HISTOCHEMICAL AND ULTRASTRUCTURAL STUDIES ON THE ISLETS OF LANGERHANS OF LEAN AND OBESE HYPERGLYCAEMIC MICE WITH AGE

by

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Summary

A histochemical and ultrastructural study has been made on the islets of Langerhans of lean and obese mice with age.

Alpha (α_2), beta (β_1 and β_2), delta (α_1) and F cells have been observed in both lean and obese mouse islets. Agranular cells were observed in the islets of old obese mice. These cells might represent atrophied β-cells.

Many morphological changes develop in the islets of old obese mice. These changes include β-cell degranulation, ceroid body accumulation, amyloid deposition, lymphocytic infiltration, platelet aggregation and islet vacuolation.

The formation of vacuoles in obese mouse islets has been examined with age. Early vacuolation is the consequence of β-cell hyperactivity, degranulation and cytoplasmic rarefaction and vacuolation. Grossly vacuolated and disrupted β-cells were observed closely applied to fragile capillaries. The latter subsequently ruptured and formed a vacuole which increased in size at the expense of the disrupted β-cell. The vacuole eventually came to occupy most of the islet volume, and was subsequently invaded by fibrous connective tissue originating from the islet capsule. This pathological deterioration of the islet tissue was probably responsible for the depressed circulating level of insulin seen in elderly obese mice.

Obesity, hyperglycaemia and raised circulating insulin levels induced in lean mice by GTG treatment suggest some mild form of insulin resistance. GTG induced obesity was not accompanied by islet vacuolation, although β-cell hyperplasia, ceroid body formation and enlargement of the Golgi apparatus and endoplasmic reticulum were observed. This would suggest that vacuolation is not a direct consequence of obesity *per se*, but the result of an interplay of hyperinsulinaemia, β-cell atrophy and some form of auto-immune phenomenon.

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C H A P T E R I

General Introduction

The first reference to the condition we now recognise as Diabetes Mellitus is to be found in the papyrus Ebers, an Egyptian medical journal already old in the time of Moses. In this journal reference is made to "the passing of frequent and large quantities of urine". The occurrence of this polyuria without pain but with emaciation and danger was recorded in a reference to diabetes by Celsus (30 B.C. - 50 A.D.). The Ionic Greek name diabetes, meaning "to run through a siphon", was coined by Aretaeus (30-90 A.D.), who also wrote of the fatal prognosis associated with the disease. Diabetes was first described as a disease of thirst by Tchang tchangking (200 A.D.), who observed a patient suffering from this disease drink ten quarts of water per day with a relative degree of polyuria.

Late in the 17th century, Willis (1675) observed the urine of the diabetic patient "to be wonderfully sweet as if imbued with honey or sugar". Dobson, in 1775, was the first to realise that the sweetness of the urine was due to sugar. A disease of the pancreas was first described as occurring in a patient dying of diabetes by Cawley (1788). The patient had multiple pancreatic calculi and widespread destruction of pancreatic tissue.

The adjective mellitus was added to diabetes by Cullen (1709-1790) to distinguish the disorder from diabetes insipidus.

As the science of histology progressed, Langerhans (1869) was able to demonstrate the presence of special areas in rabbit pancreas. In 1893 Laguesse began his studies on the cellular masses in the pancreas and he called them the "islands of Langerhans". He also suggested that the pancreatic islets constituted the organ of internal pancreatic secretion involved in carbohydrate metabolism. This view was based on the relation of the cells of the islets to the rich capillary network which surrounded them and on the presence of granules in these cells.

At about the same time, Minkowski (1889) was able to show that the removal of the pancreas from dogs produced a form of experimental diabetes.

In 1901, Opie described in detail morphological alterations in the human diabetic pancreas which included interacinar fibrosis, atrophy and varying degrees of sclerosis and hyalinisation of the islets. In addition, Weichselbaum and Stangl (1901) observed a vacuolisation in the islet cells which was termed "hydropic degeneration". These authors came to the conclusion that these morphological changes in the islets were closely related to the diabetic state.

Further progress was made by the work of Lane (1907) and Bensley (1911), who were able to separate morphologically A and B

cells in the islets. Homans (1915) investigated the development of experimental diabetes in the partially pancreatectomised dog and cat. He was able to demonstrate hydropic degeneration and eventual destruction of β-cells with permanent diabetes. The hydropic degeneration of β-cells was interpreted to be a sequela of exhaustion of this cellular system associated with excessive demand on its function. These experiments confirmed the role of the β-cells in the production of the internal secretion of the pancreas.

Various observers attempted to isolate the pancreatic hormone produced by the islets of Langerhans but were plagued by difficulties. The culmination of these endeavours was the discovery of insulin by Banting and Best (1921). These workers demonstrated an extensive lowering of blood and urinary sugar in depancreatised dogs and in many instances dramatic clinical improvement in severely diabetic animals after the administration of pancreatic extracts.

Histochemical work by Hartroft and Wrenshall (1955) and subsequent fluorescent antibody work by Lacy (1959) and Lazarus and Volk (1970) confirmed significant amounts of insulin occurred in β-cells.

The selective destruction of the β-cells of the islets of Langerhans by intravenous injections of alloxan (Jacobs, 1937;

Goldner & Gomori, 1943) provided a new approach to the study of experimental diabetes and possibly to its aetiology.

In the late 19th century, it had not gone unnoticed that many of the well-documented changes in pancreatic structure, that were characteristic of the diabetic condition, i.e. lipomatosis (fatty atrophy), islet hyalinisation and fibrosis, were present in patients, who were markedly overweight and tending towards middle-age. Consequently, it was not long before clinicians began to associate obesity with the occurrence of a maturity onset form of diabetes mellitus. Indeed, obesity, often defined as an increase in the percentage contribution of fat to the total body weight (Bray & York, 1971), is now thought to be associated with the onset of diabetes mellitus, insulin resistance (Mayer & Bates, 1953) and functional and morphological changes in the endocrine pancreas (Stern & Hirsch, 1972).

The islets of Langerhans contain at least three functionally different types of cells: alpha ($\alpha 2$) cells, which contain glucagon (Okada, Takaki & Kitagawa, 1967); beta (β) cells, which contain insulin (Hartroft & Wrenshall, 1955) and delta ($\alpha 1$) cells, which have recently been shown to contain somatostatin (Orci, 1975). In a few species, such as the dog, guinea pig and rabbit, the islets of Langerhans have been found to contain several additional cell types designated C,E,F and X (Munger, Caramia & Lacy, 1965). The

function of these additional cells has yet to be established.

The occurrence of spontaneous diabetes mellitus with or without obesity (Nakayama, Takaharo & Tsuchi, 1971; Like, Steinke & Jones, 1965) and experimentally induced diabetes (Williamson, Lacy & Grisham, 1961; Willander, 1975) is initially associated with hyperactivity of the islets of Langerhans. This hyperactivity subsequently precipitates characteristic pathological changes in islet tissue in the form of degranulation, vacuolation, hyalinisation and fibrosis.

Human diabetes mellitus has been found to occur more commonly in older individuals than in children and adolescents (Davies, 1975). Ageing can be defined as any changes in the living cell with the progression of time. The incidence of changes in the nucleus, endoplasmic reticulum, Golgi apparatus and other cytoplasmic organellae has been found to be increased with age (Franks, 1970).

Obesity has long been known to be closely associated with the occurrence of diabetes mellitus (Rabinowitz, 1970). Obesity is associated with an adipose (Salane, Knittle & Harisch, 1968) and muscle tissue (Rabinowitz & Zierler, 1962) resistance to endogenous and exogenous insulin. In addition, obesity is often accompanied by hyperinsulism, insulin resistance or insensitivity

and an impaired glucose tolerance. Diabetes, on the other hand, is often associated with the lack or insufficiency of insulin.

An important question must be answered by the authors of work associated with histochemical and histopathological research into diabetes. Does obesity influence pancreatic islet hyperfunction or vice versa? Although the majority of evidence favours obesity as the cause of pancreatic islet hyperfunction (Stern & Hirsch, 1972), the aetiology and pathogenesis of diabetes associated with obesity has not yet been satisfactorily elucidated.

Many experimental animals are characterised by a mild to moderate obesity and marked hyperglycaemia. These include the C57BL/6J mouse, the db/db mouse, the New Zealand obese mouse, the ob/ob mouse, the spiny mouse, the yellow KK mouse and the sand rat. Of special interest is the obese hyperglycaemic mouse, which is characterised by an autosomal recessive obese gene (ob/ob) with variable penetrance showing a maturity onset type of diabetes and obesity (Ingalls, Dickie & Snell, 1950; Stauffacher, Croffored, Jeanrenaud & Renold, 1967; Renold & Dulin, 1967). In this mouse, obesity can be detected by an increase in body weight at about 3-4 weeks of age (Chlouverakis, Dade & Batt, 1970). The increase in body weight continues until about 30 weeks of age. After this time, it starts to decline (Bailey, Atkins, Flatt, Best & Matty, 1977). Moderate hyperphagia, increased alimentary efficiency and

relative immobility may be responsible for the gross overweight (Mayer, Marshall, Vitale, Christevsen, Mashayekhi & Stare, 1954). Westman divided the obese mouse syndrome into three stages. The first phase is characterised by increased plasma insulin and a deteriorating glucose tolerance. In the second phase, plasma insulin reaches a peak at around 25-30 weeks of age and then begins to decline at about 30 weeks of age. In the final phase, the animal either dies or the plasma insulin and blood sugar normalise (Herberg, Major, Freytage & Gries, 1970).

Preliminary investigations into the aetiology of the obese mouse syndrome have suggested insulin resistance (Mayer & Bates, 1953; Batt & Michle, 1966). The insulin resistance is believed to be the result of reduced numbers of insulin binding sites on the plasma membranes of hepatocytes and adipocytes (Freychet, Laudat, Ressellin, Kahn, Gorden & Roth, 1972; Kahn, Neveille & Roth, 1973).

The islets of Langerhans of obese hyperglycaemic mice show many pathological changes when compared with the islets of their lean littermates. Obese mouse islets are characterised by an 8-10 fold increase in islet volume due to the hyperplasia of β-cells (Gepts, Christophe & Mayer, 1960) in response to metabolic demand. Wrenshall (1955) suggested β-cell degranulation to be an indicator of β-cell hyperactivity. Hellman and Petersson (1960) related the enlargement of β-cell nuclei and nucleoli to the

hyperactivity of these cells in the islets of obese hyperglycaemic mice. Obese islets contain some 90% β-cells (Gepts, 1960), and Hellman and Petersson (1960) suggested that this actively high proportion of β-cells in the islets of obese mice was a morphological sign of hyperactivity.

Using light microscopy, Westman (1968) was unable to detect any sign of pathological deterioration in obese mouse islets. In addition, ultrastructural studies did not reveal any marked pathological changes (Bjorkman, Hellerström & Hellman, 1963). Atkins (1972) and Atkins and Matty (1973) identified signs of pathological changes in the islets of Langerhans of old obese mouse using light microscopy. These changes included β-cell degeneration, insular fibrosis, hyalinisation and enlargement of islet β-cells. In addition, these workers observed the curious appearance of vacuoles within the islets of Langerhans of old obese mice.

The aim of this investigation was to examine the islets of Langerhans of obese hyperglycaemic mice and their lean littermates between the ages of 7 and 50 weeks of age, using light and electron microscopy, to establish an ultrastructural classification of the cell types in islet tissue and to document the cytological changes associated with hyperactivity in cells of obese mouse islets. In addition, a study by light and electron microscopy has been made to establish the sequence of morphological and ultrastructural events

that lead to the formation of islet vacuoles.

Histochemical methods have been used to demonstrate the presence of amyloid and fibrosis in the islets of ageing mice. In addition, alterations in the ultrastructural appearance of β-granules, the Golgi apparatus, endoplasmic reticulum and mitochondria in β-cells have been studied with age.

Finally, a trial study was carried out to establish whether the use of chemicals generally used to induce obesity and hyperglycaemia would produce β-cell hyperplasia and pathological lesions in lean mouse islets similar to those observed in the islets of obese hyperglycaemic mice.

CHAPTER II

Materials and Methods

2.1 Animals: the origin and characteristics of the obese hyperglycaemic syndrome in mice

Obese mice first appeared as an autosomal recessive mutation in the Jackson Laboratory in the summer of 1949, and were given the gene symbol (ob/ob) (Ingalls et al., 1950). They originated from the crossing of the offspring of V stock males and C57BL/6 females with V stock males (Mayer, Dicki, Bates & Vitale, 1951). The Aston colony was first set up in 1966 (Ariyanyagam, 1972). The original two heterozygote pairs and a number of male and female mice were obtained from the Institute of Animal Genetics, Edinburgh. They, in turn, had been obtained from Bar Harbor, U.S.A. in 1957.

Obese hyperglycaemic mice are characterised by a significantly higher body weight than their lean littermates at between 3-4 weeks of age. Obese mice are extremely slow and are not adverse to handling. They are inactive and generally do not mate, even when young, although they can be induced to mate if placed on a restricted diet from birth.

2.2 Handling and maintenance of the animals

All the animals used were housed in polythene cages. The

floor of each cage was covered with sawdust and wood shavings. The cages were cleaned out twice weekly and the water supply renewed daily. The room in which the animals were housed was regulated at an average temperature of 20°C. All animals were allowed pellet diet and water ad lib.

2.3 Isolation of pancreatic islets from exocrine tissue

Two techniques are currently available for the isolation of intact islet tissue. These are microdissection and enzyme digestion.

(a) Microdissection

Hellerström (1964) was the first to use freehand microdissection for the isolation of whole metabolically intact islets of Langerhans. His technique proved particularly suitable for use with the obese mouse pancreas, in which the islets are relatively large and easily visible within the pancreatic parenchyma.

(b) Enzyme digestion

In 1964, Moskalewski developed a technique which involved the digestion of surrounding pancreatic exocrine tissue with collagenase, and the harvesting of remaining islet tissue by sedimentation. Many modifications of the collagenase digestion method have subsequently

been described. Lacy (1967) modified the technique by sedimenting islets on a sucrose gradient. However, there is evidence to suggest that islets obtained by collagenase digestion exhibit an impaired secretion of insulin (Atkins & Matty, 1969) and cyto-morphological changes (Petkov, 1974). The isolation of islets by freehand microdissection was considered to be most suitable for the present studies.

2.4 Freehand microdissection

The microdissection procedure was carried out in a square black plastic dish containing oxgenated bicarbonate buffer (Gey & Gey, 1936). The temperature of the dissection medium was maintained at 2-4^{o}C by a water-cooled thermo-electric element. This low temperature ensured maximal viability of the tissue during microdissection. The dissecting area was viewed through an Olympus stereozoom (7-35x) microscope and illumination of the area was supplied by a single side-lamp with adjustable intensity. The dissecting tools consisted of fine watchmakers' forceps (no. 5), fine scissors, a 20-gauge hypodermic needle fixed to a glass rod and a fine pasteur pipette.

The excised pancreas was first cut into manageable portions (approximately 4mm. square) with fine scissors and all fat removed.

Half of these pieces were fixed in Bouin's fluid or buffered formalin for light microscopy. In the remaining pieces, islets were distinguished against a black background as spherical or ovoid bodies dispersed in a more translucent acinar tissue. The islets were associated with the larger blood vessels. The islets of obese mice were large, pink, highly vascularised and particularly suitable for microdissection because of their size and looseness. Microdissected islets were transferred with the aid of a finely drawn-out pasteur pipette from the dissecting dish to a black solid watchglass containing fixative for electron microscopy.

2.5 Choice of animals

Fed lean and obese mice were used between the ages of 7-45 weeks. Each experimental group contained 6 lean and 6 obese mice irrespective of sex (Table 1). All animals were killed by hyperextension of the neck. Ten islets were microdissected from each animal. Eight sections were taken from each islet and studied under the electron microscope. The physiological status of each group of animals has been summarised in Table 1.

Obese mice									Lean mice								
					E.M. Study		Light Microscopy							E.M. Study		Light Microscopy	
Age (weeks)	No. of animals	Weight (g)	Plasma insulin (ng/ml)	Plasma glucose (mg/100ml)	No. of isolated islets	No. of sections per islet	No. of blocks per animal	No. of sections per block	Age (weeks)	No. of animals	Weight (g)	Plasma insulin (ng/ml)	Plasma glucose (mg/100ml)	No. of isolated islets	No. of sections per islet	No. of blocks per animal	No. of sections per block
7	6	39 ± 6.8	15 ± 4.8	160 ± 8.3	10	8	4	50	7	6	25 ± 1.2	1 ± 0.2	130 ± 4.1	10	8	4	50
15	6	58 ± 7.5	30 ± 4.1	200 ± 10.4	10	8	4	50	15	6	28 ± 1.4	1 ± 0.18	120 ± 4.9	10	8	4	50
20	6	81 ± 8.5	32 ± 4.9	180 ± 8.9	10	8	4	50	20	6	29 ± 1.8	1 ± 0.18	120 ± 3.6	10	8	4	50
25	6	90 ± 10.2	32 ± 6.05	170 ± 8.8	10	8	4	50	25	6	30 ± 1.6	1 ± 0.24	120 ± 4.3	10	8	4	50
30	6	95 ± 12.8	32 ± 6.3	180 ± 8.4	10	8	4	50	30	6	30 ± 1.6	1 ± 0.15	120 ± 4.8	10	8	4	50
35	6	87 ± 8.3	20 ± 6.7	165 ± 8.7	10	8	4	50	35	6	30 ± 1.6	1 ± 0.23	120 ± 3.9	10	8	4	50
40	6	75 ± 8.5	14 ± 7.5	150 ± 11.6	10	8	4	50	40	6	30 ± 1.8	1 ± 0.16	120 ± 4.2	10	8	4	50
45	6	62 ± 6.8	10 ± 4.4	130 ± 10.6	10	8	4	50	45	6	30 ± 2.4	1 ± 0.18	120 ± 3.8	10	8	4	50

Table 1 Physiological status of lean and obese mice and preparative details of morphological studies.

2.6 Preparation of material for light microscopy

2.6.1. Fixation

Pieces of pancreas were fixed in the following solutions:

(a) Bouin's fluid (Bouin, 1897)

saturated aqueous picric acid	75ml.
formalin	25ml.
acetic acid	05ml.

Tissue was fixed for 24 hours at room temperature.

(b) 10% buffered formalin (Drury & Wallington, 1966)

formalin	100ml.
tap water	90ml.
sodium dihydrogen - orthophosphate ($NaH_2PO_4.H_2O$)	4g.
disodium hydrogen - orthophosphate, anhydrous (Na_2HPO_4)	6.5g.

Tissue was fixed for 24 hours at room temperature.

2.6.2 Dehydration and embedding

Specimens were treated with the following solutions:

70% ethanol, several changes (duration 24 hours)

90% ethanol, two changes (duration 1 hour)

100% ethanol, two changes (duration 1 hour)

Chloroform, two changes (duration 10-15 minutes)

50% chloroform + 50% paraffin wax, one change (duration 1 hour)

25% chloroform + 75% paraffin wax, one change (duration 1 hour)

100% paraffin wax, three changes (duration 1 hour)

2.6.3 Sectioning and staining

Serial sections 4-8 microns thick were taken from each piece of pancreas using a rotary microtome. Sections were mounted on glass slides without adhesive, cleared in xylene and hydrated by immersion in descending grades of ethanol. Sections were then stained as follows:

1. haematoxylin and eosin (Ehrlich, 1886)
2. Congo red (Putchler, 1968)
3. methyl violet (Drury & Wallington, 1966)

4.	Heidenhain's (Azan) stain	(Azan, 1916)
5.	Gomori's reticulin reaction	(Gomori, 1937)
6.	M.S.B. (Martin's, scarlet, blue)	(Lendram, 1962)
7.	P.A.S. (periodic acid, Schiff)	(Drury & Wallington, 1966)
8.	aldehyde fuchsin	(Gomori, 1950)
9.	thioflavine "T"	(Vassar & Culling, 1959)
10.	alcian blue	(Maxwell, 1963)
11.	p-dimethylaminobenzaldehyde	(Lillie, 1965)
12.	Masson's trichrome stain	(Masson, 1929)

The preparation and specificity of the above stains have been summarised in the Appendix (Page 115).

Stained sections were examined on a Zeiss photomicroscope (West Germany) and photographs were taken using Agfa colour film (din. 20).

2.7 Preparation of specimens for electron microscopy

2.7.1 Fixation

Islets obtained by freehand microdissection (Hellerström, 1964) of the excised pancreas in Gey and Gey (1936) buffer were quickly fixed in the following solutions:

1. Palade fixative (Palade, 1952)

Stock buffer solution

sodium veronal	14.7gm.
sodium acetate	9.7gm.
distilled water	500ml.

Stock osmium tetroxide solution

osmium tetroxide in distilled water	2%

Fixative

stock buffer (A)	5ml.
0.1N HCl	5ml.
distilled water	2.5ml.
2% osmium tetroxide	12.5ml.

The fixative was made up at pH 7.4. Fixation was carried out in tightly stoppered bottles at $4^{\circ}C$ for 30-60 minutes.

2. Glutaraldehyde fixation (Pease, 1964)

(a) Glutaraldehyde fixative using 6.5% glutaraldehyde in phosphate buffer

Stock solutions

i. phosphate buffer solution pH 7.2-7.4

disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$) pH 7.2 (11.872 gm./litre)	72ml.
potassium dihydrogen orthophosphate M 15($KH_2.PO_4$) (9.07 gm./litre)	28ml.

ii. 25% glutaraldehyde solution

Fixative solution (6.5% glutaraldehyde)

25% glutaraldehyde solution	6.5ml.
phosphate buffer solution (i)	18.5ml.

Rinsing solution

phosphate buffer solution (i)

Tissues were fixed in 6.5% glutaraldehyde for 4 hours, then washed in phosphate buffer solution. Fixation was carried out at room temperature ($18^{o}C$).

Postfixing solution

1% osmium tetroxide in phosphate buffer solution

After washing in phosphate buffer, tissues were postfixed in 1% osmium tetroxide/phosphate buffer solution at $4^{o}C$.

(b) Glutaraldehyde fixative using 4.5% glutaraldehyde in cacodylate solution

Stock solutions

i. cacodylate buffer solution

0.00M sodium cacodylate (10.701gm./litre)

adjusted to pH 7.2-7.4

ii. 25% glutaraldehyde solution

Fixative solution (4.5% glutaraldehyde)

25% glutaraldehyde solution	4.5ml.
cacodylate buffer solution	20.5ml.

Rinsing solution

cacodylate buffer solution

Postfixative solution

1% osmium tetroxide in cacodylate buffer solution

Fixation was carried out at room temperature (18°C), washed in rinsing solution and postfixed in 1% osmium tetroxide at 4°C for 1 hour.

2.7.2 Dehydration and embedding

Fixed islet tissue was treated with the following solutions:

50% ethanol, one change (duration 5-10 minutes)

70% ethanol, one change (duration 5-10 minutes)

90% ethanol, one change (duration 5-10 minutes)

100% ethanol, two changes (duration 5 minutes)

epoxypropane, two changes (duration 15 minutes)

50% epoxypropane + epon resin (duration 12 hours)

100% epon resin (duration 2 hours)

Islet tissue was transferred to fresh epon and cured for 48 hours at 60°C in gelatin capsules. The gelatin was removed in hot water and the block cured at 70°C for a further 2 hours. The blocks were then trimmed to a pyramidal shape with a safety razor blade prior to sectioning on an LKB ultramicrotome.

2.7.3 Sectioning and staining

Glass knives were prepared on an LKB knife maker (LKB, Stockholm, Sweden). Thin sections (500nm - 800nm) were prepared on an LKB ultramicrotome. The sections were stretched with chloroform vapour before being attached to carbon-coated copper grids. The sections were then stained as follows:

saturated aqueous uranyl acetate (duration 15 minutes)
distilled water, twice (duration 2 minutes)
lead citrate (duration 1-1.5 minutes)
0.02% NaOH (duration 45 seconds)
distilled water, twice (duration 2 minutes)

Staining was carried out in dental wax depressions and sections were examined at 50 Kv on an AEI E6B electron microscope (AEI, Manchester).

Micrographs were evaluated at magnifications between 3,000x - 7,500x on the microscope and a standard adjustment was made to the enlarger to provide a magnification from plate to print of 2x.

2.8 Choice of micrographs

Representative photomicrographs were chosen to illustrate both

the types of islet cells present and the sequence of morphological changes taking place in obese mouse islet β-cells during the manifestation of the syndrome. Where possible, the situation in the obese mouse islet β-cell has been compared with its lean mouse islet control of the same age.

2.9 Discussion

A solution of 4.5% glutaraldehyde in sodium cacodylate buffer or a solution of 6.5% glutaraldehyde in phosphate buffer with post-fixing in 1% osmium tetroxide in phosphate buffer was found suitable for the electron microscopical examination of pancreatic islet tissue from obese hyperglycaemic mice and their lean littermates. This protocol provided an acceptable technique for the preservation of the cytoplasmic organellae of pancreatic islet cells. The Palade fixative was shown to give lower contrast images in this study. Aldehyde fixation has long been used for the preservation of proteinaceous material because of its rapid penetration properties (Dawes, 1971). The use of osmium tetroxide as a post-fixative gave the cytoplasmic organellae electron density. Aldehyde, on the other hand, gave a bland image (Pease, 1964). Osmium tetroxide fixation is also known to be a superior preserver of cytological detail (Pease, 1964).

CHAPTER III

Morphological Examination of the Pancreatic Islets of Obese Hyperglycaemic Mice and their Lean Littermates

3.1 Ultrastructural examination and classification of the islet cells of obese hyperglycaemic mice and their lean littermates

3.1.1 Introduction

Since the initial identification of pancreatic islets by Paul Langerhans in 1869, many morphological studies have been carried out using light microscopical techniques. The application of histochemical techniques permitted the preliminary differentiation of islet cells into A,B,C,D and X (Lane, 1907; Bensly, 1911; Bloom, 1931; Gomori, 1939).

With the development of the electron microscope, Lacy (1957a & 1957b), Bencosme and Pease (1958), Caramia (1963), Caramia, Munger and Lacy (1965), and Munger, Caramia and Lacy (1965) documented the ultrastructural characteristics and classified the different types of pancreatic islet cells in various species, including dogs, cats, rabbits, guinea pigs and rats. Islet cells were classified as alpha, beta and delta. Other cells were classified as C,E,F and X.

The β-cell was characterised by the presence of secretory granules each consisting of a centralised electron dense granule core enclosed within a smooth membranous sac and separated from it

by a halo or clear space (Lacy, 1957a & b; Bencosme, 1958; Munger et al., 1965). The shape of the granule varied with the species of animal, being irregular in guinea pigs, bar-shaped in the dog and almost spherical in the rabbit and rat (Lacy, 1957b). β-cells were characterised by the presence of large numbers of granules, numerous mitochondria, a well-developed endoplasmic reticulum and a well-developed Golgi apparatus. β-cells occupied the bulk of the islet volume and made up some 80% of the total cells present.

Alpha (α_2) cells were characterised by their secretory granules, which were more electron dense than β-granules (Lacy, 1957a & b; Munger, 1958; Caramia et al., 1965). The limiting membranous sac of the alpha cell granule was very closely applied to the granule core. The number of mitochondria and ribosomes and the extent of the endoplasmic reticulum and Golgi apparatus were somewhat reduced compared to the β-cell.

The C-cell was first identified in the guinea pig (Bensly, 1911), and was characterised by having pale cytoplasm, few mitochondria, and a limited endoplasmic reticulum and Golgi apparatus. The C-cell contained no distinct secretory granules (Lacy, 1957). These cells were termed agranular cells in Cyclostomian and Teleostean species (Falkmer, 1964) and in higher vertebrates like the Chinese hamster (Boquist & Falkmer, 1970). The C-cell was suggested to be either the precursor of a granulated cell (Boquist &

Falkmer, 1970), or an immature islet cell (De Huyas-Guevara, 1969). In addition, it was believed that it might represent either a degranulated alpha cell (Munger et al., 1965) or a degranulated β-cell (Sato, 1966). Fujita (1968) suggested that the C-cell of the guinea pig pancreatic islet might represent the D-cell of other species.

The delta (α_1) cell was first described by Bloom in 1931. Delta cells have been found in the pancreatic islets of many species including rabbit, dog, rat and pigeon (Caramia et al., 1965; Munger et al., 1965; Kobayashi, 1969; Legg, 1967; Orci, Baetens, Doubois & Rufner, 1975; Thomas, 1975; Watanabe, Paik & Yasuda, 1975). Delta cells have been characterised by their variable electron dense granules (Bencosme & Pease, 1958). Winborn (1963) described monkey delta cell granules asempty sacs, whilst in the rabbit, delta cells were observed to contain both empty and solid types of granules (Mayer & Bencosme, 1965). The delta cell was thought to be either an early or late secretory stage of an alpha (α_2) or beta cell (Bencosme, Allen & Latta, 1963). Ferner & Stoeckenius (1963) suggested that the delta cell represented a degenerative type of cell, but this hypothesis was subsequently rejected by Caramia (1963) and Legg (1963), who suggested the delta cell to be an active, distinct cell type because of the presence of endoplasmic reticulum and Golgi apparatus.

Several different cell types have been identified in the islets of Langerhans of other animal species. An E-cell has been identified in opossum islets (Munger et al., 1965) and an F-cell has been observed in the pancreatic islets of dog (Munger et al., 1965) and mouse (De Huyas-Guevara, 1969). The presence of a G-cell has been described in human foetal pancreas (Like & Orci, 1971), whilst a W-cell has been described in the islets of Langerhans of a bat (Watari, 1968). An X-cell has been described in the pancreatic islets of Cyclostomes (Fujita, 1962) and dog (Bencosme & Liepa, 1955). The functional significance of all these different cell types has not yet been established.

The islets of Langerhans of obese hyperglycaemic mice and their lean littermates have been shown to contain a high percentage (90%) of β-cells (Gepts et al., 1960). Obese mouse islets show considerable hypertrophy and their total islet volume is some 8–10 times larger than that of their lean littermates (Gepts et al., 1960; Hellman, Hellerström, Larsson & Brolin, 1961). In the islets of the obese mouse, the β-cells are degranulated (Wrenshall, Andrus & Mayer, 1955) and contain enlarged nuclei and nucleoli (Hellman & Petersson, 1960). These characteristics have been regarded as signs of increased functional activity. The pancreas of the obese mouse contains a higher proportion of islet tissue than that of the lean littermate (Hellman et al., 1961; Westman, 1968), but obese mouse islets contain a lower proportion of alpha

cells than lean littermate islets (Hellman, 1961).

To date, the only electron microscopical work on obese mouse islets has been carried out by Bjorkman and colleagues in 1963. This study set out to describe the ultrastructural features of the cell types in the islets of Swedish ob/ob mice and their lean littermates. These workers disagreed with the preliminary classification of Lacy (1957, 1961), Munger (1962) and Caramia (1963). Bjorkman and co-workers (1963) related their ultrastructural findings to the light microscopical classification of the alpha cells into α_1 and α_2 cell types initially made by Hellerström and Hellman in 1961. They described typical alpha (α_2) cells to be syncytial, and classified the argyrophillic alpha cell as a cellular cell. Caramia (1963) and Munger and colleagues (1965) considered the argyrophillic islet cell (α_1), initially described by Hellman (1961), to be equivalent to the electron microscopically defined delta cell. However, cells having the same ultrastructural features as the syncytial α_2 cell, initially described by Bjorkman and co-workers (1963), have been described in many species as secretory granule rich β-cells (Lambert, Orci, Kanazawa, Renold & Rouiller, 1970; Creutzfeldt & Frerichs, 1970; Perrier-Barta, Felix & Jacquot, 1975; Thliveris, 1975).

The present study is designed to re-examine the ultrastructural classification of cell types in the islets of obese hyperglycaemic mice and their lean littermates.

3.1.2 Materials and methods

10 microdissected islets from each animal were processed for electron microscopy as described previously (page 18). 8 sections were cut from each islet and examined under the electron microscope.

3.1.3 Results

The islets of both lean and obese mice were either partially or completely separated from surrounding acinar tissue by a narrow connective tissue space containing collagen fibres. Two basement membranes separated the islet cells from the lumen of adjacent capillaries. One side of the basement membrane was applied to the plasma membrane of the islet cell, and the other was applied to the plasma membrane of the capillary endothelial cell. All islet cells are surrounded by a continuous but fragile plasma membrane and are closely applied to each other.

The following types of cells were identified in the islets of Langerhans of obese hyperglycaemic mice and their lean littermates.

The beta cell (β-cell)

According to the ultrastructural classification initially established by Lacy (1957, 1961) and subsequently by Munger and co-workers (1965), two types of β-cell have been identified in the islets of Langerhans of obese hyperglycaemic mice and their lean littermates:

(a) β_1-cell

β_1-cells occupy the central portion of both lean and obese mouse islets. They are uniform in appearance and characterised by the presence of numerous dense secretory granules, each enclosed by a well-defined smooth membranous sac. The β-granules vary in shape and size, most of them are spherical or ellipsoid (Plates 1 & 2). Pale and dense granules could be identified on the basis of their central core electron density. The less dense, pale granules were somewhat larger and occupied a relatively larger volume of their enclosing sacs than the smaller dense granules (Plate 2). The Golgi apparatus was well-developed and consisted of a collection of smooth membranous sacs widely dispersed throughout the cytoplasm. Coated and smooth surfaced vesicles were often associated with the Golgi apparatus (Plate 2). The larger mitochondria were either rod or ovoid in shape and contained a dense matrix with parallel christae (Plate 1). The endoplasmic reticulum (ER) was organised into a number of parallel tubular or vesicular

cisterns, with varying degrees of dilatation. Ribosomes were both attached to the endoplasmic reticulum and distributed free in the cytoplasm (Plate 1). The nuclei were spherical or ovoid in shape with one or two prominent nucleoli (Plate 1). Lysosomes were present as homogenous spherical bodies distributed throughout the cytoplasm.

(b) β_2-cell

The β_2-cell appeared to be similar to the syncytial α_2 cell originally proposed by Bjorkman, Hellerström and Hellman (1963), and resembled the polyploid beta cell recently reported to be present in the islets of Langerhans of C57 mice (Bowen & Swartz, 1976). The β_2-cells were irregular in shape, surrounded by a continuous plasma membrane (Plate 3), and often contained more than one nucleus. The nuclei were often irregular in shape, with high electron density. The nucleoli were less prominent than in other islet cells. The β_2-cell was characterised by the abundance of β-granules tightly packed together. These granules were characterised by their spherical to ovoid shape and marked electron density. There was a relatively large space between the granule and its membrane (Plates 4 & 5). The granules were packed close to the nucleus and the nuclear membrane could not normally be identified (Plates 3 & 4). Both dense and pale granules were present in the cytoplasm of the β_2-cell. The granules were so tightly packed in the cytoplasm that they tended to occlude most of the other

cytoplasmic organellae (Plate 3). The endoplasmic reticulum, when observable, consisted of fine tubules with granular membranes (Plate 3). Many large elongated mitochondria could be observed with pronounced christae (Plate 5). The Golgi apparatus was not easily visible, but, when present, consisted of a collection of small tubules (Plate 6). The β-granules of the β_2 cells were arranged in chains and orientated towards the plasma membrane.

The alpha (α_2) cell

The α_2-cells were characterised by their peripheral location in the mouse islet. Their secretory granules had a greater electron density than β-granules, were larger in size and had a regular spherical shape. The α_2-granules were enclosed in membranous sacs, but contained a minimum of space between the smooth membranous sac and the granule. There was little or no variation in electron opacity between alpha (α_2) granules (Plate 7). The Golgi apparatus of the alpha cell consisted of small irregular lamellae and vesicles (Plate 8). The endoplasmic reticulum consisted of narrow tubules dispersed through the cytoplasm and ribosomes were present both attached to the tubules and free in the cytoplasm. The mitochondria were prominent, ovoid or rod shaped, whilst the nucleus was mainly spherical to ovoid in shape (Plate 8).

The delta (α_1) cell

Delta cells were found in the islets of both lean and obese mice. These cells were generally polygonal or elongated in shape (Plate 9). The delta granules were of variable electron density, spherical, abundant and enclosed by a smooth limiting membrane very closely applied to the granule core. This limiting membrane was often indistinct (Plates 1 & 9). The Golgi apparatus was not prominent and the endoplasmic reticulum was tubular and dispersed throughout the cytoplasm (Plate 1). The mitochondria were rounded and elongated with low electron density (Plates 1 & 8). The nuclei were spherical to oval in shape (Plate 1).

The F cell

Both lean and obese mouse islets contained a cell which appeared to be similar to the "F" cell originally described by Munger and co-workers (1965). This cell was characterised by the presence of a large number of polarised, moderately electron opaque secretory granules. The granules were round or oval in shape. The granule sacs were not easily visible. The F cell was observed at the periphery of the pancreatic islets (Plate 10).

3.1.4 Discussion

The present ultrastructural study has established the presence of several well-defined cell types in the islets of Langerhans of obese hyperglycaemic mice and their lean littermates.

The ubiquitous β_1-cell is easily recognised by its secretory granules and its uniform nucleus. As regards the β_2-cell classification, Bjorkman, Hellerström and Hellman (1963) first identified these cells as syncytial α_2-cells. These workers disagreed with the alpha cell classification proposed by Lacy (1957, 1961), Bencosme and Pease (1958), Caramia (1963), and Munger and co-workers (1962, 1965), and considered the β_2 syncytial cell to be an alpha cell and the alpha cells to be cellular cells. Caramia (1963) considered the argyrophillic alpha (α_1) cell to be equivalent to the electron microscopically defined D cell. However, in the β_2-cell, the space between the granule cores and their limiting membranous sacs is very large. The cytoplasm is very dense and granule opacity is somewhat less than that of granules in the traditional alpha (α_2) cell. Both dense and pale granules can be identified in the β_2-cells. The latter tend to interdigitate between β_1-cells and occupy most of the central area of the pancreatic islet. β-cells make up the greatest proportion of cells in both lean and obese mouse islets (Westman, 1968). The β-cell contains large, well-spaced granules and dense cytoplasm (Lacy, 1961; Munger *et al.*, 1965).

The present study would suggest that the syncytial α_2-cell of Bjorkman, Hellerström and Hellman (1963) is, in fact, a β_2-cell. This assumption is consonant with ultrastructural evidence previously provided by Lacy (1957, 1961), Munger and colleagues (1962, 1965) and Caramia (1963). Lambert and colleagues (1970) and Thliveris (1975) called this cell a secretory material rich β-cell. The β_2-cell differs from the β_1-cell by virtue of its irregularly shaped nucleus and its large number of tightly packed granules. It is suggested that the β_2-cell might be an insulin storage cell. Insulin granules might pass from β_1-cells to β_2-cells. This storage hypothesis is consonant with the two pool theory of insulin biosynthesis and release described by Grodsky (1970).

The presence of insulin in β-cells has been demonstrated histochemically (Hartroft, 1955) and immunohistologically (Lang, 1973, 1975; Orci et al., 1975). Further immunohistological studies are needed to demonstrate the presence of insulin in both β_1- and β_2-cells in order to establish beyond doubt that the β_2-cell is, in fact, a β-cell and not a syncytial α_2-cell.

The present studies have also demonstrated the presence of alpha (α_2) cells in the peripheral area of both obese and lean mouse pancreatic islets. Glucagon has been demonstrated immunochemically in α_2-cells of many species, including Sprague-Dawley rats (Goldsmith, Rose, Arimura & Ganong, 1975) and pigeon

(Orci et al., 1975). The functional role of glucagon in lean and obese mouse pancreatic islets and its mechanism of secretion remains to be established.

Delta cells (D-cells) have also been identified in the islets of obese mice and their lean littermates. D-cells have been identified in the pancreatic islets of many species, including rats (Caramia, 1963), rabbits and dogs (Munger et al., 1965), guinea pigs (Legg, 1967) and pigeons (Kobayashi, 1969). Early work by Ferner and Stoeckenius (1961) suggested that the delta cell was a degenerative cell, while others suggested that the delta cell might be a precursor or a late secretory stage of either an α_2- or β-cell (Creutzfeldt, 1975). The presence of a well-defined Golgi apparatus and endoplasmic reticulum suggests that the delta cell is an active secretory cell (Caramia, 1963; Legg, 1967). It has been suggested that a gastrin-like substance is actively secreted from D-cells (Solcia & Sampieter, 1965). Lomosky, Langer and Vortel (1969) demonstrated gastrin I- and II-like activity in the D-cells of human, rat and guinea pig islets. Gastrin could not be demonstrated in the D-cells of either rat, mouse (Lacy, 1970; Dorn, Loenz, Hahn, Koch & Ziegler, 1974) or pigeon islets (Orci et al., 1975). Kimmel, Pollock and Hazelwood (1971) have isolated a pancreatic polypeptide (App) containing 36 amino acids from avian pancreata. Hazelwood (1973) suggested that "App" was another pancreatic hormone with gastrin-like activity. Using immunofluorescence

microscopy, Larsson, Sunder, Hakanson, Pollock and Kimmel (1974, 1976) demonstrated App cell granules to be similar but not identical to those of delta cells. Recently, Goldsmith and co-workers (1975) and Orci and co-workers (1975, 1976), using fluorescent antibody techniques, demonstrated the presence of somatostatin in the delta cells of rat and pigeon islets.

The ultrastructural work presented here documents the presence of delta cells in the islets of both obese hyperglycaemic mice and their lean littermates. Further immunohistological studies will be required to demonstrate the presence of somatostatin in the delta cells of obese hyperglycaemic mice and their lean littermates.

The present study has been the first to document the presence of the so-called "F-cell" in the pancreatic islets of lean and obese mice. This cell is characterised by its large size and polarised, tightly packed, grape-like, electron dense secretory granules. The functional importance of this cell has not yet been established.

3.2 Ultrastructural evidence for proposed mechanisms of insulin secretion

3.2.1 Introduction

Two probable intracellular sites of β-granule formation have been suggested. Lacy (1961), Williamson and Lacy (1961), Volk and Lazarus (1963), Bjorkman and Hellman (1964), and Williams and Ensinck (1966) suggested that secretory material was elaborated in the region of the granular endoplasmic reticulum and that the latter was subsequently transformed from a lamellar form to a vesicular form. The β-granule then appears to form within the vesicle and becomes a mature secretory granule with the disappearance of ribosomes from the endoplasmic reticulum. Other authors have suggested that β-granules are formed in the Golgi region (Morris & Richman, 1960; Lever & Findlay, 1961; Herman, Sato & Fitzgerald, 1964; Burton & Vensel, 1966). Vesicles resembling β-granule sacs are suggested to be formed within the Golgi apparatus. This is followed by a condensation of secretory material into the sac to give the secretory granule, which separates off in a dilated portion of the Golgi apparatus. Autoradiographic studies using isolated islet tissue incubated with tritiated leucine and tyrosine have provided some understanding of the complex mechanism of β-granule formation (Howell, Kostianovsky & Lacy, 1969; Orci, Lambert, Amherdt, Cameron, Kanazawa & Stauffacher, 1970; Orci, 1974).

Radio-activity, associated with the insulin precursor, proinsulin, appears to be associated with elements of the granular endoplasmic reticulum. Subsequently, radio-activity, associated with insulin, appears in the Golgi vesicles containing secretory granules. The conversion of proinsulin to insulin is believed to take place somewhere between the granular endoplasmic reticulum and the appearance of mature secretory granules in the Golgi region (Howell et al., 1969; Orci, 1974).

Many hypotheses have been put forward to explain the mechanisms by which secretory granules are released from the β-cell. The exocytosis of secretory granules into the extracellular space has been termed "emiocytosis" (Lacy, 1961; Williamson et al., 1961; Lazarus & Volk, 1962). It has been suggested that the entire granule is discharged into the extracellular space by the fusion of its limiting membrane with the plasma membrane. The rate of emiocytosis has been found to increase when islets are exposed to insulinotrophic agents such as sulphonylureas (Orci et al., 1975). Recently, Berger, Dahl and Meissner (1975) suggested that the fusion of granules with the plasma membrane might be a mechanism by which the contents of several granules could be released into the extracellular space. Emiocytotic pores in the plasma membrane have rarely been observed (Berger et al., 1975). However, cytoplasmically distributed granules must somehow move to the region of the plasma membrane before being discharged. Matthews (1970)

suggested that Brownian movement of granules in the cell cytoplasm might account for the speed of onset of insulin secretion in response to a stimulus. Lacy, Howell, Young and Fink (1968) indicated that the presence of microtubular structures in the pancreatic islet β-cell cytoplasm could be responsible for the intracellular transport of β-granules to the plasma membrane during the process of emiocytosis. Subsequent investigations have confirmed the possible role microtubules might play in the process of insulin secretion (Malaisse, Malaisse-Lagé, Baird & Lacy, 1971; Malaisse, 1973). These investigations showed that the mitotic spindle inhibitors, like colchicine and vincristine, and microtubular stabilisers, such as deuterium oxide (D20), suppressed insulin release induced by glucose or sulphonylureas. This suppression was associated with a rapid disappearance of cytoplasmic microtubules. On the other hand, Berger and co-workers (1975), using a freeze-etching technique, recently demonstrated the occurrence of membranous connections between adjacent secretory granules in β-cells. They suggested that this inter-granule coupling might represent a mechanism for the rapid and quantitative release of insulin. Another type of connection has been observed between β-granule membranes in β-cells of rat pancreatic islets. Lingualae have been reported to be present between adjacent secretory granules (Gabbay, Korf & Schneeberger, 1975). The process of linguala formation has been called "binesis" and the process is thought to account for the active release of

β-granules. Linguala formation appears to be stimulated by increasing the glucose concentration.

However, emiocytosis might not be the only mechanism available for the discharge of secretory granules from pancreatic islet β-cells. Orci, Stauffacher, Beaven, Lambert, Renold and Rouiller (1969) and, later, Orci and co-workers (1970) have described a process of β-cell degranulation (β-granulolysis) in the spiny mouse pancreatic islets. The process is best described as the intracellular destruction of mature secretory granules. This phenomenon has also been reported to occur in human islet β-cells (Nakayama et al., 1971). On the other hand, Lever and Findlay (1966) suggested that the intracytoplasmic release of secretory material might occur through hiatuses occurring in the β-granule membranes. Other investigators have postulated a diacrine type of secretion (Fujita & Matsuno, 1967; Watari, Tuskagoshi & Honma, 1970). These workers observed β-granule sacs near the plasma membrane of rabbit islet β-cells. These β-granule sacs were either empty or contained material with low electron density. It has been suggested that secretory substances might be released initially through the β-granule limiting membrane and subsequently through the β-cell plasma membrane (Watari et al., 1970).

3.2.2 Materials and methods

Pancreatic islet tissue from obese hyperglycaemic mice and their lean littermates was prepared for electron microscopy as described previously (page 18).

3.2.3 Results

The endoplasmic reticulum of the β_1 cell was seen to be organised into a number of tubular and vesicular cisternae. Ribonucleoprotein granules were attached to the outer surface of the endoplasmic reticulum as well as being dispersed in the cytoplasm. The ends of the tubules of endoplasmic reticulum were swollen and appeared to be cut off into discrete vesicles (Plate 2). These vesicles contained grey amorphous material. In addition, fully formed β-granules were often associated with the terminal ends of the endoplasmic reticulum.

The Golgi apparatus of the β_1 cell was variable in size and consisted of smooth membranous sacs. The latter contained material of varying electron density and were located within the cisternae of the Golgi apparatus (Plates 2 & 13). Ribosomes were also usually associated with these vesicles. In some instances, mature granules were observed near the Golgi apparatus (Plates 2 & 11).

Adjacent islet cell membranes were usually closely applied to each other, but well-defined intercellular spaces were also occasionally observed (Plate 12). Microvilli and cytoplasmic projections containing β-granules have been observed in these spaces. Granules were often observed attached to the plasma membranes of β_1 and β_2 cells (Plates 5 & 13). Groups of β-granules line up to form columns or tandem-like structures in the cytoplasm (Plate 6). The outer membranes of the secretory granules often seem to be fused with each other, forming semitubular-like structures (Plate 6). β-granules of varying electron density have been observed in β_2 cells. The cores appear to undergo dissolution within the granule sacs, finally disappearing completely. After the dissolution of the core, the empty granule sac remains as an electron lucent space (Plates 5 & 6). Empty granule sacs appear to be more abundant in the β_2 cells of obese mouse islets than in the same cells of lean mouse islets.

Another possible type of intracellular dissolution has been observed in β_1 and β_2 cell granules of obese mouse islets (Plates 14 & 15). The β-granules of both cells are large in size and show evidence of membrane discontinuity. This discontinuity appears as scrolling in section (Plate 15). The scrolling of β-granule membranes might be a pathological change associated with exhaustion atrophy, the result of an excessive metabolic demand for endogenous insulin in the presence of insulin resistance.

3.2.4 Discussion

It appears that there may be more than two methods of granule formation in β-cells. Many authors consider secretory granules to be formed within the granular endoplasmic reticulum (Lacy, 1961; Williamson et al., 1961). There is some support for the hypothesis that suggests that β-granules are formed within the Golgi apparatus (Logothetopoulos, 1966). In the present study, the transformation of granular endoplasmic reticulum from the lamellar form to the vesicular form and the presence of vesicles of varying electron density near the Golgi apparatus make it difficult to ascribe with certainty any form of hypothesis regarding the origin of the β-granule. However, certain observations made in the present investigation support the work of Howell, Kostianovsky and Lacy (1969), who demonstrated that amino acids are incorporated into proinsulin at the level of the endoplasmic reticulum and subsequently appear in β-granules. The Golgi apparatus would seem to participate either in the formation of β-granules and/or the translocation of granules through the cytoplasm of the cell. Orci and co-workers (1969) support this explanation and have suggested that the conversion of proinsulin to insulin occurs at some point between the granular endoplasmic reticulum and the formation of the mature granule at a time when the newly synthesised protein passes through the Golgi area and becomes encapsulated.

The present study has demonstrated the presence of junctions between β-granule membranes and the plasma membrane of both β_1 and β_2 cells. These junctions might represent the emiocytotic figures originally described by Lacy (1961). Equally well, they might represent the site of exchange of ions and metabolites (Berger et al., 1975). The fusion of the granular sac with the plasma membrane is not often observed because the point of opening of the secretory granule at the plasma membrane tends to coincide with the plane of the thin section (Orci, 1974).

In the present study, microtubules were not observed in the cytoplasm of either β_1 or β_2 cells. Microtubules have been suggested to be involved in the intracellular transport of secretory granules during the process of emiocytosis (Lacy, 1968). On the other hand, Ericson and Lundquist (1975) suggested that microtubules might only play a minor role in the insulin releasing process. However, the tandem-like arrangement of secretory granules in the β_2 cell cytoplasm of both obese and lean mouse islets and the breaking down or coalescing of adjacent β-granule membranes would support the observations of Berger, Dahl and Meissner (1975) and suggest a possible mechanism for the rapid transfer of granule contents to the plasma membrane and the rapid release of insulin.

The present work also suggests that, besides emiocytosis, secretory granules might also release their contents via intragranular dissolution. The presence of secretory granules with varying electron density and granules lacking core material suggests the intragranular dissolution of secretory material and its passage into the cell cytoplasm. This dissolution of secretory material has been called β-granulolysis (Orci et al., 1969, 1970), and appears to occur in β-cells after stimulation by sulphonylureas. Although the presence of empty granules might possibly be a function of the plane of cross-section through the granule, the definite presence of empty granules has been confirmed by serial sectioning and is only seen in β_2 cells.

The phenomena of granulolysis, emiocytosis, vacant granules and granule scrolling have been observed in animals treated with alloxan and various sulphonylureas (Lever & Findlay, 1966; Watari, 1970; Berger et al, 1975). The presence of β-granulolysis seems to occur more frequently in obese mouse islets than in lean mouse islets. The presence of β-granule scrolling has only been observed in obese mouse islets. These phenomena might be the result of an increased demand for endogenous insulin in the obese mouse.

The presence of pale granules in many β-cells is thought to be due to the rapid release of insulin from the cells inducing

rapid insulin biosynthesis (Shino et al., 1970; Nakayama et al., 1971; Wellmann, Volk & Brancato, 1971). Pale granules have been suggested to be precursors of secretory granules and the product of the polymerisation of the contained insulin (Herman et al., 1964), although Howell and co-workers (1969), using radio-active leucine, did not confirm this hypothesis. Pale granules appear to be present in increased numbers in obese mouse islets. Their presence there would reflect the rapid biosynthesis of new insulin in response to an elevated metabolic demand for endogenous insulin at the height of the syndrome.

3.3 Islet cell innervation

3.3.1 Introduction

The presence of nervous tissue in the islet has been confirmed by many authors (Lacy, 1957; Legg, 1967; Orci et al., 1970, 1974; Smith, 1975). Fluorescence microscopy has been used to demonstrate the presence of both cholinergic and adrenergic nerve fibres in the islets of several species (Esterhuizen, Spriggs & Lever, 1968; Fawcett, 1969). The number of nerve fibres varies from species to species (Lacy & Greider, 1972). Nerve fibres are usually enclosed by unmyelinated Schwann cells (Woods & Portá, 1974; Smith, 1975), although some naked axons have been

observed (Watari, 1968). Orci, Perrelet, Ravazzola, Malaisse-Lagé and Renold (1973) demonstrated the presence of extracellular spaces between islet cells and nerve endings in rat islet tissue and called them "gap junctions". The presence of islet cell nerve endings has not been confirmed in obese hyperglycaemic mice and their lean littermates. In the present work, electron micrographs have been examined for the presence of nervous tissue and a possible neural involvement in the mechanism of insulin secretion has been discussed.

3.3.2 Materials and methods

Pancreatic islets were processed for electron microscopy as described previously (page 18) and examined for the presence of nerve endings.

3.3.3 Results

Single and small bundles of myelinated and unmyelinated nerve fibres and their endings were frequently observed close to β-cell membranes and the basal membranes of islet capillaries (Plates 16 & 17). These nerve endings were frequently observed to contain mitochondria (Plates 18 & 20) and synaptic vesicles (Plate 17). Other nerve endings contained granular vesicles with

dense core material (Plate 18). Some nerve fibres were encapsulated with connective tissue (Plate 17). Axons were either completely naked (Plate 17) or partially surrounded by Schwann cell cytoplasm (Plate 19).

3.3.4 Discussion

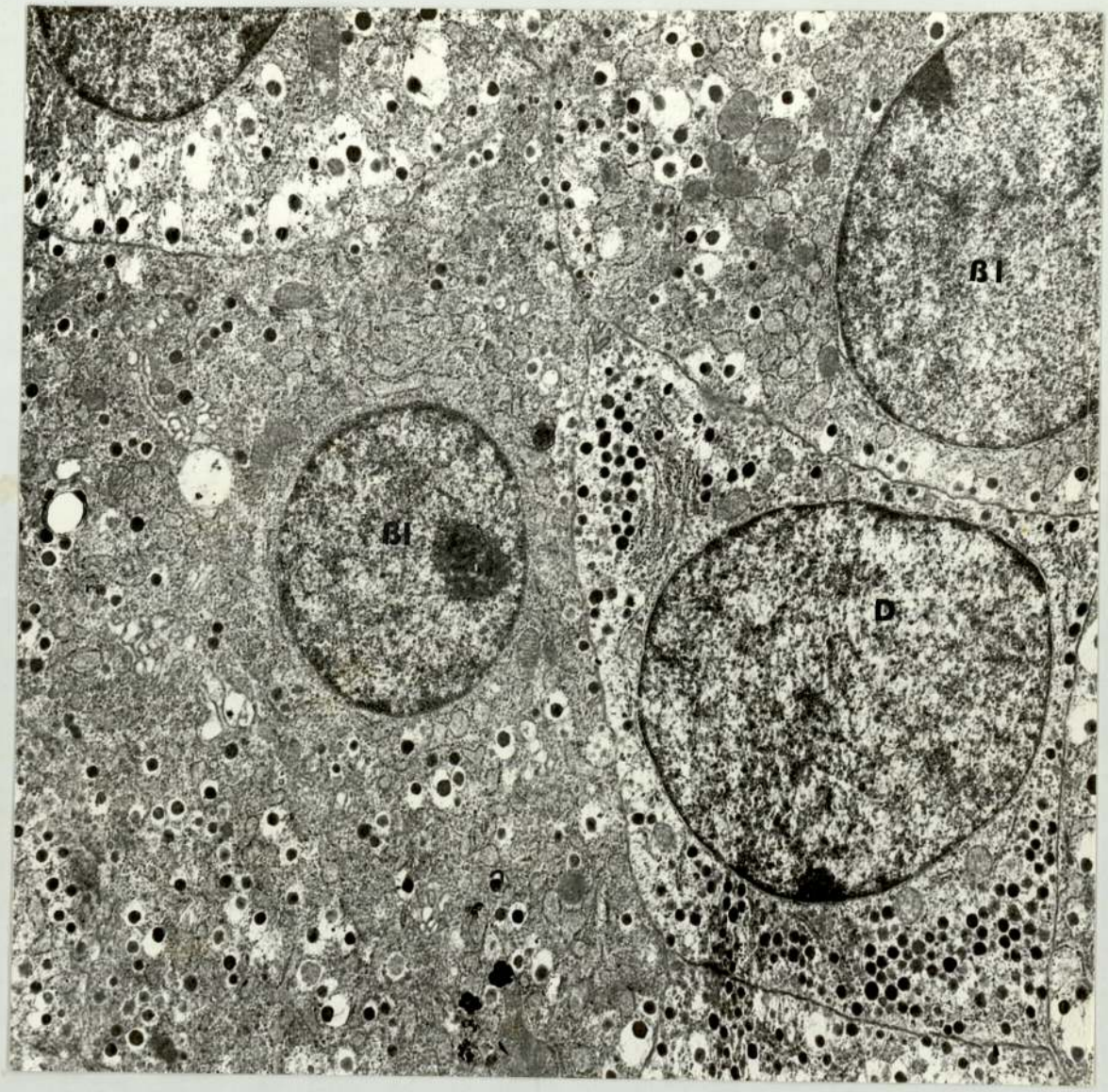
The present examination has indicated the presence of nerve endings in the islets of obese hyperglycaemic mice and their lean littermates. The presence of granular electron dense vesicles in some nerve endings and agranular vesicles in others suggests that they might be adrenergic and cholinergic nerve fibres respectively. These structures resemble the adrenergic and cholinergic nerve endings previously observed in cat pancreatic islets by Esterhuizen and co-workers (1968). Orci and co-workers (1973) observed similar nerve endings in rat islets. Adrenergic nerves are characterised by the presence of granular vesicles (Richardson, 1964; Tice & Creveling, 1975). Cholinergic nerves are characterised by the presence of empty vesicles (Orci et al., 1975). The Schwann cells observed in some micrographs might facilitate the transfer of electronic signals from the nerve fibres to the islet cell (Smith, 1975). The neural regulation of insulin secretion has yet to be confirmed. There is evidence of an increased insulin secretion following the stimulation of the vagus nerve (Kaneto,

Kosaka & Nakao, 1967). In addition, the autonomic nervous system might well be involved in the secretion of insulin from islet β-cells and the release of glucagon from the α_2 cells.

Further studies are required to differentiate the characteristics and functions of the nerve supply to the cells of the pancreatic islets of obese hyperglycaemic mice and their lean littermates.

PLATES 1-20

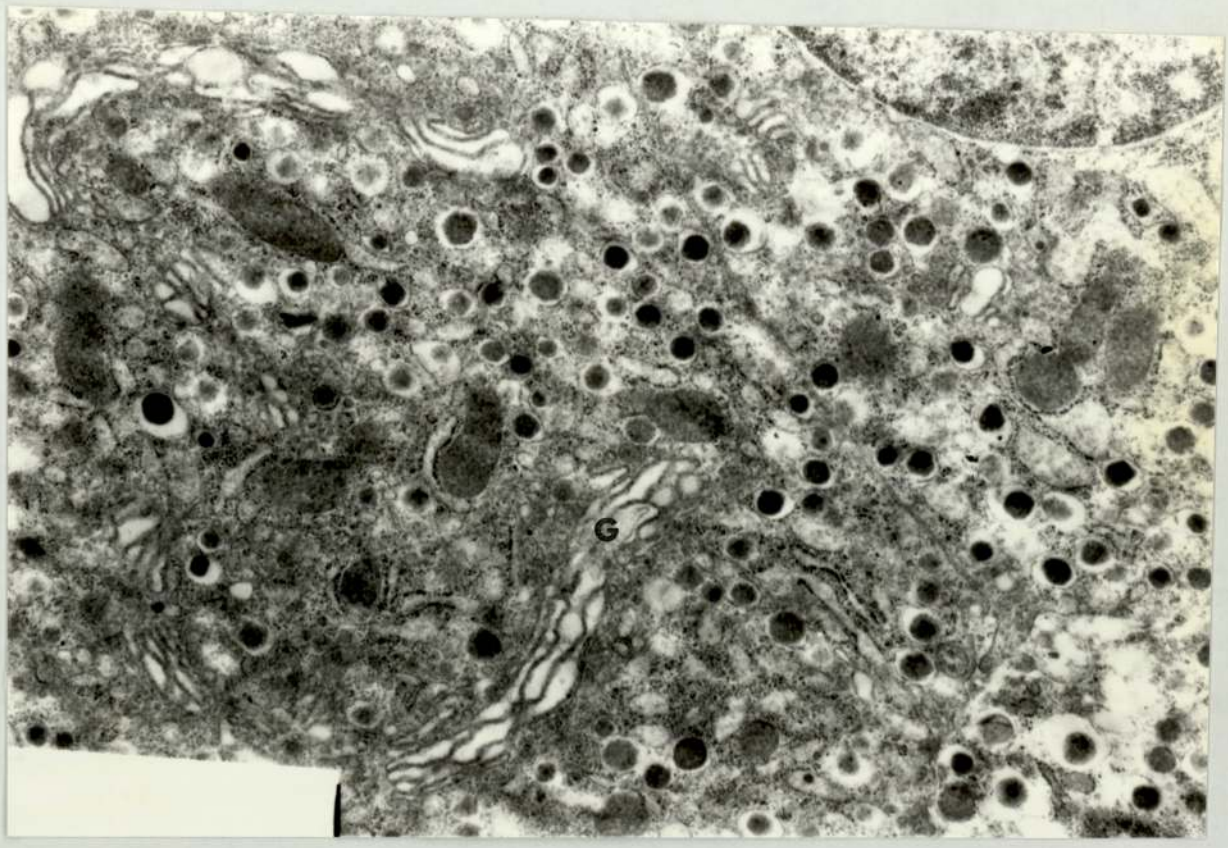
Plate 1



β_1 and α_1 (D) cells in a 7 week old obese mouse islet. Secretory granules are abundant and both pale and dense in type. The mitochondria are spherical, whilst the endoplasmic reticulum is vesicular in the β_1 cell and lamellar in the D-cell.

Glutaraldehyde-osmium x 6,000.

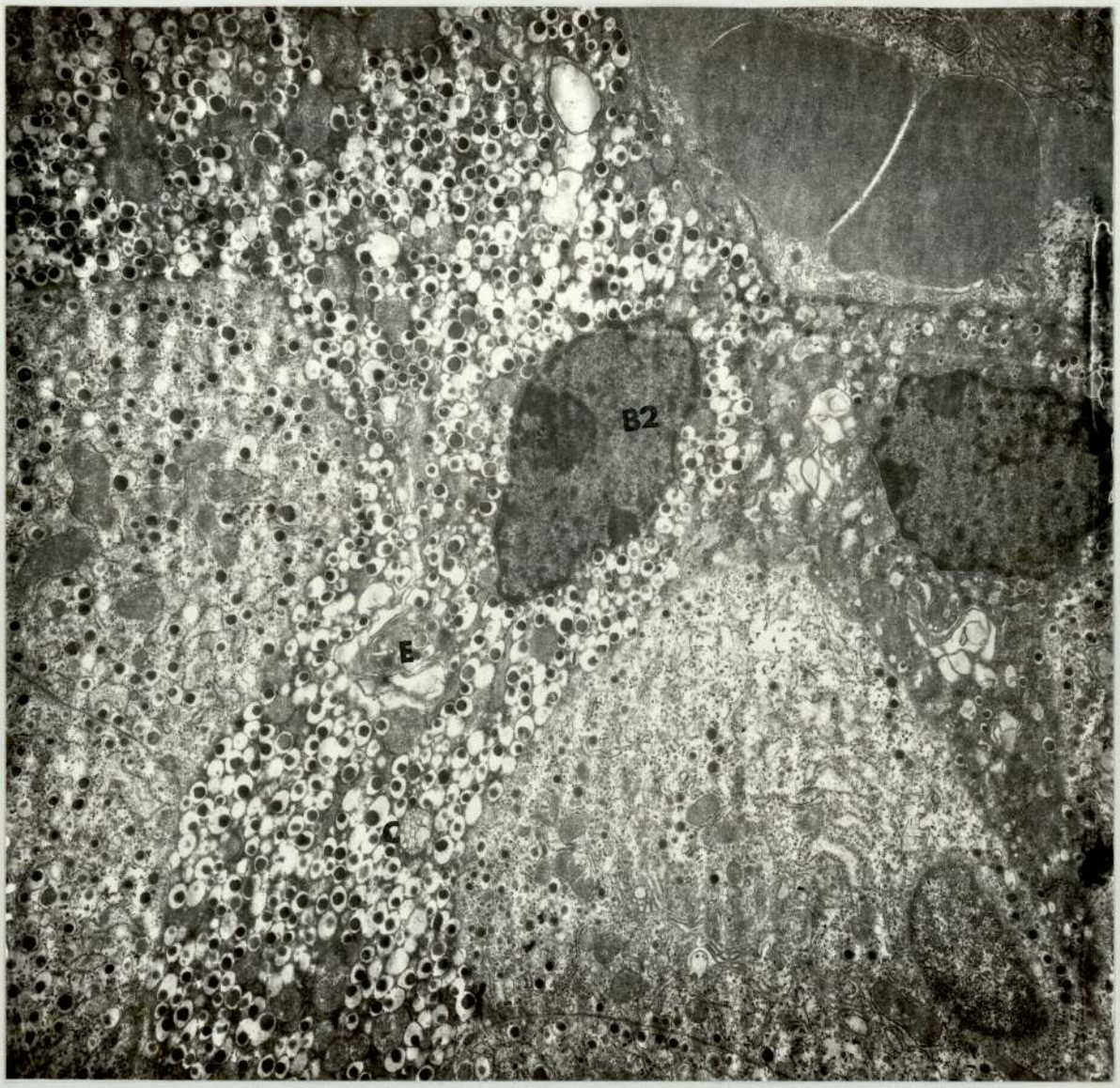
Plate 2



β_1 cell of 20 week old obese mouse islet. Notice the formation of granule membranes close to the prominent Golgi region (G) and the presence of both pale and dense granules.

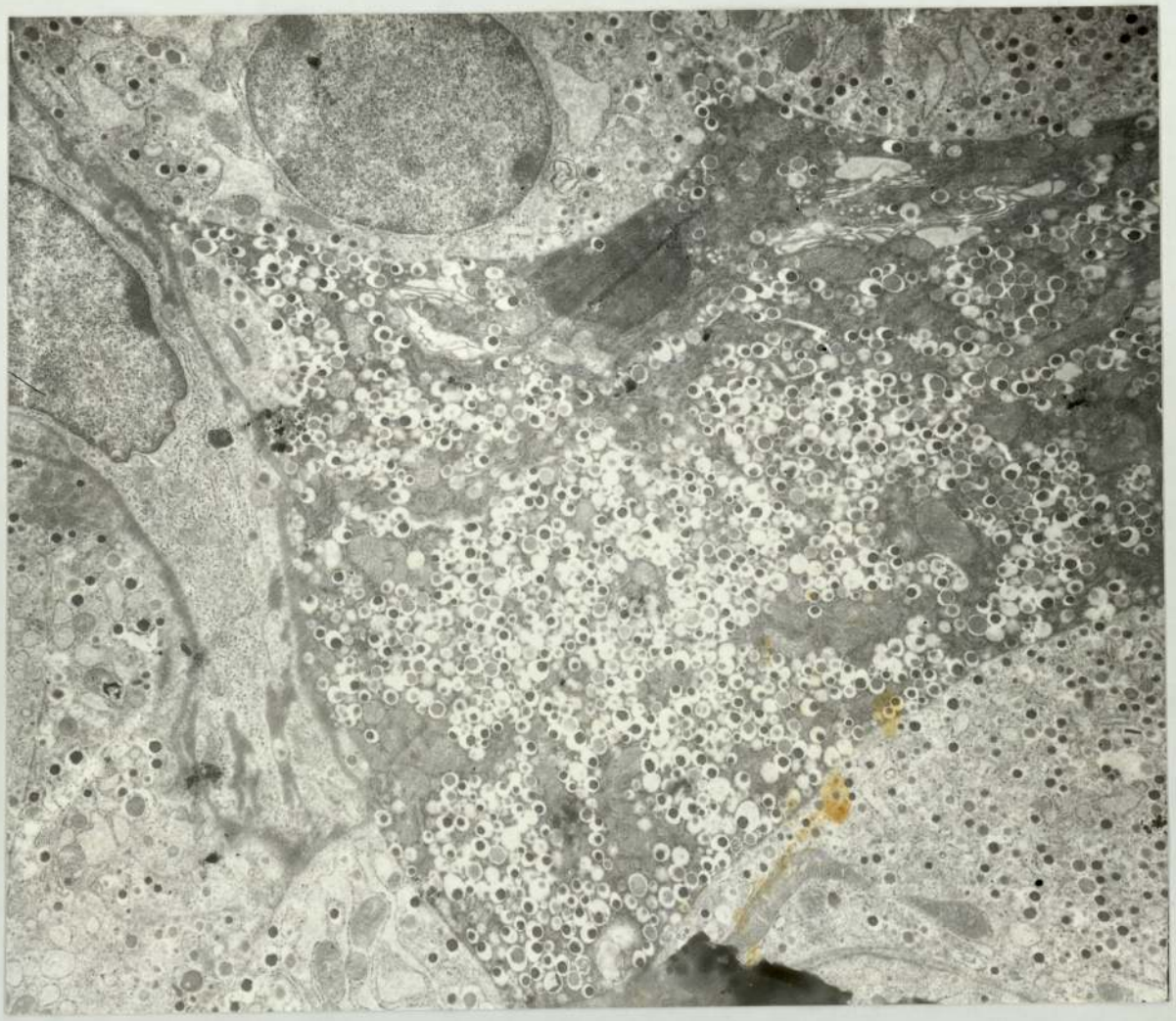
Glutaraldehyde-osmium x 25,000.

Plate 3



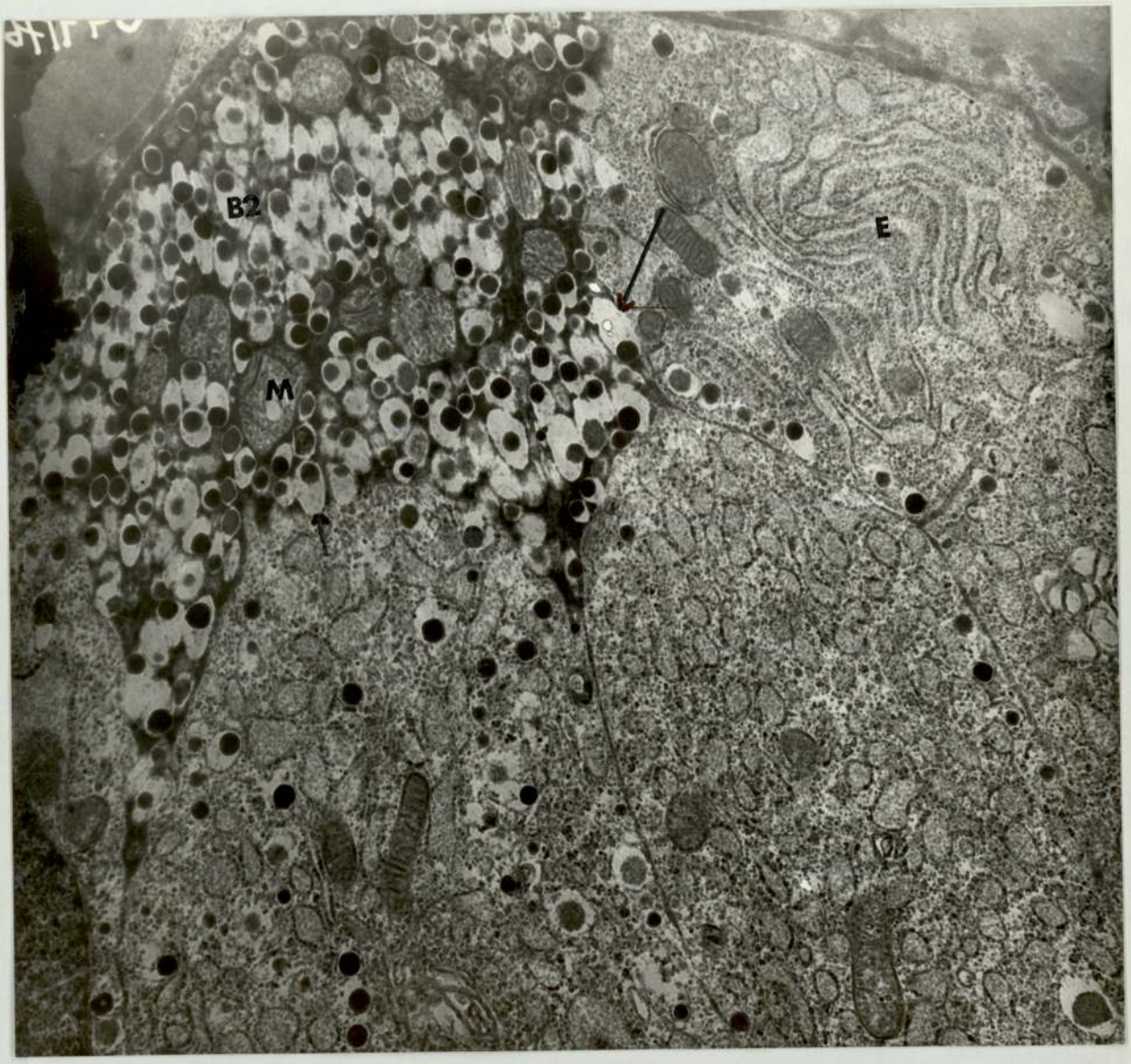
35 week old obese mouse islet. Notice the endoplasmic reticulum (E) of the β_2 cell and the presence of a ceroid body (C). Glutaraldehyde-osmium x 1,000.

Plate 4



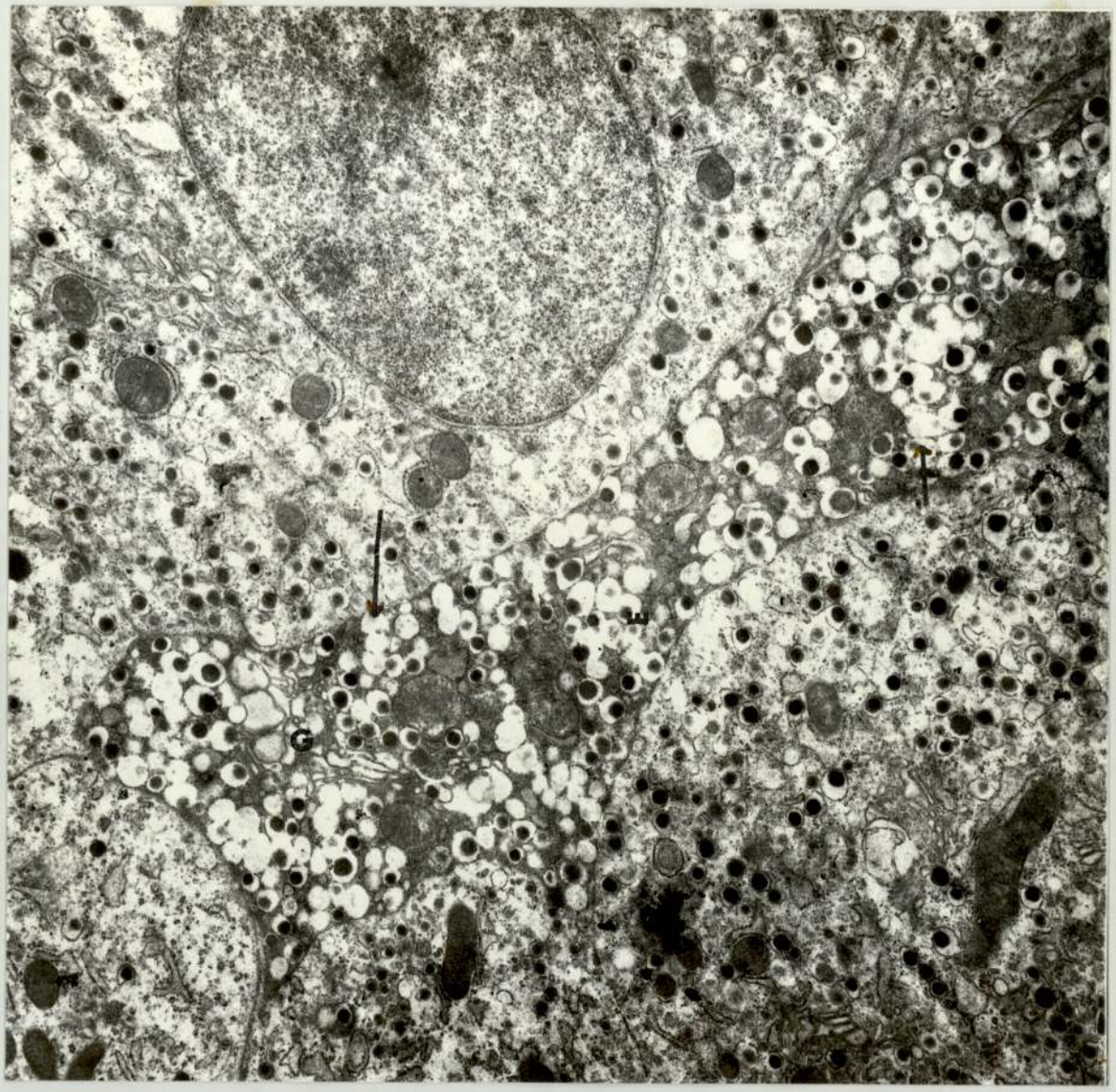
30 week old obese mouse islet. Notice the profusion of β-granules and the dark irregular shaped nucleus of the β_2 cell.
Glutaraldehyde-osmium x 6,000.

Plate 5



β_1 and β_2 cells in the islet of a 30 week old obese mouse. Notice the lamellar and vesicular forms of granular endoplasmic reticulum (E), the connection of granules to the plasma membrane of the β_1 cell (arrow) and the presence of large mitochondria (M) in the β_2 cell. Glutaraldehyde-osmium x 15,000.

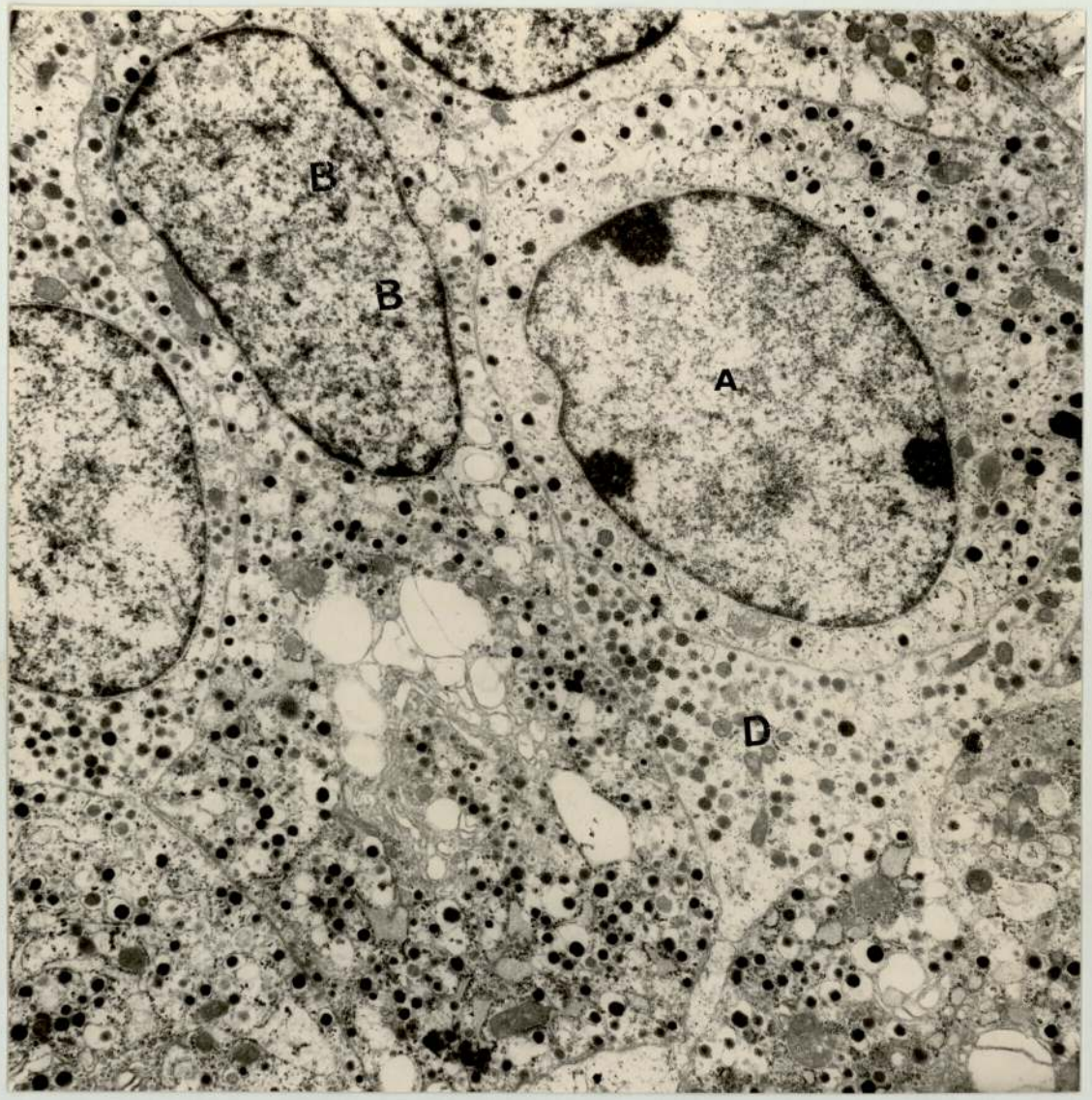
Plate 6



β_1 and β_2 cells in a 40 week old obese mouse islet. The β_2 cell contains a tubular Golgi apparatus (G) and β-granules arranged in chains in the cytoplasm (arrows).

Glutaraldehyde-osmium x 10,000.

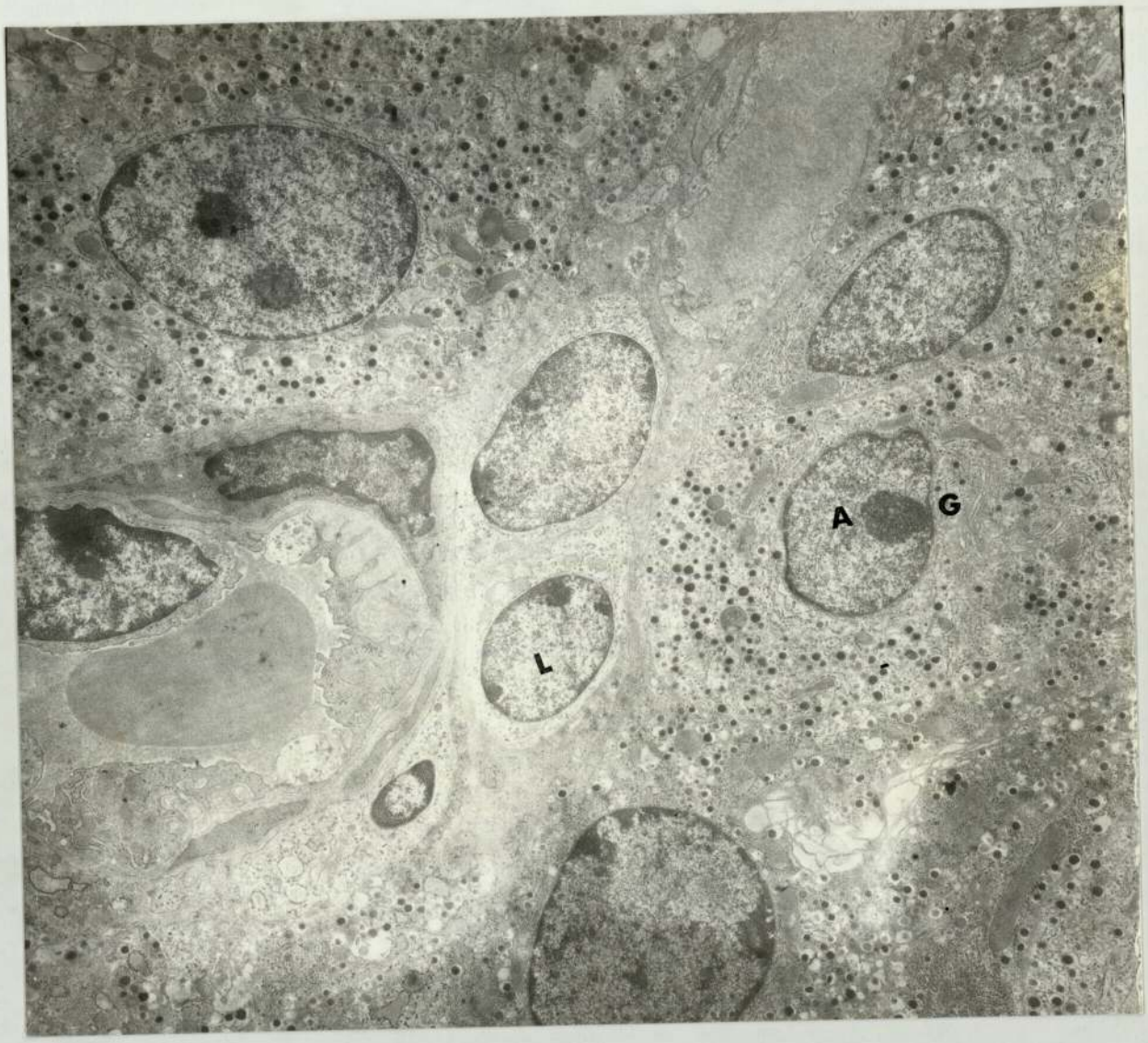
Plate 7



α_2 (A), β_1 and delta (D) cells in the islet of a 35 week old obese mouse.

Glutaraldehyde-osmium x 10,000.

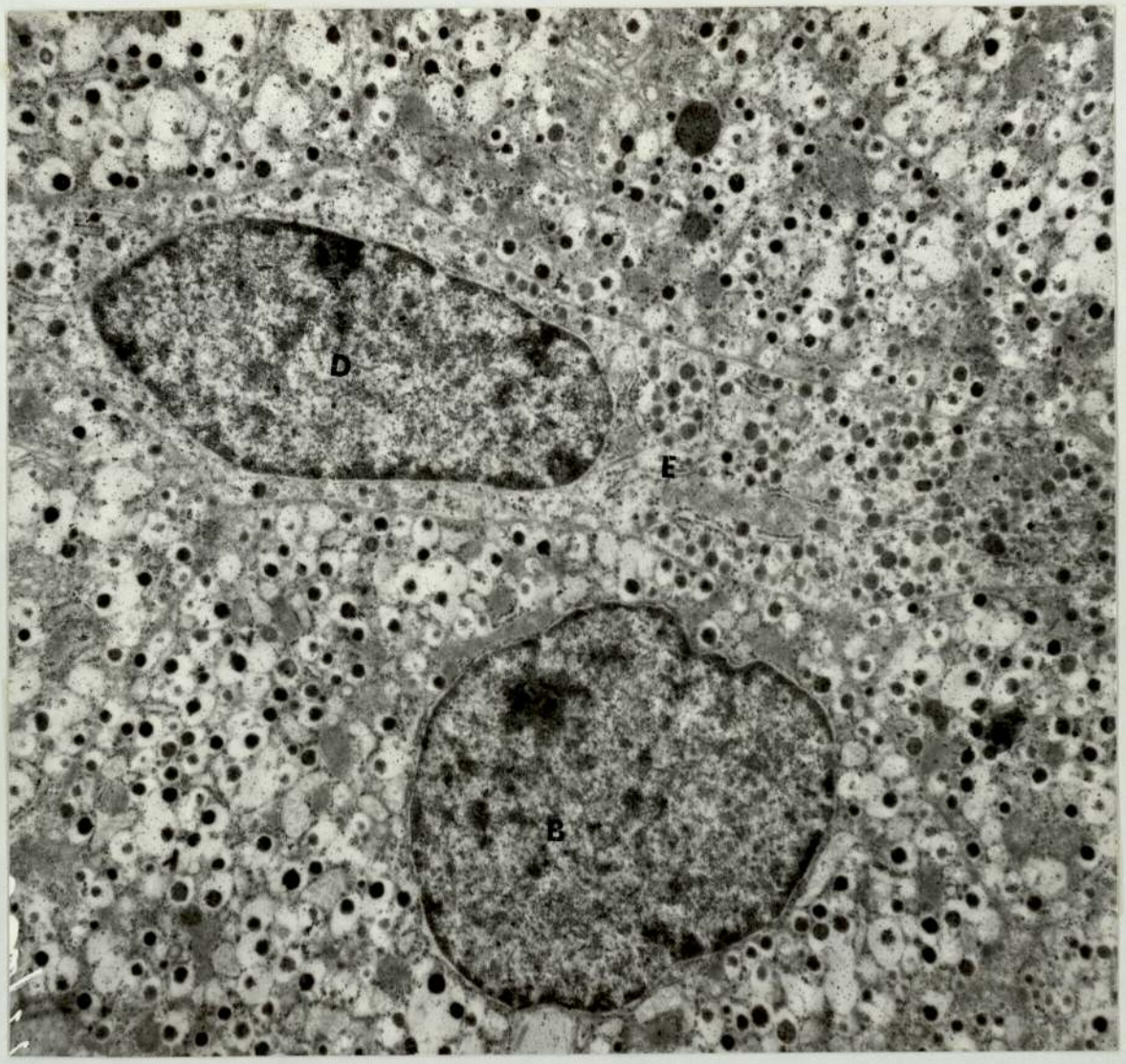
Plate 8



45 week old obese mouse islet. Note the lamellar Golgi apparatus (G) of the α_2 (A) cell and the presence of lymphocytes (L) in the capillary endothelium.

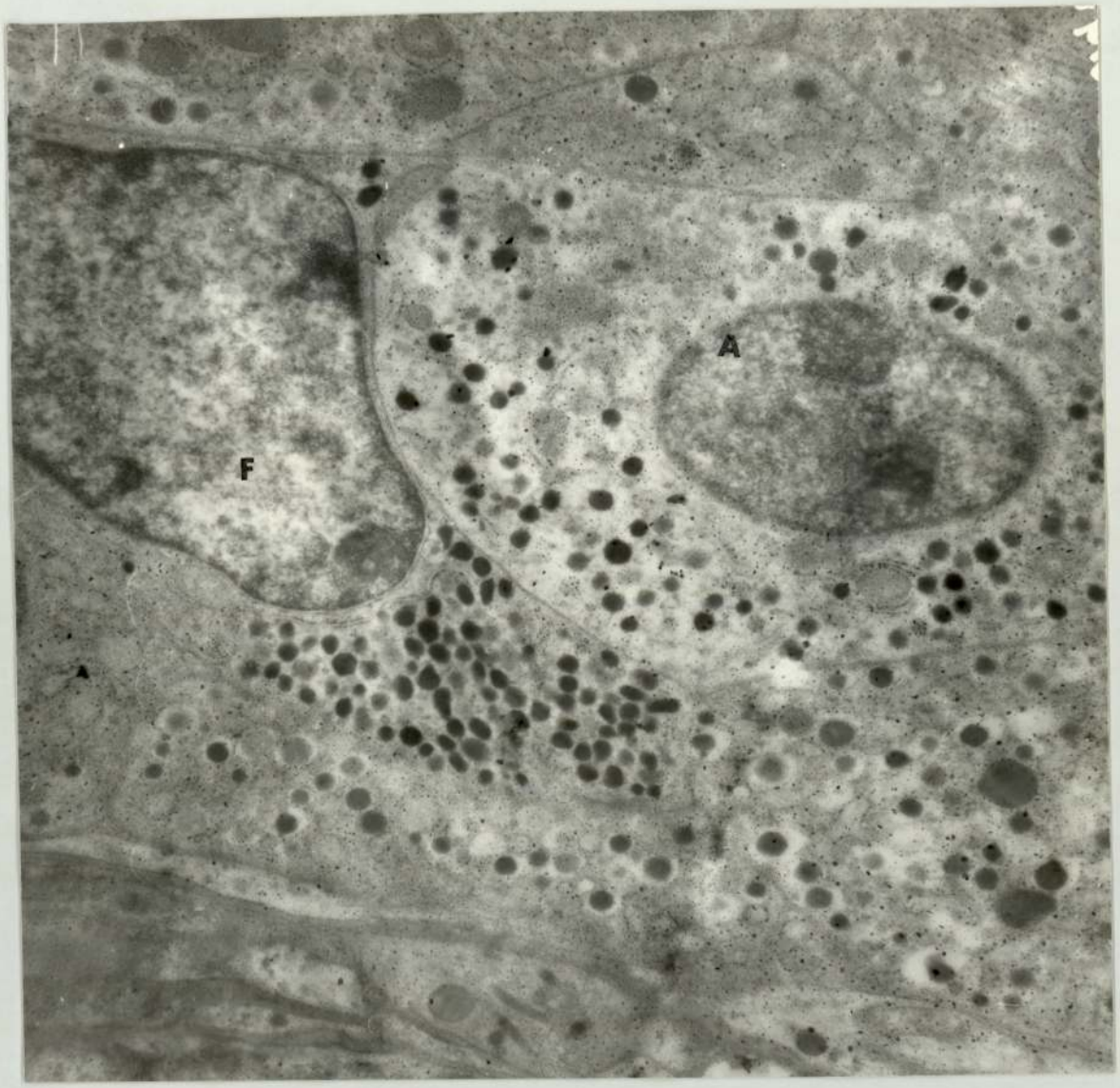
Glutaraldehyde-osmium x 6,000.

Plate 9



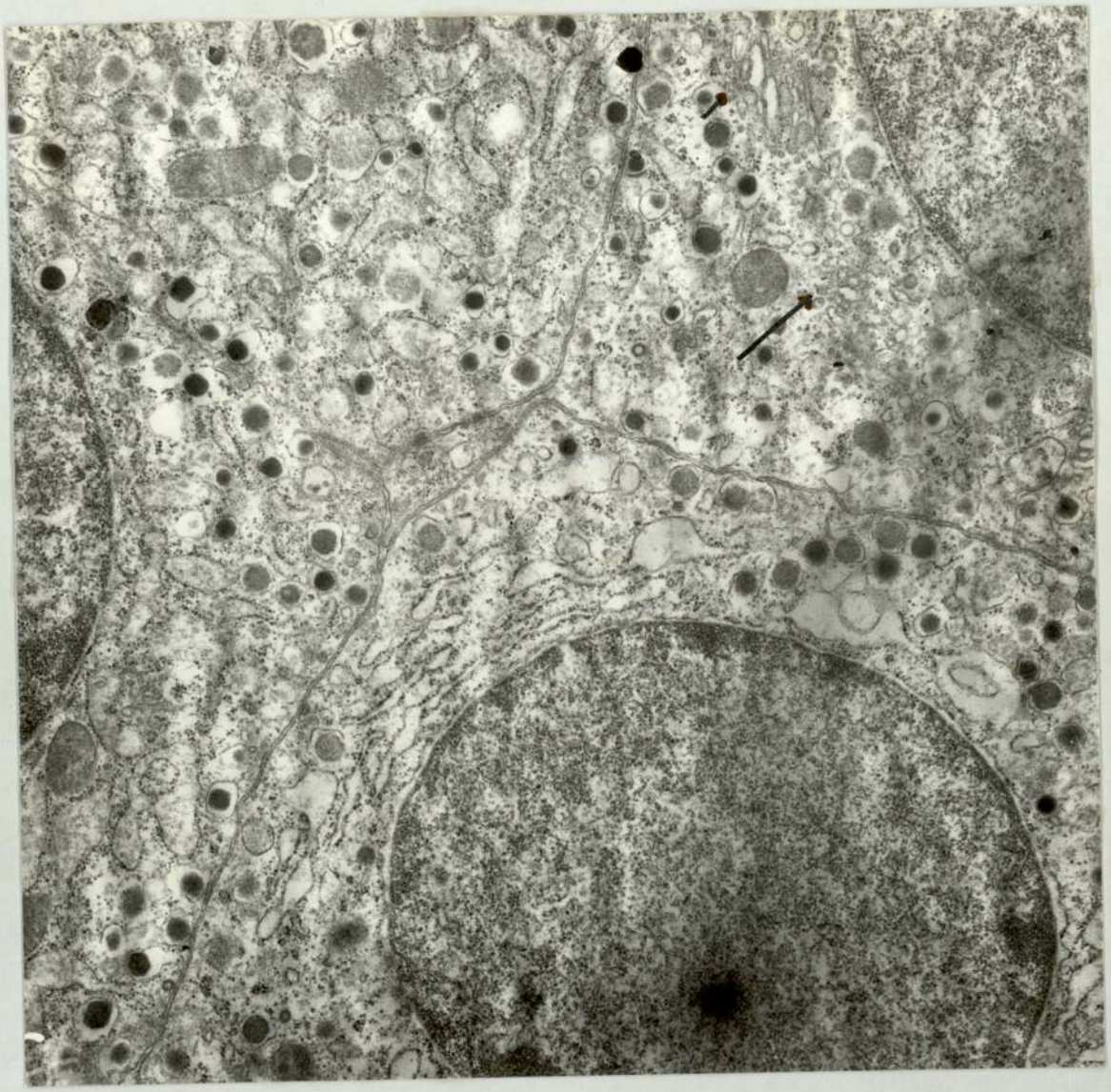
A 15 week old lean mouse islet containing a β_1 cell (B) and delta cell (D). Note the presence of lamellar endoplasmic reticulum (E) and delta granules with indistinct membranous sacs. Glutaraldehyde-osmium x 10,000.

Plate 10



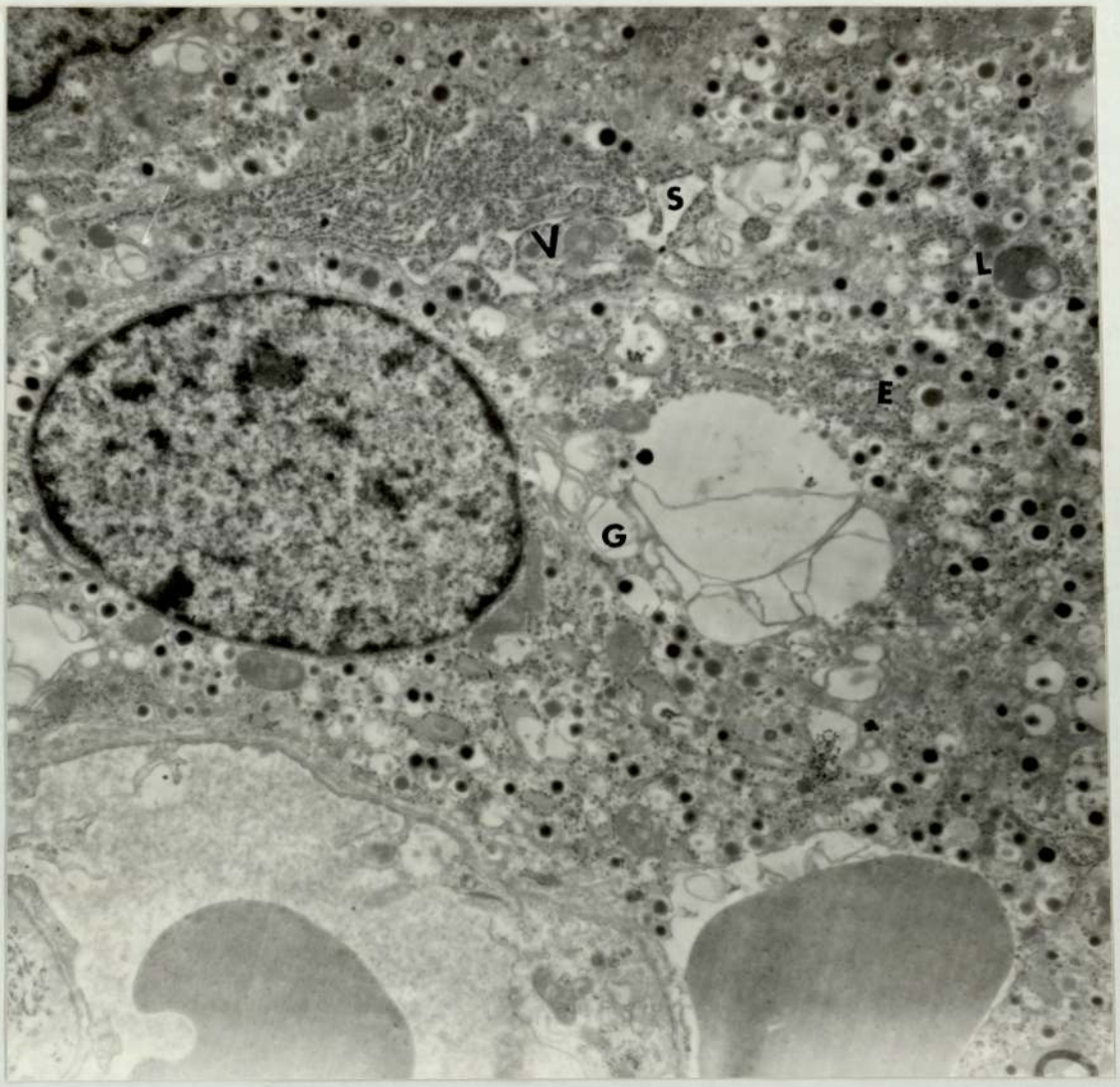
An F-cell in the islet of a 7 week old lean mouse. Note the small tightly packed polarised electron dense granules. Glutaraldehyde-osmium x 10,000.

Plate 11



A β_1 cell in the islet of a 40 week old obese mouse. Note the presence of small vesicles close to the Golgi apparatus (arrows). Glutaraldehyde-osmium x 10,000.

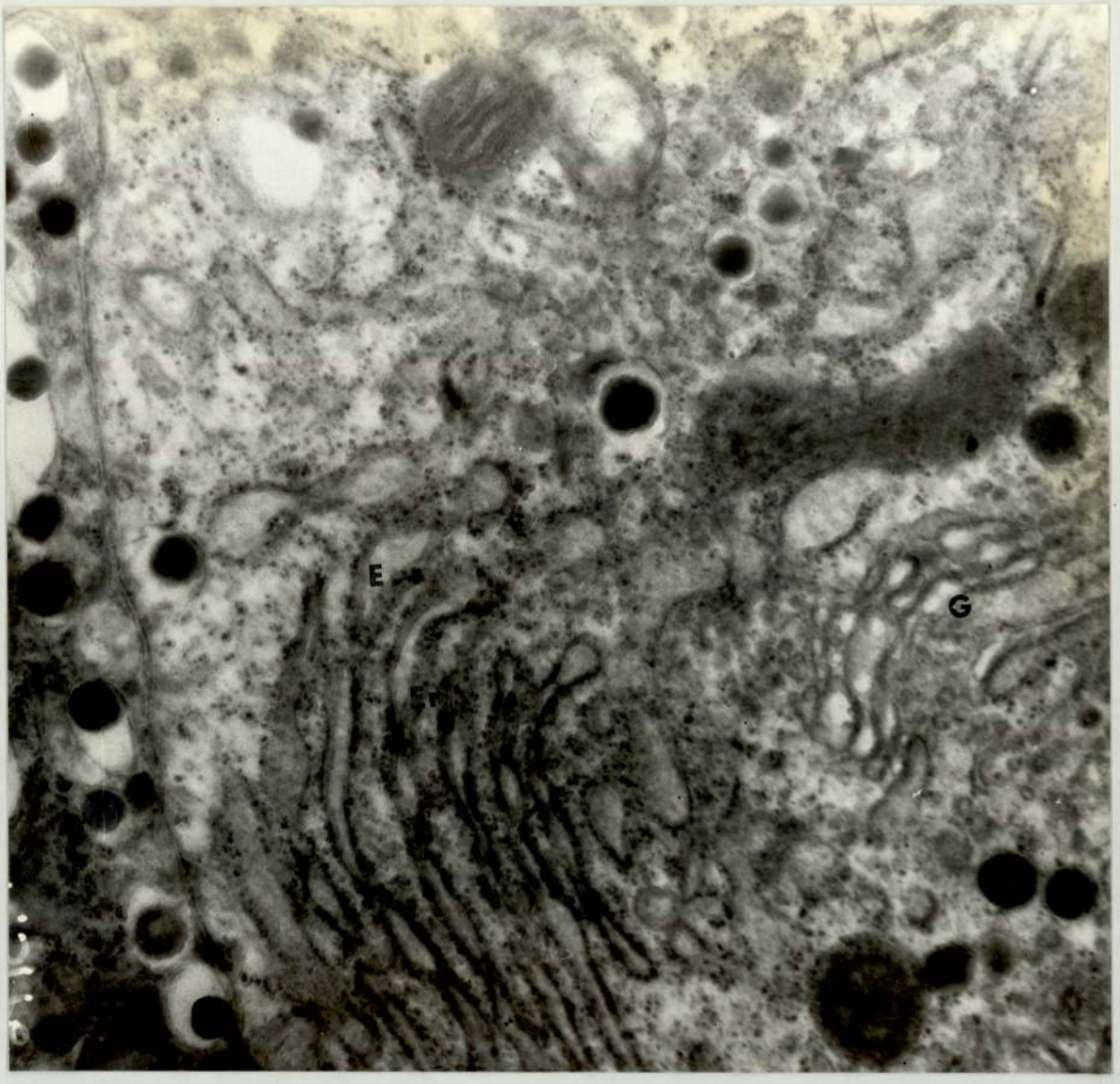
Plate 12



A β_1 cell in the islet of a 45 week old obese mouse. Notice the inflated Golgi apparatus (G) consisting of large vacuoles, the endoplasmic reticulum (E) is granular and prominent, the intercellular space (S) and microvilli (V) are also present, and the lysosome (L) appears to be in the early stages of ceroid body formation.

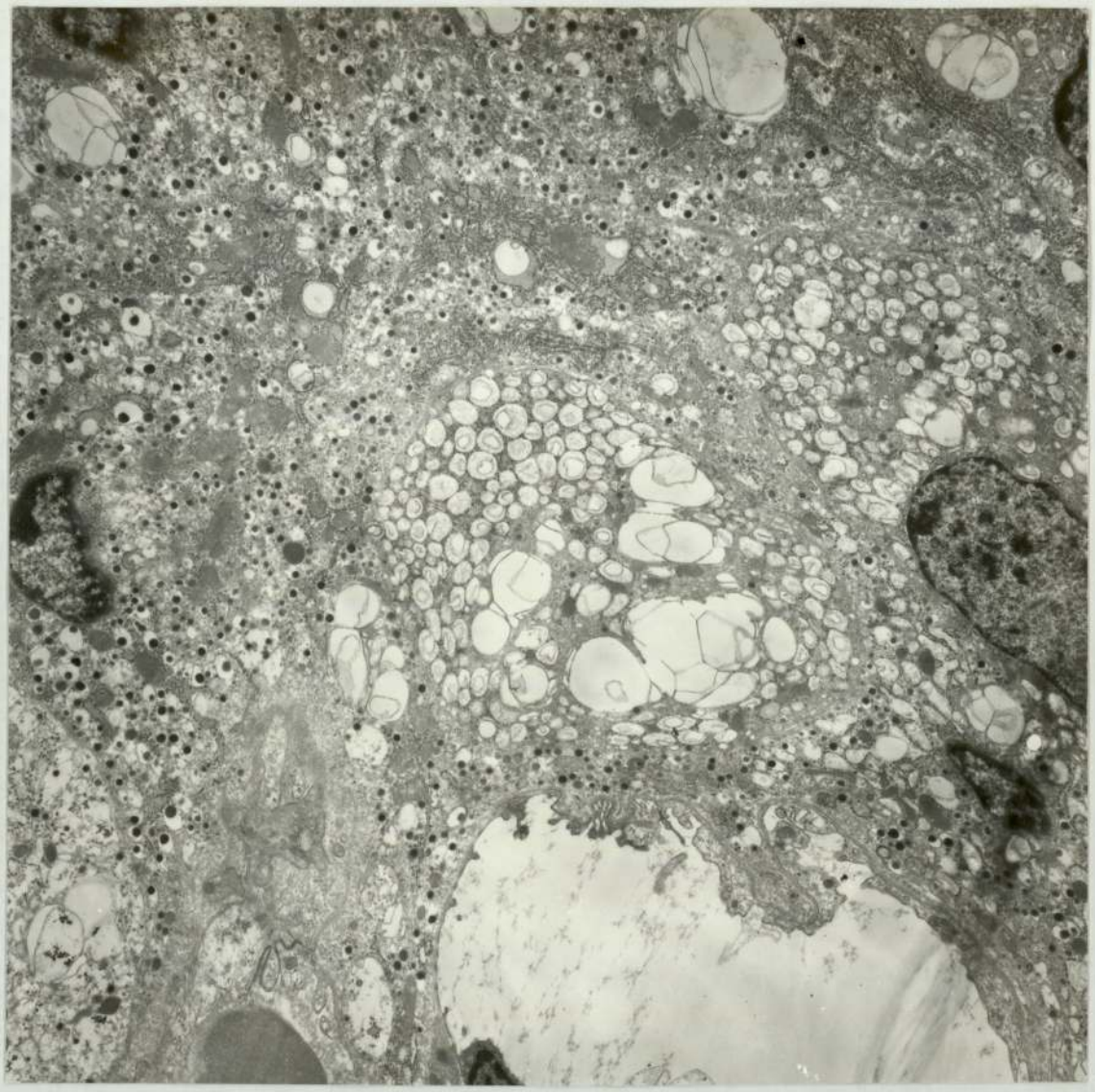
Glutaraldehyde-osmium x 10,000.

Plate 13



A β_1 cell from a 20 week lean mouse islet showing the formation of vesicles from tubular endoplasmic reticulum (E). Notice the presence of small vesicles near the Golgi apparatus (G). Glutaraldehyde-osmium x 30,000.

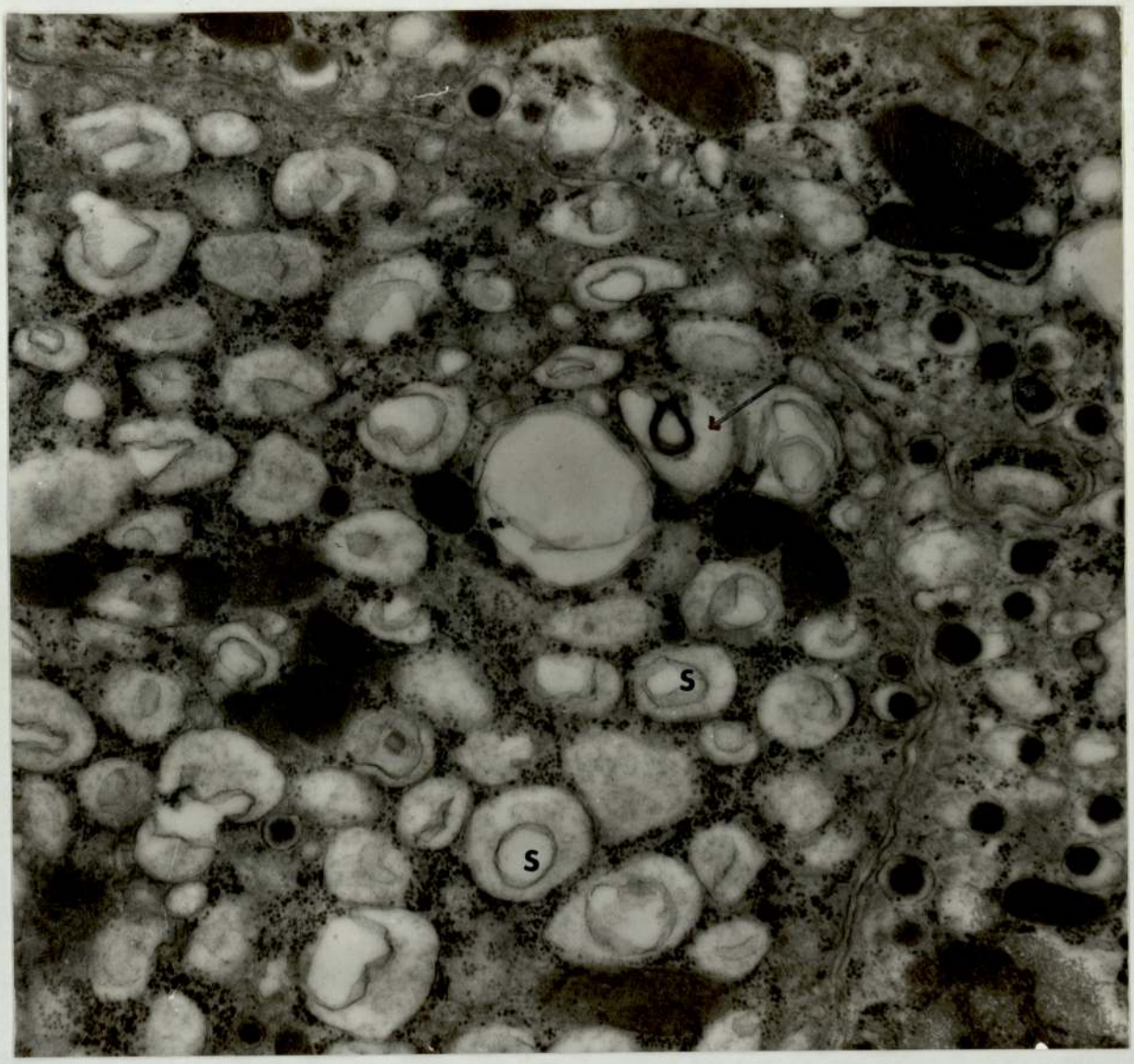
Plate 14



A β_2 cell granule scrolling in the islet of a 35 week old obese mouse.

Glutaraldehyde-osmium x 6,000.

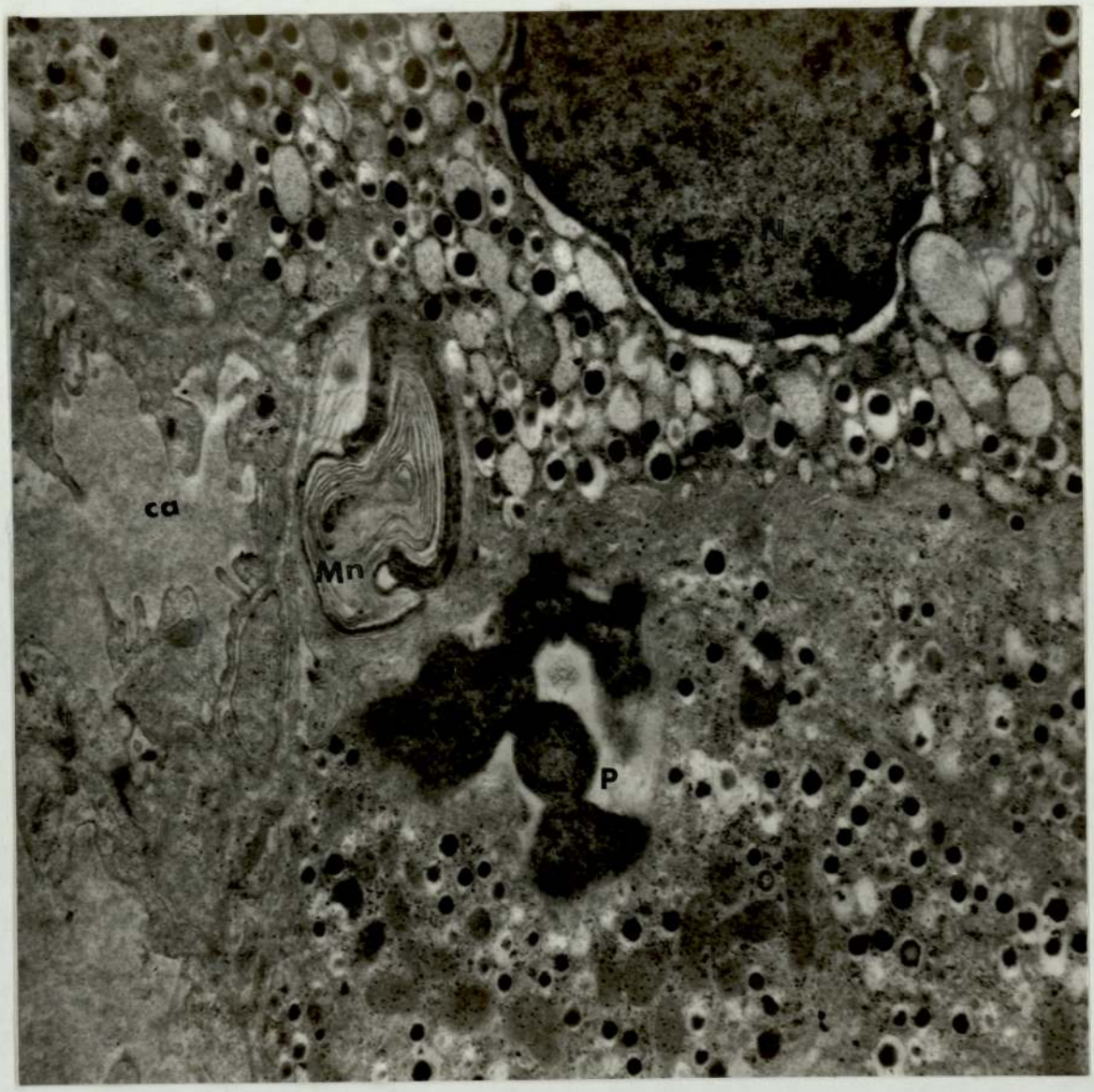
Plate 15



Enlargement of Plate 14. The invagination of granule sac (arrow) and the scrolling (S) is shown in cross-section.

Glutaraldehyde-osmium x 30,000.

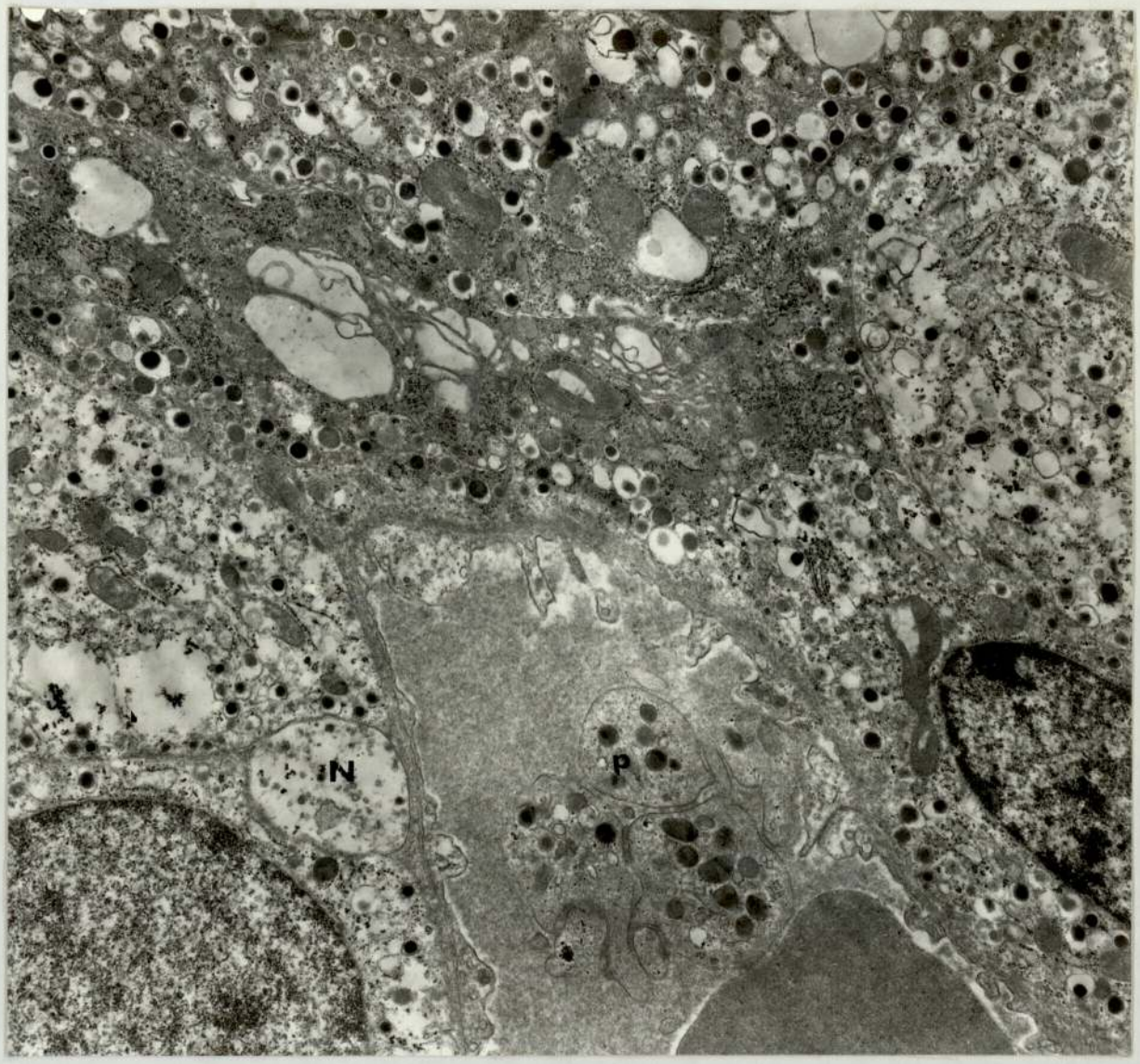
Plate 16



A myelinated axon (Mn) close to a β_2 cell and a capillary (Ca) in a 40 week old obese mouse islet. N, nucleus; P, unidentified material.

Glutaraldehyde-osmium x 15,000.

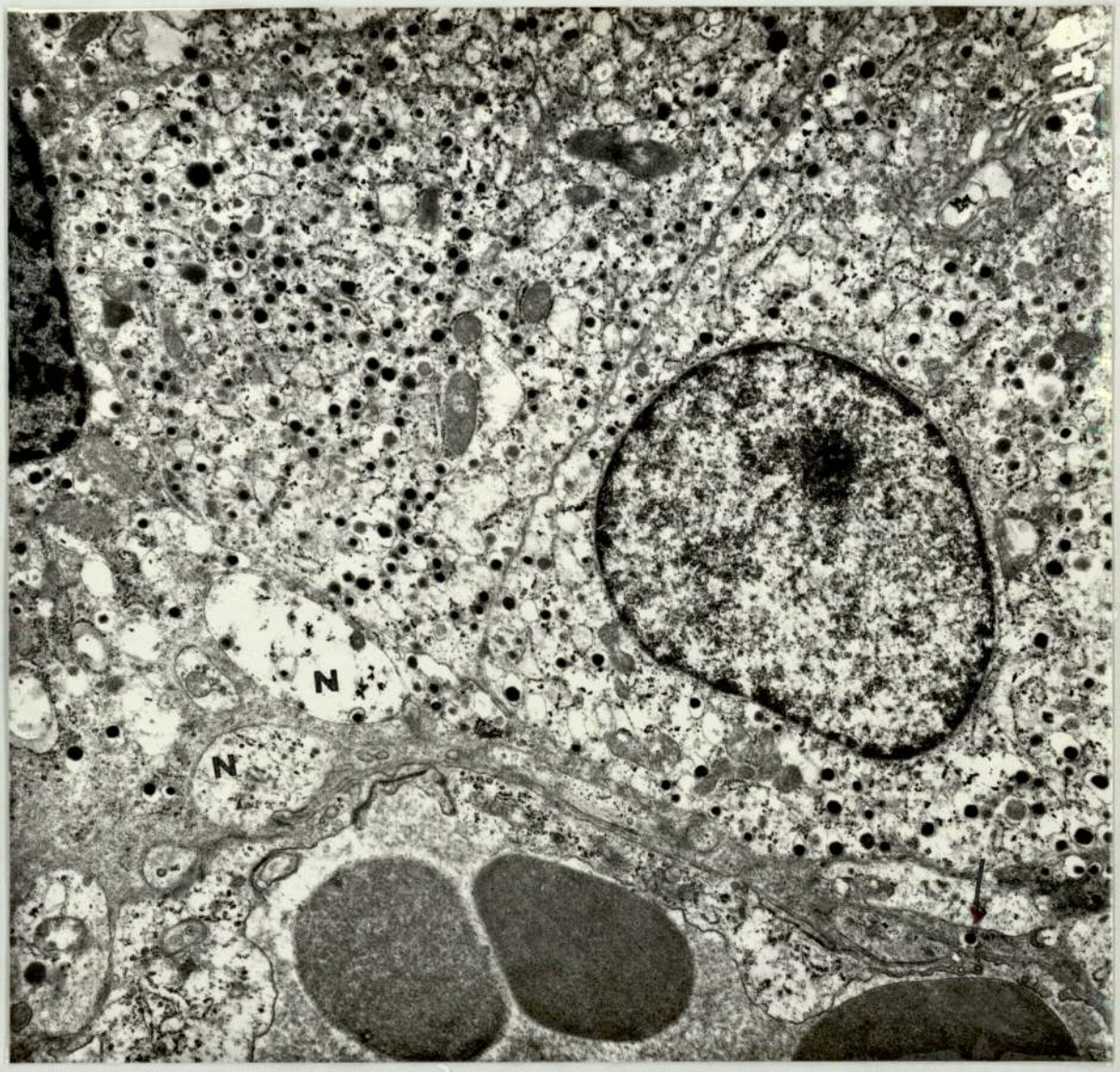
Plate 17



A β_1 cell of a 40 week old obese mouse. Notice the nerve ending (N) containing vesicular granules and blood platelet aggregation (P).

Glutaraldehyde-osmium x 10,000.

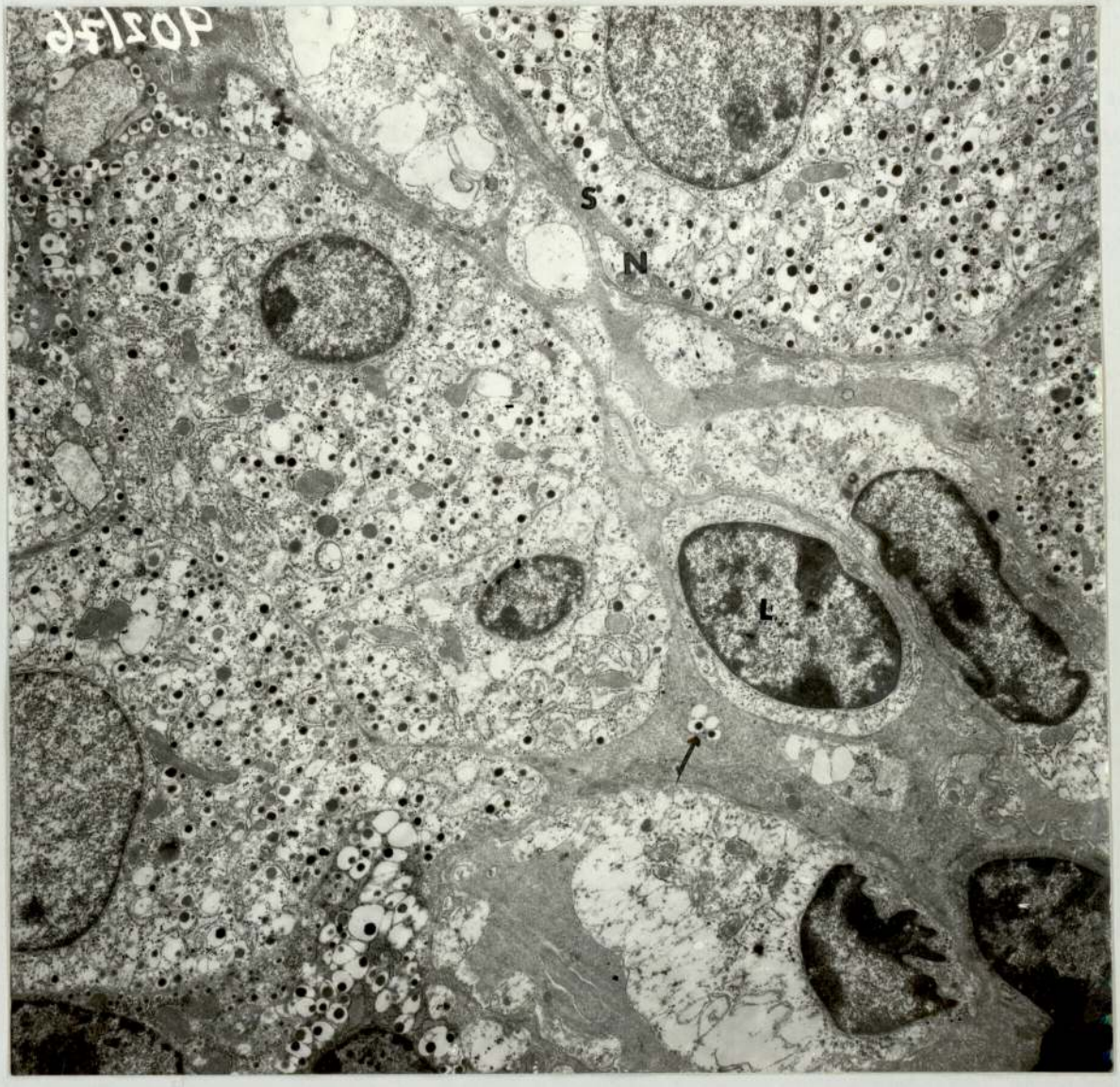
Plate 18



A β_1 cell from a 45 week old obese mouse. Notice the presence of nervous tissue (N) and the movement of a β-granule from the β_1 cell to the capillary lumen (arrow).

Glutaraldehyde-osmium x 10,000.

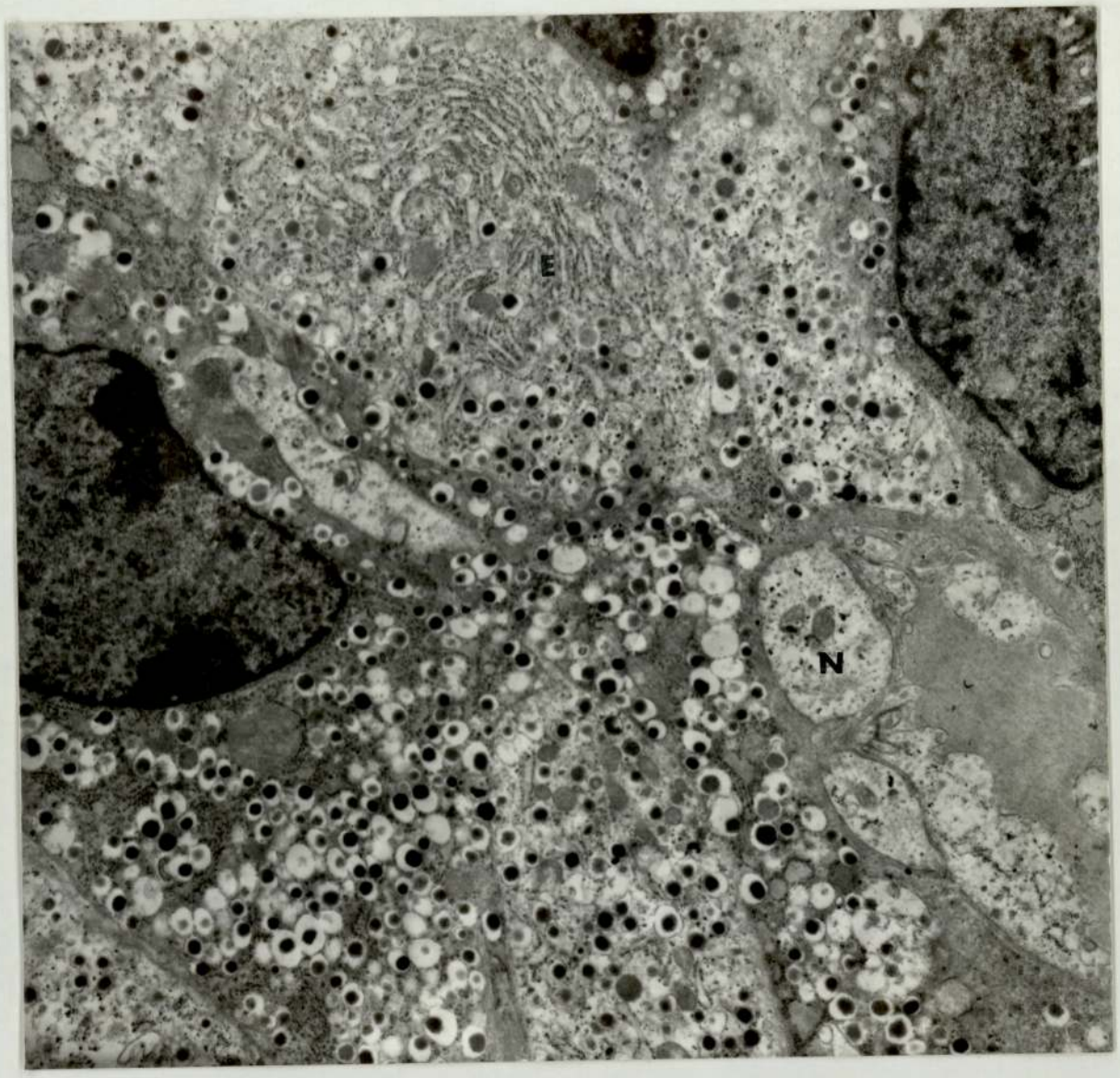
Plate 19



A 44 week old obese mouse islet showing the presence of β-granules inside the capillary lumen (arrow), several lymphocytes (L), a nerve ending (N) can be seen surrounded by a Schwann cell (S).

Glutaraldehyde-osmium x 6,000.

Plate 20



A 15 week old lean mouse islet showing normal islet β_1 and β_2 cells. E, endoplasmic reticulum; N, nerve ending. Glutaraldehyde-osmium x 5,000.

CHAPTER IV

Degenerative changes in the islets of Langerhans of obese hyperglycaemic mice and their lean littermates with age

4.1 Changes in islet morphology associated with the manifestation of human juvenile and maturity onset diabetes and the obese hyperglycaemic syndrome in mice

4.1.1 Introduction

Obesity has been defined as an over-enlargement of the adipose depot and is almost always associated with functional and morphological changes in the islets of Langerhans (Stern & Hirsch, 1972). Obesity is often associated with hyperinsulinaemia, and it has been suggested that the active synthesis and release of insulin in obesity is the response to an increased metabolic demand. The simultaneous occurrence of obesity and hyperinsulinaemia has been observed in the ob/ob mouse and KK mouse (Wrenshall, 1955; Shino et al., 1970) and in human maturity onset diabetes (Bagdade, Bierman & Portá, 1971). Islet hyperplasia and enlargement of the β-cell Golgi apparatus and endoplasmic reticulum have been used to explain the aetiology of hyperinsulinaemia (Like & Miki, 1967; Pictet et al., 1967.

Ageing per se may be defined as the deleterious reduction in physiological function which occurs in an old organism. In turn, physiological function and changes in it are recognised as being dependent upon morphological structure as well as on associated molecular reactions which occur at the cellular level and on

changes in them (Herbener, 1976).

Physiological studies of age-related alterations in the responsiveness of islets of Langerhans of old animals to glucose have shown a reduction in insulin secretory activity compared with the insulin secretory activity of islets from young animals (Coddling, Kalnins & Haist, 1975; Feldman & Plovk, 1976). Plasma insulin levels have been shown to be higher in human obese subjects than in normal lean subjects (Bagdade, 1969; Stern & Hirsch, 1972).

Morphological studies have provided evidence of structural changes in the islets of Langerhans of obese animals such as the fatty rat (Shino et al., 1976) and the spiny mouse (Gonet, Stauffacher, Pictet & Renold, 1965; Pictet et al., 1967). Degranulated β-cells have been observed in the islets of spontaneously diabetic yellow KK mice (Shino & Iwatsuka, 1970).

Glycogen deposition has also been observed in the β-cells of hyperglycaemic spiny mice (Pictet et al., 1967) and its occurrence in degranulated β-cells appears to be proportional to the extent of β-cell degranulation (Williamson & Lacy, 1961).

Morphological examination of the islets of Langerhans of aged diabetics has provided evidence of structural changes in the

β-cells. Hellman (1960) showed that the total volume of pancreatic islet tissue increased gradually with age and was a normal consequence of the ageing process.

Fibrosis has been observed in the islets of Langerhans of human diabetics (Gepts, 1965) and its extent has been shown to increase with age (Lazarus & Volk, 1962). Fibrosis and the deposition of amyloid, an extracellular, paraplastic substance (Stiller & Katenkamp, 1976) has been observed in the islets of Langerhans of aged human diabetics (Yamada, 1968; Westermark, 1972, 1973) and in the islets of spontaneously diabetic animals (Johnson, Osborne & Barnes, 1970).

Ceroid bodies, cytoplasmic lysosome-like organelles containing pigments of unsaturated fatty acids (Reed, McMillan, Hartroft & Portá, 1965; Brizzee, Harkin, Ordy & Kocck, 1976), have been observed in the pancreatic islets of human diabetics (Yokoh, Iizuka & Kano, 1959). Large numbers of ceroid bodies have been observed in islet β-cell tumours (Goldenberg, Goldenberg & Benditt, 1969) and in the β-cells of aged human diabetics (Lacy & Greider, 1972).

The nuclei of human diabetic β-cells are increased in size (Gepts, 1972). In addition, alloxan induced diabetes results in an increased size of the β-cell endoplasmic reticulum (Williamson et al., 1961) and Golgi apparatus (Orci et al., 1969). A similar

picture is seen after sulphonylurea treatment and in some spontaneously diabetic animals such as the yellow KK mouse (Shino & Iwatsuka, 1970).

Insulitis is another common pathological lesion of the pancreatic islets. This inflammatory lesion is initially characterised by the infiltration of lymphocytes and, in later stages, fibrocytic material into the islets (Von Meyenburg, 1940). Insulitis is known to occur almost exclusively in juvenile diabetics (Gepts, 1965, 1972), but it has also been reported in elderly maturity onset diabetics (Le Compte & Legg, 1972) and spontaneously diabetic animals (Barboni & Manocchio, 1962; Gepts & Toussaint, 1967).

The obese hyperglycaemic mouse is characterised by the presence of a high circulating level of insulin during a considerable period of its life-time (Wrenshall et al., 1955; Westman, 1968). Hyperglycaemia, together with islet hypertrophy (Gepts et al., 1960; Hellman et al., 1961), β-cell degranulation (Wrenshall et al., 1955; Atkins & Matty, 1973) and β-cell hyperplasia (Hellman & Petersson, 1960), have all been suggested to be phenomena associated with β-cell hyperactivity.

Previous workers in the field had been unable to demonstrate any signs of pathological change in the islets of obese mice with

age (Westman, 1968). In addition, the ultrastructural studies of Bjorkman, Hellerström and Hellman (1963) failed to demonstrate the presence of any pathological changes in the islets of Swedish obese mice. Atkins (1972) and Atkins and Matty (1973) first identified the presence of fibrosis, connective tissue capsule thickening and lymphocytic infiltration in the islets of obese mice.

The present study was designed to investigate the degenerative changes occurring in obese mouse islets with age using histochemical and ultrastructural techniques. The lean littermate was used as the age-related control.

4.1.2 Materials and methods

Islet tissue was microdissected from lean and obese mice between the ages of 7-45 weeks and prepared for electron microscopy as described previously (page 18). Ten islets were microdissected from each animal and eight sections were cut from each islet.

4.1.3 Results

Ultrastructural morphology of islets of Langerhans

The contents of β-cells show an age-related sequence of well-defined structural changes. Table 2 summarises the age-related morphological changes observed in the islets of Langerhans of obese hyperglycaemic mice and their lean littermates.

(a) Changes in lean mouse islet β-cell morphology with age

The islet β-cells of lean mice showed very little morphological change with age. There was some increase in the numbers of β-granules and the size of the granular endoplasmic reticulum. β-granulolysis was observed in old lean mouse islet β_2-cells (Plates 21-24). No other morphological changes observed in old obese mouse β-cells were ever observed in old lean mouse islet β-cells.

(b) Changes in obese mouse islet β-cell morphology with age

The granular endoplasmic reticulum of obese mouse β-cells gradually increased in size and extent with age eventually forming vacuole-like structures at 40 weeks.

In addition, there was an increase in the size of the Golgi apparatus with age, becoming very prominent at 35-45 weeks. Small

Table 2

Age	Lean mouse	Obese mouse
7-20 weeks	β-cell	β-cell
	No change	Enlargement of the endoplasmic reticulum and the formation of small intracellular vacuoles.
	Whole islet	Whole islet
	No change	Enlargement of islet size compared to lean mouse islet. Increase in number of capillaries. Early formation of intra-islet vacuoles.
20-30 weeks	β-cell	β-cell
	No change	Enlargement of the endoplasmic reticulum, Golgi apparatus and the mitochondria. Granulolysis and scrolling of β-granules. Degranulation of β-cells. Formation of intra-β-cell vacuoles.
	Whole islet	Whole islet
	No change	Further increase in islet size due to hyperplasia of β-cells. Further increase in size of islet vacuoles at the expense of β-cells. Early appearance of amyloid-like material.

Table 2 (continued)

Age	Lean mouse	Obese mouse
35-45 weeks	β-cell	β-cell
	Enlargement of the ER and Golgi apparatus.	Nuclear invagination.
		More marked degranulation.
	Granulolysis.	Necrosis.
		Increased incidence of ceroid bodies in β-cell cytoplasm.
	Whole islet	Whole islet
	No change	Dilation of blood capillaries.
		Aggregation of blood platelets in blood capillaries.
		Coalescence of dilated blood capillaries and the complete breakdown of β-cells to form large intra-islet vacuoles.
		Further loss of β-cells.
		Focal deposition of amyloid-like material.
		Lymphocytic infiltration (insulitis).
		Marked insular fibrosis.
		Appearance of mixed endocrine/exocrine cells.

Summary of the age-related morphological changes in the islets of Langerhans of obese hyperglycaemic mice and their lean littermates.

particles were often seen to be emanating from the Golgi area, forming small granular vesicles (Plate 25).

The number of β-cell mitochondria tended to increase with age and often contained a dense matrix (Plate 26). Some mitochondria appeared to be swollen (Plate 29)

The incidence of intracytoplasmic dissolution of obese mouse β-granules (granulolysis) was observed to increase with age up to 45 weeks (Plate 38). The scrolling of β-granules (discussed in Chapter III) only appeared in obese mice of 35-45 weeks of age. In addition, the frequency of degranulated β-cells increased between the ages of 35-45 weeks (Plate 26). β-granules could be observed inside the lumen capillaries (Plates 19 & 25).

Agranular cells were often observed in the islets of 35-45 week old obese mice. These cells were oval to polygonal in shape, devoid of granules, but often vacuolated. The oval nucleus often contained prominent nucleoli. The cytoplasm was low in electron density and contained elongated mitochondria and fragmented endoplasmic reticulum (Plate 26)

The nuclei of old obese mouse β-cells were often enlarged. A single electron dense rod was occasionally observed in some of these nuclei surrounded by less electron dense nucleoplasm (Plate 27).

Nuclear invaginations were observed in the β-cells of many old obese mouse islets (Plate 28). These nuclear invaginations often contained cytoplasmic organelles and granules. In some cases, the invaginated contents appeared to be completely encapsulated within the nucleus (Plate 29) and invaginated β-granules appeared to be enlarged and devoid of core material (Plate 29).

Lysosomes were observed in the pancreatic islet β-cells of both lean and obese mice. These appeared as spherical bodies with moderate homogeneous electron density. With increasing age, the lysosomes of obese mouse islet β-cells showed remarkable changes in structure. Rounded deposits of homogeneous material were often observed in the lysosome. These deposits contained focal areas of moderate electron density. These areas increased in size and number with age, and came to occupy large areas of the lysosome (Plate 25). The lysosome in this condition has been called a ceroid body (Reed et al., 1965). The size and number of obese mouse islet β-cell ceroid bodies increased with age, and as many as three of these bodies could be found in the same cell. No ceroid bodies were observed in either alpha (α_2) or D-cells of obese mice or in any islet cells of lean mice.

At 35-45 weeks of age, intermediate cells were observed in obese mouse islets containing both endocrine and zymogen granules. The cells were characterised by having a lamellar endoplasmic

reticulum and large mitochondria. These so-called perinsular acinar cells (Orci, Rufner, Renold & Rouiller, 1970) were generally located at the periphery of islets (Plates 30 & 31). Acinar cells actually surrounding obese mouse islets often contained β-granules (Plate 32)

Aggregated blood platelets were often observed in the capillaries of 30-45 week old obese mouse islets (Plates 17 & 33). Some platelets were attached to the endothelial cells of the capillaries. The incidence of platelet aggregation increased with age and was very common in the blood vessels of 45 week old obese mouse islets. Platelet aggregation was most often observed in capillaries with heavy collagen deposition. Large numbers of lymphocytes were generally associated with capillaries containing aggregated clumps of platelets (Plate 33).

Lymphocytic infiltration was often observed in the islets of old obese hyperglycaemic mice. The capillaries and adjacent tissues became infiltrated with large numbers of lymphocytes and macrophages (Plates 33,34 & 35). The macrophages contained phagocytosed β-granules (Plate 35) and adjacent β-cells showed signs of structural degeneration. β-cell degeneration was always associated with the presence of mononuclear cells (Plates 36,37 & 38). These cells were characterised by the lack of granules in their cytoplasm and the presence of a nucleus rich in peripherally distributed chromatin. In some cases, red blood cells were observed within the cytoplasm of β-cells, especially those in the islets of 40-45 week old obese mice (Plate 38).

4.1.4 Discussion

The increased size of the granular endoplasmic reticulum and Golgi apparatus and the formation of small vesicles close to the Golgi apparatus in the β-cells of 20-30 week old obese mouse islets and the increased incidence of β-granulolysis suggests the presence of active insulin biosynthesis and release. These observations are consistent with the fact that the plasma insulin level reaches its maximum at around 25 weeks of age in obese hyperglycaemic mice (Bailey et al., 1977). The granular endoplasmic reticulum and the Golgi apparatus have both been suggested to be the site of active β-granule formation (Howell et al., 1969; Orci et al., 1969). At 35-45 weeks of age, obese mouse islet β-cells show a further enlargement of the endoplasmic reticulum and Golgi apparatus as well as an increased incidence of scrolling of the β-granule membrane. This enlargement is consistent with an increased demand for insulin at this time.

Some β-cells have been observed to contain few β-granules but large areas of endoplasmic reticulum and Golgi apparatus and great numbers of unattached ribosomes. Other β-cells have been observed to contain large numbers of β-granules with reduced amounts of Golgi apparatus and endoplasmic reticulum. These observations and those of Herman and colleagues (1964) suggested that β-cell undergoes a cyclic, functional transformation.

The present study has established the occurrence of increased numbers of degenerate obese mouse islet β-cells with age. Agranular cells have been identified in lower vertebrates, such as cyclostomes (Falkmer, Hellman & Voigt, 1964; Falkmer, Boquist, Foa, Grillo, Sodoyez, Goffoux & Witty, 1969) as well as in higher vertebrates, such as Chinese hamsters and rabbits (Boquist, 1969; Boquist & Falkmer, 1970). Ultrastructurally, the agranular cell, as its name suggests, is characterised by the absence of granules and its cytoplasm shows low electron density. The agranular cells have been suggested to be immature precursors of granulated cells (Boquist & Falkmer, 1970). Equally well they might represent either degranulated alpha (α_2) cells (Lacy, 1957a) or degranulated β-cells (Sato, 1966). In the present work, agranulated cells were only observed in 40-45 week old obese mouse islets. These cells contained very few ribonucleoprotein-granules and only very small degenerate mitochondria. Degranulated β-cells have previously been observed in the islets of old obese mice (Atkins, 1972). The presence of completely degranulated β-cells and β-cells containing only a few β-granules in old obese mouse islets would suggest that the so-called agranular cells are probably degranulated β-cells.

In the present study, β-granules were observed within the lumen and cytoplasm of obese mouse islet capillary endothelial cells. It has always been assumed that the granule, once released, is

rapidly dissolved by interstitial fluids, subsequently undergoing physicochemical alterations that make it invisible (Lacy, 1961; Volk & Lazarus, 1962). Alternatively, the release of granules has been assumed to be episodic, that is, the granule is solubilised within the granule sac prior to its release (Herman et al., 1964). The present study has demonstrated the presence of cytoplasmic projections emanating from β-cells and containing β-granules. Recent scanning electron microscopical studies have supported these observations and have described the presence of irregularities on the surface of β-cells . These irregularities take the form of cytoplasmic projections and are believed to be the means by which β-granules are released from the β-cell into the extracellular and pericapillary spaces (Zimny & Blackard, 1975). However, the presence of β-granules in capillary walls of obese mouse islets is in agreement with the hypothesis that some granules leave the β-cells without undergoing prior dissolution (Bjorkman et al., 1963).

The β-cell nucleus undergoes slight morphological changes with the increasing age of the cell. There is often an invagination of the nuclear membrane and the subsequent passage of cytoplasm and organelles into the invagination. The appearance of cytoplasmic organelles within the nuclear membrane has been attributed to either abnormal mitosis (Bloom, 1967) or possibly to a swelling of the cell cytoplasm (Sobel, Schwarz & Marquest, 1969). Herbest (1976) attributed the nuclear invagination to a

reduced intranuclear pressure permitting the invagination of cytoplasm. Recently, Greider, Lacy, Kissane, Rieders and Thomas (1977) suggested that the nuclear inclusions of the rat pancreatic islets were of viral origin. The presence of cytoplasmic invaginations in the β-cell nuclei of old obese mice might be a function of age.

Intranuclear rods have been observed in nerve cells (Weindle, Schwink & Wetzstein, 1968) and more recently in the β-cells of obese hyperglycaemic mice (Boquist, 1969). Boquist suggested that these intranuclear rods might play some role in cellular activity or in cellular renewal and division. Mitotic figures have not been observed in old obese mouse islets. The presence of these rods in the nuclei of degranulated old obese mouse islet β-cells suggest that they might be associated in some way with cellular degeneration, but not β-cell renewal or division.

Increased numbers of mitochondria have been observed in the pancreatic β-cells of both spontaneously diabetic hamsters (Lacy, 1964) and long-term sulphonylurea-treated rabbits (Volk & Lazarus, 1964). The increased numbers of swollen mitochondria observed in the β-cells of obese mouse islets might reflect an increased functional activity of β-cells. In addition, irregularly shaped homogeneous bodies were often observed in the cytoplasm of obese mouse islet β-cells. These bodies are believed to be of mitochondrial origin and the result of altered mitochondrial function (Boquist, 1970). The increased incidence of these bodies in the

β-cells of old obese hyperglycaemic mice and the presence of swollen mitochondria is consonant with the observations previously made by Boquist (1970).

The β-cells of old obese mouse islets contain cytoplasmic ceroid bodies which have also been observed in the liver, uterus and skeletal muscle of man and many experimental animals including rats and mice (Hartroft & Thomas, 1957; Essner & Novikoff, 1960; Portá, 1963; Oliver, Essner, Zimring & Haimes, 1976). The site of origin and mechanism of formation of ceroid bodies is controversial. Early workers suggested mitochondria to be the site of formation of ceroid bodies (Hess, 1955; Yokoh et al., 1959; Duncan, Nall & Morales, 1960). This assumption was based on the presence of ceroid bodies adjacent to mitochondria. Subsequent histochemical and ultrastructural studies have indicated that the ceroid body is formed from the lysosome (Essner & Novikoff, 1960; Hartroft & David, 1964; Portá & Hartroft, 1964; Portá & Wilson, 1964; Hasan & Glees, 1973; Oliver et al., 1976). The ceroid body has been observed in pancreatic islet β-cells of the human diabetic (Nakayama et al., 1971) and in human β-cell tumours (Goldenberg et al., 1969), yet little information has been offered as regards their origin or function. The present ultrastructural studies are the first to document the presence of ceroid bodies in the β-cells of obese mice. Ceroid bodies were observed to be closely associated with the lysosomes of degenerating old obese mouse

islet β-cells, but were not found in the β-cells of old lean mice. The ceroid body is usually associated with necrotic and degenerating cells (Portá & Hartroft, 1963). Ceroid bodies are quite common and numerous in the islet β-cells of elderly diabetics (Lacy & Greider, 1972). In the present study, besides being observed in necrotic, degranulated and degenerating β_1- and β_2-cells of old obese mice, ceroid bodies were also observed in the islets of lean mice that had been made obese by gold thioglucose treatment. Either ceroid bodies and the enzymes they contain play a role in the later stages of β-cell degeneration, or obesity and age in some way influence their formation. However, it seems that the appearance of ceroid bodies might be associated with certain oxidative metabolic properties of the cell, and that obese mouse β-cells might exhibit ceroid bodies at an earlier age than β-cells of normal lean littermates. Ceroid bodies have been induced experimentally in liver by feeding albino mice a necrotic diet. The number of ceroid bodies present was reduced when these animals were given vitamin E (Portá & Hartroft, 1964). Indeed, ceroid body formation has been shown to be increased in the liver of mice fed vitamin E deficient diets (De Witt, 1958). Casselman (1951) suggested that the ceroid body was formed by the accumulation of unsaturated fatty acids in such a manner that there was a relative lack of biological antioxidants. The consequence of this was the auto-oxidation of the fats and their conversion to ceroid body material. Vitamin E is thought to be the antioxidant that prevents the formation of ceroid

material (Portá & Hartroft, 1964). This would suggest that vitamin E absorption and metabolism might be impaired in the obese mouse. In addition, a reduced availability of vitamin E might be responsible for the poorly developed sex organs of the obese mouse.

The presence of mixed exocrine-endocrine cells in and around islet tissue has been confirmed by many workers (Pictet et al., 1967; Orci et al., 1970; Shorr & Bloom, 1970; Atkins & Matty, 1973). Shorr called the cell containing both zymogen and β-granules an "Acino insular cell". Herman and colleagues (1964) suggested that the cytoplasm of acinar and islet cells might somehow have become intermingled. Leduc and Jones (1968) have reported the presence of acinoinsular cells in the islets of hyperglycaemic "Wellesly hybrid" mice. The presence of acinoinsular cells has been claimed to be associated with an exaggeration of the hyperglycaemic stimulation of the β-cells in spiny mice (Orci, Rufner, Renold & Rouiller, 1970). Melmed, Benitez and Holt (1972, 1973) and Setalo, Blatniczky and Vigh (1973) have demonstrated the presence of mixed cells in the pancreatic islets of rats. These workers claimed the existence of intermediate forms between alpha, beta and acinar cells. After the injection of alloxan, Setalo and colleagues observed a selective destruction of intracellular organelles morphologically similar to β-granules in these cells. The low frequency of mitotic figures among β-cells, in both foetal and postnatal islets, led to the suggestion that

proliferation of these mixed cells occurred by transformation of other islet cell types in the exocrine part of the pancreas (Bunnag, 1966). There is now evidence to suggest that both endocrine and exocrine cells arise from a common "protodifferentiated" cell type, probably of endodermal origin (Pictet & Rutter, 1972). Hellerström and Andersson (1976) suggested that the presence of acinoinsular cells might represent a regeneration of cells in diabetic islets. Atkins and Matty (1973) observed the presence of exocrine-like cells within the islets of old obese mice by light microscopy. In the present ultrastructural studies, a lamellar endoplasmic reticulum, zymogen granules and large mitochondria have often been observed in the cytoplasm of old obese mouse islet β-cells. In addition, the present study has confirmed the presence of many unidentified mixed cells at the edge of obese mouse islets containing both endocrine- and exocrine-like granules. These mixed cells might represent exocrine cells undergoing endocrine transformation.

Lymphocytic infiltration has been reported in the pancreatic islets of human diabetics and certain animal models showing inappropriate hyperglycaemia (Gepts, 1965, 1972; Craighead, 1972). Gepts (1972) observed lymphocytic infiltration in the islets of juvenile diabetics, whilst Craighead, Kanich and Kessler (1974) observed the same in the islets of Langerhans of mice injected with the encephalomyocarditis virus. Johnson (1973) reported the

coincidence of amyloid deposition and lymphocytic infiltration in the pancreatic islets of diabetic cats.

The present study has demonstrated the presence of large numbers of lymphocytes in the capillaries and β-cells of the islets of Langerhans of obese hyperglycaemic mice. The presence of these lymphocytes coincides with β-cell degeneration and islet vacuolation. Recently, Egeberg, Nerup, Anderson, Bendixen, Kromann, Gunnarsson, Hellerström and Poulsen (1976) immunised normal C57 mice with obese hyperglycaemic mouse islet extract as an antigen and observed the occurrence of β-cell degeneration and lymphocytic infiltration. Islet β-cell degeneration has also been observed in rats immunised with foetal calf pancreatic extract (Nerup, Anderson, Bendixen, Egeberg, Poulsen, Vilien & Westrup, 1976). The general picture of mononuclear and lymphocytic infiltration with partial or complete destruction of the β-cells and the occasional infiltration of fibrous tissue might suggest an infectious agent, possibly a virus (Warren et al., 1966). On the other hand, the occurrence of mononuclear cells in obese mouse islets and the subsequent degeneration of the islet cells suggests that an auto-immune mechanism might be an aetiological factor in the manifestation of the syndrome. The presence of macrophages containing phagocytosed β-granules might provide a possible source of immune complex. The possibility of viral infection in obese mouse islets needs further investigation. Viral infection may be a causative factor in insulitis (Doniach, 1974).

Erythrocytes have been observed invading the pancreatic islet tissue of obese hyperglycaemic mice. The mechanism of tissue invasion by erythrocytes has been studied by Johnson (1971). The red blood cells push against the weak endothelial lining of the capillary, forming a passage or a channel through the endothelial cytoplasm into the interstitial spaces. It has been suggested that leukocytes could possess proteolytic enzymes which might enable them to digest the basement membrane of pancreatic islet β-cells (Williamson & Grisham, 1961). The islet capillaries of obese hyperglycaemic mice have been reported to be very thin-walled (Hellman et al., 1961) and the present ultrastructural work would support this.

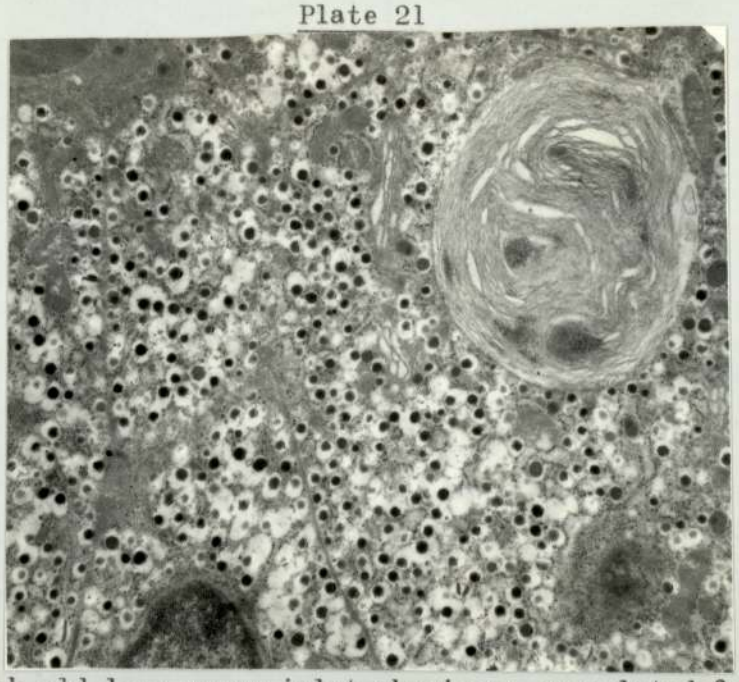
The present study has demonstrated blood platelet aggregation in the islet capillaries of 30-45 week old obese mice. In recent surveys on the pancreatic islets of man, rats, cats, dogs and mice, no incidence of pancreatic islet capillary platelet aggregation was reported. Bjorkman and colleagues (1963) believed this platelet aggregation to be protrusions of the capillary endothelial cytoplasm containing dense masses of β-cell granules. The present study confirms these structures to be blood platelets.

Adenosine diphosphate (ADP) and many other metabolites have been shown to be capable of inducing platelet aggregation. ADP itself has been shown to be synthesised and released from injured capillary endothelial cells (Honour & Mitchell, 1964), red blood cells and from platelets themselves (MacMillan, 1966). The present

study has demonstrated platelet aggregation in the thin-walled degenerating blood vessels of old obese mouse islets. Platelet aggregation has been demonstrated after injury to endothelial cells (Johnson, Webber, Wajeik & Yun, 1969). These workers consider that the ADP released by injured endothelial cells initiates the formation of thrombin, which brings about platelet aggregation. Collagen fibrils also have the capacity to aggregate platelets. Hoving (1963) established collagen fibrils as the agents responsible for platelet adherence to a connective tissue suspension. Platelets are believed to contribute directly to the inflammatory response accompanying tissue injury (Mustarod, Movat, Macmorine & Senyi, 1965). In the present work, amyloid-like material was demonstrated in the islets of old obese mice. Collagen is believed to be a precursor of these amyloid fibrils (Stiller & Katenkamp, 1976). Collagen fibrils have been demonstrated in obese mouse islet capillary walls near areas of platelet aggregation. There is evidence that collagen fibrils adhere to blood vessel walls leading to the development of thrombi (Chandler, 1971). These thrombi may impede the passage of blood through the blood vessel (Russell, 1961; Guning, 1964). The aggregation of platelets in old obese mouse islet blood vessels might form thrombi and decrease the flow of blood, thereby reducing the circulating level of insulin. Other factors, such as serotonin, adrenaline, histamine, calcium (Johnson, 1971) and even D-glucose (Bridges, Dalby, Miller & Weaver, 1965) have all been shown to enhance platelet aggregation. The role these factors might play in platelet aggregation and the effect that the latter has on the functional activity of old obese mouse islets remains to be investigated.

PLATES 21-38

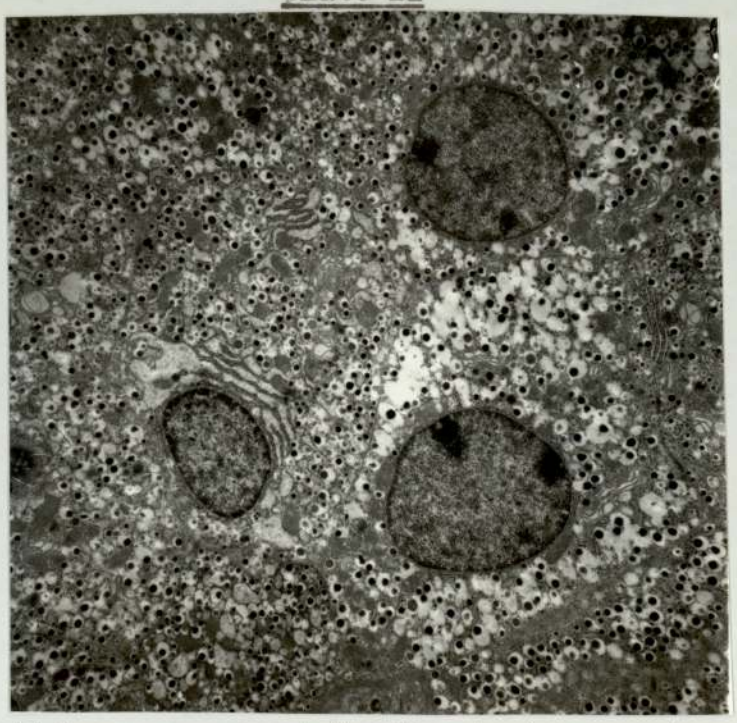
Plate 21



A 25 week old lean mouse islet showing a granulated β_1 cell and a myelinated nerve fibre.

Glutaraldehyde-osmium x 5,000.

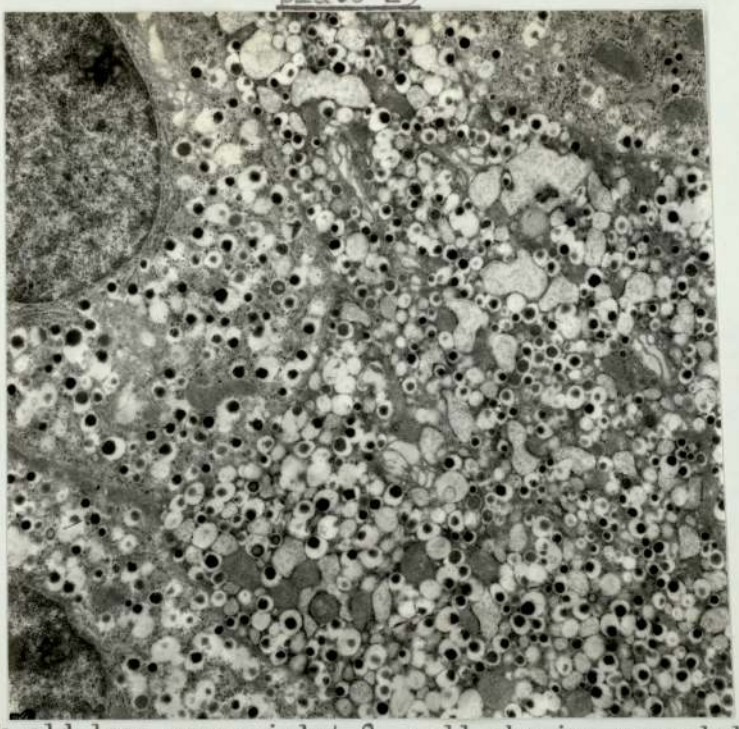
plate 22



30 week old lean mouse islet β-cells showing an enlargement of the endoplasmic reticulum and β-granulolysis.

Glutaraldehyde-osmium x 5,000.

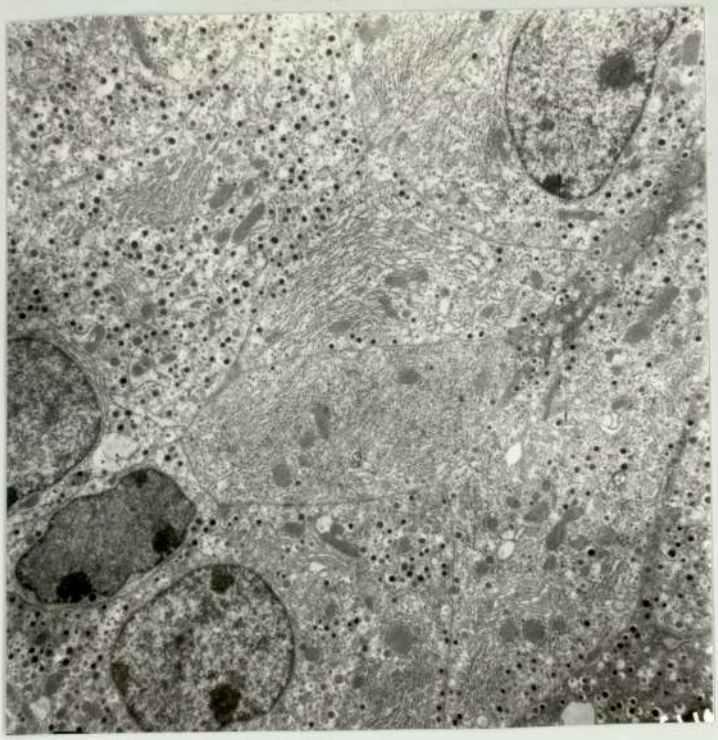
plate 23



A 40 week old lean mouse islet β_2 cell showing granulolysis.

Glutaraldehyde-osmium x 5,000.

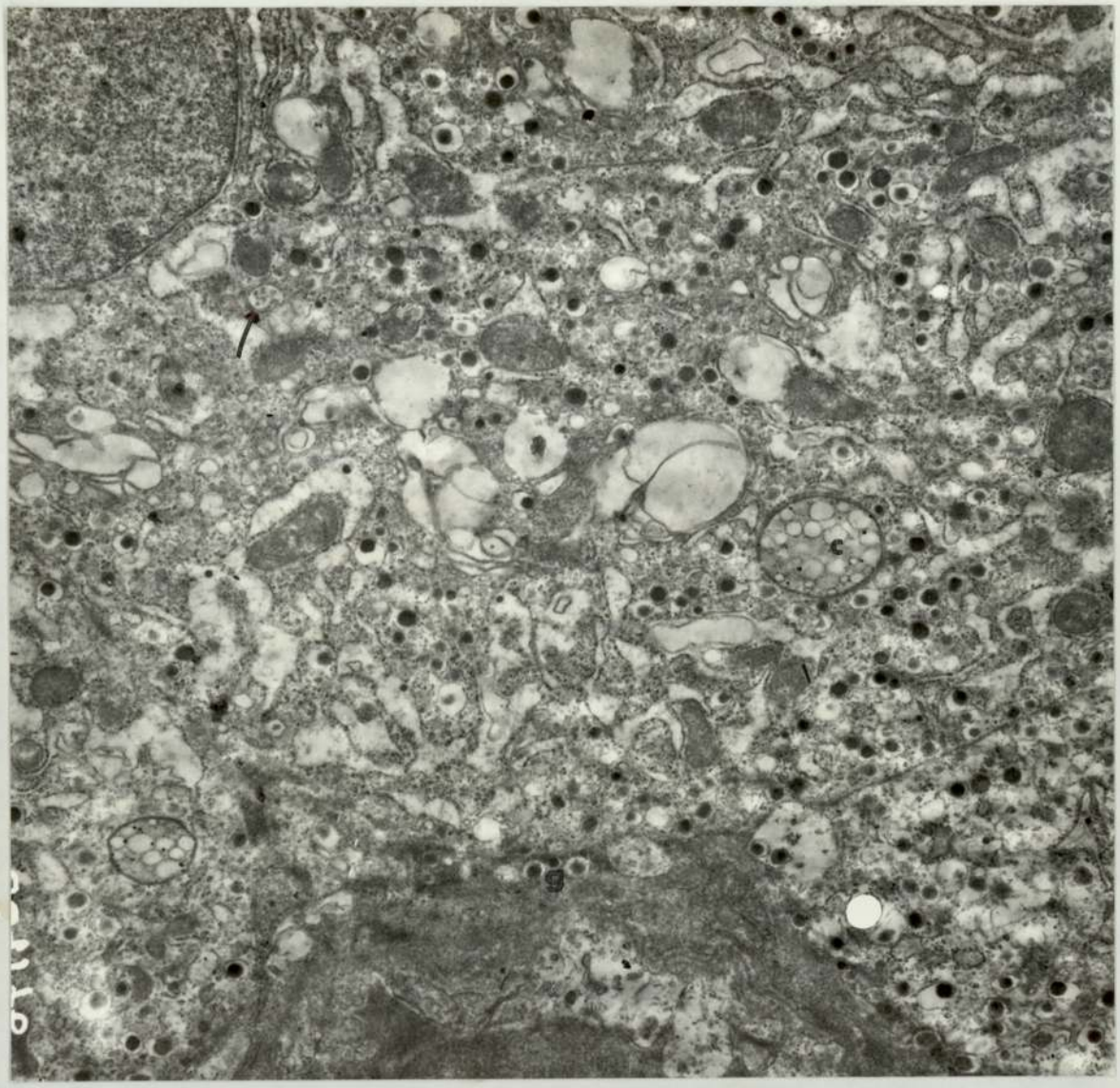
plate 24



A 45 week old lean mouse islet showing enlargement of the endoplasmic reticulum.

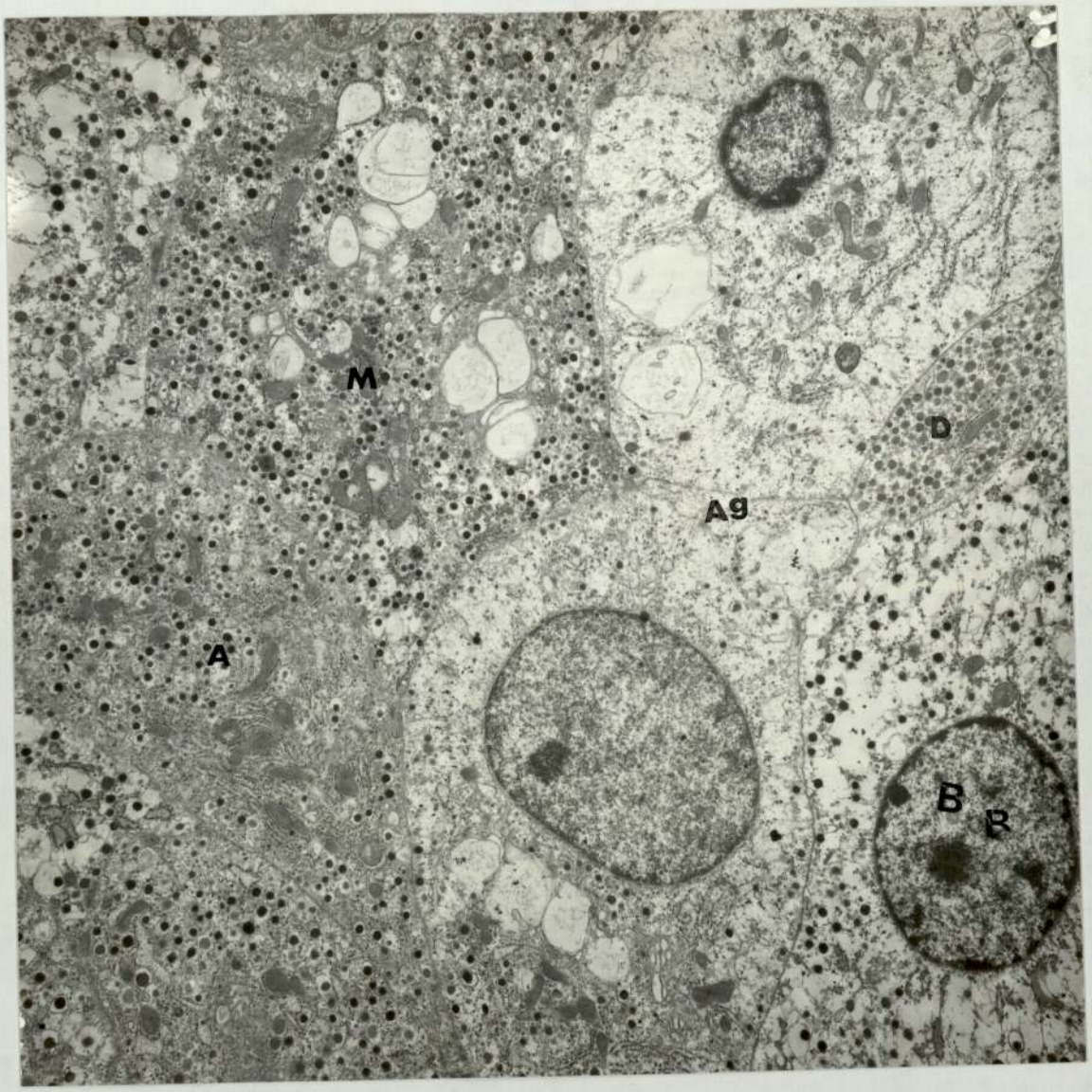
Glutaraldehyde-osmium x 5,000.

Plate 25



β_1 cell of a 42 week old obese mouse islet. Notice the β-granules (g) moving towards the capillary lumen. Many ceroid bodies (C) are present together with a granular vesicle (arrow). Glutaraldehyde-osmium x 10,000.

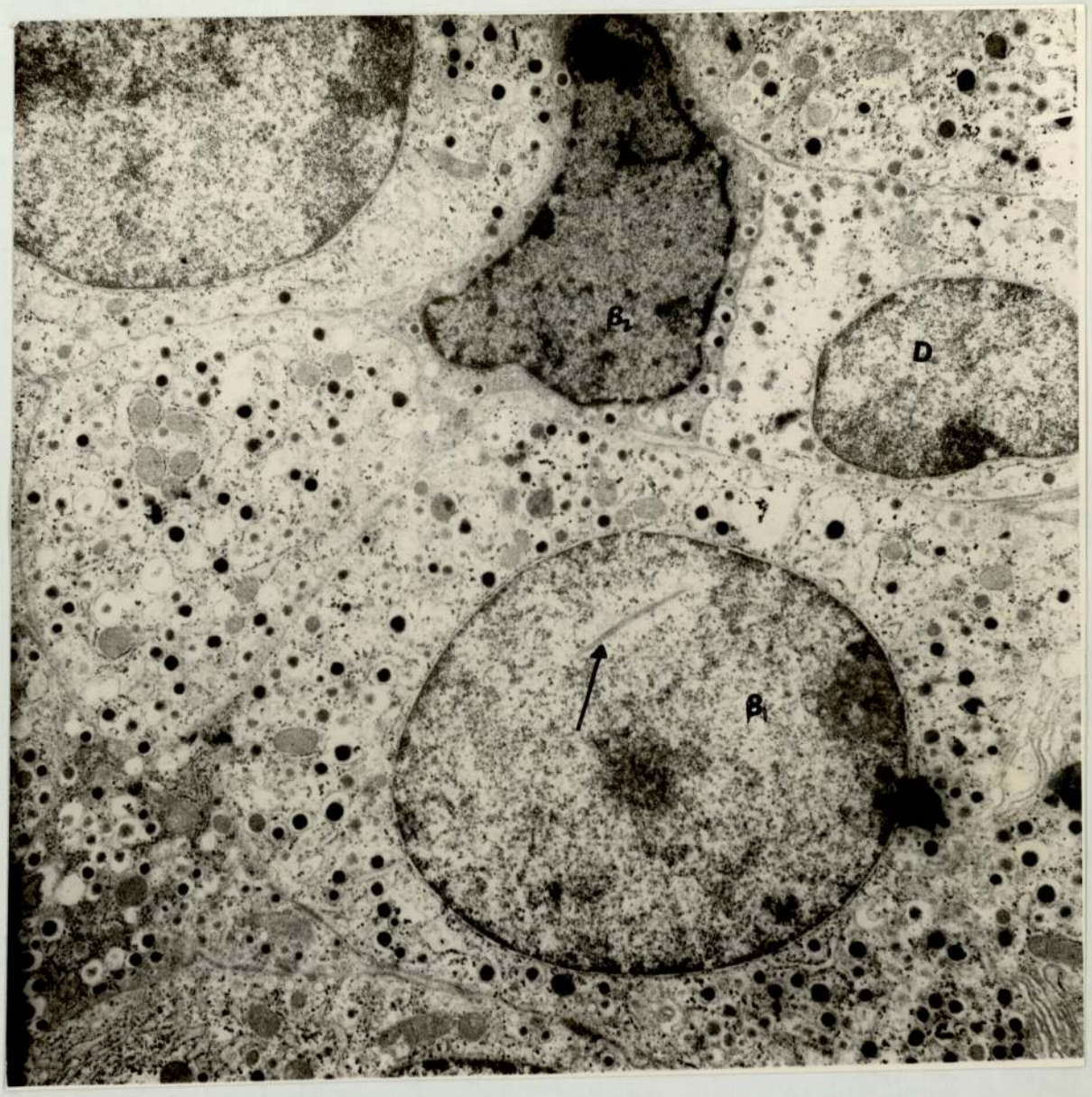
Plate 26



A 45 week old degenerating obese mouse islet containing vacuolated agranular cells (Ag). D, delta cell; β, β_1 cell; A, α_2 cell; M, increased numbers of mitochondria.

Glutaraldehyde-osmium x 6,000.

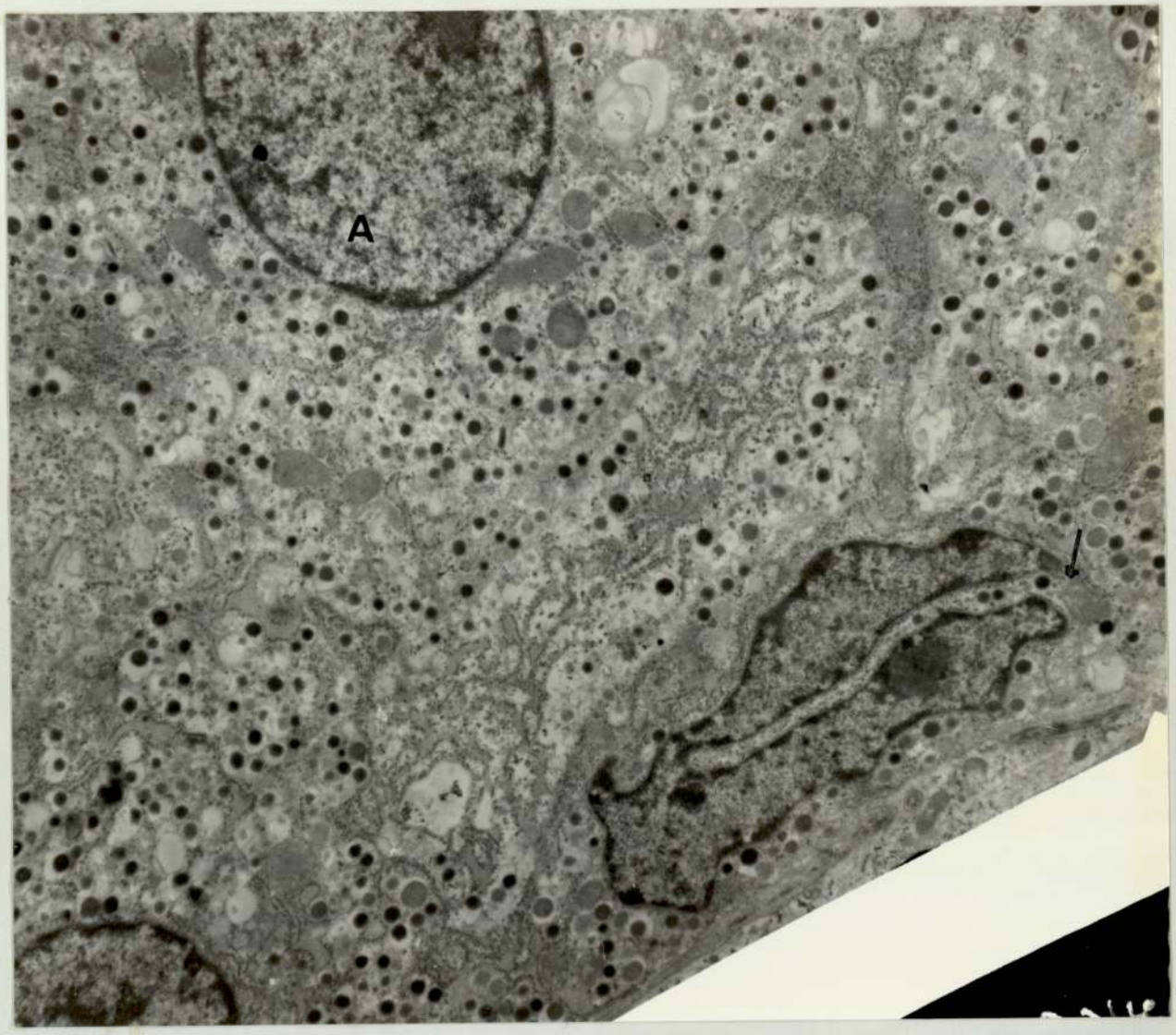
Plate 27



A 45 week old obese mouse islet. Notice the presence of an intranuclear rod (arrow) in the nucleus of a β_1 cell. β_1, β_1 cell; β_2, β_2 cell; D, delta cell.

Glutaraldehyde-osmium x 10,000.

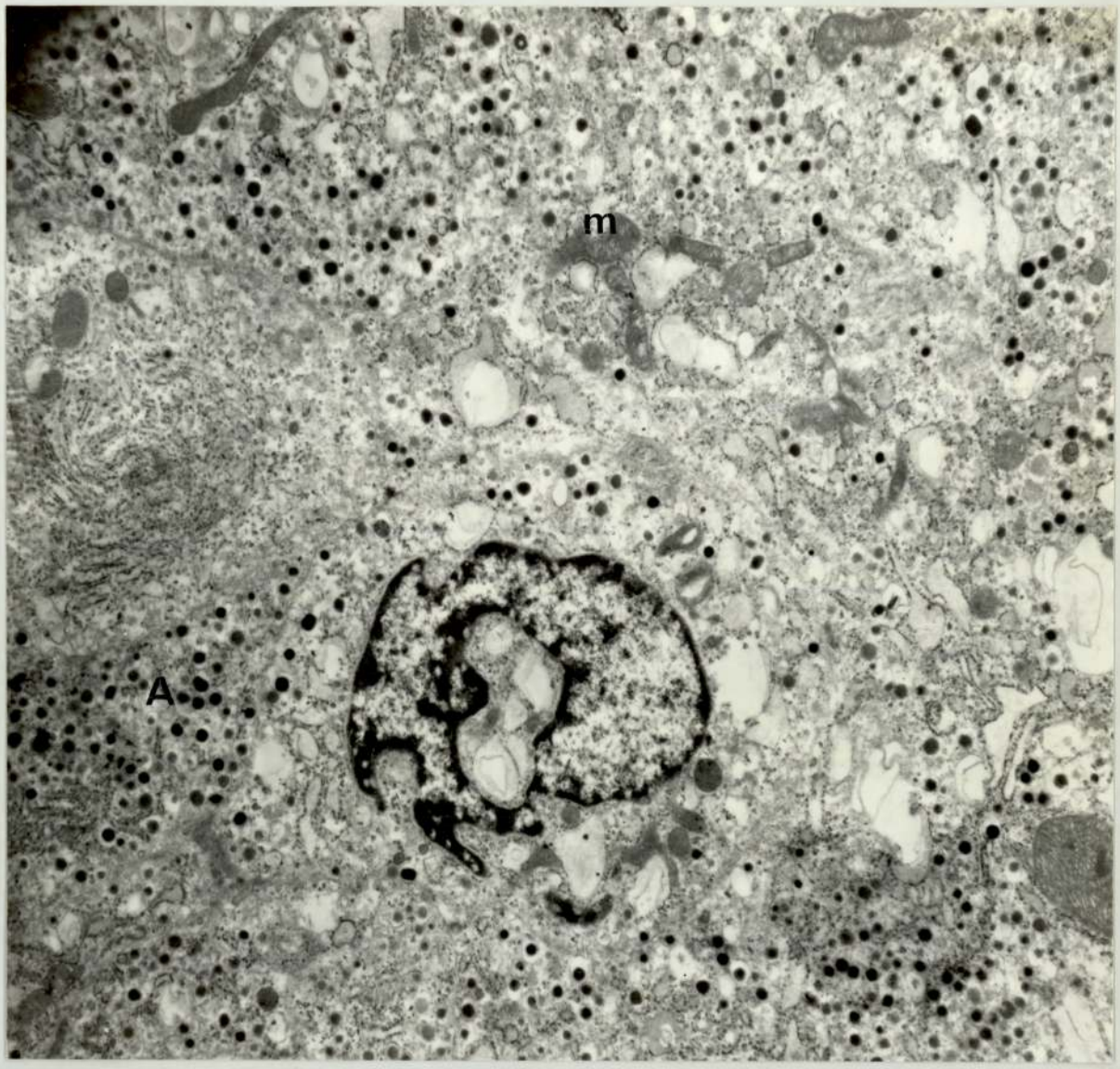
Plate 28



A 45 week old obese mouse islet showing nuclear invagination (arrow) in a β-cell. A, α_2 cell.

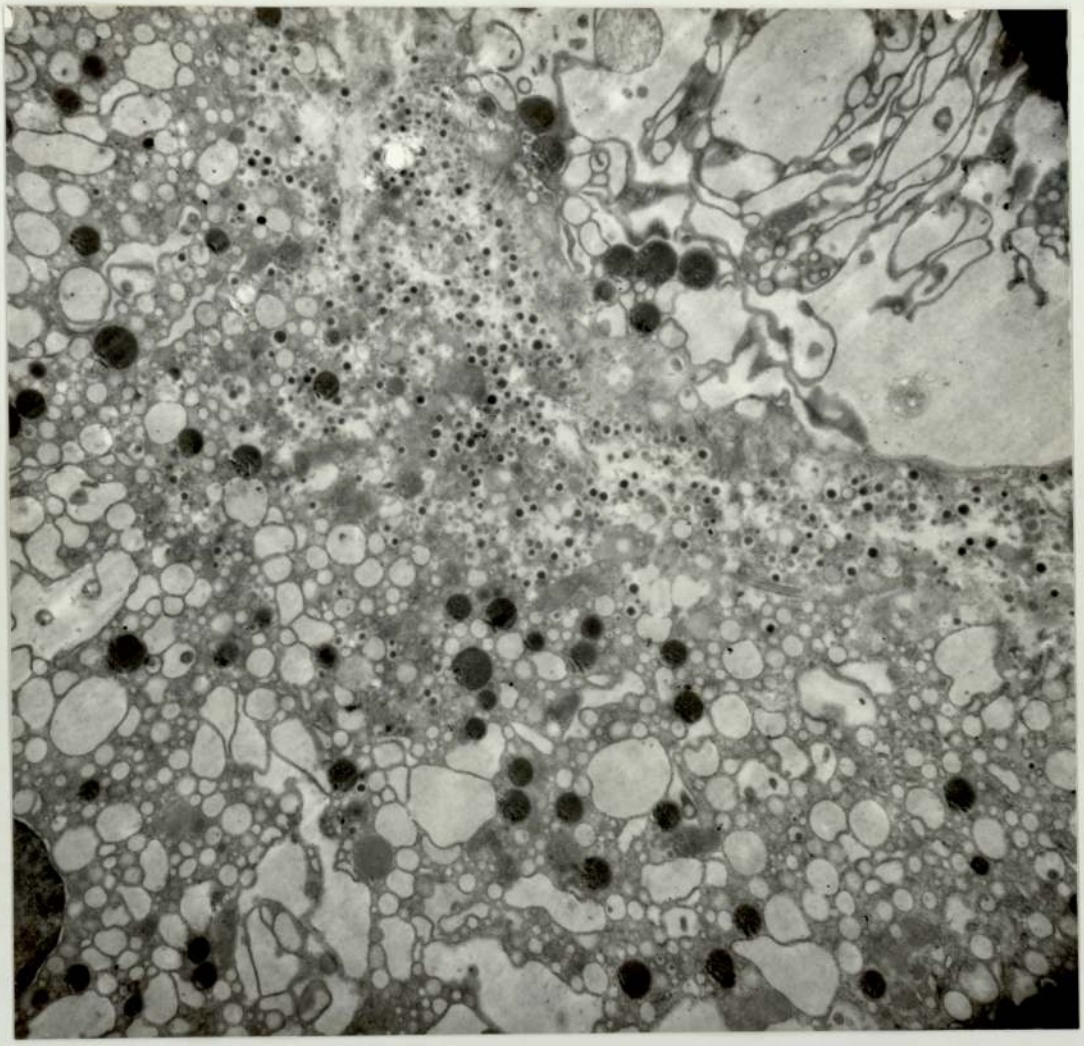
Glutaraldehyde-osmium x 10,000.

Plate 29



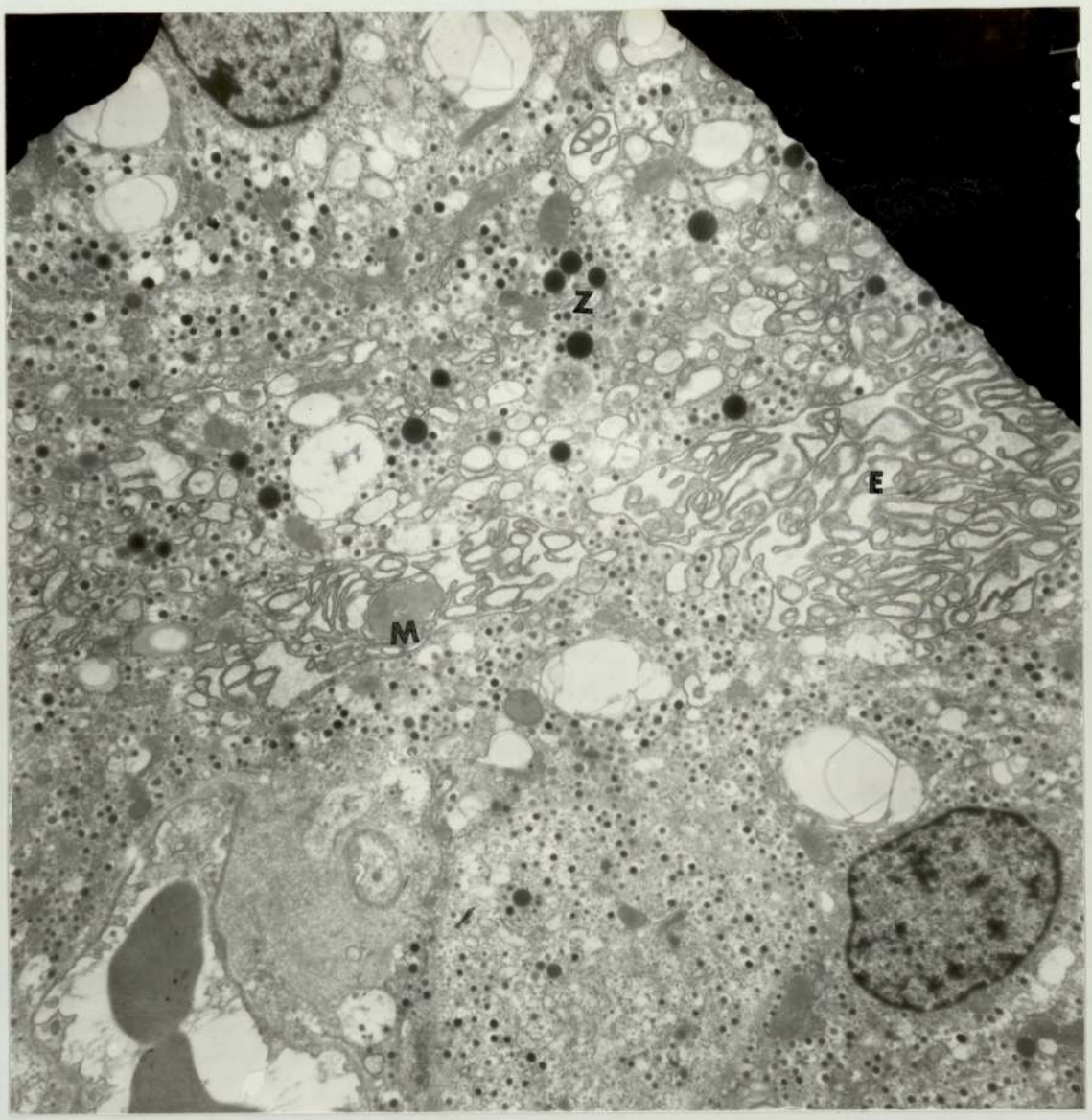
A 45 week old obese mouse islet showing nuclear invagination in a β_1 cell. A, α_2 cell; M, swollen mitochondria.
Glutaraldehyde-osmium x 10,000.

Plate 30



A 35 week old obese mouse islet. Notice the presence of the so-called perinsular acinar cells at the edge of the islet. Glutaraldehyde-osmium x 6,000.

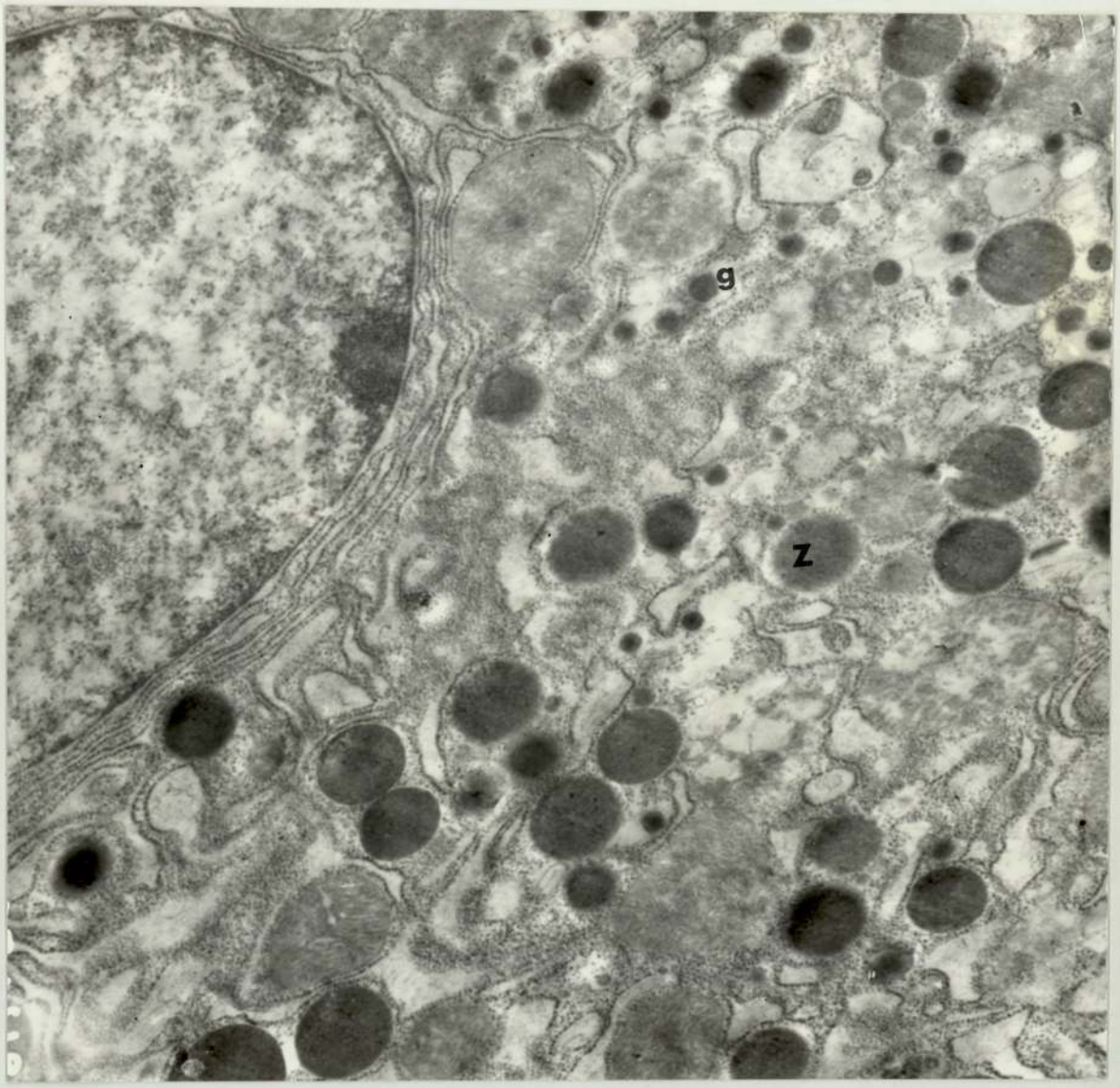
Plate 31



A 45 week old obese mouse islet containing perinsular acinar cells. Zymogen granules (Z) can be seen close to the lamellar endoplasmic reticulum (E) and large mitochondria (M). β, β_1 cell; Ca, blood capillary.

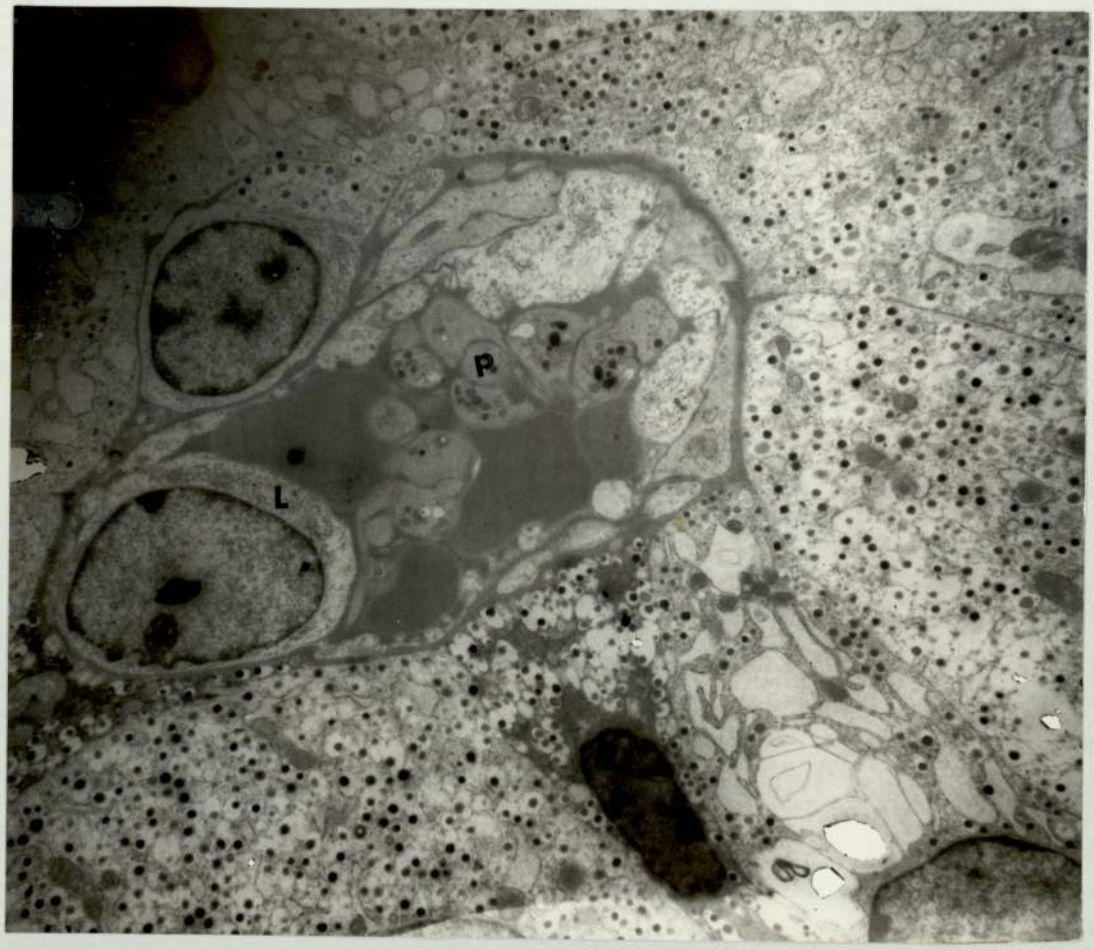
Glutaraldehyde-osmium x 6,000.

Plate 32



A 40 week old obese mouse islet. Notice the presence of endocrine granules (g) between the zymogen granules (Z) of the acinar tissue. Glutaraldehyde-osmium x 20,000.

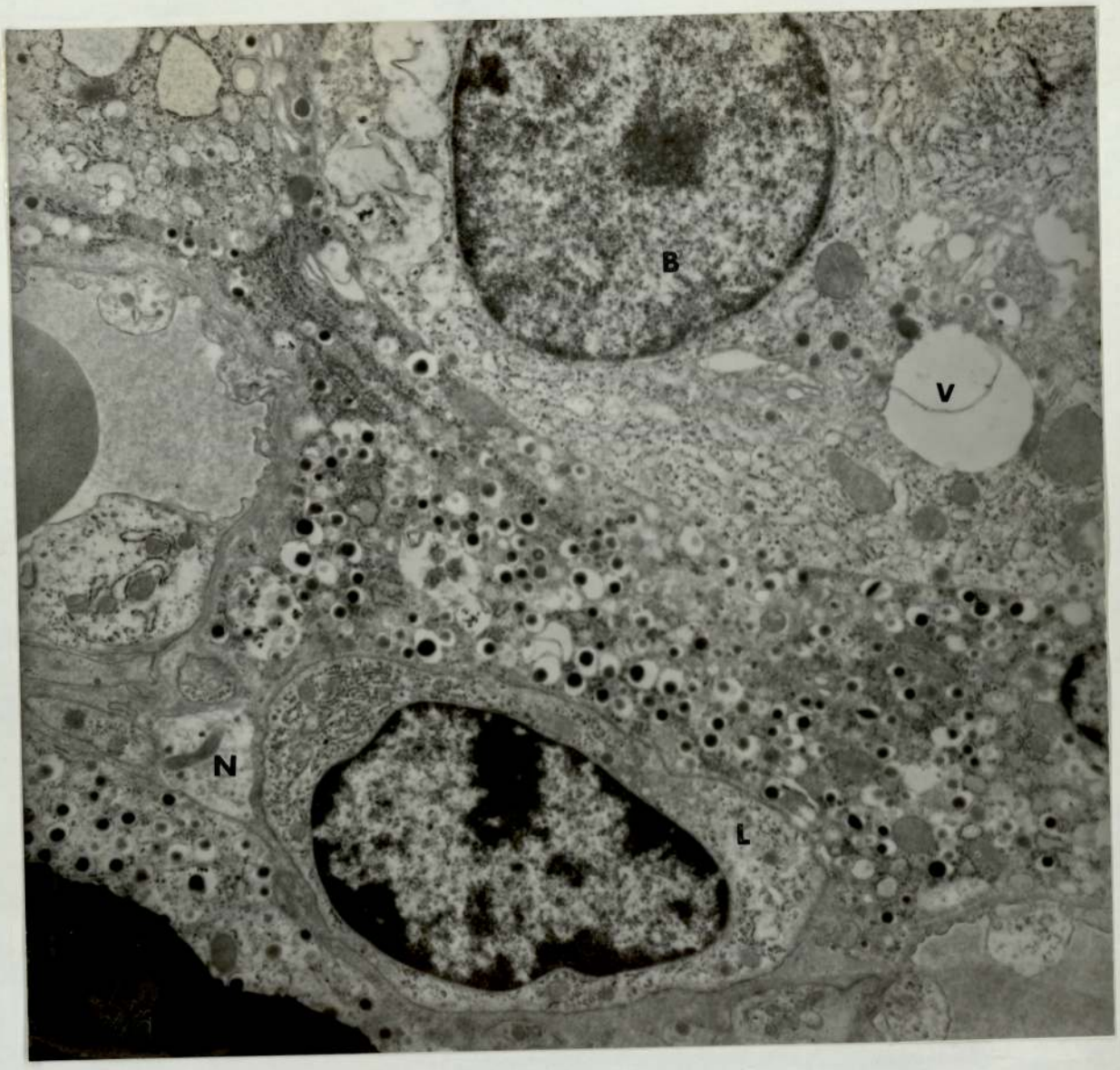
Plate 33



A 35 week old obese mouse islet showing platelet aggregation (p) and lymphocytic infiltration (L).

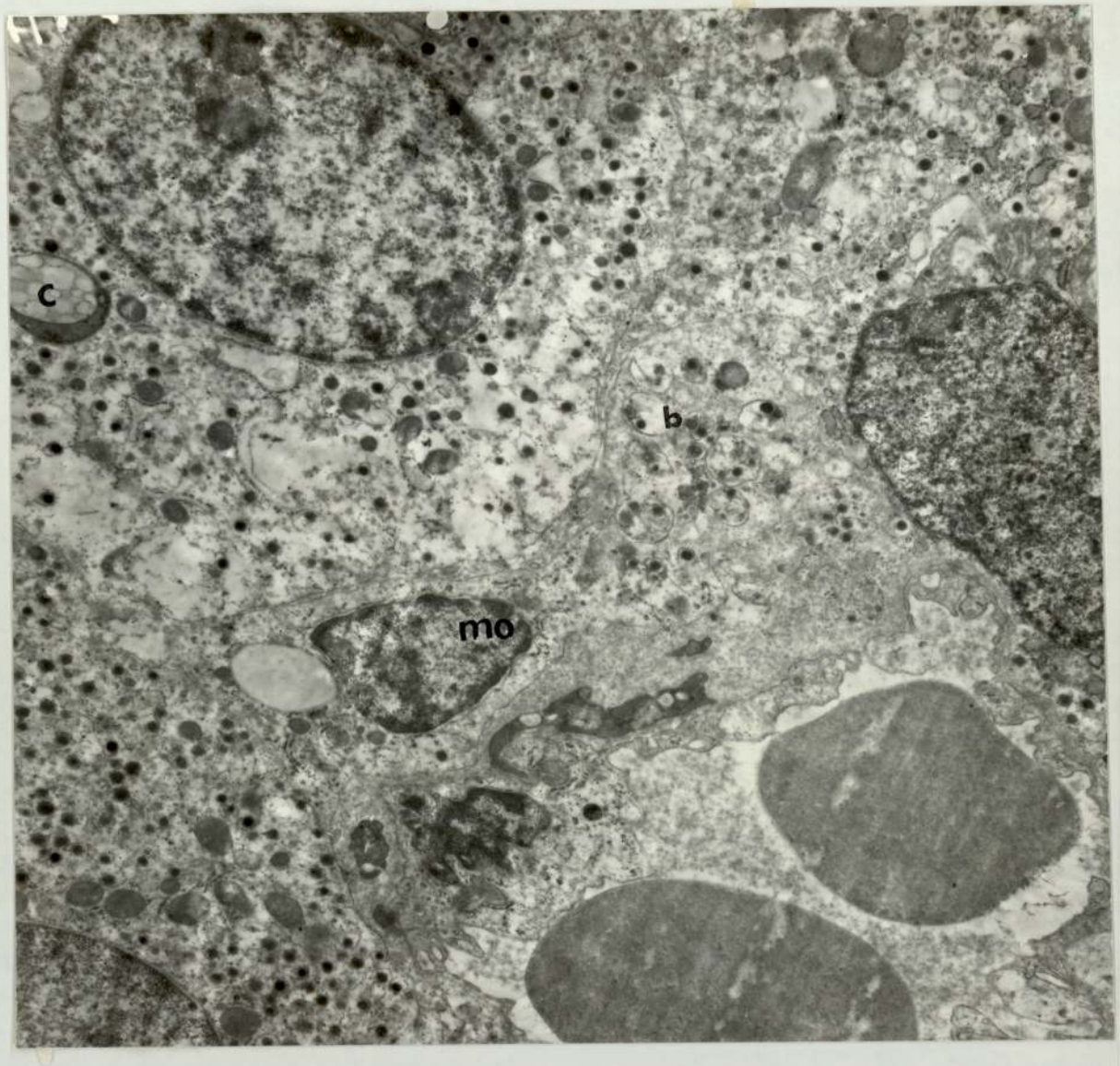
Glutaraldehyde-osmium x 6,000.

Plate 34



A 40 week old obese mouse islet showing the presence of lymphocytes (L) and a degranulated β-cell. V, vacuole. Glutaraldehyde-osmium x 10,000.

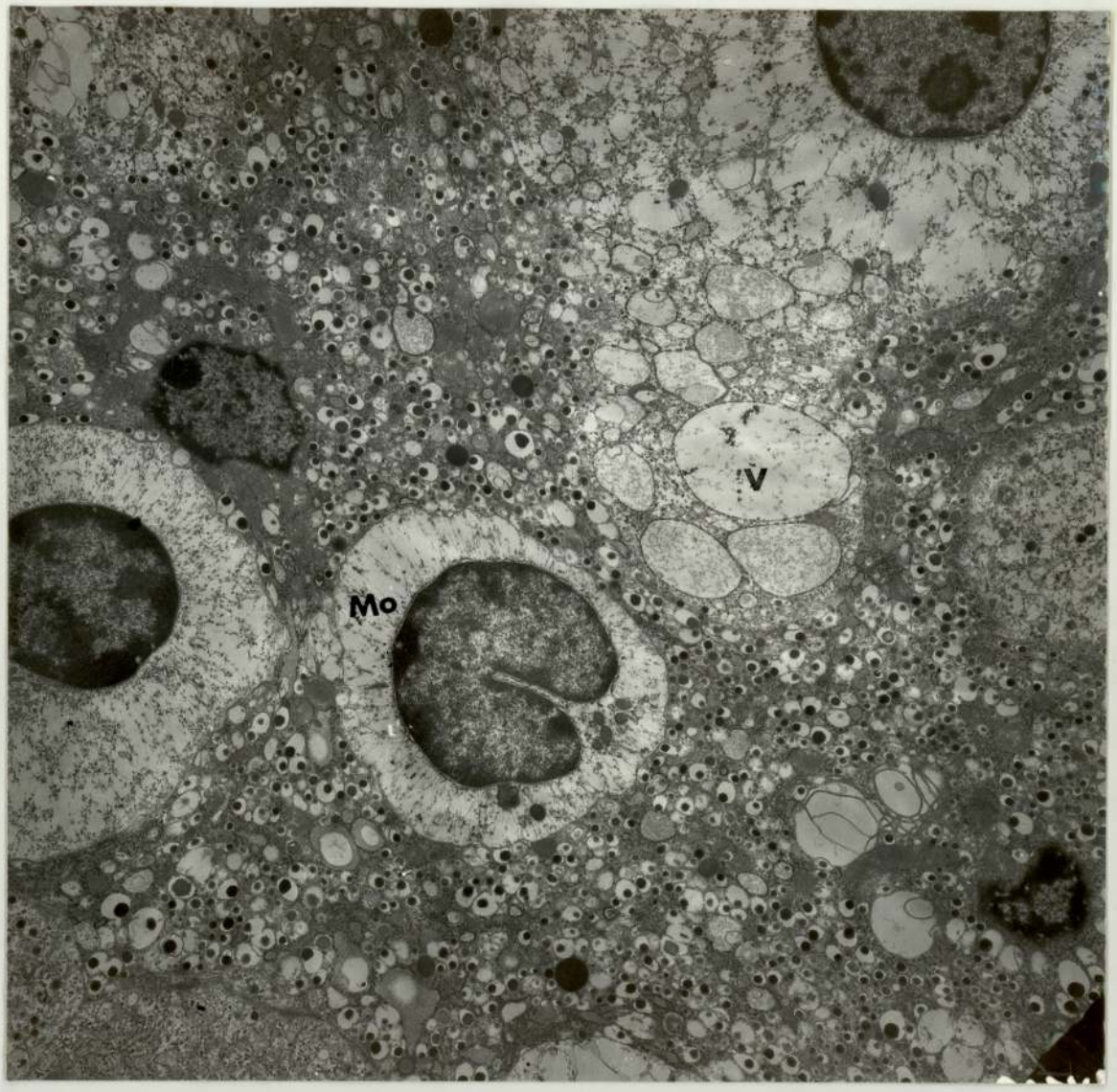
Plate 35



A 45 week old obese mouse islet showing a macrophage (Mo) engulfing β-granules (b). Notice the presence of the ceroid body (C) inside a degenerating β-cell.

Glutaraldehyde-osmium x 10,000.

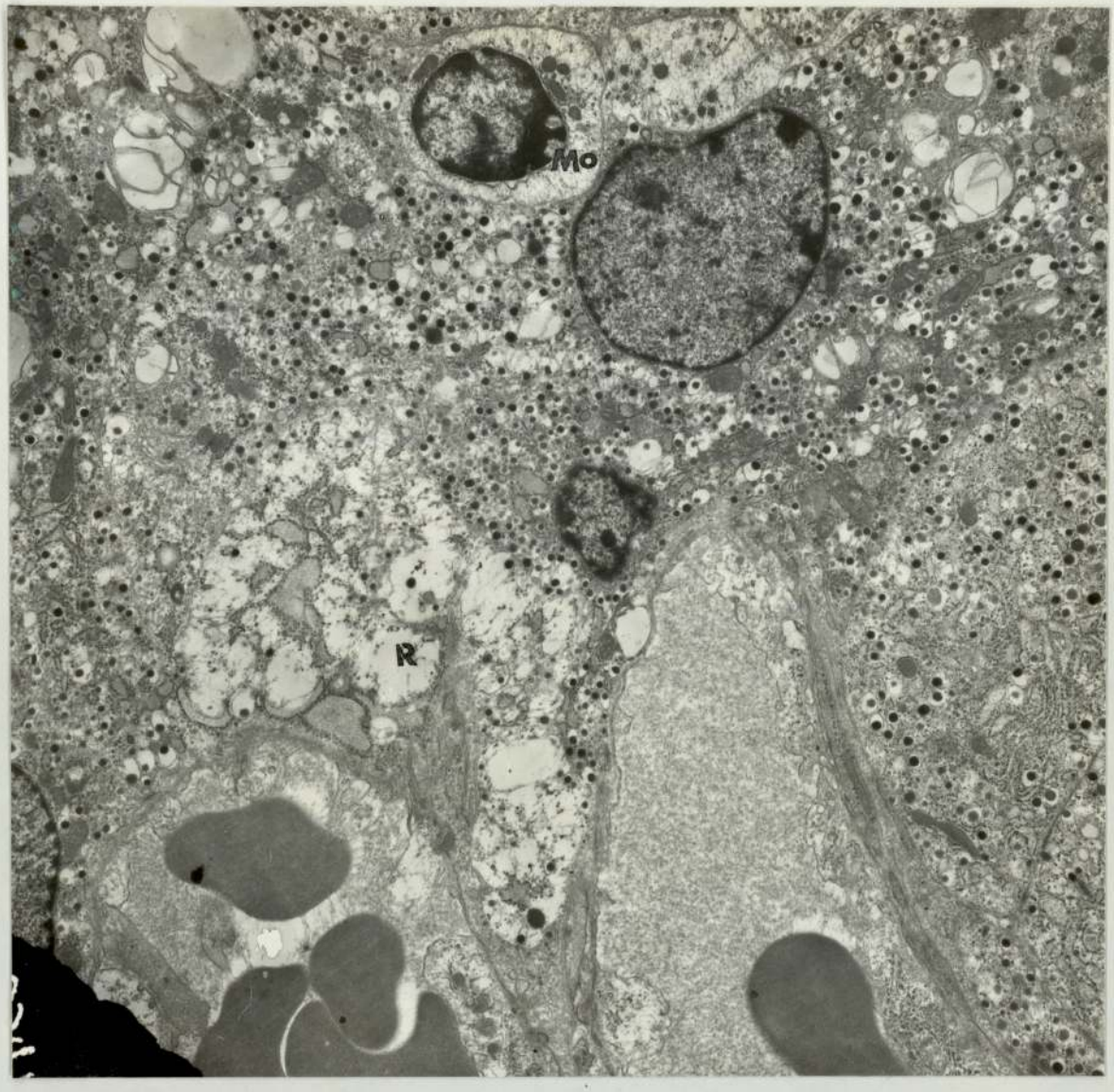
Plate 36



Monocytic (Mo) infiltration of a 45 week old obese mouse islet β-cell consistent with cellular degeneration. Large vacuoles (V) have appeared in the β-cell cytoplasm.

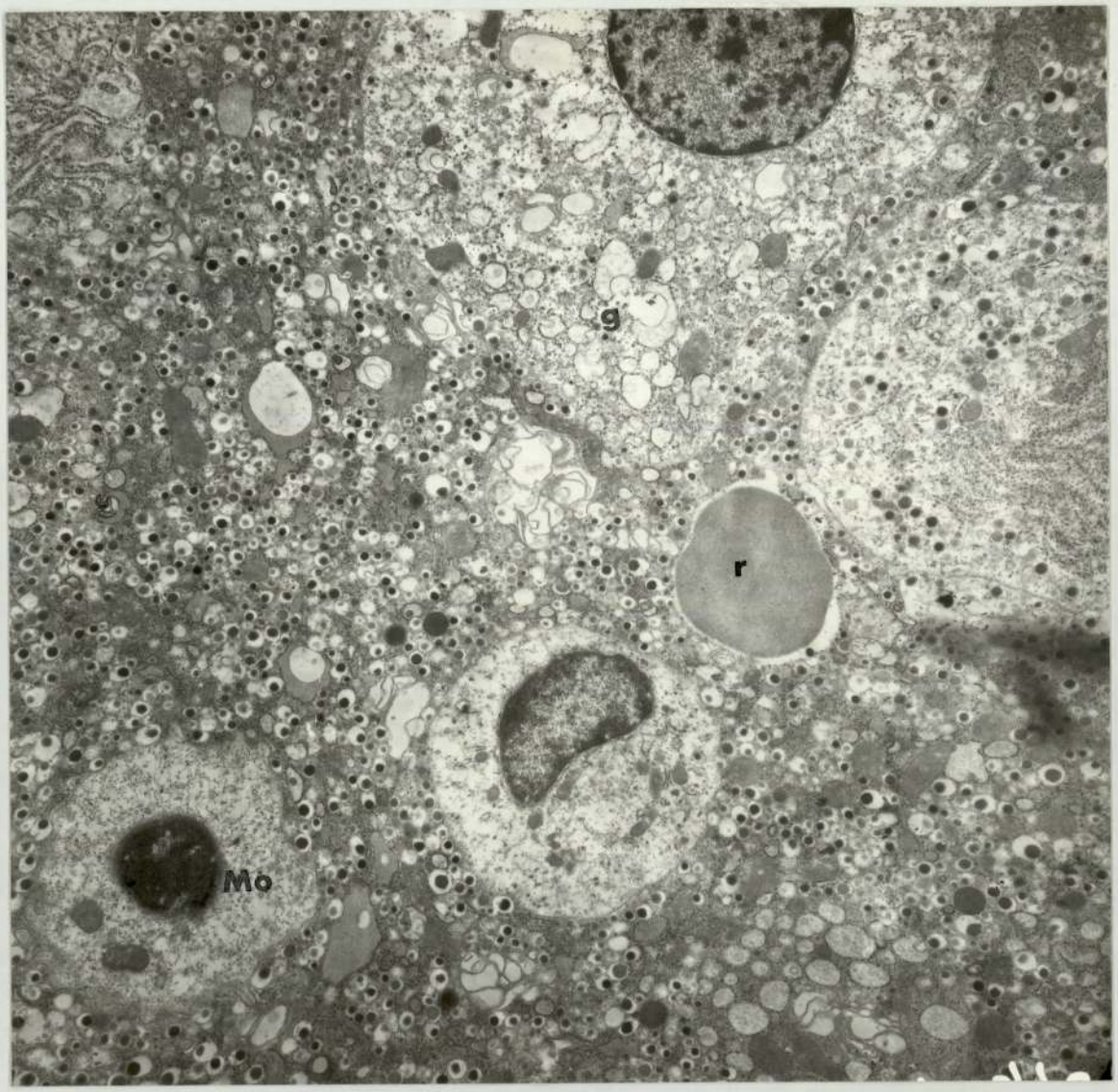
Glutaraldehyde-osmium x 6,000.

Plate 37



Islet tissue of a 45 week old obese mouse showing thin walled blood capillaries. Cytoplasmic rarefaction (R) of a β-cell and the infiltration of the tissue by a monocyte (Mo).
Glutaraldehyde-osmium x 6,000.

Plate 38



Monocytic infiltration (Mo) in the islet of a 45 week old obese mouse. Notice the degenerated β_1 cell and the enlargement and granulolysis of β-granules (g). Presence of red blood cell (r) in islet tissue.

Glutaraldehyde-osmium x 6,000.

4.2 Vacuole formation in the islets of obese hyperglycaemic mice

4.2.1 Introduction

Early work by Bjorkman and colleagues (1963) and Westman (1968) failed to demonstrate any gross pathological changes in the islets of Langerhans of obese hyperglycaemic mice with age. Atkins (1972) and Atkins and Matty (1973) presented preliminary evidence to show the presence of β-cell degeneration, fibrosis and vacuole formation in the pancreatic islets of obese mice. Islet vacuoles have been reported to occur in the Wellesly hybrid mouse (Like et al., 1965; Like & Jones, 1967) and in the yellow KK mouse (Shino & Iwatsuka, 1970), yet no information has been offered as regards either the process or aetiological consequences of their formation. The present study sets out to demonstrate the presence and formation of islet vacuoles and to examine histochemically the material they contain.

4.2.2 Materials and methods

Pancreatic tissue was excised from lean and obese mice between the ages of 7-45 weeks. Half of the pancreas was used for the microdissection of islets and the latter were processed for electron microscopy as described on page 18. The other half was processed for histochemical studies as described on page 15. Fifty sections were taken from each half pancreas and stained with congo red, thioflavine "T" and methylene blue for amyloid material; Mallory phosphotungstic acid haematoxylin (PTAH), Masson's trichrome stain, Heidenhain's azan stain, Gomori's reticulin and M.S.B. (Martin's, scarlet, blue) for connective tissue; PAS for carbohydrate and PAS with saliva for glycogen.

4.2.3 Results

Light microscopical examination of lean mouse islets with age failed to demonstrate any vacuoles (Plates 39, a-d).

Between 15 and 25 weeks of age, large central cavities begin to appear in the islets of obese mice. The size of the vacuoles gradually increased with age and the severity of the syndrome. In some islets, no central cavity was observed (Plate 40), only a large number of swollen-walled capillaries (Plate 41), which became more dilated with age. The blood vessels began to rupture

leaving small cavities or vacuoles (Plate 42). Adjacent capillaries tended to coalesce and open directly into the preformed vacuoles (Plate 42). The vacuoles tended to increase in size with age and severity of the syndrome, eventually coming to occupy most of the islet volume. As the size of the vacuole increased, the number of β-cells in the islet tended to decrease proportionately. Finally, a narrow layer of β-cells, connective tissue and α-cells remained between the edge of the vacuole and the connective tissue capsule of the islet (Plates 43 & 44). The remaining β-cells were still aldehyde fuchsin positive and, hence, contained insulin. The islet vacuole appeared to be filled with a semi-fluid like material containing lymphocytes (Plates 44 & 45), red blood cells (Plate 47) and a hyaline fibrous material (Plate 47). The islet connective tissue capsule became increasingly thicker with age and fibrocytic material was often seen invading the remaining islet space (Plates 47 & 49).

The histochemical nature of the islet vacuole contents has been summarised in Table 3. The material gave a negative reaction with amyloid stain such as congo red, thioflavine "T" and methylene blue. However, the material gave a positive reaction to connective tissue stains such as Mallory phosphotungstic acid haematoxylin (PTAH), Masson's trichromestain, Heidenhain's azan stain, Gomori's reticulin and M.S.B. In addition, the material gave a positive reaction to periodic acid Schiff (PAS), but a negative reaction to PAS after digestion with saliva (Plates 45-49).

Table 3

Summary of the histochemical characterisation of islet vacuole contents.

Stain	Reaction	Inference
Congo red	-ve	Absence of amyloid
Thioflavine "T"	-ve	
Methylene blue	-ve	
Masson's trichrome	+ve	Connective tissue; fibrin, collagen, reticulin
Azan stain	+ve	
Gomori's reticulin reaction	+ve	Reticulin
M.S.B. (Martin's, scarlet, blue)	+ve	Fibrin
P.A.S. (Periodic acid Schiff)	+ve	Carbohydrate
P.A.S. + saliva	-ve	Absence of glycogen
Aldehyde fuchsin	+ve	Insulin granules

Ultrastructural appearance of islet vacuoles

Marked ultrastructural changes were observed in islet cells adjacent to vacuoles. The extent of the granular endoplasmic reticulum and Golgi apparatus was increased. In addition to these changes and to the presence of β-cell degranulation, the cisternae of the endoplasmic reticulum were very large and swollen, appearing like intracellular cavities. These swollen cavities were either oval, circular or irregular in shape and filled with a pale-grey amorphous material (Plates 50-52). As the cisternae of the endoplasmic reticulum become distended, the other cytoplasmic organelles become compressed and ribonucleoprotein particles could be identified within the cavities (Plate 50). Flagella-like structures could be seen inside some vacuoles (Plate 53). These structures were probably the cisternae of the endoplasmic reticulum. In adjacent degranulated β-cells, there was evidence of cytoplasmic matrix rarefaction (Plates 37 & 52), the extent of which increased gradually as the β-cell degenerated. Large empty spaces appeared inside the degranulated β-cells. Cytoplasmic organelles were often observed inside these rarefied spaces (Plates 37 & 54). In old obese mouse islet β-cells, the cytoplasmic rarefaction and the coalescence of vacuoles often resulted in the production of one or two large vacuoles which came to occupy most of the cell cytoplasm. Adjacent cell organelles became compressed and the mitochondria appeared to be swollen (Plate 54). The walls of islet capillaries appeared fragile and

thin (Plates 54 & 55) and the space between the cell membrane and endothelial basement membrane became very indistinct. In areas of capillary membrane breakdown, collagen fibrils and amyloid-like material could be seen (Plate 56). The combination of cytoplasmic rarefaction, intra-β-cell cytoplasmic vacuolation, β-cell degeneration and the breakdown of thin-walled dilated capillaries leads to the formation of the islet vacuole. Many cell organelles could be seen inside these islet vacuoles (Plates 56 & 57). Free β-granules and swollen mitochondria were interdispersed with granule sacs and fibrous material inside the islet vacuole (Plate 57). Cell-free nuclei were also observed inside the vacuole, together with cytoplasmic remnants of degenerated β-cells (Plate 57).

The islet β-cells of lean mice showed very little morphological change with age. There was some increase in the numbers of β-granules and the size of the granular endoplasmic reticulum, but no vacuoles were ever observed (Plates 20-24).

4.2.4 Discussion

The present morphological studies have provided some basis for the mechanism of formation of vacuoles in the islets of obese hyperglycaemic mice. Both light and electron microscopic studies

have indicated that degenerating β-cells and thin-walled broken capillaries coalesce to form the islet vacuole with consequent reduction in β-cell numbers. Cell death and a reduction in cell number are general characteristics of the ageing process in man and other animals (Strehler, 1962). An age-related reduction in β-cell numbers has recently been reported in the islets of Langerhans of spontaneously hypertensive rats (Postnov, Gotkova & Solovyova, 1976).

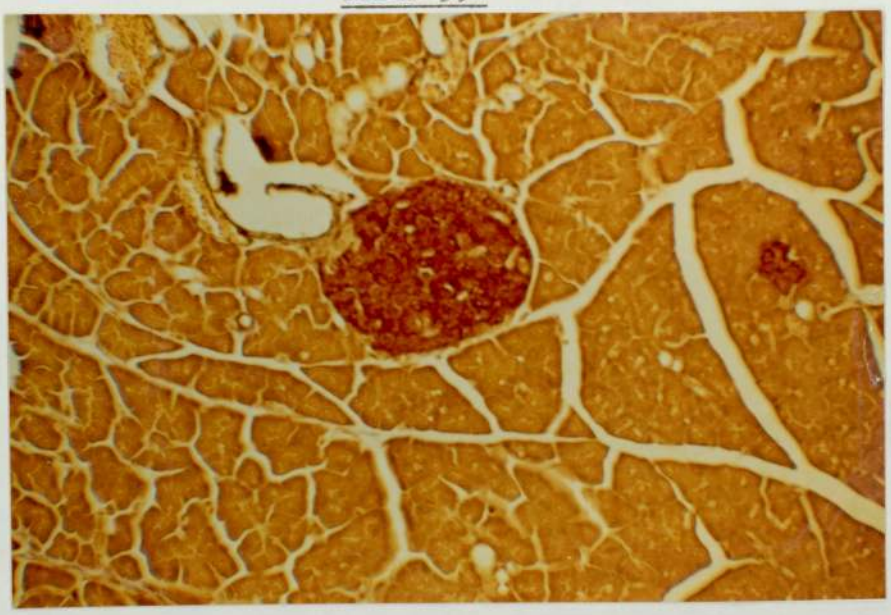
In the present study, the islet vacuoles appeared to be filled with a semifluid-like material, just observable by electron microscopy. This semifluid-like material, which had previously been called acellular material (Jones, 1969) contained blood cells and a fibrous matrix. The appearance of this fibrous matrix coincided with the thickening of the islet capsule and fibrocytic infiltration into the body of the islet. The extensive fibrosis of pancreatic islets appears to be associated with β-cell hyperplasia (Friedlander, 1961). Lazarus and Volk (1962) demonstrated that the proliferation of fibrocytic material in the pancreatic islets of dog was associated with the appearance of lymphocytes and suggested that their presence might be the result of an inflammatory process. Lymphocytes have been demonstrated in the present studies. It is possible that an auto-immune phenomenon might be involved in the degeneration of β-cells in the islets of obese hyperglycaemic mice.

The primary process in the formation of islet vacuoles, the enlargement of the β-cell endoplasmic reticulum to form intracellular vacuoles, could be the result of hyperactivity and exhaustion atrophy. Cytoplasmic rarefaction has also been observed in the β-cells of CD-1 mice infected with EMC virus (Craighead, 1974). However, rarefaction might also be a natural consequence of ageing and/or obesity where an increased metabolic demand for insulin would bring about excessive degranulation. The capillary walls of obese mouse islet β-cells are very thin (Hellman et al., 1961). Hellerström and Hellman (1961), using fluorescent dye, demonstrated an increase in the numbers and diameters of the capillaries in the obese mouse islets and suggested that this increase is manifested after a more prolonged increase in islet function. The present ultrastructural studies have confirmed the presence of capillary fragility. It is conceivable that capillary walls dilate after adjacent β-cells have degenerated. The capillaries then rupture, forming the vacuole. The latter increases in size as the β-cells degenerate and the capillaries rupture. Islet vacuoles have been observed in other species of mice. Like and Jones (1965) observed them in the islets of Wellesly hybrid mice and called them "tumours". In 1967, Like and his co-workers observed the same vacuoles and called them "central cysts", and attributed their formation to β-cell hyperactivity. Shino and Iwatsuka (1970) have observed similar vacuoles in the islets of the yellow KK mouse, and assumed them to be associated with β-cell degeneration.

Plate 39(a-d)

Islets of lean mice showing no gross morphological changes or vacuole formation with age.

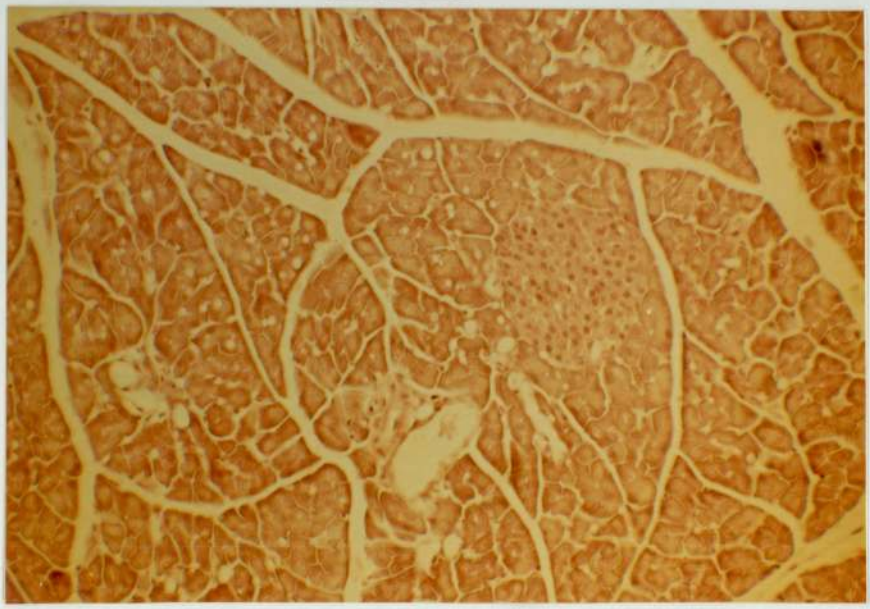
Plate 39a



A 7 week old lean mouse islet.

Aldehyde fuchsin x 180.

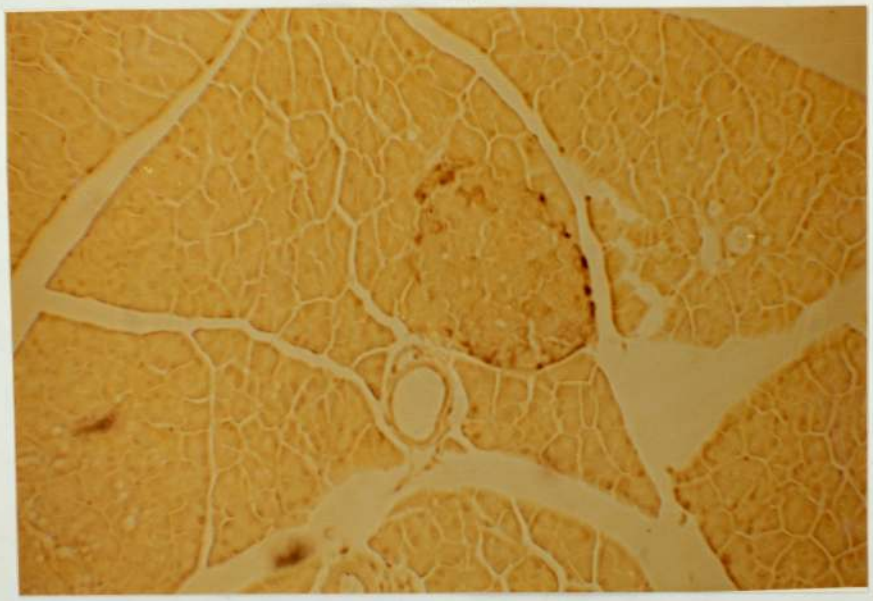
Plate 39b



A 20 week old lean mouse islet.

H. & E. x 180.

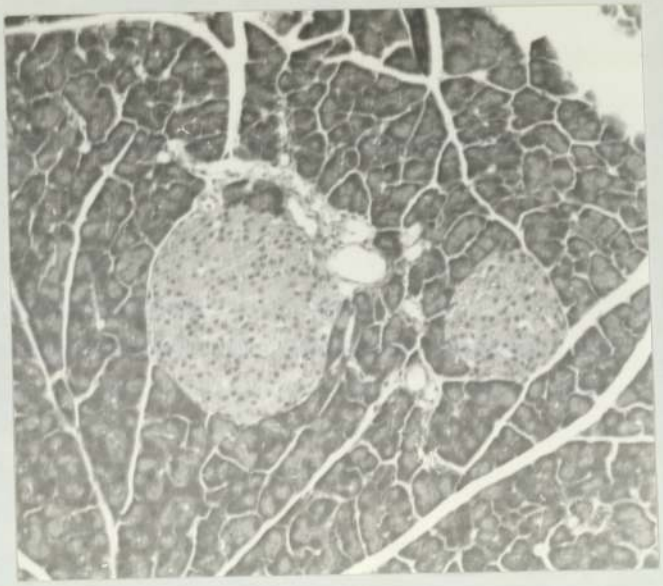
Plate 39c



A 30 week old lean mouse islet.

Silver nitrate x 180.

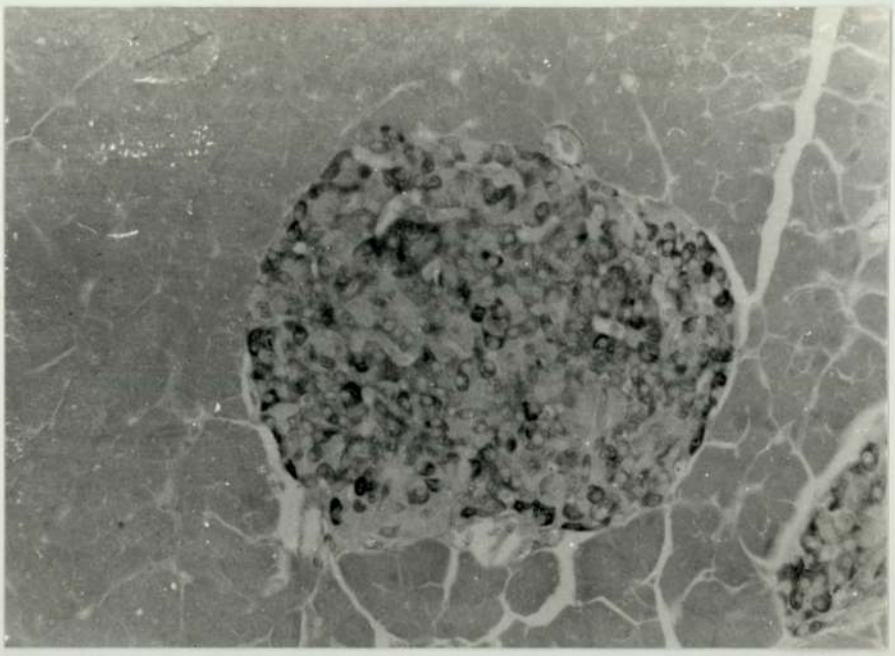
Plate 39d



40 week old lean mouse islets.

H. & E. x 180.

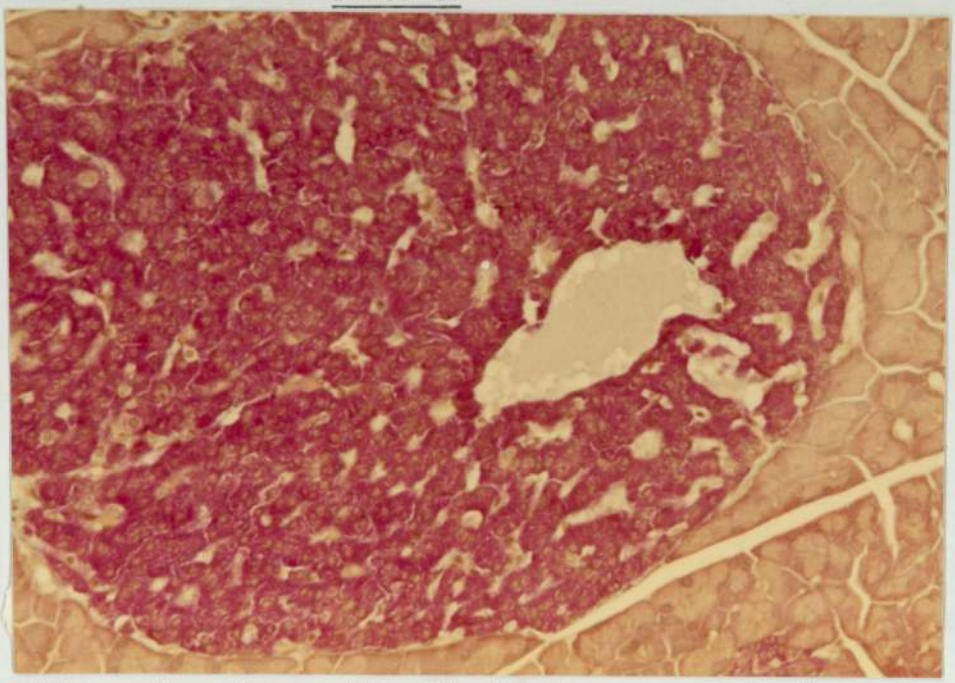
Plate 40



Islet of Langerhans of 15 week old obese mouse. Notice the pronounced islet hypertrophy and β-cell hyperplasia.

Gomori aldehyde fuchsin x 700.

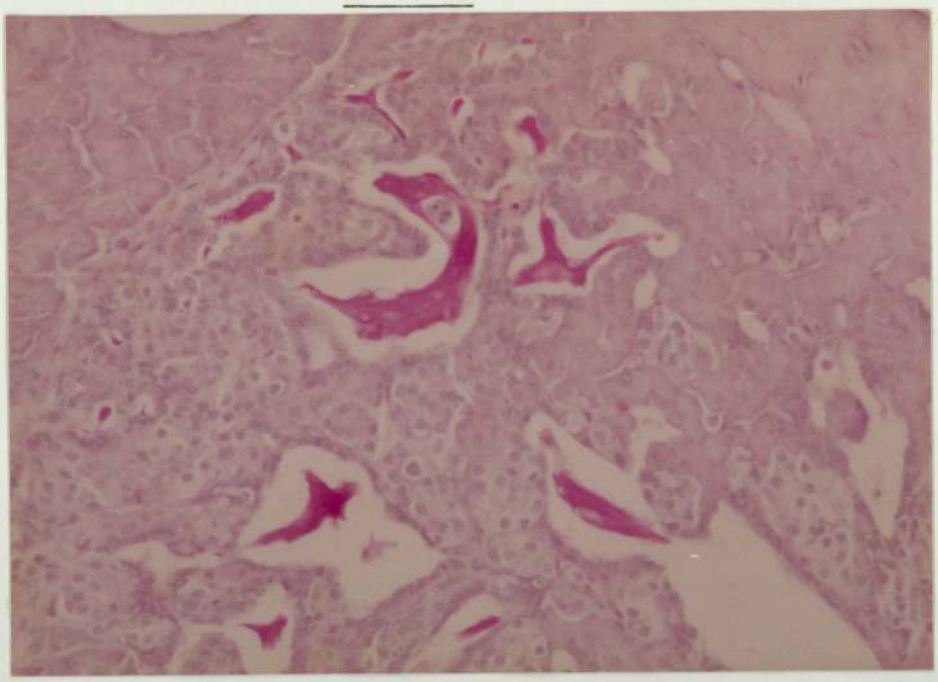
Plate 41



Islet of Langerhans from a 27 week old obese mouse showing dilated blood vessels.

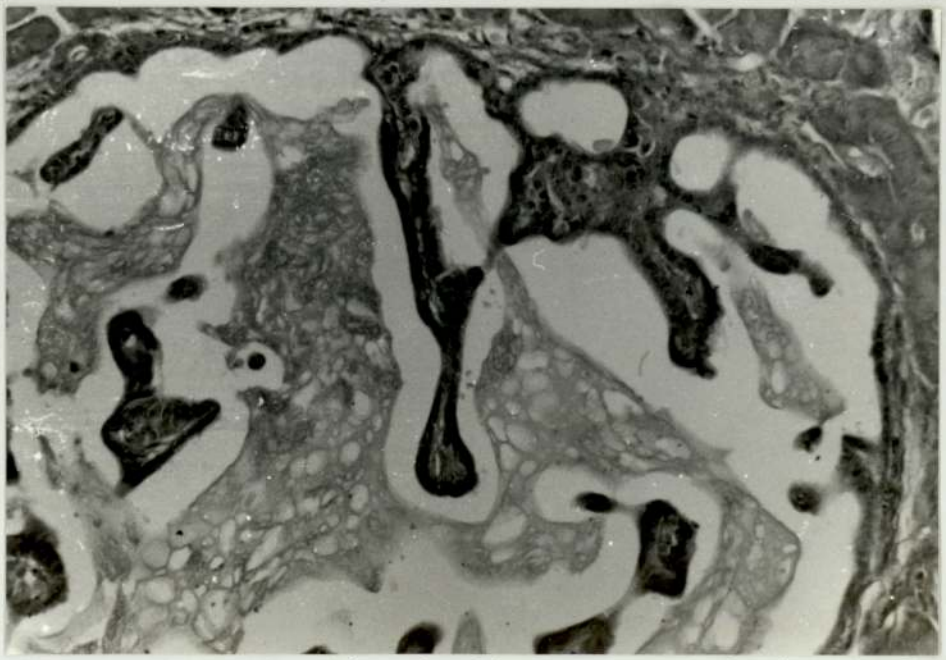
Aldehyde fuchsin x 700.

Plate 42



An islet of a 30 week old obese mouse showing enlarged blood vessels and their coalescence to form vacuoles amongst the β-cells. P.A.S. x 700.

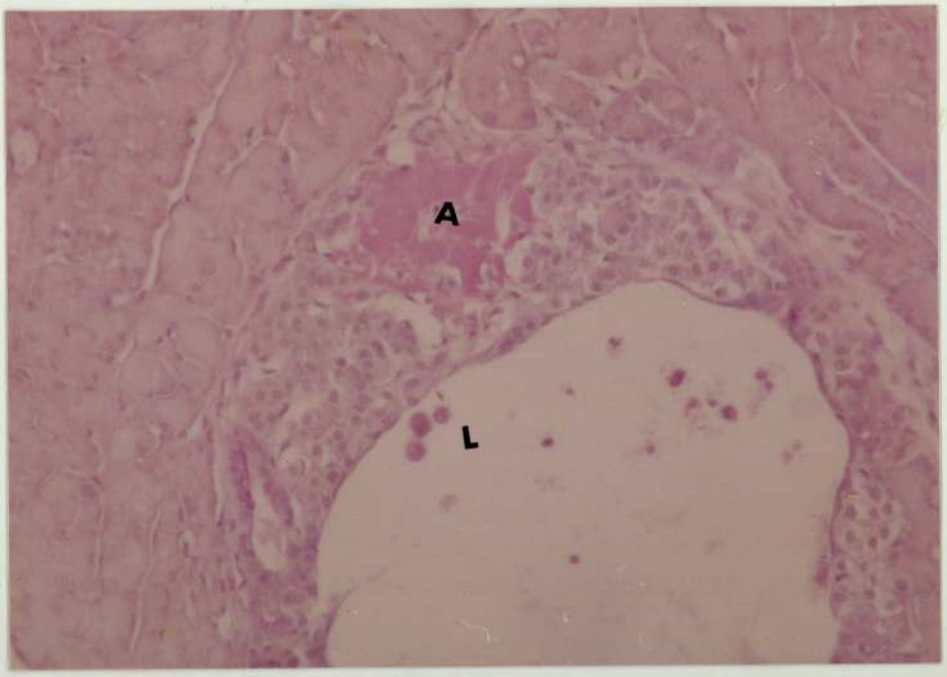
Plate 43



Islet of Langerhans of a 46 week old obese mouse showing a large vacuole occupying most of the islet. Notice the fibrous nature of the vacuole contents.

Aldehyde fuchsin x 2,800.

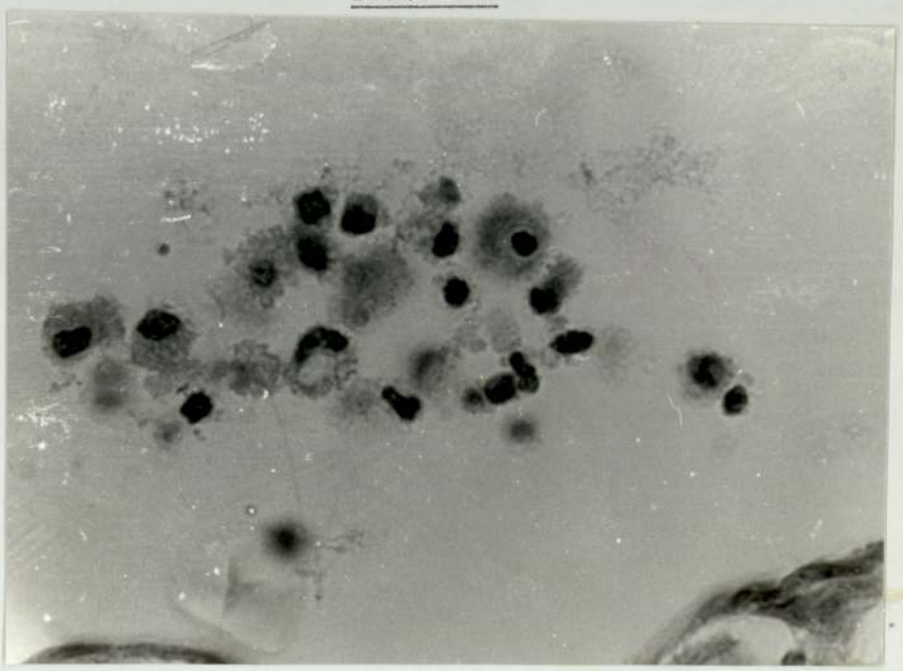
Plate 44a



Islet of Langerhans of a 42 week old obese mouse. Notice the large central vacuole containing lymphocytes (L). The deposition of amyloid (A) can be seen.

H. & E. x 700.

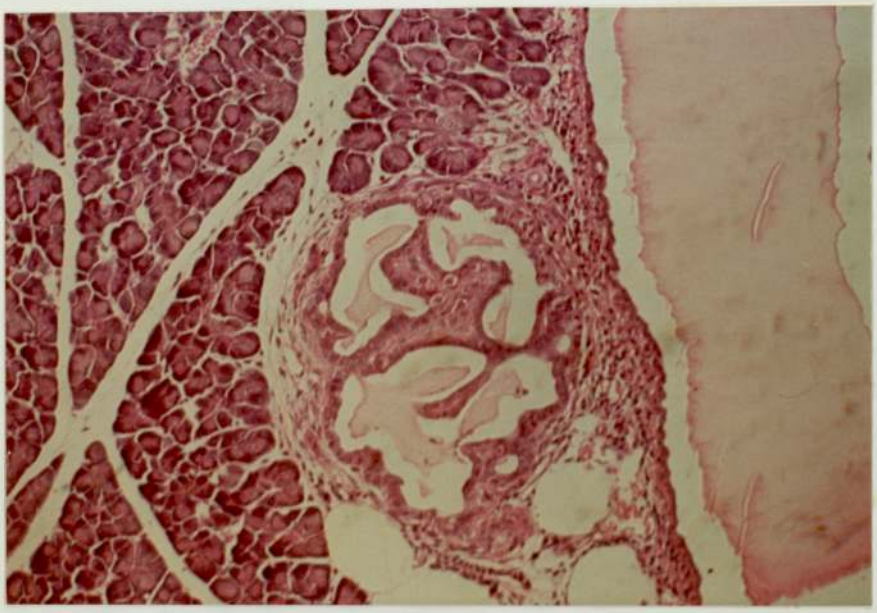
Plate 44b



Lymphocytes inside an obese mouse islet vacuole.

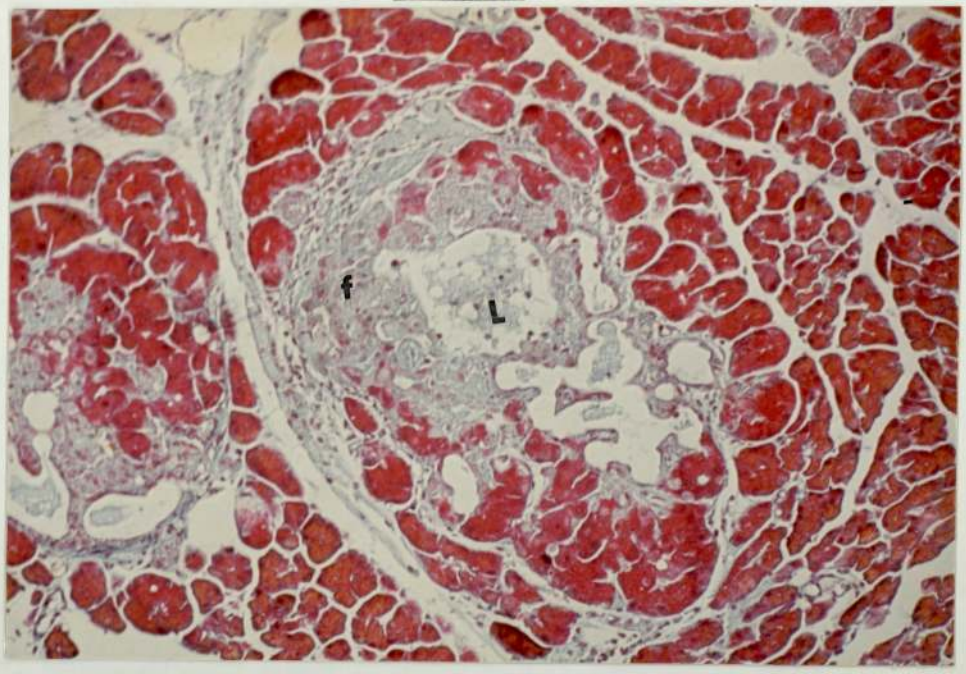
H. & E. x 2,800.

Plate 45



Islet of a 40 week old obese mouse showing the vacuole filled with a semifluid-like material. The islet capsule is thickened and each vacuole appears to be lined by a layer of columnar-like cells. P.A.S. x 450.

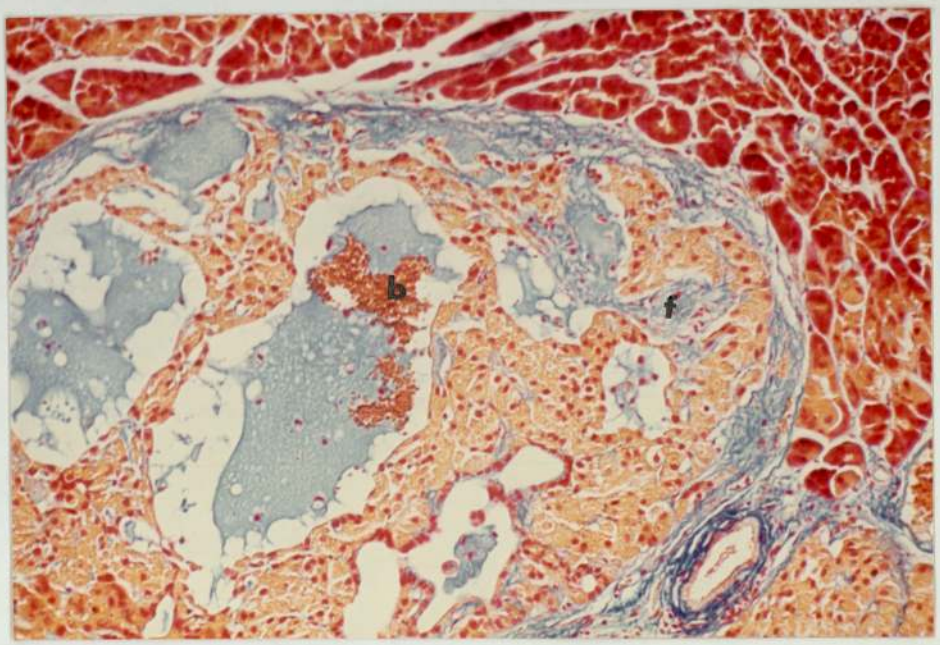
Plate 46



Islet of a 37 week old obese mouse. Notice the coalescence of small vacuoles to form a large vacuole. Lymphocytes (L) are present in the vacuole and there is heavy fibrosis (f).

Masson's trichrome x 700.

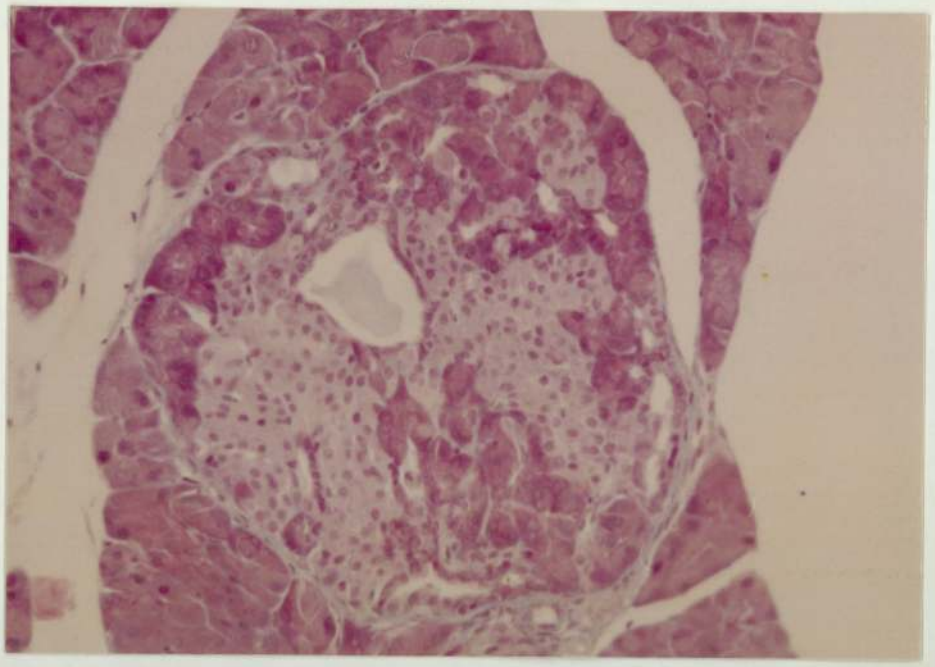
Plate 47



Islet of a 45 week old obese mouse showing large vacuoles filled with red blood cells (b) and fibrocytic infiltration (f). The islet capsule has become thickened and fibrous.

Azan stain x 700.

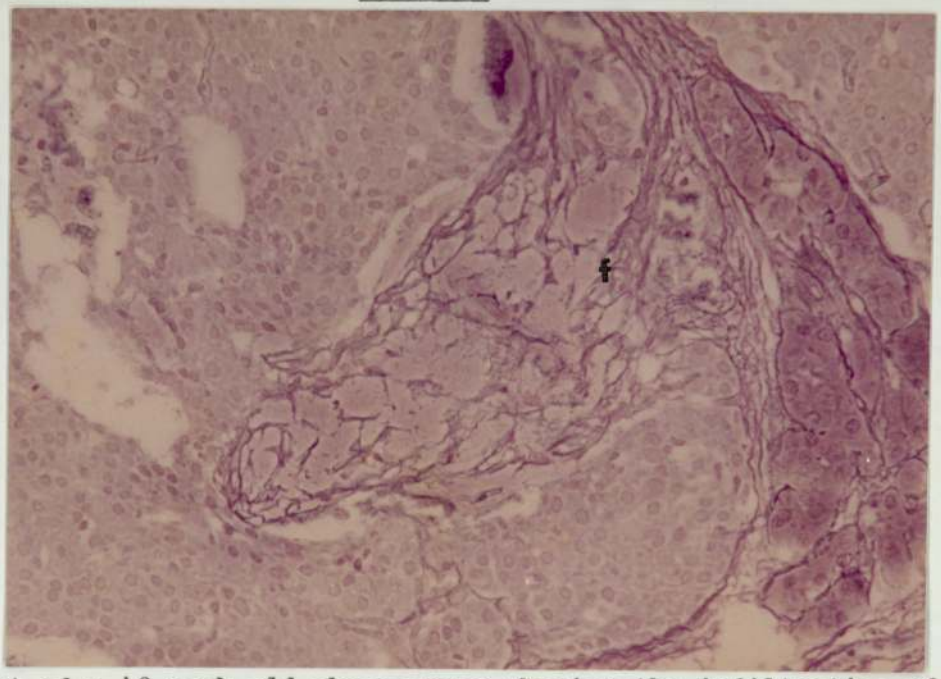
Plate 48



A 40 week old obese mouse islet showing exocrine-endocrine cell transformation.

H. & E. x 700.

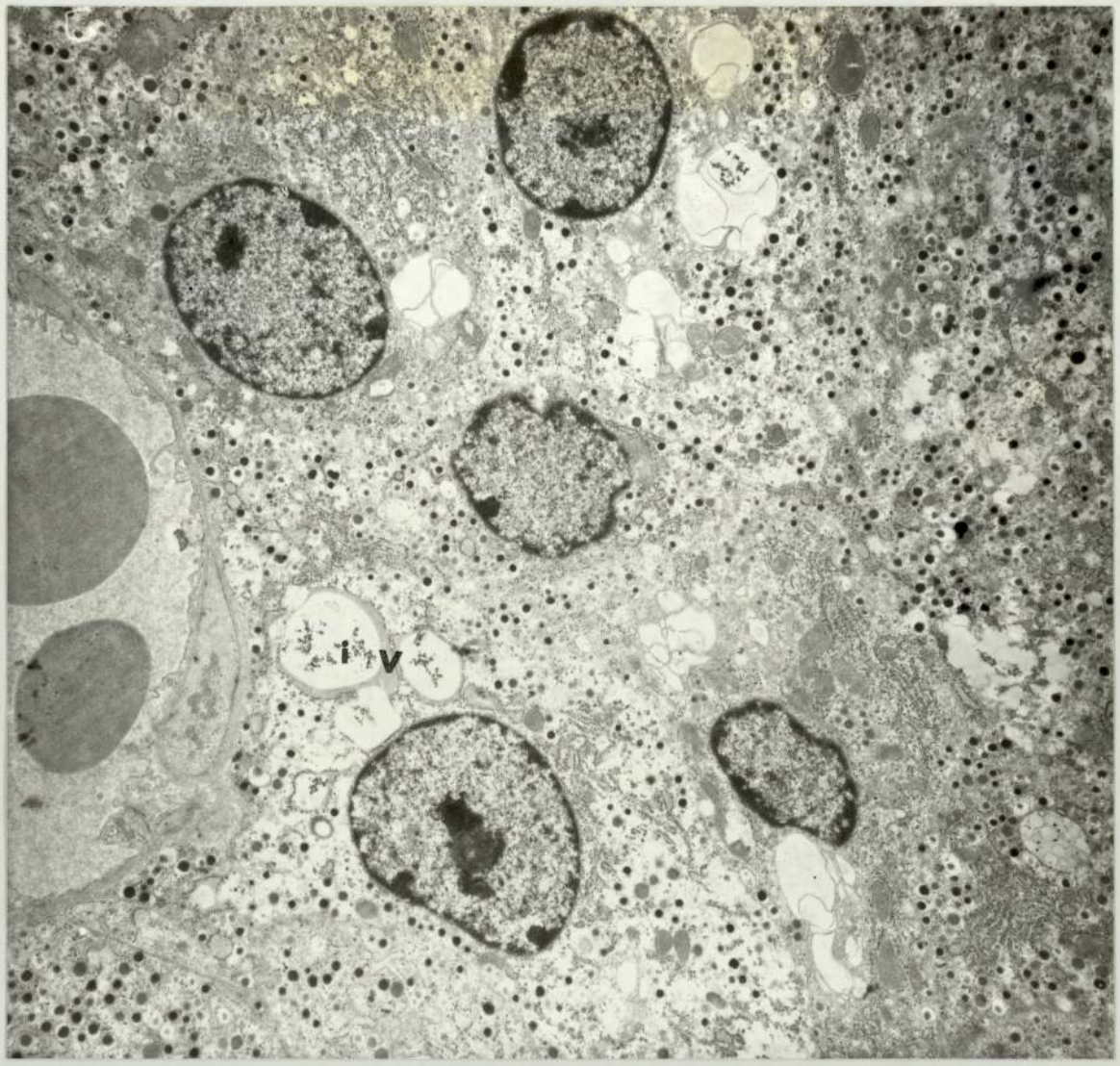
Plate 49



Islet of a 40 week old obese mouse showing the infiltration of fibrous material (f).

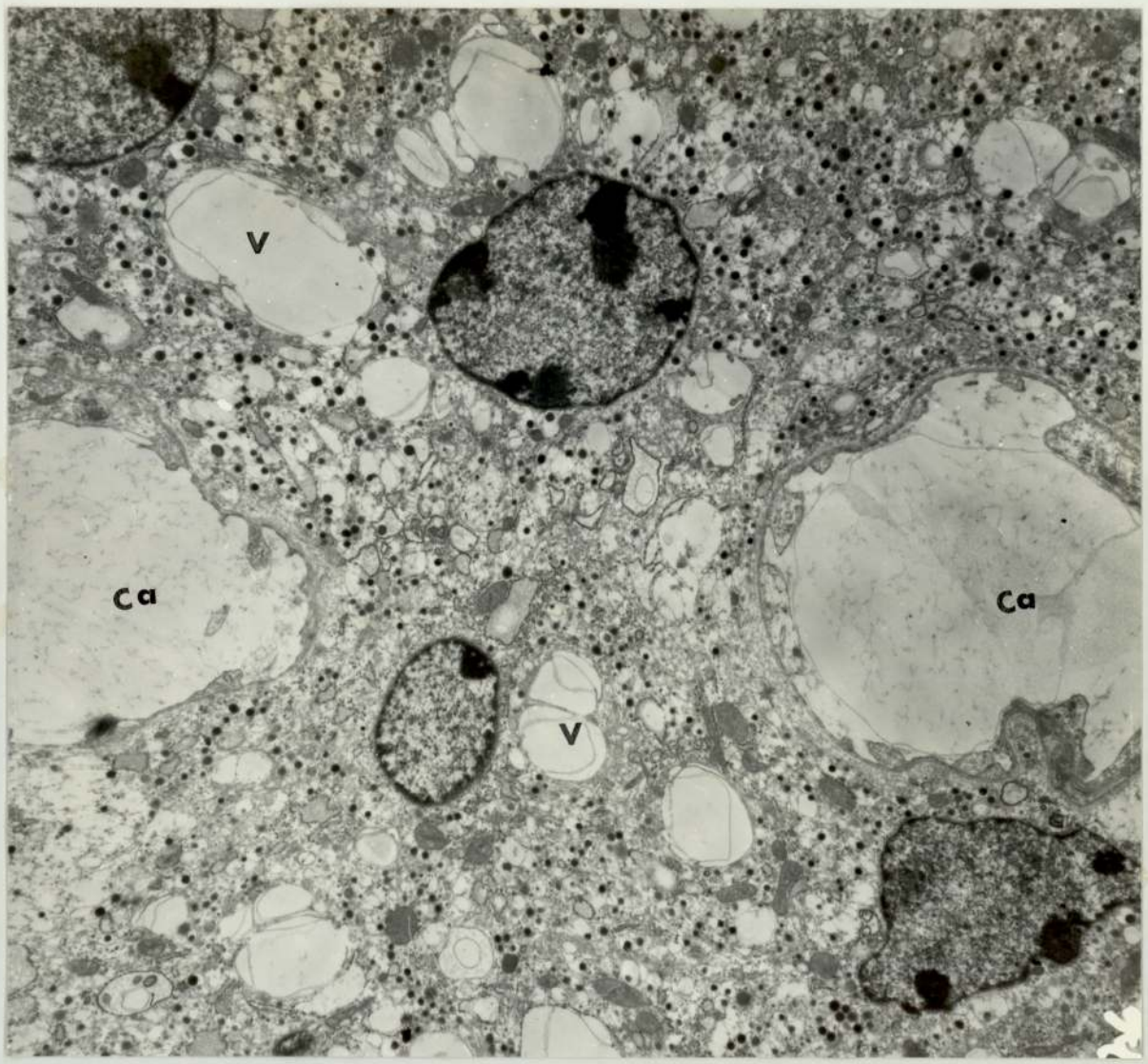
Gomori's reticulin stain x 700.

Plate 50



Islet β-cells of a 40 week old obese mouse showing the presence of distended cisterna (vacuole) of the endoplasmic reticulum. These vacuoles (v) appear to contain ribonucleoprotein particles (i). Glutaraldehyde-osmium x 6,000.

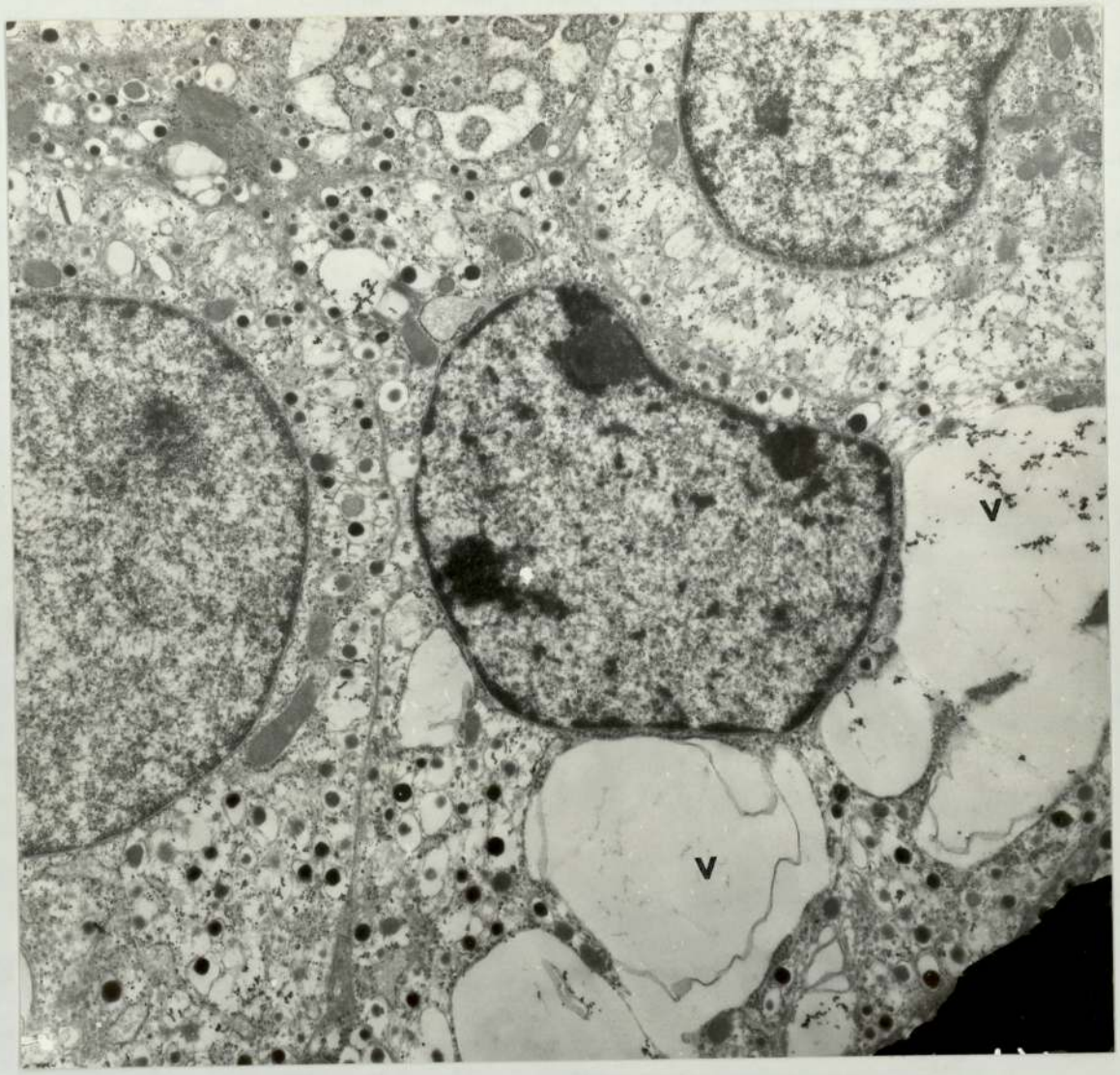
Plate 51



A 45 week old obese mouse islet β-cell showing the enlargement of the endoplasmic reticulum into vacuoles (v) and the presence of fragile thin walled capillaries (Ca).

Glutaraldehyde-osmium x 6,000.

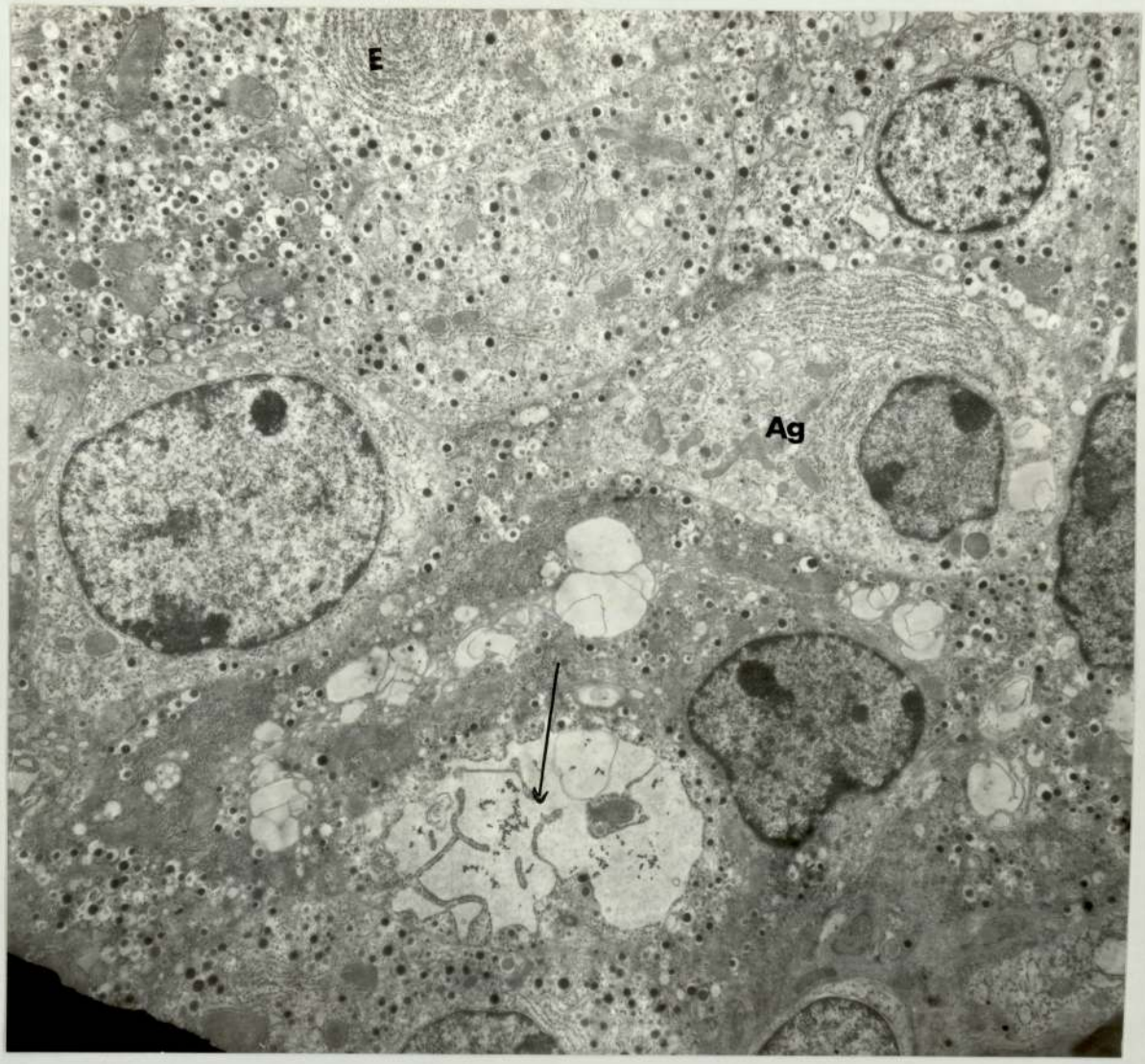
Plate 52



A 45 week old obese mouse islet showing the coalescence of adjacent degenerating β-cells containing swollen endoplasmic reticulum or intracellular vacuoles (v).

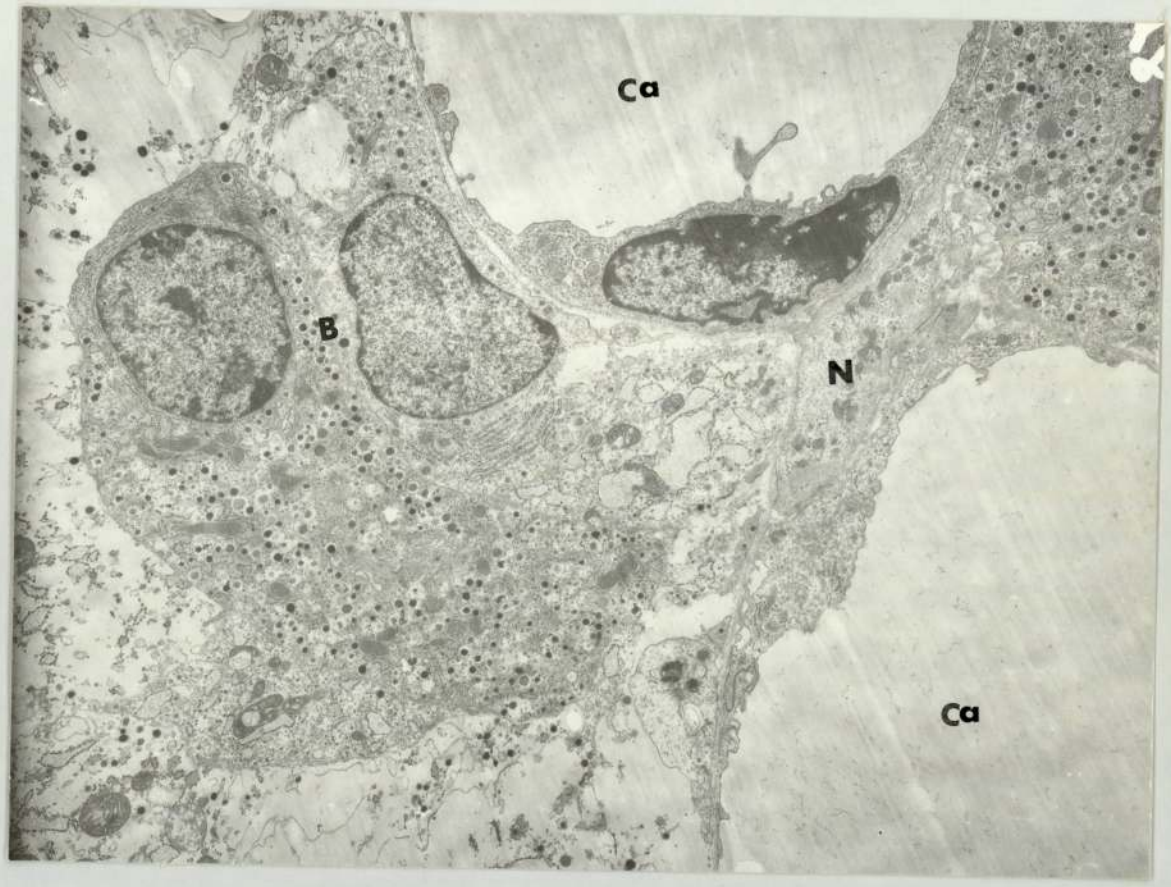
x 10,000.

Plate 53



A degenerating 45 week old obese mouse islet β-cell showing cell organelles inside a large vacuole (arrow). Notice the prominent granular endoplasmic reticulum (E) and an agranular cell (Ag). Glutaraldehyde-osmium x 6,000.

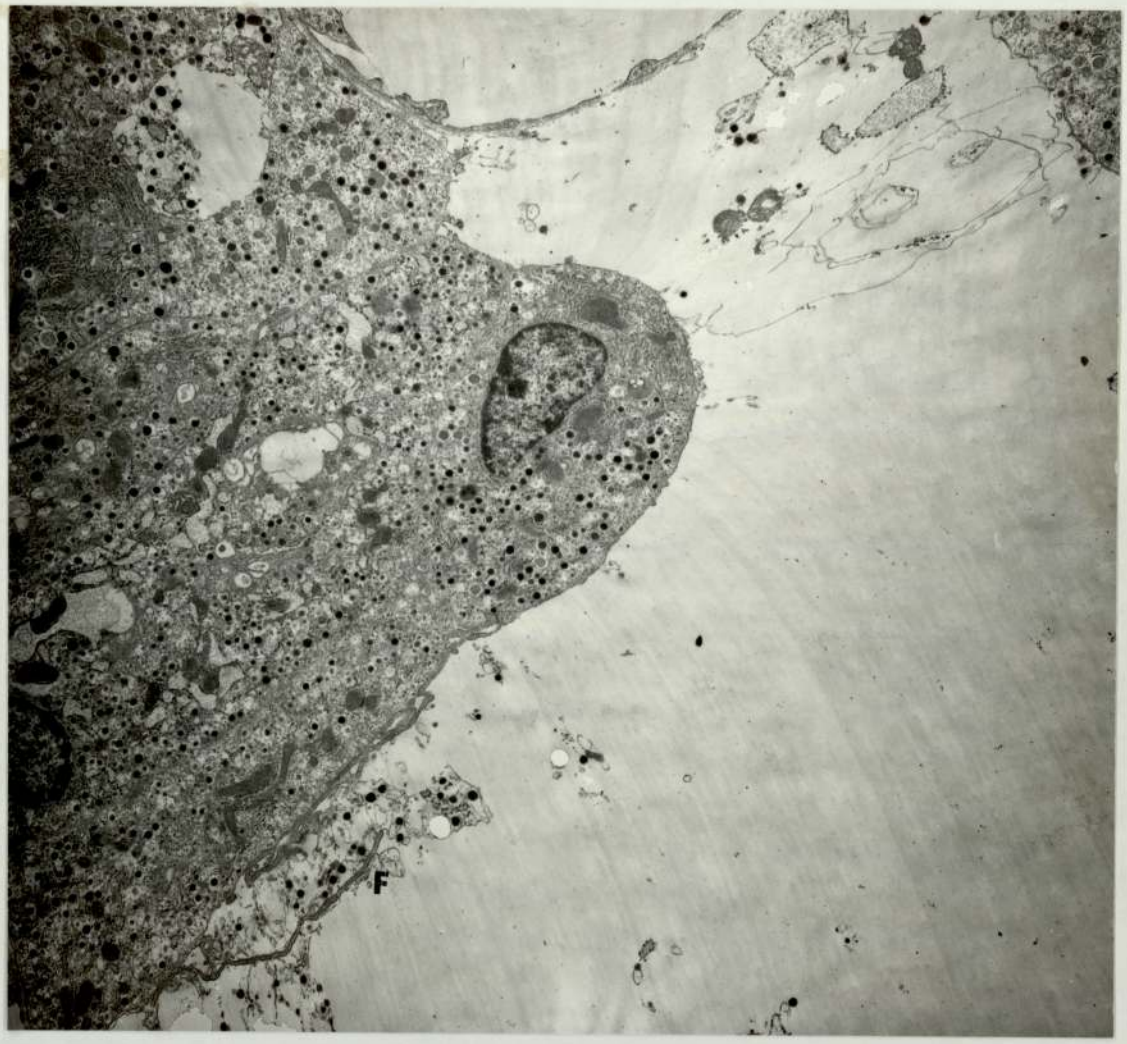
Plate 54



A 45 week old obese mouse islet showing the presence of degenerating β-cells. Notice the fragile thin walled capillaries (Ca) and the rarefied nature of the adjacent β-cell cytoplasm. β, β_1 cell; N, nerve ending.

Glutaraldehyde-osmium x 6,000.

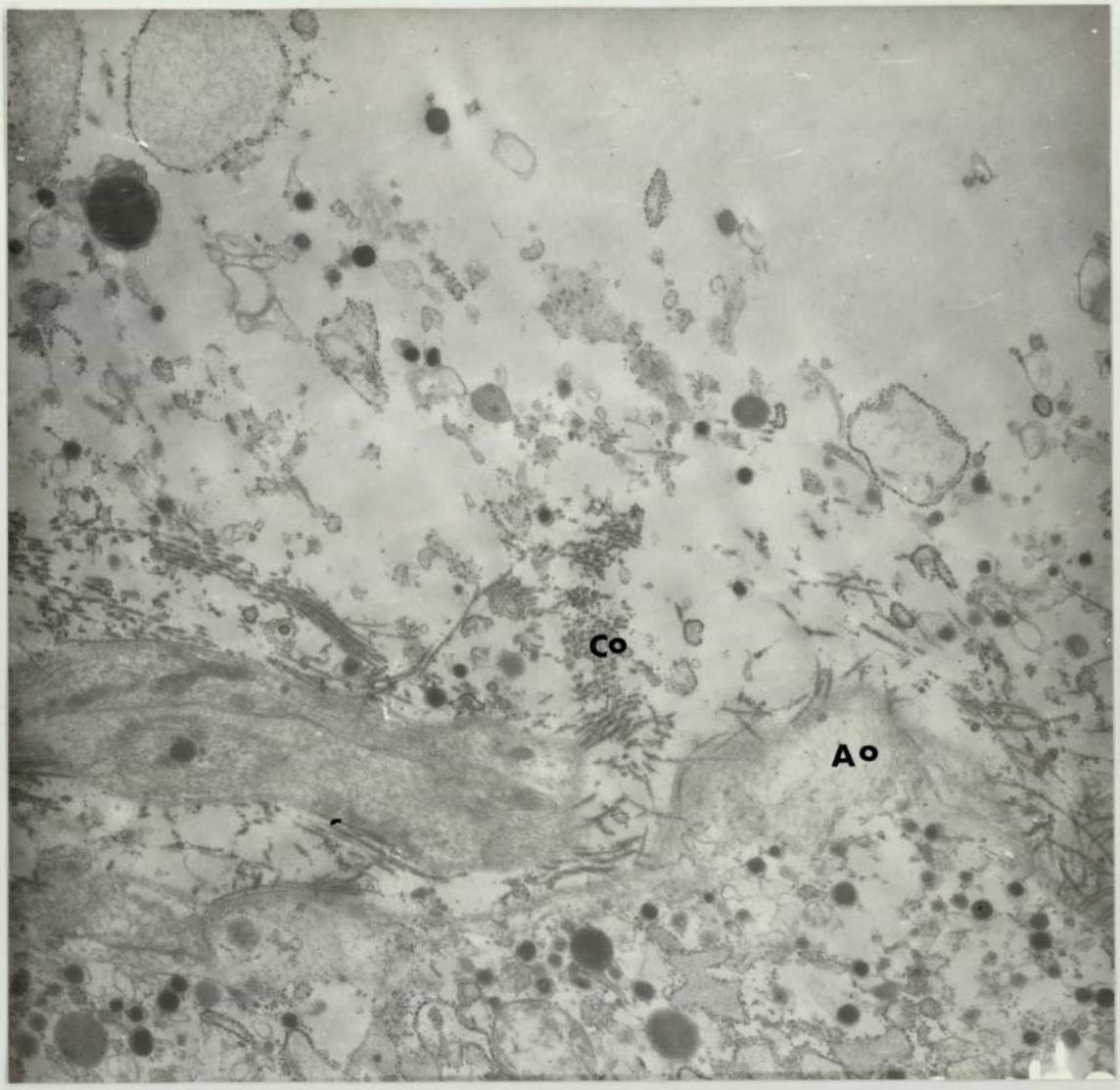
Plate 55



A 45 week old obese mouse islet showing the breakdown of the thin capillary membrane and the degeneration of adjacent β-cells at the edge of a vacuole.

Glutaraldehyde-osmium x 6,000.

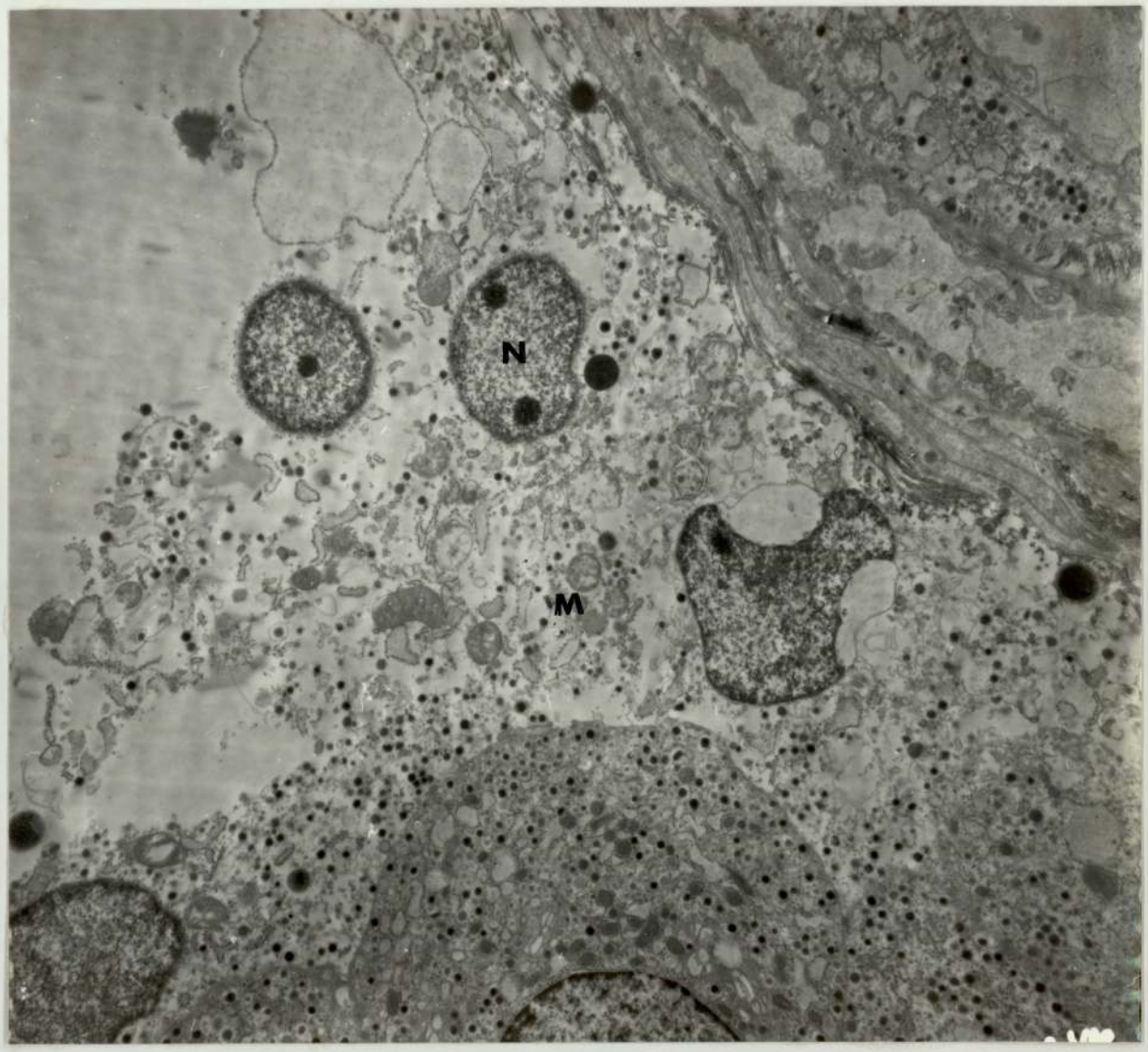
Plate 56



β-cell contents and capillary membranes at the edge of a 45 week old obese mouse islet vacuole. Notice the presence of collagen fibrils (Co) and amyloid-like material (Ao).

Glutaraldehyde-osmium x 6,000.

Plate 57



A 45 week old obese mouse islet showing the edge of an islet vacuole. Notice the swollen mitochondria (M), free nuclei (N) and the cellular debris at the periphery of the vacuole adjacent to a capillary.

Glutaraldehyde-osmium x 6,000.

4.3 The deposition of amyloid-like material in the islets of Langerhans of obese hyperglycaemic mice

4.3.1 Introduction

Amyloid has been defined as an amorphous eosinophilic, glassy, hyaline, extracellular substance, that is ubiquitous in its distribution (Cohen, 1967). Virchow (1854) applied the term "amyloid" to a hyaline substance that was observed in nervous tissue obtained at autopsy from aged patients, on the basis of its starch-like appearance when treated with iodine.

In 1901, Opie observed hyaline material in the islets of Langerhans of human diabetics. He interpreted its presence as a degenerative change and assumed it to be a part of the pathology of diabetes mellitus. Ehrlich and Ratner (1961) showed the hyaline material present in the islets of Langerhans to be amyloid. Histologically, amyloid is characterised by a green birefringence under polarised light when stained with Congo red. In addition, amyloid gives a yellow fluorescence with thioflavine "T" under ultraviolet light (Schwartz, Kurucz & Kurucz, 1965). Characteristically, amyloid contains fibrillar material (Portá, 1962; Lacy, 1964; Kawanishi, 1966; Johnson, 1970; Westermark, 1973). The fine amyloid fibrils either radiate in different directions (Lacy, 1967) or are parallel to each other (Heller, 1964). The occurrence of islet amyloidosis has been studied in both human diabetics and in experimental

animals. Westermark (1972, 1973, 1974) has been able to demonstrate the presence of amyloid in the islets of Langerhans of chronic diabetics, whilst Johnson and co-workers (1970) have demonstrated amyloid fibrils in the islets of diabetic cats. The incidence of islet amyloidosis has been found to increase with age (Thung, 1957; Bell, 1959; Yamada, 1968; Scheinberg, Cathcart, Eastcott, Skinner, Benson, Shirahama & Bennett, 1976). Amyloid has been shown to occur in 71.7% of diabetics that are over 50 years of age (Warren et al., 1968). It is still uncertain whether amyloidosis is a primary or secondary aetiological factor in diabetes. Amyloid has been found in islets of non-diabetic individuals. In the latter instance, the presence of amyloid has been thought to be due to undiagnosed instances of diabetes (Bell, 1959).

Bjorkman and co-workers (1963) and Westman (1968) have been unable to demonstrate amyloid material in the islets of old Swedish obese hyperglycaemic mice. The present study was conducted to investigate the possible deposition of amyloid material in the islets of obese hyperglycaemic mice (ob/ob).

4.3.2 Materials and methods

Pancreatic islet tissue was prepared for light and electron microscopy as described on pages 15 and 18. Material processed for light microscopy was stained with haematoxylin and eosin, Congo red, thioflavine "T", P-dimethylaminobenzaldehyde, PAS, PAS with saliva and alcian blue.

4.3.3 Results

Histological studies

Light microscopical examination of lean mouse islets with age failed to demonstrate any amyloid-like material.

Amyloid-like material was observed in the islets of Langerhans of 35-45 week old obese hyperglycaemic mice. The amyloid-like deposits appeared as homogeneous pink, eosinophilic areas when stained with haematoxylin and eosin. The deposition of amyloid-like material was primarily associated with capillary walls (Plate 58), but eventually it began to invade and displace islet tissue (Plate 58). The deposition of amyloid-like material appeared to increase with age. The histochemical reactions of islet amyloid-like material have been summarised in Table 4. Obese mouse islet amyloid-like material gave a positive fluorescent yellow reaction with thioflavine "T", a negative reaction with congo red, a positive reaction with the P-dimethylaminobenzaldehyde test for tryptophan and a positive reaction with periodic acid Schiff (PAS) for carbohydrate (Plates 44,58,59,60 & 61).

Table 4

Histochemical characteristics of obese mouse islet amyloid-like material.

Stain	Reaction	Inference
Haematoxylin and eosin	+ve	Identification of amyloid
Congo red	-ve	No green birefringence under polarised light
Thioflavine "T"	+ve	Amyloid
P-dimethylamino-benzaldehyde	+ve	Protein (tryptophan)
P.A.S.	+ve	Carbohydrate
P.A.S. + saliva	-ve	Absence of glycogen
Alcian blue	+ve	Carbohydrate

Ultrastructural studies

When viewed by electron microscopy, the obese islet amyloid-like material appeared to be composed of bundles of fine, straight or curved fibrils. These fibrils were often aggregated to form rods (Plate 62). The amyloid-like material was clearly observed to be associated with capillary endothelial cells (Plate 63). Filamentuous material could be seen invading islet β-cells through the basement membrane (Plates 63 & 64). Large numbers of collagen fibrils were also observed around the large blood vessels (Plate 65). The amyloid-like material was distributed diffusively about the cytoplasm of the islet β-cell (Plate 65). In β-cells that had been invaded with amyloid-like material, the mitochondria were poorly defined and the endoplasmic reticulum and Golgi apparatus were absent.

4.3.4 Discussion

Amyloid-like material has been demonstrated in the islets of Langerhans of old obese hyperglycaemic mice. It has not been observed in the islets of lean mice irrespective of age. The positive staining reactions of thioflavine "T" and PAS confirmed the presence of amyloid-like material in obese mouse islets. Vassor and Culling (1959) first used thioflavine "T" for the detection

of amyloid. Schwartz and co-workers (1965) confirmed thioflavine "T" as an effective stain for the detection of amyloid deposits. Cooper (1969) evaluated the methods available for the detection of amyloid and concluded that thioflavine "T" and, to a lesser extent, Congo red fluorescence and sirius red staining were very sensitive but not specific, whilst the green birefringence of Congo red was specific but not particularly sensitive. Amyloid-like material appeared to be PAS positive. PAS positive cells have been suggested to be the primary loci of amyloid formation (Teilum, 1964). The amyloid-like material in the islets of obese mice showed a negative birefringent reaction to Congo red. The green birefringence of amyloid has been attributed to the parallel orientation of dye molecule fibres and has been described as characteristic of, but not specific to, amyloid (Pearse, 1960). The absence of a birefringent reaction to amyloid with Congo red, but a positive reaction with other amyloid stains, has been related to the possible occurrence of a non-fibrillar amyloid precursor (Finn, Martin & Manns, 1970; Johnson et al., 1970). The aggregation of amyloid material into rod-like structures might contribute to the lack of Congo red staining (Brizzee, Harkin, Ordy & Kocck, 1976). The present study has demonstrated the presence of rod-like and globular amyloid deposits in the pancreatic islets of obese mice. Whether the precursor of obese mouse islet amyloid-like material is fibrillar or not has still to be established.

The demonstration of tryptophan using p-dimethylaminobenzaldehyde confirms the proteinaceous nature of obese mouse islet amyloid-like material. Amyloid protein appears to contain a large proportion of tryptophan (Pearse, 1960; Thompson, Gell & Yamada, 1961; Kawanishi, 1966; Cohen, 1967). Pearse, Ewen and Polak (1972) have distinguished two types of amyloid, immunomyloid and apudomyloid, classification depending upon the presence or absence of tryptophan. According to Pearse and co-workers (1972), the positive staining of tryptophan in obese mouse islet amyloid-like material suggests the presence of immunomyloid. The main protein component of amyloid is considered to be globulin (Teilum, 1969). Globulins generally contain a substantial carbohydrate fraction and give a positive reaction to PAS (Pearse, 1960). Amyloid has been suggested to be glycoprotein in nature (Lillie, 1965; Stiller & Katenkamp, 1976) and, in the present study, obese mouse islet amyloid-like material gave a positive reaction to PAS and alcian blue, suggesting the presence of a glycoprotein component.

The ultrastructural appearance of islet amyloid has been studied in human diabetics (Kawanishi, 1966; Nakayama, 1971; Westermark, 1973; Bilbao, 1975; Schober & Nelson, 1975), and in experimental animals (Sørenson, Heffner & Kirkpatrick, 1964; Ranløv & Wonstrup, 1967; Johnson, 1970; Scheinberg et al., 1976). Amyloid is characterised by the presence of fibrils, measuring approximately 100 A^{o} in diameter. Fibrillar amyloid isolated

from liver and spleen (Cohen & Calkins, 1964; Pras, Rimon & Franklin, 1969) and human diabetic islet tissue (Westermark, 1975, 1976) has been found to be protein in nature. Westermark concluded that human islet amyloid protein was fundamentally different from other characterised proteins in terms of its spectrophotometry and solubility. The amyloid fibrils were not separated from each other. In the present ultrastructural study, amyloid fibrils appeared both diffuse and rod-like in shape. Its protein composition remains to be investigated.

The origin of amyloid is still a controversial point. Amyloid may be formed intracellularly or from extracellular precipitates or by an interaction between the two. Teilum (1967), Bari, Pettengill and Sørenson (1969) have presented evidence for a local production of amyloid. Westermark (1972, 1975) suggested that islet amyloid was a degenerate pathological bi-product of the β-cell in chronic diabetes mellitus. Glinner, Ein and Terry (1972) and Glinner, William and Isersky (1973) suggested that amyloid fibrils might be fragments of immunoglobulin polypeptide chains. The eosinophilic and lymphocytic infiltration of hyalinised islets has also been observed (Silverman, 1963; Finn et al., 1970). This infiltration suggested that deposited amyloid had an immunoglobulin component (Johnson, 1973). Antigen-antibody precipitation has been suggested as a hypothesis for amyloid deposition and depends upon the presence of globulin as a part of

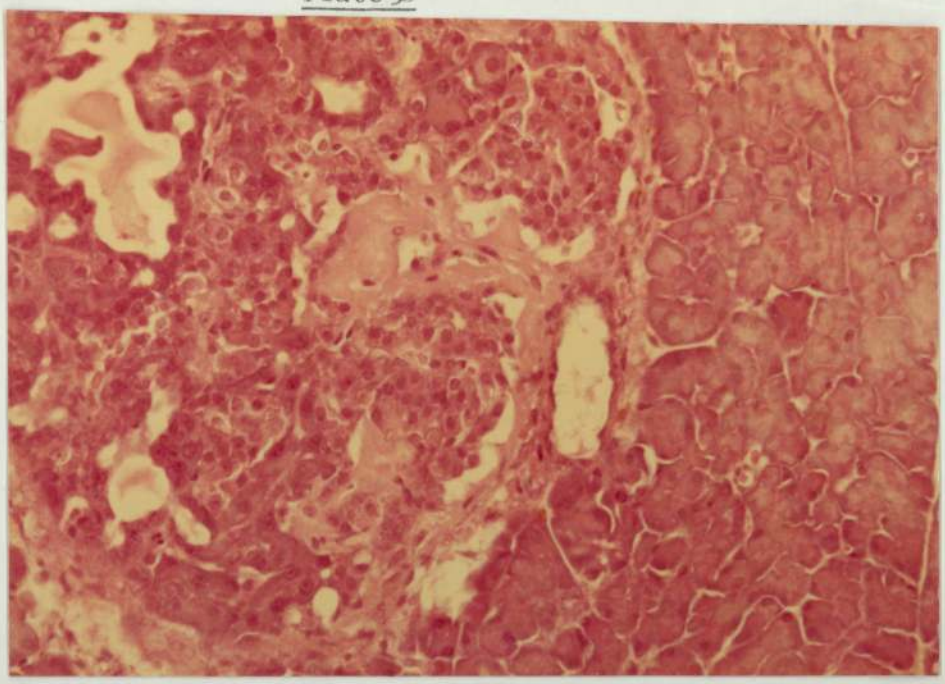
the chemical composition of amyloid (Calkins, Cohen & Glitlin, 1958; Lacy, 1964). Berns, Owen and Blumenthal (1964) suggested that islet amyloid was a precipitate of insulin and insulin antibody. Other studies have suggested amyloid to be manufactured by degenerating cells (Westermark, 1972; Bilbao et al., 1975; Schober, 1975). Teilum (1964, 1968) has presented a two-phase cellular theory that proposes that reticulo-endothelial cells (macrophages, endothelial cells, reticular cells, plasma cells and lymphoblasts) were proliferated by an antigenic stimulus, which, in the first phase, exhibited a strong pyroninophilia. After exhaustion (presumably a loss of their normal protein-synthesising capacity), these cells lost their pyroninophilia and gave a positive PAS reaction, indicating that the cells synthesised, in this second stage, a glycoprotein that eventually became amyloid. In the present studies, amyloid-like material was deposited from outside the β-cell. The pathogenesis of obese mouse islet amyloid-like material might be consistent with the two-phase theory.

The circulating insulin level of old obese hyperglycaemic mice has been found to be depressed at 35 weeks of age (Bailey et al., 1977). It is possible that the deposition of amyloid-like material in the capillary walls of old obese mouse islets might provide a barrier to the passage of insulin from the β-cells to the capillary lumen.

The relationship between amyloid deposition, the occurrence of diabetes and the normal ageing process has been studied by many workers (Thung, 1957; Bell, 1959; Gepts, 1967; Finn et al., 1970; Scheinberg et al., 1976). Amyloid deposition occurs as a consequence of the normal ageing process in brain, heart and the islets of Langerhans (Schwartz, 1968). Bell (1959) suggested that the hyalinisation of islets in diabetics was a function of age rather than the diabetes. On the other hand, Ehrlich and Ratner (1961) related the amyloidosis of pancreatic islets to the incidence of diabetes mellitus and subsequent work by Gepts (1967) suggested that islet hyalinisation only occurred after the prolonged hypersecretion of insulin. Yamada (1968) suggested that ageing, the occurrence of diabetes mellitus and the integrity of the capillary endothelial cells were in some way related to islet amyloidosis. The formation and deposition of hyaline material in pancreatic islets appears to be associated in some way with the capillary endothelial cells (Lacy, 1964; Warren et al., 1966). In the present study, the deposition of amyloid fibres close to capillary endothelia in the islets of old obese hyperglycaemic mice and the absence of amyloid from islets of lean mice irrespective of age would suggest that amyloid deposition is a product of the manifestation of the obese hyperglycaemic syndrome and associated in some way with the integrity of islet capillary endothelial cells.

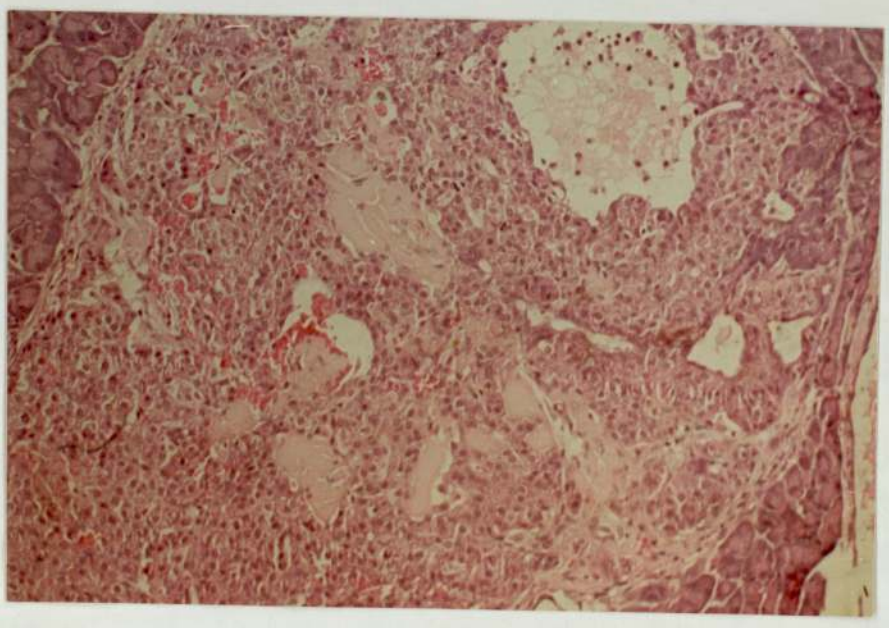
PLATES 58–65

Plate 58



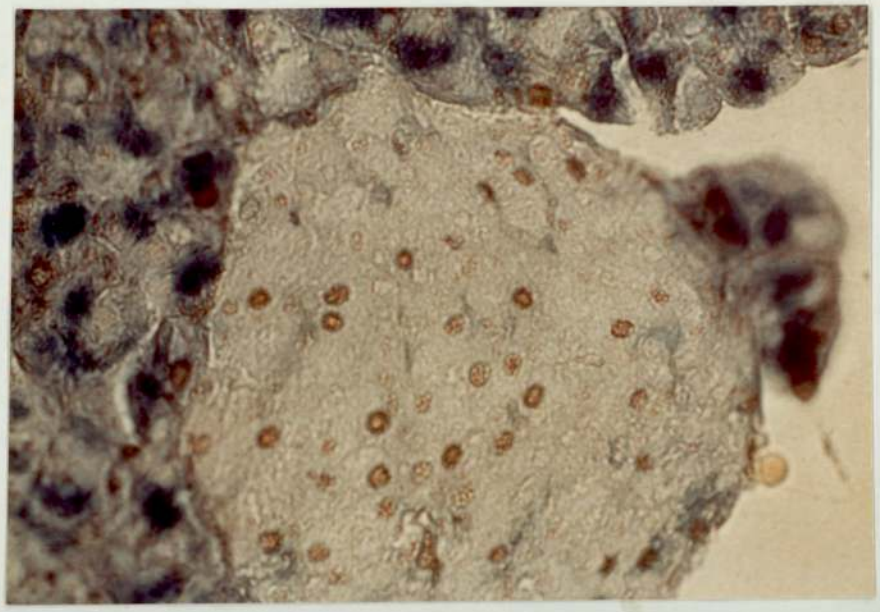
Islet of a 35 week old obese mouse showing the deposition of amyloid-like material around blood capillaries and between β-cells.
H. & E. x 700.

Plate 59



Islet of a 42 week old obese mouse showing the deposition of amyloid-like material around blood vessels and between β-cells. The vacuole contains many lymphocytes.
H. & E. x 700.

Plate 60



Islet of a 40 week old obese mouse showing the deposition of amyloid-like material around blood vessels.

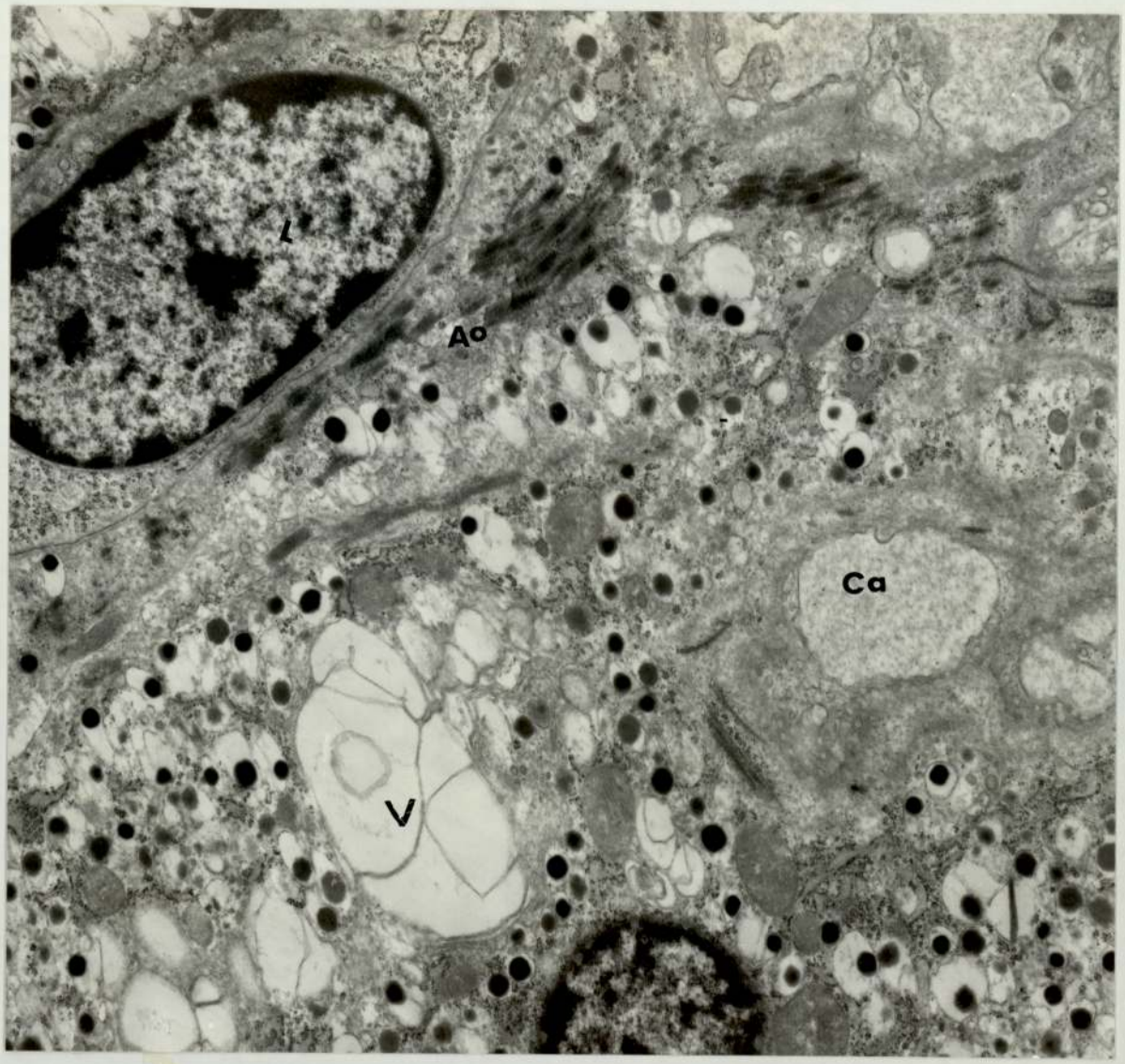
p-dimethylaminobenzaldehyde x 700.

Plate 61

Islet of a 40 week old obese mouse showing the yellow fluorescence of amyloid-like material.

Thioflovine "T" under U/V light x 2,800.

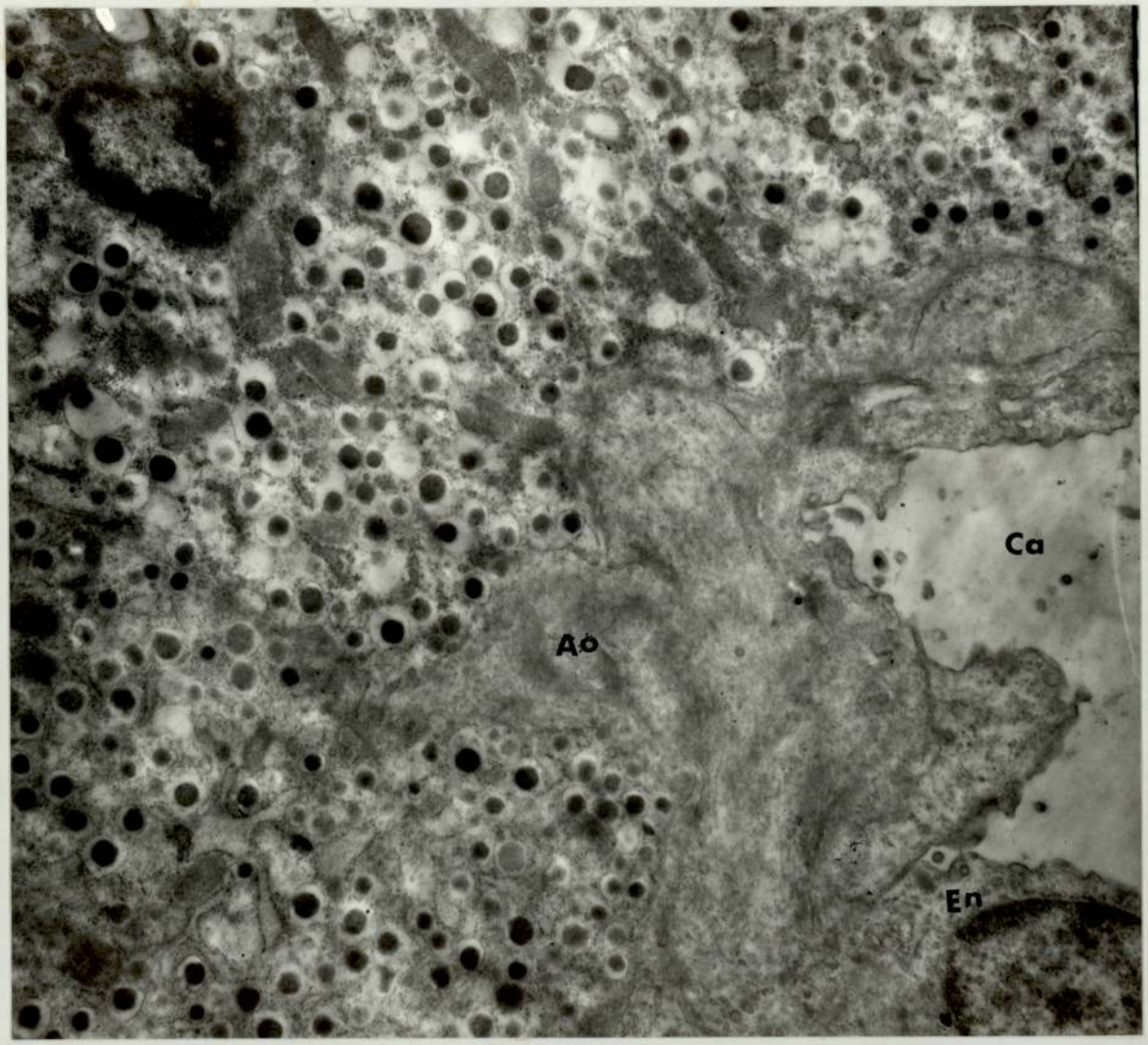
Plate 62



A 40 week old obese mouse islet showing the presence of rod-like accumulations of amyloid-like material (Ao) close to capillaries (Ca). L, lymphocyte; V, vacuole.

Glutaraldehyde-osmium x 15,000.

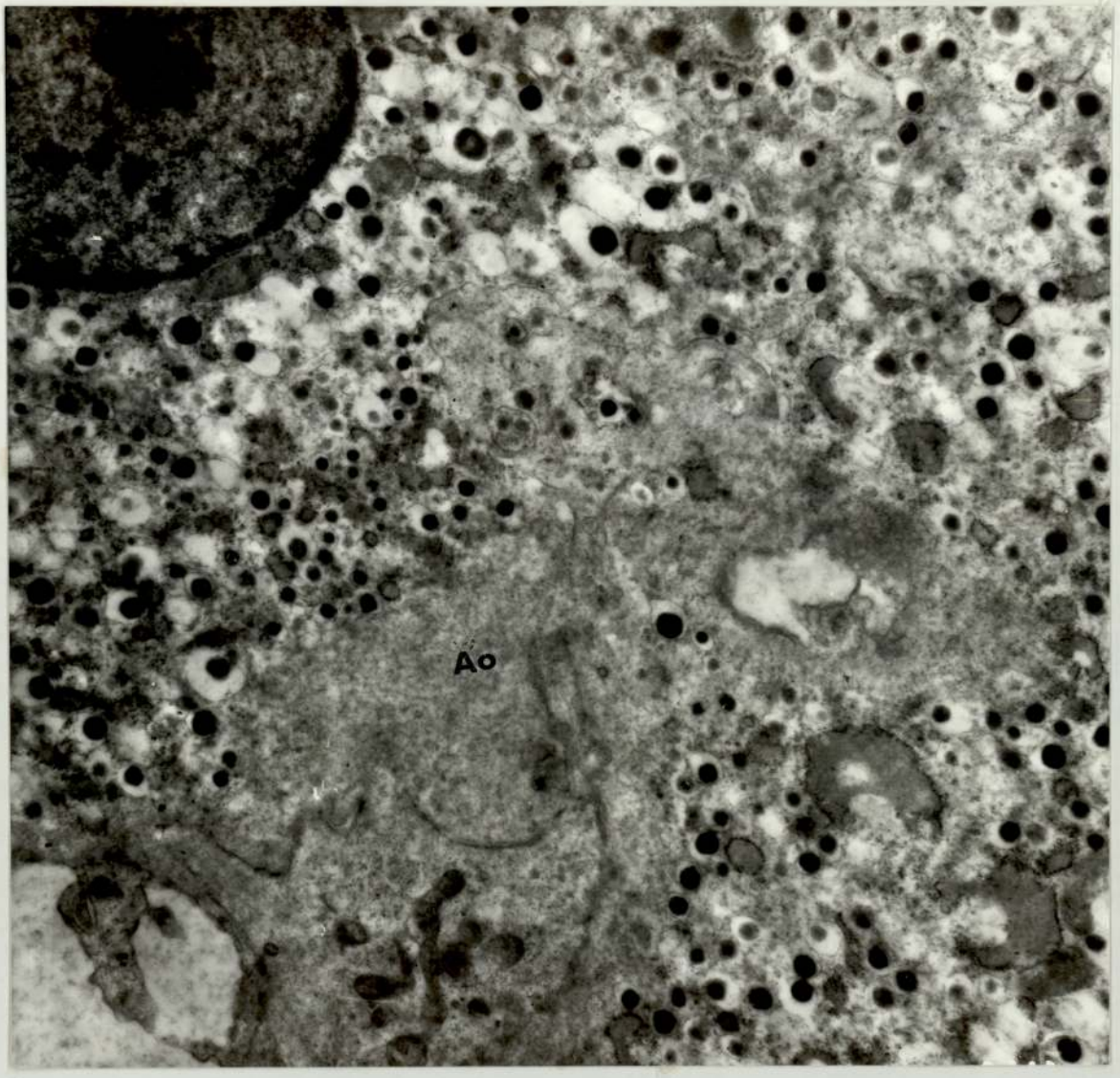
Plate 63



A 35 week old obese mouse islet showing the deposition of amyloid-like material (Ao) associated with the endothelial lining (En) of a capillary (Ca).

Glutaraldehyde-osmium x 10,000.

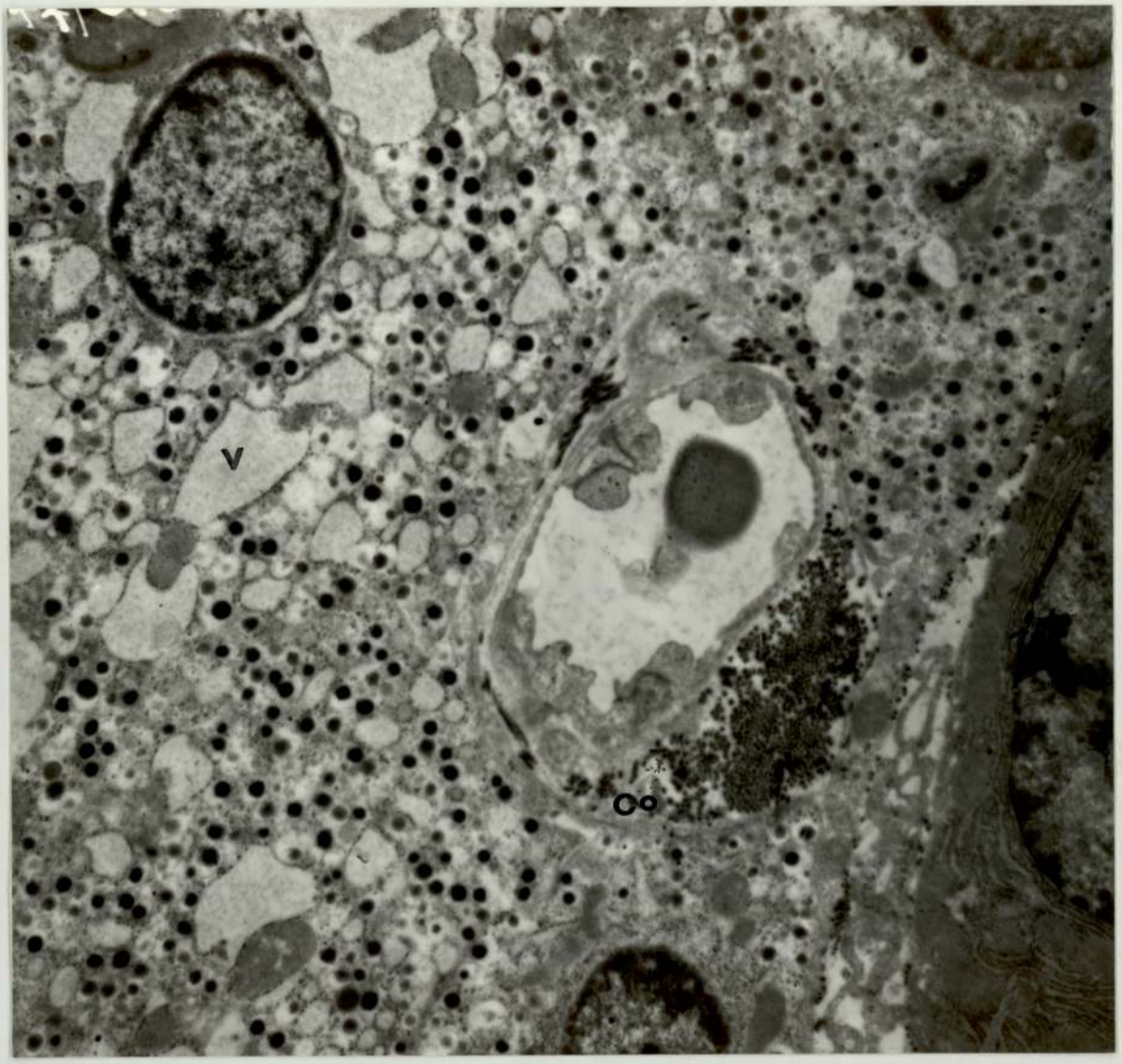
Plate 64



A 35 week old obese mouse islet showing the deposition of amyloid-like material (Ao) and its intrusion into the β-cell cytoplasm.

Glutaraldehyde-osmium x 10,000.

Plate 65



A 40 week old obese mouse islet containing degranulated β-cells. The endoplasmic reticulum is enlarged into vacuoles (V) and collagen fibrils can be closely associated with the capillary wall.

Glutaraldehyde-osmium x 10,000.

CHAPTER V

The Effect of Chemically-induced Obesity and Hyperglycaemia on Lean Mouse Islet Morphology

5.1 Introduction

Obesity can be induced in animals by gold thioglucose (GTG) injection (Katsuki, 1962; Colman, 1970; Debons, Kkimsky, From & Pattinian, 1974), electrical lesioning of the ventromedial hypothalamus (Kennedy, 1963) or by feeding the animal with a high fat diet (Lemonnier, Suquet, Aubert & Pequignot, 1975). Obesity induced by these means is characterised by concomitantly elevated levels of blood glucose and plasma insulin (Katsuki, 1962; Colman, 1970; Chlouverakis, 1971).

Hyperphagia is unquestionably involved in the genesis of obesity, especially in spontaneously hyperglycaemic rodents (Cameron, Stauffacher & Renold, 1972). Mice made obese by GTG treatment (Stevenson, 1969) or hypothalamic lesioning (Kennedy, 1963) show an increased food intake and massive weight gain. Increased food intake would invariably stimulate pancreatic insulin synthesis and secretion (Creutzfeldt, Feurle & Ketterer, 1970). Indeed, the enlargement of the pancreatic islets of GTG treated or electrically lesioned obese mice has been attributed to islet β-cell hyperactivity (Katsuki, 1962; Kennedy, 1963).

Obese hyperglycaemic mice are characterised by hyperinsulinaemia and hyperglycaemia (Mayer et al., 1953; Westman, 1968; Bailey et al., 1977). In addition, these animals show

hyperphagia, insulin resistance (Mayer et al., 1953; Batt & Michle, 1966) and hypertrophy of the islets (Gepts et al., 1960; Atkins & Matty, 1973).

In the present ultrastructural study, the islets of gold thioglucose treated lean mice have been examined to establish whether hyperglycaemia and obesity induced by GTG treatment results in the appearance of islet vacuoles and other morphological changes characteristic of the obese hyperglycaemic syndrome in mice.

5.2 Materials and methods

Animals

The animals used in this study were male lean littermates of obese hyperglycaemic mice from the Aston colony. Preliminary experiments were carried out on 10 animals to establish the most suitable dose of GTG. Subsequently, obesity and hyperglycaemia were induced in 15 lean mice by interperitoneal injection of a solution of gold thioglucose (Sigma) in sterile double distilled water (60mg/ml). Each mouse received a single injection of 0.6mg per gram of body weight.

These animals were allowed free access to food and water

during the course of the experiments. A similar number of lean mice were given an i.p. injection of sterile double distilled water alone and these animals served as controls. All animals were housed and cleaned as described on page 10.

Glucose tolerance test (IPGTT)

32 week old GTG treated mice and their controls were each injected with a weight related glucose load (2g/Kg body weight) and blood was subsequently milked from the cut end of each animal's tail at 0, 30 and 60 minutes. Glucose was measured by glucose oxidase on a Beckman glucoseanalyser (Stevens, 1972).

Insulin radio-immunoassay

The insulin immunoassay system adopted in the present study was based on the double antibody system of Hales and Randle (1963).

Morphological studies

5 surviving 32 week old GTG treated lean mice were killed by hyperextension of the neck. Islets were microdissected from the excised pancreata and processed for light and electron microscopy using the methods described on page 18. Ten islets were taken from each pancreas for electron microscopy and eight sections were

cut from each islet. The remainder of each pancreas was used for light microscopy.

5.3 Results

After one month, 10 of the 15 GTG treated animals had significantly increased their body weight (45gm) compared to lean controls (25gm). This increase in body weight continued slowly and reached a maximum of around 60gm at about 6 months. The GTG treated mice had elevated levels of blood glucose (165 ± 18.5mg/100ml) (Table 5), and plasma insulin (11.3 ± 1.25ng/ml) compared with untreated lean control animals of the same age. In glucose tolerance tests, the blood glucose level of GTG treated mice rose to 340 ± 21mg/100ml after 30 minutes and decreased to 280 ± 29mg/100ml after 60 minutes. By contrast, the mean blood glucose level of control lean mice was 164 ± 18mg/100ml after 30 minutes and 154 ± 8mg/100ml after 60 minutes (Figure 1). These observations suggested that glucose tolerance was impaired in the GTG treated mice.

TABLE 5

Blood glucose levels of 32 week old fed lean control, GTG treated lean and obese hyperglycaemic mice. For comparison (mean ± SEM).

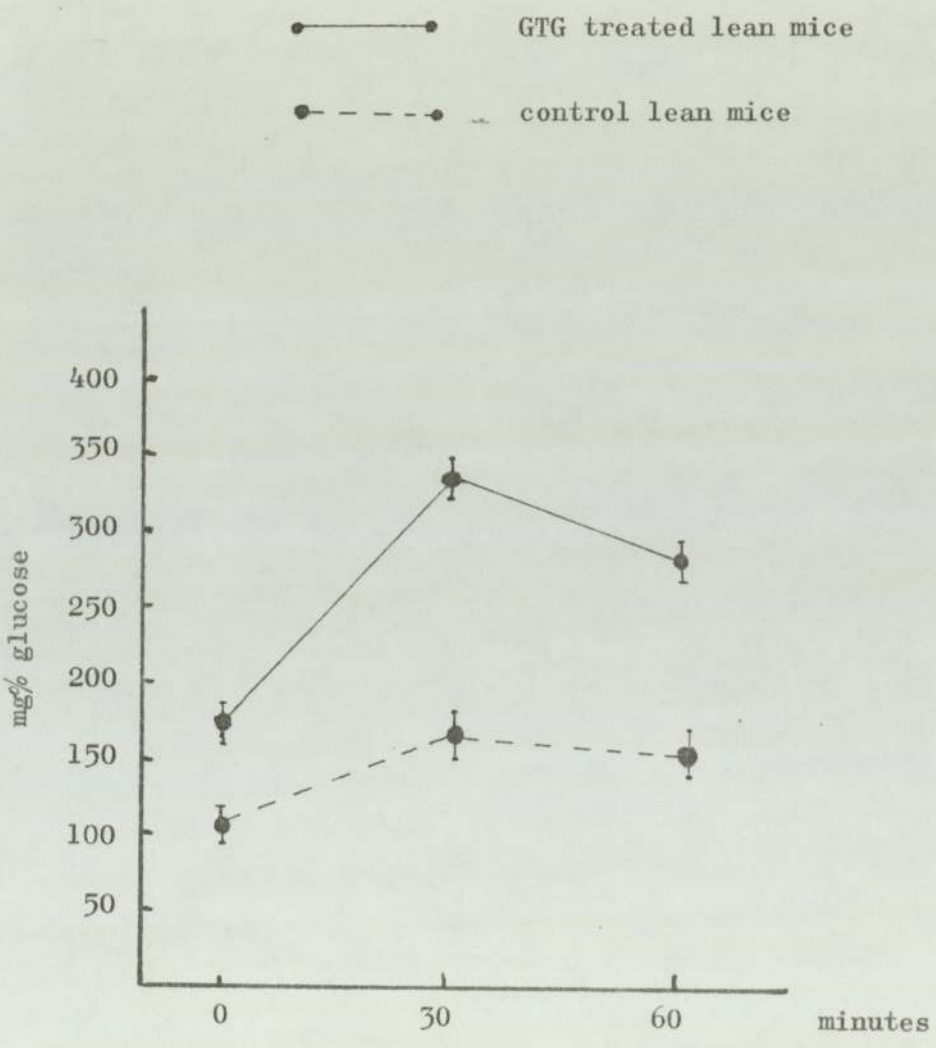
	Lean mice	GTG treated mice	Obese hyperglycaemic mice
glucose mg/100ml	125±16.7	165.8±18.5	193±16.1
number of mice	15	15	15

Lean and GTG treated mice P<0.001

Obese hyperglycaemic and GTG treated mice P<0.01

Figure 1

i.p. glucose tolerance test on 32 week old GTG treated and control lean mice.



Histological observations

Light microscopy

Most of the islets of GTG treated mice appeared to be enlarged, although no quantitative measurements were made. There were no signs of degeneration, necrosis or vacuole formation (Plates 66 & 67). The increased plasma insulin in GTG treated mice might have been the result of a functional compensatory hypertrophy of the islets in response to the prevailing hyperglycaemia.

Ultrastructural examination

The islets of Langerhans of GTG treated mice showed changes in the structure of granular endoplasmic reticulum and the Golgi apparatus, both of which appeared to be increased in size. Many β-granules appeared to have lost their core material. This could have been the result of either the dissolution of secretory material inside the granule membranous sac or degranulation (Plate 68). The endoplasmic reticulum of some β-cells was occasionally enlarged, suggesting an increased synthetic activity (Plate 69). Platelet aggregation was often observed in islet capillaries (Plate 70), together with the deposition of amyloid-like material (Plate 71) and the appearance of large ceroid bodies in the β-cells (Plates 68 & 69).

5.4 Discussion

The present study confirms that the injection of lean mice with gold thioglucose subsequently results in a marked increase in body weight and elevated circulating levels of blood glucose and plasma insulin. Many other reports have also demonstrated elevated blood glucose and insulin levels in mice previously injected with gold thioglucose (Colman & Hummel, 1970). The presence of an elevated plasma insulin concomitant with hyperglycaemia would suggest the presence of a tissue resistance to insulin in the GTG treated mice (Chlouverakis, 1971).

The present study also confirms that GTG treated animals develop an impaired tolerance to exogenous glucose injected intraperitoneally. This observation supports the previous work of Katsuki and co-workers (1962), who were able to demonstrate an abnormal response by GTG treated lean mice to glucose injection. This inability to respond to glucose load was suggested to be due either to insulin antagonism (Smith & Hall, 1973) or a decreased tissue sensitivity to insulin (Lang, Anders, Poxefsky & Gregerman, 1966), especially by fat, whose proportion of the total body mass appears to increase after GTG treatment.

The present study provided evidence of an alteration in β-cell morphology after GTG treatment. The islets of GTG treated lean mice

appeared to be slightly enlarged compared to the islets of untreated control animals. Indeed, the total pancreatic islet volume has been shown to be increased in GTG treated lean mice (Petersson & Hellman, 1962). The enlargement of the Golgi apparatus, endoplasmic reticulum and mitochondria would suggest an increased synthetic and secretory activity of the β-cells. The elevated plasma insulin of the GTG treated mice might have been the result of a functional compensatory hypertrophy of the islets in response to the prevailing hyperglycaemia.

Certainly GTG induced hyperglycaemia, hyperinsulinaemia and obesity did not induce islet vacuolation in lean mice. It might be that this treatment did not aggravate islet hyperactivity sufficiently to promote islet vacuolation. Islet vacuolation per se, being the final expression of events that occur subsequent to functional exhaustion of the β-cells vis à vis, increased capillary fragility and coalescence combined with β-cell degeneration.

The appearance of ceroid bodies in the β-cell cytoplasm of GTG treated lean mice suggested that obesity and hyperglycaemia might be causative factors in their formation. The appearance of ceroid bodies might reflect exhaustion atrophy of the β-cell in response to tissue insulin resistance. Indeed, degranulation and vacuolar degeneration were observed in the β-cells of GTG treated animals. In addition, amyloid deposition and platelet aggregation

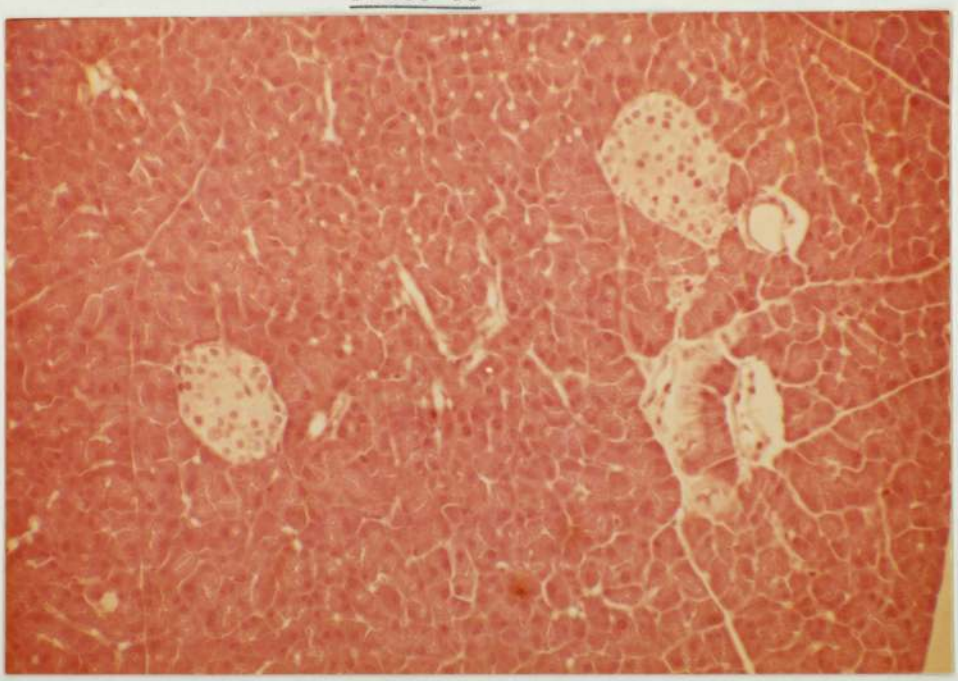
were observed in the capillaries of GTG treated mice, and, in this respect, the situation resembled that seen in old spontaneously obese mice. Few lymphocytes were observed in the islets of Langerhans of GTG treated lean mice. Craighead (1976) has observed the presence of insulitis in mice injected with gold thioglucose and suggested that GTG treatment increased the susceptibility of mice to viral infection.

Necrotic lesions have been observed in the hypothalamic regions of GTG treated mice (Marshall, Barnet & Mayer, 1955; Katsuki et al., 1962). Hypothalamic lesions have also been observed in mice made obese by electrical stimulation of the ventromedial centre of the hypothalamus (Kennedy, 1963; Mayer & Thomas, 1967). These workers suggested that there was a relationship between the appearance of hypothalamic lesions and the incidence of obesity and hyperglycaemia, since hypothalamic lesioned animals became hyperphagic, hyperglycaemic, hyperinsulinaemic and showed an overall increase in body weight. Although these changes may be associated with hypothalamic lesions induced chemically or electrically, it has yet to be established whether the hypothalamus has a direct or indirect effect upon pancreatic islet function. Damage to the hypothalamus has been shown to elevate the hormonal threshold necessary for the production of both hyperglycaemia and glucosuria (Katsuki et al., 1962). However, there is no evidence to suggest that a hypothalamic lesion is responsible for the obesity and hyperglycaemia observed in genetically obese mice.

On the other hand, increased food intake may result in a syndrome characterised by obesity, glucose intolerance and hyperinsulinaemia (Sims & Horton, 1968). Hyperphagia might stimulate insulin biosynthesis and release directly or indirectly through the stimulating effects of gastro-intestinal hormones (Creutzfeldt et al., 1970). In the present study, hyperphagia, hyperinsulinaemia, islet hypertrophy and the increased body weight of lean mice treated with GTG suggest that obesity per se may be a contributing factor to islet hyperfunction.

PLATES 66–71

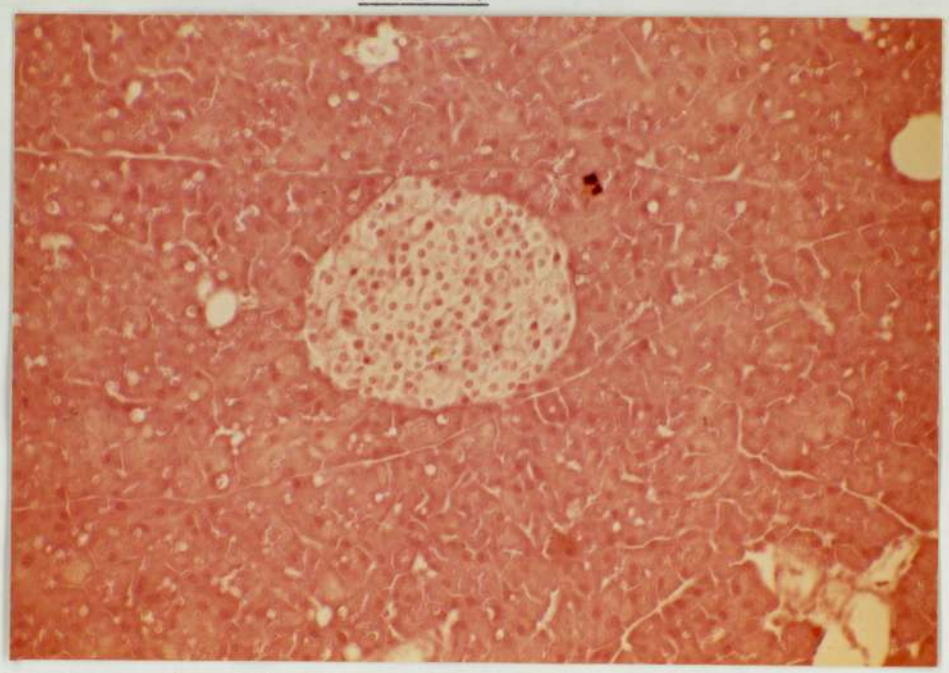
Plate 66



Islets of a 30 week old lean control mouse.

H. & E. x 180.

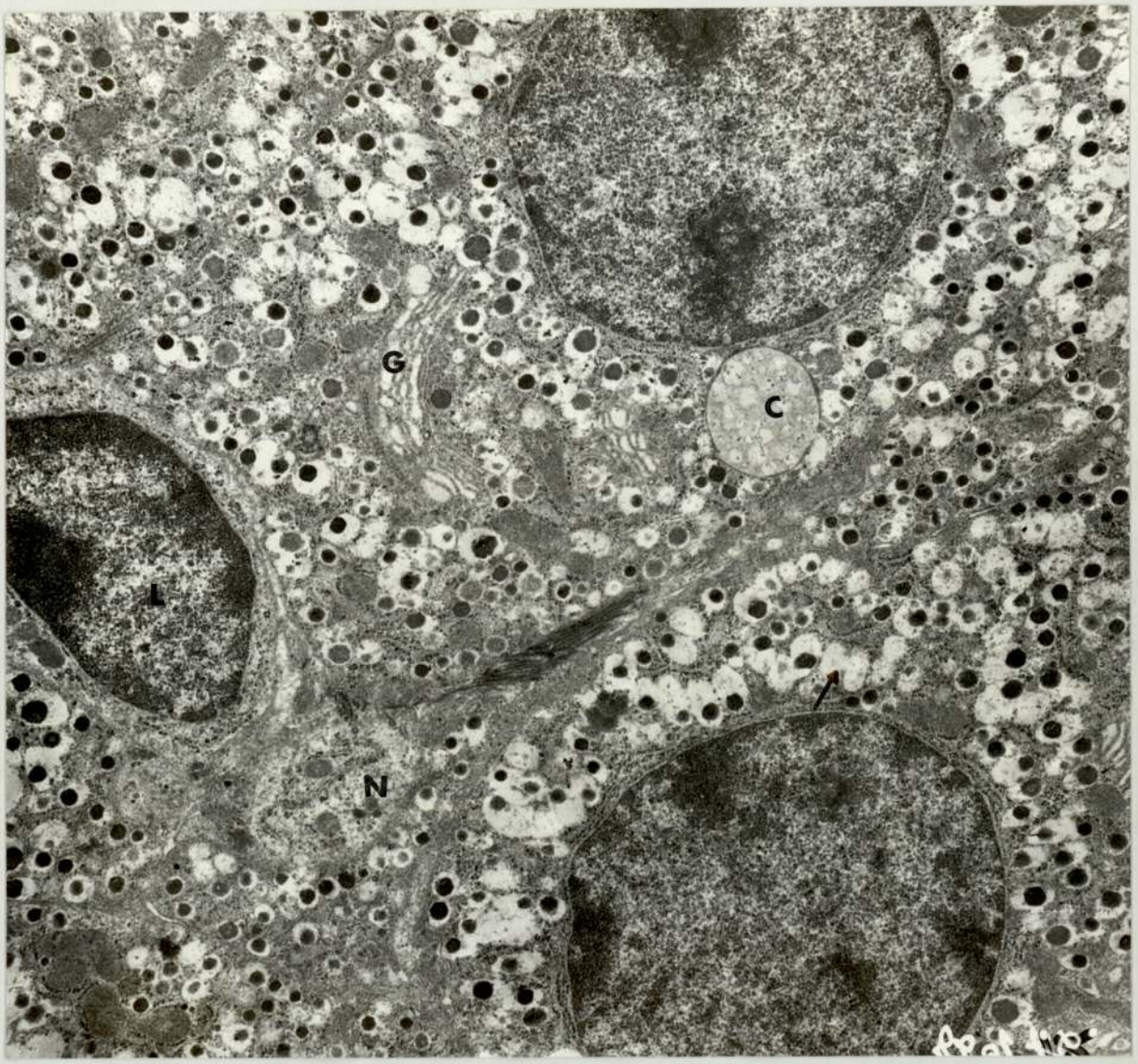
Plate 67



Islet from a 32 week old GTG treated lean mouse.

H. & E. x 180.

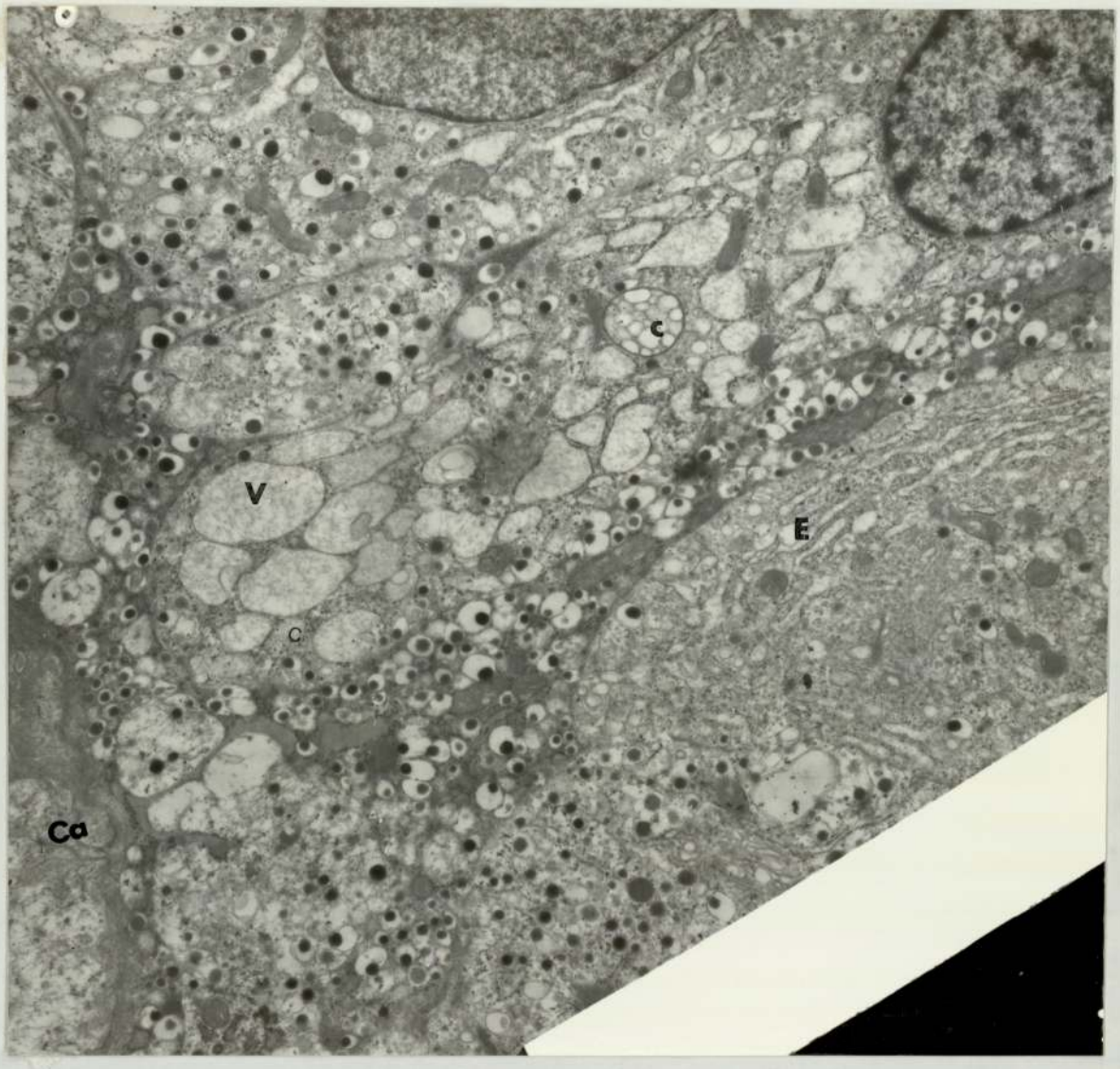
Plate 68



β_1 cells of lean mouse injected with gold thioglucose. Notice the ceroid body (C) and Golgi apparatus (G). In addition, the micrograph shows the presence of a lymphocyte (L), large empty β-granules (arrows) and a nerve ending (N) situated between the plasma membranes of adjacent β-cells.

Glutaraldehyde-osmium x 10,000.

Plate 69

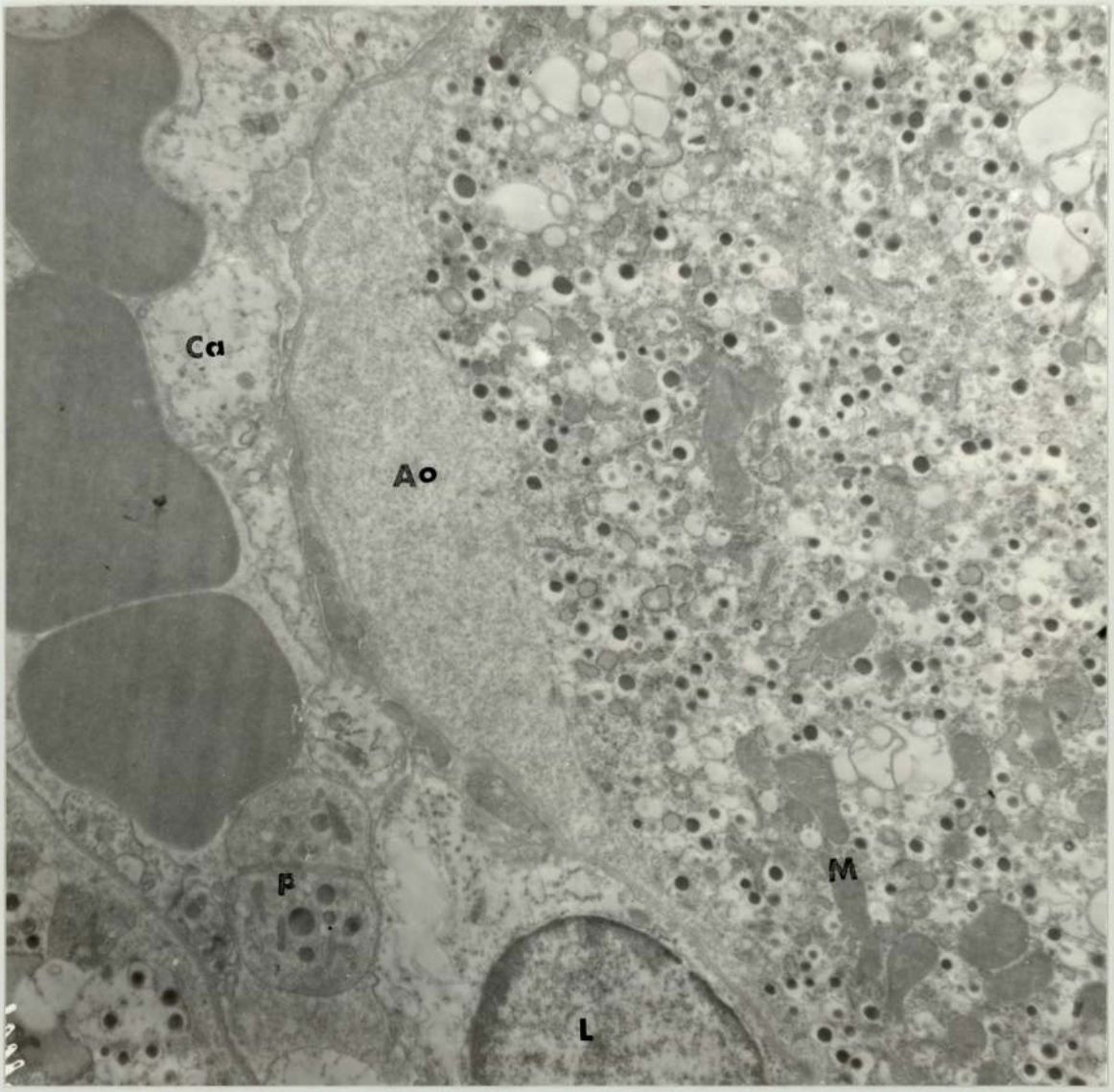


GTG treated lean mouse islet β-cell showing the enlargement of the endoplasmic reticulum to form vacuoles (V), a ceroid body (C) and increased quantities of endoplasmic reticulum (E).

Ca, capillary.

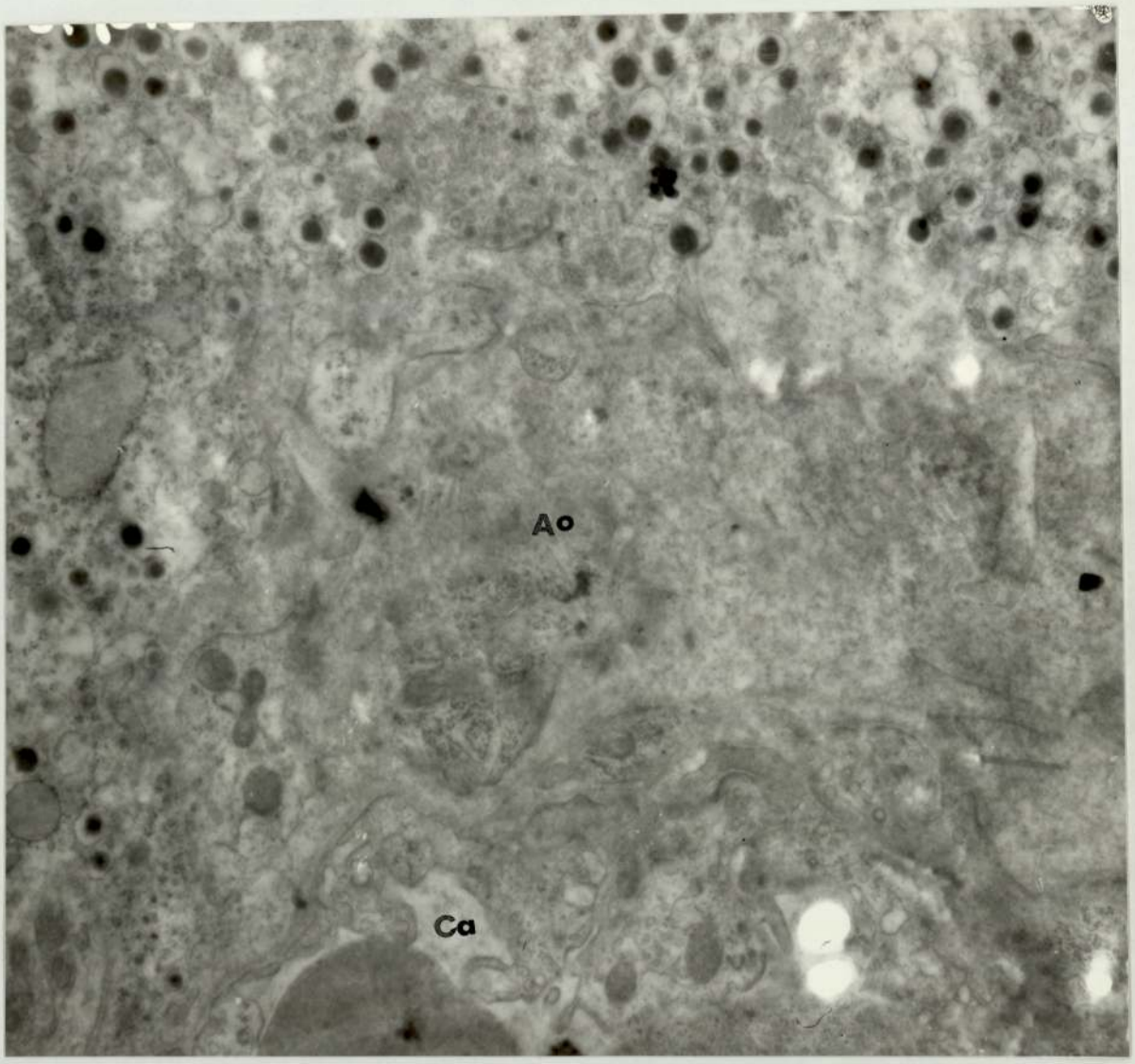
Glutaraldehyde-osmium x 6,000.

Plate 70



A capillary–β–cell junction in the islet of a GTG treated lean mouse showing platelet aggregation (P), large mitochondria (M) and amyloid-like material (Ao). L, lymphocyte; Ca, capillary. Glutaraldehyde-osmium x 10,000.

Plate 71



Deposition of amyloid-like material (Ao) and the junction of a β-cell with a capillary in the islet of a GTG treated lean mouse. Glutaraldehyde-osmium x 15,000.

GENERAL CONCLUSION

Experimental work presented here has shown the islets of Langerhans of obese hyperglycaemic mice and their lean littermates to be composed of at least three morphologically distinct cell types. These are the alpha (α_2) cell, the beta (β_1 and β_2) and the delta (α_1) cell. In addition, a few granular cells have also been observed. One of these is the so-called "F" cell, first described by Munger and co-workers (1965) and later by Fujita (1974). The F cell is characterised by its variably dense cytoplasmically polarised granules.

The β_2-cell was previously called a "syncytial α_2-cell" (Bjorkman et al., 1963). This cell resembles morphologically a type of β-cell originally described in the rat by Lacy (1957, 1961) and Caramia (1963). It has also been identified in the spiny mouse (Pictet et al., 1967) and in the C57 mouse (Bowen & Schwartz, 1976). The β_2-cell is characterised by a large number of β-granules. Each granule contains a large space or halo separating the granule core from its membranous sac. The granule cores range from spherical to rectangular in shape and may be dense or pale. The cytoplasm of the centrally located β_2-cell is dense and filled with closely packed β-granules and an irregular shaped nucleus. Characteristics of this nature suggest that the so-called "syncytial α_2-cell" is a β-cell. An agranular cell type has been observed in the islets of old obese hyperglycaemic mice. These cells contained degenerate mitochondria and endoplasmic reticulum and a few

β-granules. The present study suggests that these cells are atrophic degenerating β-cells.

The present study has confirmed that pancreatic islets of obese hyperglycaemic mice and their lean littermates are supplied with both myelinated and non-myelinated nerve endings. These nerves were either adrenergic or cholinergic and terminated at both α- and β-cells. Nerve endings were frequently observed between islet cells and capillary endothelia.

The presence of pale and dense granules in β-cells is of functional importance. The pale granules have been shown to represent an immediately available pool of insulin (Goldenberg et al., 1969; Moleson et al., 1973; Aerts & Van-Assch, 1975). The increased numbers of pale granules in the islet β-cells of 20-25 week old obese mice is consistent with functional hyperactivity and a markedly elevated plasma insulin level.

The insulin granule is believed to be released by a process of emiocytosis (Lacy, 1961). In this regard, the present study has provided some evidence of β-granule fusion with the β-cell membrane, both in lean and obese mouse islets. However, the observation of emiocytotic figures at the level of the β-cell membrane depends upon the angle of sectioning. The fusion of β-granules with the β-cell plasma membrane has been suggested to

be a primary event in the release of insulin from the β-cell (Berger et al., 1975). In addition to emiocytosis, insulin also appears to be released from β-granules into the β-cell cytoplasm by a process of intracellular dissolution and by β-granule scrolling. Cytoplasmic insulin may then be subsequently released into the extracellular space via the plasma membrane. However, this process might be responsible for the intracellular disposal of excess stored insulin, and may be a function of its over-production. The increased intracellular dissolution of β-granules in the β-cells of old obese hyperglycaemic mice and the incidence of β-granule scrolling exclusively in these animals may be interpreted as a pathological consequence of an earlier, but no longer required, rapid production and release of insulin in response to an exaggerated metabolic demand.

Obese mouse islets show well-defined pathological changes with age. At 3-45 weeks of age, obese mouse islets of Langerhans show well-defined changes in morphology. There is an increased occurrence of degranulated β-cells and signs of their degeneration leading to the formation of agranular cells. Increased numbers of lysosomes appear in the cytoplasm with increasing age. These lysosomes undergo remarkable changes leading to the formation of ceroid bodies. Ceroid body formation appears to be a secondary consequence of cell degeneration, necrosis, fibrosis and/or hyalinisation (Reed et al., 1965).

Insulitis is a condition in the islets of Langerhans characterised by the presence of inflammatory cells, mostly lymphocytes (Gepts, 1965). An auto-immune reaction is one of several different agents that are believed to cause lymphocytic infiltration (Egeberg, Junker, Kromann & Nerup, 1976). Insulitis is characterised by variability in the size and shape of islet cells, degranulation and degeneration of β-cells, fibrosis and distortion of the islets, reduction in the number of β-cells and lymphocytic infiltration (LeCompte & Legg, 1972; Freytage & Kloppel, 1974; Egeberg et al., 1976). In the present studies, lymphocytic infiltration associated with degranulation and degeneration of β-cells and islet fibrosis was observed in the islet tissue of the old obese mice. Macrophages containing phagocytosed β-granules associated with degenerate β-cells were observed in the islets of Langerhans of obese mice. This would suggest that the degeneration of β-cells by exhaustion atrophy may be the result of an inflammatory auto-immune phenomenon.

Amyloid-like material has been demonstrated in the capillary walls of 30-45 week old obese mouse islets, either within or closely applied to β-cells. The deposition of amyloid-like material in capillary walls and between β-cells and capillaries could form a barrier to the passage of insulin from the β-cell into the capillary lumen. The deposition of amyloid-like material in the islets of Langerhans of old obese mice is external and consistent

with the two-phase theory (Teilum, 1968). Amyloid deposition might in part be responsible for the reduced circulating level of plasma insulin seen by Bailey and co-workers (1977) in old obese mice. The biochemical characteristics of the amyloid-like material in old obese mouse islets remains to be investigated.

Zymogen granules have been observed in cells at the periphery of the islets of old obese mice. These cells are probably acinoinsular cells. In old obese mouse islets, exocrine-like tissue was observed within the islets. This would suggest the possibility of exocrine-endocrine cell transformation.

In old obese mouse islets, blood platelets were aggregated in thin-walled fragile capillaries. Platelet aggregation occurred close to deposits of collagen fibrils. Collagen fibrils have the capacity to aggregate platelets (Hoving, 1963). It is possible that the aggregation of platelets in small capillaries of old obese mouse islets might impede the passage of insulin through the capillary system of the islet.

As the obese hyperglycaemic syndrome manifests itself, vacuoles appear within the islets. These vacuoles are the result of a series of pathological changes that occur in the islets from about 15 weeks of age (Figure 2).

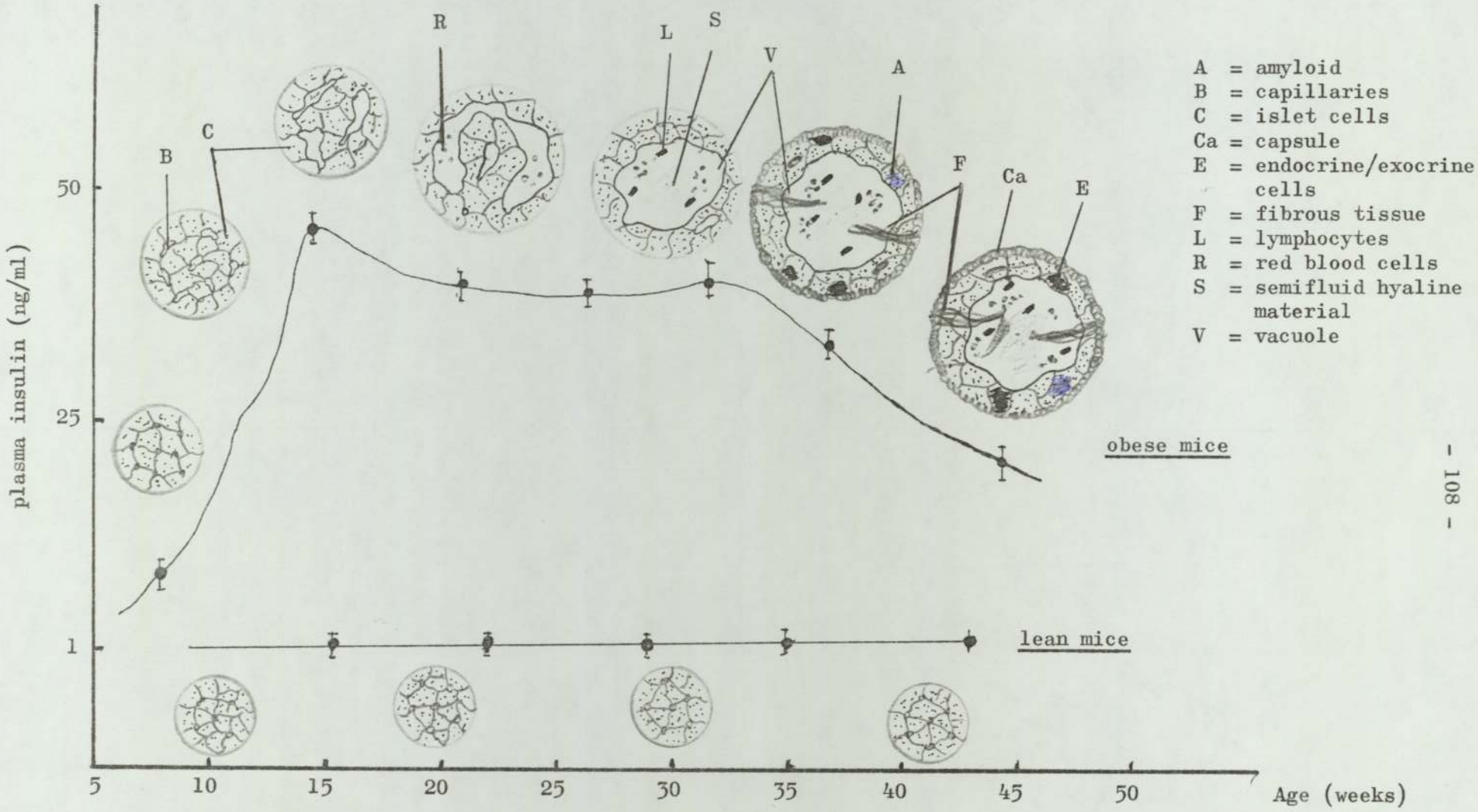


Figure 2 A schematic profile of obese mouse islet vacuole formation and the corresponding level of plasma insulin.

1. Ultrastructural studies have shown that the primary morphological event is the appearance of vacuoles (swollen cisternae of the ER) in the cytoplasm of β-cells. Adjacent vacuoles coalesce and form larger pockets which eventually come to occupy most of the β-cell cytoplasm. There is a loss of β-granules and subsequent cytoplasmic rarefaction.

2. The walls of swollen obese mouse islet capillaries appear thin and fragile.

3. β-cells adjacent to fragile dilated capillaries begin to degenerate. The capillaries finally rupture, forming a small vacuole (Figure 2).

4. As more capillaries rupture, the vacuole increases in size and this is assisted by the continuous degeneration of adjacent β-cells. In old obese mice, the vacuole comes to occupy most of the islet. A layer of columnar cells appears around the edge of the vacuole. There may be an invasion of the remaining islet tissue with fibrous material from the connective tissue capsule of the islet. The vacuole becomes filled with a semifluid fibrous material. Lymphocytes and macrophages can be seen close to degenerating β-cells and actually inside the islet vacuoles.

Freytage and Kloppel (1974) have shown that β-cell degeneration is invariably associated with lymphocytic infiltration. Muntefening (1974) has observed β-cell decay and dilated blood capillaries in mice innoculated with EMC virus. In the present study, the prevalence of lymphocytes in old obese mouse islets, the degeneration of β-cells and the presence of dilated blood capillaries suggest the possibility of an immunological involvement in obese mouse islet vacuole formation.

The increase in vacuole size and the concomitant reduction in the number of islet β-cells, coupled with the deposition of amyloid-like material, could account for the reduced circulating insulin levels observed in 35-45 week old obese mice. Insulin deficiency is known to result from a profound reduction in the number of β-cells (Gepts, 1972). Lazarus and Volk (1962) suggested the cause of the secretory inactivity of β-cells of aged diabetics to be due to a vascular lesion, which provided an anatomical barrier to the release of insulin. Gepts (1972) has reported that β-cells usually contain only 50% of their usual complement of insulin in aged diabetics. Degranulated and degenerate β-cells have been demonstrated in the islets of old obese mice.

Gold thioglucose treatment of homozygous (+/+) lean littermates of obese mice resulted in an increased body weight, hyper-

glycaemia and hyperinsulinaemia. Certain morphological changes characteristic of genetically obese (ob/ob) mouse islets were observed in the islets of GTG treated lean mice. These included increased β-granulolysis, the appearance of ceroid bodies and islet hypertrophy, but islet vacuolation was not observed. Table 6 compares the morphological changes induced in lean mouse islets by GTG treatment with the well-characterised morphological changes observed in obese hyperglycaemic mouse islets. These changes indicate a proportionality between the level of deterioration in the pancreatic islets and an increase in body weight and insulin secretion. This proportionality would suggest that obesity is an aetiological factor in the promotion of islet hyperfunction.

The development of the obese hyperglycaemic syndrome in mice can be divided into three stages (Herberg et al., 1970; Bailey et al., 1977). The first phase is characterised by rapid weight gain and an increasing plasma insulin level in response to hyperglycaemia. During the second phase, serum insulin and blood glucose reach a peak, and in the final phase the plasma insulin and blood glucose begin to fall and the body weight decreases. The present study has documented many morphological changes that occur in obese mouse pancreatic islets with age (Table 2, page 57). Islet vacuolation, fibrosis and amyloid deposition would account for the depressed plasma insulin levels of old obese mice (Figure 2).

Table 6

Comparison of the morphological characteristics of GTG treated lean mouse islets and obese mouse islets.

Morphological characteristics	GTG treated mice	obese (ob/ob) mice
β-cell degranulation	+	+++
Enlargement of Golgi apparatus and ER	+	+++
β-granulolysis	+	+++
β-cell degeneration	0	++
Ceroid bodies	+	++
β-cell hyperplasia	+	+++
Islet hypertrophy	+	+++
Islet vacuolation	0	++
Fibrosis	0	++
Amyloid-like material	+	++
Mixed cell (endocrine/exocrine)	0	++
Insulitis	0	+

0 = absent; + = mild; ++ = marked; +++ = very marked

The present study has also demonstrated very limited changes in the β-cells of old lean mice. These changes included increased numbers of β-granules and enlargement of the endoplasmic reticulum. However, amyloid deposition, islet fibrosis, vacuolation, ceroid body accumulation and hypertrophy were not observed.

GTG treatment of lean mice did induce obesity, hyperglycaemia and ceroid body formation, pathologies that are characteristically observed in the obese mouse. However, GTG treatment did not induce islet vacuolation in lean mouse islets. It may be that this treatment did not aggravate islet hyperactivity sufficiently to promote islet vacuolation.

Age associated changes in cells may be expressed as changes in morphology and function (Franks, 1970). The central importance of insulin producing pancreatic β-cells in the syndromes associated with inappropriate hyperglycaemia in rodents is attested to by the presence of some anomaly of either β-cell morphology or pancreatic insulin content (Cameron et al., 1972). The present study has demonstrated many morphological changes in the islets of Langerhans of old obese mouse.

Hyperphagia has been described as possibly the major factor in the genesis of obesity (Cameron et al., 1972) and ageing (Everitt, 1973). It is known that each organ or function has a

finite lifespan and, after being used a certain number of times, it starts to break down. Everitt concluded that the greater the quantity of food consumed, the faster is the rate of ageing, the earlier the onset of age-related pathology and the shorter the lifespan. Obese mice are certainly hyperphagic. The increased food intake imposes an insuperable constraint on the β-cell, stimulating it to hypersecrete insulin. Hyperphagia and the associated hyperglycaemia that it produces might accelerate the ageing process in the islet β-cell. Accelerated ageing could lead to the degeneration of β-cells as observed in old obese mice, although the presence of lymphocytes would suggest some form of auto-immune phenomena.

In conclusion, one might suggest that the obese mouse syndrome is the product of the interaction of several well-defined aetiological factors. These include hyperphagia, hyperglycaemia, hyperinsulinaemia, auto-immune phenomena and the ageing process.

In the present study, the close examination of the sequence of pathological changes occurring in the islets of Langerhans of obese mice has enabled us to understand a little more clearly the complex interplay of aetiological factors that make up the maturity onset diabetic syndrome.

A P P E N D I X

Preparation of Stains used in Morphological Studies

1. Haematoxylin & Eosin

Ehrlich's haematoxylin

Haematoxylin	2g.
Absolute alcohol	100ml.
Glycerine	100ml.
Distilled water	100ml.
Glacial acetic acid	10ml.

Technique

1. Stain in haematoxylin for 15 minutes.
2. Wash in running tap water for 2-3 minutes.
3. Remove excess stain by decolourising in 1% acid alcohol.
4. Wash in tap water for 10 minutes.
5. Stain in 1% aqueous eosin for 3 minutes.
6. Wash in water, dehydrate in alcohol, clear in xylene and mount in Canada balsam.

Results

Nuclei	- blue to blue-black
Cytoplasm	- shades of pink
Red blood cells and granules	- bright orange-red
Fibres	- deep pink
Collagen	- light pink

2. Congo Red

1. Stain for 10 minutes in Mayer's haemalum (haematoxylin 1g., sodium iodate 0.2g., potassium alum 50g., citric acid 1g., chloral hydrate 50g. and distilled water 1,000ml.).
2. Wash in 3 changes of distilled water.
3. Transfer to alkaline NaCl/alcohol for 20 minutes. (To 40ml. saturated NaCl solution in 80% alcohol, add 0.4ml 1% NaOH.)
4. Stain in saturated congo red solution.
5. Dehydrate quickly in 3 changes of absolute alcohol, clear and mount.

Results

Amyloid	- red to pink
Elastic tissue	- lighter red
Nuclei	- blue
Amyloid	- green birefringence under plane polarised light

3. Methyl Violet

1. Stain in 1% aqueous methyl violet for 5 minutes.
2. Wash in water and differentiate in 1% acetic acid.
3. Wash in running tap water for 5-10 minutes.
4. Drain the slide and mount with Apathy's gum syrup (Arabic gum 50g., cane sugar 50g., distilled water 50ml., thymol 0.05g.).

Results

Amyloid	-	purplish-red to red
Nuclei, cytoplasm and connective tissue	-	blue to violet

4. Gomori's Reticulin Method

1. Take section to water.
2. Oxidise with 1% potassium permanganate for 2 minutes.
3. Rinse in tap water and bleach with 3% potassium metabisulphite.
4. Wash in tap water.
5. Treat with 2% iron alum for 1 minute.
6. Wash with tap water and two changes of distilled water.
7. Treat with ammonical silver solution for 1 minute (4ml. of 10% KOH in 20ml. of 10% silver nitrate).
8. Rinse with distilled water.
9. Treat with 10% neutral formalin for 3 minutes.
10. Wash in tap water.
11. Treat with 0.2% gold chloride for 10 minutes.
12. Rinse with distilled water and treat with 3% potassium bisulphite for 1 minute.
13. Rinse with distilled water and treat with sodium thiosulphate for 2 minutes.
14. Wash, dehydrate, clear and mount in resin medium.

Results

Reticulin fibres	-	black
Nuclei	-	greyish
Collagen	-	dark greyish-purple

5. M.S.B. (Martin's, Scarlet, Blue)

1. Stain nuclei with celestine blue-haemalum (celestine blue B 0.5g., ferric ammonium sulphate 5g., glycerine 14ml., distilled water 100ml.).
2. Rinse in tap water, differentiate in acid alcohol and wash in tap water.
3. Rinse in 95% alcohol and stain with 0.5% martius yellow in 95% alcohol containing 2% phosphotungstic acid for 2 minutes.
4. Rinse in distilled water and stain in 1% crystal scarlet 6R in 2.5% acetic acid for 10 minutes.
5. Rinse in distilled water and treat with 1% phosphotungstic acid for 5 minutes.
6. Rinse with distilled water and stain in 0.5% soluble blue in 1% acetic acid for 10 minutes.
7. Rinse in 1% acetic acid, dehydrate, clear and mount.

Results

Nuclei	- black
Fibrin	- red
Connective tissue	- blue

7. Aldehyde Fuchsin (AF)

1. Oxidise with Lugol's iodine for 10 minutes (iodine 1g., potassium iodide 2g. and distilled water 12ml.).
2. Rinse in tap water and bleach with 2.5% sodium thiosulphate.
3. Wash in tap water followed by 70% alcohol.
4. Stain in jar of aldehyde fuchsin for 20 minutes.
5. Wash in 95% alcohol followed by water.
6. Stain in celestine blue-haemalum.
7. Wash in water, differentiate in acid alcohol and wash in water.
8. Counterstain with orange G for 45 seconds.
9. Rinse with 0.2% acetic acid followed by 95% alcohol.
10. Dehydrate, clear and mount.

Results

Beta cells	- purple-violet
Alpha (α_2) cells	- yellow
Delta (α_1) cells	- blue-black
Collagen	- green

8. Thioflavine "T"

1. Stain with alum haematoxylin for 2 minutes.
2. Wash in water and stain in freshly filtered 1% aqueous thioflavine "T" for 3 minutes.
3. Rinse in water and treat with 1% acetic acid for 20 minutes.
4. Wash in running water and mount in Apathy's gum syrup.

Results

Amyloid - fluorescent bright yellow against an olive-green background.

9. Alcian Blue

1. Stain in alcian blue solution for 10-30 minutes (alcian blue 8GS 0.5g., glacial acetic acid 3ml., distilled water 100ml.).
2. Wash in running water for 5 minutes.
3. Dehydrate, clear and mount.

Results

Acid mucopolysaccharides - blue

Nuclei - red

10. P-dimethylaminobenzaldehyde

1. Take sections to 100% alcohol and dry briefly.
2. Immerse in 1g. P-dimethylaminobenzaldehyde in 10ml. concentrated hydrochloric and 30ml. glacial acetic acid for 5 minutes at 25°C.
3. Glacial acetic acid, 3 washes, 30, 60 and 60 seconds.
4. Add 1ml. freshly diazotised S acid or safranine to 40ml. cold (15-20°C) glacial acetic acid, mix and pour into cold Coplin jar. Sections were immersed for 5 minutes.
5. Wash in 2 changes glacial acetic acid.
6. Stain for 5 minutes in 0.05% new fuchsin in glacial acetic acid and wash in 2 changes glacial acetic acid for 1 minute each.
7. Acetic acid + xylene, 3-4 changes of xylene, cellulose caprate.

Results

Tryptophan - blue

11. Masson's Trichrome Stain

1. Stain with celestine blue.
2. Wash in water and differentiate with acid alcohol.
3. Wash in tap water and rinse in distilled water.
4. Stain for 10 minutes in a solution of 1% ponceau de xylidine in 1% acetic acid and 1% acid fuchsin in 1% acetic acid.
5. Rinse in distilled water and differentiate in 1% phosphomolybdic acid.
6. Rinse in distilled water and stain in aniline blue (2% in 2% acetic acid).
7. Wash in 1% acetic acid for 1 minute.
8. Blot, dehydrate, clear and mount.

Results

Nuclei	- black
Muscle	- red
Blood cells, fibrin and some cytoplasmic granules	- red
Collagen, reticulin, amyloid and mucin	- blue

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