AN INVESTIGATION OF GLUCAGON BIOSYNTHESIS IN ISOLATED PANCREATIC ISLETS OF GUINEA PIGS

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1. Introduction

It is well established that glucagon is stored within the A_2-cells of the pancreatic islets, although up till now little is known about the cellular mechanisms involved in the biosynthesis of this hormone. The possibility that glucagon, like insulin, may be initially synthesized as a larger precursor has been considered on the basis of the recent demonstration of glucagon-like immunoreactive material of at least twice the molecular size of glucagon in pancreatic extracts of dog and several other species including man [1]. In addition, recent reports have indicated the occurrence of a possible precursor molecule of glucagon in fish [2] and in pigeon islets [3], but there appears to date to be little information available about the mechanisms of glucagon biosynthesis and its regulation in mammalian islets.

We report here investigations of the biosynthesis of glucagon in isolated guinea-pig islets of Langerhans during incubation *in vitro* for 17 hr or in tissue culture for up to 6 days. Tryptophan, labelled with tritium, was used as a marker for newly synthesized glucagon in the guinea-pig islets since this amino acid is present in the glucagon molecule [4] but not in the proinsulin [5] or insulin [6] molecule of any species studied so far. After incubation labelled proteins were extracted from the islets and analysed by gel filtration. At each time examined the extracts contained labelled proteins eluting in the void volume and in addition a protein of molecular weight about 18,000 which could not be dissociated by gel filtration in the presence of 8 M urea or 3 M acetic acid. The elution volume of this protein corresponded with that of a peak of glucagon-like immunoreactivity present in islet extracts which were analysed on the same column. Small amounts of labelled protein co-eluting with glucagon on gel filtration were detected only after maintaining islets in tissue culture for six days. The possible significance of these results in relation to postulated mechanisms of glucagon biosynthesis is briefly discussed.

2. Methods

2.1. *Incubation of islets*

Isolated islets were obtained from male guinea-pigs by collagenase digestion according to Howell and Taylor [7]. Incubations were performed for 1 to 17 hr at 37° in tightly capped glass vials, each containing 100 to 200 islets in 1 ml of incubation medium and with a gas phase of 95% O_2 + 5% CO_2. The incubation medium consisted of a bicarbonate-buffered salt solution [8] containing a mixture of essential amino acids [9], 50 U/ml of penicillin and an equal amount of streptomycin, 10 μg/ml phenol red and, if not otherwise stated, 17 mM D-glucose. L-[^{3}H] tryptophan (3.7 Ci/mmole; The Radiochemical Centre, Amersham) was added to the medium at a concentration of 20 μCi/ml (final specific activity 0.8 Ci/mmole).

To make possible a study of islet protein synthesis over prolonged periods of time, isolated islets were maintained in tissue culture for up to six days according to techniques previously described in detail [10]. [^{3}H] Tryptophan was added to the culture medium at a concentration of 40 μCi/ml. Dilution by non-labelled tryptophan in the medium decreased the final specific activity to 0.4 Ci/mmole.

2.2. *Extraction procedures*

After incubation or tissue culture the islets were washed thoroughly in incubation medium containing a 500-fold excess of unlabelled L-tryptophan. The islets were subsequently homogenized in an all-glass homogenizer with 1 ml 5% trichloroacetic acid (TCA) supplemented with 2 mg bovine plasma albumin (BPA) and 0.5 mg beef-pork glucagon (Sigma). The resulting precipitate was washed twice with 5% TCA and then extracted with acid alcohol followed by precipitation with ether–alcohol according to Kenny [11]. In some experiments islets and incubation medium were homogenized together and the resulting precipitate washed 4 times in 5% TCA before further extraction. In this case the medium was supplemented with 2 mg/ml BPA and 1000 U/ml Trasylol throughout the incubation period.

2.3. *Gel filtration*

Islet extracts were subjected to gel chromatography on 65 × 1 cm columns of Biogel P-10 (Bio-Rad Laboratories, Richmond, Calif., USA) or Sephadex G-100 (Pharmacia, Uppsala, Sweden) equilibrated with 0.05 M NH_4HCO_3 (adjusted to pH 8.8 with NaOH), or with 3 M acetic acid. Eluates were collected in 1.0 or 1.15 ml fractions and the protein content of each fraction determined spectrophotometrically at 280 nm. Aliquots of 0.4 ml were counted for radioactivity in a liquid scintillation spectrometer after addition of Triton X-100:toluene:PPO scintillant (700:300:5, v/v/w). Columns were calibrated with blue dextran 200, BPA, alpha-chymotrypsinogen and beef-pork glucagon. In some experiments pooled fractions were rechromatographed after dialysis at 4° against distilled water followed by lyophilization and treatment for 5 hr in 0.05 M NH_4HCO_3 containing 8 M urea. In these experiments the column (Sephadex G-100) was equilibrated with 0.05 M NH_4HCO_3 also containing 8 M urea.

2.4. *Immunoassay*

Glucagon-like immunoreactivity was determined using the radioimmunoassay for pancreatic glucagon described previously [12]. Standards were prepared from beef-pork glucagon dissolved in 0.05 M NH_4HCO_3.

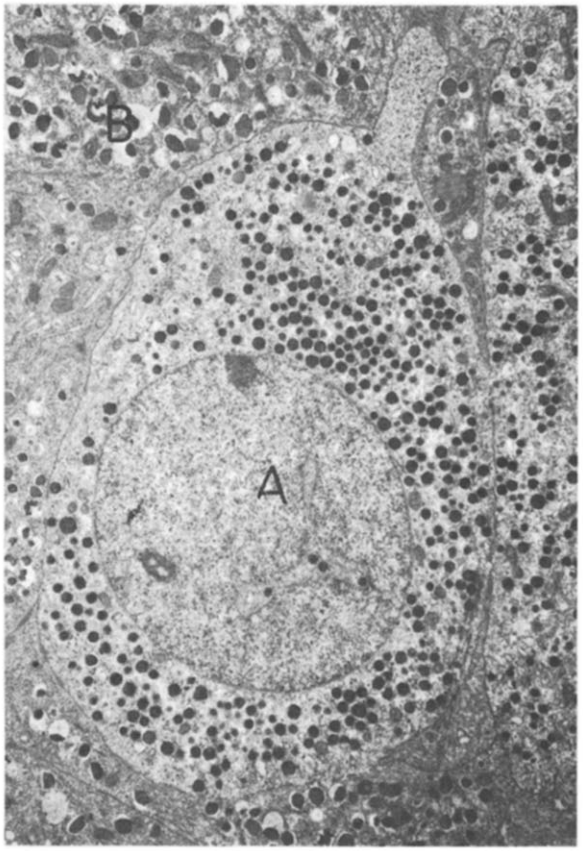

Fig. 1. Electron micrograph of A and B cells of guinea pig islet of Langerhans after incubation *in vitro* for 17 hr in the conditions described in the text. Tissue was fixed in 3% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated in ethanol and embedded in an epoxy resin. Thin sections were stained in a saturated solution of uranyl acetate in 50% ethanol before examination. Magnification × 5000 approx.

3. Results

Initial experiments showed that incorporation of [^{3}H] tryptophan into acid–alcohol soluble islet proteins was relatively slow and incubations were therefore extended for up to 17 hr. In order to maintain a linear rate of amino acid incorporation over this period of time it was necessary to supplement the incubation medium with essential amino acids according to Eagle [9]. Ultrastructural examination of islets after such prolonged incubation showed an excellent morphological preservation of the great majority of islet cells (fig. 1).

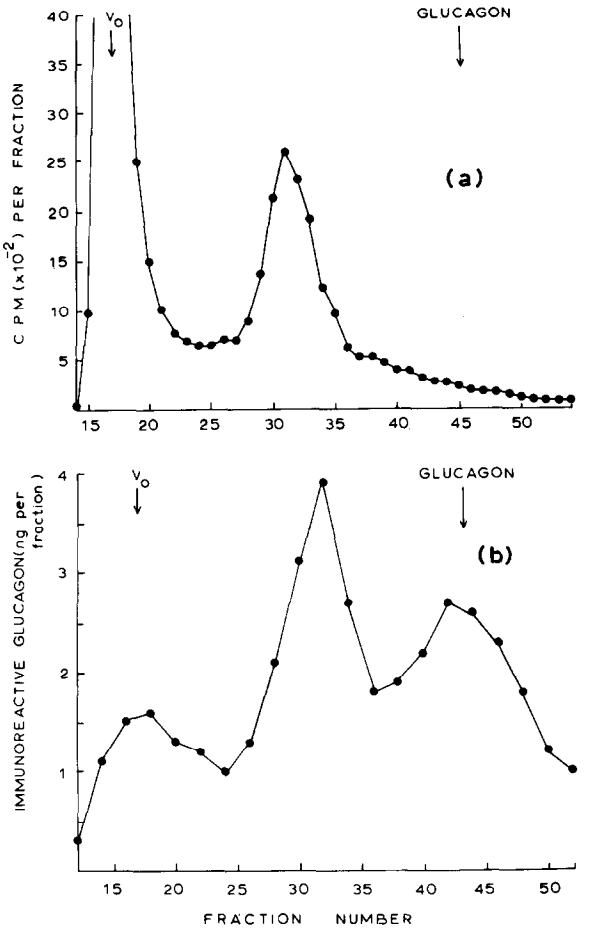

Fig. 2. a) Gel filtration on Sephadex G-100 of an extract obtained from 133 islets incubated *in vitro* for 17 hr with [^{3}H] tryptophan. V_0 indicates position of void volume. Fraction volume 1.0 ml. b) Glucagon-like immunoreactivity in islet extracts subjected to gel filtration on Sephadex G-100. Alternate fractions were assayed and each point represents the mean of 3 experiments. Fraction volume 1.0 ml.

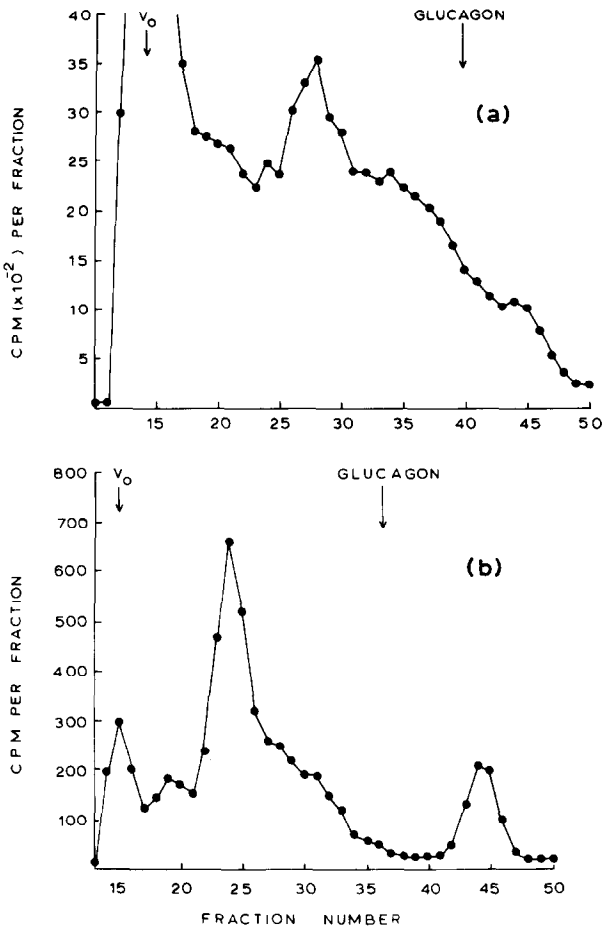

Fig. 3. a) Gel filtration on Sephadex G-100 of an extract obtained from 160 islets maintained in tissue culture for 6 days. ^{3}H-labelled protein now elutes also in the glucagon region. Fraction volume 1.15 ml. b) Elution pattern after rechromatography on Sephadex G-100 in 8 M urea of pooled eluates containing 18,000 molecular weight protein. Most labelled material migrates in a region corresponding to this protein. The small peak eluting after glucagon presumably represents free amino acid. Fraction volume 1.15 ml.

An increase of the glucose concentration of the medium from 3 to 17 mM considerably enhanced incorporation of labelled tryptophan into protein, whereas an increase of the concentration of arginine appeared inhibitory. This was the case also when islets were homogenized together with the incubation medium, suggesting that the apparent decrease in incorporation of amino acid was not due to increased release of labelled secretory products into the medium. Almost total inhibition of [^{3}H] tryptophan incorporation was achieved by addition to the medium of cycloheximide (100 μg/ml) or by incubation at 4° for 17 hr.

Fractionation of acid–alcohol soluble islet proteins on a Biogel P-10 column produced only one discrete peak of labelled proteins, which eluted in the void

volume. Further separation of the labelled islet proteins was therefore attempted on a Sephadex G-100 column. Two peaks of [^{3}H] tryptophan-containing proteins were then resolved, one corresponding to the void volume and one migrating between this region and that of the carrier glucagon. Only minimal quantities of labelled protein eluted with the carrier glucagon even when incubations were carried out for 17 hr (fig. 2a). Isolated islets were therefore maintained in tissue culture for 6 days in the presence of [^{3}H] tryptophan and in these experiments also, radioactivity eluted as two discrete peaks corresponding to those found after 17 hr of incubation, but in addition labelled proteins were now present in the glucagon region (fig. 3a).

Calibration of the Sephadex G-100 columns with proteins of known molecular weight suggested that the discrete peak of tryptophan-containing protein eluting in fractions 28–35 (fig. 2a) had a molecular weight of 18,000 ± 2000. In order to determine whether the 18,000 M.W. material was a polypeptide complex held together by noncovalent forces, eluates from this region of the chromatograms were pooled and rechromatographed in the presence of 8 M urea. As seen in fig. 3b this treatment caused no major shift in the elution pattern of the labelled proteins.

The glucagon-like immunoreactivity in fractions of islet extracts after gel filtration on Sephadex G-100 is shown in fig. 2b. A prominent peak eluted in the same region as the 18,000 M.W. protein, whereas the immunoreactivity in the glucagon region was considerably smaller.

4. Discussion

We were surprised that there was an almost complete absence of radioactivity associated with fractions containing marker glucagon after gel filtration of partially purified extracts of islets or incubation media, which had previously been incubated for periods of up to 17 hr with [^{3}H] tryptophan. This result is unlikely to be due to lack of viability of the A_2-cells, since ultrastructural examination as well as continuing incorporation of amino acid into protein both suggested that islet function remained normal over this 17 hr period. It also seems improbable that guinea-pig glucagon would be lost during the extraction procedure, since carrier beef-pork glucagon, added immediately after homogenization of the islets, could readily be recovered in the column eluates on gel filtration, or that guinea-pig glucagon should have a sequence so different from the other species so far studied that it was not extracted along with the carrier into the relatively crude extracts used in this study. Indeed, labelled protein co-eluting with glucagon on gel filtration was found after a 6 day incubation in tissue culture, suggesting both that guinea-pig glucagon may contain tryptophan, and that it is extracted by the procedures which are described above.

The tryptophan-containing protein of approximate molecular weight 18,000 which extracted with glucagon from guinea-pig islets of Langerhans, appears to be very similar in molecular weight to an unidentified protein, whose presence was reported in studies of the biosynthesis of mouse islet proteins [13]. We have found that this protein is not disaggregated on gel filtration in the presence of 8 M urea or 3 M acetic acid and it therefore seems unlikely to represent an aggregate or polymer of glucagon. However, the protein co-elutes with a peak of glucagon-like immunoreactivity on gel filtration and this observation suggests the possibility that some of the immunological determinant groups of glucagon are present within its structure. In addition, recent unpublished observations in this laboratory indicate that guinea-pig islets, which had been deprived of 95% of their B-cells by a previous treatment *in vivo* with streptozotocin and subsequently incubated *in vitro* with [^{3}H] tryptophan produced an elution pattern on gel filtration which was qualitatively and quantitatively very similar to that found in the present study. Furthermore electron microscopic autoradiographic analysis of the distribution of [^{3}H] tryptophan-containing proteins in guinea-pig islets after incubation for 17 hr has shown a significant concentration of these proteins within A_2-cell granules (S.L. Howell and C. Hellerstrom, unpublished).

Further experiments will be required to clarify the role of the 18,000 molecular weight protein in A_2-cell function, and to explore the mechanisms involved in the biosynthesis and storage of glucagon in the guinea-pig.

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