Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum

Characterization of the peptide

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A peptide isolated from porcine gut according to its glucagon-like activity in liver (bioactive enteroglucagon) has been characterized immunologically, biologically and chemically: its potency relative to pancreatic glucagon in interacting with an antiglucagon antibody, hepatic glucagon-binding sites and hepatic adenylate cyclase was ~100%, 20% and 10%, respectively. In contrast, it is ~20-times more potent than glucagon in oxyntic glands, justifying the term 'oxyntomodulin'. Chemically, it consists in the 29 amino acid-peptide glucagon elongated at its C-terminal end by the octapeptide Lys–Arg–Asn–Lys–Asn–Asn–Ile–Ala; accordingly, it is called 'glucagon-37'.

Glucagon-37 *Oxyntomodulin* *Bioactive enteroglucagon* *Amino acid sequence* *Immunology* *Biological activity*

1. INTRODUCTION

A peptide consisting of 37 amino-acid residues has been isolated [1] from porcine jejuno-ileum on the basis of its glucagon-like activity in liver (interaction with hepatic glucagon-receptors and activation of adenylate cyclase). Owing to its biological characteristics [2,3], this peptide is likely to represent the hyperglycemic–glycogenolytic factor discovered in intestinal extracts [4]. Accordingly, the term 'bioactive enteroglucagon' appears to be convenient. However, the discovery of a tissue-specificity of this peptide at the level of oxyntic glands isolated from the rat stomach led us to propose the term 'oxyntomodulin' for it [5,6]. This report aims to describe the primary structure of this intestinal peptide as well as its basic immunological and biological features. According to its chemical structure, this peptide may be called 'glucagon-37'.

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Abbreviations: PTH, phenylthiohydantoin; dansyl, dimethylaminonaphtalene sulfonyl

2. MATERIALS AND METHODS

2.1. *Isolation procedure*

The peptide was isolated as in [1].

2.2. *Amino acid analysis*

Amino acid analyses were carried out on a Beckman 121M instrument after hydrolysis for 24 h at 110°C with 6 N HCl containing 0.5% phenol.

2.3. *Enzymatic digestions*

The peptides were hydrolyzed with two different enzymes:

(1) *Armillaria mellea* protease (a gift from Dr P.L. Walton, ICI Laboratories, Cheshire) in 1% NH_4HCO_3 (pH 7.8) at an enzyme/substrate ratio of 1/100 (w/w) for 2 h at 37°C, conditions that resulted in a complete cleavage at the amino group of the lysine residue [7];

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(2) TLCK-treated α-chymotrypsin (EC 3.4.21.1) from Worthington (USA) in the same buffer for 7 min at 25°C with an enzyme/substrate ratio of 2/100 (w/w); these conditions were chosen because they allowed one to obtain cleavage of pancreatic glucagon at specific bounds (section 3).

After the incubation time, the tubes containing the samples were placed in boiling water and kept boiling for 3 min. The digests were injected onto the HPLC column (see below) either directly after a 2-fold dilution with the chromatography solvent or after lyophilization and reconstitution into the solvent.

2.4. *High performance liquid chromatography (HPLC) of the peptide fragments*

After enzymic digestion, the peptidic fragments were separated by HPLC using a chromatographic apparatus in [1] fitted with a μ Bondapak C18 0.4 × 30 cm column (Waters Assoc.). Aqueous solvent delivered by pump A consisted of 0.13% (w/w) CF_3COOH (TFA) in water; organic solvent, delivered by pump B, was CH_3CN containing 0.05% $CH_3COOC_2H_5$. Peaks detected at 215 or 280 nm were integrated with a computing integrator (model 4100 Spectra Physics), collected into silanized glass tubes and lyophilized after evaporation in vacuo of the organic solvent.

2.5. *Determination of the amino-acid sequence of the fragments obtained*

We used the direct Edman degradation procedure [8] as in [9]. The method was miniaturized to handle micro samples: Essentially, all volumes were reduced to 1/5th and the glassware chosen accordingly. The PTH-amino acids obtained were identified by HPLC [10] with minor modifications. We also used for some residues the dansyl-Edman procedure [11].

2.6. *Radioimmunoassay*

Glucagon-like immunoreactivity (GLI) was measured as in [3] using an antibody (666-6) produced in our laboratory that recognizes similarly glucagon-like immunoreactivity of either pancreatic or intestinal origin. Specific (C-terminal) pancreatic glucagon-like immunoreactivity was measured with different antisera, K47 (a gift from Dr L. Heding, Novo Res. Inst., Copenhagen) and 30K (a gift from Dr R. Unger, University of Texas, Dallas).

2.7. *Radioreceptorassay in rat liver membranes*

This was performed with rat liver plasma membranes and ^{125}I-pancreatic glucagon as in [3].

2.8. *Study of adenylate cyclase activity in rat liver membranes*

The procedure used was described in [3].

2.9. *Chemicals*

TFA was sequence grade from Pierce (USA) in 1 g ampules. CH_3CN and $CH_3COOC_2H_5$ were spectroscopic grade from Merck (Darmstadt). Chemicals used for sequence work were sequence grade, mostly from Pierce (USA). Peroxide-free ether [9] was from Mallinckrodt (USA). Other chemicals were pro analysi grade.

3. RESULTS

3.1. *Immunological and biological characterization*

On a molar basis, the intestinal peptide (referred to as G-37) possesses the same affinity as pancreatic glucagon (referred to as G-29) for our anti-glucagon antibody (666-6) (fig.1).

On the contrary, the affinity of G-37 for antibodies directed against the C-terminal portion of glucagon such as K47 or 30K, (usually referred to as 'specific' for pancreatic glucagon), was ~1% of that of G-29 (not shown).

Fig.2 displays the interaction of pure G-37 with the glucagon (G-29)-receptors present in rat liver membranes. It may be seen that the apparent potency of G-37 was ~20% of that of G-29 all along the dilution curves.

Fig.3 shows that at all concentrations tested, G-37 was ~10% as potent as G-29 in stimulating hepatic adenylate cyclase; the efficacy was identical for G-37 and G-29.

3.2. *Structural studies*

Fig.4 displays the HPLC-separation of the peptidic fragments obtained from either pancreatic glucagon (upper panel) or the intestinal peptide (lower panel) after enzymic digestion with *Armillaria mellea* protease. Two peptidic fragments were obtained from pancreatic glucagon: G-19, representing the 1–11 fragment; and G-39, correspond-

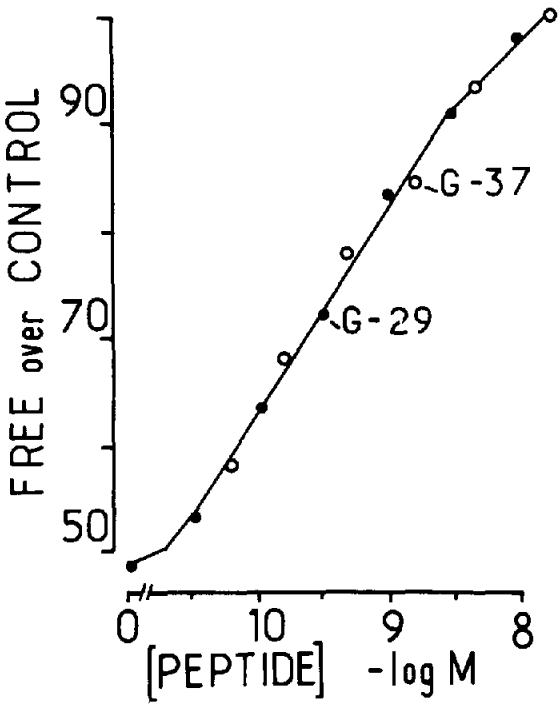

Fig.1. Effect of glucagon (G-29) and the intestinal peptide (G-37) on the binding of ^{125}I-glucagon to an anti-glucagon antibody produced in our laboratory (section 2), the two peptides are compared on a molar basis. Conditions are indicated in section 2.

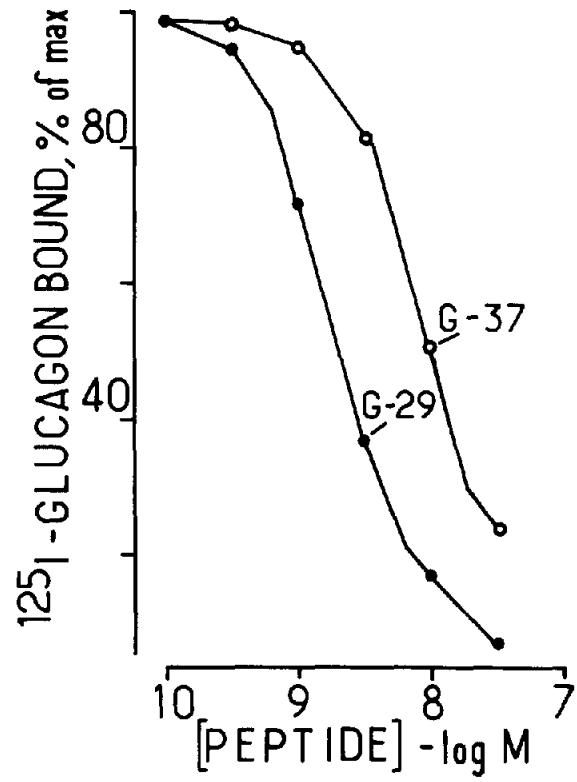

Fig.2. Effect of glucagon (G-29) and the intestinal peptide (G-37) on the binding of ^{125}I-glucagon to rat liver plasma membranes [3]. The 2 peptides are compared on a molar basis. For details see section 2.

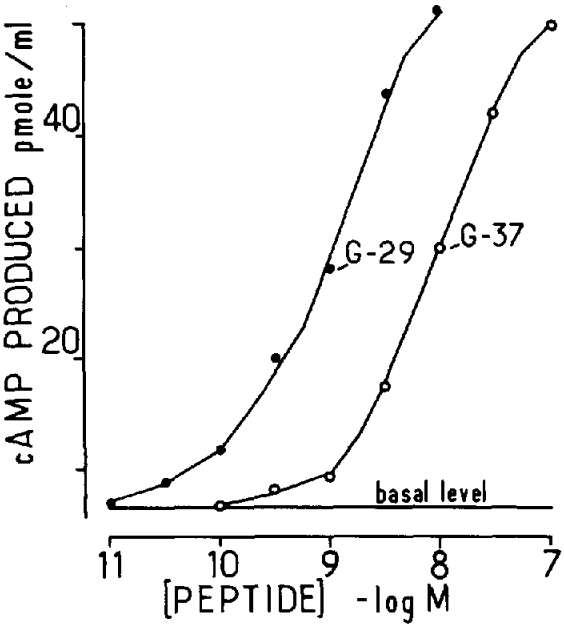

Fig.3. Effect of glucagon (G-29) and the intestinal peptide (G-37) on cyclic AMP accumulation in rat liver plasma membranes. Peptides are compared on a molar basis. Conditions are given in section 2.

ing to the 12–29 fragment. From the intestinal peptide, 4 peptidic fragments were obtained (EG-3, EG-10, EG-19, EG-39). It must be noticed that the relative peak heights and/or peak areas calculated by the computing integrator between G-19 and G-39 on the one hand and between EG-19 and EG-39 on the other were not significantly different, whatever the wavelength (215 or 280 nm) used (not shown).

Fig.5 shows the separation of HPLC of the peptidic fragments obtained from either pancreatic glucagon (upper panel) or the intestinal peptide (lower panel) after enzymic digestion with α-chymotrypsin in conditions where specific bonds

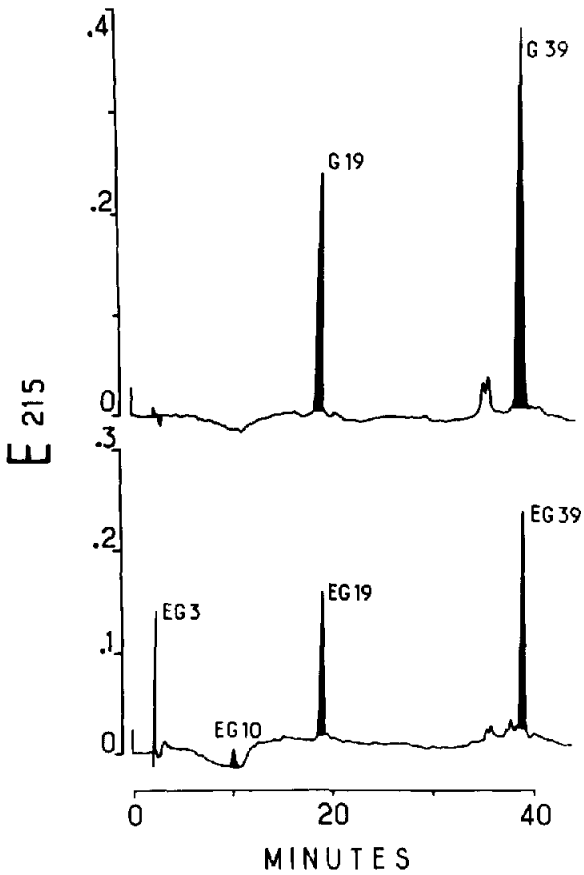

Fig.4. HPLC-separation of the peptide fragments obtained after digestion with *Armillaria mellea* protease of glucagon (upper panel, G-peaks) and the intestinal peptide (lower panel, EG-peaks). Conditions for the digestion and for the HPLC-separation are in section 2. The data and absorbance of the peaks at 280 nm are given in table 1.

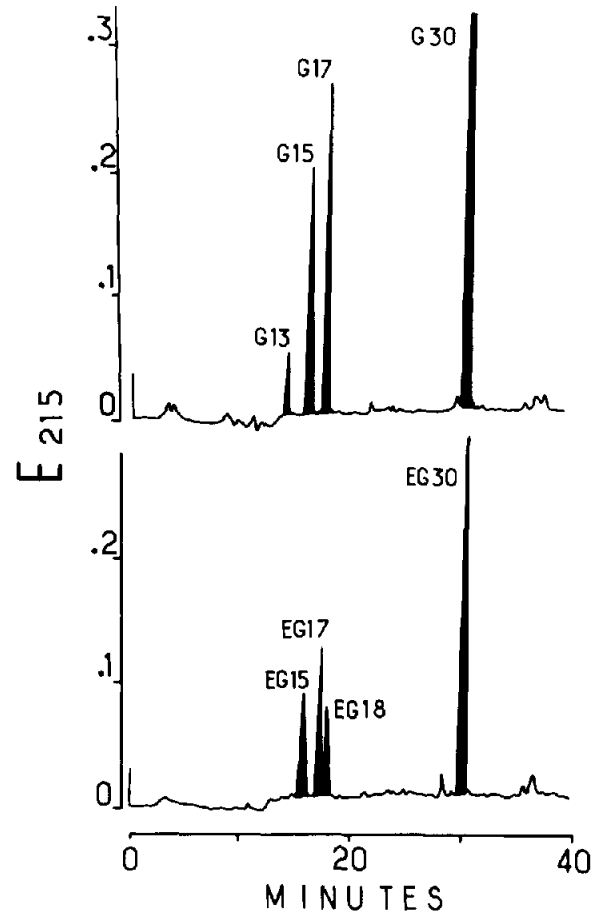

Fig.5. HPLC-separation of the peptidic fragments obtained after digestion with α-chymotrypsin under specially designed conditions (sections 2 and 3) of glucagon (upper panel, G-peaks) and the intestinal peptide (lower panel, EG-peaks). Conditions for the digestion and for the HPLC-separation are given in section 2. The absorbances of the peaks at 280 nm are given in table 1.

are attacked. Indeed, no detectable splits were observed in our conditions at bonds 10–11 or 22–23, known to be more resistant than bonds 6–7, 13–14 and 25–26 [12]; in our conditions, the latter bonds were totally cleaved. Several points have to be stressed:

(i) To 3 of the 4 fragments obtained from glucagon (G-15, G-17, G-30) corresponded 3 equivalent fragments from the intestinal peptide (EG-15, EG-17, EG-30);

(ii) Similarly to the corresponding fragment from glucagon (G-15), EG-15 did not display any measurable absorbance at 280 nm, whereas, like G-17 and G-30, EG-17 and EG-30 did;

(iii) The relative peak heights or peak areas calculated between G-15, G-17 and G-30 were not significantly different from the relative values calculated between EG-15, EG-17 and EG-30, whatever the wavelength (215 or 280 nm);

(iv) The single difference between the two runs (fig.5) was the presence in the digest of the intestinal peptide of a fragment at 18 min (EG-18) that replaced the glucagon fragment G-13. Furthermore, EG-18 displayed relatively to the other peaks a higher absorbance at 215 nm than did G-13.

Table 1

Data obtained from digestion, followed by separation of the peptidic fragments by HPLC, of glucagon (G-peaks) and the intestinal peptide (EG-peaks)

Armillaria mellea digest			α-Chymotrypsin digest		
Fragment	HPLC peak	A_{280}	Fragment	HPLC peak	A_{280}
1–11	G-19	+	1–6	G-15	–
1–11	EG-19	+	1–6	EG-15	–
12–29	G-39	+	7–13	G-17	+
12–29	EG-39	+	7–13	EG-17	+
30–32	EG-3	–	14–25	G-30	+
33–37	EG-10	–	14–25	EG-30	+
			26–29	G-13	–
			26–37	EG-18	–

Digestion of the native peptides was performed by *Armillaria mellea* protease (left panel) or α-chymotrypsin (right panel). Conditions are described in section 2

Table 1 summarizes the data obtained after enzymic digestion of the peptides.

Fig.6 displays the compared chemical structure of glucagon (glucagon-29) and the intestinal peptide (glucagon-37) according to all the data available, including the amino acid sequence of the fragments.

Table 2 shows the amino acid composition of the intact peptide. If we except the relatively low values obtained for Asx and Ser (91% and 85% of that expected, respectively), the data are in keeping with the sequence displayed in fig.6.

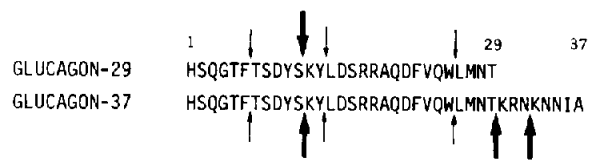

Fig.6. Comparison of the primary structure of glucagon (glucagon-29) and of the intestinal peptide (glucagon-37) according to the data available from the digestion with the 2 enzymes (fig.4,5) followed by amino acid sequence determination of the fragments obtained. Thick arrows indicate the sites of action of *Armillaria mellea* protease; thin arrows indicate the sites of action of α-chymotrypsin used under the conditions in section 2. Amino acids are indicated by one-letter symbols [23].

Table 2

Amino acid analysis of the intact G-37 molecule (as in section 2)

Res.	Calc. value	Nearest integer	Theor. value
Cys	0	0	0
Asx	6.41	6	7
Thr	2.55	3	3
Ser	3.41	3	4
Glx	3.47	3	3
Pro	0.31	0	0
Gly	1.2	1	1
Ala	2.16	2	2
Val	0.92	1	1
Met	0.97	1	1
Ile	0.99	1	1
Leu	2.18	2	2
Tyr	2.02	2	2
Phe	1.69	2	2
Trp	–	–	1
Lys	3.2	3	3
His	1.11	1	1
Arg	3.46	3	3

The relatively low values obtained with Ser and Asx may be attributed to some degradation of the sensitive serine residue and to a slight problem in integration of the Asx peak, respectively. The tryptophan residue cannot be identified using the present methods (destroyed by acid hydrolysis)

4. DISCUSSION

On a molar basis, the newly isolated intestinal peptide possessed for our anti-glucagon antibody, which is representative of the so called 'non-specific anti-glucagon antibodies', an affinity which was similar to that of pancreatic glucagon. On the contrary, its affinity for the anti-glucagon antibodies directed against the C-terminal portion of the glucagon molecules was ~ 1% of that of pancreatic glucagon. These data are in keeping with the structure of the molecule (fig.6); indeed, the C-terminal extension is likely to hinder the interaction between the molecule and the antibodies directed against the C-terminal portion of glucagon.

On the same molar basis, the calculated affinity of the intestinal peptide for the hepatic glucagon-receptor was 4–5-times lower than that of pan-

creatic glucagon, whereas the former was about one order of magnitude less potent than the latter in stimulating hepatic adenylate cyclase. This poor (~2-fold) difference between the 2 relative potencies rules out the possibility pointed out in [13,14] that the intestinal glucagon-like peptide that displays an affinity for the hepatic glucagon-receptors, thus inhibiting the glycogenolytic effect of pancreatic glucagon and leading to hypoglycemia. On the contrary, bioactive enteroglucagon is a good agonist of glucagon at the hepatic level and a possibility exists that in some physiological and/or pathological states, where high levels of the intestinal peptide together with low levels of pancreatic glucagon are present in the portal vein, the former peptide may be of some importance in regulating blood glucose through glycogenolysis/gluconeogenesis. In any case, the action of the pure intestinal peptide on the hepatic glucagon-sensitive systems and the fact that this peptide is the main, if not the single, intestinal peptide that possesses these characteristics [1] strongly supports the idea that the 37 amino acid glucagon-like peptide from intestine is the hyperglycemic–glycogenolytic peptide discovered in [4]. Accordingly, the term 'bioactive enteroglucagon' appears to be convenient.

The demonstration [5] that bioactive enteroglucagon possesses, in addition to its glucagon-like effect in liver, a tissue-specific action at the level of gastric glands isolated from the fundic part of the rat stomach, suggests that the main physiological role of the intestinal peptide is not at the hepatic level but at the level of the acid-secreting gastric area that contains the oxyntic glands. Accordingly, we have proposed the term 'oxyntomodulin' for this peptide [5,6]. This tissue-specificity is further supported by the demonstration [15] that oxyntomodulin is much more potent than pancreatic glucagon in inhibiting pentagastrin-induced gastric acid secretion in rat in vivo; the relative potency of the intestinal peptide vs that of the pancreatic peptide is similar (15–20 times in favor of the former) both in the in vitro [5] and the in vivo [15] models. If we compare the potency of the intestinal peptide to that of glucagon in the 2 rat tissues, it appears that oxyntomodulin is ~200-times more potent relative to glucagon in stomach than in liver. This large difference between the 2 peptides in their tissue-specificity together with the fact that the intestinal glucagon-like peptides are probably released into blood under physiological conditions that are quite different to those under which pancreatic glucagon is released (review [16]) strongly suggest that the 2 peptides are different physiological entities; according to its high potency in inhibiting gastric acid secretion[15], it is reasonable to suggest an enterogastrone role for oxyntomodulin.

As far as the chemical structure of the intestinal peptide is concerned, we have suggested [6] that the peptide contains the whole (1–29) glucagon molecule elongated at its C-terminal end by a basic octapeptide (Lys–Arg–Asn–Lys–Asn–Asn–Ile–Ala). The doubt concerning a possible modification in the N-terminal portion of the glucagon moiety [6] may be ruled out according to these data. Indeed, we can confirm that, in [6], a modification (e.g., a deamidation of the glutamine residue in position 3) had occurred during the storage of the peptide used. Thus the single structural difference between G-29 and G-37 is the presence in G-37 of the basic octapeptide. The presence of this extension explains not only the hindrance observed of the interaction with some antibodies (see above) but also with hepatic glucagon-sensitive systems. However, it is noteworthy that despite the 8 amino acid extension, the decrease in affinity for the glucagon-receptors is not striking; this contrasts with the dramatic loss of affinity of the C-terminally modified glucagon molecules [17].

In sharp contrast to what is observed in liver, G-37 is ~20-times more potent than glucagon in stomach both in vitro [5] and in vivo [15]; it is thus very likely that the oxyntic glands contain a receptor which recognizes with a much higher affinity the 37 amino-acid peptide than the 29 amino-acid peptide; the basic octapeptide is thus most probably deeply involved in the interaction with the gastric receptor.

Two peptides that contain glucagon 1–29 plus a C-terminal extension in the form of a basic octapeptide have been isolated: one was the 37 amino-acid peptide 'proglucagon fragment' [18] of pancreatic origin; the other was the 69 amino-acid 'glicentin' or 'proglucagon' [19] that contains an additional 32 amino-acid N-terminal extension. A structural difference appears in the published sequences: residues 4 and 5 of the C-terminal extension were Asn–Lys in the 'proglucagon fragment' when the order was Lys–Asn in 'glicentin'. As reported [6], we found in the 37 amino-acid intesti-

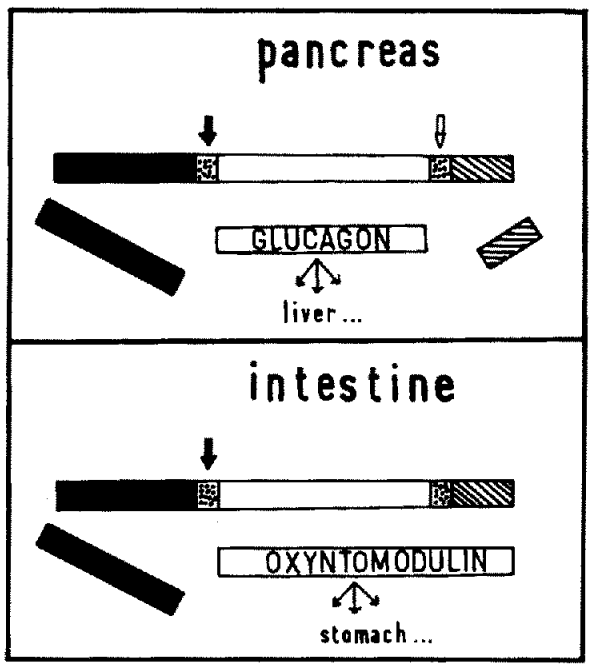

Fig.7. Schematic processing of proglucagon supposed to occur in pancreas [19] and in intestine (according to these results). For details, see section 4.

nal peptide the same order Lys–Asn as in the 'glicentin' sequence. Some technical artefact [6,19] is likely to explain the sequence in [18]. This is further supported by the observation (unpublished) that the pancreas contains, in addition to G-29, small amounts of a peptide resembling in all ways the intestinal G-37.

According to the fact that both the N-terminal and the C-terminal extensions of glucagon-29 begin with a basic dipeptide, as in most of the prohormones, glucagon-29 may be released from proglucagon by enzymatic cleavage of the basic dipeptide at both the N-terminal and the C-terminal extensions [19].

According to our data, we can speculate (fig.7) that:

(1) The mechanism above [19] is operative at the pancreatic level (upper panel) leading to the release into the blood stream of the G-29 molecule that will act on its target tissues, particularly liver.

(2) In contrast, (lower panel) the processing enzyme that cleaves proglucagon at the amino end (Lys–Arg dipeptide) of the glucagon molecule appears to be present also in intestine, but the processing enzyme that cleaves at position 29 of glucagon (Lys–Arg dipeptide) is probably lacking or inactive in intestine*; accordingly, the 37 amino-acid peptide 'oxyntomodulin' is released and, owing to its tissue specificity, acts on its target tissues, particularly stomach. It remains, however, to be established what are the exact physiological conditions under which this peptide is released and the regulatory mechanisms involved.

Owing to the largely confusing terminology for describing the peptides of the glucagon-family, we propose for the 3 known peptides the chemical structure-related terms glucagon-29 for pancreatic glucagon, glucagon-37 for the peptide that we have isolated from porcine intestine as well as that isolated from pancreas [18] (these 2 peptides being most probably identical) and glucagon-69 for the so-called 'glicentin' or 'proglucagon'. This terminology is related to the fact that all these peptides contain the whole glucagon molecule and describes the number of amino-acid residues, whatever the type of extension (N- and/or C-terminal). Such a terminology is now commonly used for the N-terminally extended molecules, e.g., the somatostatin family: somatostatin-14, somatostatin-28 [20,21]. On the other hand, a universal denomination of the peptides structurally related to a previously named one will await an internal consensus.

In conclusion, the isolation of this glucagon-related intestinal peptide [1], the determination of its chemical structure (this paper) and the discovery of its tissue-specificity [5,15] is expected to shed new light on the biological significance of the peptides of the glucagon-family.

* These differences between pancreas and intestine are not absolute since small amounts of G-37 are present in rat pancreas (unpublished) as well as small amounts of G-29 in intestine of rat (unpublished) and human [22]

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