

# ISOLATION OF AN INSULIN SECRETION GRANULE FRACTION

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## ABSTRACT

Goosefish islets were homogenized in 0.25 M sucrose and separated into nuclear, mitochondrial + secretion granule, microsomal, and supernatant fractions. Eighty per cent of the cytochrome oxidase activity and 75 per cent of the bioassayed insulin activity were found in the mitochondrial + secretion granule fraction (6000 g for 10 minutes). The mitochondrial + secretion granule fraction was further subfractionated by centrifugation (2 hours at 100,000 g and 0°C) using a continuous linear density gradient (1.0–2.0 M sucrose). Eighteen to 20 subfractions were collected by piercing the bottom of the tube and collecting drops. The total protein was distributed into a bimodal curve consisting of a high density component, which contained 90 per cent of the insulin (secretion granules), and a lower density component, which contained the cytochrome oxidase activity (mitochondria).

For many years, histologists have described subcellular structures believed to be the storage site of cellular secretion products. In the past few years, differential centrifugation has been employed to separate such secretion granules from other cellular elements. Only with the recent utilization of density gradient fractionation, however, have secretion granules been separable from other subcellular particles. Siekevitz and Palade (18) employed discontinuous density gradients in their studies on zymogen granules. A chromaffin granule fraction was isolated by Blaschko *et al.* (1) from ox adrenal glands. Recently, pituitary secretion granules corresponding to acidophilic and basophilic granules have been isolated by combined centrifugation and filtration (15). Subcellular particles corresponding to nerve endings also have been isolated (4, 20).

Workers have carefully studied the beta cell secretion granules of the islets of Langerhans for many years, utilizing their specific staining properties. Lacy (8) has recently extended these

morphological and physiological studies using the electron microscope. The physical isolation of the insulin-containing secretion granule has been complicated by the fact that the islets of Langerhans in mammals constitute only about 1 per cent of the total pancreatic tissue. Many teleost fish, however, have a principal islet which is separate from the acinar pancreatic tissue (14). Such fish make ideal material for physical isolation of insulin-containing granules. Maske (13) first reported observations on fractionated islet tissue from the flounder (*Pleuronectes*). He found that 62 per cent of the insulin was isolated with the mitochondria, but he could not resolve the question of whether or not the insulin was contained in a separate secretion granule. Lazarow (9) also found that the majority of the insulin in the islet of the toadfish (*Opsanus tau*) could be sedimented with low gravitational forces.

In our present study, islet tissue homogenates of the goosefish (*Lophius piscatorius*) were fractionated by differential centrifugation; the

mitochondrial fraction was then subfractionated in a continuous linear density gradient, and an insulin-containing fraction was separated from the mitochondria. A preliminary report of these results has been published (11).

## MATERIALS AND METHODS

### Fractionation

The goosfish were caught and stored for several days in tanks of cold running sea water. Principal islets, weighing 30 to 100 mg, were obtained from the goosfish and used immediately. In a typical fractionation experiment, 30 to 100 mg of islet were

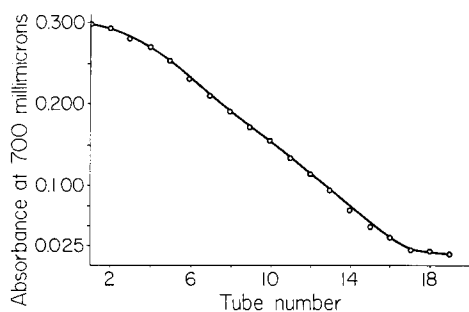


FIGURE 1 The density gradient produced by the device described in the text. Cupric sulfate was included in one of the two syringes and distributed in the same manner as the denser of the two sucrose solutions used to form the gradient. After formation of the gradient column, the contents of the tube were immediately separated by piercing the bottom of the tube and collecting 19 subfractions of 10 drops each. The samples were then diluted, and the absorbance determined at 700  $\mu$ .

homogenized in 0.25 ml of cold 0.25 M sucrose. This was diluted with an additional one ml of 0.25 M sucrose solution. One ml of homogenate was transferred to a 6  $\times$  50 mm nitrocellulose tube for fractionation at 0°C in the Servall RC-2 centrifuge. The following fractions were collected:

Fraction I—the sediment from 600 *g* for 5 minutes, washed once.

Fraction II—the sediment from 6,000 *g* for 10 minutes, washed once.

Fraction III—the sediment from 25,000 *g* for 2 hours.

Fraction IV—the supernatant.

In the density gradient experiments, a continuous gradient ranging from 1.0–2.0 M sucrose was formed by a cam-driven device. This device utilizes two cam-driven syringes leading into a common mixing chamber. One syringe contained 1.0 M sucrose and

the other, 2.0 M sucrose. The cams were devised to operate the respective syringes at such rates as to deliver a linear density gradient into a 10  $\times$  50 mm nitrocellulose centrifuge tube. Similar devices have been described (5). Fig. 1 indicates the characteristics of such a gradient, which is essentially linear in the range of interest. Fraction II was dispersed in 0.5 ml of 0.25 M sucrose solution, layered over the cold gradient, and centrifuged in the SW39 rotor of the Spinco model L preparative ultracentrifuge, at 100,000 *g* (average) for 2 hours at a temperature of 0°C. The contents of the tube were immediately separated by piercing the bottom of the tube and collecting about 20 subfractions of 10 drops each (2).

### Cytochrome Oxidase Determination

Cytochrome oxidase analyses were done by the method of Cooperstein and Lazarow (3). Type III cytochrome *c* was obtained from Sigma Chemical Company, St. Louis.

### Protein Extraction and Purification

The protein in the fractions was precipitated with equal volumes of 10 per cent trichloroacetic acid (TCA) and the precipitate was collected by centrifugation; the TCA precipitate was then extracted with acid alcohol (0.18 N HCl in 75 per cent ethyl alcohol (17)). Protein determinations by Lowry's method (12) were made on the TCA residue, which was dissolved in 0.1 N NaOH, and on dried aliquots of the acid alcohol extract (acid alcohol-soluble protein (AASP)). In all cases, bovine serum albumin was used as the standard. The AASP was purified by the following procedure. The alcohol was removed by evaporation under nitrogen and the residue was washed once with acetone and twice with ether in order to remove lipids. The residue was then dissolved in acid alcohol and the pH of the solution adjusted to 9.0 with  $\text{NH}_4\text{OH}$ . After centrifugation, the supernatant was removed, dried, and the residue was redissolved in acid alcohol. This material was designated as the purified acid alcohol-soluble protein (PAASP), and contained from 25 to 35 per cent insulin on bioassay.

### Chromatography and Bioassay of Insulin

In some experiments, chromatography of the PAASP was carried out (6, 7, 10) in 2-butanol-1 per cent acetic acid- $\text{H}_2\text{O}$  (1:1 by volume) using Whatman 3 MM paper. The chromatograms were stained with 0.05 per cent bromphenol blue in 1 per cent  $\text{HgCl}_2$  and 2 per cent acetic acid. Washing was done in 2 per cent acetic acid. In other cases, the material was bioassayed using the epididymal fat pad method (16). The results obtained by chromatography agreed well with bioassay values.

## RESULTS

### Standard Fractionation

Although many fractionation studies were carried out, only the most complete experiment will be reported. When the cytochrome oxidase activity was expressed as the percentage of the total activity present in each of the various fractions (Fig. 2 *A*), it was found that 80 per cent of the activity was present in fraction II. Only 13

Fig. 2 *B*. Fraction II contained somewhat more, and fraction III somewhat less, protein than did the other fractions.

In the original homogenate, 38 per cent of the total protein was soluble in acid alcohol (2.5 mg/72 mg of tissue). Of the acid alcohol-soluble protein present in all fractions, 49 per cent appeared in fraction II, while only 20, 17, and 12 per cent appeared in fractions I, III, and IV, respectively (Fig. 2 *C*). From these fractions, a

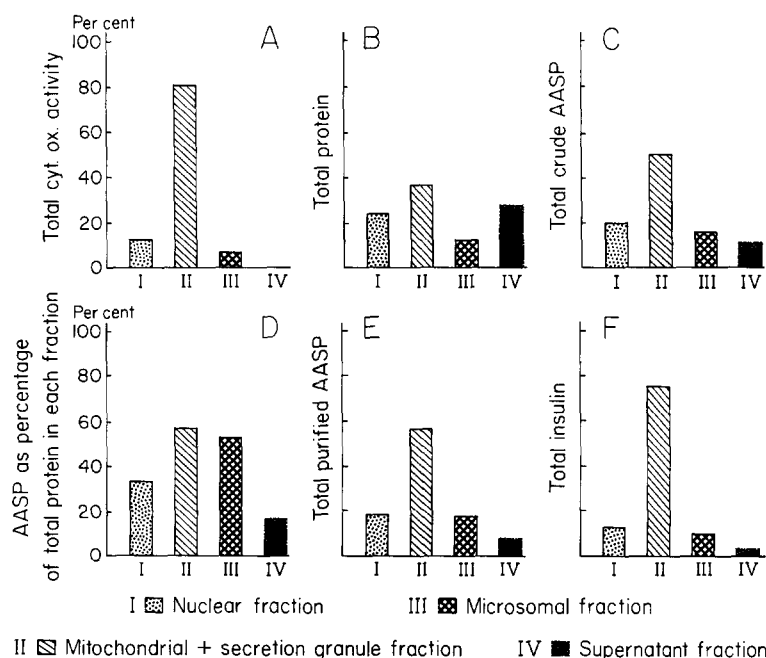


FIGURE 2 The percentage distribution of various constituents in the subcellular fractions of goosfish islet tissue. *A*. Percentage distribution of the total cytochrome oxidase activity. *B*. Percentage distribution of the total TCA-precipitable protein. *C*. Percentage distribution of the crude acid alcohol-soluble protein (AASP). *D*. Percentage of the total protein in each fraction which is soluble in acid alcohol. *E*. Percentage distribution of purified acid alcohol-soluble protein. *F*. Percentage distribution of insulin as measured by epididymal fat pad bioassay.

and 8 per cent of the activity were present in fractions I and III, respectively, while no activity was observed in the supernatant fraction (IV). It is assumed that these results correspond to the distribution of the mitochondria within the fractions.

The original homogenate contained 6.56 mg of protein/72 mg of tissue, which is 9.1 per cent of the wet weight. The amount of protein recovered from the four fractions (72 mg of tissue) was 5.82 mg (89 per cent recovery). The percentage distribution of total protein is shown in

total of 2.3 mg of crude AASP was recovered from 72 mg of tissue (92 per cent recovery).

Fig. 2 *D* shows the percentages of the total protein present in each fraction which was acid alcohol-soluble. These were 60 per cent in fraction II and 53 per cent in fraction III. Upon further purification the percentage of acid alcohol-soluble protein in fraction II increased to 56 per cent of the total PAASP present in all fractions (Fig. 2 *E*). Purification removed 50 per cent of the crude AASP. A total of 1.15 mg PAASP (72 mg tissue) was recovered.

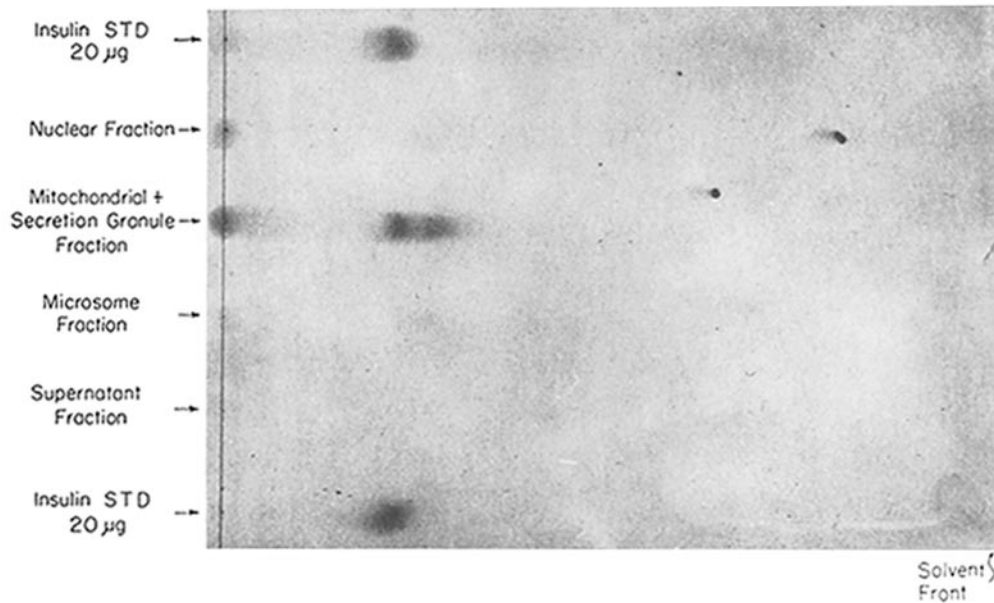


FIGURE 3 A photograph of an ascending paper chromatogram of purified acid alcohol-soluble protein from subcellular fractions of goosfish islet tissue. Chromatography was carried out in 2-butanol-1 per cent acetic acid-H<sub>2</sub>O for 12 hours at 23°C. Magnification,  $\times \frac{2}{3}$ .

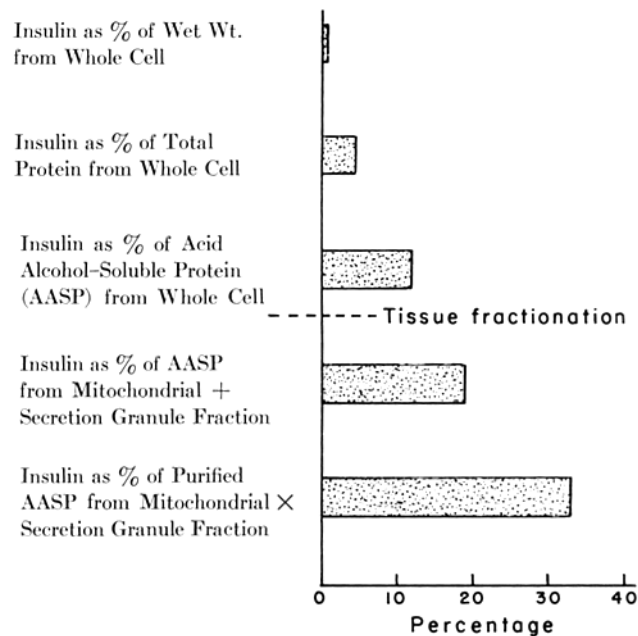


FIGURE 4 Insulin content of various extracts from goosfish islet tissue.

The purified acid alcohol-soluble protein was subjected to ascending paper chromatography (Fig. 3). A large quantity (approximately  $\frac{1}{3}$ ) of the total protein in fraction II migrated with

the same  $R_f$  as that of the beef insulin standard, while only traces were seen in fractions I and III. Some of the protein in fraction II remained at the origin and some migrated faster than insulin.

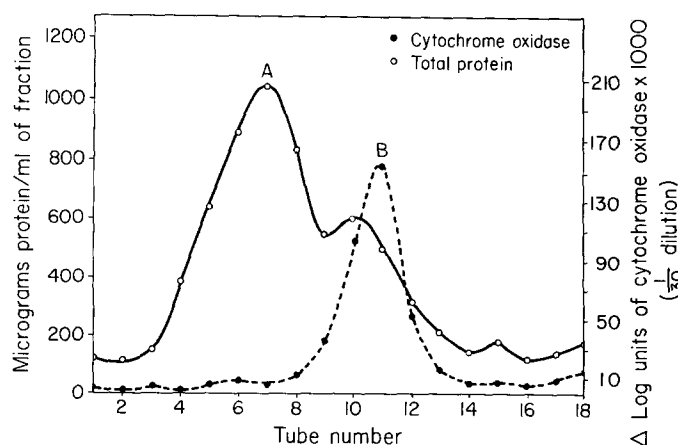
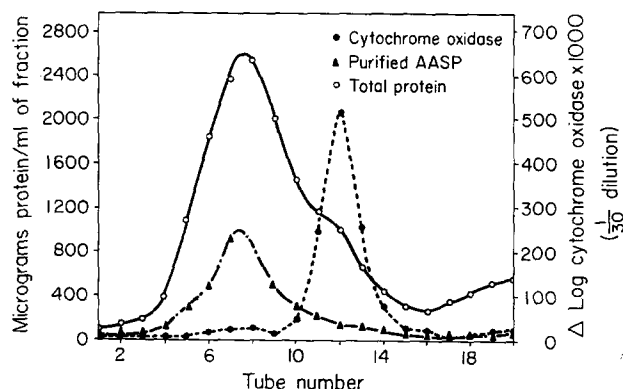


FIGURE 5 The density gradient subfractionation of the mitochondrial + secretion granule fraction (II) from goosefish islet. The solid line represents the total protein in micrograms/ml of each subfraction. The dashed line represents the cytochrome oxidase activity in each subfraction expressed as  $\Delta$  log units  $\times 1000$  for a  $1/30$  dilution.

A  $\Delta$  log unit represents the change of the logarithm of the absorbance of reduced cytochrome *c* per minute at  $550 m\mu$ .

FIGURE 6 The density gradient subfractionation of the mitochondrial + secretion granule fraction (II) from goosefish islet. The solid line represents the total protein in micrograms/ml of subfraction. The dashed line represents cytochrome oxidase activity in  $\Delta$  log units (see legend of Fig. 5). The dotted and dashed line represents the purified acid alcohol-soluble protein in micrograms/ml of subfraction.



We cannot yet say which of the two closely related moving proteins is insulin. However, Taylor *et al.* (19) found in the toadfish that the more slowly moving protein was insulin. In any case, fraction II clearly contains most of the protein that migrated with an  $R_f$  similar to that of beef insulin.

In addition, the purified material was bioassayed. Fig. 2 *F* shows that 75 per cent of the insulin was in fraction II. We recovered 0.920, 5.490, 0.716, and 0.273 units from fractions I through IV, respectively. The total (7.40 units from 72 mg of tissue) is equivalent to 102 units/gm tissue. On the basis of assuming 25 units/mg, the tissue contained 0.4 per cent insulin on a wet weight basis.

The PAASP from fraction II contained about

32 per cent insulin. This agrees well with qualitative estimates from the chromatogram (Fig. 3). The progressive purification of insulin by fractionation, extraction with acid alcohol, and further purification is shown in Fig. 4.

#### Subfractionation of the Mitochondrial + Secretion Granule Fraction (II).

When fraction II was recentrifuged in a density gradient (1.0 to 2.0 *M* sucrose), two major components could be visualized as distinct layers. The distribution of total protein within the gradient (Fig. 5) showed two major components (*A* and *B*) with maxima in tubes seven and ten. The largest component (*A*) corresponded to a

sucrose concentration of about 1.66 M (density 1.21). The smaller protein peak (*B*) corresponded to a sucrose concentration of about 1.4 M (density 1.17). The cytochrome oxidase distribution had its maximum in tube number 11 and coincided with protein component *B*. Note that the maximum of protein peak *B* differs from that of the cytochrome oxidase peak by one tube. This probably results from displacement of the maximum of component *B* by summation of the normal distributions of two overlapping components. If a normal distribution of each of the two components (*A* and *B*) is assumed, the mitochondrial protein

the fat pad method (tubes 5 to 15), 91 per cent of the insulin was contained in tubes 5 to 10, whereas only 5 per cent was found in the tubes corresponding to the mitochondrial component (tubes 11 to 15). About 4 units of insulin were recovered from the mitochondria + secretion granule fraction and, since about 75 per cent of the islet's insulin is contained in this fraction, there would have been about 53 units recovered per gram of islet tissue. This recovery is excellent, considering the number of manipulations carried out. In this experiment, the insulin represented 10 per cent of the total protein and 25 per cent

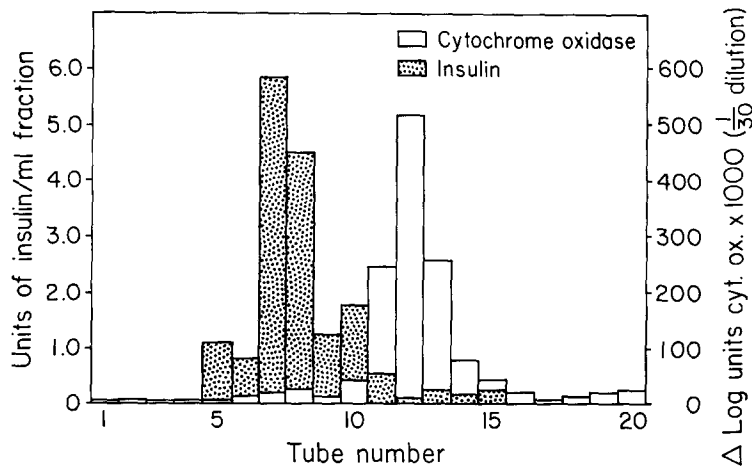


FIGURE 7 The distribution of insulin and cytochrome oxidase in subfractions obtained from the density gradient centrifugation of fraction II (mitochondrial + secretion granules) from goosfish islet. Insulin is expressed in units/ml of subfraction. Cytochrome oxidase activity is expressed in  $\Delta$  log units (see legend of Fig. 5).

represents 26 per cent of the total protein present in tubes 1 through 13.

The gradient fractionation was repeated with the mitochondrial + secretion granule fraction from 100 mg of islet. Fig. 6 shows the distribution of total protein, cytochrome oxidase activity, and purified acid alcohol-soluble protein. In Fig. 7, the insulin bioassay values on the PAASP from tubes 5 through 15 are compared to the cytochrome oxidase distribution. These results show that the PAASP was distributed beneath protein peak *A* and was separated from the cytochrome oxidase activity. Paper chromatography of equivalent volumes of tubes 7 and 12 showed that a protein with an  $R_f$  similar to that of beef insulin was present in tube number 7, but not in 12. Of the subfractions bioassayed by

of the PAASP in the subfraction (tube 7) containing the heavier particulate component (*A*).

#### DISCUSSION

The results verify earlier studies in which insulin was found to sediment with the mitochondria by standard fractionation procedures. Despite the fact that both particles have different sedimentation constants, they both happen to sediment at 6000 *g* for 10 minutes in 0.25 M sucrose. Our results clearly show that an insulin-containing granule fraction can be separated from the mitochondrial fraction by the gradient centrifugation technique. Maske found that insulin constituted 4.7 per cent of the protein in his mitochondrial fraction; our results show that insulin comprises about 10 per cent of the protein in the

secretion granule subfraction. The 10 per cent value, however, represents only a minimum concentration, since fish islet tissue contains alpha cell secretion granules, which may have contaminated our insulin granule subfraction.

The synthesis of insulin granules has been studied by electron microscopy in rat islets (21). These morphological studies suggest that, under conditions in which insulin synthesis is presumably taking place, the endoplasmic reticulum of the beta cell forms vesicles within which an amorphous material is deposited. According to Lacy's interpretation, as the amount of material increases, the definitive secretion granules are formed. We have done preliminary H<sup>3</sup>-leucine incorporation studies which suggest that the microsomes fraction (endoplasmic reticulum) may be involved in the synthesis of insulin. Preliminary evidence also suggests that a lipoprotein material may be associated with the insulin secretion granule: 1 per cent saponin released as much AASP from

the secretion granule + mitochondrial fraction (II) as did 0.1 N HCl. Following saponin treatment, however, about 50 per cent of the protein in this fraction can still be sedimented at 6000 g for 10 minutes. By contrast, 0.1 N HCl completely solubilizes this fraction.

The separation of an insulin-containing secretion granule will permit a study of the physical and chemical characteristics of the insulin stored within the secretion granule. It may provide pertinent information on the nature of the native insulin-protein complex within the granules. Such studies are now in progress.

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