ISOLATION AND PROPERTIES OF SECRETORY GRANULES FROM RAT ISLETS OF LANGERHANS

I. Isolation of a Secretory Granule Fraction

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ABSTRACT

A method has been devised for the isolation of a secretory granule fraction from isolated rat islets of Langerhans. The islets were homogenized in buffered sucrose, and the homogenate was separated into nuclear, mitochondrial, secretory granule, and microsomal fractions by differential centrifugation. The secretory granule fraction was purified by differential centrifugation in discontinuous sucrose density gradients. A greater degree of purification could be achieved by the use of two successive gradients of this type, although the final yield was greatly reduced. Biochemical and morphological characterization of the fractions was obtained; the secretory granule fraction contained both insulin and glucagon. The limiting membranes of the granules remained intact and the general appearance of the granules was similar to that seen within the whole islet cells.

INTRODUCTION

The islets of Langerhans in mammals comprise only a very small proportion (1–2%) of the total volume of the pancreas, which has rendered separation of their secretory granules from intact pancreas very difficult (Randall and Shaw, 1964). However, a secretory granule fraction has been isolated from goosefish pancreas in which a large principal islet is obtainable free from exocrine tissue (Lindall, Bauer, Dixit, and Lazarow, 1963).

Methods have recently become available for the isolation of islets from mammals in large numbers by incubation of the pancreas with collagenase (Moskalewski, 1965; Kostianovsky and Lacy, 1966; Howell and Taylor, 1966), and the availability of preparation of this type has greatly facilitated biochemical studies of the islet cells. In the present paper, a method is described for the isolation of a secretory granule fraction from rat islets of Langerhans by the use of differential and density-gradient centrifugation; biochemical and morphological characterization of the fractions is presented. In the accompanying papers, the results of investigations of some biochemical properties, and of ultrastructural studies of the insulin-containing beta granules, are described.

MATERIALS

Sucrose and trichloroacetic acid were obtained from Fisher Scientific Co., Fairlawn, New Jersey. Crystalline-serum albumin, sodium phosphate, yeast ribonucleic acid (Type XI), and horse-heart cytochrome *c* (Type III) were obtained from Sigma

Chemical Co., St. Louis. Cellulose nitrate centrifuge tubes were obtained from Beckman Ltd., Palo Alto, California. Crystalline beef insulin (23.6 units per mg) and beef-pork glucagon were kindly donated by Dr. Walter Shaw, Lilly Research Laboratories, Indianapolis.

METHODS

Isolation of Islets of Langerhans

Pancreatic tissue from 500–700-g male Wistar rats was used; food and water were available ad lib prior to the experiments. Islets were isolated after collagenase digestion of the pancreas, by the technique described by Lacy and Kostianovsky (1967). Yields of 150–200 islets were obtained from each rat, providing an initial wet weight of 3–6 mg of tissue.

Homogenization

After isolation, the islets were placed into a 2-ml capacity Tenbroeck tissue grinder (Kontes Ltd., Vineland, New Jersey) which contained 0.4 ml of an aqueous solution of 0.3 M sucrose and 5 mM sodium phosphate, pH 6.0, at 4°C. Homogenization was effected manually by means of six vigorous passes of the plunger. The homogenate was allowed to stand in the tissue grinder for 2 min before the supernatant was removed; the large fragments remaining were then rehomogenized in 0.2 ml of buffered sucrose, and these homogenates were pooled before centrifugation. This and each subsequent procedure was performed at 4°C.

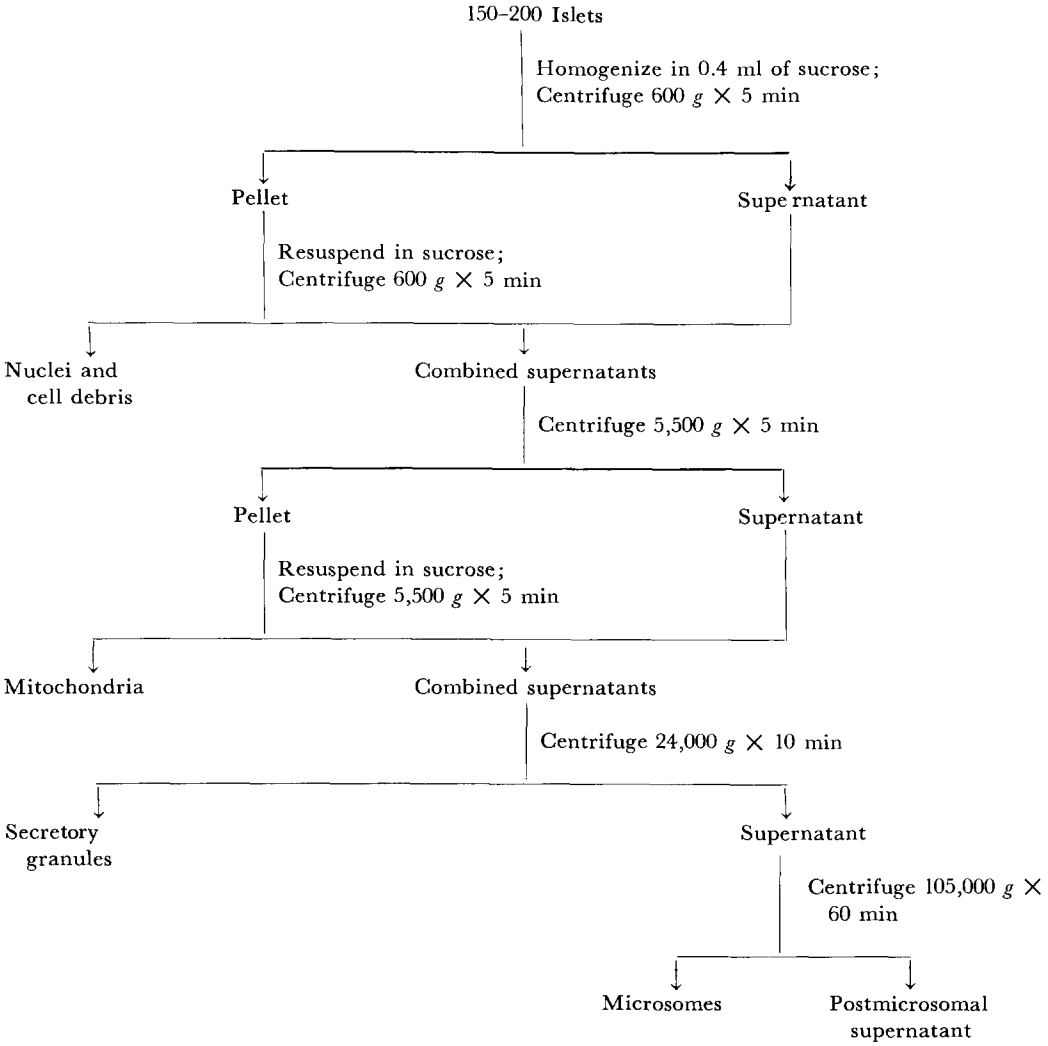

FIGURE 1 Differential centrifugation procedure utilized for fractionation of rat islets.

Differential Centrifugation

The differential centrifugation scheme used is outlined in Fig. 1. Nuclei and cell debris were removed by centrifugation of the homogenate at 600 *g* for 5 min: *g* values quoted throughout are averages obtained at the center of the tubes. The supernatant was decanted while the pellet was resuspended in buffered sucrose and again centrifuged at 600 *g* for 5 min. The supernatants were combined and centrifuged at 5,500 *g* for 5 min, the supernatant being decanted and the pellet resuspended and centrifuged as before. The pellet obtained was designated the mitochondrial fraction.

The pooled supernatants were then centrifuged at 24,000 *g* for 10 min for provision of a secretory granule fraction. The microsomal fraction was prepared by centrifugation of the supernatants at 105,000 *g* for 60 min in a Spinco Model L centrifuge, with the SW 39 rotor. Mitochondrial and secretory granule fractions were obtained by the use of the SS 34 rotor of the Sorvall RC2-B centrifuge. The pellets obtained were resuspended in 0.01 N HCl and stored at −20°C for analysis at a later date.

Density Gradient Centrifugation

The secretory granule fraction obtained in this way was purified by centrifugation in a discontinuous sucrose density gradient. All solutions were prepared in 5 mM sodium phosphate, and were adjusted to pH 6.0. Gradients were prepared 2 hr before use in $1\frac{15}{16} \times \frac{5}{16}$-inch cellulose nitrate centrifuge tubes. They consisted of successive layers of 0.2 ml of 1.7 M sucrose (bottom), 0.3 ml of 1.60 M sucrose, 0.3 ml of 1.55 M sucrose, 0.2 ml of 1.4 M sucrose. After 2 hr the sample, which was resuspended in 0.3 ml of buffered 0.3 M sucrose (pH 6.0) by repeated passages into a finely drawn Pasteur pipette, was layered on top of the gradient (Fig. 2). The tube was immediately centrifuged for 60 min at 105,000 *g* in the SW 39 rotor of the Spinco Model L ultracentrifuge.

After centrifugation, successive layers were removed from the top of the gradient by the use of a J-shaped pipette. For biochemical studies, the fractions were diluted to 0.8 M sucrose with distilled water, disrupted by repeated freezing and thawing, and stored at −20°C for assay. Alternatively, they were diluted in the same way and centrifuged at 40,000 *g* for 20 min to provide pellets which were fixed for electron microscopy; the supernatants were retained for hormone assay in these experiments.

In some experiments, the insulin-rich fraction obtained was diluted to 0.8 M sucrose with distilled water and then layered onto a second identical gradient and centrifuged for a further 60 min under the conditions previously described. Fractions were then withdrawn and treated in the same way.

		% Protein	% Insulin	% Glucagon	% Cytochrome-C-oxidase
SAMPLE					
1.40	I	17 ± 3	12 ± 2	23 ± 5	73 ± 9
1.55	II	44 ± 4	29 ± 5	27 ± 4	<10
1.60	III	28 ± 3	47 ± 6	40 ± 7	<10
1.70	IV	11 ± 3	12 ± 2	10 ± 3	<10
Recoveries:		104 ± 6	93 ± 4	109 ± 5	–

FIGURE 2 Composition of sucrose density gradient. Results are given as mean ± standard error of the mean of four determinations of the per cent distribution in each fraction.

Chemical Analysis of Fractions

Insulin was determined by the immunoassay procedure of Morgan and Lazarow (1963), with beef insulin standards.

Glucagon was determined by an immunoassay method in which antibody-bound glucagon was separated by precipitation with 66% ethanol (Howell, unpublished method). Anti-glucagon sera were produced in rabbits by the method of Assan et al. (1965).

Protein was determined in 10% trichloroacetic acid (TCA) precipitates of the fractions redissolved in 1 N sodium hydroxide, by the method of Lowry et al. (1951), with crystalline serum albumin standards.

Ribonucleic acid (RNA) was extracted from TCA precipitates by the method of heating with 5% TCA at 90°C for 20 min. The RNA content of the extracts was estimated by the orcinol reaction (Mejbaum, 1939), with a purified yeast RNA standard. Cytochrome *c* oxidase was determined by the method of Cooperstein and Lazarow (1955).

Since the total quantities of tissue in each fraction were very small, a complete analysis of each component could not be performed on a single fraction. Thus, separate pellets were prepared for estimations of cytochrome *c* oxidase and RNA levels; this permitted estimation of all components in the differential centrifugation fractions. However, reliable RNA and cytochrome *c* oxidase estimations could not be obtained in many of the fractions obtained after density-gradient centrifugation.

Electron Microscopy

Pellets were fixed in situ with 5% glutaraldehyde in 0.15 M phosphate buffer, pH 7.4, for 30 min,

TABLE I

*Analyses of Fractions Obtained by Differential Centrifugation of Homogenates of Rat Islets**

	Protein	Insulin	Glucagon	RNA	Cytochrome *c* oxidase
Nuclei + cell debris	27 ± 2	9 ± 2	10 ± 4	16 ± 4	14 ± 3
Mitochondria	21 ± 3	18 ± 4	19 ± 5	15 ± 3	59 ± 4
Secretory granules	14 ± 2	61 ± 5	56 ± 7	17 ± 2	18 ± 4
Microsomes + cell supernatant	38 ± 3	12 ± 2	15 ± 5	52 ± 3	9 ± 2
% Recovery of total present in homogenate	109 ± 3	107 ± 4	94 ± 7	91 ± 6	89 ± 4

* Results are given as Mean ± SEM of six determinations of the per cent distribution in each fraction.

and postfixed for 1 hr with 2% OsO_4 in the same buffer. After dehydration, the pellets were embedded in an epoxy resin either in the original centrifuge tubes, if horizontal sections across the pellet were required, or alternatively in flattened molds in which the pellets were oriented so as to give sections in the other plane. Thin sections were mounted on 300-mesh copper grids and stained with lead citrate (Reynolds, 1963) and uranyl acetate (Watson, 1958). They were examined in an RCA EMU 3F or a Siemens Elmiskop I electron microscope.

RESULTS

Fractions Obtained by Differential Centrifugation

The composition of the subcellular fractions obtained by differential centrifugation is shown in Table I. Means of 61% of the insulin and 56% of the glucagon content of the whole homogenate were present in the secretory granule fraction. It is of interest that the centrifugal forces required to sediment the secretory granules were closely similar to those used in studies of ox (Randall and Shaw, 1964) and flounder (Maske, 1957) pancreas. This fraction was contaminated with mitochondria as evidenced by its cytochrome *c* oxidase activity, and by rough endoplasmic reticulum, as shown by the presence of RNA. It was not possible to reduce the contamination of the granule fraction by these organelles without substantial loss of recovery of insulin.

A proportion of the insulin remained associated with the mitochondrial fraction, although this proportion could be reduced to about 15% by repeated washings. Similarly, 2–3% of the insulin was sedimented in the microsomal fraction while approximately 10% remained in the final supernatant. This relatively small degree of solubilization was achieved by the use of gentle homogenization (which also permitted numbers of cells to remain intact in the nuclei + debris fraction), and by the use of optimal pH and temperature conditions for the preservation of the granules. Factors affecting the stability of the isolated beta granules are discussed in the subsequent paper.

TABLE II

Specific Activity of Insulin in Subcellular Fractions

	Milliunits Insulin/μg Protein	Recovery
		%
Whole homogenate	1.33	100
Granule fraction from differential centrifugation	2.59	63
Fraction III from first density gradient	5.38	30
Fraction III from second density gradient	6.25	12

Fractions Obtained by Density Gradient Centrifugation

In preliminary experiments, a large number of subfractions was withdrawn from the tube after centrifugation and analyzed; it was found that the gradient was resolved into four main zones, each of which was relatively homogeneous within itself. Thus, only these four main fractions, the position and composition of which are shown in Fig. 2, were routinely separated and analyzed. Fraction I was rich in cytochrome *c* oxidase

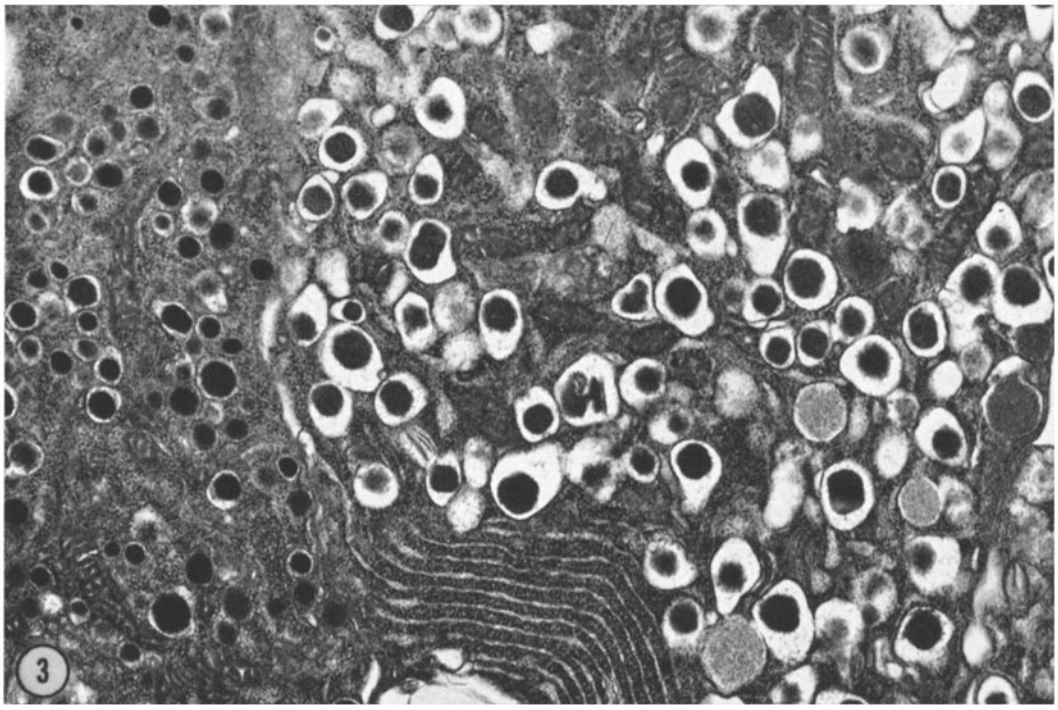

FIGURE 3 Portions of alpha cell (left) and beta cell of rat islet. The alpha cells have electron-opaque granules with closely applied membranes. The beta cells contain a mixture of dense granules separated by a space from the surrounding membrane and of pale amorphous granules. × 24,700.

activity, and contained some hormone activity. However, sedimentation of the components of the fraction followed by analysis of the supernatant and pellet indicated that about 80% of the insulin in this fraction was present in free solution. Fraction II possessed the highest protein content, and was shown morphologically to consist of microsomal material; RNA concentrations could not be reliably determined on the small quantities of tissue available in any of these fractions. Fraction III contained most of the insulin and glucagon content of the gradient: after further centrifugation of the fraction, some 75% of its hormone content was sedimentable and presumably present within the granules.

In some experiments the material obtained in Fraction III was diluted with distilled water to 0.8 M sucrose and layered onto the top of a second sucrose density gradient of similar composition (Fig. 2). After a further 60 min of centrifugation at 105,000 *g*, the four layers were withdrawn and analyzed. While some further purification of the granule fraction could be achieved by this means (Table II), the loss of over-all yield as a result of solubilization of the granules during more prolonged exposure to high-density sucrose solutions was considerable. This second gradient was, therefore, not routinely used.

Results of calculations of the degree of purification of the insulin-rich fractions obtained by these methods, on the basis of the specific activity of insulin (milliUnits insulin μg protein) in each fraction, are shown in Table II. These values were derived from measurements of rat insulin by the use of beef insulin standards; these two species of insulin have been demonstrated to possess comparable cross-reactivities with guinea pig anti-beef insulin serum (Malaisse, Malaisse-Lagae, Lacy, and Wright, 1967).

The use of a single density-gradient centrifugation resulted in an approximately 30% yield of insulin, which had been purified fourfold from the whole homogenate (Table II).[1] Further fractiona-

[1] A yield of glucagon of 23% was achieved with a similar degree of purification.

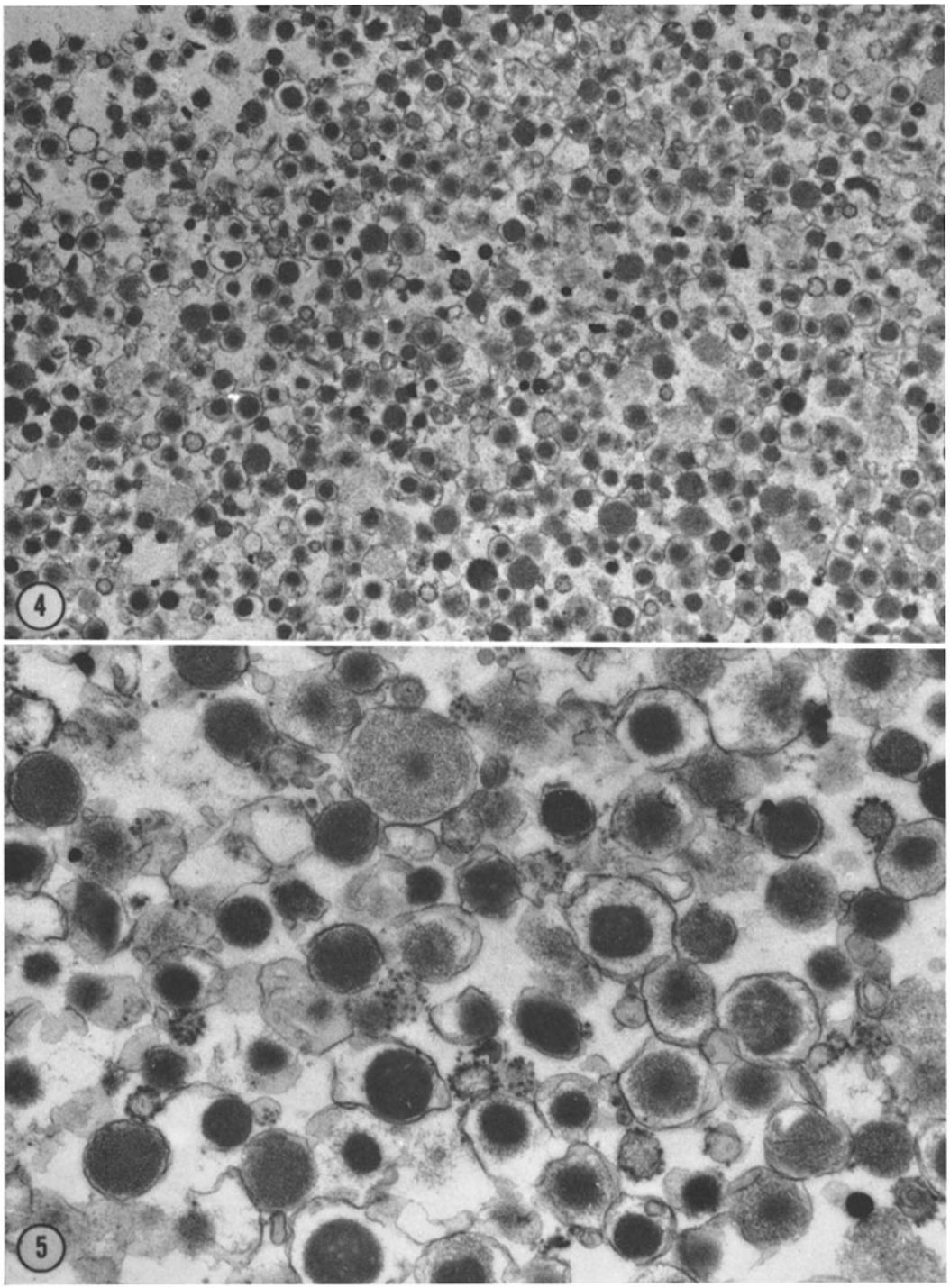

FIGURE 4 Secretory granule fraction obtained after density gradient centrifugation of rat islets. × 11,700.

FIGURE 5 Secretory granule fraction obtained after density gradient centrifugation. The three types of granule identifiable in thin sections are identifiable in this fraction. × 40,000.

tion resulted in a great reduction of yield of both hormones with little additional purification.

Morphology of the Subcellular Fractions

Fig. 3 shows portions of alpha and beta cells of an islet of Langerhans of the rat. The alpha cells contain glucagon stored within electron-opaque granules with closely applied membranes, while the beta cells contain dense granules separated by a space from the surrounding membrane and pale granules, both of which granules are assumed to contain insulin (Lacy, 1961; Herman, Sato, and Fitzgerald, 1963). Fig. 4 shows a low magnification view of a secretory granule fraction obtained after the density-gradient centrifugation procedure described above. A different area from the same pellet is shown at higher magnification in Fig. 5; it contains dense granules with closely or loosely applied membranes intact, which may tentatively be identified as alpha or beta granules, and also pale granules of the type seen within the intact cells. Some microsomal contamination is also present.

DISCUSSION

Separation of the Secretory Granules

Numerous previous attempts to achieve separation of subcellular organelles in a state of great purity have shown that this separation can be achieved only at the expense of a loss of over-all yield (for discussion see de Duve, 1964). Thus in the present study, it was possible to obtain routinely a four-fold purification of the insulin content of the tissue homogenate with an approximately 30% yield (Table II). Attempts to achieve further purification by the use of a second density gradient, an approach used with some success in the isolation of adrenal medullary granules (Delarue, 1968), resulted in a large loss of yield with only a small increase in specific activity of the fraction (Table II).

It has been pointed out by de Duve, Berthet, and Beaufay (1959) and by Dean and Hope (1968) that particles of the dimensions of the secretory granules (180–250 mμ) do not reach an equilibrium position in the density gradient during a 1-hr period of ultracentrifugation. However, the relative instability of the secretory granules from islet tissue renders them unsuitable for the 4–5-hr centrifugation required to reach equilibrium, and experiments in which this approach was attempted resulted in increased solubilization of the granules which tended to offset any greater degree of purification achieved.

Separation of Alpha and Beta Granules

Assay of insulin and glucagon content of 12 subfractions derived from the sucrose density gradients suggested that there was little difference between the densities of the alpha and beta granules, and that it was not possible to achieve adequate separation of the two types of granule in this species by these techniques.

However, there was a direct correlation between the specific activity of the hormones in each subcellular fraction and their content of secretory granules as revealed by electron microscopy. This provides further indirect evidence that the alpha and beta granules may represent the major intracellular storage sites of glucagon and insulin.

We thank Dr. Marie Greider and Mrs. Gene Thomas for advice and help with electron microscope studies.

This project was supported in part by National Institutes of Health grant numbers AM 03373, AM 01226, AM 06181 and GM 08574.

Dr. Howell thanks Eli Lilly and Co. for a grant-in-aid, and the Wellcome Trust for a travel grant.

Received for publication 23 August 1968, and in revised form 7 November 1968.

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