true glucagon" is also found in the duodenum, confirming the findings of Sasaki et al. [3], but only in minute amounts. On the other hand small amounts are found also in the small intestine and considerable amounts in the colon. Considering the size of the gastrointestinal mucosa in the pig, the gastrointestinal tract must be considered as an important source of extrapancreatic glucagon in this species. An apparently low molecular weight PTG component in duodenal extracts was also described by Sasaki et al. [3]. As this component was detected only after acid-ethanol extraction it may represent an extraction artefact. The duodenal GLI components are in any case, probably of minor physiological importance, because of their very low concentration. Furthermore, by immunofluorescence no glucagon-containing cells can be found in the porcine duodenum [4].

3. A peptide which is immunologically similar to glucagon, but eluting much earlier from the columns ($K_{av} = 0.30$, Figs. 5, 7, 8), was found in most gastrointestinal tissue, and in considerable amounts in the ileum and the colon. This peptide which probably corresponds to pancreatic "large glucagon", described by Rigoupoulo et al. [26], did not bind to the glucagon receptor on liver cells.

4. As expected [27] two forms of gut type glucagon were found in most tissues, and both in large amounts in the ileum and colon. The large GTG, however, appears heterogeneous, as repeated fractionation of large amounts of large GTG shows it to be composed of three components, a major component of $K_{av} = 0.22$, a smaller component of 0.25, and variable amounts of a component with a K_{av} of 0.36. Also, by polyacrylamide gel electrophoresis in urea containing 15% gels, large GTG is separable into three components (to be published). The canine large GTG was also heterogeneous [4], and so was the purified large GTG described by Murphy et al. [28]. The K_{av}: 0.22 – component of large GTG is believed to be identical to the GLI I purified by Sundby [14], which was selected for purification because it constituted the major part of the GLI in the gut extracts; furthermore, the GLI I elutes with a K_{av} of 0.22. Neither of the components of large GTG interacted with ^{125}I-labelled glucagon at the hepatic receptor, and the same is true for the GLI I. Furthermore, GLI I does not appear to posess a hepatic receptor of its own, because ^{125}I-labelled GLI does not bind to the porcine liver cell membranes under conditions in which labelled glucagon is strongly bound. Small GTG, regardless of gastrointestinal source, constantly competed with the labelled glucagon for binding to the membranes, consistent with earlier reports [15, 29] and consistent with the proposed glycogenolytic effect of this component [27, 30].

The physiological implications of these observations are difficult to assess. Obviously, if it can be proven that small GTG has an intrinsic glycogenolytic activity [30], and that it is being secreted in concentrations [31] sufficient to excert this activity, it may be of some importance for the control of glucose metabolism. A biological activity of large GTG has not yet been defined [28, 30, 32]. It may represent a pro- or storage form of one of the smaller peptides, but if this is the case it is present in remarkably high concentration compared to the end-products. Its putative physiological activity may lie in other areas of the gastrointestinal physiology [9, 33, 34].

The importance of the complete system of PTG peptides in the gut is more easily appreciated. Inappropriate secretion of glucagon has been held responsible for the hyperglycaemia of diabetes mellitus [35]. The diabetic syndrome resulting from pancreatectomy was explained by persistent secretion of extrapancreatic glucagon [36–38]. While the hyperglucagonaemia after pancreatectomy in dogs appears well documented [36–41], much controversy exists concerning the results of pancreatectomy in man [42–45].

In the present study the pig was chosen because of its physiological similarity to man and because earlier search had established that its gastric content of PTG was similar to that of the primates [4, 7]. In the pig persistent glucagonaemia and even hyperglucagonaemia, associated with hyperglycaemia, is seen several weeks after pancreatectomy (unpublished observations). In this species, also, extrapancreatic glucagon may be of considerable importance.

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