Studies on the Biosynthesis of Pancreatic Glucagon in the Pigeon (*Columba livia*)

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(Received 27 May 1975)

The biosynthesis of glucagon was studied in microdissected pigeon pancreatic islets. [^3H]-Tryptophan and [^3H]leucine were incorporated into big and little glucagon. No precursor-product relationship was evident between big and little glucagon after radioactive pulse-chase and immunoreactive chase incubations. Radioactive and immunoreactive little glucagon and immunoreactive big glucagon were actively secreted and the synthesis of both glucagons was inhibited by high concentrations of glucose. [^3H]Tryptophan and [^3H]leucine were incorporated into an islet protein of about 20000 mol.wt. Gel filtration of extracts of turkey pancreas revealed the presence of an immunoreactive peak of mol.wt. approx. 20000. This glucagon-immunoreactive component was also present in dog and ox pancreas and was stable to chaotropic agents and elution at various pH values. A similar-sized glucagon-immunoreactive species was present in the dog circulation. These results are discussed in the light of the presently accepted mechanisms of glucagon biosynthesis.

The biosynthesis of little glucagon has been reported by a number of workers (Noe & Bauer, 1971; Tung & Zerega, 1971; Hellerström *et al.*, 1974). O'Connor *et al.* (1973), using the perfused rat pancreas, showed that at least two species of glucagon were synthesized, a moiety of approx. 8200 mol.wt. (big glucagon) and a 3500-mol.wt. moiety (little glucagon). Big glucagon is lipolytic, probably insulinogenic and is present in the circulation (O'Connor & Lazarus, 1976). The present study was designed to investigate whether big glucagon is a glucagon-like hormone distinct from little glucagon or a biosynthetic precursor of little glucagon and to examine islet and pancreas extracts for further radioactive and immunoreactive glucagon species. The glucagon content of avian islets appears to be about 10 times that in the mammalian pancreas (Vuylsteke & de Duve, 1953) and the pigeon pancreas has a population of large islets of Langerhans rich in α cells (Roth, 1968). Glucagon biosynthesis was therefore studied in pigeon islets that had been isolated by microdissection.

Experimental

Materials

Sephadex G-50 and G-100 (superfine grade) were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. DL-[^3H]Tryptophan was from Becton Dickinson, Wembley, Middx., U.K., and L-[^3H]leucine from New England Nuclear Chemicals, Reading, Berks., U.K. Human serum albumin was from Kabi Pharma-

ceuticals, Stockholm, Sweden. NE 220 scintillation fluid was purchased from Nuclear Enterprises, Edinburgh, Scotland, U.K., and pigeons were from W. H. Alderwood, London N.W.1, U.K.

Methods

Microdissection of islets. Pigeons, starved overnight, were killed by cervical dislocation and the pancreas was excised. The pancreas was sliced once by a hand microtome and the slices were examined under a dissecting microscope (Nikon SMZ-2) at $\times 8$ magnification. The larger islets were whiter than the surrounding acinar tissue and could be dissected out by gently teasing off the surrounding acinar tissue with watchmaker's forceps. The islets could be obtained virtually free of contamination by acinar tissue. Up to 150 islets could be obtained from ten pigeons.

Incubation procedures. Islets (65–150) were incubated in 0.5 ml of bicarbonate-buffered Ringer solution, pH 7.4 (Gey & Gey, 1936), supplemented with human serum albumin (1 mg/ml) and all the protein amino acids at 10 $\mu\text{g}/\text{ml}$, excluding the radioactive amino acid. [^3H]Tryptophan (25 Ci/mmol) or [^3H]leucine (30 Ci/mmol) were added at 200 $\mu\text{Ci}/\text{ml}$. Aprotinin (1000 kallekrein inactivator units/ml) and penicillin (100 units/ml), were present in some incubations.

The islets were incubated in a shaking incubator under an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5), at 42°C which is the pigeon body temperature.

Pulse-chase incubations. Islets (approx. 100 per incubation) were incubated with [^3H]tryptophan for 3.5 h (pulse) followed by incubation in a chase medium that contained non-radioactive tryptophan (10 $\mu\text{g}/\text{ml}$) and cycloheximide (18 mM). This concentration of cycloheximide was sufficient to block incorporation of radioactive amino acids into proteins. Separate batches of islets were incubated in the chase medium for 1.5, 4, 5.5, 8, 16 and 20 h. In all but the 1.5 h chase the chase medium contained penicillin. Immunoreactivity chase incubations generally had radioactive amino acid and cycloheximide present as a check on protein-synthesis blockage. No pulse-incubation period was required, since the ratio of the immunoreactive big to little glucagon present in the islets at the time of islet isolation was compared with the ratio present after the chase-incubation period.

Extraction procedure. The islets were either separated from the medium, or the medium with the islets was precipitated by trichloroacetic acid. Islets extracted alone were washed three times with 1 ml of ice-cold 10% (w/v) trichloroacetic acid and then homogenized in a 1 ml glass homogenizer with 1 ml of acid/ethanol (0.75 ml of ethanol, 0.25 ml of water, 5 μl of conc. HCl), and stored overnight at 4°C. The insoluble residue was then removed by centrifugation and the supernatant freeze-dried. The extract was taken up in 1 ml of 0.05 M- NH_4HCO_3 , pH 8.8, and dialysed against 10 litres of the same solution for 8 h. The solution was acidified to 1 M with 50 μl of acetic acid before gel filtration.

Incubation media were precipitated by 0.5 ml of cold 20% (w/v) trichloroacetic acid and then desalted on Sephadex G-25 in 1 M-acetic acid to remove unincorporated radioactive amino acid. When bovine big glucagon and porcine crystalline little glucagon were separately taken through the islet-extraction procedure no immunoreactive breakdown products were detectable.

Gel filtration. Islet extracts and media were gel filtered on Sephadex G-50 (superfine grade) in 1 M-acetic acid, in acrylic columns (0.9 cm $\times$ 60 cm); 0.8 ml fractions were collected, at 4°C. Re-fractionations were on columns (0.9 cm $\times$ 30 cm) of Sephadex G-50 and G-100 in 1 M-acetic acid, and 0.5 ml fractions were collected. A sample (0.6 ml) of alternate 0.8 ml fractions, and 0.4 ml of all 0.5 ml fractions were mixed with 10 ml of scintillant (NE 220) and counted for radioactivity to a counting error of $\pm 5\%$, after subtraction of background count. Pancreas extractions, gel filtrations and immunoassay procedures were carried out as described elsewhere (O'Connor & Lazarus, 1976).

Circulating glucagon species. Glucagon secretion was stimulated in a mongrel dog by a 10 min infusion of adrenaline (1 mg in 10 ml of 0.9% NaCl) (Iversen, 1973), under Nembutal anaesthesia, and then

approx. 250 ml of blood was removed. The plasma was fractionated unextracted to eliminate possible breakdown of high-molecular-weight glucagon species which would give artifacts. Plasma (100 ml) was acidified with acetic acid to 1 M, the precipitate removed by centrifugation at 1000g for 30 min, and gel filtered on a column (10 cm $\times$ 100 cm) of Sephadex G-50 (superfine grade), in 1 M-acetic acid, fraction size 50 ml. The void-volume and salt-peak areas were discarded, including the little-glucagon area, and the remaining material was pooled and re-fractionated in glucagon immunoassay buffer (O'Connor & Lazarus, 1976), on a column (5 cm $\times$ 60 cm) of Sephadex G-50 (superfine grade); 5 ml fractions were collected and immunoassayed for immunoreactive glucagon.

Results

Incorporation of [^3H]tryptophan

The profile on Sephadex G-50 of [^3H]tryptophan incorporation into 65 isolated pigeon islets is shown in Fig. 1(a). Incubation was for 3.5 h and glucose was omitted from the medium. The salt-peak area contained molecules smaller than 1500 mol.wt., including unincorporated [^3H]tryptophan. Two main fractions of incorporated radioactivity were eluted between the void-volume (V_0) and salt-peak areas, coincident with the elution position of pigeon big glucagon, $V_e/V_0 = 1.65$, and little glucagon, $V_e/V_0 = 2.1$. The positive identification of biosynthesized fractions of big and little glucagon has been described (O'Connor *et al.*, 1973). The glucagon-immunoreactivity profile of pigeon islet extract is shown in Fig. 5. The peak fractions of ^3H -labelled little glucagon (fractions 40–47; Fig. 1a), were pooled and re-fractionated on a column (0.9 cm $\times$ 30 cm) of Sephadex G-50 (results not shown). The radioactive little glucagon from pigeon was eluted as a clearly resolved peak of material coincident with the porcine little glucagon marker. After extended incubation periods (20 h), incorporation into little glucagon was much greater than into big glucagon (Fig. 1b). In these islet incubations the media were extracted with the islets.

Pigeon islets were incubated for 20 h with [^3H]tryptophan in the presence of 28 mM-glucose, an inhibitor of glucagon secretion (Ohneda *et al.*, 1969). No defined fraction of either big or little glucagon was obtained. In an effort to increase radioactive incorporation into glucagon, 20 h incubations were carried out in the presence of 5 mM-arginine and 1.67 mM-glucose, a potent stimulant of glucagon release (Unger *et al.*, 1969). This resulted in the synthesis of little glucagon, qualitatively similar to that obtained from a 20 h incubation in the absence of arginine and glucose (results not shown). Thus increased synthesis of glucagon was not seen in the presence of a potent secretagogue.

Incorporation of [^3H]leucine

Pigeon islets in the absence of glucose were incubated with [^3H]leucine for 3.5 h. The peak areas of material corresponding to the proinsulin/big-glucagon and insulin/little-glucagon fractions were separately pooled and re-fractionated (Fig. 2). Fig. 2(a) shows the incorporation of [^3H]leucine into a peak of material coincident with pigeon proinsulin immunoreactivity, $V_e/V_0 = 1.5$, and material was eluted between insulin and proinsulin, the elution

position of big glucagon. Fig. 2(b) shows the elution profile of the insulin/little glucagon fraction. Two fractions of radioactive material are present, which eluted coincident with pigeon insulin, $V_e/V_0 = 1.75$, and pigeon little glucagon.

Radioactive pulse-chase incubations

To ascertain whether big glucagon was a biosynthetic precursor of little glucagon, pigeon islets were incubated for 3.5 h in the presence of [^3H]tryptophan and chased in fresh medium containing unlabelled tryptophan and cycloheximide for periods ranging from 1.5 to 16 h.

The islets were extracted separately from the medium. Incorporation into glucagon was less than when the medium was co-extracted with the islets (cf. Figs. 1a and 3a). After a 5.5 h chase (Fig. 3b), and also extended periods of chase (16 h; Fig. 3c), the ratio of ^3H -labelled big glucagon to little glucagon remained unaltered. However, the absolute incorporation declined in both glucagon fractions, indicating degradation and/or secretion of the hormones.

Incubation media

Pigeon islets were incubated with [^3H]tryptophan for 20 h to allow substantial little glucagon biosynthesis to occur, and then chased for 20 h. Virtually no little glucagon was present in the islets, and only a small amount of big glucagon was present. On gel

Fig. 1. Incorporation of [^3H]tryptophan into pigeon islet proteins

(a) 3.5 h incubation. Pigeon islets (65) were incubated with [^3H]tryptophan in the presence of 1.7 mM-glucose. The islets were extracted as indicated in the text and gel filtered on a column (0.9 cm $\times$ 60 cm) of Sephadex G-50 in 1 M-acetic acid; 0.8 ml fractions were collected. The radioactivity profile represents the c.p.m. present in 0.6 ml of alternate 0.8 ml fractions. The glucagon immunoreactivity marker elution positions were determined as indicated in the legend to Fig. 4 and the remaining markers were determined by their E_{276} in a calibration gel filtration. Fractions 19–23 were re-fractionated on Sephadex G-100 (Fig. 6b) and fractions 24–32 were re-fractionated on Sephadex G-50 (Fig. 6a). Fractions 40–47 constituting the ^3H -labelled little glucagon were pooled and re-fractionated as indicated in the text. (b) 20 h incubation. Pigeon islets (90) were incubated for 20 h with [^3H]tryptophan and gel filtered on the same column. After 20 h incubation the ratio of little glucagon/big glucagon, had been reversed, compared with the ratio at 3.5 h shown in (a). Abbreviations of the markers: V_0 , void volume (elution position of Blue Dextran 2000); BG, bovine big-glucagon and pigeon big-glucagon immunoreactivity; LG, porcine little-glucagon and pigeon-little glucagon immunoreactivity.

Fig. 2. Incorporation of [³H]leucine into pigeon islet proteins in the absence of glucose

(a) Extract from 100 pigeon islets was gel filtered preparatively on a column (0.9cm × 60cm) of Sephadex G-50 in 1M-acetic acid; 0.8ml fractions were collected. The fractions representing the proinsulin and big-glucagon elution positions were combined and re-fractionated on the same column. Portions (0.6ml) of alternate fractions were counted for radioactivity. The radioactivity profile shows [³H]leucine incorporation into pigeon proinsulin and pigeon big glucagon. (b) Fractions comprising the insulin and little glucagon elution positions in the preparative gel filtration were combined and re-fractionated on a column (0.9cm × 30cm) of Sephadex G-50 in 1M-acetic acid. Fractions (0.5ml) were collected and 0.4ml of consecutive fractions were counted for radioactivity. The radioactivity profile shows [³H]leucine incorporation into pigeon insulin and pigeon little glucagon. Calibration markers: PI, porcine proinsulin; I, bovine insulin; LG, porcine little glucagon.

filtering the chase medium (Fig. 4a), a large amount of radioactive little glucagon was found, representing secretion of the newly synthesized hormone. The medium was gel filtered on Sephadex G-50 in 1M-acetic acid and freeze-dried portions of each fraction were assayed for glucagon content. The presence of both big- and little-glucagon immunoreactivity was demonstrated (Fig. 4b) indicating that big glucagon is also actively secreted by the islet α_2 cell.

Immunoreactivity chase incubations

The possibility that big glucagon represented a pool of little-glucagon precursor which had a very rapid rate of turnover was studied. Islets were incubated in the presence of cycloheximide for 16h and then the islet extract was gel filtered. Comparison of Figs. 5(a) and 5(b) shows that the amount of big glucagon had not decreased after 16h in comparison with the immunoreactive big glucagon present in unincubated islets.

Incorporation of tryptophan and leucine into molecules larger than proinsulin

Pigeon islets incorporated [³H]tryptophan into big and little glucagon as well as into material eluted at the void volume of the Sephadex G-50 column. The latter material (fractions 19–23, Fig. 1) was re-fractionated on Sephadex G-100 (Fig. 6b) and a small fraction of radioactive material was obtained with $V_e/V_0 = 2.0$ and apparent mol.wt. approx. 20000. The area immediately after the void volume of Sephadex G-50, (fractions 24–32, Fig. 1) was re-fractionated on Sephadex G-50, and the 20000-

mol.wt. material was re-eluted just after the void volume, $V_e/V_0 = 1.2$ (Fig. 6a). Identical elution positions for the 20000-mol.wt. material were obtained with [³H]leucine (Figs. 6c and 6d).

Radioactive material eluted at the void volume of Sephadex G-100, when refiltered on Sephadex G-200 was eluted as free tryptophan. More free tryptophan than leucine was eluted at the void volumes of Sephadex G-50 and G-100, presumably because of greater non-specific binding attributable to the aromatic nucleus of tryptophan.

Glucagon immunoreactivity larger than big glucagon

To examine the glucagon immunoreactivity in avian islet extracts that was eluted at the void volume of Sephadex G-50 (see Fig. 5), 1.4kg of turkey pancreas was extracted by acid/ethanol and fractionated on Sephadex G-50, as described for ox pancreas (O'Connor & Lazarus, 1976). The immunoreactive fraction that was eluted at the void volume was pooled and re-fractionated on a column (2.5cm × 100cm) of Sephadex G-100 in 1M-acetic acid, at a flow rate of 14ml/h, fraction size 7.5ml. Fig. 7(a) shows the glucagon-immunoreactivity profile. The majority of the immunoreactivity consisted of previously unfractionated little glucagon and was eluted near the salt peak. A fraction of glucagon immunoreactivity was eluted with $V_e/V_0 = 2.1$ and apparent mol.wt. approx. 20000. To establish the elution position of this molecule relative to big and little glucagon, the immunoreactivity was pooled and re-fractionated on a column (1.6cm × 70cm) of Sephadex G-50; 0.8ml fractions were collected (Fig.

7b). The molecule was eluted with $V_e/V_0 = 1.2$ (cf. big glucagon, $V_e/V_0 = 1.65$, and little glucagon, $V_e/V_0 = 2.1$).

Glucagon with mol.wt. 20000

Dog and ox pancreas extracts were examined for the presence of 20000-mol.wt. glucagon and the molecule was found to be present in both species (Figs. 8a and 8b).

The elution position of the immunoreactive material present in the Sephadex G-50 void-volume fractions from bovine pancreas extract was studied in the presence of the chaotropic agent trimethylurea on columns of Sephadex G-100 in order to assess whether the 20000-mol.wt. glucagon molecule contained the little-glucagon immunoreactive site covalently bonded to a large molecule. As shown in Fig. 8(b), the elution position of 20000-mol.wt. glucagon was stable to the chaotropic agent. The small amount of immunoreactive material that was eluted at the void volume of Sephadex G-100 in 1M-acetic acid, however, dissociated and was eluted as little glucagon.

The stability of the elution position of 20000-mol.wt. glucagon was examined further by re-fractionation of pooled fractions from a preparative gel filtration of turkey pancreas extract at alkaline pH (8.8) and fractionation of ox pancreas extract at neutral pH (7.4). The elution position was stable at all pH values examined.

Presence of 20000-mol.wt. glucagon molecules in the circulation

Examination of dog plasma for the presence of glucagon species other than little glucagon revealed the presence of two other areas of glucagon immunoreactivity, within the fractionation range of Sephadex G-50 (Fig. 9). These studies were conducted with an enteroglucagon cross-reacting antiserum so that the

Fig. 3. Pulse-chase incubations of pigeon glucagon labelled with [^3H]tryptophan

(a) Pigeon islets (100) were incubated with [^3H]tryptophan for a 3.5h pulse and then extracted immediately and gel filtered on a column (0.9cm $\times$ 60cm) of Sephadex G-50 in 1M-acetic acid. Portions (0.6ml) of alternate fractions were counted for radioactivity. After the pulse-labelling period 100 islets were chased for 5.5 (b) or 16(c) h in the presence of non-radioactive tryptophan, cycloheximide and penicillin, and then gel filtered as described above. The ratio of the amounts of little glucagon to big glucagon shows no alteration with increasing chase periods, but the absolute size of both peaks has declined. Markers: BG, big-glucagon immunoreactivity; LG, little-glucagon immunoreactivity.

Fig. 4. Analysis of pigeon islet incubation media

(a) Incorporation of [^3H]tryptophan into newly secreted little glucagon. Islets (100) were incubated for 20h with [^3H]tryptophan and then chased for 20h in the presence of cycloheximide. The medium was desalted on Sephadex G-25 in 1M-acetic acid; fraction size was 1 ml, and the void volume was freeze-dried. The residue was reconstituted in 0.5 ml of 1M-acetic acid and then fractionated on a column (0.9cm $\times$ 30cm) of Sephadex G-50 in 1M-acetic acid; fraction size 0.5 ml. Portions (0.4 ml) of each fraction were counted for radioactivity. (b) To examine the glucagon-immunoreactive species secreted by pigeon islets the chase incubation medium was examined by gel filtration on a column (0.9cm $\times$ 60cm) of Sephadex G-50 in 1M-acetic acid, and 0.8 ml fractions were collected. A portion (20 μ l) of each fraction was freeze-dried and reconstituted in 0.1 ml of the glucagon assay buffer for glucagon immunoassay. The little-glucagon fractions were diluted a further 1:4 before assay. The buffer composition and immunoassay method have been described (O'Connor & Lazarus, 1976). Markers: V_0 , void volume; BG, big glucagon; LG, little glucagon; S/P, salt peak.

organ of origin cannot be specified. A glucagon-immunoreactive fraction was eluted in the position of pancreatic big glucagon and gut peak I glucagon-like

immunoreactivity (Valverde *et al.*, 1970) and a further peak was eluted with $V_e/V_0 = 1.2$, coincident with the pancreatic 20000-mol.wt. glucagon elution position.

Discussion

Since the report of the presence of big glucagon in pancreas extract (Rigopoulou *et al.*, 1970), it has been widely assumed (Tager & Steiner, 1973; Melani, 1974) that this molecule is a proglucagon, i.e. a biosynthetic precursor of little glucagon. The prefix pro- should be reserved for those hormonal forms for which quantitative transfer of material from a precursor pool into its presumed product in the presence of protein synthesis inhibition has been unequivocally demonstrated. Only proinsulin and proparathyrin have fulfilled these requirements. Limited proteolysis with trypsin has been used in an attempt to demonstrate precursor relationships but can only represent corroborative evidence.

Trypsin treatment of bovine big glucagon produces not little glucagon, but a slightly smaller immunoreactive molecule. Limited proteolysis of little glucagon apparently produces this same molecule, indicating that the little-glucagon structure is present within the big-glucagon molecule. However, this result by itself furnishes no direct evidence of a precursor-product relationship. Trypsin treatment of anglerfish big glucagon produces intact little glucagon because the latter molecule is more resistant to proteolytic attack than is mammalian little glucagon (Trakatellis *et al.*, 1975). In the present paper we have directed our attention to the precursor role of big glucagon.

Pigeon islets readily incorporated [^3H]tryptophan and [^3H]leucine into little glucagon and [^3H]tryptophan-labelled glucagon was secreted into the medium. The synthesis of [^3H]tryptophan-containing glucagon was decreased in the presence of a high (28 mM) glucose concentration, an inhibitor of glucagon secretion. [^3H]Leucine, in the presence of zero glucose concentration was incorporated into proinsulin and insulin as well as into glucagon. [^3H]Tryptophan and [^3H]leucine were also incorporated into a fraction of mol.wt. 8200, which was eluted in the position of pigeon big-glucagon immunoreactivity. Pigeon big-glucagon biosynthesis began immediately, whereas a lag of about 2 h occurred before incorporation into little glucagon could be detected. Incorporation into little glucagon once commenced was considerably faster than incorporation into big glucagon, so that after 20h incubation the big-glucagon fraction was far smaller than that of combined stored and secreted little glucagon.

Purification on Sephadex G-100 of the radioactive fraction that was eluted at the void volume on Sephadex G-50 showed that both [^3H]tryptophan and [^3H]leucine were incorporated into material eluted

Fig. 5. Glucagon immunoreactivity chase incubations of pigeon islets

(a) Pigeon islets (100 per incubation) were isolated and extracted immediately (the zero chase), or (b) incubated for a 16h chase period in the presence of cycloheximide before extraction. They were then fractionated on a column (0.9cm×60cm) of Sephadex G-50 in 1M-acetic acid, and 0.8ml fractions were collected. The column fractions were immunoassayed for glucagon content and the ratios of the sizes of the big glucagon to the little glucagon fractions before and after the 16h chase period were compared. No significant difference was observed. Markers: V_0 , void volume; BG, big glucagon; LG, little glucagon.

Fig. 6. Incorporation of [^3H]tryptophan and [^3H]leucine into 20000-mol.wt. glucagon

(a) [^3H]Tryptophan incorporation: fractions 24–32 (Fig. 1) were pooled and re-fractionated on a column (0.9cm×30cm) of Sephadex G-50 in 1M-acetic acid. (b) [^3H]Tryptophan incorporation: fractions 19–23, the void volume of the Sephadex G-50 column (Fig. 1) were re-fractionated on a column (0.9cm×30cm) of Sephadex G-100 in 1M-acetic acid. (c) [^3H]Leucine incorporation: the 20000-mol.wt. glucagon area was pooled from the preparative gel-filtration column of Sephadex G-50 described in the legend to Fig. 2(a) and re-fractionated on a column (0.9cm×30cm) of Sephadex G-50 in 1M-acetic acid. (d) [^3H]Leucine incorporation: the void-volume fractions from the preparative gel-filtration column of Sephadex G-50 referred to in (c) were re-fractionated on a column (0.9cm×30cm) of Sephadex G-100 in 1M-acetic acid. In all re-fractionations, 0.5ml fractions were collected at 4°C, and 0.4ml of consecutive fractions were counted for radioactivity. A component of apparent mol.wt. 20000 present in pigeon islets was labelled by both tryptophan and leucine. Markers: V_0 , void volume; 2×10^4 mol.wt., 20000-mol.wt. glucagon; LG, little glucagon; S/P, salt peak.

Fig. 7. Turkey pancreas 20000-mol.wt. glucagon immunoreactivity

(a) Turkey pancreas extract was gel filtered on a column (5cm $\times$ 100cm) of Sephadex G-50 in 1M-acetic acid. The extraction and fractionation procedures were as described for bovine pancreas extract (O'Connor & Lazarus, 1976). The glucagon immunoreactivity eluting near the void volume of the above column was pooled and re-fractionated on a column (2.5cm $\times$ 100cm) of Sephadex G-100 in 1M-acetic acid. Fractions (5ml) were collected and portions (0.1ml) of alternate fractions were immunoassayed for glucagon content. This glucagon-immunoreactivity profile is shown. The majority of the immunoreactivity elutes coincident with the little-glucagon marker elution position. A smaller fraction containing glucagon immunoreactivity elutes between fractions 40–50 with apparent mol.wt. 20000. (b) Turkey 20000-mol.wt. glucagon (a portion of the pool of fractions 40–50 from a) was re-fractionated on a column (1.6cm $\times$ 70cm) of Sephadex G-50 in 1M-acetic acid. Fractions (0.8ml) were collected, and portions (0.1 ml) of alternate fractions were immunoassayed for glucagon content. A single glucagon-immunoreactive fraction was obtained with apparent mol.wt. 20000. Markers: V_0 , void volume; 2×10^4 mol.wt., 20000-mol.wt. glucagon; LG, little glucagon.

between chymotrypsin A (mol.wt. 25000) and cytochrome *c* (mol.wt. 12500). The elution position of this radioactive fraction corresponded to that of turkey 20000-mol.wt. glucagon, which had been located by immunoreactivity. Poffenbarger *et al.* (1971) and Schatz *et al.* (1973), using mouse islets, described

incorporation of [3 H]leucine into a protein eluted with similar molecular-weight characteristics to 20000-mol.wt. glucagon, and Petersen *et al.* (1975), using a 3 H-labelled amino acid mixture, obtained incorporation into an approx. 16000-mol.wt. molecule in mouse islets which could be bound by glucagon antibodies. No synthesis of little glucagon, however, was obtained by any of these authors. [3 H]Tryptophan has thus been incorporated into three molecules: little glucagon (mol.wt. 3500), big glucagon (mol.wt. 8500) and a 20000-mol.wt. glucagon.

No pulse-chase relationship between big and little glucagon could be demonstrated with either short or long pulse times. With extended periods of chase the little glucagon fraction declined and this was correlated with the secretion of 3 H-labelled little glucagon. It is conceivable that a precursor role for big glucagon would not have been detected by radioactive pulse-chase experiments if the pool of big glucagon was extremely small and the rate of transfer into product was extremely rapid. If the synthesized material eluted in the big glucagon fraction was heterogeneous, this would also mask a small amount of incorporation into big glucagon. This proposition was examined by immunoreactivity chase experiments. In the presence of effective blockage of protein synthesis, no diminution of the pool of big glucagon immunoreactivity could be detected over a 16h incubation period. The quantity of big glucagon present in pancreas extracts is not significantly underestimated by immunoassay (O'Connor & Lazarus, 1976). Therefore if the above precursor-product relationship existed, this incubation period would have allowed ample time for nearly all of the pool of big glucagon immunoreactivity to be transferred into little glucagon.

Three areas of glucagon immunoreactivity were present in the Sephadex G-50 profiles of pigeon islet extract, namely little glucagon, big glucagon and a small fraction of immunoreactivity at the void volume. Extraction and fractionation of turkey pancreas enabled examination of the properties of each of these fractions. Turkey little glucagon resembled bovine little glucagon in molecular weight and charge but showed virtually no cross-reactivity with antisera specific for the C-terminus of mammalian little glucagon. This agrees with the replacement of serine-28 by asparagine (Markussen *et al.*, 1972). Turkey big glucagon was present in higher concentration (120 μ g/kg of pancreas) than the bovine hormone (33 μ g/kg) and was of similar mol.wt. (approx. 8200). Both immunoreactive big and little glucagon are actively secreted by the α_2 cell and the ratio of big to little glucagon is decreased relative to the intracellular ratio.

Turkey pancreas extract contained an immunoreactive glucagon with apparent mol.wt. 20000. Examination of ox and dog pancreas extracts demonstrated the presence of this immunoreactive

Fig. 8. Presence of 20000-mol.wt. glucagon in dog and ox pancreas and stability of the molecular weight to a chaotropic agent

(a) A dog pancreas was extracted with acid/ethanol and gel filtered on a column (2.5cm $\times$ 100cm) of Sephadex G-50 in 1M-acetic acid. Glucagon immunoreactivity was eluted near the void volume, and was pooled and re-fractionated on a column (2.5cm $\times$ 100cm) of Sephadex G-100 in 1M-acetic acid. Fractions (5ml) were collected and portions (0.1ml) were immunoassayed for glucagon content. This glucagon immunoreactivity profile is shown and a peak of immunoreactivity is present with apparent mol.wt. 20000. (b) A portion of the pooled void volume fractions from gel filtration of ox pancreas extract on the Sephadex G-50 column described in (a) was re-fractionated on a column (0.9cm $\times$ 30cm) of Sephadex G-100 in 1M-trimethylurea. Fractions (0.5ml) were collected, and portions (0.1ml) of consecutive fractions were freeze-dried and immunoassayed for glucagon content. The void-volume position was marked by elution of a fraction of high-molecular-weight protein, but the chaotropic agent eliminated the glucagon immunoreactive fraction which would have eluted at the void volume in 1M-acetic acid (cf. a) indicating that the later material consisted of unfractionated little glucagon. The elution position of 20000-mol.wt. glucagon was unaffected. Markers: V_0 , void volume; 2×10^4 mol.wt., 20000-mol.wt. glucagon; LG, little glucagon.

glucagon species in both, and it thus appears to be common to the mammalian and avian pancreas. This 20000-mol.wt. glucagon was stable to chaotropic agents and to elution at different pH values. It cross-reacted with both non-specific and C-terminus-specific glucagon antibodies. Examination of dog plasma indicated the presence of an immunoreactive species in the circulation of the same size as the glucagon of 20000mol.wt.

Previous biosynthetic studies of glucagon have produced a wide range of molecules (mol.wt. 70000-9000), all of which have been proposed for the role of a proglucagon. Noe & Bauer (1971), using anglerfish principal islet tissue, demonstrated incorporation of [3 H]tryptophan into material of 11400 mol.wt., shown to be heterogeneous by gel filtration. Trakatellis *et al.* (1975) found that less than 50% of this [3 H]tryptophan incorporation was present in anglerfish big glucagon. Subsequently Noe & Bauer (1975) showed a pulse-chase relationship between the 11400-mol.wt. fraction and little glucagon,

and have proposed that the 9000-mol.wt. big glucagon isolated by Trakatellis *et al.* (1975) is not involved in the synthesis of anglerfish little glucagon or at most has a transient intermediate role. It is noteworthy that the major species of glucagon in the cod has an apparent molecular weight on gel filtration similar to that of big glucagon and no little-glucagon immunoreactivity eluted coincident with the porcine little-glucagon marker (O'Connor & Lazarus, 1976). This suggests that mammalian little glucagon may have been derived from a similar larger molecule.

Tung (1973), using pigeon islets isolated by collagenase treatment, obtained incorporation of [3 H]tryptophan into material with an apparent mol. wt. 69000. Whether this material and 20000-mol.wt. glucagon are related is not apparent. Hellerström *et al.* (1974) reported [3 H]tryptophan incorporation into material of 18000mol.wt. in guinea-pig islets after 17h incubation. Incorporation into little glucagon required incubations for at least 6 days. The 18000-mol.wt. material when fractionated at low pH

Fig. 9. Presence of a 20000-mol.wt. glucagon species in the dog circulation

Approx. 100 ml of dog plasma was adjusted to pH 3 with acetic acid and the precipitate discarded. The sample was then gel filtered without further extraction on a column (10 cm $\times$ 100 cm) of Sephadex G-50 in 1 M-acetic acid and 12.5 ml fractions were collected. Eluates comprising the void-volume protein fraction and the little glucagon/salt fraction, determined from the E_{276} of the eluates, were discarded and the remaining fractions were pooled and freeze-dried. The residue was taken up in 40 ml of glucagon immunoassay buffer which had been diluted 1:20 with water and re-fractionated on a column (5 cm $\times$ 60 cm) of Sephadex G-50 in glucagon assay buffer diluted 1:20 and 5 ml fractions were collected. Portions (2 ml) of alternate fractions were freeze-dried, reconstituted in 0.1 ml of water and immunoassayed for glucagon content. An antiserum cross-reacting with enteroglucagon was used, giving the summation of circulating pancreatic and gut glucagon species. Glucagon immunoreactivity was eluted as two fractions with apparent mol.wts. of 20000 and 8000. Markers: V_0 , void volume; this was marked by the elution position of residual high-molecular weight protein; 2×10^4 mol.wt., 20000-mol.wt. glucagon; BG, big glucagon and the elution position of gut 'peak I glucagon-like-immunoreactivity'; LG, little glucagon.

dissociated into material of 9000 mol.wt. Hellerström *et al.* (1974) were unable to demonstrate that the 9000-mol.wt. material was a precursor of glucagon. The behaviour of the 18000-mol.wt. material was unlike that of 20000-mol.wt. of glucagon, which shows stability to changes in pH. Howell *et al.* (1974), using radioautography, showed rapid transfer of [3 H]tryptophan from the guinea-pig α_2 -cell rough endoplasmic reticulum into the secretion granules, but no labelled little glucagon could be extracted. In rat pancreas, pigeon islets and fish islets, incorporation into little glucagon occurs within 4 h. The reasons for the extended period necessary to achieve little glucagon biosynthesis in isolated guinea-pig

islets are unclear, but may reflect a decreased ability of mammalian islets to synthesize glucagon, since similar time-periods of incubation were necessary to synthesize little glucagon in isolated mouse islets (K. J. O'Connor, unpublished work).

Pulse-chase incubations in pigeon islets could not demonstrate that big glucagon is a biosynthetic precursor of little glucagon. As yet no pulse-chase information is available relating the 20000-mol.wt. glucagon to the other two glucagons. The inter-relationships between the various known glucagon species in the pancreas thus remain to be elucidated.

We gratefully acknowledge the technical assistance of Mr. R. A. Forder.

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