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Evidence that Glicentin Contains the Entire Sequence of Glucagon

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Glicentin (a highly purified 100-amino-acid peptide with glucagon-like immunoreactivity from porcine gut) was subjected to limited digestion with trypsin and carboxypeptidase B, and the resulting peptides were studied by gel filtration and region-specific glucagon radioimmunoassays. Similar digests of glucagon and purified fragments of glucagon were studied in parallel. Glicentin gave rise to peptides that corresponded closely to the 1–17 and 19–29 fragments of glucagon. Also, ^{125}I-labelled glicentin and ^{125}I-labelled glucagon gave rise to identical fragments after trypsin treatment. On the basis of this and other evidence [Jacobsen, Demandt, Moody & Sundby (1977) *Biochim. Biophys. Acta* **493**, 452–459] it is concluded that glicentin contains the entire glucagon sequence at residues number 64–92 and thus fulfills one of the requirements for being a 'proglucagon'.

A family of peptides with glucagon-like immunoreactivity can be extracted from the intestinal mucosa of a number of vertebrate and invertebrate species including man and pig; one of the peptides is probably identical with true pancreatic glucagon (mol.wt. 3485), but the majority of the extractable immunoreactivity stems from peptides with higher mol.wt., which show immunoreactivity towards antisera directed against the mid- to *N*-terminal sequence of the glucagon molecule only (Holst, 1978). One of these peptides, provisionally named 'glicentin', was purified from porcine intestine (Sundby *et al.*, 1976) and was shown to consist of 100 amino acid residues. The sequence of its 10 *C*-terminal amino acids corresponds closely to the *C*-terminal sequence of the so-called 'proglucagon fragment' isolated by Tager & Steiner (1973), and on the basis of this and other indirect evidence it was hypotheszied that the glicentin molecule contained the entire glucagon sequence (Jacobsen *et al.*, 1977).

We here report the results of immunochemical studies of the structure of glicentin, which strongly support this hypothesis.

Experimental

Materials

Highly purified glicentin ('glucagon-like immunoreactivity 1') from porcine intestinal mucosa (Sundby *et al.*, 1976; Jacobsen *et al.*, 1977) was a generous gift from Drs. F. Sundby and A. Moody, the NOVO Research Institute, Bagsværd, Denmark. Highly purified porcine glucagon and (monoiodinated) ^{125}I-labelled glucagon (Jörgensen *et al.*, 1972) and ^{125}I-labelled glicentin (Moody *et al.*, 1978) were also gifts from the NOVO Research Institute. Purified tryptic fragments of porcine glucagon were gifts from Dr. A. Salokangas, National Institute for Medical Research, London NW7, U.K. A synthetic peptide corresponding to the 10 *C*-terminal amino acids of glicentin (Jacobsen *et al.*, 1977) plus the 17–26 sequence of glucagon (in agreement with the postulated structure of part of glicentin) was prepared by N. Yanaihara, Shizuoka College of Pharmacy, Shizuoka, Japan and obtained through the NOVO Research Institute. 3-L-tosylamidobutan-2-one (TPCK)-treated bovine trypsin was from Worthington, Freehold, NJ, U.S.A.: phenylmethanesulphonyl fluoride-treated carboxypeptidase B was from Merck (catalogue no. 2300), Darmstadt, W. Germany; soya-bean trypsin inhibitor (T 9003) was from Sigma, U.S.A.; human serum albumin (reinst., trocken) was from Behringwerke, Marburg, W. Germany, and aprotinin was from NOVO Research Insitute. ^{125}I-labelled albumin and ^{22}NaCl were from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were analytical grade, except methylmaleic anhydride (no. 801363, Merck) which was reagent grade. Sephadex G-50 (fine grade) and columns (catalogue no. K 16/100) were from Pharmacia Fine Chemicals, Uppsala, Sweden.

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Methods

Gel filtrations were performed in columns (K 16/100; 1000mm × 16mm) of Sephadex G-50 (fine grade) equilibrated and eluted at 4°C with 0.125M-NH_4HCO_3 adjusted to pH9.0 and supplemented with human serum albumin (2g/l), NaCl (0.1M) and thiomersal (0.6mM) at a constant flow of approx. 20ml/h. Fractions corresponding to approx. 0.02 bed vol. were collected automatically. Sample size never exceeded 2% of bed volume.

Radioimmunological determination of glucagon-like immunoreactivity was performed as previously described (Holst & Aasted, 1974; Holst, 1977*a*,*b*) using antiserum 4304, which binds to the 6–15 sequence of glucagon, and antiserum 4305, which binds to the 19–29 sequence. (Antiserum 4304 does not bind any of the tryptic fragments of glucagon, but readily binds the 1–17 fragment, and also binds duck glucagon, which is modified at position 16; assuming that the binding site can accommodate at most a chain of eight amino acids, antiserum 4304 should bind to the 6–15 sequence of glucagon. Antiserum 4305 binds fragments 19–29, but no other fragments, and does not bind turkey glucagon, which is modified at position 28.)

Antiserum 4304 binds labelled and unlabelled glucagon and glicentin with identical affinity (Holst, 1977*a*), whereas antiserum 4305 does not bind intact glicentin.

The assays were modified as follows. Antiserum

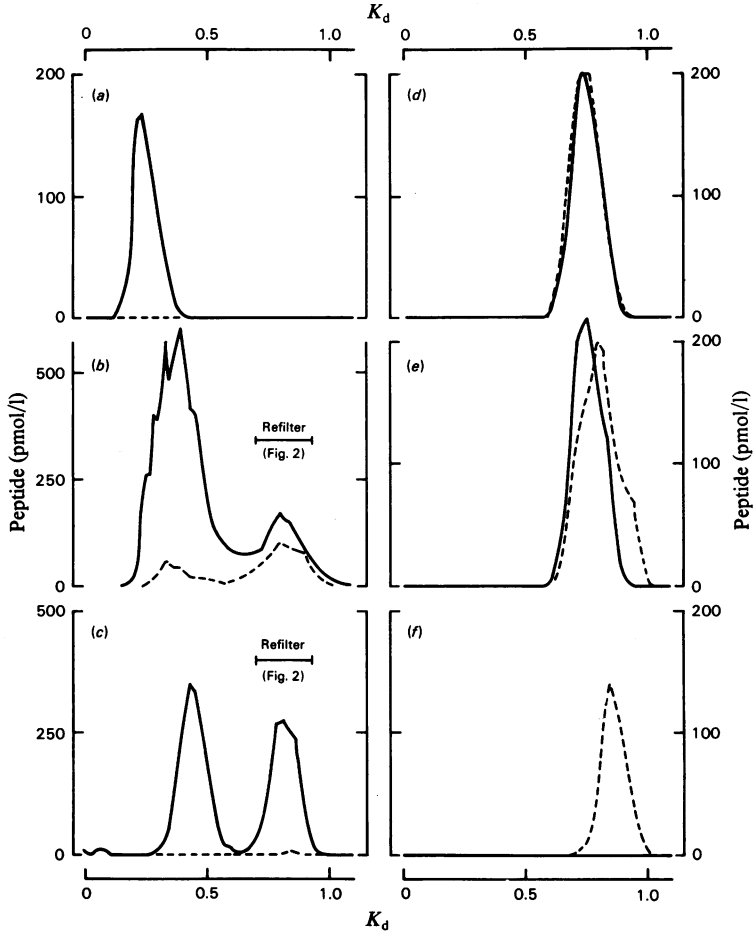

Fig. 1. *Gel filtration of glicentin (a, b and c) and glucagon (d, e and f) before and after trypsin treatment as measured by 6–15 immunoreactive* (——) *and 19–29 immunoreactive* (– – – –) *antisera to glucagon and plotted against coefficient of distribution, K_d*

(*a*) Glicentin (2pmol); (*b*) glicentin (150pmol) + trypsin (12.5μg/ml for 30min); (*c*) glicentin (20pmol) + trypsin (10μg/ml for 2h); (*d*) glucagon (6pmol); (*e*) glucagon (6pmol) + trypsin (10μg/ml for 30min); (*f*) glucagon (6pmol) + trypsin (20μg/ml for 1h).

4305 was used instead of the previously described antiserum 4317, because of its identical specificity and superior avidity (K for glucagon binding is 5×10^{11} l/mol); the assay buffer was the buffer described above for column experiments; separation was performed using plasma-coated charcoal (Stadil & Rehfeld, 1973). Detection limits of the assays were 2–3 pM and the within-assay coefficient of variation was better than 10%.

Enzymic degradations were performed at 37°C for 30–120 min using trypsin at 10–100 μg/ml. When followed by a carboxypeptidase (20 μg/ml) incubation, trypsin activity was inhibited by a 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.

Glucagon fragment 1–17 was prepared by trypsin treatment of methylmaleic anhydride-treated glucagon as described by Senyk *et al.* (1971) and by Dixon & Perham (1968).

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:

$$K_d = \frac{V_e - V_0}{V_i}$$

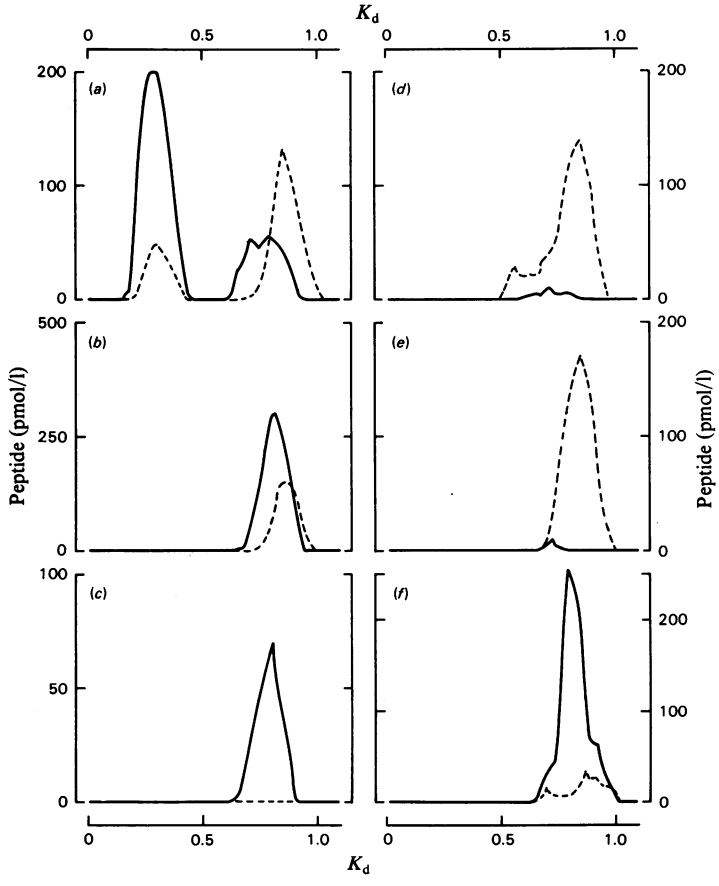

Fig. 2. *Gel filtration of enzyme-digested glicentin (a, b and c) and glucagon-related peptides (d, e and f) as measured by 6–15 immunoreactive* (——) *and 19–29 immunoreactive antisera to glucagon* (– – – –), *and plotted against coefficient of distribution,* K_d

(*a*) Glicentinn (30 pmol) + trypsin (20 μg/ml for 1 h) followed by addition of soya-bean trypsin inhibitor (20 μg/ml) and carboxypeptidase B (20 μg/ml) for 1 h. (*b*) Glicentin + trypsin. Refiltration of fractions (Fig. 1*b*) eluted at K_d 0.70–0.95. (*c*) Glicentin + trypsin. Refiltration of fractions (Fig. 1*c*) eluted at K_d 0.70–0.95. (*d*) A synthetic peptide (0.1 μg) corresponding to glucagon 17–29 + eight additional amino acids corresponding to the proposed *C*-terminal sequence of glicentin (see Fig. 5) treated with trypsin and carboxypeptidase B as in (*a*). (*e*) Glucagon 19–29 (200 pmol). (*f*) Glucagon 1–17 (30 pmol; estimated amount, without correction for losses during preparation).

where V_e is the elution volume for the substance in question, V_0 is the exclusion volume and V_1 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

All left-hand columns of Figs. 1, 2 and 4 show the results of experiments with glicentin or derivatives therefore, and the right-hand columns show similar experiments with glucagon or glucagon derivatives. In Figs. 1(*a*) and 1(*d*) are shown the elution positions of glicentin ($K_d = 0.23$; range of 10 experiments 0.22–0.26) and glucagon ($K_d = 0.74$; range of 8 determinations 0.71–0.76) as determined by using antisera 4305 and 4304 which are reactive with sequences 19–29 and 6–15 respectively. Note the homogeneity of these controls, the inability of antiserum 4305 to detect immunoreactivity in intact glicentin, and the exact symmetry of the two assays of the glucagon peak.

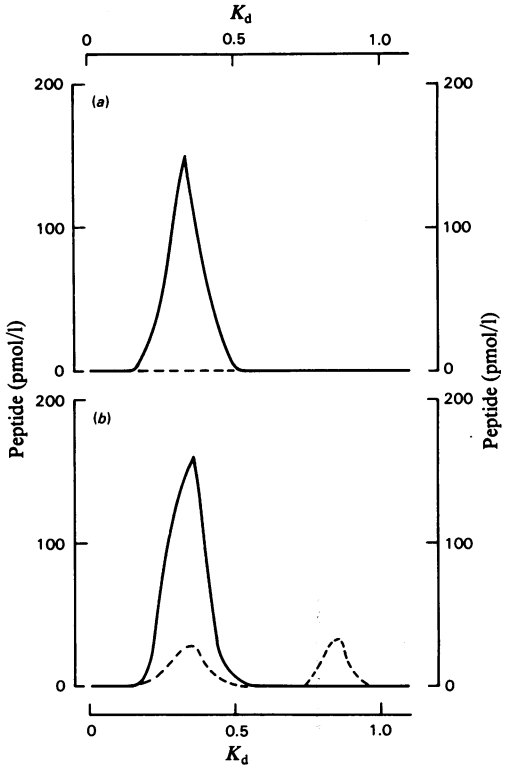

Fig. 3. *Gel filtration of enzyme-digested glicentin as measured by 6–15 immunoreactive* (——) *and 19–29 immunoreactive* (– – – –) *antisera to glucagon, and plotted against coefficient of distribution,* K_d

(*a*) Glicentin (30 pmol) was incubated with trypsin (100 μg/ml) for 120 min followed by addition of soyabean trypsin inhibitor (100 μg/ml), and a sample was removed for gel filtration. (*b*) Carboxypeptidase B (20 μg/ml) was added to the remainder, and incubation was continued for a further 60 min.

Figs. 1(*b*) and 1(*e*) show the results of limited tryptic digestion (10 μg/ml, 30 min, peptide/enzyme ratio approx. 1). Glucagon is now no longer homogeneous, there is a surplus of 6–15 immunoreactivity, and part of the 19–29 immunoreactivity has moved to the right. Glicentin is now markedly heterogeneous, the main peak has moved to the right, and a new peak of 6–15 immunoreactive material has appeared at K_d 0.80; simultaneously, 19–29-immunoreactive material has appeared at K_d 0.33 and at K_d 0.8–0.85.

Further tryptic digestion (10–20 μg/ml for 60–120 min) breaks down glucagon to one peak of immunoreactive homogeneous material at K_d 0.85, with only 19–29 immunoreactivity (Fig. 1), whereas glicentin (Fig. 1*c*) is broken down to homogeneous peaks at K_d 0.45 and 0.8, almost without 19–29 immunoreactivity. After prolonged trypsin treatment, the peak at K_d 0.8 disappears (Fig. 3*a*), and, after incubation overnight with trypsin at 100 μg/ml, the immunoreactivity of glicentin disappears altogether (results not shown). However, if glicentin is treated with carboxypeptidase B after limited or prolonged tryptic digestion, a large homogeneous peak containing 19–29-immunoreactive material appears at K_d 0.85 (Figs. 2*a* and 3). Fig. 2(*b*) shows a refiltration (after freeze-drying) of the fractions collected between K_d 0.7 and 0.95 in the experiment shown in Fig. 1(*b*); the eluted immunoreactivity clearly consists of two homogeneous peaks of material, one with 6–15 immunoreactivity and a K_d of 0.80, and another with only 19–29 immunoreactivity and a K_d of 0.85. In Fig. 2(*c*) is shown a refiltration of similar fractions from the experiment in Fig. 1(*c*); the immunoreactive material eluted in this area clearly consists of one homogeneous moiety with K_d 0.8 and exclusively 6–15 immunoreactivity.

Fig. 2(*d*) shows the results of treatment with trypsin and carboxypeptidase B of a synthetic peptide consisting of the glucagon sequence from residue 17 to 29 plus another eight amino acids corresponding to the proposed sequence of the *C*-terminus of glicentin (Jacobsen *et al.*, 1977). This peptide, which untreated shows neither 6–15 immunoreactivity nor 19–29 immunoreactivity (upon gel-filtration of 35 nmol), and which does not give rise to immunoreactive fragments upon trypsin treatment alone (results not shown), produces by the combined enzyme digestion a moiety that is clearly 19–29-immunoreactive, with a K_d of 0.85. Fig. 2(*e*)

shows the gel-filtration pattern of the purified *C*-terminal (19–29) tryptic fragment of glucagon; the fragment is eluted as a single homogeneous peak of 18–29-immunoreactive material with K_d 0.85. Finally, Fig. 2(*f*) shows the elution profile of glucagon fragment 1–17, which is eluted as a homogeneous peak of 6–15-immunoreactive material at K_d 0.80.

Fig. 4 shows the results of a series of experiments in which the elution profiles and immunoreactivities of labelled glicentin and glucagon were studied before and after trypsin treatment. In Figs. 4(*a*) and 4(*d*) are shown the radioactivity elution profiles of the intact peptides; in addition is shown the elution profile of $^{22}Na^+$ used for calibration. In Figs. 4(*b*) and 4(*e*) are shown the radioactivity elution profiles after trypsin treatment of the two peptides. Note the similarity of the two profiles, with one peak at K_d 0.90 and another at K_d 1.05. Samples of the eluted fractions were then incubated with surplus 6–15- and 19–29-immunoreactive antisera. After separation of bound and free moieties, the antibody-bound radioactivity was plotted against K_d. Again, glucagon and glicentin showed a similar pattern; in both cases a single homogeneous exclusively 6–15-immunoreactive fragment with K_d 0.90 was found.

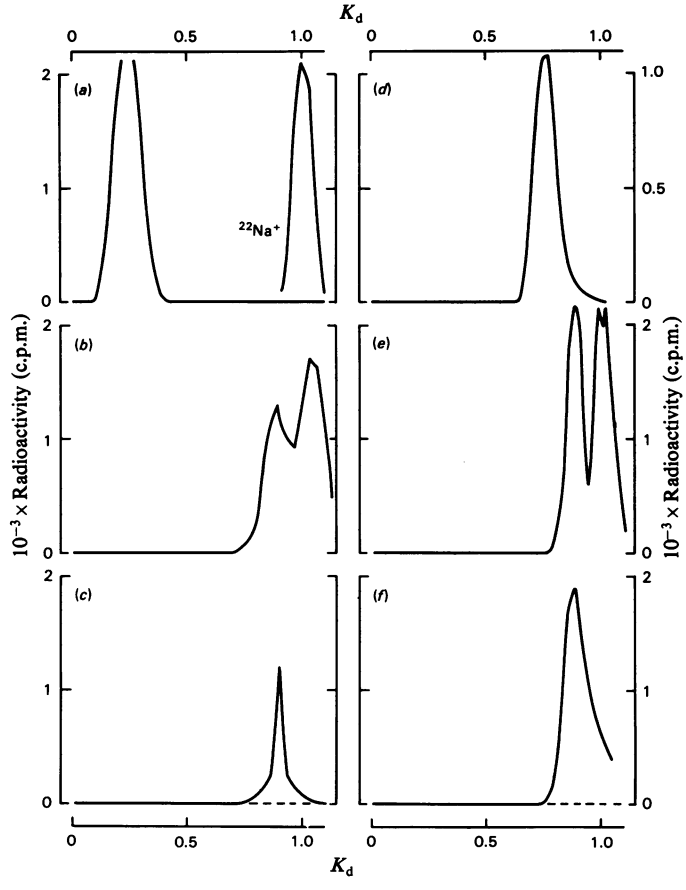

Fig. 4. *Gel filtration of ^{125}I-labelled glicentin (a, b and c) and glucagon (d, e and f) with or without trypsin treatment*
(*a*) Radioactivity elution profile of ^{125}I-labelled glicentin. The elution profile of $^{22}Na^+$ added for internal calibration is also shown. (*b*) ^{125}I-labelled glicentin + trypsin (20 μg/ml for 1 h) radioactivity elution profile. (*c*) Samples of the eluted fractions from (*b*) were incubated with excess antiserum (5 μl, undiluted) for 24 h, and bound and free moieties were separated with plasma-coated charcoal. Antibody-bound radioactivity is plotted against K_d. ——, 6–15 immunoreactive antiserum; – – – –, 19–29 immunoreactive antiserum. (*d*) Radioactivity elution profile of ^{125}I-labelled glucagon. (*e*) ^{125}I-labelled glucagon after treatment with trypsin as in (*b*). (*f*) The fractions from (*e*) were treated as in (*c*), and antibody-bound radioactivity was plotted against K_d.

Discussion

Our gel-filtration results have been given in terms of the coefficients of distribution, K_d, since this value is supposed to be relatively independent of geometry and packing density of the column (Determann, 1969), but the K_d of a given substance still varies somewhat, and we have therefore tried to minimize all sources of variability in the column set-up (see the Experimental section); in this way the standard deviation for the K_d of glucagon was held below 0.02 K_d units. We are therefore confident that differences of 0.05 K_d unit are highly significant and are caused by true differences in hydrodynamic volumes of the eluted molecules. On the basis of these considerations we offer the following interpretation of our results.

Glucagon (Fig. 5) contains lysine or arginine residues at positions 12, 17 and 18, and is therefore susceptible to tryptic cleavage at these sites; limited tryptic digestion of glucagon could therefore lead to the formation of a mixture of the following peptides: 1–12, 1–17(18), 13–17(18), 13–29 and 18(19)–29. Of these, our 5–16-immunoreactive antiserum (4304) will react with peptide 1–17(18) only, whereas the 19–29-immunoreactive antiserum will react with peptides 13–29 and 18(19)–29 only. The 13–29 peptide has not been available to us, and we do not know its elution position on our columns. However, there is reason to believe that the bonds between residues 17–19 are more susceptible to tryptic cleavage than is the 12–13 bond (see below) and relatively little peptide 13–29 may therefore be formed. Thus limited tryptic digestion should give rise to the following immunoreactive fragments: 1–17(18), 18(19)–29, and possibly remaining intact glucagon, whereas more extensive digestion should lead to the appearance of only the 19–29 fragment. Our results are in excellent agreement with this prediction. In the calibration experiments the 1–17 and 19–29 fragments show exactly the expected immunoreactivity and the elution positions were at K_d 0.8 and 0.85 respectively. Extensive degradation of glucagon led to the formation of a single 19–29 immunoreactive fragment, eluted at K_d 0.85, and the limited digestion led to a mixture of fragments as shown in Fig. 1(*e*); this Figure can be resolved to show the elution positions of each of the three measurable resulting peptides (including glucagon), taking their elution positions from the calibrations into consideration (Fig. 6).

The synthetic peptide consisting of glucagon 17–29 plus the *C*-terminal sequence of glicentin (see Fig. 5) could not be measured by either antiserum; this shows that the *C*-terminal sequence of glucagon must be free in order to be measurable by 19–29-immunoreactive antisera. Tryptic digestion should not lead to the formation of measurable fragments, in agreement with our negative findings. A combination of trypsin and carboxypeptidase B treatment, however, should lead to the formation of a measurable 19–29 peptide, and this was also found (Fig. 2*d*).

The radioactive label ^{125}I is uniformly distributed between the two tyrosine residues in mono-iodinated glucagon (von Schenck & Jeppsson, 1977). By trypsin treatment, again assuming that the 12–13 bond is less susceptible than the 17–19 bonds, labelled glucagon should give rise to the following radioactive fragments: 1–12, 1–17(18), 13–17(18) and 13–29; of these the 13–17(18) fragment, because of the iodine and its size, would be expected to be eluted at $K_d \geq 1.0$. Of these fragments, only 1–17(18) and 13–29 would be expected to be immunoreactive. In our experiments radioactivity was eluted at K_d 0.9 and 1.05, but only the peak at K_d 0.90 was immunoreactive, and, as expected,

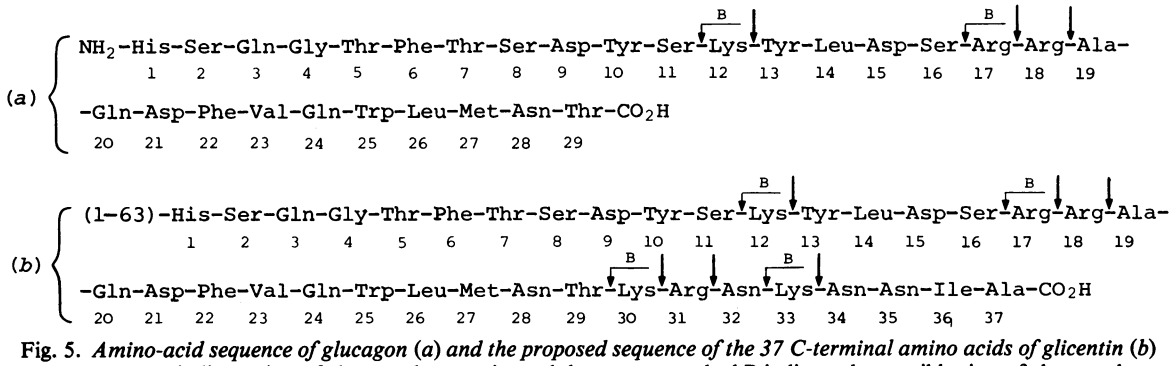

Fig. 5. *Amino-acid sequence of glucagon (a) and the proposed sequence of the 37 C-terminal amino acids of glicentin (b)* Heavy arrows indicate sites of cleavage by trypsin, and the arrows marked B indicate the possible sites of cleavage by carboxypeptidase B after trypsin treatment.

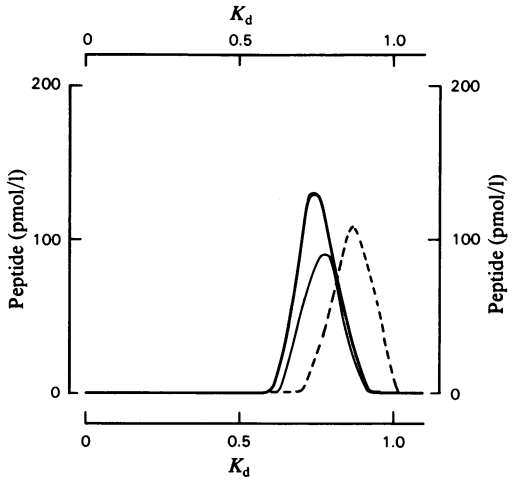

Fig. 6. *Elution pattern of immunoreactive fragments of glucagon after limited tryptic digestion*

A redrawing of the experiment from Fig. 1*e*, in which the elution positions of the known tryptic fragments of glucagon have been taken into consideration. Thus, it was possible to calculate the individual contributions to the cumulative elution pattern of each of the three immunoreactive moieties: ——, remaining intact glucagon; ——, glucagon 1–17(18); – – – –, glucagon 18(19)–29. By (vertical) addition of —— and —— (the total 6–15 immunoreactivity), and addition of —— and – – – – (the total 19–29 immunoreactivity) Fig. 1*e* is obtained.

displayed 5–16 immunoreactivity. The peak at K_d 0.90 must therefore represent labelled 1–17(18) glucagon, and the peak at K_d 1.05 the 13–17(18) fragment; labelled fragment 13–29 was not found (since the 19–29-immunoreactive antiserum would be expected to bind such a fragment).

Upon trypsin treatment, glicentin gave rise to immunoreactive fragments which were remarkably similar to those of glucagon digestion. If the proposed sequence of its 37 *C*-terminal amino acids is correct (Fig. 5), extensive trypsin treatment should lead to complete disappearance of immunoreactivity, since the 19–30 fragment formed (Fig. 5*b*) would be immeasurable (cf. the experiments with the synthetic '17–37' peptide); this was also found. Limited trypsin treatment should lead to the formation of immunoreactive 1–17(18) glucagon, and such a peptide was indeed formed (Fig. 1*b* and Fig. 2*b*); in some experiments small amounts of a peptide corresponding to the 19–29 fragment were also formed as shown in Figs. 1 and 2(*b*); how this peptide is formed is not clear. On the other hand, a combination of trypsin and carboxypeptidase B treatment should lead to the formation of a clearly distinguishable peak of 19–29 fragment; this was also the case (Figs. 2*a* and 3). Labelled glicentin gave rise to radioactive, as well as immunoreactive, fragments which correspond closely to those obtained from labelled glucagon (Fig. 4).

In conclusion, our results strongly suggest that glicentin contains sequences corresponding to the glucagon sequences 1–17(18) and 18(19)–29; on the basis of this evidence it is tempting to conclude that glicentin contains the entire glucagon sequence.

The importance of this assumption is obvious; glicentin is extracted from porcine intestinal mucosa, but glicentin-like immunoreactivity has recently been demonstrated in the pancreatic A-cells of several species (Ravazzola *et al.*, 1979). It has therefore been proposed that glicentin is the proper proglucagon (Moody *et al.*, 1978); this hypothesis gains considerable support from the present results.

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References

Determann, H. (1969) *Gel Chromatography*, 2nd edn., pp. 64–70, Springer-Verlag, Berlin

Dixon, H. B. F. & Perham, R. N. (1968) *Biochem. J.* **109**, 312–314

Holst, J. J. (1977*a*) *Diabetologia* **13**, 159–169

Holst, J. J. (1977*b*) in *Glucagon, Its Role in Physiology and Clinical Medicine* (Foa, P. P., Bajaj, J. S. & Foa, N. L., eds.), pp. 287–303, Springer-Verlag, New York

Holst, J. J. (1978) *Digestion* **17**, 168–190

Holst, J. J. & Aasted, B. (1974) *Acta Endocrinol. (Copenhagen)* **77**, 715–726

Jacobsen, H., Demandt, A., Moody, A. J. & Sundby, F. (1977) *Biochim. Biophys. Acta* **493**, 452–459

Jörgensen, K. H. & Larsen, U. D. (1972) *Horm. Metab. Res.* **4**, 223–224

Moody, A. J., Jacobsen, H. & Sundby, F. (1978) in *Gut Hormones* (Bloom, S. R., ed.), pp. 369–378, Churchill Livingstone, Edinburgh

Ravazzola, M., Siperstein, A., Moody, A. J., Sundby, F., Jacobsen, H. & Orci, L. (1979) *Endocrinology* **105**, 499–508

Senyk, G., Williams, E. B., Nitecki, D. E. & Goodman, J. W. (1971) *J. Exp. Med.* **133**, 1294–1308

Stadil, F. & Rehfeld, J. F. (1973) *Scand. J. Gastroenterol.* **8**, 101–112

Sundby, F., Jacobsen, H. & Moody, A. J. (1976) *Horm. Metab. Res.* **8**, 366–371

Tager, H. S. & Steiner, D.F. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2321–2325

von Schenck, H. & Jeppsson, J.-O. (1977) *Biochim. Biophys. Acta* **491**, 503–508