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Biosynthesis of Glucagon in Isolated Pancreatic Islets of Guinea Pigs

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1. The biosynthesis of glucagon in guinea-pig A_2 cells was investigated by incubation of isolated islets of Langerhans in the presence of [^{3}H]tryptophan for periods of up to 14 days. Proteins were extracted from islets and incubation media and analysed by gel filtration. 2. In addition to very-high-molecular-weight (100000) proteins, the principal tryptophan-containing biosynthetic product after incubation for up to 17h was a protein of minimum mol.wt. 9000, which co-eluted on gel filtration with a peak of glucagon-like immunoreactivity, but was apparently devoid of biological activity in a fat-cell assay. A discrete peak of labelled glucagon was only recovered after incubation for at least 6 days. Losses of glucagon during the extraction and rapid secretion of newly synthesized glucagon into incubation media were excluded as reasons for the lack of recovery of labelled hormone from islets after shorter incubations. 3. The 9000-mol.wt. protein was localized to A_2 cells in experiments using B-cell-depleted islets, and to A_2-cell granules by subcellular fractionation and electron-microscopic radioautography. Only glucagon was secreted into the incubation medium. 4. Possible relationships between the 9000-mol.wt. protein and glucagon are discussed in the light of postulated mechanisms of glucagon biosynthesis.

There is much evidence to indicate that glucagon originates in the A_2 cell of the pancreatic islets, but little is known so far of the mechanism and regulation of glucagon biosynthesis. Extracts of mammalian pancreas contain two species of glucagon-like immunoreactivity, one large with an estimated molecular weight of at least 7000 and one small with a molecular weight of about 3500, corresponding to that of crystalline glucagon (Rigopoulou *et al.*, 1970). This observation was interpreted to suggest that glucagon, like insulin, may be synthesized via a larger precursor molecule. In further support of this, other reports have indicated the occurrence of a possible precursor molecule of glucagon in fish (Noe & Bauer, 1971, 1973) and in pigeon islets (Tung & Zerega, 1971). However, large glucagon-like immunoreactivity has so far not been converted into biologically and immunologically active glucagon and the existence of a precursor–product relationship therefore remains to be demonstrated.

In the present study we have investigated the biosynthesis of glucagon in isolated guinea-pig islets, which have been incubated with [^{3}H]tryptophan as a marker for newly synthesized glucagon. A preliminary report on part of this work has been published (Hellerström *et al.*, 1972).

Experimental

Materials

Sephadex G-100, G-50 (fine) and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. L-[^{3}H]Tryptophan (sp. radioactivity 5.3Ci/mmol) and L-[4,5-^{3}H]leucine (sp. radioactivity 36Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Collagenase was supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A. Bovine plasma albumin (fraction V) was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Glycerol was assayed with the commercially available kit Biochemical Test Combination no. 15989 from Boehringer, Mannheim, Germany. Trasylol (aprotinin) was purchased from Bayer Farma AB, Stockholm 4, Sweden. Bovine/porcine glucagon, α-chymotrypsinogen (type II), cycloheximide and cytochrome *c* (type VI) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Tissue-culture media, calf serum and Hanks solution were supplied by Statens Bakteriologiska Laboratorium, 105 21 Stockholm, Sweden, and penicillin and streptomycin by Glaxo Laboratories Ltd., Greenford, U.K. Streptozotocin (lot 9164-VDV-132) was

generously given by Upjohn Co., Kalamazoo, Mich., U.S.A. Other reagents were of the purest grade commercially available.

Methods

Isolation of islets and incubation procedure. Adult male guinea pigs were killed by a sharp blow on the head and the pancreas was quickly removed. In some cases the animals received an intraperitoneal injection of streptozotocin (350mg/kg body wt.) 1 week before being killed. Islets were isolated by collagenase digestion as described in detail by Howell & Taylor (1968). In each experiment 150–350 isolated islets were incubated in 0.5ml of bicarbonate buffer (Gey & Gey, 1936) supplemented with amino acids as described by Eagle (1959), 100 units of penicillin/ml, 0.1mg of streptomycin/ml, 20μCi of [^{3}H]tryptophan or [^{3}H]leucine/ml and 10μg of Phenol Red/ml. Non-labelled tryptophan and leucine were omitted when the medium contained the corresponding radioactive amino acid. Glucose was added to a concentration of 16.7mM if not otherwise stated. Incubations were performed with shaking in silicone-treated Warburg vessels (total volume 5ml) at 37°C and a gas phase of O_2+CO_2 (95:5). Each incubation lasted between 2 and 17h as described below. After incubations the islets were transferred together with the incubation medium to a 10ml centrifuge tube and allowed to sediment to the bottom of the tube. They were subsequently washed three times with 5ml of non-radioactive medium containing 0.5mg of tryptophan or leucine/ml before being homogenized and extracted as described below.

Tissue culture of isolated islets. Islets were isolated from adult male guinea pigs with collagenase under aseptic conditions and cultured *in vitro* for 6 or 14 days as described in detail by Andersson & Hellerström (1972). The culture medium consisted of TCM 199 supplemented with 10% (w/v) calf serum, 40μCi of [^{3}H]tryptophan/ml, 100 units of penicillin/ml and 0.1mg of streptomycin/ml. The glucose concentration was adjusted to 16.7mM. After the culture period islets were harvested by trypsin treatment for 15min (Andersson & Hellerström, 1972), washed in Hanks solution containing excess of non-labelled tryptophan and further extracted as described below.

Extraction procedure. The incubated and washed islets were homogenized in an all-glass homogenizer together with 1ml of 5% (w/v) trichloroacetic acid supplemented with 2mg of bovine plasma albumin and 0.5mg of bovine/porcine glucagon. The resulting precipitate was washed twice with 5% trichloroacetic acid and extracted with acid ethanol [15ml of conc. HCl per litre of 70% (v/v) ethanol] for at least 2h at 4°C. The acid-ethanol-soluble proteins were then precipitated with ether–ethanol (Kenny, 1955) before being subjected to gel filtration (see below). In some experiments the incubation medium was extracted either along with the homogenized islets or separately. In these experiments the medium was supplemented with 2mg of bovine plasma albumin/ml and 500 units of Trasylol/ml throughout the incubation period.

Gel-filtration procedure. Islet or medium extracts were subjected to gel chromatography on columns of either Sephadex G-100 or G-50 with flow rates of approx. 15ml/h. The dimensions of each column are specified below. Columns packed with Sephadex G-100 were equilibrated with 0.05M-NH_4HCO_3 (adjusted to pH8.8 with 1M-NaOH) and those containing Sephadex G-50 with 3M-acetic acid. The precipitated islet and medium extracts were dissolved in 300μl of the relevant buffer before application to the column. Eluates were collected in 1ml fractions with continuous recording of the extinction at 280nm by an absorptiometer (Uvicord II, LKB-Produkter AB, Stockholm, Sweden). From each fraction a portion (0.4ml) was counted for radioactivity in a liquid-scintillation spectrometer (Packard Tri-Carb model 3380). Columns were calibrated with Blue Dextran 2000 (mol.wt.~2000000), bovine plasma albumin (mol.wt. 66000), α-chymotrypsinogen (mol.-wt. 22500), cytochrome *c* (mol.wt. 12400) and bovine/porcine glucagon (mol.wt. 3485).

Subcellular fractionation of islet homogenates. Groups of 150 islets were incubated with [^{3}H]-tryptophan for 17h as described above. The islets were washed thoroughly in medium containing 0.5mg of unlabelled tryptophan/ml and then homogenized manually in 0.3M-sucrose–10mM-sodium phosphate buffer, pH6.0, in a Kontes all-glass homogenizer by using six passes of the grinder. This and each subsequent centrifugation procedure were performed at 4°C. Nuclei and cell debris were removed by centrifugation of the homogenate at 700*g* for 5min and the supernatant was removed and re-centrifuged at 24000*g* for 10min to provide a crude storage-granule-containing fraction. The supernatant from this centrifugation, which contained soluble proteins together with microsomal fractions and ribosomes, was retained for analysis with the storage-granule fraction. The storage-granule fraction was resuspended in 0.02M-NaOH before application to a Sephadex G-100 column (50cm×1cm), whereas the supernatant containing the soluble proteins was applied directly to a similar column. The columns were eluted with 0.05M-NH_4HCO_3 and the radioactivity was determined in the fractions (1ml) that were collected.

Measurement of glucagon immunoreactivity. Glucagon was measured by the radioimmunoassay described by Edwards *et al.* (1970). Standards were prepared from bovine/porcine glucagon (Sigma) dissolved in 0.05M-NH_4HCO_3 (pH8.8). Two anti-glucagon sera were used, one of which was specific for pancreatic glucagon with no cross-reaction from

proteins of gastrointestinal origin (kindly supplied by Dr. K. D. Buchanan, Queen's University, Belfast).

Glucagon immunoreactivity was determined in groups of 15 homogenized guinea-pig islets after isolation by collagenase digestion. The islets were homogenized in 0.5ml of 0.02M-NaOH in an all-glass homogenizer, and the homogenate was applied directly to a column of Sephadex G-100, which was eluted with 0.05M-NH_4HCO_3, pH8.8. Fractions (1ml) were collected and assayed for glucagon content.

The secretion of glucagon immunoreactivity was measured from groups of 100 isolated islets. Two groups of 100 islets were taken from the same preparation of isolated islets and preincubated at 37°C for 1h in bicarbonate buffer (Gey & Gey, 1936) supplemented with amino acids (Eagle, 1959) and 12.5mM-glucose. The islets were then washed in fresh medium and incubated for a further 2h at 37°C. One group of islets was incubated in 1ml of the medium containing amino acids and 12.5mM-glucose, and the other group in the same medium but containing in addition 2.5mM-arginine and 10μM-adrenaline. After 2h incubation, the medium was removed from the islets and either applied directly to a Sephadex G-100 column eluted with 0.05M-NH_4HCO_3, pH8.8, or stored at −20°C until application to the same column. Fractions (1ml) were collected and assayed for glucagon content. After the incubation, the two groups of islets were homogenized in 1ml volumes of 0.1M-NaOH and samples were taken for assay of protein content by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Bioassay of glucagon. The biological activity of glucagon in islet extracts was assayed by the isolated chicken fat-cell technique, mainly as described by Langslow & Hales (1970). Isolated fat-cells were prepared from white Leghorn chickens by incubating pieces of subcutaneous adipose tissue (1–2g wet wt.) in 5ml of Krebs–Ringer bicarbonate buffer (DeZuka & Cohen, 1964), supplemented with 4% (w/v) bovine plasma albumin, 5.5mM-glucose and 1.4mg of collagenase/ml. The temperature was 37°C and the gas phase was O_2+CO_2 (95:5). Incubations were performed for 30–40min in a shaking thermostat (140 oscillations/min) and every 10min each incubation vial was shaken vigorously by hand. After filtration of the tissue suspension through gauze, fat-cells were separated by centrifugation for 2min at 1000*g*, washed twice and finally resuspended in Krebs–Ringer bicarbonate buffer supplemented with 4% (w/v) bovine plasma albumin and 16.7mM-glucose. Glycerol release was measured in 1ml fractions of fat-cell suspension incubated with the unknown sample or a known amount of bovine/porcine glucagon standard. A standard curve was constructed in each experiment by dissolving glucagon in 0.5M-acetic acid, pH2.7, containing 4% (w/v) bovine plasma albumin. Serial dilutions of the glucagon solution (10μl each) were then added to the fat-cell suspension. The unknown samples consisted of gel-chromatographed fractions of islet extract which had been freeze-dried and redissolved in 0.5ml of Krebs–Ringer bicarbonate buffer before being added to the fat-cell suspension. Incubations were performed for 1h at 37°C in a gas phase of O_2+CO_2 (95:5). The incubations were terminated by addition of 1ml of 10% trichloroacetic acid and the glycerol was measured spectrophotometrically (Garland & Randle, 1962) with the Biochemical Test Combination kit.

Results

Biosynthetic labelling of islet proteins after incubation with [³H]tryptophan

The mean rate of incorporation of [^{3}H]tryptophan into acid-alcohol-soluble islet proteins was 62 ± 15 c.p.m./h per islet (mean±S.E.M.; $n=11$). The corresponding value for [^{3}H]leucine was 139 ± 9c.p.m./h per islet ($n=3$). These values have not been corrected for possible secretion of labelled material into the incubation medium. Because of the relatively slow incorporation rate with [^{3}H]tryptophan (see also below) incubations were extended for up to 17h in incubation media supplemented with amino acids and antibiotics. During this prolonged period of incubation there was a very good ultrastructural preservation of the islets (see plate 1 in the Appendix, Howell *et al.*, 1974) and the rate of amino acid incorporation remained linear. When cycloheximide (140μg/ml) was added to the medium or incubations were performed at 4°C incorporation was almost completely abolished.

The gel-filtration patterns of radioactive acid-alcohol-soluble proteins extracted from islets incubated for various periods of time up to 17h are given in Fig. 1. At all incubation times studied the radioactivity migrating with the carrier glucagon was low or negligible and showed no tendency to form peaks. After incubation for 2h in the presence of radioactive tryptophan labelled proteins were eluted mainly in the void volume of the Sephadex G-50 column (Fig. 1*a*). After incubation for 6h, however, an additional small peak of radioactivity appeared immediately after the void-volume proteins (Fig. 1*b*). This peak had reached a considerable size after 17h incubation (Fig. 1*c*) and will subsequently be referred to as peak II. The approximate molecular weight of the labelled material in this peak was calculated as 9000, when gel filtration was performed in 3M-acetic acid on a Sephadex G-50 column. However, when gel filtrations were carried out in NH_4HCO_3 buffer

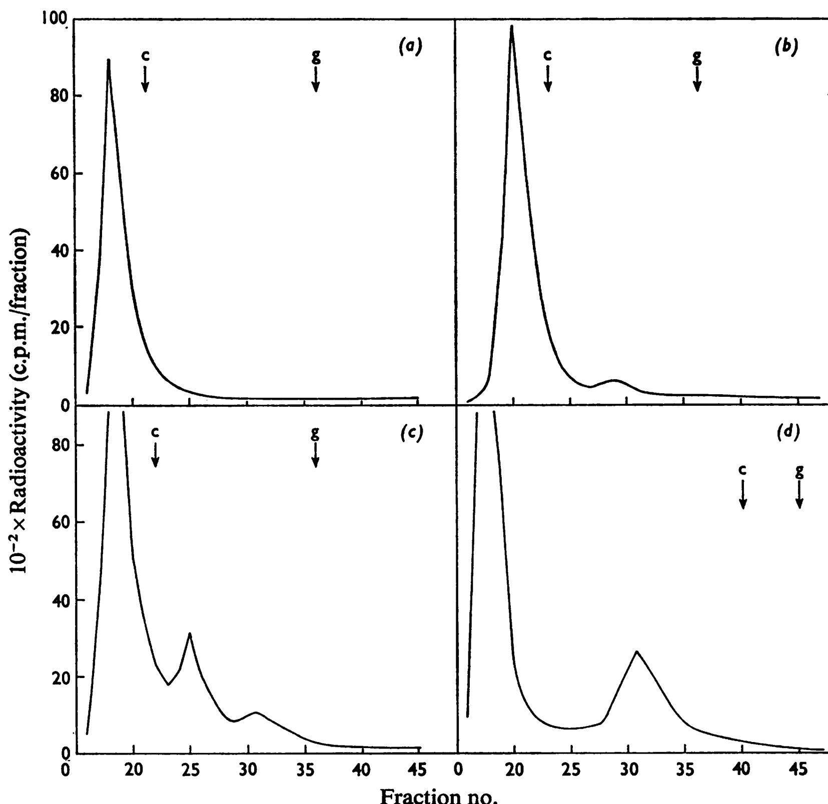


Fig. 1. *Gel-filtration profiles of [^{3}H]tryptophan incorporation into acid-ethanol-soluble proteins from isolated guinea-pig islets incubated for various time-periods*

Islet extracts were eluted in 3M-acetic acid on a Sephadex G-50 column (1 cm × 67 cm) in Figs. 1(*a*), 1(*b*) and 1(*c*) and in 0.05M-NH_4HCO_3 on a Sephadex G-100 column (1 cm × 67 cm) in Fig. 1(*d*) (volume of fractions was 1 ml). Incubations were performed for 2h (Fig. 1*a*), 6h (Fig. 1*b*) and 17h (Figs. 1*c* and 1*d*) in media containing 20 μCi of [^{3}H]tryptophan/ml and 16.7 mM-glucose. The elution volume of molecular-weight markers, cytochrome (c) and glucagon (g), is indicated by arrows.

(pH 8.8) on Sephadex G-100 there was instead a discrete radioactive peak in the region corresponding to approx. mol.wt. 18000 (Fig. 1*d*). This suggested that labelled protein in peak II aggregated and was eluted as a dimer at an alkaline pH value. In further support of this notion refiltration in 3M-acetic acid of such pooled and freeze-dried material produced a distinct peak exactly corresponding to peak II on the Sephadex G-50 column (results not shown).

The results referred to above were obtained with a glucose concentration of 16.7 mM in the incubation medium. When the glucose concentration was decreased to 3.3 mM there was a considerable decrease of incorporation into acid-alcohol-soluble proteins. As shown in Fig. 2 this reflected a decreased incorporation into proteins eluted both in the void volume and in peak II. It is also seen that a decreased glucose concentration did not affect the elution pattern in the glucagon region to any marked extent.

Labelling of islet proteins after tissue culture of islets in the presence of [^{3}H]tryptophan

The radioactive gel-filtration profiles of acid-alcohol-soluble proteins from islets maintained in tissue culture for 6 or 14 days are shown in Figs. 3(*a*) and 3(*b*). After both 6 and 14 days of culture there was a considerable incorporation of label into both void-volume proteins and peak II proteins. In addition, there was a marked peak of labelled protein eluted in the area of marker glucagon.

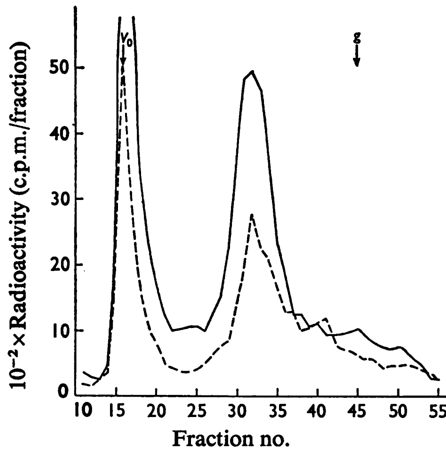


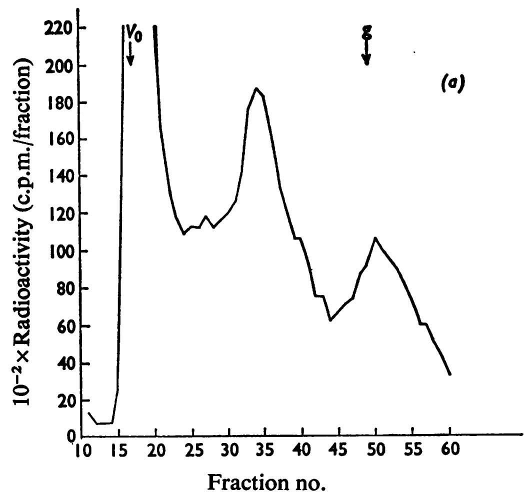
Fig. 2. *Gel-filtration profiles of [³H]tryptophan incorporation into acid-ethanol-soluble proteins from isolated guinea-pig islets incubated with different glucose concentrations*

The islets were incubated for 17h in media containing either 3.3mM-glucose (----) or 16.7mM-glucose (——). At each glucose concentration 200 islets were incubated. Gel-filtration was on Sephadex G-50 (1cm×67cm); vol. of fractions was 1ml. The elution position of glucagon (g) and the void volume (v_0) are indicated by arrows.

Cellular and subcellular distribution of [³H]tryptophan-labelled islet proteins

A radioautographic investigation of the distribution of label over the B and A_2 cells is given in the Appendix (Howell *et al.*, 1974). The incorporation of labelled tryptophan into islet proteins after destruction of the B cells with streptozotocin is presented in Fig. 4. Incubation for 17h of such islets, which are known to consist of at least 60–70% A_2 cells (Petersson *et al.*, 1970; Howell *et al.*, 1971) produced a gel-filtration pattern very similar to that obtained with extracts of normal guinea-pig islets. Thus the radioactivity in peak II was prominent, whereas little labelling was found in the glucagon area.

Subcellular fractionation of islets showed that most of the radioactivity incorporated into the 9000-mol.wt protein of peak II was associated with the crude secretory-granule preparation with little associated with the soluble proteins in the supernatant fraction.



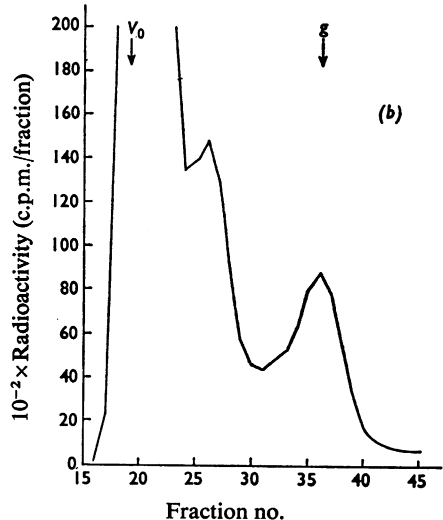


Fig. 3. *Gel-filtration profiles of [³H]tryptophan incorporation into proteins extracted from isolated islets maintained in tissue culture*

(*a*) Extract of 180 islets cultured for 6 days. Gel filtration was carried out on a Sephadex G-100 column (1cm×67cm) in 0.05M-NH_4HCO_3, pH8.8. (*b*) Extract of 240 islets cultured for 14 days. Gel filtration was carried out on a Sephadex G-50 column (1cm×67cm) in 3M-acetic acid. The elution position of glucagon (g) and the void volume (v_0) are indicated by arrows. Vol. of fractions was 1ml.

Gel-chromatographic fractionation of glucagon-like immunoreactivity in islets and in incubation media

As shown in Fig. 5, gel filtration in NH_4HCO_3 of homogenates of 15 isolated islets produced two distinct and prominent peaks of immunoreactivity, one eluted in a position approximately corresponding to a mol.wt. of 18000 (peak II) and the other to that of native pancreatic glucagon with mol.wt. approx. 3500. Peak II contained about half of the immunoreactivity recovered within the glucagon region. This relationship may not reflect the situation within the

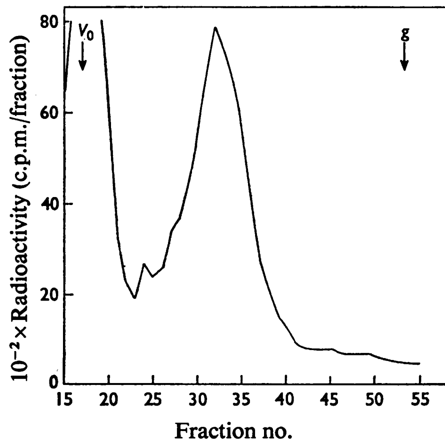


Fig. 4. *Gel-filtration profile of [^3H]tryptophan incorporation into proteins extracted from streptozotocin-treated guinea pigs*

Altogether 130 islets were isolated from four guinea pigs which were made diabetic with streptozotocin 1 week before the experiment. The islets were incubated for 17h and gel filtration of the islet extract was carried out on a Sephadex G-100 column (1cm × 67cm) in 0.05M-NH_4HCO_3, pH 8.8. The elution position of glucagon (g) and the void volume (v_0) are indicated by arrows. (Vol. of fractions 1 ml).

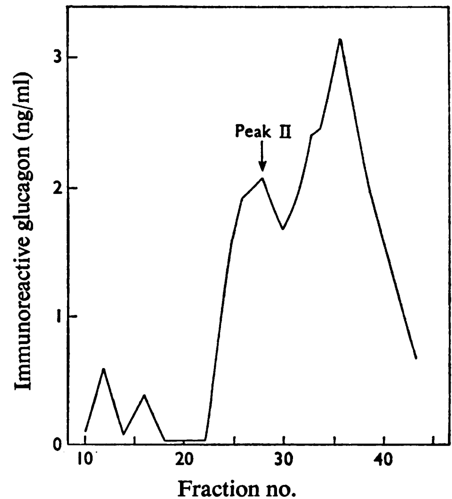


Fig. 5. *Elution pattern of glucagon immunoreactivity in islet homogenates*

Isolated guinea-pig islets were homogenized in 0.02M-NaOH and the homogenate was directly applied to a column of Sephadex G-100 (1 cm × 50cm) and eluted with 0.05M-NH_4HCO_3, pH 8.8 (vol. of fractions 1 ml). The results are the mean of three experiments with homogenates of 15 islets used in each experiment.

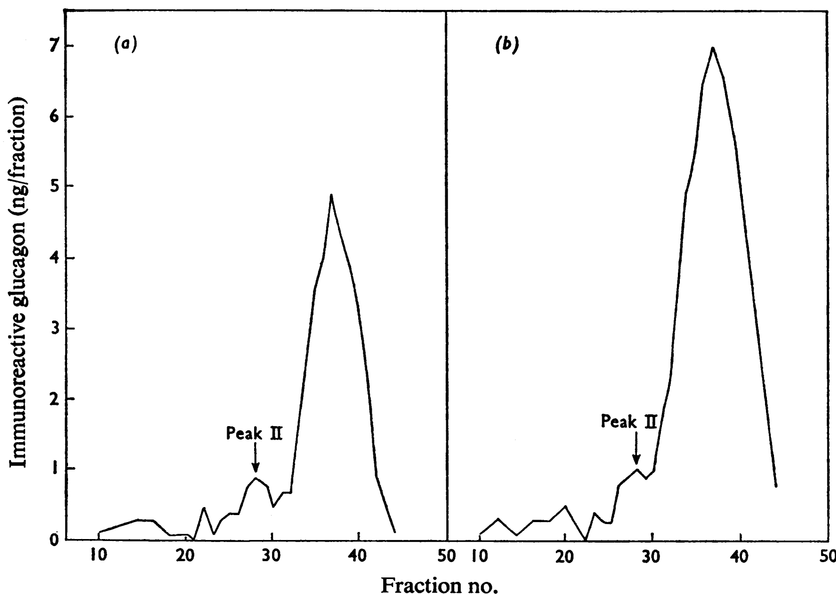


Fig. 6. *Elution pattern of glucagon immunoreactivity secreted from isolated guinea-pig islets*

Batches of 100 islets were preincubated for 1 h and then incubated for 2 h in 1 ml of bicarbonate buffer containing amino acids (Eagle, 1959). (*a*) The incubation medium was supplemented with 12.5mM-glucose only. (*b*) The incubation medium was supplemented with 12.5mM-glucose plus 2.5mM-arginine and 10μM-adrenaline. The incubation medium was removed from the islets and applied to a column of Sephadex G-100 (1 cm × 50cm), which was eluted with 0.05M-NH_4HCO_3, pH 8.8 (vol. of fractions 1 ml).

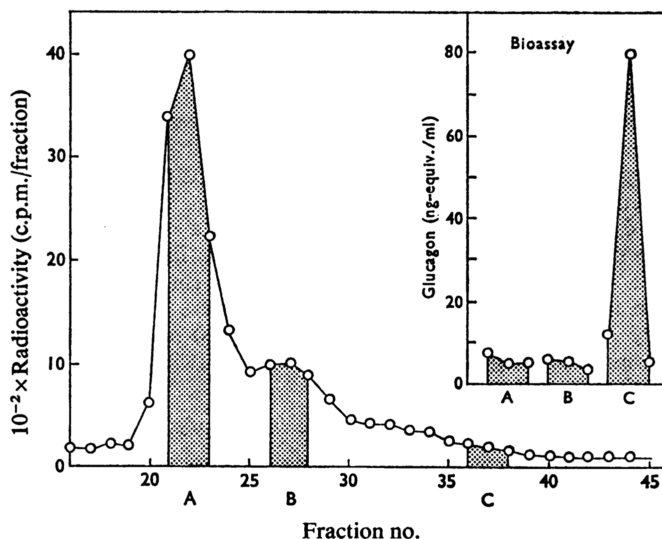


Fig. 7. *Bioassay of glucagon-like activity in fractions after gel filtration of an islet extract*

Isolated islets (500) were incubated with [^{3}H]tryptophan (20μCi/ml) for 6h and extracted as described in the text. After gel filtration in 3M-acetic acid on a Sephadex G-50 column (1 cm × 67 cm) fractions (1 ml) within the hatched areas (A–C on the large graph) were freeze-dried and redissolved in the bioassay buffer. After appropriate dilution individual fractions were incubated with isolated fat-cells and the glycerol release was determined. The result for each fraction is given in the inset and expressed as glucagon equivalents after comparison with a standard curve.

intact islet, however, since no corrections could be made for possible differences in the cross-reactivity of the protein in peak II and glucagon with the antisera used in the immunoassay. Both antisera used in the assay, one specific for pancreatic glucagon, gave the same distribution of glucagon immunoreactivity from homogenized islets.

The distribution of glucagon immunoreactivity after gel filtration of proteins secreted by 100 isolated islets into the incubation medium is shown in Figs. 6(*a*) and 6(*b*). The mean protein content of each of the groups of 100 islets was 350 μg, and each result was corrected to give the protein secreted from 100 islets of this protein content. The results are means of three experiments. Both when the islets released glucagon in the control incubations, i.e. in medium containing amino acids and 12.5mM-glucose, and after further stimulation with arginine and adrenaline, only one prominent peak of glucagon-immunoreactive protein was eluted, and this was located in the region corresponding to native glucagon. Only small amounts of immunoreactive material were recovered in the region corresponding to peak II, regardless of whether the glucagon release was stimulated or not, and may reflect a small degree of cell breakage during the incubation.

Bioassay of glucagon in islet extracts after gel filtration

The glucagon-like biological activity in various fractions of a gel-filtered islet extract is shown in Fig. 7. Only a small lipolytic activity was present in proteins migrating at the void volume or with peak II. By contrast, the glucagon region showed a pronounced activity. This pattern of response was present without exception, although in some experiments there was barely any lipolytic activity either in the void volume or in peak II.

Discussion

Absence of labelled glucagon after short periods of incubation

It seems possible to exclude a number of explanations for the absence of labelled glucagon in islet extracts even after prolonged incubation of isolated islets with [^{3}H]tryptophan. Thus the extraction procedure utilized was shown to be effective in recovering ^{125}I-labelled porcine glucagon added to islet extracts after homogenization of the islets. Similarly, ^{3}H-labelled glucagon present after incubation for 6 days was recoverable in column eluates after an exactly comparable extraction, whereas further

experiments in which the extraction procedure was completely omitted and the islets were homogenized in 0.02 M-NaOH before running on the column also failed to show the presence of labelled glucagon after 17 h incubation.

Another possibility appeared to be that guinea-pig glucagon does not contain tryptophan, but this can be discounted, since labelled glucagon could be detected after incubation for 6 days with tryptophan and since guinea-pig A_2 cells stain strongly positive with a specific tryptophan stain (Petersson & Hellman, 1963). Tryptophan is present within the glucagon molecule of every known species, and although the amino acid composition of guinea-pig glucagon has not been investigated, the amino acid sequence of all five mammalian glucagons studied to date is in fact identical (Bromer *et al.*, 1957, 1971; Sundby & Markussen, 1971*a*, *b*; Thomsen *et al.*, 1972). Moreover, incubation of the islets in the presence of other amino acids (leucine, tyrosine) instead of tryptophan, although demonstrating the presence of guinea-pig proinsulin and insulin, did not provide evidence of label in the glucagon region of the column eluates after short-term incubations (J. C. Edwards, S. L. Howell & C. Hellerström, unpublished work). Finally, investigation of the media after incubation of islets in the presence of [^{3}H]tryptophan failed to reveal the presence of labelled glucagon, and it was thus excluded that the newly synthesized molecule was secreted from the A_2 cell very rapidly after its synthesis.

Taken together this evidence suggests that in the conditions of these experiments there was no detectable incorporation of radioactivity into 3500-mol.wt. glucagon during the incubation of isolated guinea-pig islets in amino acid-containing media for periods up to 17 h. Another protein of minimum mol.wt. 9000 (peak II), which contained [^{3}H]tryptophan and co-eluted with a peak of glucagon-like immunoreactivity, was, however, consistently recovered from the columns.

Regulation of protein biosynthesis in the A_2 cell

In some preliminary experiments attempts were made to alter the rate of biosynthesis of glucagon by utilizing agents known to alter rates of secretion. Rates of glucagon secretion are increased during incubation of isolated islets with arginine (Edwards *et al.*, 1970), or in some species in the presence of low glucose concentrations (Vance *et al.*, 1968; Chesney & Schofield, 1969; Iversen, 1971). However, addition of arginine (Hellerström *et al.*, 1972) or decreasing the glucose concentration in the incubation media containing [^{3}H]tryptophan during a 4 h period of incubation resulted in little effect or even a decrease in incorporation of label into tryptophan-containing proteins. High concentrations of glucose may inhibit glucagon secretion in some species (Vance *et al.*, 1968; Chesney & Schofield, 1969), but incorporation of label into tryptophan-containing proteins, and specifically into the 9000-mol.wt. component, was increased during incubation in the presence of 16.7 mM-glucose. Prolonged hyperglycaemia would therefore be expected to result in an accumulation of exportable protein in A_2-cell granules as a result of increased synthesis without concomitant increase in rates of secretion. It is perhaps not surprising that in this situation destruction of excess of granule material by crinophagy (granulolysis) has been observed within the A_2 cell (Orci *et al.*, 1968).

Mechanism of glucagon biosynthesis

Our results suggest that, although there is no immediate incorporation of labelled tryptophan into glucagon, there appears to be a rapid incorporation into a protein of mol.wt. 9000 which is associated with a peak of glucagon immunoreactivity. After incubation of isolated islets with labelled tryptophan for several days radioactivity can be detected in a protein of mol. wt. 3500 corresponding to glucagon. In a study of glucagon biosynthesis in the perfused rat pancreas (O'Connor *et al.*, 1973) radioactivity was incorporated into glucagon after 4 h incubation, but even in this case the incorporation was small and was only detectable when the whole pancreas was extracted for glucagon. Similarly, studies of glucagon biosynthesis in islets from the angler-fish (Noe & Bauer, 1971, 1973) and the pigeon (Tung & Zerega, 1971) have shown a rapid incorporation of labelled tryptophan into a protein of mol.wt. 9000 with little incorporation into glucagon.

Although [^{3}H]tryptophan is incorporated initially into the 9000-mol.wt. protein, none of this labelled protein is actively secreted from the A_2 cell, all of the secreted immunoreactivity appearing in the form of glucagon. However, despite the absence of convincing evidence of a precursor–product relationship between the 9000-mol.wt. protein and glucagon, it is tempting to suppose that this protein, which apparently possessed at least some glucagon-like immunoreactivity although devoid of biological activity on fat-cells, may represent the initial form of the exportable biosynthetic product of the A_2 cell. Without information about the specific immunoreactivity of the 9000-mol.wt. protein it is difficult to determine the relative sizes of the pools of this protein and glucagon. Extractions of whole pancreas have suggested that the higher-molecular-weight material might be only a minor component, although degradation and losses which would be expected during the extraction of whole pancreas might minimize the yield. Thus analysis of isolated guinea-pig islets in which degradation by proteolytic enzymes

of the exocrine pancreas should be avoided, and in which no preliminary extraction was performed, show that approx. 40% of the immunoreactivity was present in the 9000-mol.wt. form. Similarly, analysis by gel filtration of crystalline glucagon has revealed only a very low yield (less than 1%) of a presumed precursor protein which had a molecular weight of only about 4000 (Tager & Steiner, 1973), and it seems possible that this material may thus be already extensively degraded during the extraction procedure.

The long delay between biosynthesis and the appearance of labelled glucagon would suggest that there might exist in the guinea pig a large pool of the 9000-mol. wt. precursor material. The results suggest that this protein is stored in the granules, and that conversion may occur only slowly over a period of days. There is no doubt that the glucagon-like immunoreactivity which is secreted by the islets consists almost exclusively of 3500-mol.wt. glucagon, suggesting perhaps that the 'older', fully transformed granules might be preferentially secreted. The situation in the A_2 cell might thus be intermediate between that in the B cell, where biosynthesis of proinsulin is followed with a half-time of 1h by conversion into insulin and storage in the granules (Howell *et al.*, 1969; Kemmler *et al.*, 1972), and that of the exocrine pancreas in which the precursors (trypsinogen and chymotrypsinogen) are stored and secreted in the precursor form. Reported heterogeneity of the granule core after special fixation of A_2 cells, and in particular of the presence of glucagon immunoreactivity detected by a peroxidase-labelled-antibody procedure only at the periphery of the A_2 granule (Bussolati & Solcia, 1971), might suggest the presence of both precursor and glucagon within the storage granules. However, in the absence of direct confirmation of a precursor–product relationship between peak II protein and glucagon, and of information about the specific immunoreactivity of peak II, such an interpretation must remain a matter for speculation.

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