ABSTRACT

William G. Masius. A MORPHOLOGICAL STUDY OF THE PREFERENTIAL RELEASE OF NEWLY SYNTHESIZED INSULIN AND THE "MARKING" EFFECT (under the direction of Ronald W. Dudek). Department of Biology, June 1987.

Recent studies have demonstrated two pools of insulin within the pancreatic beta cell. The first is the older stored pool and the second contains the newly synthesized hormone. It has been shown using radiolabeled insulin that it is the newly synthesized insulin pool that is secreted preferentially from the beta cell in response to prolonged glucose stimulation. The cellular mechanism responsible for selecting the newly synthesized insulin for preferential release is termed "marking". This is a regulated process that occurs during the transport of the newly synthesized hormone and its precursor through the Golgi apparatus.

Rat islets are isolated, cultured, and placed in groups of 25 to be "marked" with either 100nM phorbol ester (TPA), 25mM alpha ketoisocaproic acid (KIC), 20mM glucose, or they are left unmarked in 2mM glucose for 75 minutes. Islets at this point are either fixed, using quick freezing, and prepared for electronmicroscopy or both "marked" and unmarked groups are subsequently stimulated with 20mM glucose for 20 minutes. After the additional glucose stimulation, the islets undergo quick freeze fixation and are prepared for electronmicroscopy. The media from "marking" and subsequent stimulation is saved and

insulin radioimmunoassay is performed. Insulin values from the "marked" islets showed a large elevation of those that were from unmarked islets even after subsequent stimulation with high glucose. Morphologically, two observations of the "marked" beta cell could possibly explain the preferential release of newly synthesized insulin. First, endoplasmic reticulum is observed approaching and possibly binding to the beta cell membrane. The endoplasmic reticulum might play a role in an alternate pathway for the release of newly synthesized insulin. The second observation is the presence of two granule populations within the beta cell, but which granule population contains the newly synthesized is uncertain. The preferential release of the newly synthesized insulin could be through either of these two routes, or by a combination of the two, or by some other mechanism.

# A Morphological Study on the Preferential Release of Newly Synthesized Insulin from Rat Islets and the "Marking" Effect.

A Thesis Presented to the Faculty
of the Department of Biology
East Carolina University

In Partial Fulfillment of
the Requirements for the Degree
of Masters of Science in Biology

by
William G. Masius
July 20, 1987

# A MORPHOLOGICAL STUDY ON THE PREFERENTIAL RELEASE OF NEWLY SYNTHESIZED INSULIN FROM RAT ISLETS AND THE "MARKING" EFFECT.

by

William G. Masius

APPROVED BY:

SUPERVISORS OF THESIS

Ronald W. Dudek, Ph.D.

Gerhard W. Kalmus, Ph.D.

COMMITTEE MEMBERS

Everett C. Simpson, Ph.D.

Charles E. Bland, Ph.D.

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

Charles E. Bland, Ph.D.

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette, Ph.D.

ACKNOWLEDGEMENTS

I would like to thank Dr. Ronald Dudek for directing my thesis and serving as a co-chairman of my thesis committee. Without his time, interest, and resources this project would not have been possible. I would like to thank Dr. Gehard Kalmus for serving as a co-chairman of my thesis committee and for taking interest in my graduate work. I would also like to thank him for his guidance while I was an undergraduate and graduate student. I would like to thank Dr. Everett Simpson and Dr. Charles Bland for taking the time to serve as members of my thesis committee. Finally, I would like to thank Mr. Phil Scates, Ms. Meg Stover, and Mr. Dan Whitehead for their technical assistance on this project.

TABLE OF CONTENTS

## INTRODUCTION

The endocrine portion of the pancreas is composed of the islets of Langerhans. The islets are made up of several cell types: the alpha cell, the beta cell, the delta cell, and the F cell. The alpha cell is located on the periphery of the islets and accounts for 20% of the total cell population. It is also responsible for the synthesis and secretion of glucagon. The insulin secreting beta cells make up 75% of the islets and are located in the central portion of the islets. The delta cells which comprise less than 5% of the islets, are found scattered throughout the islets, and produce and secrete the hormone, somatostatin. Small numbers of pancreatic polypeptide secreting F cells are also distributed between the more prominent islet cells.

The synthesis of preproinsulin is the initial event in the life of insulin (Orci, 1985). Preproinsulin is synthesized on the rough endoplasmic reticulum and transported across the organelle membrane into the cisternae. The signal peptide is cleaved from the preproinsulin molecule as it enters the cisternae of the rough endoplasmic reticulum leaving proinsulin. The proinsulin molecule consists of a single polypeptide chain of 81 amino acids. The polypeptide is divided into three segments: 1) the A chain which is located at the COOH terminus; 2) the B chain which is located at the $NH_2$ terminus; and 3) the C-peptide which connects the two chains (Permutt, 1981). The endoplasmic reticulum is the first of three membrane compartments involved with the secretion of insulin. The proinsulin travels through the endoplasmic reticulum until it is transported to the Golgi appartus via transfer vessicles. Using

tritiated leucine, pulse chase experiments have shown that it takes approximately 20 to 35 minutes for immunoreactivity to appear in the Golgi apparatus (Gold and Grodsky, 1984).

The Golgi apparatus is the second membrane compartment involved in the secretory process and functions in the packaging and sorting of newly synthesized insulin and its precursor proinsulin into secretory granules (Orci, 1982). Fletcher and coworkers (1982) have demonstrated that the conversion of proinsulin to insulin begins in the endoplasmic reticulum. Other studies have shown that the proteolytic conversion of proinsulin to insulin begins within the membranes of the Golgi apparatus (Steiner et al., 1974). The Golgi apparatus receives the newly synthesized protein on the cis surface from the transfer vessicles of the rough endoplasmic reticulum. The newly synthesized insulin will then pass through the Golgi stacks until it reaches the dilated cisternae of the trans surface of the Golgi apparatus (Orci et al., 1984). The dilated ends of the Golgi stacks have morphologically been shown to produce the secretory granules in the beta cell (Orci, 1985).

The secretory granules are the third and final membrane compartment involved in the storage and secretion of insulin. A majority of conversion of proinsulin into insulin occurs within the secretory granules. The conversion of proinsulin to insulin involves the cleavage of C-peptide from the A and B chains by a trypsin like enzyme (Permutt, 1981). After the removal of the C-peptide, the two chains are connected by two inter chain disulfide bridges and the C-peptide remains in the granules. In the pulse chase experiments, radiolabeled insulin was first observed within secretory granules approximately 60 minutes after the tritiated leucine

was added to the islets. These results demonstrate that insulin secretion from the beta cell follow first order kinetics with a half life of 60 minutes (Steiner et al., 1974).

The pancreatic beta cell contains two pools of its secretory product. An older, mature pool of insulin is located within the secretory granules of the cell. The newly synthesized pool which consists of freshly converted insulin and its precursor proinsulin has yet to be fully elucidated within the cell (Gold and Grodsky, 1984). There have been several speculations on the morphologic location of newly synthesized insulin in the beta cell. Several proposals suggests that there is a separate granule population (Orci et al.,1984). Orci and coworkers (1984) have reported the presence of a coated granule population within the beta cell. The endoplasmic reticulum has also been proposed as a pool of newly synthesized insulin which may act as an accelerated pathway for its preferential release from the beta cell (Dudek et al., 1983). Most of the data at the present would favor a subpopulation or a separate population of secretory granules in the beta cell.

A recent morphological study indicates the presence of two secretory granule populations (Orci, 1985). First, the older and more established granule population consists of the mature secretory granules that contain a dense core surrounded by a clear halo filled with filamentous material. Second, the other granule population is very similar morphologically to the first with the exception of a coating on the membrane of this new granule population. This coat has been shown to consist of clathrin using the protein A-gold immunocytochemistry technique. The clathrin coating has

also been located on the terminal ends of the Golgi apparatus. It has been proposed that the coated granules are the site of conversion of prohormone into its mature form and any subsequent storage takes place in the mature, non-coated granule population (Orci, 1985).

Using quick freeze fixation, Dudek and Boyne (1986) have also morphologically described two granule populations existing within the beta cell. The first granule population, which is the same as the first population described above, contains a dense core surrounded by an translucent halo containing filamentous material. Protein-A gold immunocytochemistry showed insulin immunoreactivity present within the core and filamentous material (Dudek et al., 1984). The second granule population described by Dudek and Boyne (1986) was completely electron dense with the halo and filamentous material absent in this granule population.

The importance of two pools of insulin is that one of them is secreted preferentially. Surprisingly, recent studies have shown that newly synthesized insulin is preferentially secreted from the pancreatic beta cell upon glucose stimulation (Halban, 1982). This has been demonstrated by using pulse chase experiments to trace secretory material through the secretory pathway of the beta cell. Approximately 60 minutes after tritiated leucine was introduced to the islets under stimulatory amounts of glucose, radiolabeled hormone was being secreted from the cell with a higher specific activity than the unlabeled insulin. These results indicate that newly synthesized insulin is being secreted preferentially from the beta cell and it was independent of the islet size or location within the pancreas (Gold and Grodsky, 1984).

The cellular mechanism which determines whether the newly synthesized insulin is preferentially secreted or subsequently stored is termed "marking" (Gold and Grodsky, 1984b). The exact mechanism is not known at this time, but preferential release has been reported for several other proteins, which include: gonadotropin (Hoff et al., 1977), parathyroid hormone MacGregor et al., 1975), renin (Katz and Malvin, 1982), and prolactin Osamura et al., 1982). It has been determined that "marking" is a regulated process that occurs during the cellular transport of the protein to, or through the Golgi apparatus (Gold et al., 1984). "Marking" does not affect the rate of biosynthesis of insulin, but rather it is responsible for selection of the newly synthesized pool for preferential release. The period corresponding to the transport of insulin and proinsulin through the Golgi apparatus is refered to as the "marking" period and has been determined to be glucose sensitive. High levels of glucose present during the "marking" period result in the preferential secretion of newly synthesized insulin. When islets were exposed to low glucose concentrations "marking" did not occur and insulin was randomly secreted from the islets (Gold et al., 1982). Three cellular events occur during the "marking" period. The first is a lag time that corresponds to the transport of the protein from the rough endoplasmic reticulum to the Golgi apparatus . Second is the conversion of proinsulin to insulin which begins midway into the "marking" period regardless of the glucose concentration. Third is the initial secretion of the newly synthesized hormone which starts approximately the same time as conversion (Gold et al., 1982).

Glucose functions as a natural secretagogue, and also as a "marking" agent (Gold et al., 1982). All secretagogues do not function as "marking" agents. A "marking" agent must be able to "mark" the newly synthesized insulin for immediate preferential release from the beta cell. Alpha ketoisocaproic acid, which is a leucine metabolite like glucose, has been shown to be an effective "marking" agent and a secretagogue for insulin (Gold and Grodsky, 1984b). The tumor promoting phorbol ester, 12-0-tetradecanylphorbol-13-acetate (TPA), has also proven to be a powerful "marking" agent. The exact action of TPA in "marking" is unknown, but the phorbol ester is known to effect the beta cell by altering the ionic fluxes, primarily calcium efflux, within the cell (Malaisse et al., 1980). This change in ionic flux in the beta cell leads to an increase in cytosolic calcium. Cyclic AMP levels increase in the presence of TPA which is probably a result of the rise in cytosolic calcium levels (Virji et al., 1978).

The purpose of this study is to examine the morphological aspects of the "marking" process in the pancreatic beta cell. As previously mentioned, "marking" is the physiological process in the beta cell where the newly synthesized insulin is preferentially secreted. Since the cellular events of the "marking" process are unclear, experiments were designed to elucidate the morphological correlates of this physiological process (diagramed below). Briefly, the protocol consists of taking islets of Langerhans that are obtained from fresh rat pancreata and placing them in culture for seven days. After culturing, the islets are incubated in culture media containing one of three "marking" agents; 100nM phorbol ester (TPA), 25mM alpha ketoisocaproic acid (KIC), and 20mM glucose, or

the islets are left unmarked in a media containing 2mM glucose. The marking period was determined to be approximately 75 min. from pulse chase experiments performed by Gold and Grodsky (1984). After the islets are marked, they are divided into two groups. The first group of "marked" islets are immediately quick frozen so as to arrest the islets during the "marking" process. The second group of islets are washed and incubated in 20mM glucose for 20 minutes. After the second incubation, this group of islets is quick frozen so as to arrest them during their secretory phase. All of the media from the "marked", unmarked, and the second incubation conditions is collected and assayed for insulin content. The quick frozen islets are prepared for electron microscopy. The islets are examined with the electron microsope for differences between the "marked" and unmarked groups.

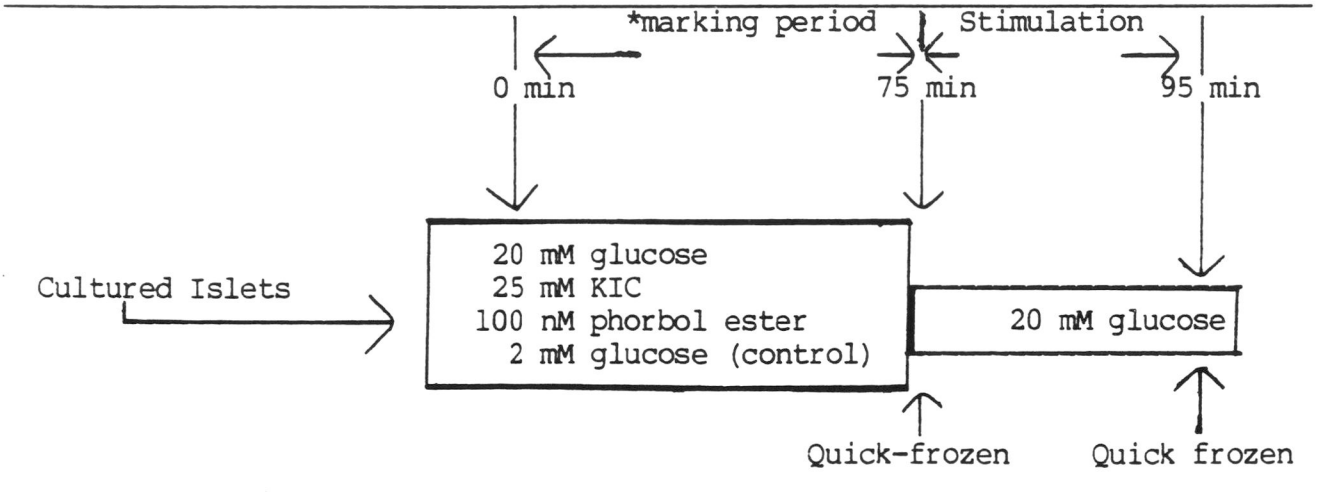



Diagram 1. Experimental design proposed by Gold and Coworkers (1982) for "marking" islets and subsequent stimulation. At each point where the islets are quick frozen, media is collected and assayed for insulin.

## MATERIALS and METHODS

### Preparation of Islets of Langerhans

Male Sprague-Dawley rats weighing approximately 225g to 250g, obtained from Taconic Farm Laboratories (Germantown, NY), are used in the experiments. The rats are allowed free access to food and water until they are sacrificed. After the animals are sacrificed by decapitation, the pancreata are dilated with approximately 20ml of Hanks Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY) and removed. Excess fat, blood vessels, and ducts are trimmed away. The pancreata are placed in 10ml beakers, two per beaker, and chopped into fine pieces using three pairs of scissors. The minced tissue is transfered to scintillation vials where 11mg of collagenase (Sigma Chemical Co., St.Louis, Mo) is added to each vial. The vials are shaken, separately, for approximately 15 minutes in a water bath at 37$^{O}$C in order to digest away the exocrine tissue and thus leaving only the islets of Langerhans. At the end of this period, ice cold HBSS is added to stop the digestive activity of the collagenase (Lacy and Kostianovsky, 1967). The remaining islets are then washed three times with cold HBSS. After washing, the islets are hand picked three times under a dissecting microscope using thin, siliconized pasteur pipets. The islets are divided into groups of fifty and placed in test tubes and washed three times with RPMI 1640 culture media containing 11.1mM glucose, 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, N.Y.), 20 mM hydroxyethyl piperazine-N'-2 ethane sulfonic acid (HEPES) buffer, and antibiotics (pencillin 1000 U/ml and streptomycin 0.1mg/ml). The islets are placed in 60mm culture dishes containing 4.5ml of RPMI 1640 culture media. The cultured for 7 days in a 95% $CO_2$ - 5% air atmosphere. After

the 7 days in culture, an almost pure population of beta cells remained within the islets.

The islets are removed after the incubation period and 25 islets are placed in each of eight 35mm petri dishes containing 2ml of RPMI 1640 culture media without glucose. The media is supplemented with one of the following "marking" agents: 20 mM glucose, or 25 alpha-ketoisocaproic acid, or 100nM phorbol ester (Sigma Chemical Co., St. Louis, MO), or the islets were left unmarked by supplementing the media with 2 mM glucose. The islets are "marked" for 75 minutes according to Gold and coworkers (1982). After the "marking" period, the islets from one plate of each agent are removed and physically fixed using a quick freeze fixation technique. The media from dishes is saved for radioimmunoassay of insulin at a later time. The culture media containing the "marking" or nonmarking agents is removed from the dishes and replaced with RPMI 1640 culture media containing 20 mM glucose. After a 20 minute stimulatory period, the islets are removed from the medium and prepared for electron microscopy. Again the media is saved for radioimmunoassay for insulin at a later time.

Quick Freeze Fixation

The procedure for quick freeze fixation of pancreatic islets has been previosly described by Dudek and Charles (1984). The following is a general outline of the methods involved in the quick freeze fixation of islets.

a) Preparation of Tissue-Holding Probe Tips

A piece of foam (No. P-990; 0.25-in. thick; Illbruk USA, Minneapolis,MN) is fitted into the slot of the probe tip which is part of

the quick-freeze assembly. A small square of aluminum foil is glued to the surface of the foam which extends 1-2 mm above the probe tip. A new piece of foam with foil is prepared for each freeze.

b) Prepartion of Copper Bars

The surface of the copper bars are polished using either diamond paste and a nylon cloth (Ted Pella Inc., Tustin, Ca.) for rough polishing or alumina number 3 micropolish and a MICROCLOTH (Ted Pella Inc., Tustin, Ca) for fine polishing; both are used in conjunction with a polishing wheel. The surface of the copper bar is rinsed with distilled water and carfully dried using filter paper.

c) Preperation of Quick-Freezing Device

The "Gentleman Jim" quick-freezing device (Quick Freezing Devices Inc., Baltimore, MD) is used in these experiments. The device is leveled on a lab bench top. The bounce of the device is checked using a digital bounce monitor and if the monitor does not read zero, adjustments need to be made on the device. The stainless steel dewar is then filled with liquid nitrogen and the polished copper bars are dropped into the dewar of liquid nitrogen. The copper bars are super cooled when a rush of nitrogen vapor is observed from the top of the dewar. This rush of nitrogen vapor is known as nucleate boiling. The dewar is then aligned in the device so that the probe tip is centered over the copper bar. At this point, the device is ready for quick-freezing.

d) Freezing of the Islets

After the islets are removed from the media using a finely drawn pipette under a dark-field stereomicroscope, they are placed on a Millipore membrane which has been placed on the bottom half of a perfusion

chamber soaked in culture medium. The upper right hand corner has been cut diagonally so that the islet side of the membrane can be distinguished.

As the islets are placed on the membrane, the level of culture media is decrease by lowering a piece of polyethylene tubing that is connected to the perfusion chamber. The membrane is then picked up with a pair of fine tipped forceps and placed on the aluminum foil and the sponge of the probe tip. Any excess media is removed by carefully and quickly blotting the edge of the membrane. The probe tip is then attached to the striker which in turn is released subsequently quick-freezing the islets in the process. The probe tip is then carefully removed from the striker and quickly transfered to a container of liquid nitrogen where the sponge, containing the frozen islets, is stored until freeze drying. This procedure is repeated for each set of islets to be quick-frozen. Between each set of freezes the copper bars are removed from the dewar and their face is thawed to remove any ice crystals that might have formed during the previous freeze. The dewar is refilled with liquid nitrogen and the bars are placed back into the dewar. Once nucleate boiling occurs, the quick-freeze apparatus is ready for another set of freezes.

**Freeze Drying**

After the islets are quick frozen, they are rapidly transfered to a container of liquid nitrogen where they are stored until all the quick freezing is completed. The millipore filters containing the islets are removed from the sponges and placed in a holding device so the filters will not float away. Once all the specimens are put in their respective

holding chambers, they are quickly transfered to the specimen chamber of the Coulter-Terracio freeze drying apparatus (Ted Pella INC., Burlington, VT) and immediately transfered to a dewar of liquid nitrogen. Freeze drying allows for the dehydration of the frozen islets through the sublimation of ice. The specimen chamber of the apparatus is contained within the condenser tube. The condenser/specimen tube is placed under a vacuum of 0 Torr. The condenser/specimen tube remains in the dewar of liquid nitrogen for 3 days with a temperature reading taken each day. After the third day, a vycor heater (Corning, NY) connected to a rheostat is placed in the condenser/specimen tube. The rheostat is set at 1 volt and raised in increments of 1 volt every 12 hours for three days. A temperature reading is taken with each increase in voltage. On day 7, the temperature is monitored closely and when the temperature reaches 30$^{o}$C the condenser/specimen tube is placed in a graduated cylinder containing 1100 ml of ETOH at -110$^{o}$C. As the temperature increases the vacuum will deteriorate because of the water being evacuated from the walls of the condenser tube. When the vacuum returns to 0 Torr the condenser/specimen tube is removed from the graduated cylinder and when the condenser/specimen tube returns to room temperature remove the vycor heater.

The islets are osmicated while still in the freeze drying apparatus. Osmium tetroxide ($OsO_4$) vapors are introduced to the islets by opening the valve to the osmium tube. The islets are exposed to the osmium vapors for approximately 30 minutes. After osmication, the vapors are condensed into the osmium tube by placing a container of liquid nitrogen around the bottom of the tube for approximately 60 minutes.

The islets are then infiltrated with Epon 812/Araldite 502 resin for 12 to 24 hours. The exact formula for the resin is as follows: Araldite 502, 4.3 gm; Epon 812, 5.6 gm; dibutyl phtalate, 0.6 gm; DDSA, 11.25 gm; DMP-30, 0.3 gm. The resin is slowly introduced to the specimen chamber through the resin intake tube. After the islets are infiltrated, they are removed from the holding chambers and placed into flat embedding molds contaianing Epon 812/Araldite 502 resin. Flat embedding molds are used because it is easier to orient the islet containing membrane in them. The resin is cured for 48 hours at 60$^{o}$C in a curing oven. The resin blocks, containing the islet, are then removed from the molds and they are trimmed so the islets can be sectioned on the ultramicrotome. Pale gold sections (90 nm) are cut on the Reichart ultracut microtome and placed on copper mesh grids (Polaron Equipment Ltd., Watford, England). Each grid is placed section face down on a drop of uranyl magnesium acetate for 45 min at 45$^{o}$C followed by lead citrate for 2 min at room temperature (23$^{o}$C). The sections are now ready for electron microscopy.

Immunocytochemistry

Silver-gold sections (70-90 nm thick) are mounted on nickel grids. The grids are incubated in 1% ovalbumin and 0.04 M Phosphate buffered saline (PBS) at room temperature for 48 hours. The grids are then incubated overnight at 4$^{o}$C in beem capsules containing 50 ul of rat insulin antisera. After exposure to the primary antisera, the grids are jet washed with 0.04 M PBS and placed on drops of 0.04 M PBS for 3-5 min. The grids are blotted on filter paper and floated section face down on a drop of Protein A-gold (Janssen Pharmaceutica, Beerse, Belgium) for 1

hour at room temperature (23°C). The size of the gold particles is 10 nm. After exposure to the protein A-gold, grids are jet washed with 0.04 M PBS and placed on drops of 0.04 M PBS for 3-5 min, followed by jet washing with distilled water and placing them on drops of distilled water for 10 min. After the grids are allowed to air dry for 20 min, the side of the grid not exposed to the Protein A-gold is counterstained with uranyl magnesium acetate for 1 hour at room temperature followed by a jet wash with distilled water. The grids are then placed on a drop of lead citrate for 2 min at room temperature. The grids are allowed to air dry before use or storage.

Electron Microscopy

After staining, the ultrastructure of the "marked" and unmarked islets is examined on the JEOL 1200 EX electron microscope. Electron photomicrographs are taken of desired structures using Kodak electron microscopy film. Stereo pairs are taken at various angles using the goniometer. All negatives are printed on Kodak Polycontrast Rapid II RC paper.

DNA Assay

The islets are removed from the incubation media and placed into 12x75 mm culture tubes containing 1 ml PBS. The islets were disrupted for the DNA assay using a sonicator (Heatsystems-Ultrasonics Inc., Plainview, NY). A DNA standard stock is made by dissolving 20 mg of thymus DNA in 20 ml of buffer, pH 7.4. The standard stock is diluted to make standard concentrations of 200, 150, 100, 75, 50, and 25 ng/ml and buffer is used for the 0 concentration. Aliquots of 25, 50, or 100 ul are removed from the sonicated samples and placed into tubes containing 3.975, 3.950, and

3.900 ml of working BBI respectively. The tubes are then vortexed. The standards and the samples are placed in a flourometer and their transmittance is recorded. The transmittance is correlated with a DNA value (ng/ml) using a computer program.

Radioimmunoassay

Groups of 10 islets were cultured and incubated under the same conditions as those that were frozen. The islets were removed and saved for DNA assay and the media was assayed for insulin. A standard curve was set up by serially diluting 20.50 ng/ml of stock insulin standard to 10.25, 5.12, 2.56, 1.28, 0.64, 0.32, 0.16 ng/ml. An aliquot of the sample is placed into a disposable culture tube and borate buffer is added to bring the volume to 0.2 ml. Four total count tubes are setup by first placing 0.3 ml of borate buffer into culture tubes. Four nonspecific binding tubes were set up by placing 0.2ml of buffer into culture tubes. To all the tubes, 0.1 ml of $^{125}$I-insulin label was added. The primary antibody, AINS-GP (1:75,000), was added to all the tubes except the nonspecific binding tubes. Normal guinea pig serum (1:1,000) was added to the nonspecific binding tubes in place of the AINS-GP. The tubes are then vortexed and stored for 48 hours at 4$^{o}$C.

After storage, second antibody was added to all the tubes except for the counting tubes. Once the second antibody was added, the tubes were vortexed and incubated for 24 hours at 4$^{o}$C. The tubes were centrifuged at 4$^{o}$C for 30 min at 3,000 RPM. After centrifugation, the tubes were aspirated with a vacuum system and placed on a Beckman gamma counter. Information obtained from the gamma counter was recorded onto a computer

disc for analysis at a later date.

## RESULTS

### RIA

The insulin values, expressed as pg/ngDNA/min, of the media from "marked" and unmarked islets are given in Table 1. The insulin values are expressed in terms of the quantity of insulin per quantity of DNA to compensated for any size difference in the islets. The insulin values were determined by RIA while the DNA values were determined by DNA assay. The insulin secretion values for each of the "marking" agents is as follows: 100nM TPA was 3.2±0.35 pg/ngDNA/min, 25mM KIC was 1.8±0.16 pg/ngDNA/min, and 20mM glucose was 1.2±0.11 pg/ngDNA/min. The insulin secretion value for the unmarked group was 0.13±0.014 pg/ngDNA/min. The 100 nM phorbol ester (TPA) causes the greatest increase in secretion by exhibiting a 25 fold increase over the unmarked (2 mM glucose) group of islets. The 25 mM alpha ketoisocaproic acid (KIC) also demonstrated its ability as a marking agent by causing a 14 fold increase in the secretory rate. The final marking agent, 20 mM glucose, did not show as great an increase in insulin secretion as TPA and KIC, but did show a 9 fold increase over the unmarked islets.

The insulin values of the media from the islets that were "marked", washed, and then stimulated with 20 mM glucose are given in Table 2. The insulin values for the islets that were "marked" and stimulated are as follows: 100nM TPA was 4.6±0.48 pg/ngDNA/min, 25mM KIC was 2.8±0.29 pg/ngDNA/min, and 20mM glucose was 0.85±0.09 pg/ngDNA/min. The insulin value for the islets that were unmarked and stimulated with 20mM glucose was 0.75±0.15 pg/ngDNA/min. After all the groups were treated with 20mM

glucose, the insulin secretion values again showed that the islets "marked" with TPA and KIC are secreting more insulin. In comparing the insulin secretion values of Table 2 with those of Table 1, the islets that were "marked" or unmarked followed by treatment with 20mM glucose (Table 2) show higher values, with the exception of the 20mM group, than do the islets that were only "marked"(Table 1). Since the radioimmunoassay only measures total insulin values, there is no way to determine how much of the inulin that was secreted under each condition was newly synthesized or the older, stored form. Gold and his coworkers (1982) found through pulse chase experiment that newly synthesized insulin was being secreted preferentially to the older, stored form. The large differences in insulin secretion between "marked" and unmarked groups of islets is possibly due to increased mobilization and the preferential release of the newly synthesized insulin.

Electron microscopy

The islets were first examined by light microscopy to ensure that a quality quick freeze fixation had taken place (fig. 1 and 2). Initially, the islets of the different "marking" conditions as well as the unmarked islets were examined by electron microscopy and photomicrographed (figs. 3, 4, 5, and 6). After initial examination of photomicrographs and assay data, the islets "marked" with 100nM phorbol ester was determined to show the greatest effect due to "marking" and studied in the greatest detail and compared to the 2mM glucose, unmarked group. Islets "marked" with TPA demonstrated the endoplasmic reticulum to be in close orientation with the beta cell membrane (fig. 9 and 17). The endoplasmic reticulum appeared to

attach to the cell wall and form an opening between the cisternae and the extracellular space when the islets were treated with TPA (fig. 7). The endoplasmic reticulum was also observed closely associated with secretory granules near the cell membrane in these "marked" islets (fig. 8 and 9). The unmarked islets did not show any of these characteristics demonstrated in "marked" islets (fig. 11 and 13).

Two populations of secretory granules were found in both "marked" and unmarked islets and the groups that were subsequently treated with 20mM glucose. The first secretory granule population, designated granule population 1, is characterized by an electron dense core surrounded by a translucent halo that contains filamentous material (fig. 12 and 18). This granule population 1 can be described as having a wagon wheel appearance. Using Protein A-gold immunocytochemistry, the dense core and filamentous material of granule population 1 demonstrate immunoreactivity for insulin (figs. 13, 14, 15, 16, and 19). The second secretory granule population of the beta cell, designated granule population 2, is completely electron dense with no translucent halo or filamentous present (fig. 12). The secretory granules in populations 1 and 2 are approximately the same size (30-40 um). The entire electron dense granule of population 2 exhibits insulin immunoreactivity when treated with Protein A-gold immunocytochemistry (figs. 9, 10, 14 and 15).

TABLE 1

INSULIN SECRETION
"Marking"

| Agents | n | Insulin [pg/ngDNA/min.] |
|---|---|---|
| ** 100nM TPA | 5 | 3.2±0.35 * |
| 25Mm KIC | 5 | 1.8±0.16 * |
| 20mM Glucose | 5 | 1.2±0.11 * |
| ** 2mM Glucose | 4 | 0.13±.014 |

*Indicates significance from control [p 0.05]
Data X± S.E.M.
**Indicates conditions studied by E.M.

TABLE 2

INSULIN SECRETION
"Marking" + 20mM glucose stimulated

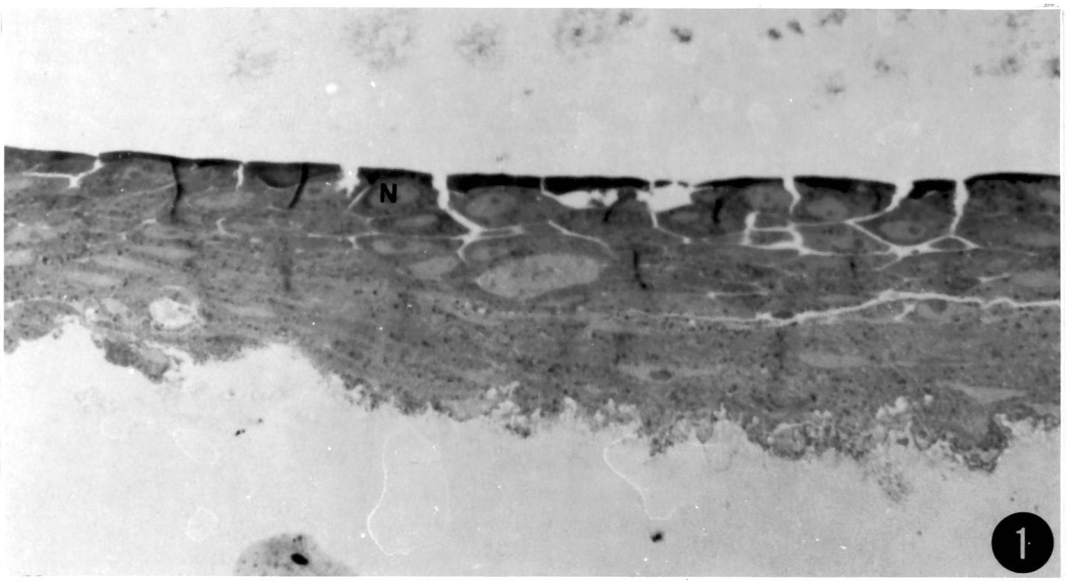
| Agent | n | Insulin [pg/pgDNA/min.] |
|---|---|---|
| ** 100nM TPA | 5 | 4.6±0.48 * |
| 25mM KIC | 5 | 2.8±0.29 * |
| 20mM Glucose | 4 | 0.85±0.09 |
| ** 2mM Glucose | 3 | 0.78±0.15 |

Data represents islet stimulation with 20mM glucose immediately following "marking".

*Indicates significance from control [p 0.05]
Data X± S.E.M.
**Indicates conditions studied by E.M.

Figure 1. This is a light micrograph of islets of Langerhans that have been "marked" with TPA. The islets were fixed by the quick freeze method followed by freeze drying. The photomicrograph demostrates that proper freezing took place. Frozen edge on top. Magnification 200x.

Figure 2. This is a light micrograph of unmarked islets of Langerhans that again demonstrates a good freeze fixation. The frozen edge is on the top. Magnification 200x.


N
1

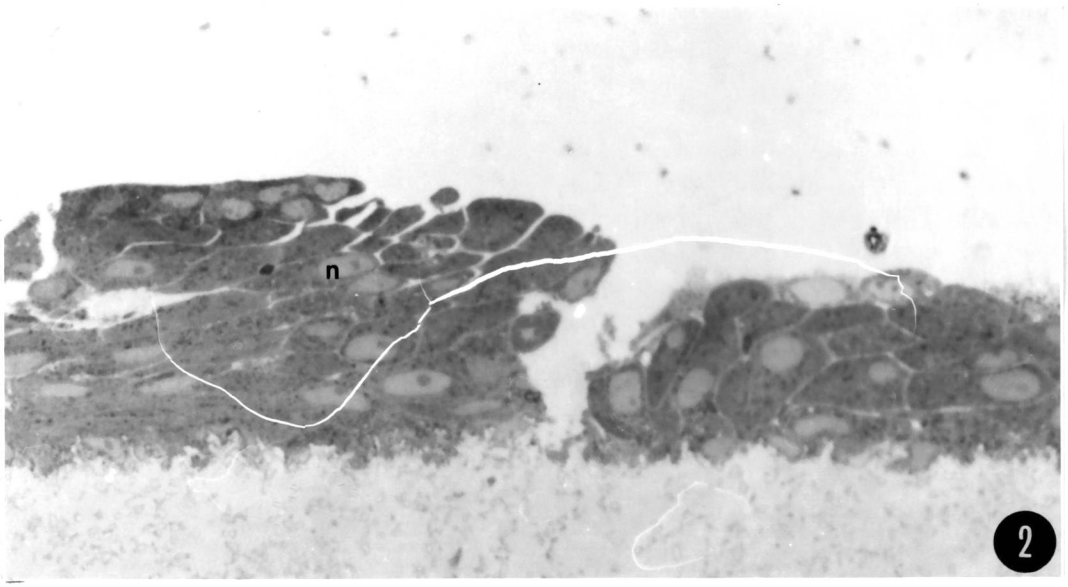
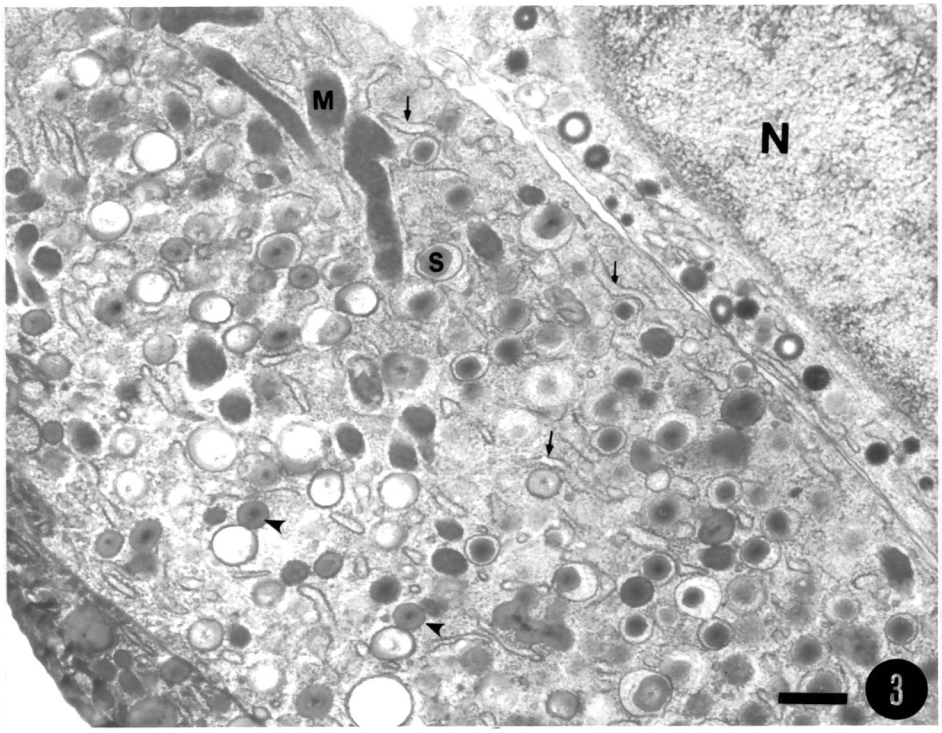

n
2

Figure 3. Beta cell "marked" with 100 nM TPA. The cell contains a large nucleus (N) and several mitochondria (m). The beta cell also contains a large amount of endoplasmic reticulum (arrows) which appears to be closely associated with the secretory granules (s). This cell possesses a second granule population (arrowheads) which is electron dense. Marker equals 0.5 micrometers. Magnification 20,000x.

Figure 4. Unmarked beta cell. The unmarked cell also has a large nucleus (N) andthe insulin biosynthetic and secretory components which include the Golgi apparatus (g), the endoplasmic reticulum (ER), and the Secretory granules (S). Marker equals 0.5 micrometers. Magnification 20,000x.


M
N
S
3

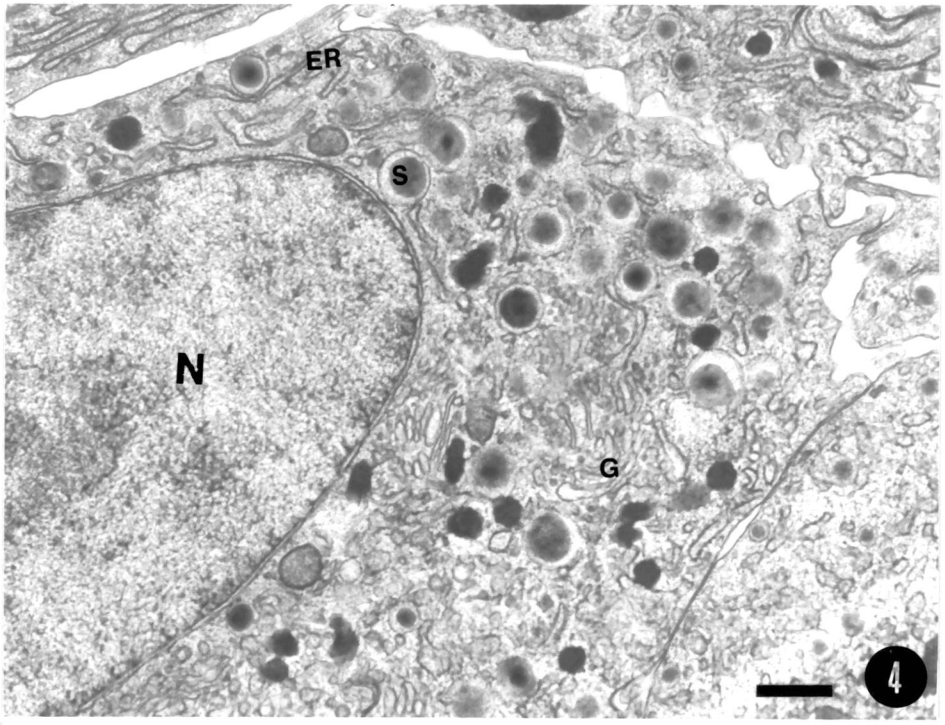
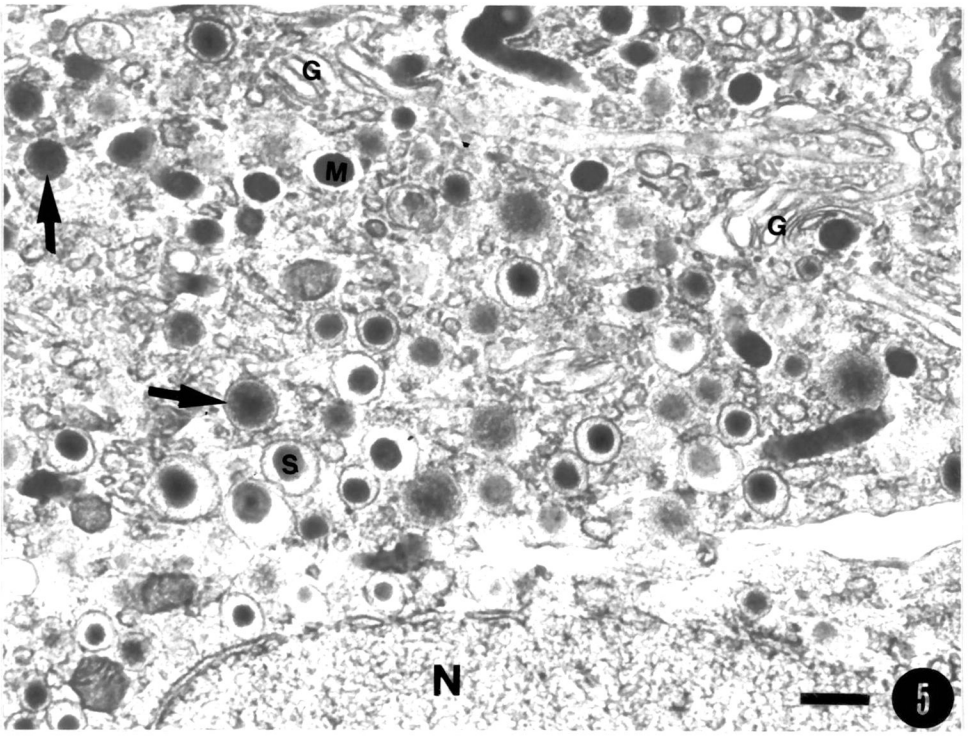

ER
S
N
G
4

Figure 5. Beta cell "marked" with 25 mM KIC. This beta cell demonstrates an expansive Golgi (G) and a large number of mature secretory granules (S). The arrows indicate the presence of population 2 granules. The cell also contains a nucleus (N) and mitochondria (m). The marker equals 0.5 micometers. Magnification 24,000x.

Figure 6. Beta cell "marked" with 20 mM glucose. The cell contains a Golgi (G), endoplasmic reticulum (arrows), mitochondria (m) and a large nucleus (N). The population 1 granules (S) and the population 2 granules (arrowheads) are lined up along the cell membrane as well as being scattered throughout the cell. Marker equals 0.5 micrometers. Magnification 16,000x.


G
M
G
S
N
5

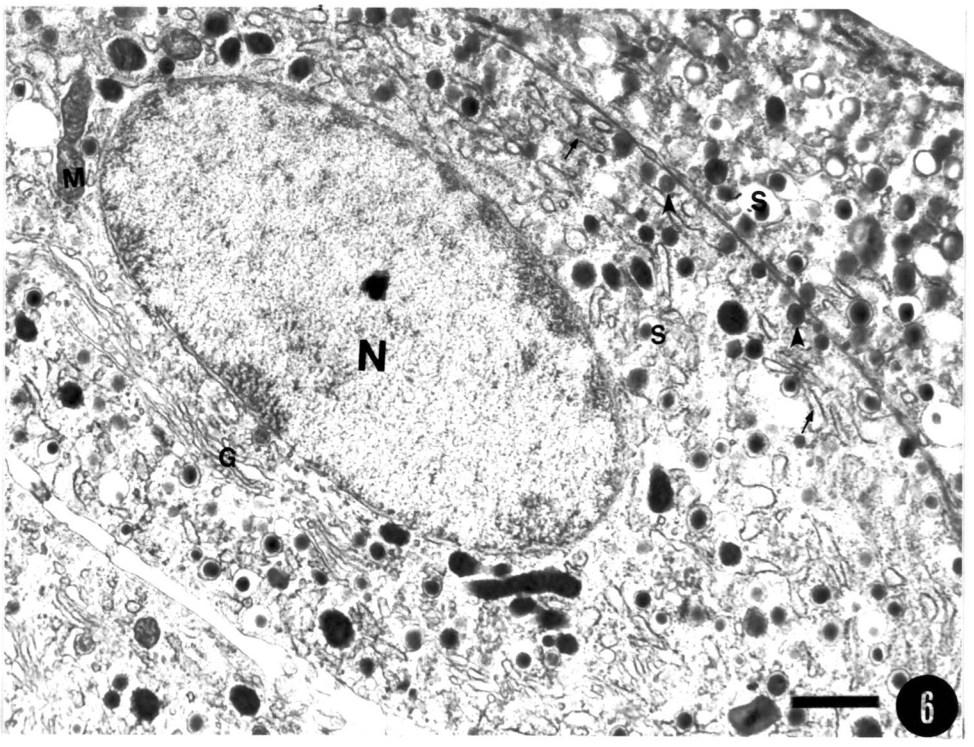
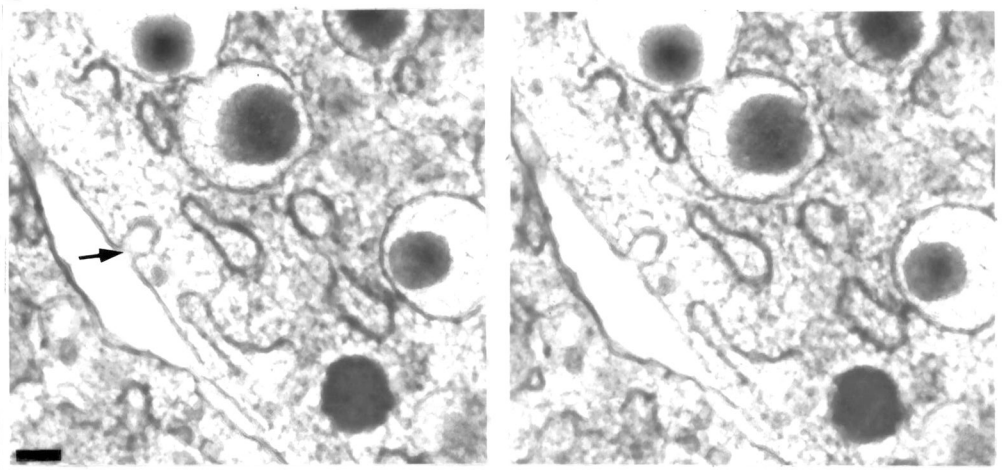

M
S
S
N
G
6

Figure 7a and 7b. TPA "marked". Stereo pairs. The "marked" pancreatic beta cell shows the binding of endoplasmic reticulum to the cell membrane and the formation of an opening (arrow). Marker equals 0.1 micrometer. Magnification 55,000x.



7a

7b

Figure 8. TPA "marked". Stereo pair. The beta cell demonstrates an endoplasmic reticular extension (arrow) leading from a secretory granule towards the cell membrane. Marker equals 0.1 micrometer. Magnification 110,000x.

8a
8b

Figure 9. TPA "marked". Immunochemically stained for insulin using the protein A-gold technique. Both the population 1 secretory granules (G) and the population 2 secretory granules demonstrated insulin immunoreactivity. A large amount of endoplasmic reticulum (ER) is present in the cell and is in close association with granules and the cell membrane at some points (arrow). Marker equals 0.2 micrometers. Magnification 30,000x.

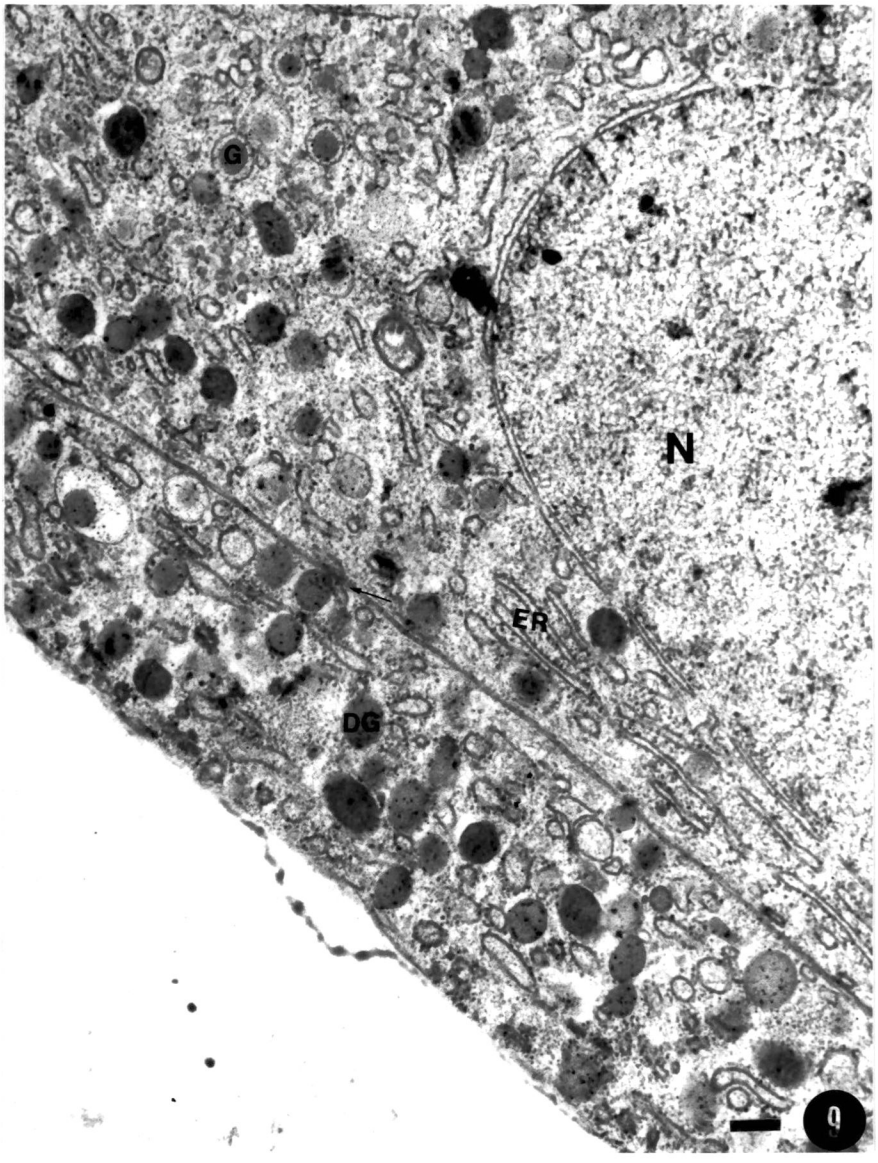

G
N
ER
DG
9

Figure 10. TPA "marked". Immunocytochemically stained for insulin. The population 2 secretory granules (DG) demonstrate insulin immunoreactivity by the presence of gold particles. The population 2 secretory granule (G) of the pancreatic beta cell also exhibits insulin immunoreactivity. Marker equals 0.1 micrometer. Magnification 62,500x.

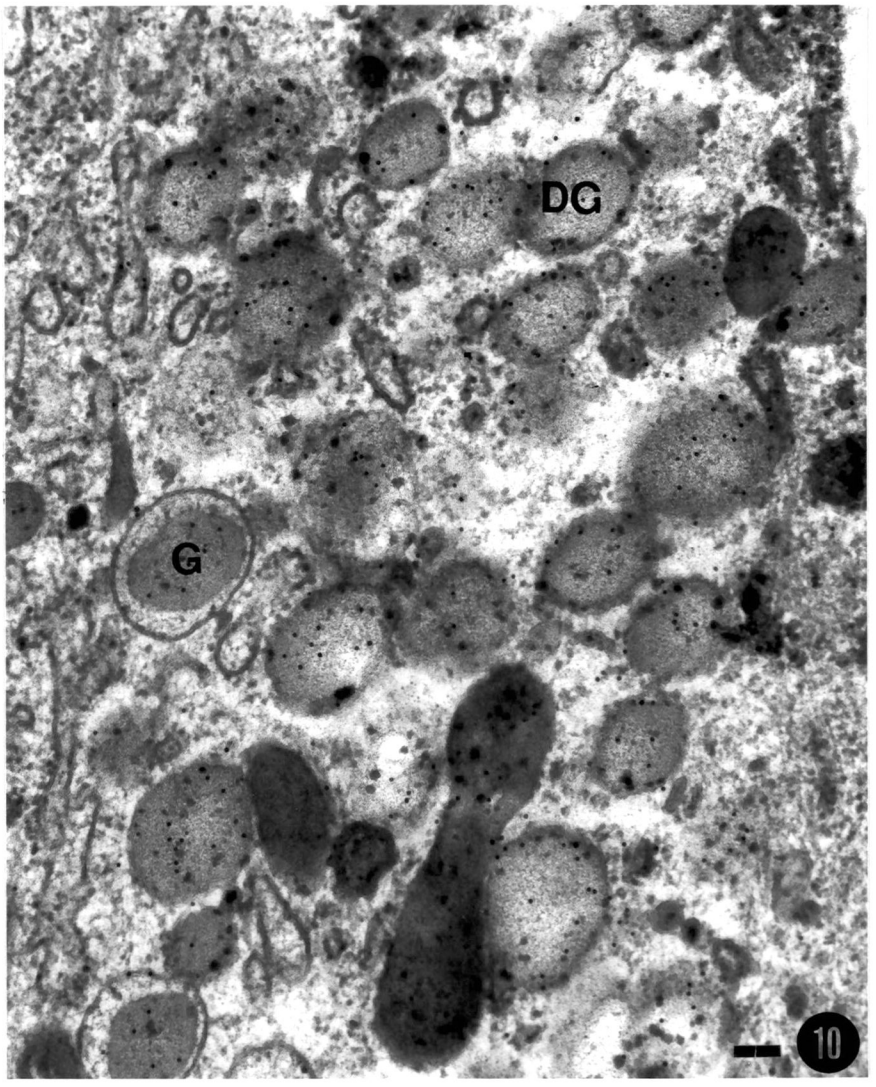

DG
G
10

Figure 11, Unmarked. There is a large number of population 1 secretory granules (G) and many are located at the periphery of the cell (arrowheads). Scattered among the granules are mitochondria (m) and endoplasmic reticulum (arrows). Marker equals 0.2 micrometers. Magnification 23,000x.

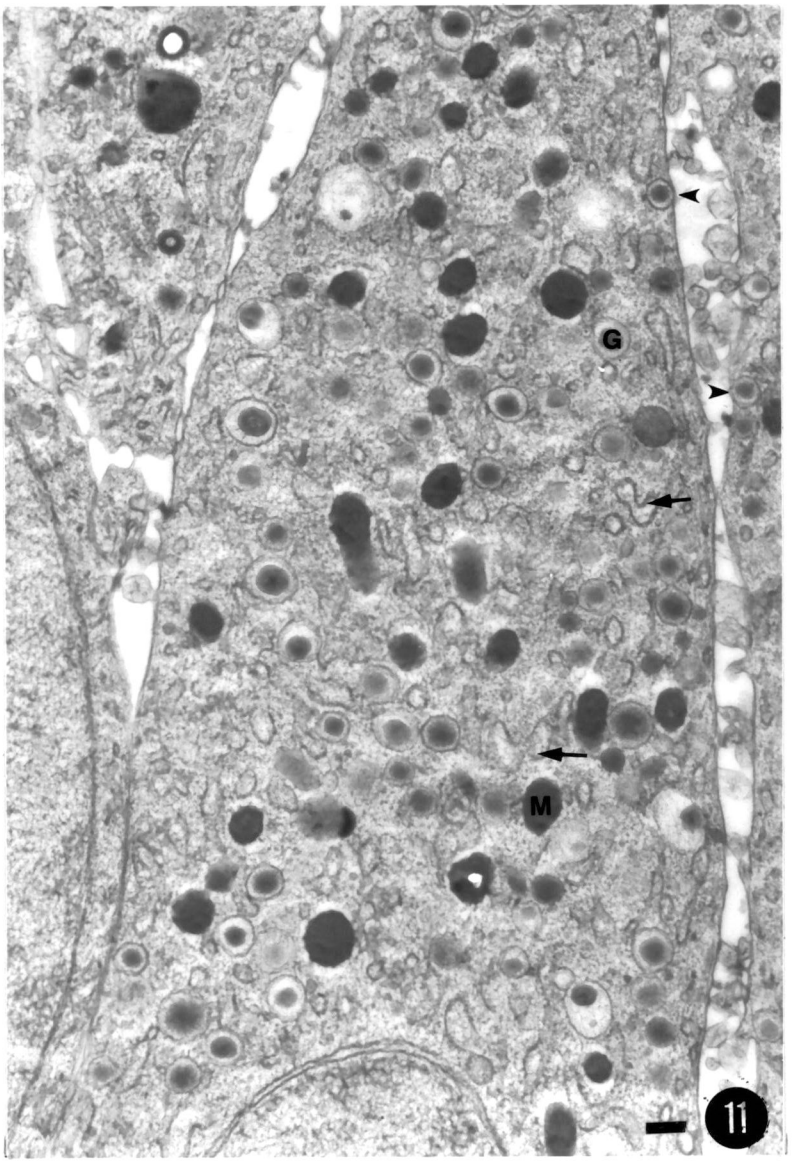

G
M
11

Figure 12. Unmarked. This electron micrograph demonstrates the presence of both population 1 secretory granules and the population 2 granules within the unmarked pancreatic beta cell. A filamentous material (arrows) is observed projecting outward from the core of granule in population 1. Endoplasmic reticulum (ER) is observed passing between granules. Marker equals 0.1 micrometers. Magnification 60,000x.

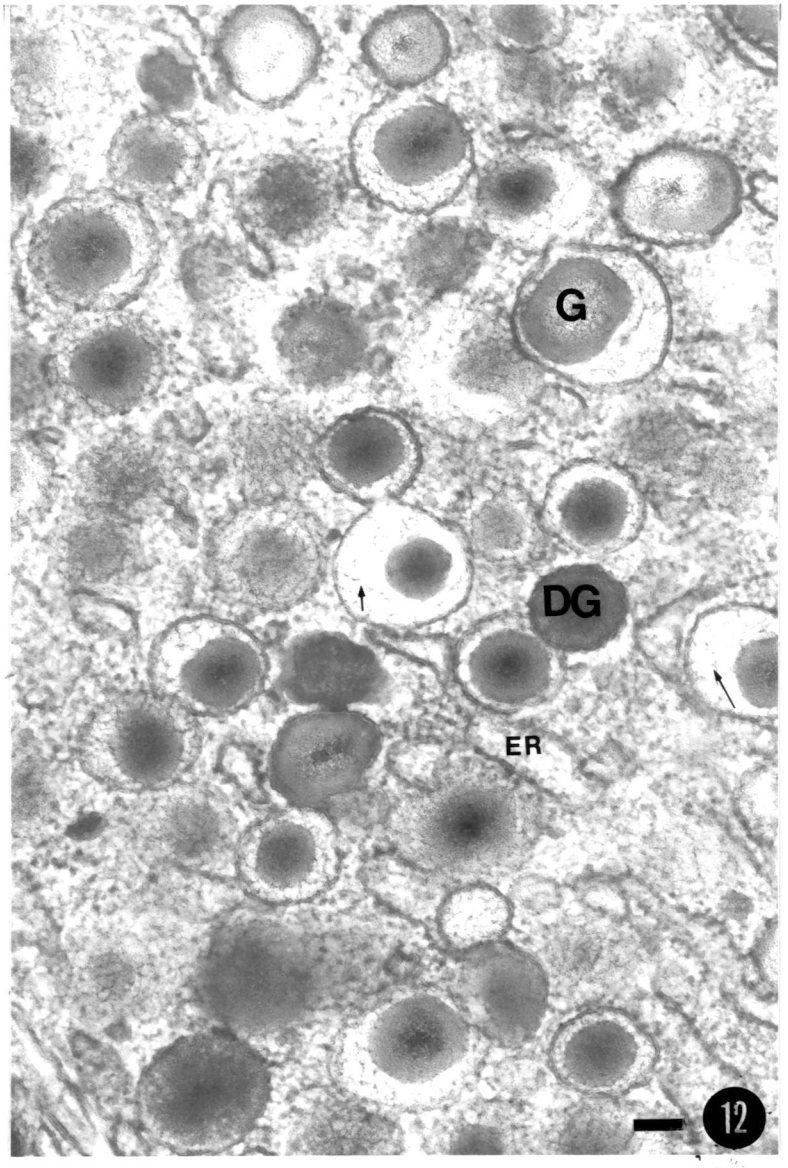
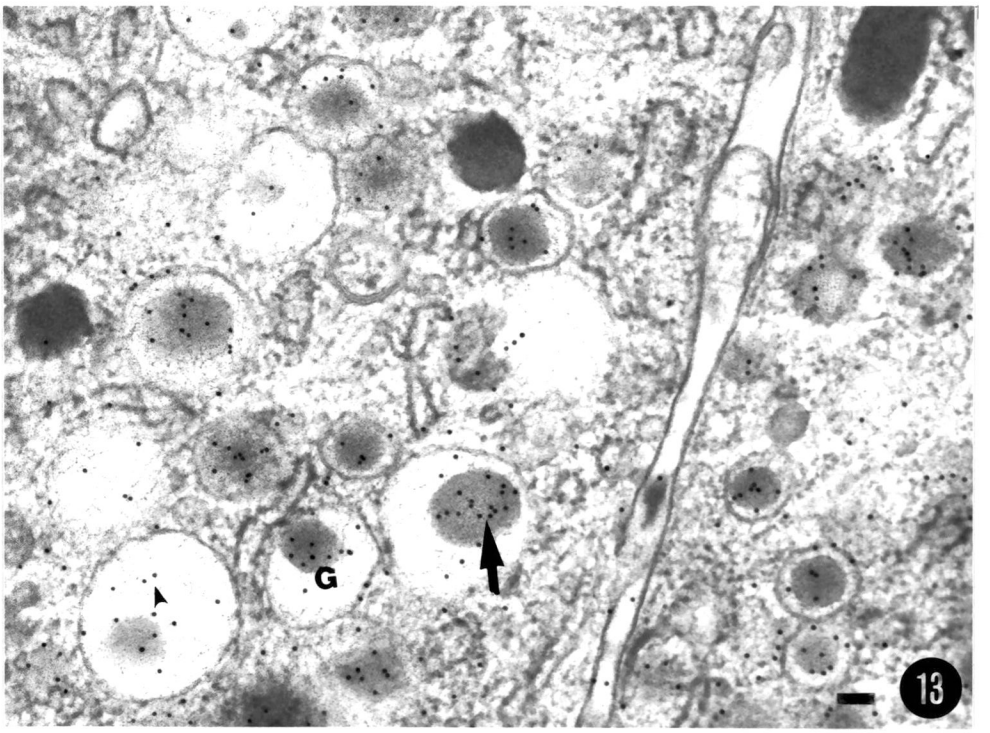

G
DG
ER
12

Figure 13. Unmarked. Immunocytochemically stained for insulin. Immunoreactivity for insulin is present in both the dense core (arrow) and the filametous material (arrowhead) of the population 1 secretory granule (G). Marker equals 0.1 micrometer. Magnification 50,000x.

Figure 14. Unmarked. Immunocytochemically stained for insulin This beta cell demonstrates insulin immunoreactivity (arrowheads) in both population 1 secretory granules (G) and population 2 secretory granules (DG). A large amount of endoplasmic reticulum (ER) is present within the cell. Marker equals 0.2 micrometers. Magnification 24,000x.


G
13

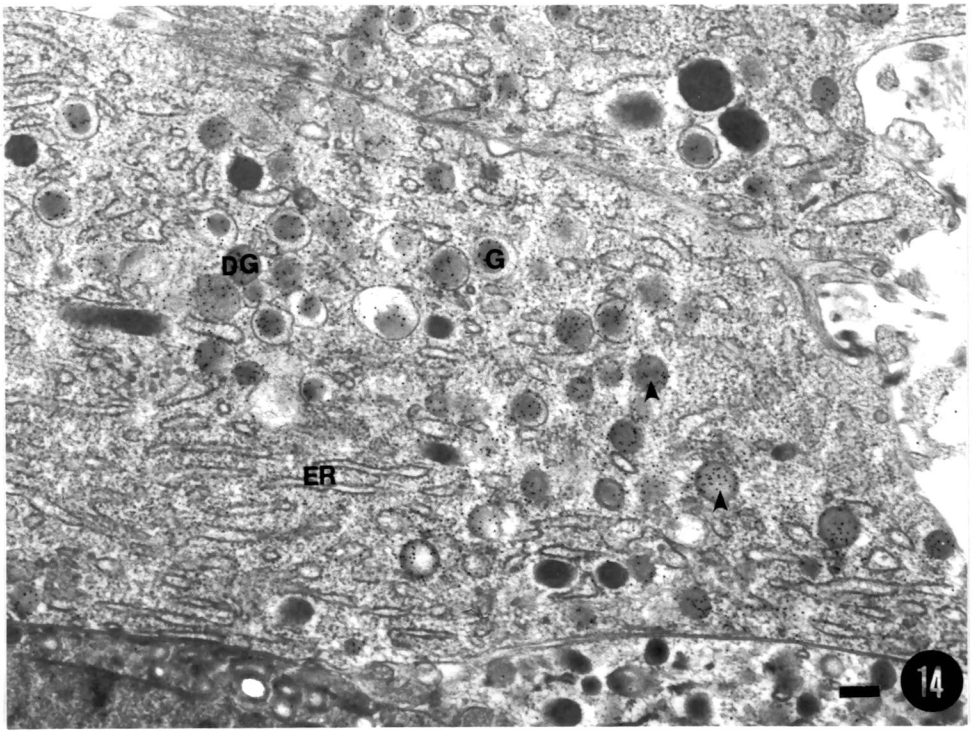
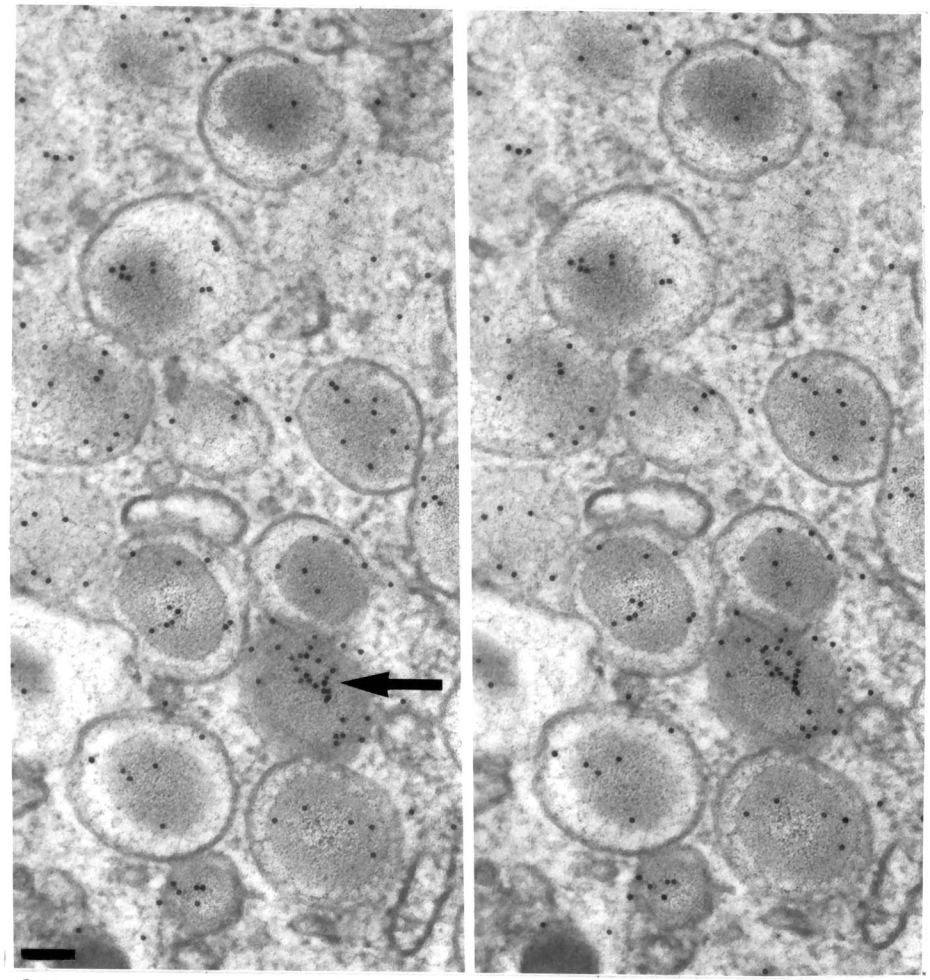

DG
G
ER
14

Figure 15. TPA "marked" followed by 20mM glucose treatment. Immunocytochemically stained for insulin. Stereo pair. The arrow indicates insulin immunoreactivity in the population 2 secretory granules. Marker equals 0.1 micrometer. Magnification 75,000x.



15a

15b

Figure 16. TPA "marked" followed by 20mM glucose treatment. Immunocytochemically stained for insulin. The presence of insulin in the population 1 beta cell secretory granules (G) and the population 2 secretory granules is demonstrated by the presence of gold particles. Marker equals 0.2 micrometers. Magnification 33,750x.

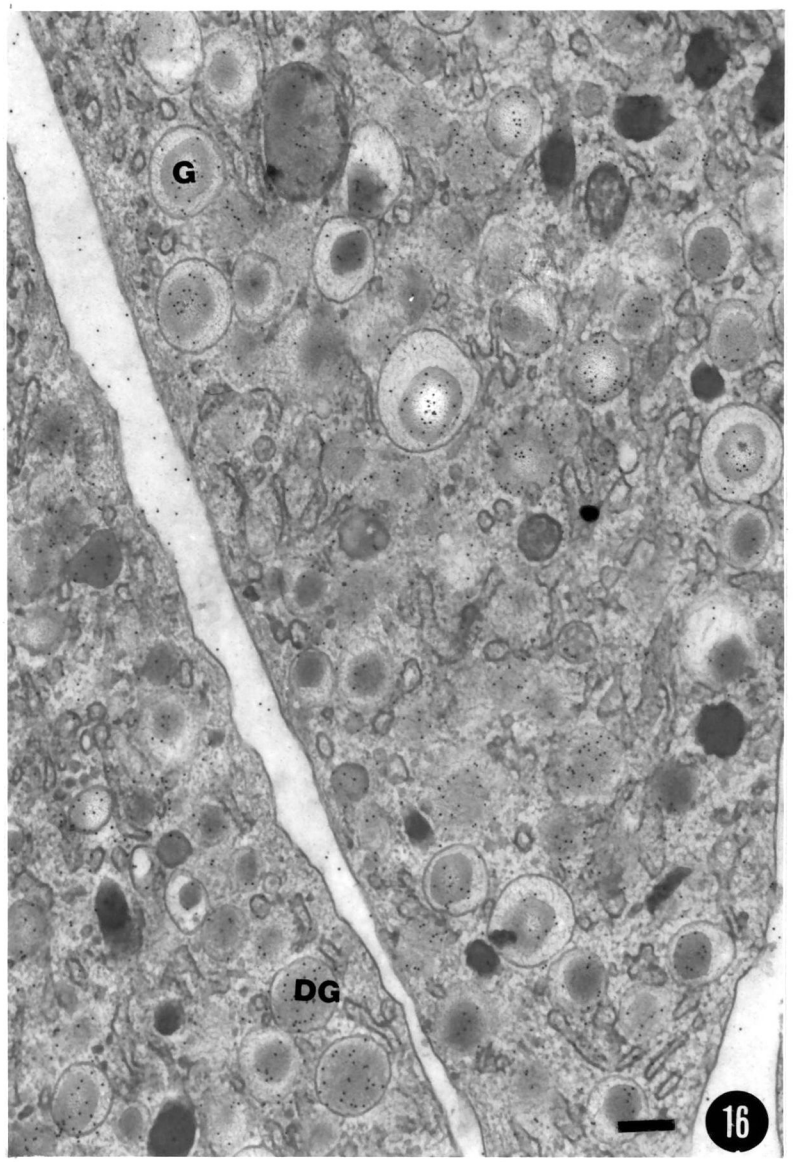

G
DG
16

Figure 17. TPA "marked" followed by 20mM glucose treatment. This micrograph demonstrates the presence of endoplasmic reticulum (ER) associated with the plasma membrane of the beta cell. population 1 secretory granules (G) and population 2 granules (DG) are randomly distributed throughout the cytoplasm. Marker equals 0.2 micrometers. Magnification 33,750x.

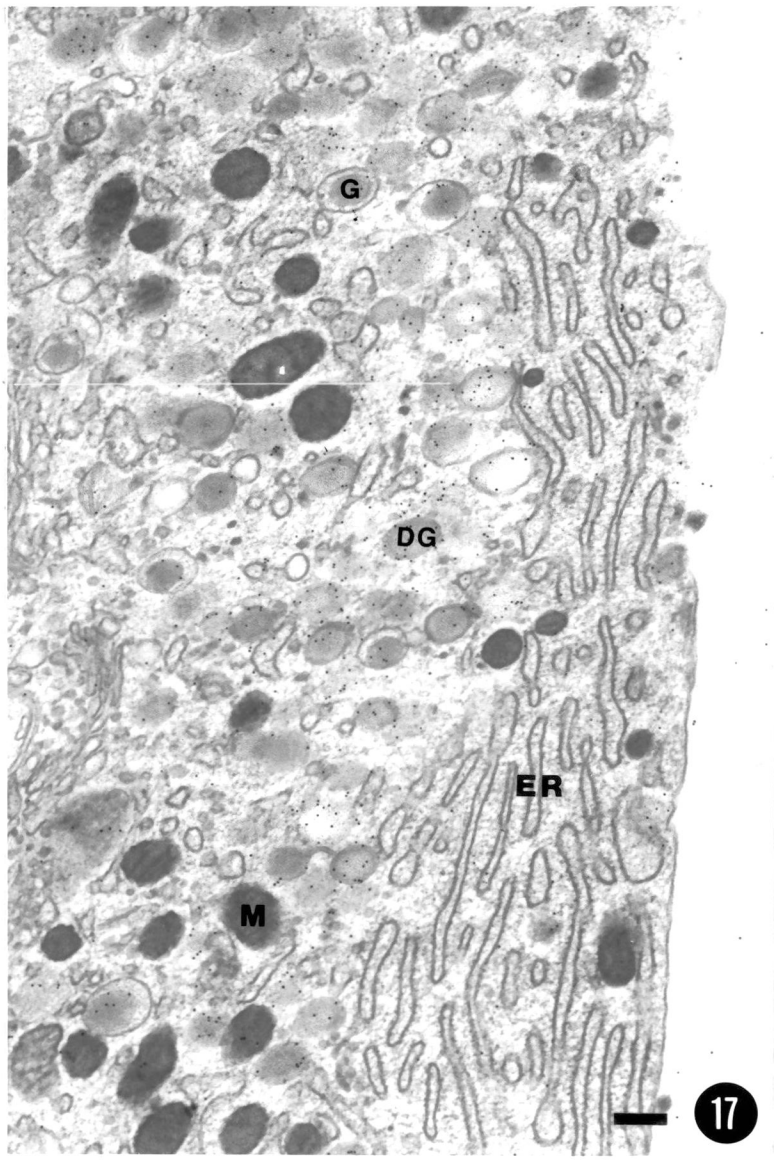
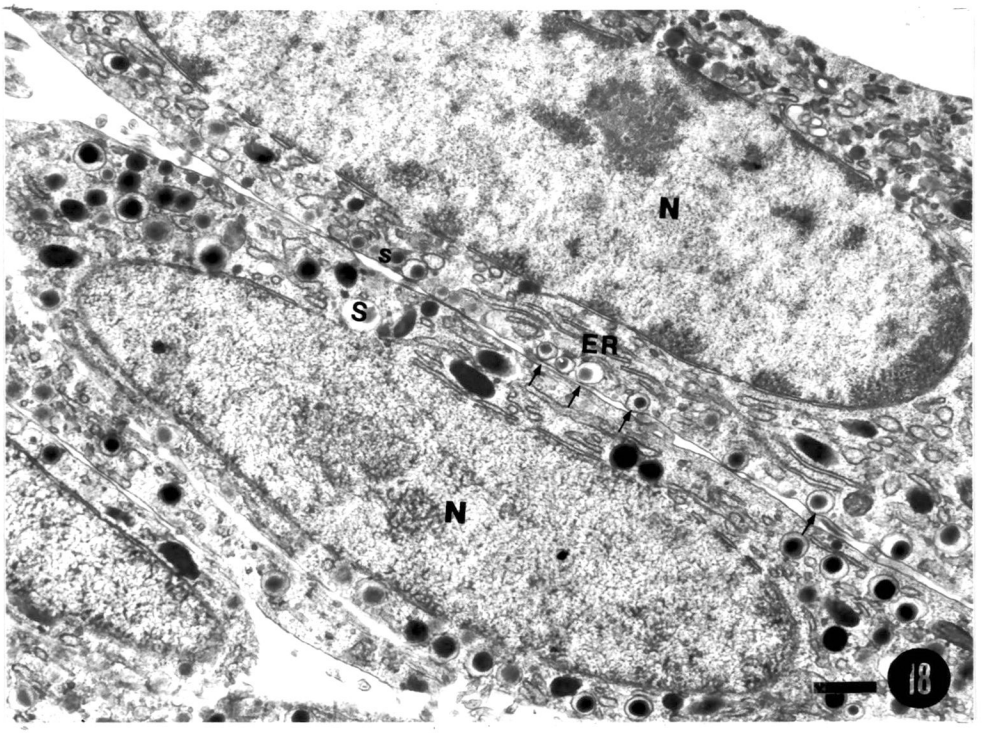
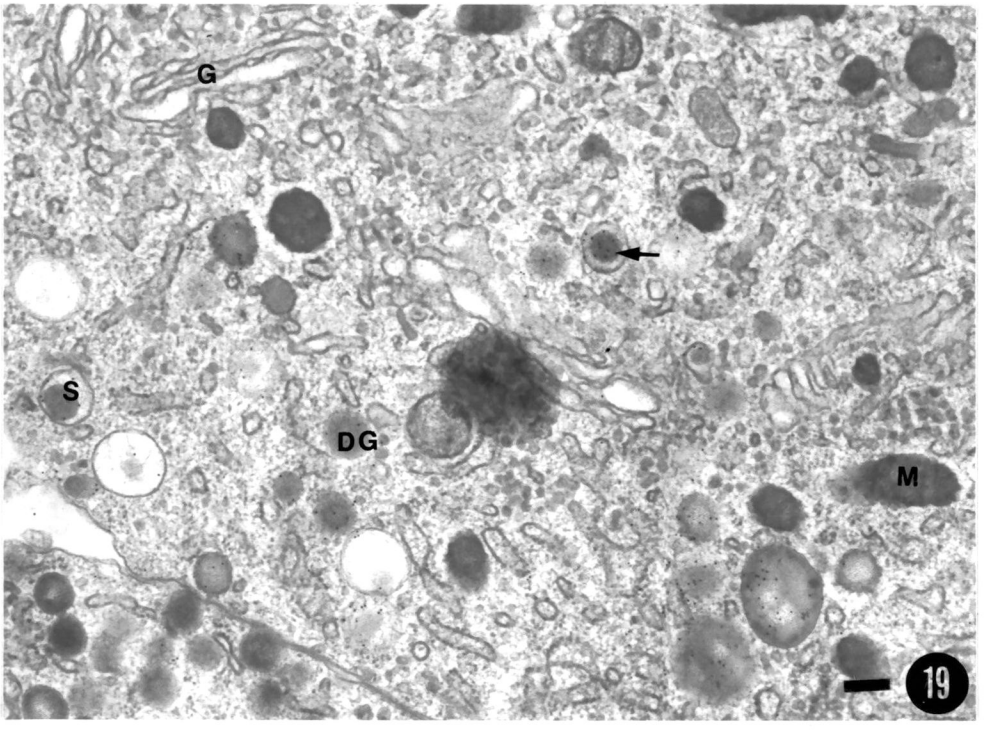

G
DG
ER
M
17

Figure 18. Unmarked followed by 20mM glucose treatment. The secretory granules (S) lining up along the beta cell plasma membrane (arrows). The beta cells exhibit a large nucleus (N) and an extensive endoplasmic reticulum (ER). Marker equals 0.5 micrometers. Magnification 16,000x.

Figure 19. Unmarked followed by 20mM glucose treatment. Immunocytochemically stained for insulin. Insulin immunoreactivity (arrows) is shown in both population 1 (S) and population 2 (DG) secretory granules. Golgi apparatus (G) and mitochondria (m) are present. Marker equals 0.2 micrometers. Magnification 30,000x.


N
S
S
ER
N
18


G
S
DG
M
19

## DISCUSSION

The morphological mechanism by which newly synthesized insulin is preferentially selected for secretion has not been elucidated at this time. When the islets are "marked", they secrete the newly synthesized hormone preferentially to the older stored hormone, but the morphological question remains from which compartment in the beta cell is the newly synthesized insulin secreted. There have been several possibilities proposed by previous investigators. The results from the experiments in this thesis support three possible mechanisms. The first possiblity is that there is a subpopulation of beta secretory granules responsible for the preferential release of newly synthesized insulin. The second is that the newly synthesized insulin is released from an accelerated pathway involving the endoplasmic reticulum binding to the cell membrane and secretion occuring at this site. The third is a combination of the two mechanisms or the release of newly synthesize insulin may occur by some completely different mechanism.

A mechanism involving two secretory granule populations appears to be more feasible for the release of newly synthesized insulin than an accelerated pathway involving the endoplasmic reticulum. There are two possible mechanisms for the secretion of newly synthesized insulin that involve two granule populations. The first is that the newly synthesized insulin is contained within the granules in population 1 while the older, stored insulin is found within the electron dense granules of population 2. Both granule populations demonstrated insulin immunoreactivity, but at different locations within the granule. The granules in population 1 only exhibited insulin immunoreactivity within core and filametous material

while granules in population 2 were insulin immunoreactive throughout. Steiner and coworkers (1974) reported that insulin forms a crystalline complex with zinc and this complex is what forms the dense core of granule population 1. They also reported that proinsulin does not complex as readily with zinc as does insulin. Since the granules in population 2 have an appearance similar to the core of the granules in population 1, it could be assumed that this granule population contained only the mature form of insulin. If this is the correct mechanism then the granules in population 1 contain and secrete newly synthesized insulin.

The second possible mechanism is similar to the one presented above, but with the function of the two granule populations reversed. In this mechanism, the granules of population 2 would contain proinsulin and newly synthesized insulin at random throughout the granule. The random location of the newly synthesized insulin might explain the electron dense appearance of the granule and would account for the insulin immunoreactivity throughout the granule of this population. As the proinsulin is converted into newly synthesized insulin, the insulin begins to form a complex with zinc and a dense core forms as is seen in granule population 1. If these are the events that occur during the conversion of proinsulin to newly synthesized insulin then the granules of population 2 are responsible for the release of newly synthesized insulin and the granules of population 1 store the older hormone.

In either mechanism, the second granule population may be part of the time dependent maturation of insulin. Insulin kinetic studies have shown that proinsulin to insulin conversion requires approximately 1 hour

(Gold and Grodsky, 1984). As conversion occurs, the newly synthesized insulin begins to crystallize and form a complex with zinc and a core would begin to form within the granule (Steiner et al., 1974). The appearance of the granule could possibly change from a completely dense structure to a dense core surrounded by a translucent halo, or vice versa as conversion progresses. Whichever granule population is the site of conversion is most likely the location of newly synthesized insulin within the beta cell.

Work presented in this thesis only postulates the function of granule population 2 and the location of the newly synthesized hormone in the beta cell, but does not address the mechanism for the preferential release of newly synthesized insulin. The reason the work only postulates the function of granule population 2 is that this population was found in both "marked" and unmarked islets. Gold and coworkers (1982) have biochemically demonstrated that newly synthesized insulin is preferentially secreted from the beta cell by "marking" it. If the electron dense granule population is indeed the compartment for the newly synthesized insulin then it would follow that secretion occurs from these granules. The mechanism for how the beta cell, or any other cell, preferentially secretes its newly synthesized product remains unclear. Orci (1985) has demonstrated the presence of a clathrin coating on the dilated ends of the Golgi apparatus and some of the granules. He also favors a receptor mediated exocytotic mechanism for the release of the coated granules. Electron microscopic investigations in these experiments did not reveal the presence of a coating on either granule population. These results, however, do not rule out the possibility of a receptor

mediated exocytotic mechanism for the release of newly synthesized insulin.

The second possible morphological mechanism for the preferential secretion of the newly synthesized hormone involves a shorter, accelerated route via the endoplasmic reticulum. This pathway results in the release of insulin directly from the endoplasmic reticulum which forms a junction and a pore with the plasma membrane of the cell. Electron micrographs of islets "marked" with TPA (fig. 7) demonstrate the binding of the endoplasmic reticulum with the plasma membrane. The endoplasmic reticulum was repeatedly observed to be located near the plasma membrane. Ultrastructural investigations have also revealed that the endoplasmic reticulum is closely associated with secretory granules that are approaching the plama membrane (fig. 8). A short, accelerated pathway for the preferential secretion of newly synthesized involving the endoplasmic reticulum remains a possibility due to the morphological evidence obtained in this study.

Several other authors have proposed a constituitive pathway involving the endoplasmic reticulum for the release of secretory products. An accelerated pathway has been reported for the prolactin secreting cells in the anterior pituitary. It has been proposed that this pathway results in the secretion of prolactin directly from the endoplasmic reticulum (Osamura et. al., 1982). Moriarity and Tobin (1976) proposed that the endoplasmic reticulum that lied closest to the plasma membrane in thyroidectomized cells might be responsible for the secretion of thyroid stimulating hormone. The endoplasmic reticulum has been shown to approach

and bind to the plasma membrane of the mast cell (Chandler and Heuser, 1980). Recent studies indicate that the newly synthesized amylase in the exocrine pancreas might be secreted via an alternate, nonregulated pathway. The newly synthesized pancreatic amylase would bypass the granules and be released from endoplasmic reticular derived vessicles (Beaudoin et. al., 1983). With reference to these alternate secretory pathways, it is possible that the beta cell of the endocrine pancreas has a constituitive pathway as well as a regulated one. The constituitive pathway in the beta cell would involve the release of the newly synthesized insulin from the endoplasmic reticulum or possibly one of its derivitives.

The results from this study leaves three possible morphlogical explanations for the preferential release of newly synthesized insulin in response to the "marking" effect. First, a mechanism involving two populations of secretory granules was determined to be possible through electron microscopic investigations in this study. Second, these investigations also revealed that the endoplasmic reticulum may play a role in the secretion of newly synthesized insulin from the beta cell. Third, the mechanism for preferential release may be a combination of the two mechanisms proposed above.

## LITERATURE CITED


Beaudoin, A. R., Vachereau, A. and St.-Jean, P. Evidence that amylase is released from two distinct pools of secretory proteins in the pancreas. Biochima et Biophysica Acta, 75:302-305, 1983.

Chandler, D. E. and Heuser, J. E. Arrest of membrane fusion events in mast cells by quick freezing. The Journal of Cell Biology, 86:666-674, 1980.

Dudek, R. W., Charles, T. M. and Boyne, A. F. Preferential secretion of newly synthesized insulin is predictable from the ultrastructure of simulated physically fixed beta cells. Journal of Histochemistry and Cytochemistry, 31:1067-1072, 1983.

Dudek, R. W. and Charles, T. M. Physical fixation of pancreatic islets for electron microscopy. In: Methods in Diabetes Research, Vol. 1; Part A, J. Larner and S. Pohl, eds., John Wiley and Sons, New York, pp. 257-267, 1984.

Dudek, R. W., Boyne, A. F., and Charles, T. M. Novel secretory granule morphology in physically fixed pancreatic islets. Journal of Histochemistry and Cytochemistry ,929-934, 1984.

Dudek, R. W. and Boyne, A. F. An excursion through the ultrastructural world of quick frozen pancreatic islets. The American Journal of Anatomy, 175:217-243, 1986.

Fletcher, D. E., Quigley, J. P., Baver, G. E. and Noe, B. D. Characterization of proinsulin and proglucagon convertin activities in isolated islet secretory granules. Journal of Cell Biology, 90:312-322, 1981.

Gold, G., Grodsky, G. M. and Gishizy, M.C. Evidence that glucose "marks" B cells resulting in preferential release of newly synthesized insulin. Science, 218:56-58, 1982.

Gold, G. and Grodsky, G. M. Kinetic aspects of compartmental storage and secretion of insulin and zinc. Experientia, 40:1105-1114, 1984.

Gold, G., Pou, J., Nowlain, R. M. and Grodsky, G. M. Effects of monensin on conversion of prosinsulin and secretion of newly synthesized insulin isolated rat islets. Diabetes, 33:1019-1024, 1984.

Halban, P. A. Differential ratios of newly synthesized and of stored insulin from pancreatic islets. Endocrinology, 110:1183-1188, 1982.

Hoff, J. D., Lasley, B. L. Wang, C. F. and Yen, S. S. The two pools of pituitary gonadrotropin: regulation during the menstrual cycle. Journal of Clinical Endocrinology and Metabolism 44:302-312, 1977.

Katz, S. A. and Malvin, R. L. Secretion of newly synthesized renin. Endocrinology, 111:201-207, 1982.

Lacy, P. E., and Kostianovsky, M. Method of the isolation of intact islets of Langerhans from the rat pancreas. Diabetes, 16:35-39, 1967.

MacGregor, R. R., Hamilton, J. W. and Cohn, D. V. The by-pass of tissue hormone stores during the secretion of newly synthesized parathyroid hormone. Endocrinology, 97:178-188, 1975.

Malaise, W. J., Sever, A., Herchvelz, A., Carpinelli, A. R., Poloczek, P., Winland, J. and Castagna, M. Insulinatropic effect of tumor promoter 12-0 tetradecanoylphorbol-13-acetate in rat pancreatic islets. Cancer Research, 40:3827-3831, 1980

Moriarity,G. C. and Tobin, R. B. An immunocytochemical study of TSH storage in rat thyroidectomy cells with and without D orL thyroxine treatment. Journal of Histochemistry Cytochemistry, 24:1140-1149, 1976.

Orci, L. Macro- and micro-domains in the endocrine pancreas. Diabetes, 31:538-565, 1982.

Orci, L., Ravazzola, M. and Perrelet. (Pro)insulin associates with Golgi membranes of pancreatic B cells. Proc. Natl. Acad. Sci. USA, 81: 6743-6746, 1984.

Orci, L. The insulin factory: a tour of the plant surroundings and a visit to the assembly line. Diabetologia, 28:528-546, 1985.

Osamura, R. Y., Komatsu, N., Izumi, S., Yoshimura, S. and Watanabe, K. Ultrastructural localization prolactin in the rat anterior pituitary glands by preembedding peroxidase-labeled antibody method: observations in normal, castrated or estrogen-stimulated specimen. The Journal of Histochemistry and Cytochemistry, 30:919-925, 1982.

Permutt, M. Alan. Biosynthesis of insulin. In: The Islets of Langerhans S. J. Cooperstein and D. Watkins, eds., Academic Press, New York, pp. 75-96, 1981.

Steiner, D. F., Kemmler, W., Tager, H. S. and Peterson, T. D. Proteolytic processing in the biosynthesis of insulin and other proteins. Federation Proceedings, 33:2105-2115, 1974.

Virji, M. A. G., Steffes, M. W., and Estensen, R. D. phorbol myristate acetate: effect of a tumor promoter on insulin release from isolated rat islets of Langerhans. Endocrinology, 102:706-711, 1978.