BETA GRANULE FORMATION IN ISOLATED ISLETS OF LANGERHANS

A Study By Electron Microscopic Radioautography

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ABSTRACT

The distribution of radioautographic grains over organelles within the beta cells of rat islets of Langerhans was investigated at various times after pulse labeling of the isolated islets with tritium-labeled amino acids. Ten minutes after the start of labeling most of the grains were situated over the endoplasmic reticulum and cytoplasm; by contrast, 60 min from the start of labeling the majority of the grains were associated with the beta granules. At 20, 30, and 45 minutes after pulse labeling the proportion of grains associated with the Golgi complex was increased two- to three-fold over the 10- or 60-minute values. The distribution of radioautographic grains over granules in the intact cells did not suggest that the electron-lucent type of secretory granules were precursors of the electron-opaque granules. Furthermore, studies of the pattern of grains over granules isolated by centrifugation 60 min after pulse labeling showed no preferential labeling of the electron-lucent type of granule. It is concluded that labeled amino acids are incorporated initially in the endoplasmic reticulum, and that the label subsequently appears in the beta granules. The Golgi complex participates either in the formation of the beta granule or in the translocation of the granule through the cytoplasm of the cell.

INTRODUCTION

Previous ultrastructural investigations have suggested that beta granule formation in the islets of Langerhans may occur without the participation of the Golgi complex (Lacy, 1961), although evidence of this type has been conflicting (Ferreira, 1957; Falkmer and Olsson, 1962; Logothetopoulos, 1968). Definitive studies of this process can best be attempted by electron microscopic radioautography of islet tissue maintained in vitro for varying periods following pulse labeling. The availability of a technique for the isolation of mammalian islets in large numbers (Lacy and Kostianovsky, 1967) has made possible a study of this type.

In these experiments islets were isolated from rat pancreas and incubated for 5 min with tritiated amino acids. They were then incubated in chase[1] medium for periods of 10, 20, 30, 45, or 60 min from the start of labeling, prior to fixation of the tissue or separation and fixation of the subcellular components. Quantitative studies

[1] Incubation medium containing excess unlabeled amino acids.

of the distribution of radioactivity in various organelles was undertaken at each time by means of electron microscopic radioautography.

METHODS

Isolation of Islets of Langerhans

Islets were isolated from 500–700 g male Wistar rats after incubation of the pancreas with collagenase; this procedure has been described in detail elsewhere (Lacy and Kostianovsky, 1967).

Incubation Procedure

Groups of 60 isolated islets were transferred to vials containing 1 ml of a bicarbonate-buffered saline medium (Renold et al., 1960) which was previously gassed to pH 7.4 with a 95% oxygen:5% carbon dioxide mixture and which contained 2.5 mg/ml glucose and 2.5% (v/v) normal rat serum. Each vial was incubated at 37°C for 15 min before the injection of L-leucine-4,5-^{3}H (specific activity 5.0 c/mmole; New England Nuclear Corp., Boston, Mass.) and L-tyrosine-3,5-^{3}H (specific activity 26.5 c/mmole; New England Nuclear Corp.) to give a final concentration of each isotope of 100 μc/ml (20 μM leucine; 5.3 μM tyrosine). After a further 5 min incubation, the vials were unsealed and the radioactive medium was removed; the tissue remaining was washed rapidly three times with previously warmed and gassed incubation medium which contained glucose and serum as before and, in addition, unlabeled L-leucine (2 mM) and L-tyrosine (2 mM). After decantation of these chase media, 2 ml of medium containing 2.5 mg/ml glucose, 2.5% rat serum and 0.2 mM L-leucine and L-tyrosine was added and incubation was continued for 10, 20, 30, 45, or 60 min after the start of labeling. The vials were gassed continuously with 95% O_2:5% CO_2 throughout this incubation period.

Subcellular Fractionation of Islets

In some experiments the islets were fractionated after 10, 30, or 60 min incubation into nuclear, mitochondrial, secretory granule, and microsomal fractions by the use of differential and density-gradient centrifugation. This procedure has been described in detail elsewhere (Howell, Fink, and Lacy, 1969).

Fixation of Tissue

At the appropriate times 7–10 islets were removed from the incubation vials by the use of a Pasteur pipette and placed immediately in 5% glutaraldehyde solution in 0.15 M phosphate buffer, pH 7.4, for 30 min. Postfixation was performed in 2% OsO_4 in the same buffer for 2 hr. After dehydration, islets were embedded singly in an epoxy resin.

Pellets obtained by fractionation of whole islets were fixed in a similar way, but were embedded in situ in the centrifuge tubes in which they were prepared.

Radioautographic Procedure

The technique employed for electron microscopic radioautography of the tissues was similar to that described by Lever, Spriggs, and Graham (1968). Ilford L4 emulsion and Kodak D19b developer were used.

Microscope slides (3″ × 1″) were cleaned in chromic acid, rinsed in distilled water, and dried before coating with a 1.4% solution of collodion in amyl acetate and drying vertically at room temperature. Ribbons of sections (silver-grey) were then collected on copper specimen grids and transferred to drops of deionized water placed on the surface of the collodion-coated slides. The sections were floated off the grids and onto the droplets of water, four such ribbons being placed on each slide. The water was evaporated at 37°C and the sections remained firmly attached to the slide. Subsequent location of each ribbon of sections was facilitated by scoring the lower surface of the slides to indicate their approximate positions.

The sections on the slides were stained with lead citrate (Reynolds, 1963) for 16 min. Excess lead was removed by washing with 0.02 N NaOH and deionized water. After drying, the section-bearing surface of each of the slides was coated with a thin layer of carbon in a carbon evaporator. Ilford L4 emulsion (Ilford Ltd., Essex, England) was applied to the sections by dipping the slides in a bath of emulsion at 34°C and withdrawing them mechanically by the use of an electric motor. The emulsion had been previously diluted 1:3 with filtered deionized water, and a withdrawal speed of approximately 5 cm/min was used. Small alterations of the thickness of the emulsion layer were achieved by altering the rate of withdrawal of the slides, and it was possible to obtain reproducibly emulsion films close to a monolayer of silver grains. A similar method of application of emulsion layers was described by Kopriwa (1967). After being dried in a vertical position, the slides were placed in light-proof boxes which contained a drying agent, and were stored at 4°C for periods of up to 3 months.

Development of the emulsion was carried out for 2 min in Kodak D19b developer; the stop bath contained distilled water and fixation was carried out in 20% sodium thiosulphate for 2 min. The slides were washed for 30 sec in four successive baths of distilled water. Copper grids (200 mesh) were then located over the sections on the slides under a dissecting microscope and the collodion film, together with the sections, emulsion, and grids, was floated carefully onto the surface of a trough of deionized water. The film was then recaptured onto a second clean glass slide with the grids in direct contact with the slide. After drying, the grids were cut free from the sur-

rounding collodion film and dipped in amyl acetate for 1½ minutes to remove excess collodion. The sections and emulsion layer remained on the grids, which were ready for examination in the electron microscope.

The distribution of silver grains over organelles was determined and tabulated by two authors, one of whom had no prior knowledge of the time of sampling of the tissue, without further treatment of the sections. The assessment of the distribution was very similar in each case. While the contrast obtained was sufficient for identification of cellular components, it was not adequate for illustrative purposes. Therefore, after quantitation of the grains the grids were treated with 0.06 N sodium hydroxide for 25 min in order to remove excess gelatin, and the sections were restained with uranyl acetate (15 min) and lead citrate (15 min). Since this treatment occasionally resulted in partial loss of radioautographic grains, the restained sections were used only for illustration.

RESULTS

Distribution of Radioautographic Grains

Determination of background grains over areas of epoxy resin which did not contain tissue, indicated that the background remained at a low level throughout the 6–8 wk required for adequate exposure of the radioautographs. No evidence was obtained of binding of radioactivity by the fixa-

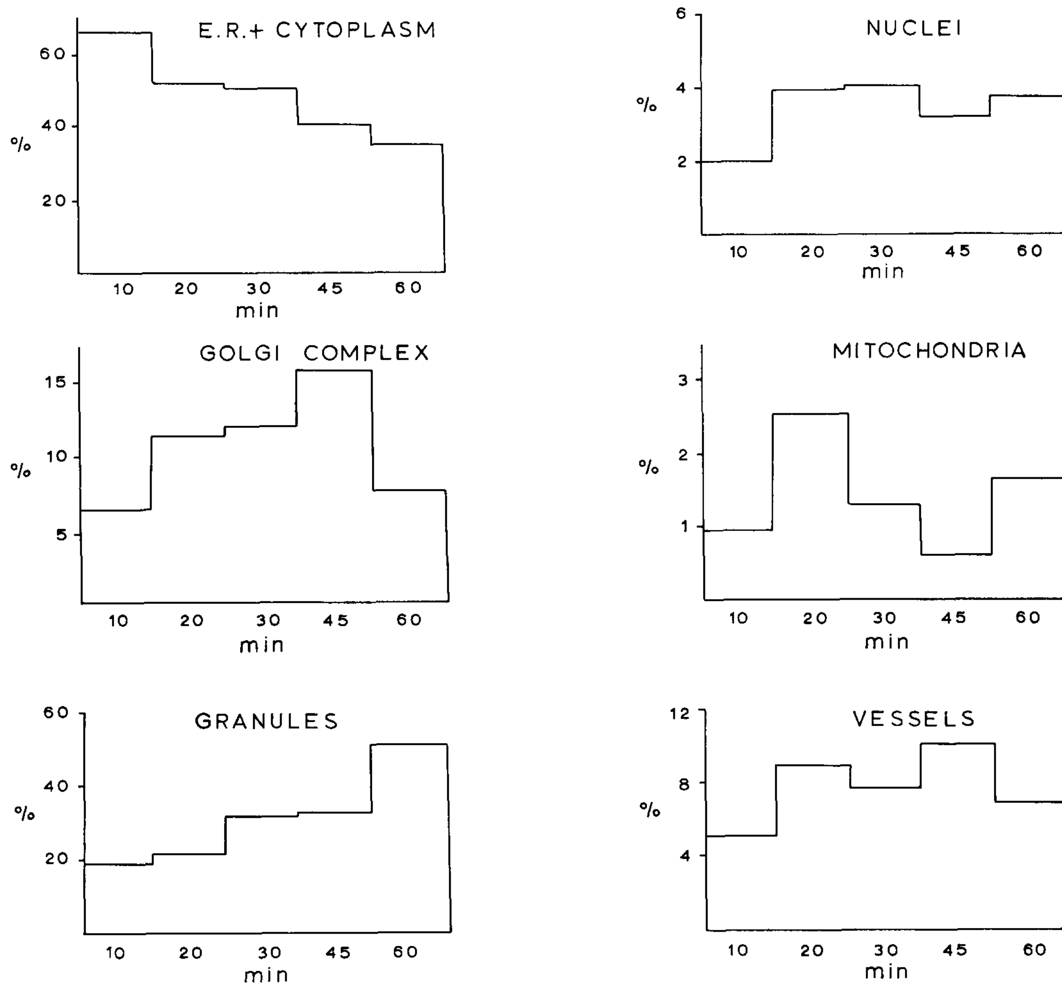
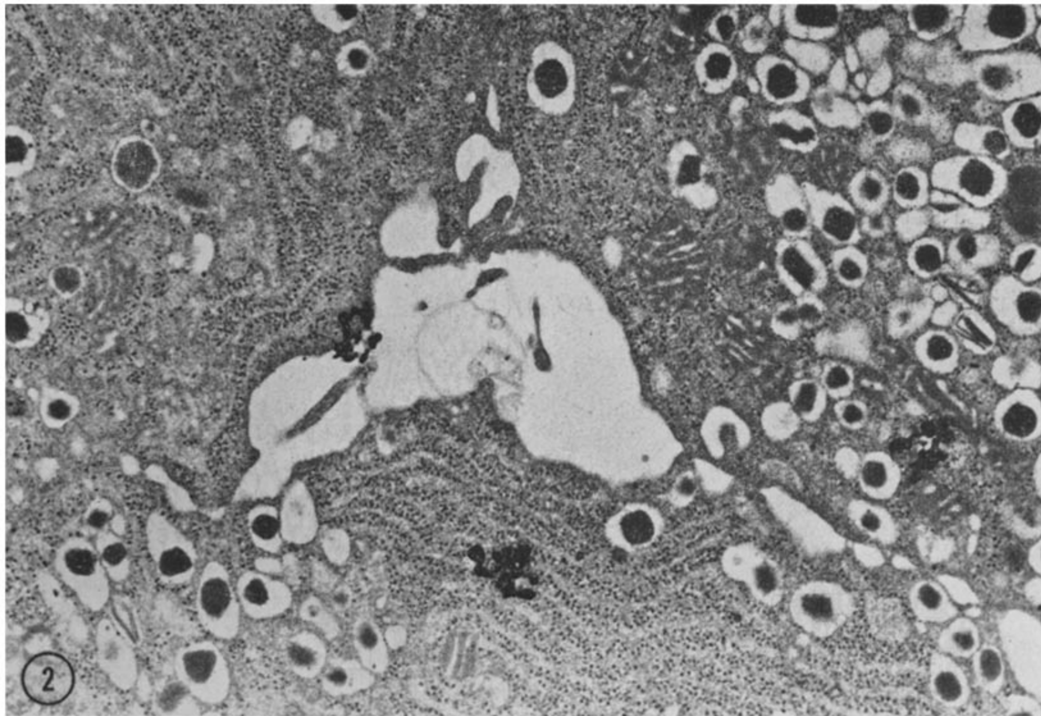


FIGURE 1 Distribution of radioautographic grains over organelles at various times after the start of a 5 min period of labelling with [^{3}H]-amino acids. Results are expressed as a percentage of the total number of grains counted at each time.



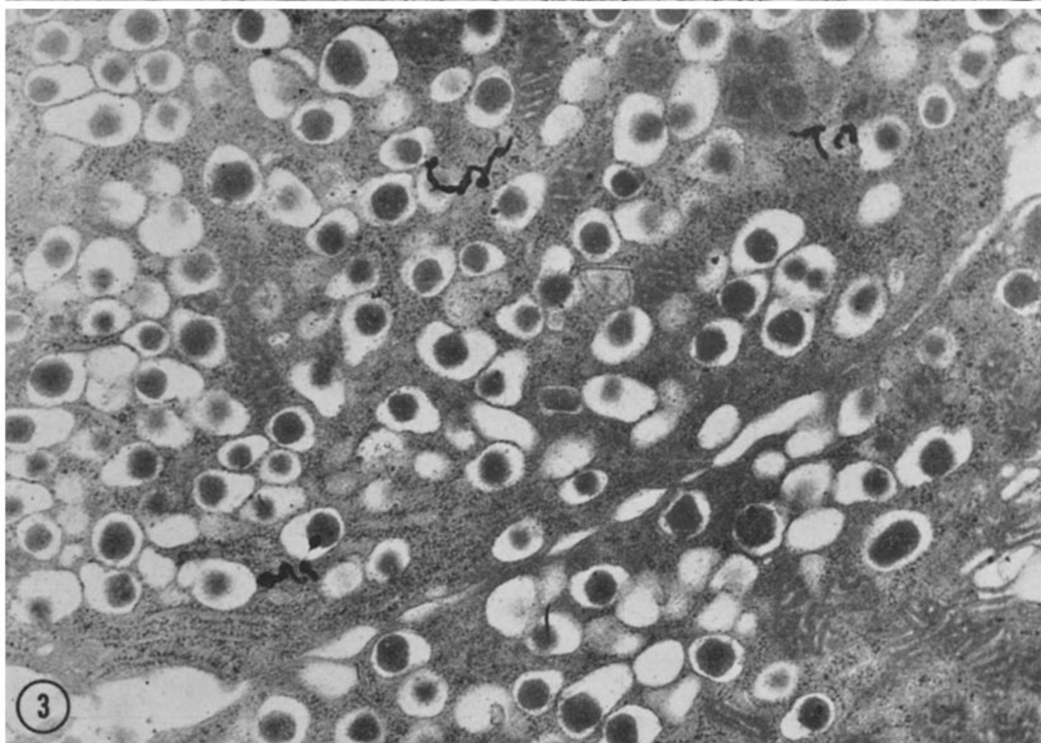


FIGURE 2 Electron microscope radioautograph of beta cell of rat islet incubated for 5 min with labeled amino acids and for 5 min in chase medium. The grains are associated predominantly with the rough surfaced endoplasmic reticulum. × 25,000.

FIGURE 3 Electron microscope radioautograph of beta cell of rat islet incubated for 5 min with labeled amino acids and for 15 min in chase medium. Grains are localized over the ground cytoplasm as well as over the rough surfaced endoplasmic reticulum. × 30,000.

tives; the number of grains over areas of tissue which would be expected to remain unlabeled (e.g. blood vessels and erythrocytes) did not exceed 5–10% of the total, despite the relatively large area of the sections occupied by these structures.

For the purposes of determining the distribution of grains, the islet was divided into six areas: a) endoplasmic reticulum and cytoplasm, b) Golgi complex, c) secretory granules, d) mitochondria, e) nuclei, and f) blood vessels and red cells. A grain was arbitrarily recorded as being associated with an organelle if >50% of the developed grain was superimposed upon it. A total number of 180–210 grains was counted at each of the five times studied; the relative distribution of the grains over the various organelles is shown in Figure 1.

ENDOPLASMIC RETICULUM AND CYTOPLASM: The proportion of grains associated with these areas fell progressively and approximately linearly from a value of 67% at 10 min to 33% at 60 min after the start of the pulse labeling. It is assumed that the radioactivity was progressively transferred to other compartments in the cell.

GOLGI COMPLEX: At the 10 min and 60 min observation times, 5–6% of the grains were associated with this organelle. However, at 20 and 30 min this proportion was 11–12% and at 45 min from the start of pulse labeling it reached a level three times that of the 10 min and 60 min values.

SECRETORY GRANULES: Grains were localized over the granules at all times studied, which amounted to 18–22% at 10 or 20 min. However, from 30 min onwards, a rise in the proportion of grains associated with the granules was seen, which increased sharply from 33 to 51% between 45 and 60 min after the start of labeling.

NUCLEI AND MITOCHONDRIA: The distribution of grains over these organelles was similar at all the times studied; the proportion of grains associated with the nuclei fluctuated between 1.9 and 3.9% whereas that associated with the mitochondria was 0–2.5%.

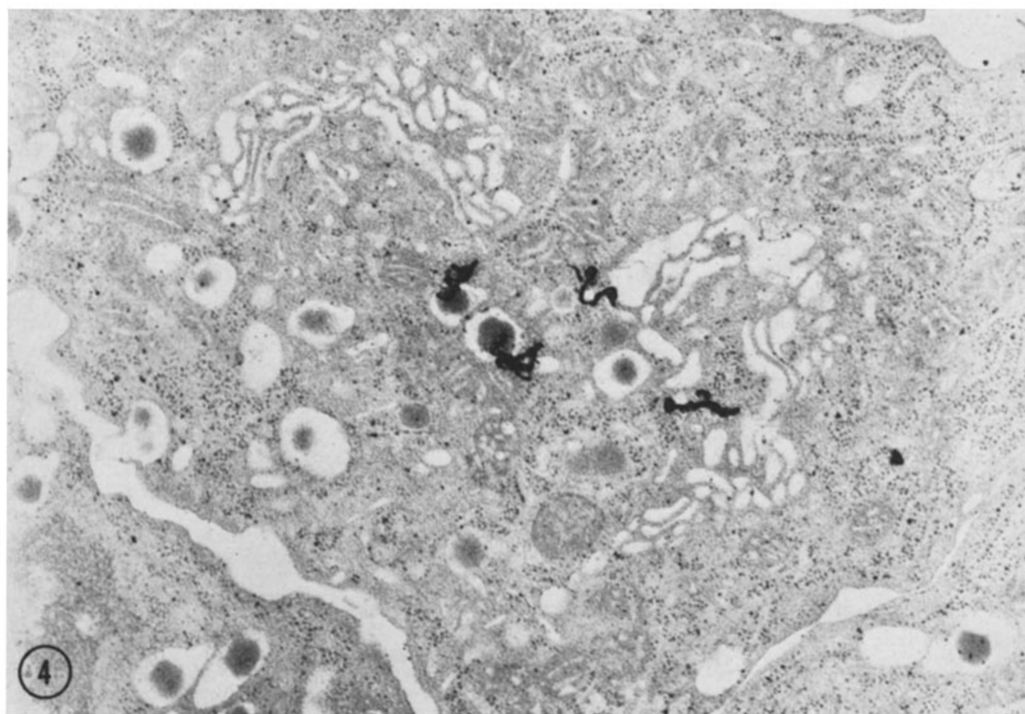


FIGURE 4 Electron microscope radioautograph of beta cell of rat islet incubated 5 min in labeled amino acids and for a further 40 min in chase medium. Grains are associated both with the Golgi complex and with typical electron-opaque secretory granules at the periphery of the complex. × 27,000.

Figures 2–6 show radioautographs of beta cells at each of the times studied. The figures were selected to illustrate the changes in localization which were demonstrable by quantitative determination of grain distribution.

Distribution of Grains over Organelles in Subcellular Fractions

MICROSOMES: Radioautography of the microsomal pellet obtained 10 and 30 min after pulse labeling of the islets revealed labeling of structures within the pellet at both times. However, the large size of the developed radioautographic grains in relation to the size of the subcellular particles made it impossible to draw firm conclusions about the localization of the radioactivity within this subcellular fraction.

SECRETORY GRANULE FRACTION: A low magnification view of the secretory granule pellet isolated 55 min after a 5 min pulse label with amino acids is shown in Fig. 7. A portion of the same pellet is shown at higher magnification in Fig. 8. It was clear that there was no preferential labeling of the "pale" electron-lucent granules, since most of the grains were associated with the electron-opaque "dense" granules. Examination of a comparable fraction isolated 30 min after pulse labeling showed only a very few grains associated with the secretory granules, and it was not possible to derive useful data from a study of pellets prepared at that time.

DISCUSSION

It is clearly shown in Figures 2–6 that islets isolated by collagenase digestion of the pancreas may maintain an entirely normal ultrastructure during a 1 hr incubation under the conditions used in these experiments. The insulin secretory responses of the isolated islets (Lacy and Kostianovsky, 1967) appear very similar to those of pancreas slices incubated in vitro (Coore and Randle, 1964) and to those of perfused pancreas preparations (Grodsky, Batts, Bennett, Vcella, McWilliams, and Smith, 1963). Furthermore, results of biochemical analyses of islets obtained from frozen sections of whole pancreas (Matschinsky, Kauffman, and Ellerman, 1968) and from collagenase-isolated islets (Montague and Taylor, 1968) show marked similarities. Therefore, it is assumed that the metabolism of the isolated islets is comparable to that of the islet cells in vivo.

The use of an in vitro preparation provides great advantages in experiments of this type, i.e., a true pulse label can be delivered to the tissue while a much higher specific activity of labeled amino acids can be made available than that which can be achieved by injection of the amino acids in vivo.

Incubation of the isolated islets in the presence of high concentrations of glucose has previously been shown to cause a substantial (8–10 fold) increase in the rate of incorporation of amino acids into insulin (Howell and Taylor, 1966) as well as a comparable increase in rates of insulin secretion (Lacy and Kostianovsky, 1967). In the experiments reported above, islets were incubated for 15 min in a medium of high glucose content (2.5 mg/ml) before pulse labeling; under these conditions rates of insulin biosynthesis should be maximal throughout the period of labeling, and the rate of incorporation of radioactivity into insulin should far exceed the rate of incorporation into the sedentary proteins synthesized by the cell. Therefore, it has been assumed that the grains observed in the radioautographs represent, predominantly, radioactivity incorporated into exportable protein. Secretion of labeled insulin into the incubation medium occurs about 1 hr after pulse labeling with [^{3}H]-amino acids of isolated islets or pancreas slices maintained in vitro (Howell and Taylor, 1967; Steiner, 1967). Thus, the total radioactivity present in the beta cell should remain constant during this 1 hr interval which was the period of incubation utilized in this study.

The high proportion of silver grains found over the rough surfaced endoplasmic reticulum at the earliest times after labeling indicates that the rough endoplasmic reticulum may be the site of insulin synthesis, as suggested by Lacy (1961) from in vivo ultrastructural studies of the rat pancreas, and by Bauer et al. (1966) from studies of the pattern of radioactivity of microsomal fractions from goosefish islets incubated in vitro with a radioactively labeled amino acid. This newly synthesized material is apparently transferred to the Golgi complex since grain counts over this structure were elevated at the 20, 30, and 45 min intervals. The reasons for the extended interval of increased labeling over the Golgi complex is not clear from these studies. This may reflect a lack of synchrony amongst different cells, resulting from a physiological variation in activity, as well as from the relatively long 5 min

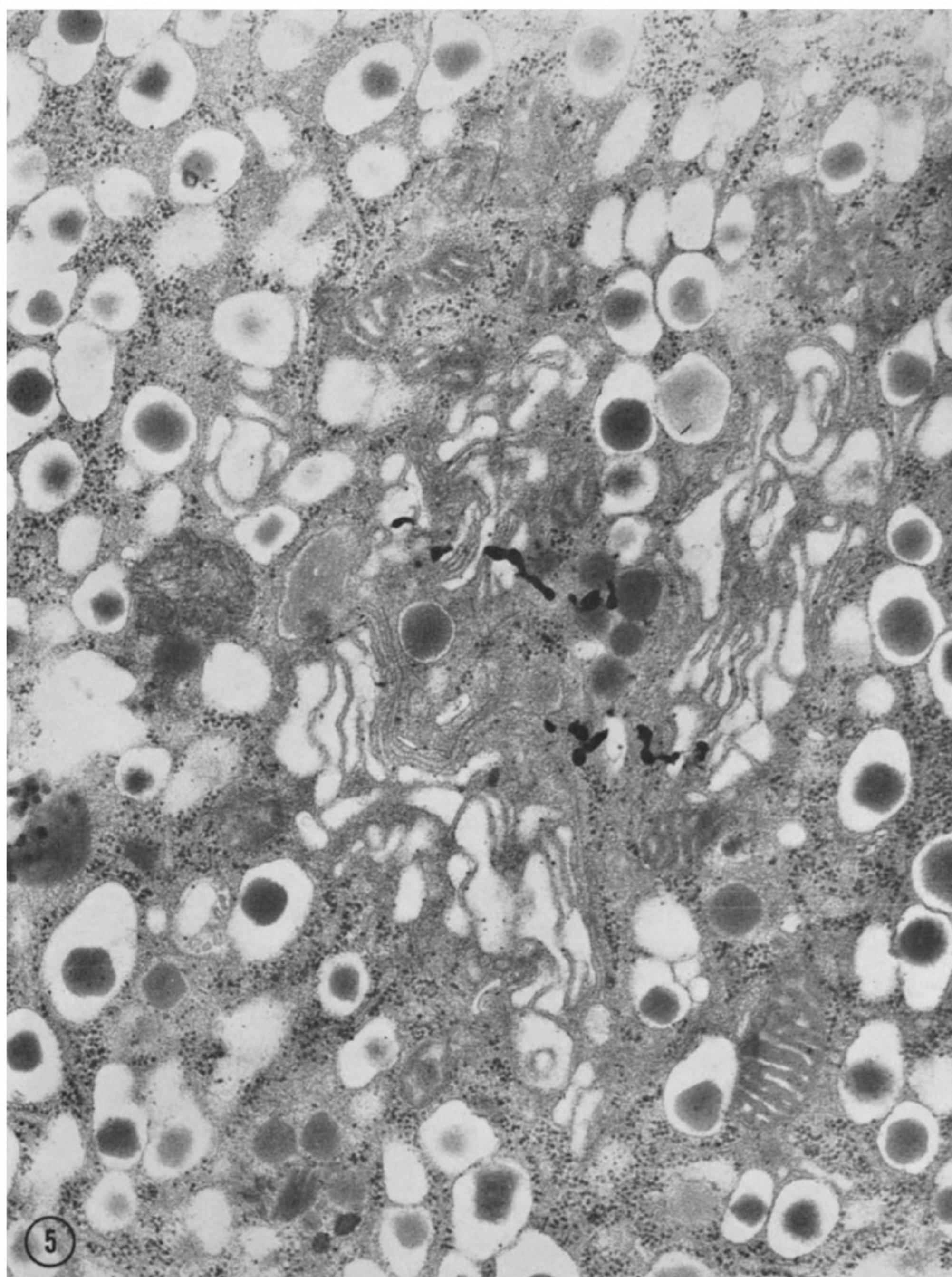
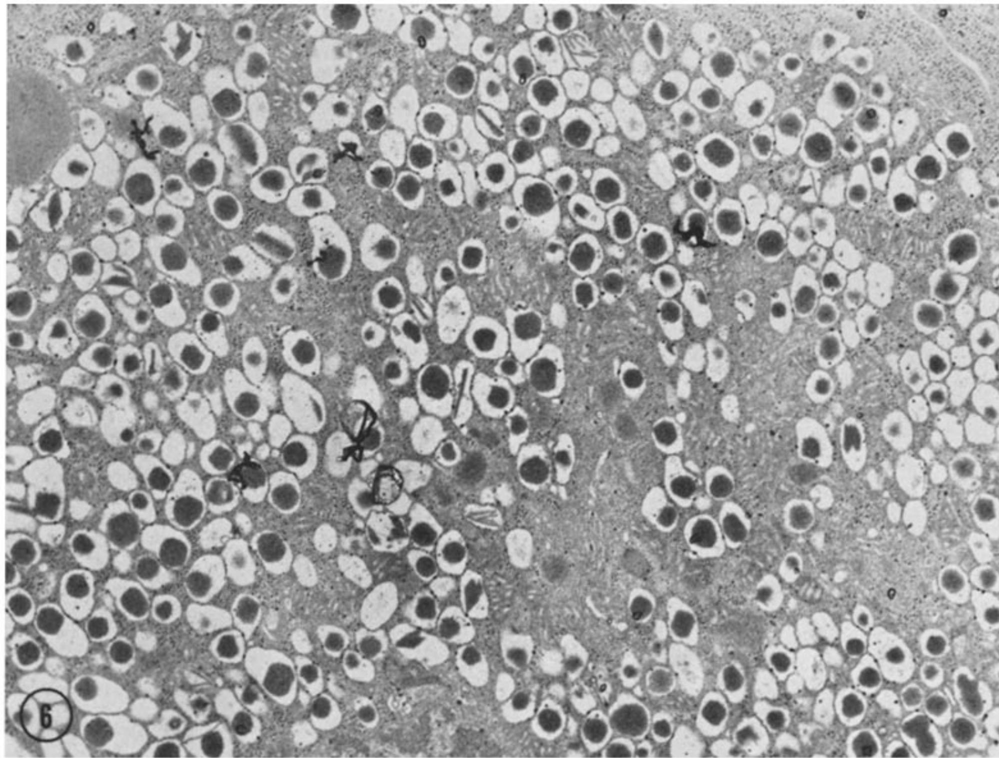


FIGURE 5 Electron microscope radioautograph of beta cell of rat islet incubated for 5 min with labeled amino acids and for a further 40 min with chase medium. Grains are localized over the lamellae of the Golgi complex and also over electron-opaque, membrane-free granules at the centre of the organelle. $\times$ 36, 000.



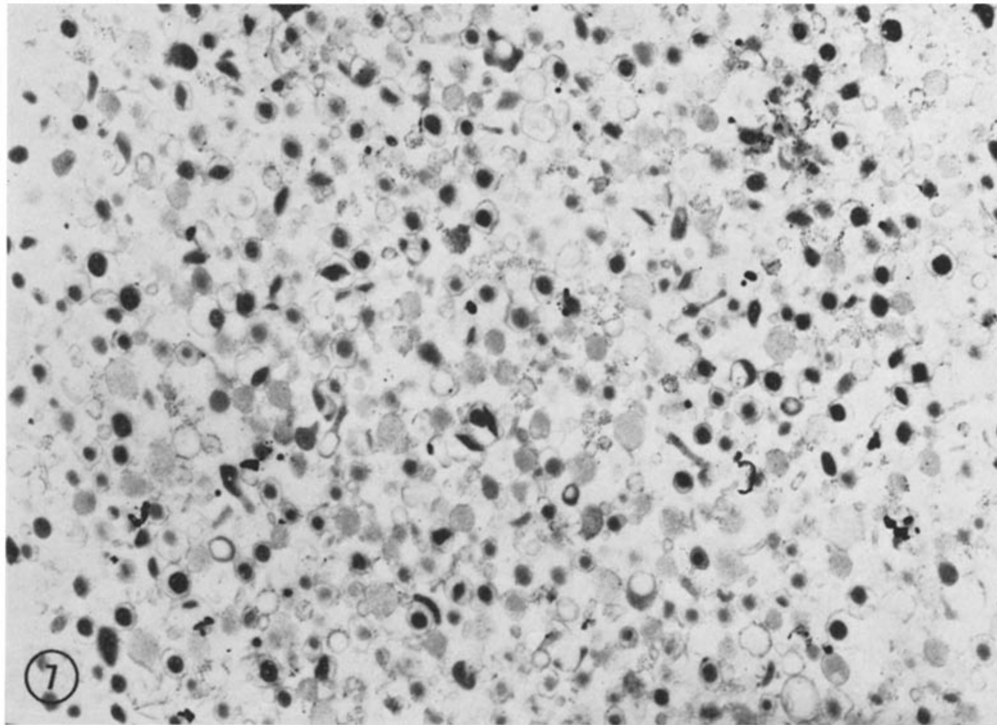


FIGURE 6 Electron microscope radioautograph of beta cell of rat islet after incubation for 5 min in labeled amino acids and for 55 min in chase medium. The grains are associated with secretory granules distributed widely in the cytoplasm. × 22,000.

FIGURE 7 Electron microscope radioautograph of secretory granule pellet prepared from rat islets after 5 min incubation with labeled amino acids and 55 min incubation in chase medium. A low magnification field showing the composition and degree of labeling of the pellet. × 13,000.

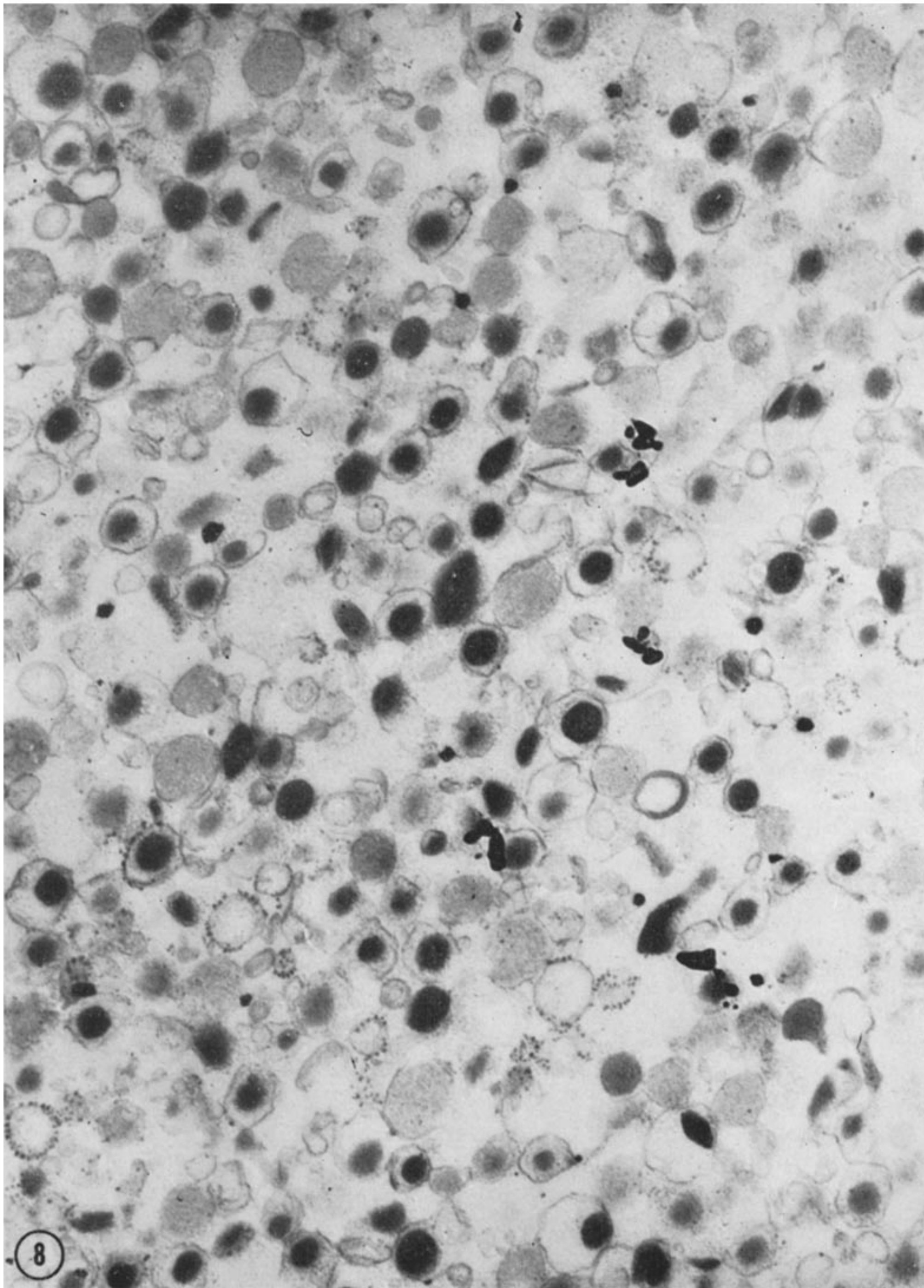


FIGURE 8 High magnification view of the pellet shown in Fig. 8. Radioautographic grains are associated predominantly with the electron-opaque type of granule. No label appears to be localized over occasional contaminating rough microsomes. × 30,000.

"pulse" to which they are subjected. Significant labeling of beta granules was present prior to the increased labeling over the Golgi complex. It does not seem likely that this early labeling of beta granules is due to the binding or absorption of newly synthesized insulin to the granule since isolated beta granules fail to bind exogenous insulin in vitro (Howell, Young and Lacy, 1969). Control experiments have not yet been performed to determine the time course of labeling of the granules after incubation with a labeled amino acid not present in the insulin molecule. The patterns of transfer of radioactivity demonstrated by radioautography were confirmed in parallel experiments in which the radioactivity present in isolated microsomal and granule fractions was determined at various times.

The silver grains observed over the Golgi complex were not related to any particular structure within this organelle. Grains were present over "pale" and "dense" granules as well as over cisternae and membranes of the Golgi complex. In order to investigate a possible relationship between "pale" and "dense" beta granules utilizing a larger randomized sample of the two types, radioautography was performed on secretory granules isolated from intact cells 60 min after labeling. Despite a fairly high proportion (approximately 35%) of electron-lucent "pale" granules in the pellet, the silver grains were localized predominantly over the electron-opaque granules (Figs. 7, 8). The amount of labeling in radioautographs of the granular fraction at 30 min was not sufficient to permit quantitative studies of differences in labeling of "pale" and "dense" granules. Thus, the content and the possible function of the "pale" granules and the precise role of the Golgi complex in the formation of the beta granules remain unclear.

The present radioautographic studies indicate that the labeled amino acids are incorporated initially into the endoplasmic reticulum and are transferred via the Golgi complex to beta granules. This apparently represents the intracellular pathway of insulin synthesis. Steiner, Cunningham, Spigelman, and Aten (1967) have reported that insulin is synthesized in the form of a single chain precursor, proinsulin, which is subsequently converted to the insulin molecule in the beta cell. Studies are in progress to determine the localization of proinsulin and insulin in subcellular fractions of islets after incubation in vitro in the presence of labeled amino acids.

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