The Biosynthesis of Glucagon in Perfused Rat Pancreas

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The biosynthesis of glucagon was studied by using the recirculated, isolated perfused rat pancreas. [³H]Tryptophan was initially incorporated into acid-ethanol-extractable protein, which on gel filtration was eluted with a molecular weight of about 9000 and contained a small amount of glucagon immunoreactivity. With longer incubation [³H]tryptophan incorporation into a second peak was obtained in an identical position with that of the majority of rat glucagon immunoreactivity. This peak of labelled protein exhibited migration characteristics on polyacrylamide-gel electrophoresis identical with those of rat glucagon and was identified as newly synthesized glucagon by demonstration of specific binding and dissociation behaviour with glucagon antibodies. The incorporation of [³H]tryptophan into acid-ethanol-extractable protein was inhibited by cycloheximide. High concentrations of glucose increased [³H]tryptophan incorporation into high-molecular-weight protein but decreased incorporation into proteins smaller than cytochrome *c*. The pattern of [³H]leucine incorporation into protein was similar to that of [³H]tryptophan.

Rigopoulou *et al.* (1970) have reported the existence of a molecule more than twice the size of glucagon, containing glucagon immunoreactivity in extracts of dog pancreas. Noe & Bauer (1971) used Angler-fish principal islet tissue to demonstrate incorporation of [³H]tryptophan into fish glucagon and into a second molecule more than twice the size of glucagon. They were unable to demonstrate significant immunoreactivity in this larger molecule, nor was it found possible to incorporate [³H]leucine into fish glucagon in either the presence or the absence of glucose. Tung & Zerega (1971) reported the incorporation of labelled tryptophan into bird glucagon and higher-molecular-weight proteins in isolated pigeon islets. It appeared that in this system all tryptophan incorporation was virtually abolished by high concentrations of glucose.

Little is known of the pattern and control of glucagon secretion and of the storage of the hormone, and nothing is known of the mechanism of glucagon biosynthesis in mammalian systems. The biosynthesis of glucagon in mammalian islets isolated with collagenase appears to be extremely difficult to accomplish considering the ease with which insulin can be synthesized in these islets. Biosynthesis of glucagon *in vitro* was therefore undertaken in a recirculated perfused rat pancreas preparation.

Materials

Sephadex G-50 and G-75 (superfine grade) were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K.; Trasylol proteolytic enzyme inhibitor from F.B.A. Pharmaceuticals, Haywards Heath, Sussex,

U.K.; DL-[³H]tryptophan from Becton Dickinson, Wembley, Middx., U.K.; L-[³H]leucine from New England Nuclear Chemicals, Reading, Berks., U.K.; Na¹²⁵I from The Radiochemical Centre, Amersham, Bucks., U.K.; ¹²⁵I-labelled glucagon from Isoserve, Cambridge Nuclear, Mass., U.S.A.; bovine serum albumin from Sigma (London) Chemical Co., London S.W.6, U.K.; cytochrome *c* from Boehringer Corp. (London) Ltd., London W.5, U.K.; bovine insulin from Burroughs Wellcome and Co., Dartford, Kent, U.K.; bovine/porcine glucagon from Eli Lilly, Basingstoke, Hants., U.K.; Ne 220 scintillant from Nuclear Enterprises, Edinburgh, U.K.; acrylamide (electrophoresis grade) from Eastman Kodak Co., Rochester, N.Y., U.S.A.; ethylene diacrylate from Borden Chemical Inc., Philadelphia, Pa., U.S.A.; Coomassie Brilliant Blue from G. T. Gurr Limited, London N.W.9, U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Operative and incubation techniques

Male Wistar rats (250 g) were starved overnight, anaesthetized by nembutal (50 mg/kg, intraperitoneally) and the pancreas was dissected free of the gut and spleen. The ventral aorta was dissected free of all surrounding tissue, cannulated, the portal vein cut and the entire pancreas removed from the animal. The pancreas was perfused with an incubation medium consisting of bicarbonate-buffered Ringer, pH 7.4 (Gey & Gey, 1936), supplemented with dextran (4%, w/v), and Trasylol (1000 Kallikrein inactivator units/

ml). All protein amino acids at a concentration of $10\text{ }\mu\text{g/ml}$, excluding the radioactive amino acid under study, were added to 100ml of the perfusate. DL-[^3H]Tryptophan (present in all known glucagons but no known proinsulin or insulin) (25 Ci/mmol) and L-[^3H]leucine (50 Ci/mmol) were used at a concentration of $250\text{ }\mu\text{Ci}$ in 100ml of perfusate. Glucose was added to a concentration of 1.67 mM in the majority of experiments. Penicillin (100 units/ml) was added to perfusate used for incubations of more than 2h. The perfusate was gassed continuously with $\text{O}_2 + \text{CO}_2$ (95:5), which was moistened by bubbling through water. The pancreas and perfusate were maintained at 37°C by means of a Dubnoff metabolic shaker bath. The perfusate was continuously circulated from the perfusate reservoir through the pancreas by means of a Serva peristaltic pump, which maintained pulsed flow at a rate of 5 ml/min . pH monitoring throughout initial experiments indicated that a constant pH of 7.4 was maintained in this system. The pancreas was cleared of blood by preincubation for 30min with perfusate containing 1.67 mM -glucose without recirculation. After incubation the pancreas was washed for 30min by perfusion with perfusate containing 16.7 mM -glucose to remove the majority of unincorporated radioactivity and at the same time to minimize glucagon secretion. Up to three pancreas preparations were perfused simultaneously.

The isolated perfused pancreas system was tested for glucagon release during a recirculated perfusion. The medium was sampled at 15min intervals for 4h into an equal volume of glucagon-assay buffer and immediately deep-frozen at -80°C until assayed. A cumulative linear release of glucagon was demonstrated. A linear increase in incorporation of radioactivity into acid-ethanol-soluble protein was obtained for more than 4h thus demonstrating viability of the preparation under the experimental conditions employed.

Perfusion of pancreas of streptozotocin-diabetic rat

Male rats (200g) were rapidly injected intravenously with 13.5 mg of streptozotocin (kindly supplied by Upjohn Ltd., Crawley, Sussex, U.K.) in 1 ml of acid saline (0.3 g of *O*-cresol and 10 ml of 1 M-HCl /litre of 0.9% NaCl). The animals were left 36h before operation and fed *ad libitum* before being starved overnight. Controls were injected with acid saline alone. The operative and pancreas perfusion techniques remained unaltered.

Extraction procedure

The perfused pancreata were extracted by the method of Kenny (1955) for glucagon. The pancreas was dissected free of extraneous tissue and ligatures, homogenized in acid-ethanol (750 ml of ethanol, 250 ml of water and 5 ml of conc. HCl) by using a

Willems polytron (Kinematica G.m.b.H., Lucerne, Switzerland) homogenizer and extracted overnight at 4°C . Two re-extractions with acid-ethanol for 1h and $\frac{1}{2}\text{ h}$ respectively were followed by adjustment of the pooled supernatants to pH 7.5 with aq. NH_3 (sp. gravity 0.880) and centrifugation to remove the residue. Protein was precipitated by alcohol-ether (6.8 ml of ethanol and 11.2 ml of diethyl ether/ 4 ml of extract) overnight at 4°C . The precipitate was recovered by centrifugation and dried by vacuum desiccation at 4°C . The residue was dissolved in 9 ml of chloride-phosphate buffer (200 ml of $0.1\text{ M-Na}_2\text{HPO}_4$, pH 7.4, and 800 ml of 0.9% NaCl/litre) and dialysed against 10 litres of the same solution for 48h at 4°C . The dialysis residue was then freeze-dried and the residue taken up in 1.5 ml of 1 M -acetic acid in preparation for gel filtration.

Gel filtration

Gel filtration of labelled pancreas extract was carried out in 1 M -acetic acid on columns ($1.6\text{ cm} \times 70\text{ cm}$) of Sephadex G-50 (superfine grade) at 4°C .

Refractionations were carried out on columns of $0.9\text{ cm} \times 30\text{ cm}$. Flow rate was adjusted to 4 ml/h and the eluate was collected in 15 drop fractions ($\approx 0.8\text{ ml}$), or 10 drops ($\approx 0.5\text{ ml}$) for the smaller columns in an LKB Ultrarac fraction collector. Protein was determined by a Uvicord II absorptiometer connected to an LKB chopper-bar recorder, or by micro-Lowry protein assay (Lowry *et al.*, 1951) with bovine serum albumin as standard protein. The void-volume peak fraction contained less than $75\text{ }\mu\text{g}$ of protein. Columns were calibrated with bovine serum albumin, cytochrome *c*, bovine insulin, bovine/porcine glucagon and $^{125}\text{I}^-$ or Cl^- , the last being detected by AgNO_3 . Marker proteins were not added to pancreatic extracts because of uncertainties introduced by non-specific absorption of radioactive amino acid. Similarly, ^{131}I -labelled protein markers were not added because of uncertainties introduced when counting for ^3H in the presence of ^{131}I . Radioimmunoassays of insulin and glucagon were used as internal calibration markers during test-extract filtrations. Samples (0.5 ml) of alternate fractions from 70 cm columns and 0.4 ml samples of all fractions from 30 cm columns were mixed with 10 ml of NE 220 scintillant and radioactivity was measured in a Nuclear Enterprises automatic liquid-scintillation spectrometer. Radioactivity was measured until sufficient counts had accumulated to give a counting error of $\pm 5\%$ after subtraction of the background count.

Electrophoresis

The peaks were pooled, freeze-dried and resuspended in 0.2 ml of 0.01 M-HCl - 15% (w/v) sucrose, with a trace of Bromophenol Blue tracker dye.

The 5cm running gels, containing 15% (w/v) polyacrylamide and ethylene diacrylate cross-linking agent were polymerized by persulphate. The stacking gel (2.5% polyacrylamide) was polymerized above the running gel. Samples were subjected to electrophoresis in 0.1 M-Tris-0.077 M-glycine buffer, pH 8.6. Under standard running conditions electrophoresis was stopped when the solvent front had migrated to the bottom of the gel. Extended electrophoresis times were obtained by continuation of electrophoresis for a further 2h.

Gels to be stained were fixed in 10% (w/v) trichloroacetic acid for 30min, then stained overnight in aq. 1% (w/v) Coomassie Brilliant Blue in 12.5% (w/v) trichloroacetic acid. Gels for staining and slicing were stained within the running tube by injection of trichloroacetic acid fixative and stain through and around the gel. Gels for counting of ^3H radioactivity were sliced in a Gilson Gel Slicer directly from the running tubes into 1 or 2mm slices, with 0.4 M-aq. NH_3 as diluent. The samples were eluted for 48h at 4°C and then 10ml of scintillant was added to each. Slices intended for radioimmunoassay were eluted with 0.25ml of 0.01 M-HCl overnight at 4°C . Two 100 μl samples were then freeze-dried in assay tubes and reconstituted in 100 μl of glucagon-assay buffer.

Insulin and glucagon immunoassay

Insulin was assayed by the double-antibody method with the Wellcome/Amersham Kit (Wellcome Biological Reagents, Beckenham, Kent, U.K.).

Glucagon was assayed by using two antisera, a pancreatic-specific (K 814 kindly donated by L. Heding, Novo Research Institute, Copenhagen, Denmark), and a cross-reacting antiserum. Initial assays were carried out according to the method of Heding (1971) by using ethanol separation. Later assays employed a double-antibody technique with anti-rabbit IgG precipitating serum (Wellcome Biological Reagents) as second antibody. Assay standard curves were constructed by a plot of the ratio of radioactivity in the blank to radioactivity in the sample (C_0/C_p) versus bovine/porcine glucagon standards from 2ng to 125pg. Binding to antisera of pooled radioactive peaks was carried out by incubation with glucagon antiserum, (undiluted or 1/100), for 24h at 4°C followed by freeze-drying, reconstitution in 0.5 ml of 0.1 M-sodium barbitone buffer, pH 8.6, and separation of the bound and free forms on Sephadex G-75 in the same buffer. Non-immune serum was used for controls.

Results

Column calibration

The relative elution positions of protein standards are shown in Figs. 1 and 2. Bovine serum albumin

has an approximate molecular weight of 66000 and elutes at the void volume. Cytochrome *c*, molecular weight 12400, and bovine insulin, molecular weight 5750, elute in the positions indicated. Bovine/porcine glucagon, molecular weight 3480, elutes approx. 10.5ml after insulin on a 70cm column and approx. 2ml after insulin on a 30cm column.

Calibration of the experimental extract was obtained from the insulin and glucagon immunoassays of the profile. Insulin immunoassay indicated the elution volumes of rat insulin and rat proinsulin. Glucagon immunoassay of alternate fractions of eluate indicated two peaks of glucagon immuno-reactivity (Fig. 1); rat glucagon identical with bovine/porcine glucagon of molecular weight 3480 and rat pancreatic large glucagon of molecular weight similar to rat proinsulin, molecular weight approx. 9000.

Pattern of [^3H]tryptophan incorporation into protein

Fig. 1 shows the radioactivity elution profile of an extract of rat pancreas incubated with [^3H]tryptophan for 4h. A complex pattern of incorporation eluted in a position near to or before cytochrome *c*. These proteins represent pancreatic proteins of high molecular weight. A large peak of [^3H]tryptophan incorporation eluted between cytochrome *c* and insulin in a position coincident with that of the large immunoreactive glucagon of pancreatic origin. A small peak of [^3H]tryptophan incorporation eluted coincident with the elution volume of glucagon marker. The vast majority of the immunoreactive glucagon eluted coincident with the smaller [^3H]tryptophan-labelled protein, supporting the view that this peptide was radioactively labelled rat glucagon. With incubation times of less than 4h [^3H]tryptophan incorporation could be detected only in the large-glucagon area.

After rechromatography on a 0.9cm $\times$ 30cm column, the large [^3H]tryptophan peak (fractions 100-125, Fig. 1), re-eluted in the position of the large glucagon of pancreatic origin (Fig. 2a). Refractionation of the glucagon peak area (fractions 126-138, Fig. 1), gave a well-resolved peak of [^3H]tryptophan incorporation eluting in an identical position with that of rat glucagon (Fig. 2b).

The identification of the [^3H]tryptophan peak eluting in the glucagon-marker position was established as newly biosynthesized rat glucagon by incubation of the pooled fractions with glucagon antiserum and separation of antibody bound from free glucagon by gel filtration. ^3H radioactivity was shifted from the glucagon elution position to the void volume of the column, the elution position of an antibody complex (Fig. 3a). Incubation of the antibody complex so formed with 0.1 M-HCl for 16h dissociated the majority of the glucagon, which was then re-eluted in the position of glucagon (Fig. 3b).

Pattern of [^3H]leucine incorporation into protein

[^3H]Leucine incorporation in the presence of zero glucose (Fig. 4a) was very similar to that of

[^3H]tryptophan. Fig. 4(c) shows the refractionation profile of the rat glucagon area and Fig. 4(b) shows the refractionation profile of the large peak of

Fig. 1. Gel-filtration pattern of [^3H]tryptophan incorporation into acid-ethanol-soluble proteins from rat pancreas

Column characteristics: 1.6cm $\times$ 70cm; Sephadex G-50 (superfine grade) in 1 M-acetic acid; volume of fractions, 0.8ml. Radioactivity (—) was determined on 0.5ml samples of alternate fractions. Samples (0.1ml) of alternate fractions were radioimmunoassayed for immunoreactive glucagon content (----). The immunoreactive peak indicated by the bar (fractions 100–116) is designated large-glucagon immunoreactivity. The elution position of molecular-weight markers, bovine/porcine glucagon, bovine insulin, cytochrome c, and bovine serum albumin are indicated by arrows.

Fig. 2. [^3H]Tryptophan-incorporation pattern of pancreas extract after refractionation of the large-glucagon-immunoreactivity area and glucagon area

Column dimensions: 0.9cm $\times$ 30cm; vol. of fractions, 0.5ml. Samples (0.4ml) of all fractions were counted for radioactivity. (a) Refractionation of the large-glucagon-immunoreactivity area of Fig. 1 (fractions 100–116). (b) Refractionation of the glucagon area of Fig. 1 (fractions 120–140).

Fig. 3. Binding of synthesized glucagon to glucagon antibodies and dissociation of the glucagon-antibody complex

(a) Gel-filtration elution profile of the separation of antibody-bound synthesized [^3H]glucagon from unbound glucagon. Column characteristics: 0.9 cm $\times$ 30 cm; Sephadex G-75 in barbitone buffer, pH 8.6, 0.4 ml samples of alternate fractions (vol. 0.5 ml) were counted. (b) Gel-filtration elution profile of acid-dissociated glucagon-antibody complex (incubated in 0.1 M-HCl for 16 h at 4°C). Column characteristics: 0.9 cm $\times$ 30 cm; Sephadex G-50 in 1 M-acetic acid; vol. of fractions 0.5 ml.

incorporation. Glucagon immunoassay of this peak demonstrated that simultaneously the large-glucagon immunoreactivity had been further purified.

Effect of cycloheximide and glucose concentration on [^3H]tryptophan incorporation

In the presence of cycloheximide, a specific inhibitor of protein synthesis at the ribosome level, [^3H]tryptophan incorporation was markedly inhibited (Fig. 5). The addition of 11 mM- and 16.7 mM-glucose decreased incorporation into both peaks, this effect being more marked at the higher concentration.

At the same time increased incorporation of radioactive tryptophan occurred into excluded protein (compare Figs. 1 and 5). Perfusion of pancreas of streptozotocin-diabetic rat with [^3H]tryptophan in the presence of 16.7 mM-glucose gave an incorporation pattern comparable with low glucose incorporation (results not shown).

Polyacrylamide-gel electrophoresis

Rat [^3H]glucagon was subjected to electrophoresis on polyacrylamide gel and the radioactivity and glucagon immunoreactivity of the sliced gels determined.

After standard gel electrophoresis, biosynthesized [^3H]glucagon migrated in an identical position with that of rat glucagon immunoreactivity and

bovine/porcine glucagon standard (Figs. 6a and 6b). The peak of ^3H eluting in the immunoreactive-large-glucagon position migrated on gel electrophoresis in

Fig. 4. Gel-filtration pattern of [^3H]leucine incorporation in acid-ethanol soluble proteins from pancreas

(a) Column dimensions: 1.6cm $\times$ 70cm; vol. of fractions, 0.8ml. —, [^3H]Leucine radioactivity of alternate fractions. Glucagon immunoreactivity (----) is plotted from the ratio of counts bound in the control to the counts bound in the sample (for details see the text). ·····, Insulin immunoreactivity. (b) Refractionation of the large-glucagon-immunoreactivity area (fractions 100–115) of Fig. 4(a). Column dimensions: 0.9cm $\times$ 30cm; vol. of fractions, 0.5ml. —, Radioactivity of all fractions; ----, glucagon immunoreactivity. (c) Refractionation of the glucagon area (fractions 116–136) of Fig. 4(a). Volume of column fractions, 0.5ml. —, Radioactivity; ·····, insulin immunoreactivity (C_0/C_1).

Fig. 5. Effect of cycloheximide and high concentrations of glucose on $[^3\text{H}]$ tryptophan incorporation into acid-ethanol-soluble proteins from pancreas

For details see the text. Column fraction vol., 0.8 ml. —, Glucose, 11 mM, 2h incubation; ····, glucose, 16.7 mM, 3h incubation; ----, cycloheximide, 0.66 mM, 2h incubation.

the same position as rat glucagon immunoreactivity and large-glucagon immunoreactivity (results not shown). When extended electrophoresis times were used, glucagon migrated to the positive (bottom) end of the gel. Under these conditions the large peak migrated only a small distance further into the gel (Fig. 6c).

Discussion

Results presented here indicate that rat pancreas perfused *in vitro* incorporates $[^3\text{H}]$ tryptophan into acid-ethanol-extractable proteins eluting in the position of large glucagon and more slowly into a protein eluting in a position characteristic of glucagon.

The identification of the latter protein as newly synthesized rat glucagon is based on the following observations. The synthesized material is extracted by the method of Kenny (1955), a standard glucagon-extraction procedure; tryptophan, a rare amino acid, present in glucagon is actively incorporated; the synthesized material elutes in a position identical with that of the majority of rat glucagon immunoreactivity; incorporation is decreased by the presence of cycloheximide and high concentrations of glucose; on gel electrophoresis it migrates to the same position as glucagon and is not separable from the glucagon immunoreactivity. Finally, the synthesized material was positively identified as newly synthesized rat glucagon by demonstration of specific binding to glucagon antibodies and the dissociability of the complex by low pH. Gastrin of pancreatic origin which contains tryptophan elutes later than glucagon

Fig. 6. Polyacrylamide-gel electrophoresis of the radioactive peak in the large-glucagon-immunoreactivity area and of synthesized $[^3\text{H}]$ glucagon

Gels counted for radioactivity were sliced directly from the running tubes in 1 mm sections; gels for immunoassay were sliced into 2 mm sections. (a) Electrophoresis under standard conditions (see the text). Immunoreactivity (C_0/C_s) of 10 ng of bovine/porcine glucagon standard. (b) Electrophoresis of a pooled peak of synthesized rat glucagon. —, $[^3\text{H}]$ -Tryptophan radioactivity; ----, immunoreactivity of rat glucagon. (c) Extended electrophoresis of the peak of $[^3\text{H}]$ tryptophan incorporation eluting in the large-glucagon-immunoreactivity area. —, $[^3\text{H}]$ -Tryptophan radioactivity. Bovine/porcine glucagon standard (100 μg) was stained by Coomassie Blue within the running tube and sliced in 1 mm sections. ----, Extinction of the eluted slices at 600 nm.

from Sephadex G-50 and does not cross-react with glucagon antibodies.

The synthesized [^3H]tryptophan peak containing large-glucagon immunoreactivity had a molecular weight comparable with that of rat proinsulin. This material appears to be synthesized in a way similar to that of rat glucagon in the presence of cycloheximide and high concentrations of glucose. It migrates on gel electrophoresis in a similar position to that of glucagon on 5cm gels unless extended running times are employed. The molecule is then less electronegative than glucagon. This synthesized material can also be partially bound by, and dissociated from, glucagon antibodies (see Fig. 3b).

A time-lag of about 4h occurs before [^3H]tryptophan incorporation into glucagon can be detected. This observation may be explained either in terms of technical difficulties (i.e. the presence of just one tryptophan residue in the molecule, the relatively low specific radioactivity of the labelled amino acid, etc.), or by the existence of a precursor with slow or retarded conversion in the biosynthesis of glucagon.

High concentrations of glucose impaired [^3H]tryptophan incorporation into both the large-glucagon-immunoreactivity area and the glucagon area. Thus high concentrations of glucose may inhibit glucagon biosynthesis *per se*, or, as suggested by the streptozotocin results, via an insulin-mediated step. This latter possibility requires further substantiation.

In contrast with insulin biosynthesis, which is

virtually eliminated in the absence of glucose (K. J. O'Connor, A. Gay & N. Lazarus, unpublished work), optimum glucagon biosynthesis appeared to occur at low (or zero) concentrations of glucose. Incorporation of leucine into glucagon occurred in the presence of zero glucose whereas leucine was preferentially utilized for insulin biosynthesis in the presence of high concentrations of glucose.

Investigations into the nature of the synthesized peak containing large-glucagon immunoreactivity should help to define whether a precursor-product relationship exists in the biosynthesis of glucagon or whether two separate hormonal entities with glucagon-like structure are present in pancreas.

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