STUDIES ON PROINSULIN AND PROGLUCAGON BIOSYNTHESIS AND CONVERSION AT THE SUBCELLULAR LEVEL

I. Fractionation Procedure and Characterization of the Subcellular Fractions

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ABSTRACT

Anglerfish islets were homogenized in 0.25 M sucrose and separated into seven separate subcellular fractions by differential and discontinuous density gradient centrifugation. The objective was to isolate microsomes and secretory granules in a highly purified state. The fractions were characterized by electron microscopy and chemical analyses. Each fraction was assayed for its content of protein, RNA, DNA, immunoreactive insulin (IRI), and immunoreactive glucagon (IRG). Ultrastructural examination showed that two of the seven subcellular fractions contain primarily mitochondria, and that two others consist almost exclusively of secretory granules. A fifth fraction contains rough and smooth microsomal vesicles. The remaining two fractions are the cell supernate and the nuclei and cell debris. The content of DNA and RNA in all fractions is consistent with the observed ultrastructure. More than 82% of the total cellular IRI and 89% of the total cellular IRG are found in the fractions of secretory granules. The combined fractions of secretory granules and microsomes consistently yield >93% of the total IRG. These results indicate that the fractionation procedure employed yields fractions of microsomes and secretory granules that contain nearly all the immunoassayable insulin and glucagon found in whole islet tissue. These fractions are thus considered suitable for study of proinsulin and proglucagon biosynthesis and their metabolic conversion at the subcellular level.

Subcellular fractionation of pancreatic islet tissue has been accomplished successfully with islet tissue from rats (7, 11, 14, 24, 25), mice (2), codfish (4, 5), anglerfish (1, 13, 23), and human islet adenomas (15, 16). In many of these procedures, especially those in which mammalian islets are used, it is difficult to obtain microsome and secretory granule fractions that are not appreciably contaminated with components from other fractions. Before beginning a comprehensive research effort to study islet hormone synthesis and metabolic cleavage at the subcellular level, we improved existing techniques (1, 13, 23) for fractionation of anglerfish islet tissue. In this communication, a modified method for isolating microsomes and secretory granules from anglerfish islet tissue...
is described. The contents of fractions were analyzed both ultrastructurally and chemically. This paper is the first in a series describing studies of anglerfish proinsulin and proglucagon biosynthesis and conversion at the subcellular level.

MATERIALS AND METHODS

Fractionation Procedure

Anglerfish (Lophius americanus) were obtained during the summer months at the Marine Biological Laboratory, Woods Hole, Massachusetts. Principal and secondary islets were decapsulated and prepared for incubation or fractionation as previously described (1, 20). Anglerfish pancreatic islets are anatomically separated from exocrine pancreatic tissue. One can therefore obtain from each animal, with very little mechanical (decapsulation) and no chemical perturbation, 50-250 mg of islet tissue that is free from contamination with exocrine pancreatic tissue. The fractionation procedure was a modification of a technique developed specifically for the isolation of subcellular components in anglerfish islet (1, 13, 23). Fig. 1 is a flow diagram depicting the procedure. All preparations were kept at 4°C throughout the fractionation. Glassware which came into contact with the prepared fractions was coated with Siliclad (Clay Adams, Inc., Div. of Becton, Dickinson & Co., Parsippany, N. J.).

The islet tissue was homogenized in 1.0 ml of 0.25 M sucrose by 20 vertical strokes in a teflon pestle-glass homogenizer; the pestle was driven at 400 rpm (homogenizers and variable-speed motor from Tri-R Instruments, Inc., Rockville Centre, N. Y.). Homogenates were then transferred to 2-ml cellulose nitrate tubes (Beckman Instruments, Inc., Fullerton, Calif.) for centrifugation at 600 g for 5 min in an SS 34 rotor of a Sorvall RC-2 centrifuge. After one wash, the combined pellet from these precipitations (nuclei and cell debris) comprised Fraction I (F-I). Centrifugation of the resulting super- nate at 6,000 g for 10 min in the same nitrocellulose tube and rotor combination yielded a crude preparation of secretory granules and mitochondria (precipitate, crude F-II) and a crude microsomal fraction (supernate, crude F-III). The precipitates were suspended in 2.5 ml of 1.5 M sucrose in a 5-ml cellulose nitrate tube. This suspension was then subjected to centrifugation concurrent with the crude F-II preparation. The band that appeared at the 0.25-1.4 M sucrose interface (microsomes) and the pellet were removed by careful aspiration. As shown in Fig. 1, this procedure yielded seven subcellular fractions.

Chemical Analysis of Fractions

In many experiments, particularly those that involved isotope incorporation, insulin, glucagon, and their respective precursors were extracted from the subcellular fractions by a modification of the acid ethanol-trisodium citrate-methylene chloride technique as previously described (21). Determination of protein content in the subcellular fractions and in extracts of fractions was made by the micro-method of Lowry et al. (17). The content of RNA and DNA in the fractions was determined by the fluorometric method of Prasad et al. (22).

Antibody against anglerfish insulin (Novo, Copenhagen) was developed in guinea pigs. 2 Iodination of anglerfish insulin was accomplished by the method of Greenwood and Hunter (6). Appropriately diluted aliquots of 100 μl of 100 μl of a bovine serum albumin (BBB) and 15 μl 125I-anglerfish insulin and 200 μl BBB containing antibody at a dilution of 1:2,000 for 1 h at room temperature and then overnight at 4°C. After addition of 100 μl of horse serum and 600 μl of 1% BBB, antibody-bound 125I-insulin was separated from free radioactivity by adding 500 μl of a 40% solution of polyethylene oxide (Polysciences, Inc., Warrington, Pa.) followed by centrifugation.

Determination of immunoreactive glucagon in the subcellular fractions was made, as previously described (21), with the Unger 30K antiserum which selectively binds near the C-terminus of glucagon.

Electron Microscopy

Before fixation, all fractions that formed at interfaces on discontinuous density gradients were diluted to 5 ml with 0.25 M sucrose and then precipitated by centrifugation at 125,000 g for 1 h in the ultracentrifuge. For electron microscopy, fractions and subfractions as well as small blocks of whole islet tissue (1 mm3) were fixed for 1 h in cold paraformaldehyde-glutaraldehyde fixative (10) in 0.1 M phosphate buffer, pH 7.2. After being rinsed in the same phosphate buffer, all preparations were post-fixed for 1 1/2 h in cold 2% OsO4, in 0.1 M phosphate buffer. Samples were then dehydrated through a graded series of alcohols and propylene oxide and embedded in Maraglas (3; Polysciences, Inc.). Thin sections were cut with an LKB Ultratome III, stained with uranyl acetate

1 Gravitational forces are average values for the center of the tubes.

2 Graciously supplied by Dr. D. T. Watkins, University of Connecticut.
and lead citrate, and examined with an RCA EMU-3D or a Philips 300 electron microscope.

RESULTS

Ultrastructural Analysis of Whole Tissue and Fraction Contents

Fig. 2 is an electron micrograph of intact anglerfish islet tissue. The appearance is similar to that of islet tissue from other species. The presence of several cell types is indicated by the observation of groups of cells with morphologically differing populations of secretory granules. The morphology of the granules in one of the cells shown in the inset of Fig. 2 is similar to that observed in alpha2 cells from several species, including humans (12, 18). Most of the cells appear to possess large numbers of secretory granules whereas the endoplasmic reticulum is relatively inconspicuous. Golgi complexes are readily identifiable. The relative paucity of rough endoplasmic reticulum may reflect the lower overall metabolic and synthetic rates (when compared to mammalian islet tissue) which have been observed in anglerfish islet tissue (20).

The ultrastructural appearance of all seven subcellular fractions was assessed. F-I was found to consist primarily of intact or broken nuclei and cellular debris. Mitochondria are the predominant component of the F-II_{top} subfraction of the crude F-II. The intermediate subfraction of the crude F-II includes mitochondria, cell fragments and debris, and a few free secretory granules. Sorenson et al. (23) showed in studies with the original fractionation procedure that the F-II_{top} and F-
FIGURE 2 Electron micrograph of intact anglerfish islet tissue. N, nucleus; SG, secretory granules; Go, Golgi complex. The inset shows adjacent cells (1, 2) which have distinctly different populations of secretory granules. Cell 2 possesses granules with morphology similar to that observed in A₂ cells from several species. × 12,500; inset, × 7,500.
IIFRACTION fractions possess 98 and 2%, respectively, of all the cytochrome C oxidase activity in the crude F-II. Inasmuch as the ultrastructural examinations in the present study substantiate this finding, we chose not to determine cytochrome oxidase activity.

Secretory granules comprise the F-II_{BOTTOM} subfraction of the crude F-II (Fig. 3). Only very small amounts of material other than secretory granules appear in this fraction. This material is composed of small mitochondria and, very rarely, rather large vesicles, probably mitochondrial debris. The secretory granules in this fraction range in average diameter from 220 nm for the smaller granules to 310 nm for the larger granules. No single type of granule appears to predominate.

In earlier work involving fractionation of anglerfish islet tissue, the microsome fraction was prepared simply by submitting the supernate from the crude F-II preparation to rigorous centrifugation (1, 13, 23). After analysis of extracts of microsomes prepared in this manner, it was observed that substantial amounts of insulin were present. This suggested contamination of the microsome fraction with secretory granules. Ultrastructural examination of microsomes that were prepared without subfractionation confirmed that secretory granules were present (Fig. 4). The contaminating secretory granules were removed effectively in the modified procedure by subfractionation of the crude F-III, as described in Methods and shown in Fig. 1. Fig. 5 shows that the F-III_{CONTAMINANT} subfraction consists primarily of secretory granules and ribosomes. The ribosomes are probably free cytoplasmic ribosomes or are stripped from rough microsomal vesicles as they arrest at the 0.25-1.4 M sucrose interface. The range in average diameter of secretory granules in this fraction is from 150 nm for the smaller granules to 200 nm for the larger granules. It is of interest that this range of sizes is lower than that which characterizes the secretory granules in the F-II_{TOP} subfraction. The smaller granules of the F-III_{C} subfraction apparently have a lower sedimentation velocity in 0.25 M sucrose. However, as is the case for the F-II_{TOP} subfraction, no single type of granule appears to predominate in the F-III_{C} subfraction.
Fig. 6 shows the contents of the microsome (F-III) fraction after subfractionation of the crude F-III preparation and removal of the F-IIIc subfraction. All contaminating secretory granules apparently are removed by the subfractionation procedure. The purified F-III appears to contain only ribosome-studded and smooth membranous microsomal vesicles.

**Chemical Analysis of Fractions**

The chemical analyses of the contents of the fractions corroborate the ultrastructural findings. The recovery of protein in the subcellular fractions (data not shown) compared favorably with results from previous studies. If the amounts of protein found in the F-IIg, IIh, IIi, and F-IIIc subfractions are combined in order to estimate the total protein content of the crude F-II, the protein values correlate very well with those obtained in other laboratories where islet tissue from anglerfish (1, 13, 23), cod fish (4), and rats (7, 14, 24) was subjected to fractionation.

Fig. 7 shows the distribution of RNA and DNA in the subcellular fractions from anglerfish islet tissue. The distribution of these nucleic acids correlates well with the ultrastructural analysis of fraction contents. The finding that 27% of the total RNA appears in the F-IIIc (secretory granule) subfraction is corroborated by electron microscope examination of this fraction (Fig. 5). If the ribosomes in the F-IIIc are considered to be free ribosomes or ribosomes that have become detached from membranes in the crude F-III, it then can be stated that the crude F-III contains 81.5% of the total RNA (F-IIIc plus F-III). DNA is present predominantly in the nuclear fraction, but appreciable amounts appear in the microsome fraction and in both fractions of secretory granules. Inasmuch as the DNA content in the technique used (22) is computed as the difference from total nucleic acids after digestion of RNA by RNase, it is possible that removal of RNA was incomplete. However, prolonged incubation in the presence of RNase does not significantly alter the proportion of DNA in the various fractions. An alternate explanation for the appearance of DNA in the fractions of microsomes and granules is that DNA from nuclei fractured during homoge-
nization becomes incorporated artifactually into these fractions during ultracentrifugation. An indication that the latter explanation may be accurate is that nearly 5% of the total DNA is found in the cell supernate (F-IV). This could represent smaller fragments of released nuclear DNA.

The distribution of immunoreactive insulin (IRI) and immunoreactive glucagon (IRG) in the subcellular fractions is shown in Fig. 8. 82% of the total IRI and 89% of the total IRG are found in the two secretory granule fractions. The microsome fraction contains 2.4% of the total IRI and 6.8% of the total IRG. The presence of 14.2% of the total IRI in F-I probably indicates the presence of a significant number of unbroken beta cells or beta cell fragments. If this component of F-I had been more completely disrupted during homogenization, the recovery of IRI in the fractions of secretory granules and microsomes might have been even higher.

Because only a small quantity of anglerfish insulin antibody was available, the data shown in Fig. 8 are the mean values of two determinations on a single fraction. However, an indication that the fractionation procedure consistently yields preparations of microsomes and secretory granules having minimal amounts of contamination comes from the determination of IRG content in acid ethanol extracts of fractions made after tissue incubations (19). In six such determinations, the IRG content in the microsome and secretory granule fractions combined was 93.5 ± 1.1% (mean ± SEM). This result was consistent for tissue incubated for 20 min, 1 h, or 3 h before fractionation. In addition, a recent preliminary assessment of the distribution of immunoreactive somatostatin in subcellular fractions showed that 96.3% of the somatostatin appears in the microsome and secretory granule fractions. Somatostatin is the product of the islet delta cell (9). Thus, nearly all the recovered insulin, glucagon, and somatostatin is found in the fractions of secretory granules and microsomes.

DISCUSSION

As a prelude to the study of islet hormone biosynthesis at the subcellular level, it was necessary to perfect a cellular fractionation procedure that ef-
The F-III obtained by subfractionating the crude F-III is predominantly a microsomal fraction. × 29,500.

Preliminary examination of fraction contents after fractionation by a procedure developed previously for anglerfish islet tissue (1, 13, 23) proved that the microsome fraction was contaminated with secretory granules (Fig. 4). The contaminating

FIGURE 6

DNA

RNA

Percent Total RNA or DNA

FIGURE 7 Distribution of RNA and DNA in the subcellular fractions determined before extraction. The data are mean values from two determinations.
granules can be effectively removed by centrifugation of the crude F-III over 1.4 M sucrose. Ultrastructural examination of the seven subcellular fractions that were derived from the fractionation procedure indicated that the fractions of secretory granules and microsomes obtained were contaminated very little with components from other fractions (Figs. 3, 5, and 6).

This observation was confirmed in part by analysis of the distribution of RNA and DNA and immunoreactive insulin and glucagon. That the mitochondria (F-IIc and F-IIc2) and secretory granules (F-IIa) have very little nuclear or microsomal contamination is indicated by the very low DNA and RNA content of these fractions. Howell et al. (7) reported finding 15% of total cellular RNA in a mitochondrial fraction isolated from rat islets. By contrast, only 4.1% of the total DNA and 3.0% of the total RNA were found in the combined mitochondrial fractions (F-IIT and F-IIM) in the present study (Fig. 7). As suggested in Results, the appearance of small amounts of DNA in the F-III and secretory granule fractions reflects the presence of DNA released from nuclei or remaining attached to fragments of nuclear membranes from nuclei that were ruptured at the time of homogenization.

The most striking portion of the data from the chemical analyses of fraction contents is the distribution of IRI and IRG (Fig. 8). The recovery of more than 82% of the total IRI and more than 89% of the total IRG in the fractions of secretory granules and the consistent recovery of more than 93% of the IRG in the combined F-III and granular fractions indicate that the procedure utilized results in the preparation of microsome and secretory granule fractions that show little loss of hormonal products into the other subcellular fractions. The values for hormone recovery in the secretory granules and microsomes reported here are considerably higher than those for recoveries reported after fractionation of rat islets (7, 14), islets from the obese hyperglycemic mice (2), cod fish islets (5), anglerfish islets (1, 13), and human islet adenomas (15, 16). If one assumes that the largest proportion of the intracellular insulin, glucagon and their biosynthetic precursors would exist in vivo primarily in a membrane-bound state, the effectiveness of any islet fractionation procedure might be measured by the proportion of peptide hormone retained in fractions containing vesicular components. By this criterion, the fractionation procedure reported here is indeed effective.

It thus seems reasonable to postulate that fractions of microsomes and secretory granules from anglerfish islet tissue that were isolated, as described here, after incorporation of radioactively
labeled amino acids might be excellent preparations for analysis of islet hormone biosynthesis and intracellular precursor-to-product conversion. In support of this contention, results from experiments in which fractionation was done after incubation for the incorporation of [(14)C]isoleucine and [(3)H]tryptophan into insulin, glucagon, and their precursors indicate that most of the radioactivity incorporated into these peptides is sequestered in the fractions of microsomes and secretory granules. Only a very small proportion of the labeled hormones and precursors are found in any of the other subcellular fractions (19). The most important factor in obtaining microsome and secretory granule fractions yielding such high proportions of sequestered islet hormones is very likely directly related to the ease of obtaining relatively large quantities of pure pancreatic islet tissue from the anglerfish.

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