STUDIES ON INSULIN BIOSYNTHESIS

Subcellular Distribution of Leucine-H^3 Radioactivity During Incubation of Goosefish Islet Tissue

G. ERIC BAUER, ARNOLD W. LINDALL, JR., PADMAKER K. DIXIT, GORDON LESTER, and ARNOLD LAZAROW

From the Department of Anatomy, University of Minnesota, Minneapolis, and the Marine Biological Laboratory, Woods Hole, Massachusetts

ABSTRACT

Islet tissue from the goosefish (*Lophius piscatorius*) was incubated in the presence of leucine-H^3. The tissue was then separated into subcellular fractions and the radioactivity determined in total acid alcohol–soluble proteins, insoluble proteins, and insulin. At any time, microsomal protein had a higher activity than secretion granule protein. Pulse-chase experiments further suggest the microsomes as primary sites of protein synthesis. The data are evidence for microsomal synthesis of insulin and for its subsequent transfer into the secretion granules.

INTRODUCTION

The incorporation of labeled amino acids into proteins of subcellular fractions (9) has been frequently utilized in determining the sites of protein synthesis within the cell. The technique also has been applied in studying the synthesis and storage of specific proteins. Siekevitz and Palade (20) traced the subcellular distribution of radioactivity in the proteolytic enzymes of guinea pig pancreas following injection of leucine-C^{14}. They demonstrated that these enzymes are synthesized by microsomal constituents, and then are transferred to the zymogen granule fraction.

At present, little is known about the production of insulin in islet tissue. Light and Simpson (13) and others (22–24) have demonstrated amino acid incorporation into insulin by mammalian pancreas slices. Wagle has presented evidence for insulin synthesis in a cell-free system derived from dog pancreas (25). Since the principal islet of the goosefish is free of acinar tissue (17), we have used it in preference to the mammalian pancreas (in which the islets represent only 1 to 2 % of the total volume). Amino acid incorporation into insulin by the isolated islet tissue from the goosefish has been demonstrated (2, 4). Humbel has reported similar studies using islet tissues from toadfish (10) and goosefish (11).

In a previous report (14) it was shown that, upon homogenization and fractionation of goosefish islets, 75% of the insulin in the tissue sedimented in the classical mitochondrial fraction; a small but significant percentage of the insulin was found in the microsomal fraction. Further, an insulin granule subfraction was separated from mitochondria by sucrose density gradient centrifugation. This work demonstrated that most of the insulin in the islet is present in a storage (or secretion) granule, which corresponds to the B granule visible by light and electron microscopy (12). By combining the methods of incorporation of labeled amino acid into islet tissue in vitro and fractionation of the tissue by centrifugation, we

have investigated the sites of synthesis and storage of islet proteins, including insulin. Preliminary reports on the progress of this work have been presented (3, 4).

MATERIALS AND METHODS

INCUBATION: Goosefish (*Lophius piscatorius*) were obtained during July and August at Woods Hole, Massachusetts, and maintained in tanks supplied with running sea water. The principal islet was removed from the fish, freed of its connective tissue capsule, and cut into 5-mg pieces. Approximately 100 mg of tissue, from 1 to 3 fish, were used in each experiment. The weighed islet pieces were incubated for various time periods in 1 ml of a Krebs-Ringer bicarbonate medium (2) containing DL-leucine-4, 5-H^3 (5.4 c/mmole). The incubation vessel consisted of a 15-ml vial fitted with a syringe cap. Just prior to incubation, and for several minutes following introduction of tissue, the capped vessel was gassed with an oxygen and carbon dioxide mixture (95:5). At the end of the incubation period, the tissue was removed from the medium and homogenized in a loose-fitting glass homogenizer containing 1 ml of cold 0.25 M sucrose. The diluted homogenate was separated into the following fractions by differential centrifugation:

I, nuclei, cellular debris (the sediment from 600 *g*,[1] 5 min, washed once).

II, mitochondria, secretion granules (the sediment from 6000 *g*, 10 min, washed once).

III, microsomes, (the sediment from 105,000 *g*, 60 min).

IV, cell supernatant, (the 105,000 *g* supernatant).

In some experiments, the mitochondria + secretion granule fraction (Fr. II) was separated into a mitochondria-rich subfraction and a secretion granule-rich subfraction by the following procedure. The washed 6000 *g* pellet was resuspended in 0.25 M sucrose and layered upon a two-step sucrose gradient (1.5 ml of 2.0 M sucrose-2.5 ml of 1.5 M sucrose) in a 5-ml centrifuge tube. The suspension was centrifuged at 125,000 *g* for 1 hr in a Spinco 39 L swinging bucket rotor. The resulting particulate bands, at the 0.25 to 1.5 M interface (mitochondria) and the 1.5 to 2.0 M interface (secretion granules), were removed with a capillary pipette. The subfractionation procedure is based on the calculated densities of the mitochondria peak (ca. 1.4 M sucrose) and the granule peak (ca. 1.66 M sucrose) observed by fractionation on a continuous sucrose density gradient (14). By this procedure, 90 per cent of the cytochrome *c* oxidase (a mitochondrial enzyme) was recovered in the mitochondrial subfraction.

EXTRACTION OF PROTEINS: Proteins of the isolated fractions were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 5 per cent. The precipitated proteins were washed 3 to 5 times with 5 per cent TCA and then extracted twice with acid alcohol (19). The resulting residue was extracted further with alcohol-ether (1:1) and ether. The pooled acid alcohol extracts were designated the acid alcohol–soluble fraction (AASF), and the defatted protein–nucleic acid fraction was designated the TCA-precipitable protein residue (TPR). Insulin from the goosefish islet is recovered from the TCA precipitate by acid alcohol treatment (4). The TPR of nuclear, microsomal, and supernatant fractions were further extracted with 5% TCA for 15 min at 90° according to the method of Schneider (18). The centrifuged precipitate (washed once with 5% TCA) was designated the insoluble protein (IP). Protein contents of the various samples were determined (in duplicate) by the micromethod of Lowry et al. (16). Unless noted to the contrary, bovine serum albumin was used as protein standard. For radioactivity determinations, duplicate 25-μl aliquots of the samples were dried in counting vials, and redissolved in 0.5 ml of Hyamine 10X (Rohm & Haas Co., Philadelphia) followed by 10 ml of toluene containing 0.4% 2,5-diphenyloxazole and 0.02% 1,4-bis-2-(5-phenyloxazolyl)benzene. Samples were counted in a Packard Tri-Carb Liquid Scintillation Counter at 10% efficiency. Incorporation data were expressed as CPM/mg of protein (specific activity).

ELECTROPHORESIS: Acid alcohol–soluble extracts of microsome and secretion granule fractions from tissue incubated for 2 hr were prepared for acrylamide gel electrophoresis in the following way. The extracts were concentrated by evaporation and adjusted to pH 5 by adding NH_4OH. The major lipid fraction was removed by two ether extractions, and the resulting residue was redissolved in 1 ml of acid alcohol. Immediately before electrophoresis, duplicate aliquots were dried and redissolved in Tris-glycine buffer (0.05 M Tris + 3.84 mM glycine, pH 8.3). Equal volumes of upper gel solution (2½% polyacrylamide) were mixed with the samples, and photopolymerized upon a spacer gel of 2½% polyacrylamide. Electrophoresis was carried out on a 7½% polyacrylamide gel for 40 min at 5 ma per column in Tris-glycine buffer, pH 8.3. Following the run (at 5°), the gels were removed and frozen for subsequent fractionation, or stained with amido black. After destaining, these samples were used to locate protein bands in the unstained samples, which then were separated and extracted for several hours with acid alcohol. Aliquots of these fractions were used to determine radioactivity and biological activity. Apparatus and reagents for acrylamide gel electrophoresis were supplied by Canalco (Bethesda, Maryland). DL-leucine-4,5-H^3 was obtained from New England Nuclear Corp., Boston. Crystalline

[1] All gravitational forces are average.

bovine zinc insulin and crystalline porcine glucagon were gifts from Dr. Mary Root, Eli Lilly Company, Indianapolis.

RESULTS

Protein Distribution among Subcellular Fractions

Fig. 1 shows the distribution of protein among subcellular fractions isolated from islet tissue after 15 min of incubation. The secretion granule subfraction contained approximately 30% of the total acid alcohol–soluble protein (AASP) and 8% of the insoluble protein (IP), while the mitochondrial subfraction contained 18% AASP and 12% IP. The combined AASP from secretion granules and mitochondria totaled 46%. The microsome fraction contained approximately equal quantities of AASP and IP (17 and 23%, respectively). These protein distributions were essentially unchanged during prolonged incubation of islet tissue, and are similar to those reported for unincubated tissue (14).

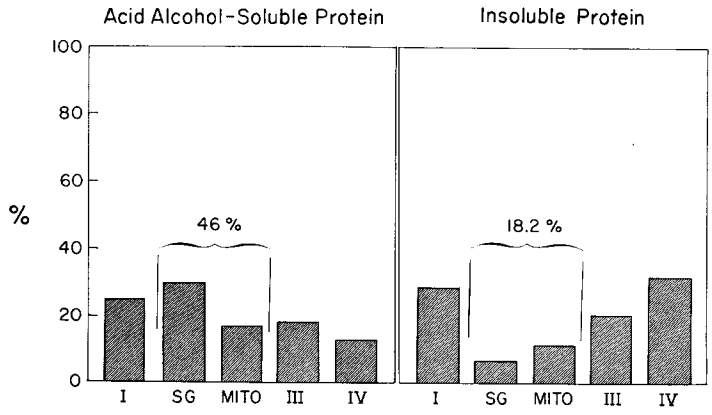

FIGURE 1 The percentage distribution of proteins isolated from the subcellular fractions of goosefish islet tissue. The tissue had been incubated in vitro for 15 min in a Krebs-Ringer bicarbonate medium.

Incorporation into Standard Subcellular Fractions

Preliminary in vitro and in vivo incorporation studies were carried out to determine the pattern of incorporation of leucine-H^3 into subcellular fractions. Leucine-H^3 (200 μmoles/l) was added to the incubation medium, or injected into the fish intracardially. After various time periods, the tissue samples were homogenized in cold 0.25 M sucrose and fractionated into nuclear, mitochondrial, microsomal, and supernatant fractions. We found that the distributions of radioactivity among the cell fractions were similar in vivo and in vitro (Table I). This similarity indicates that the pattern of incorporation in vitro reflects a metabolic activity of islet tissue, rather than an artifact of the incubation procedure.

The distributions of radioactivity in acid alcohol–soluble proteins after 15 and 120 min of in vitro incubation are compared in Fig. 2. Following 15 min of incubation with leucine-H^3, the specific activity of the acid alcohol–soluble protein (AASP) of the microsomal fraction was higher than that of any other fraction, whereas that of the mitochondria + secretion granule fraction (Fr. II) was lowest. This pattern of incorporation was observed also during shorter incubation periods of 2, 5, and 10 min (not illustrated).

After 120 min of incubation with leucine-H^3, the specific activity of microsomal AASP increased 8-fold with respect to the 15-min value. The specific activity of Fraction II AASP, however, increased 30-fold during the same period (Fig. 2).

In another experiment, islet tissue was incubated with leucine-H^3 for an initial 15 min, rinsed in medium containing unlabeled leucine, and incubated for an additional 105 min in unlabeled medium ("pulse-chase"). The cell fractions were isolated and the specific activities of AASP determined, as above. A comparison of these values with those obtained after 15 min of incubation (Fig. 2) shows that the specific activities of AASP of the nuclei (Fr. I) and supernatant (Fr. IV) decreased slightly, while that of the microsomes

increased slightly. In contrast, the radioactivity in the AASP of Fraction II increased 5-fold during the same period. These data suggest that microsomal protein, labeled during 15 min of incubation with leucine-H^3, was transferred to the mitochondria + secretion granule fraction.

TABLE I

A Comparison of Radioactivity Incorporated into Subcellular Fractions of Goosefish Islet Tissue Following in Vivo and in Vitro Administration of Leucine-H^3

Fraction	Radioactivity recovered	
	In vivo	In vitro
	%	%
Nuclei + debris	18.6	18.6
Mitochondria + secr. granules	25.2	26.7
Microsomes	46.7	43.5
Supernatant	9.5	11.2

In the in vivo experiment, 1 ml of Krebs-Ringer bicarbonate medium containing DL-leucine-H^3 (200 μmoles/l; 5.4 c/mmole) was injected into the fish 90 min prior to isolation of the islet tissue. In the in vitro experiment, the islet tissue was incubated for 120 min in Krebs-Ringer bicarbonate medium containing DL-leucine-H^3 (200 μmoles/l; 5.4 c/mmole). Both tissue samples were processed simultaneously.

Incorporation Studies Involving Mitochondrial and Secretion Granule Subfractions

In order to test this hypothesis, we performed more extensive incorporation studies (covering 15-, 30-, 60-, and 120-min periods), involving the separation of Fraction II into secretion granule and mitochondrial subfractions.

A. *Acid Alcohol–Soluble Protein (AASP)*

When islet tissue was incubated with leucine-H^3 for intervals up to 120 min, the specific activities of AASP of all fractions increased linearly (Fig. 3 *A*). The microsome AASP had the highest activity at any time, while the secretion granule AASP had the lowest. If the 15-min and 120-min values are compared, however, the secretion granule fraction showed the greatest increase in specific activity of AASP (cf. Table II).

A pulse-chase incubation was carried out in the same series of experiments. Under these conditions, the radioactivity incorporated into AASP of Fractions I, III, and IV was markedly diminished with respect to the corresponding 120-min values; the AASP of secretion granule and mitochondrial subfractions were less affected (compare Figs. 3 *A* and 4 *A*).

These data are summarized in Table II. The ratios of activities of the various fractions indicate

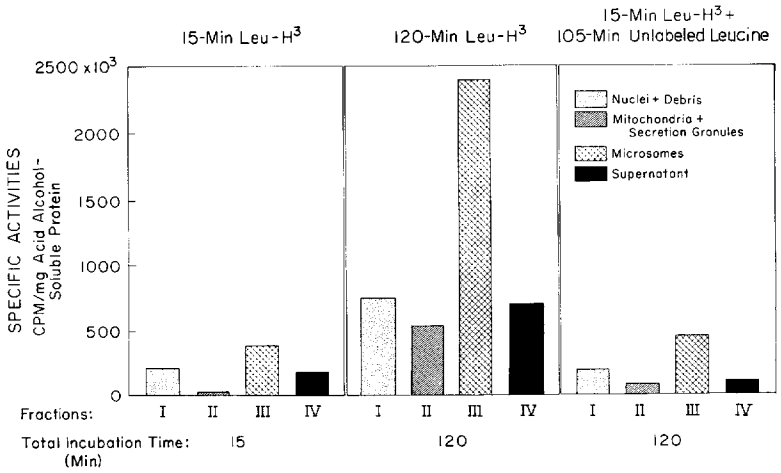

FIGURE 2 Distribution of radioactivity incorporated into AASP from subcellular fractions following in vitro incubation of goosefish islet tissue with DL-leucine-4,5,-H^3 (200 μmoles/l; 5.4 c/mmole). In the pulse-chase experiment, tissue was incubated with leucine-H^3 for 15 min; the medium then was replaced with medium containing unlabeled leucine, and the tissue incubated for an additional 105 min.

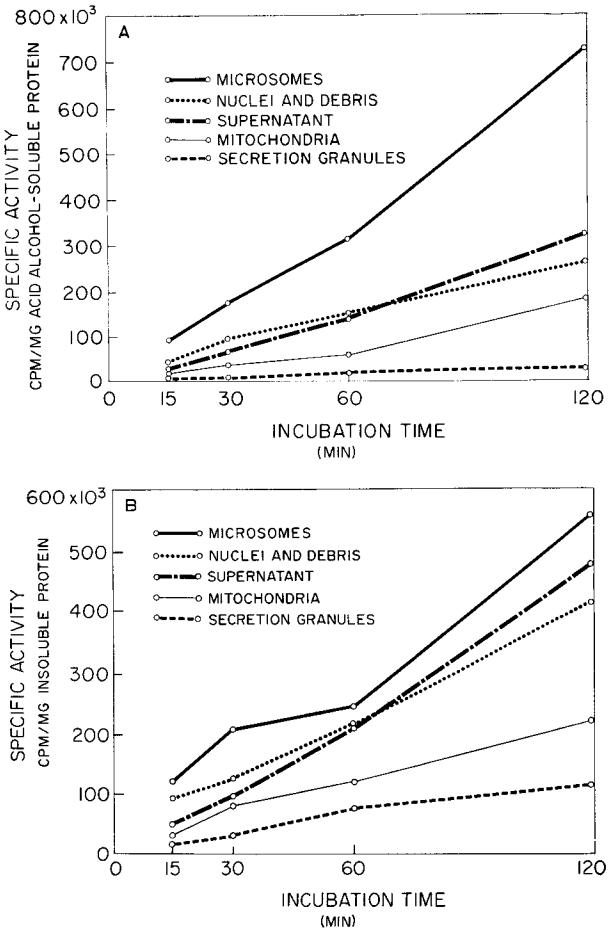

FIGURE 3 Distribution of radioactivity incorporated into various components of islet subcellular fractions following incubation with DL-leucine-4,5-H^3 (80 μmoles/l; 5.4 c/mmole). *A*, Specific activities of acid alcohol-soluble proteins (AASP). *B*, Specific activities of insoluble proteins (IP). For further details of the experiment, see text.

that the secretion granule AASP was least affected by the concentration of free leucine-H^3 in the incubation medium, which suggests that it was more directly dependent upon receipt of labeled proteins from other cell fractions.

B. *Insoluble Protein (IP)*

The pattern of leucine-H^3 incorporation into insoluble protein differed in some respects from that seen in the AASP extracts. Again, the activity of microsomal protein was highest at any time, while that of the secretion granules was lowest. The activity of IP of each cell fraction increased during 120 min of incubation (Fig. 3 *B*). However, when the 15-min and pulse-chase experiments are compared, we find decreases in the activities of microsomal and nuclear insoluble proteins, but continued increases in the activities of IP of other fractions (Fig. 4 *B*). The dissimilarity in the chasing of radioactivity from the AASP and IP of the microsomes is also noteworthy (compare *A* and *B* in Fig. 4).

Evidence for the Microsomal Site of Insulin Synthesis

In order to isolate insulin, the acid alcohol–soluble proteins of microsome and secretion granule fractions were separated by acrylamide gel electrophoresis (Fig. 5, *E* and *F*). Identification of the proteins which migrated into the separatory gel was done by comparison with insulin and glucagon standards. In Fig. 5, *A* and *D* are electrophoretograms of pure bovine insulin (i.e., 25 units of biological activity/mg) and porcine glucagon. The heterogeneity of bovine insulin may be the result of polymerization or partial denaturation during its isolation (*see* references 5, 7). The glucagon

TABLE II

Changes in Distribution of Radioactivity in Acid Alcohol-Soluble Proteins Following in Vitro Incubation with Leucine-H^3

Fraction	Specific activities (CPM/μg AASP)			Ratios of specific activities		
	A	B	C	C/A	B/A	C/B
I	44.8	49.6	249	5.6	1.1	5.0
Secretion granule	1.80	9.18	26.5	15	5.7	2.9
Mitochondria	13.3	52.9	177	13	4.0	3.4
III	98.5	166	716	7.3	1.7	4.3
IV	32.2	61.0	311	9.7	1.9	5.1

A, 15 min of incubation with leucine-H^3. B, Pulse-chase (15-min incubation with leucine-H^3 followed by 105-min incubation with unlabeled leucine). C, 120-min incubation with leucine-H^3. DL-leucine-4,5-H^3 (80 μmoles/l; 5.4 c/mmole) was used.

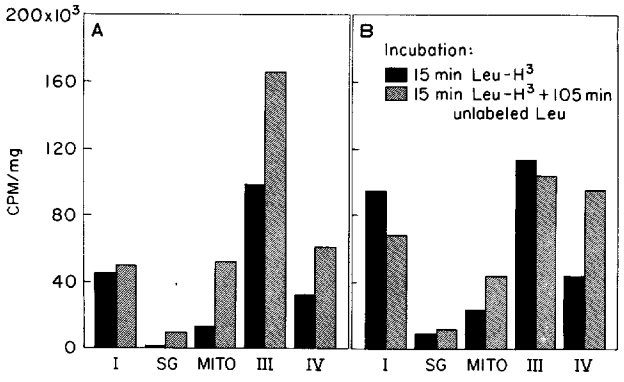

FIGURE 4 Distribution of radioactivity incorporated into various components of islet subcellular fractions following pulse-chase incubation in vitro. DL-leucine-4,5,-H^3 (80 μmoles/l; 5.4 c/mmole) was used. A, Acid alcohol-soluble protein. B, Insoluble protein.

sample was reported to contain a trace of insulin, which may account for the presence of protein bands *2* and *4* in Fig. 5 *D*. Fig. 5 *C* shows an electrophoretogram of goosefish insulin, purified as described previously (4, 12). Fig. 5 *B* illustrates that impure preparations of insulin from other teleost fish exhibit protein bands that differ from those of bovine insulin, and that impurities are readily apparent upon electrophoresis.

On the basis of these comparisons, protein bands *2*, *3*, and *4* were considered to represent insulin, and band *1* to be glucagon (Fig. 5, *E* and *F*). The absence of other proteins from electrophoretograms of the microsomes and secretion granules indicates that these nonhormonal proteins were excluded from the 7½% polyacrylamide gel. Electrophoresis of aqueous extracts of secretion granules (15) and microsomes (unpublished data) show much more complex protein patterns.

As shown in Table III, the radioactivity of protein bands *2*, *3*, and *4* (which had similar insulinlike activities) was 62 times higher in the microsomes than in the secretion granules. The microsomal "glucagon" band had a specific activity (CPM/mg protein) 24 times higher than the corresponding band in the secretion granules. These data suggest that the incorporation of leucine-H^3 into insulin and glucagon, as well as into total AASP, occurs primarily in the microsome fraction.

DISCUSSION

The present study represents a biochemical determination of the sites of protein synthesis and storage in islet tissue. Separation of the tissue into its subcellular fractions permitted us to follow the distribution of radioactivity incorporated into protein under various conditions of incubation.

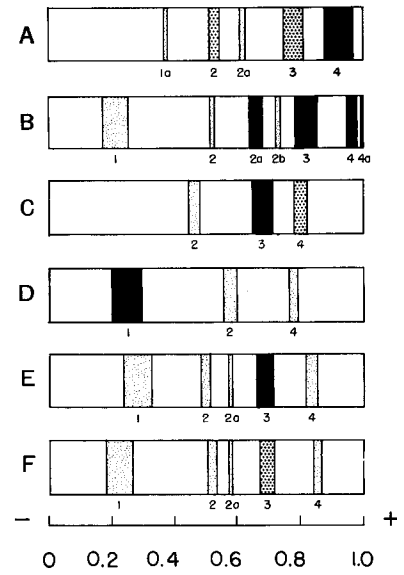

FIGURE 5 Diagrammatic representation of acrylamide gels following electrophoresis of protein standards (*A* to *D*), AASP of secretion granules (*E*) and microsomes (*F*). Only proteins which migrated into the lower gels are illustrated. The shadings (solid, heavy stipple, light stipple) roughly mimic the densities of protein bands observed following staining with amido black dye. The scale indicates the migration of stained protein bands relative to the tracking dye front (=1.0). A, Beef insulin (25 U/mg). B, Bonito-tuna insulin (16 U/mg). C, Goosefish insulin (>21 U/mg). D, Pork glucagon. E, Granule Fr. AASP (120-min incubation). F, Microsome Fr. AASP (120-min incubation).

Two groups of proteins were isolated from the subcellular fractions on the basis of their differing solubilities. One group (the acid alcohol–soluble protein) contains insulin and glucagon, which are destined for storage and, ultimately, secretion from the islet cells. In these experiments, we presented evidence for the microsomal synthesis of acid alcohol–soluble proteins and their subsequent transfer to other cell fractions. Since the specific activity of insulin isolated from the microsomes was many times higher than that from the secretion granules, we may infer that the microsomes are also the primary site of insulin synthesis.

The second group of proteins (insoluble protein) corresponds to the standard protein fraction which has been isolated from tissues (i.e., it is insoluble in 5% TCA, and alcohol, and remains insoluble during extraction of nucleic acids). On the basis

TABLE III

Specific Activities of Insulin Bands from Microsomal and Secretion Granule Fractions Isolated by Acrylamide Gel Electrophoresis

Specific activity		Ratio
(CPM/U insulin)		Microsomes/ Secretion granules
Secretion granules	Microsomes	
1,410	88,000	62.3

The subcellular fractions were isolated from islet tissue after 2 hr of in vitro incubation in medium containing DL-leucine-4,5-H^3 (80 μmoles/l, 5.4 c/mmole). One unit (U) of insulinlike biological activity is equivalent to 0.04 mg of protein.

of the incorporation data presented above (Fig. 3 and 4), the microsome fraction appears to be the major site of synthesis of the insoluble protein of islet tissue. This protein also appears to be transferred, in part, to the secretion granule fraction. On the basis of electron microscope observations, Williamson et al. (26) presented evidence that the secretion granule of the B cell is elaborated within the rough-surfaced endoplasmic reticulum. Our data suggest that the secretion granules of islet tissue are composed not only of insulin and glucagon, but of other proteins as well.

The specific activity of labeled insulin from whole islet tissue was not determined in these experiments. However, calculated on the basis of insulin isolated from the microsome and secretion granule fractions (which contain over 85% of the total insulin of the cells), we have estimated this to be approximately 200,000 CPM/mg insulin. A previously reported value for tritium-labeled insulin, isolated by paper chromatography from tissue incubated for 2 hr in vitro, was 418,000 CPM/mg protein (4). Allowing for differences in amino acid concentration in the two experiments (80 μmoles/l vs. 240 μmoles/l), and counting efficiencies (10% vs. 8%), these values agree reasonably well.

An active incorporation of radioactivity into the proteins of the mitochondria was observed in these studies. Whether or not this represents independent protein synthesis by these organelles (*see*. 8, 21, 6) must be clarified by use of a cell-free incorporation system.

The presence of glucagon in goosefish islets has been reported by others (1). Although our data on the incorporation of leucine-H^3 into glucagon are preliminary, they indicate that a detailed investigation of glucagon synthesis in goosefish islet tissue would be feasible.

Separation of the subcellular sites of islet hormone synthesis and storage will facilitate studies on the factors responsible for the control of these processes.

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