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Evidence that enteroglucagon (II) is identical with the *C*-terminal sequence (residues 33–69) of glicentin

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Enteroglucagon (II) was isolated from extracts of pig ileum mucosa by repeated gel filtrations, and its immunochemical and chromatographic characteristics were compared with those of a synthetic peptide corresponding to the 33–69 sequence of pig glicentin, before and after digestion with trypsin or trypsin followed by carboxypeptidase B, by using five region-specific assays covering most of the glicentin sequence. Enteroglucagon (II) and the synthetic peptide behave identically under three different conditions of chromatography as determined with all five assays (including a highly specific radioreceptor assay), and gave rise to similar fragments after enzyme digestion. It was therefore concluded that enteroglucagon (II) and the 33–69 sequence of glicentin are most probably identical.

Apart from glicentin (Sundby *et al.*, 1976; Holst, 1980; Thim & Moody, 1981), the L-cells of the pig intestinal mucosa produce a number of other peptides with glucagon-like immunoreactivity (GLI), including the so-called 'peak II GLI' or 'basic GLI', termed 'enteroglucagon (II)' in the present paper, which resembles glicentin in that it reacts exclusively with glucagon antibodies directed against the mid-sequence of the glucagon molecule (Holst, 1978), but is distinguished from glicentin by its smaller molecular size (Holst, 1977). Enteroglucagon (II) is present in high concentrations, but glicentin is the predominant form. In further contradistinction to glicentin, enteroglucagon (II) seems to possess glucagon-like bioactivity (Holst, 1978); furthermore, it has been suggested that enteroglucagon (II) is circulated in the plasma, and may thus fulfil some of the requirements of a true hormone (Holst, 1982*a*).

In the present paper I describe studies of the immunological and chromatographic characteristics of enteroglucagon (II) from pig ileal mucosa with the use of assays that are specific for different regions of the glucagon and glicentin molecules; my results strongly suggest that the sequence of enteroglucagon (II) is identical with the sequence of the *C*-terminal 37 amino acid residues of the glicentin molecule (Thim & Moody, 1981; Fig. 1). Enteroglucagon (II) thus in all probability contains the entire glucagon sequence (Holst, 1980; Moody & Thim, 1981) without *N*-terminal prolongations, a fact that may explain its glucagon-like biological activities (Holst, 1982*b*).

Experimental

Materials

Highly purified glicentin (Sundby *et al.*, 1976; Thim & Moody, 1981) was generously given by Dr. A. J. Moody, Novo Research Institute, Bagsværd, Denmark. Highly purified porcine glucagon and (monoiodinated) ^{125}I-labelled glucagon (Jørgensen & Larsen, 1972) and ^{125}I-labelled glicentin (Moody *et al.*, 1981) were also gifts from the Novo Research Institute. A synthetic peptide corresponding to the 37 *C*-terminal amino acid residues of glicentin (Yanaihara *et al.*, 1979) was a gift from Professor N. Yanaihara, Shizuoka College of Pharmacy, Shizuoka, Japan, and so was antiserum 4804, raised against a synthetic peptide corresponding to the last 20 amino acids of glicentin (Yanaihara *et al.*, 1981). Antiserum R 64, which was raised against glicentin, was a gift from Dr. A. J. Moody, Novo Research Institute.

Bovine trypsin [L-1-chloro-2-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated] was from Worthington Corp., Freehold, NJ, U.S.A.; carboxypeptidase B (phenylmethanesulphonyl fluoride-

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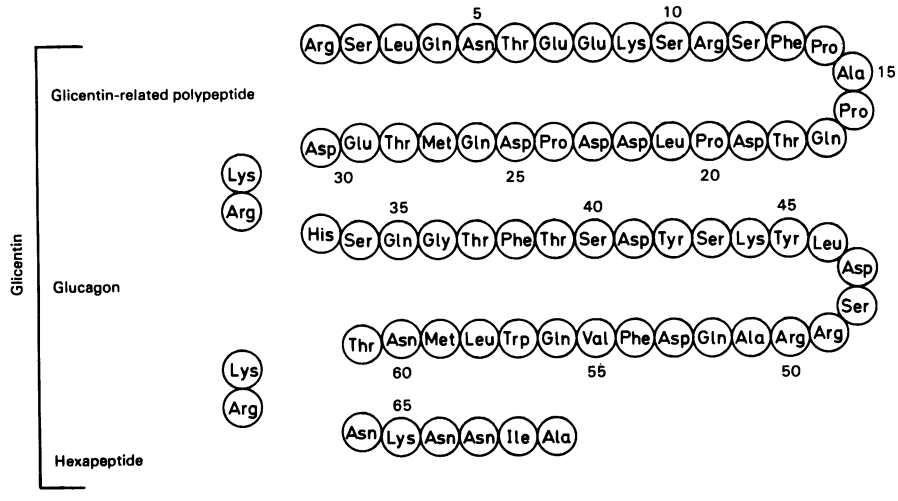

Fig. 1. *Amino acid sequence of pig glicentin (Thim & Moody, 1981)*
The suggested sites of cleavage for the generation of glucagon and glicentin-related polypeptide (Thim & Moody, 1981) are indicated to the left.

treated) was from Merck (catalogue no. 2300), Darmstadt, W. Germany; soya-bean trypsin inhibitor (T 9003) was from Sigma Chemical Co., St. Louis, MO, U.S.A.; human serum albumin (reinst, trocken) was from Behringwerke, Marburg, W. Germany, and aprotinin was from the Novo Research Institute. ^{125}I-labelled albumin and ^{22}NaCl were from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were analytical grade. Sephadex G-50 (fine grade and superfine grade) and columns (catalogue nos. K 50/100 and 16/100) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Polygelinum (Haemaccel) was from Behringwerke.

Methods

Gel filtrations were performed in two different column systems: (1) K 16/100 columns (1000 mm × 16 mm) of Sephadex G-50 (fine grade) equilibrated and eluted at 4°C with 125 mM-NH_4HCO_3 adjusted to pH 9.0 and supplemented with human serum albumin (2 g/l), NaCl (0.1 M) and thiomersal (0.6 mM) at a flow rate of approx. 20 ml/h; (2) K 50/100 columns (1000 mm × 50 mm) of Sephadex G-50 (superfine grade) equilibrated and eluted at 4°C with 0.5 M-acetic acid at a flow rate of approx. 60 ml/h.

For both systems fractions corresponding to approx. 0.02 bed vol. were collected automatically. Sample size never exceeded 2% of bed volume. Radioimmunological analyses were performed with the following five assay systems: (*a*) assays for the glucagon sequence 6–15 were made with antiserum 4304, glucagon standards and ^{125}I-labelled glucagon as previously described (Holst, 1980); (*b*) assays for the glucagon sequence 19–29 were made with antiserum 4305, glucagon standards and ^{125}I-labelled glucagon as previously described (Holst, 1977, 1980); (*c*) assays for the 15–30 sequence of the glicentin molecule (Thim & Moody, 1981; Fig. 1) were performed with antiserum R 64, glicentin standards and ^{125}I-labelled glicentin as previously described (Moody *et al.*, 1981); (*d*) assays for the 61–69 sequence of the glicentin molecule (Thim & Moody, 1981) were made with antiserum 4804, which was raised against synthetic glicentin-(49–69)-peptide [and therefore does not recognize intact glucagon or glucagon-(19–29)-peptide (Yanaihara *et al.*, 1981; J. J. Holst, unpublished work)], glicentin standards and ^{125}I-labelled glicentin as tracer; incubation and separation methods were similar to those employed for the glucagon assays; (*e*) a radioreceptor assay for glucagon based on the binding of ^{125}I-labelled glucagon to pig liver cell membranes for determination of a part of the glucagon sequence, which probably comprises the 21 *N*-terminal amino acids (Holst, 1975, 1977, 1982*b*).

These five different assays cover most of the glicentin sequence, as indicated schematically in Fig. 2; parts of the molecule are displayed separately to illustrate the binding regions of the assays as well as possible products of glicentin biotransformation (Moody & Thim, 1981; Holst, 1982*b*). Note that the

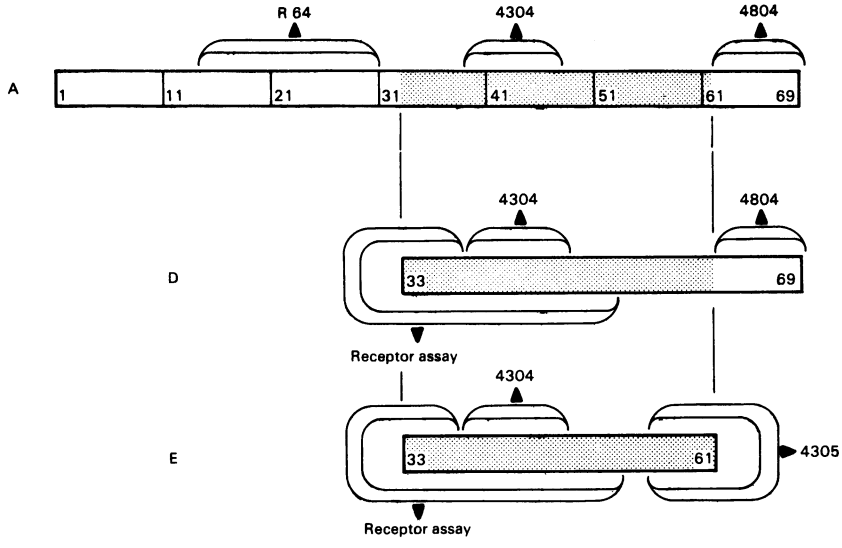

Fig. 2. *Schematic representation of the binding regions of the assay systems employed as illustrated against the sequence of the glicentin molecule*

'A' represents the entire glicentin molecule. 'D' represents the 33–69 sequence of glicentin, and 'E' the 33–61 sequence, which is identical with glucagon (stippled area). Note that the assay for the C-terminal glucagon sequence (with antiserum 4305) and the receptor assay require unextended terminals of the glucagon sequence for binding.

assay for glucagon-(19–29)-peptide (antiserum 4305) requires the absence of C-terminal extensions for recognition of the 19–29 sequence (Holst, 1980); likewise the radioreceptor assay probably requires a free and exposed N-terminal sequence of glucagon for recognition of a ligand (Holst, 1982*b*).

Extractions were by an acid/ethanol method as previously detailed (Method II described by Newgard & Holst, 1981). Pig ileal mucosa was obtained from anaesthetized pigs and frozen immediately as described previously (Holst, 1977); the frozen tissue was homogenized and extracted with acid/ethanol at −20°C and centrifuged, and the supernatant was mixed with ice-cold diethyl ether. The aqueous proteinaceous phase was isolated at −50°C, redissolved in distilled water and subjected to gel filtration in acetic acid.

Enzymic degradations were performed either at 22°C or at 37°C for 30–120min, with trypsin at 10–100μg/ml. When this was followed by a carboxypeptidase incubation, trypsin activity was inhibited by addition of soya-bean trypsin inhibitor. The incubations were terminated by the addition of aprotinin (5000 kallikrein-inhibiting units at 0°C), and the mixture was applied immediately to the column. All experiments were performed at least in triplicate, and only representative examples of reproducible experiments are shown.

Elution positions are referred to by the coefficient of distribution: $K_d = (V_e - V_0)/V_i$, where V_e is the elution volume for the substance in question, V_0 is the exclusion volume and V_i is the available inner volume, determined as the difference between the elution volumes of ^{125}I-labelled albumin and $^{22}Na^+$, which were added to all samples to be filtered, as internal standards.

Results

Fig. 3 shows the results of the analyses of one of the extracts of pig ileal mucosa ($n = 6$). Enteroglucagon (II), which is defined as the second most predominant component with glucagon-like immunoreactivity corresponding to the middle part of the glucagon sequence (assay with antiserum 4304), is located as a well-defined peak at K_d 0.5. The antiserum against the C-terminal glucagon sequence does not recognize this component (Fig. 3*b*), nor does the antiserum against the 15–30 sequence of glicentin, which clearly reacts with glicentin at K_d 0.25 (Fig. 3*c*). The assay against the glicentin 61–69 sequence (with antiserum 4804) and the receptor assay both recognize the component at K_d 0.5, the receptor assay, however, with apparently lower affinity, in that it measures about 1/50th of, e.g., the assay for the 6–15 sequence (with antiserum 4304).

The left-hand sections of Figs. 4 and 5 show the

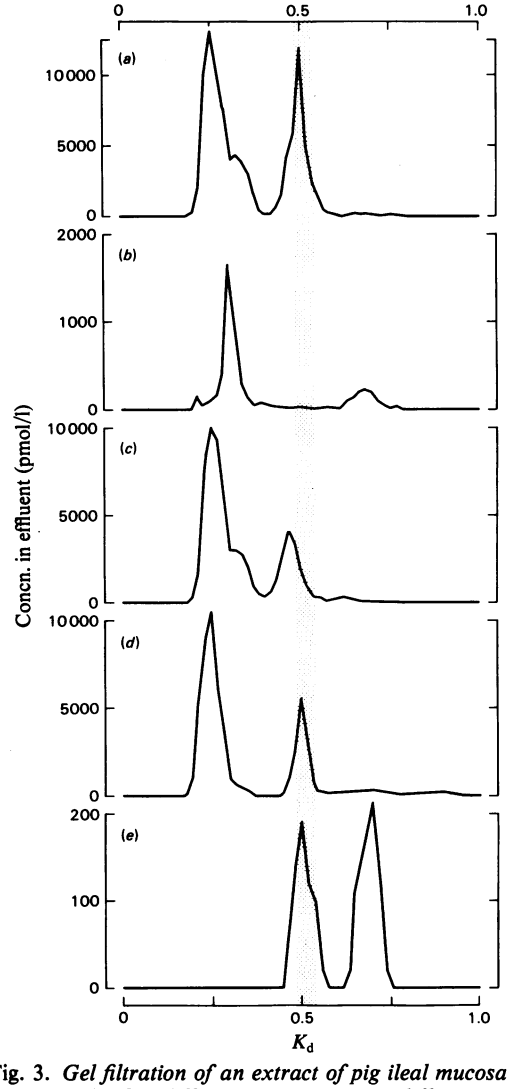

Fig. 3. *Gel filtration of an extract of pig ileal mucosa as measured by five different assays against different parts of the sequence of glicentin (see Fig. 2)*

The ordinates show effluent concentrations as measured with the glucagon 6–15 assay (with antiserum 4304, panel *a*), the glucagon 19–29 assay (with antiserum 4305, panel *b*), the glicentin 15–30 assay (with antiserum R 64, panel *c*), the glicentin 61–69 assay (with antiserum 4804, panel *d*) and the radioreceptor assay (panel *e*). The effluent concentrations were plotted against coefficient of distribution, K_d. Enteroglucagon (II) is eluted at K_d 0.5. The effluent corresponding to the stippled area was pooled and used for the experiments illustrated in Figs. 4 and 5. Column system 2 was employed.

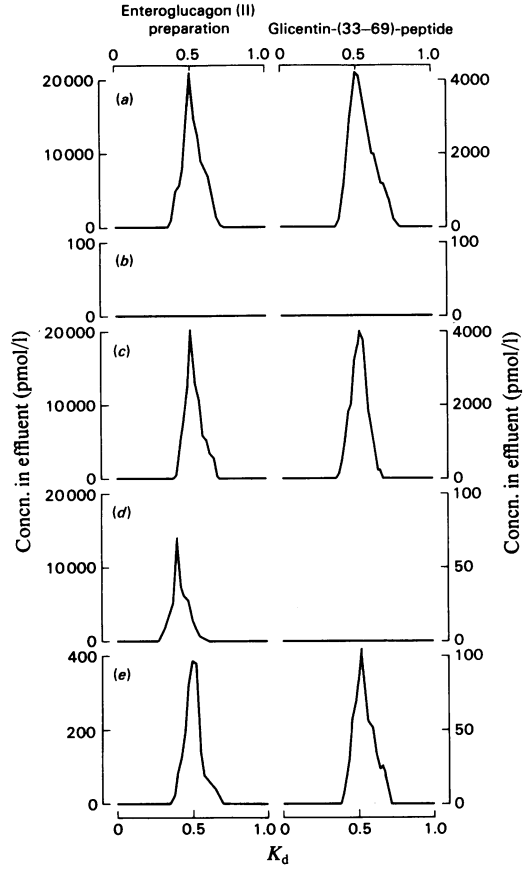

Fig. 4. *Gel filtration of enteroglucagon (II) (K_d 0.5 effluent from gel filtration of pig ileal extracts; see Fig. 3) (left-hand sections) and synthetic glicentin-(33–69)-peptide (right-hand sections) in acetic acid, as measured by assays for different parts of the glicentin sequence (see Fig. 2)*

The ordinates show the effluent concentrations as measured with the glucagon 6–15 assay (panel *a*), the glucagon 19–29 assay (panel *b*), the glicentin 61–69 assay (panel *c*), the glicentin 15–30 assay (panel *d*) and the radioreceptor assay (panel *e*). The concentrations were plotted against coefficient of distribution, K_d. Column system 2 was employed.

results of further experiments with enteroglucagon (II) isolated from the gel filtrations shown in Fig. 3 at the K_d interval 0.48–0.54. The right-hand sections show similar experiments performed with a synthetic peptide corresponding to the sequence of the *C*-terminal 37 amino acids of glicentin.

Fig. 4 illustrates the identical behaviour of enteroglucagon (II) and the synthetic peptide on gel filtration in acetic acid as determined with the glucagon 6–15 assay (with antiserum 4304), the glicentin 61–69 assay (with antiserum 4804) and the receptor assay. The glucagon 19–29 assay (with antiserum 4305) was negative in both experiments. The glicentin 15–30 assay (with antiserum R 64) identifies a component in the enteroglucagon (II)

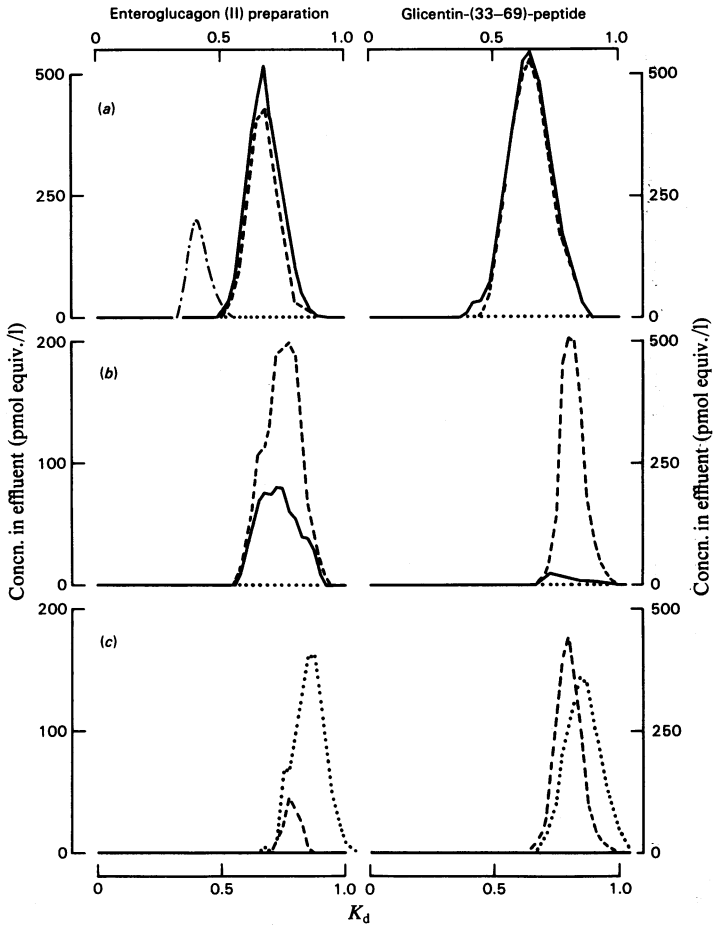

Fig. 5. *Gel filtration of enteroglucagon (II) (left-hand sections) and synthetic glicentin-(33–69)-peptide (right-hand sections) before and after digestion with trypsin or trypsin + carboxypeptidase B, as measured by assays for different parts of the glicentin sequence (see Fig. 2) and plotted against coefficient of distribution, K_d*

Enteroglucagon (II) (K_d 0.5 effluent from gel filtration of pig ileal extracts; see Fig. 3) and 10μg of synthetic glicentin-(33–69)-peptide (the purity of which was uncertain) were applied to the columns (column system 1) in what appeared to be immunochemically similar amounts before (*a*) or after (*b*) incubation with trypsin (20μg/ml for 1 h) or (*c*) incubation with trypsin (100μg/ml for 1 h) followed by addition of soya-bean trypsin inhibitor (20μg/ml) and carboxypeptidase B (20μg/ml) for 1 h. ——, Glucagon 6–15 assay (with antiserum 4304); ·····, glucagon 19–29 assay (with antiserum 4305); – – – –, glicentin 61–69 assay (with antiserum 4804); —·—, glicentin 15–30 assay (with antiserum R 64).

preparation, but its elution position differs from enteroglucagon (II) (K_d 0.40 versus 0.50); from the results in Fig. 3(*c*) it is clear that this is a component with glicentin-like immunoreactivity contaminating the enteroglucagon (II) preparation.

Fig. 5 shows gel filtrations of enteroglucagon (II) and the synthetic peptide at pH 9 (note the change in elution position from 0.50 in acetic acid to 0.65 in NH_4HCO_3). Again the glucagon 6–15 assay and the glicentin 61–69 assay (with antisera 4304 and 4804 respectively) identify the two moieties in a similar manner, whereas the glucagon 19–29 assay (with antiserum 4305) is negative. The contaminating glicentin-like peptide is even better separated in this column system (Fig. 5*a*).

Fig. 5(*b*) shows the results of tryptic digestion. Both moieties gave rise to a new component with immunoreactivity exclusively in the glicentin 61–69 assay at K_d 0.80, whereas other antigenic determinants were much diminished or lost. By further tryptic digestion (200μg/ml for 2 h at 37°C), the component active in the glicentin 61–69 assay as

well as the one active in the glucagon 6–15 assay are further degraded and finally disappear (results not shown). Fig. 5(*c*) shows the results of limited tryptic digestion (similar to that shown in Fig. 5*b*) and subsequently carboxypeptidase treatment. Again the reactive component in the glicentin 61–69 assay at K_d 0.80 is seen, but a component that is active in the glucagon 19–21 assay (with antiserum 4305) at K_d 0.85 appears in addition.

In further gel-filtration experiments with enteroglucagon (II) and the synthetic peptide, but this time in acetic acid containing 0.2% Polygelinum, the elution position of both moieties changed to K_d 0.59.

Discussion

The analytical tool employed in the present investigation was a combination between high-resolution gel filtration and region-specific radio-(immuno)analysis.

The gel-filtration system employed (Sephadex G-50) shows pronounced sorption effects (Eaker & Porath, 1967), whereby the separation system must be regarded as a system that combines gel-permeation with ion-exchange and hydrophobic chromatography. Similar elution positions, as observed in the present work with enteroglucagon (II) and synthetic glicentin-(33–69)-peptide, therefore do not necessarily reflect identical molecular sizes. However, the identical changes in elution positions after filtration of the two moieties under different conditions (acetic acid, acetic acid + Polygelinum and NH_4HCO_3 at pH 9) strongly suggest that the two have similar molecular size as well as charge and apolar properties. The changes in elution positions must be regarded as being highly significant, in view of my finding that the standard deviation of the K_d values on repeated gel filtration is below $0.02 K_d$ (Holst, 1980).

The other half of the analytical system was chosen so that the greatest possible part of the glicentin sequence was covered by region-specific assays. Particularly useful were the receptor assay and the *C*-terminal glucagon assay (with antiserum 4305), since both assays required intact and free, i.e. non-extended, *N*- and *C*-terminal parts of the glucagon sequence respectively (Holst, 1980, 1982*b*); the least extension or minor modification at the terminals leads to complete or severe losses of reactivity in both assay systems.

The assay with antiserum R 64 showed that the present enteroglucagon (II) preparation did not contain the antigenic determinant of the glicentin molecule, against which this antiserum is directed. As previously suggested, this determinant is found in the *N*-terminal sequence of glicentin (Moody & Thim, 1981; Thim & Moody, 1981), probably at the 15–30 sequence region.

A peptide with K_d 0.40, which exhibited reactivity solely in the assay with antiserum R 64, was found in the original extracts ($n = 6$) as well as contaminating the enteroglucagon (II) preparation; after refiltration it was well separated from enteroglucagon (II), however. This component reactive in the assay with antiserum R 64 probably corresponds to the glicentin-related polypeptide, previously isolated from the pancreas (Moody *et al.*, 1981; Moody & Thim, 1981), and believed to be secreted from the pancreas synchronously and in equimolar amounts with glucagon, as a consequence of cleavage of the glicentin molecule at the basic residues 31 and 32, whereby two peptides are generated, glicentin-related polypeptide and glicentin-(33–69)-peptide (see Fig. 1).

If glicentin acts as the biosynthetic precursor in the intestinal mucosa, as well as in the pancreas (Moody *et al.*, 1981), then the presence of glicentin-related polypeptide in extracts of pig ileal mucosa provides further evidence that a peptide corresponding to glicentin-(33–69)-peptide must be present in the same tissue.

The reactivity of the enteroglucagon (II) preparation in the assay with antiserum 4304 proves that it contains the antigenic determinant of the 6–15 sequence of glucagon, in which respect it behaves like the synthetic peptide. The reactivity with antiserum 4804, which was raised against synthetic glicentin-(50–69)-peptide and does not react with glucagon (Yanaihara *et al.*, 1981), shows that enteroglucagon (II) also contained the antigenic determinant of the *C*-terminal octapeptide of glicentin; in this respect also enteroglucagon (II) behaved identically with the synthetic peptide.

The radioreceptor assay probably requires a rather long intact sequence of glucagon for recognition, as discussed in detail by Holst (1982*b*); it is likely that as much as the 1–23 sequence must be present to provide a binding energy to the receptor that amounts to just a small fraction of that of glucagon; furthermore, the slightest *N*-terminal prolongation leads to complete loss of reactivity (see Holst, 1982*b*). The positive identification of enteroglucagon (II) by the receptor assay, which is in perfect agreement with my previously published results (Holst, 1977), strongly suggests that enteroglucagon (II) does indeed contain the *N*-terminal glucagon sequence up to maybe residue 20, and without *N*-terminal extensions. Again, the reactivity of the synthetic peptide in this assay was very similar to that of enteroglucagon (II).

As expected, because of the suspected prolongation of the molecule at the *C*-terminus, the assay against the *C*-terminal glucagon sequence (with antiserum 4305) was completely negative with enteroglucagon (II) as well as the synthetic peptide. According to the hypothesis, however, entero-

glucagon (II) should contain the entire glucagon sequence, and to expose the C-terminal glucagon sequence I subjected enteroglucagon (II) as well as the synthetic peptide to trypsin and carboxypeptidase digestion as previously described (Holst, 1980), whereby a fragment corresponding to glucagon-(19–29)-peptide should be generated; according to previous experience with the same column system, the elution position of this fragment, which should be identified solely by the C-terminal glucagon assay (with antiserum 4305), should be K_d 0.85 ± 0.02 (mean $\pm$ S.D.). Indeed, after carboxypeptidase treatment of enteroglucagon (II) as well as the synthetic peptide, such a component appeared at that position. Most probably, therefore, the glucagon sequence 19–29 was present in the peak II preparation. Incubations with trypsin (or carboxypeptidase B) alone did not generate C-terminal glucagon immunoreactivity, but the glucagon 6–15 immunoreactivity (with antiserum 4304) disappeared rapidly with low trypsin concentrations, and the elution position of C-terminal glicentin immunoreactivity (with antiserum 4804) shifted more and more to the right, indicating progressive cleavage of the molecules from the N-terminus; with the synthetic peptide such cleavage might take place between residues 44 and 45, whereby the glucagon 6–15 immunoreactivity is lost, and between residues 49, 50 and 51, residues 62, 63 and 64 and residues 65 and 66; this is in agreement with the fact that extended trypsin degradation leads to smaller and smaller fragments and also that the reactivity in the glicentin 61–69 assay (with antiserum 4804) disappears last. Also in these experiments the enteroglucagon (II) and the synthetic peptide gave rise to remarkably similar degradation products; somewhat higher trypsin concentrations were necessary with enteroglucagon (II), in agreement with the fact that no attempts had been made to purify the material beyond that obtained by the repeated gel filtrations.

I have here provided evidence that enteroglucagon (II) contains the entire glucagon sequence and the same C-terminal 8-amino acid-residue extension of glicentin. The exactly identical elution positions of enteroglucagon (II) and the synthetic model glicentin-(33–69)-peptide, as well as the identical chromatographic behaviour of their enzymic digests, strongly suggest that the two moieties are indeed identical.

It may be noted that the present immunochemical sequence analysis was performed on the quantity of enteroglucagon (II) present in extracts from a few grams of ileal mucosa (less than 1 nmol of peptide). The requirements for chemical sequence analysis would probably have been several orders of magnitude greater with a peptide of this size and would imply complete purification, which has proven particularly difficult with the gut peptides, which are present in comparatively low concentrations because of the sporadic occurrence of the particular cells in the gut mucosa. Thus immunochemical sequence analysis of intestinal peptides may be accomplished with 10^5 times less tissue than required for chemical analysis.

The structure of enteroglucagon (II), already suspected for some time (Holst, 1978; Moody & Thim, 1981), explains why glucagon-like bioactivity has been ascribed to this component in various systems (for review see Holst, 1978); with the N-terminal sequence exposed, it is probable that enteroglucagon (II) possesses most of the biological activities of glucagon, although the C-terminal extension may weaken its potency compared with glucagon. On the other hand, the particular structure of enteroglucagon (II) may be responsible for its 20-fold-stronger interaction with receptors on rat gastric glands (Bataille *et al.*, 1981); furthermore, the possibility exists that enteroglucagon (II) may be transformed *in vivo* into intact glucagon (Koranyi *et al.*, 1981). The question then arises, whether enteroglucagon (II) is found in the circulation; my finding that enteroglucagon (II) is eluted from basic columns at a position that is very close to the position of pancreatic glucagon (K_d 0.72; Holst, 1980) may explain the preponderance of 6–15-sequence immunoreactivity over C-terminal immunoreactivity regularly found in studies of the glucagon profile in plasma (Holst, 1981, 1982*a*), and explain why this preponderance has not immediately been interpreted to be of intestinal origin.

Enteroglucagon (II) may thus be an important hormonal peptide of the glucagon family.

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