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STUDIES ON INSULIN SECRETION AND GLUCOSE TOLERANCE IN EXPERIMENTAL OBESITY AND DIABETES

By

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Thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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DEDICATION

This thesis is dedicated to my husband Jack. His love, his patience and his clever technical help were of uttermost importance in the completion of this research work.

Cette thèse est également dédiée à ma mère, Eugénie Dalpé. Elle a été un catalyseur dans la conduite de mes études universitaires et n'a jamais cessé de m'encourager.

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ABBREVIATIONS

a.c.	ante cibum (before a meal)
AGG	anti-(guinea-pig γ-globulin)
AIS	guinea-pig anti-insulin serum
BB rat	spontaneously diabetic Wistar rat
BSA	bovine serum albumin
CaM	calmodulin
CTL	control
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene-bis-(oxyethylenenitrilo)tetraacetic acid or ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetraacetic acid
FR	food restriction
GPS	guinea-pig serum
IDDM	insulin-dependent diabetes mellitus
I.M.	intramuscular
I.P.	intraperitoneal
IRI	immunoreactive insulin
I.V.	intravenous
KRB	Krebs-Ringer bicarbonate
Ln	lean
NIDDM	non-insulin-dependent diabetes mellitus
OTC	oxytetracycline
p.c.	post cibum (after a meal)
PDE	phosphodiesterase
RIA	radioimmunoassay
TFP	trifluoperazine

USEFUL CONVERSIONS

Glucose mM x 18 = mg/dl

Insulin uU x 40 = pg (on the basis of 25 U/mg)

SUMMARY

Studies were carried out on insulin secretion and glucose tolerance in animal models of obesity and diabetes. The animal models used were the ob/ob mouse, a model of Type II (non-insulin dependent) diabetes and the BB rat, a model of Type I (insulin-dependent) diabetes. The antibiotic oxytetracycline (OTC) has been used in the past to study the nature of the metabolic derangement in the obese-hyperglycemic syndrome. OTC is known to result in a reduction of the severity of many of the metabolic abnormalities shown by the ob/ob mouse. It was not known, however, whether the effects of oxytetracycline are pancreatic, extrapancreatic or both.

The first part of this thesis focuses on studies done to investigate the extrapancreatic action of oxytetracycline further. The spontaneously diabetic BB rat was used in these studies because the syndrome is associated with very small and rare islets containing virtually no B-cells and thus allows a study which is not confounded by the presence of a functioning pancreas. Chronic OTC-treatment was found to alter the diabetic status of the BB rat. The treatment led to lowered plasma glucose levels in the fed as well as in the fasted state. These results indicated that the oxytetracycline treatment was effective in lowering the insulin requirements as well as in improving the handling of glucose. The effects of the drug were not secondary to the decreased food intake as a food-restricted control group did not show the improvements in glycemia or glucose and insulin tolerance. These results are a further indication that oxytetracycline enhances the response of peripheral tissues to insulin and thus favors better control of glycemia.

The second part of this thesis deals with studies designed to investigate the exaggerated insulin secretory response of the ob/ob mouse. The effect of oxytetracycline treatment on the response of the obese mouse

to various secretagogues was investigated *in vivo* and *in vitro*. Lean mice and their isolated islets responded to glucose, glucagon and aminophylline as expected from data already in the literature. Obese mice had an exaggerated insulin response to these stimuli whether applied *in vivo* or *in vitro*, but that response was decreased by prior treatment of the animal with OTC. *In vivo* and *in vitro* studies done in parallel on obese mice, food-restricted to the intake of OTC-treated obese mice, indicated that food-restriction did not diminish insulin secretion as much as OTC-treatment. Furthermore, it did not result in a significant decrease in the glycemic responses to glucose, glucagon or aminophylline. The *in vivo* data support the previous conclusion that OTC-treatment increases the insulin sensitivity of peripheral tissues. The *in vitro* data lead me to postulate that the greatly reduced release of insulin by the islets of the OTC-treated ob/ob mouse could result from a restoration of the sensitivity of the islets to the feedback inhibition by insulin.

Zinc has been shown to have an important role in the process of insulin storage and secretion. Previous studies had suggested a state of relative zinc deficiency for the ob/ob mouse. Dietary zinc supplementation of the obese mouse resulted in a significant decrease in the *in vitro* insulin secretion in response to glucose and a significant increase in the islet insulin content. The combination of zinc feeding and OTC treatment was better than that of either treatment alone in correcting the exaggerated release of insulin from the ob/ob mouse islets and in increasing the insulin content of those islets. This suggests that zinc and OTC have additive effects on the improvement of the insulin sensitivity of tissues.

The last part of this thesis was designed to investigate if the exaggerated insulin secretory response of the islets of the obese mouse to

secretagogues may be related to alterations in the Ca^{2+}-calmodulin system. Major differences were found between the effects of the Ca^{2+}-calmodulin inhibitor trifluoperazine (TFP) on islets of lean and obese mice. The lean mouse islets were more resistant than the obese mouse islets to the inhibitory effect of TFP on the insulin-secretory response (20 mM glucose). Furthermore, under certain experimental conditions, TFP had a significant stimulatory effect on basal insulin release (3 mM glucose) which was most readily noticeable in islets of ob/ob mice. On a per islet basis, the islets of the obese mouse were found to contain more calmodulin than the islets of the lean mouse. Thus the greater inhibitory effect of TFP on ob/ob versus lean mouse islets is unlikely to be due to a depletion of calmodulin in the former group. These results indicate qualitative as well as quantitative differences in the insulin secretory process in lean and ob/ob mice.

CHAPTER 1. INTRODUCTION

"La fixité du milieu intérieur est la condition de la vie libre".

Claude Bernard, 1859.

During the mid-nineteenth century, one of the most important investigative contributions of Claude Bernard was the discovery of a glucoregulatory system that maintains the constancy of glucose in the "milieu intérieur" (Unger, 1981). Since then, progress has been made toward the elucidation of the mechanisms governing this glucose constancy.

Glucose homeostasis depends on many hormones to increase the blood glucose concentration (glucagon, catecholamines, growth hormone, glucocorticoids) but depends almost entirely on one hormone, insulin, to effect a decrease in blood glucose concentration (Lambert, 1976; Unger et al, 1978). The fine tuning of the rate of insulin release is the result of an interplay between substrates and hormones reaching the islets of Langerhans via the circulation, and direct influences via the autonomic nerve fibers ending in the pancreatic islets (Lambert, 1976; Unger et al, 1978).

In mammals, one of the most important controls over insulin release is exerted by variations of the glucose concentration in the extracellular fluid. Alterations in the sensitivity of the B-cells to glucose as well as in the dynamics of glucose-stimulated insulin release may result in a deterioration of the glucose tolerance of the entire body leading to diabetes mellitus.

This thesis is a report on studies done on the insulin secretion and the glucose tolerance in experimental obesity and diabetes. The literature review is divided into sections corresponding to the subject areas dealt with in the study. A nomenclature and classification system for diabetes mellitus is followed by a presentation of the characteristics of the two

animal models of diabetes that were used in this work, namely the spontaneously diabetic BB rat and the C57Bl/6J obese (ob/ob) mouse. A summary of the present knowledge of the role of the pancreatic islets in insulin biosynthesis and in insulin release is then presented. Finally the literature on the antibiotic oxytetracycline is reviewed.

I. REVIEW OF LITERATURE

I. A. Diabetes Mellitus

I. A. 1. Nomenclature and classification

Diabetes mellitus can be generally defined as carbohydrate intolerance. It has been classified into the following two main groups by the National Diabetes Data Group (NDDG) of the N.I.H. (1979): Type I- insulin-dependent diabetes mellitus (IDDM) and Type II- non-insulin-dependent diabetes mellitus (NIDDM). This separation into two broad categories was made on the basis of several factors. As reviewed by Irvine (1977, 1980), phenomena that are present in IDDM but not in NIDDM are:

(1) circulating organ-specific islet cell antibodies;

(2) a high frequency of other organ-specific auto-antibodies (thyroid and gastric);

(3) insulitis (or isletitis) which is a lymphocytic infiltration of the pancreatic islets which leads to a marked decrease in the number or even total loss of B-cells;

(4) evidence for cell-mediated immunity against pancreatic islets;

(5) higher prevalence of certain HLA antigens (such as HLA-B8, HLA-B15, DW3, DW4) than in the normal population. [The term HLA refers to :Human Leucocyte system A. HLA is the major histocompatibility complex in man; it is a genetic system of closely linked genes on the short arm of the 6th chromosome].

In diabetes in general (both in IDDM and NIDDM) the etiology is

considered multifactorial i.e. both genetic and environmental factors play a role and the genetic basis is thought to be polygenic (Irvine, 1980). Although a large body of information has been gathered by physicians and scientists, human diabetes mellitus is still a poorly understood disease. Considering that both genetic and environmental factors contribute to and modify the pathogenesis of human diabetes, researchers have been interested in studying examples of spontaneous diabetes in laboratory animals where it would be more feasible to distinguish between the genetic and the environmental contributions (such as the effects of diet, drugs, toxins, infectious agents etc).

I. A. 2. Diabetes mellitus in laboratory animals

Spontaneous diabetes among animals has been recognized for some time. For example, Leblanc (1851) documented diabetes in a monkey. The disease has been well characterized in only a few species, mostly rodents. Since the report by Ingalls et al (1950) describing a mutation in the mouse characterized by profound obesity, numerous articles have been published describing the physiology and pathology of a number of spontaneous diabetic syndromes occurring in laboratory rodents (Cameron et al, 1972a; Like, 1977; Mordes and Rossini, 1981; Rossini et al, 1980). These small laboratory rodents by virtue of their large number and low cost, have yielded an enormous amount of data.

The major diabetic syndromes found in animal models has been classified by Like (1977) into lethal and non lethal syndromes. This classification is based upon the hypothesis that the ability of the organism to produce new pancreatic B-cells determines whether the syndrome will or will not be lethal. In the lethal category are animal models, such as the BB rat, in which the diabetic syndrome is severe and significantly reduces life expectancy; examination of the pancreatic islets of such

animals reveals destruction of B-cells with transient and/or inadequate replacement. In contrast, in the non-lethal group, animals, such as the ob/ob mouse, either experience a full life span with the disease or may in fact eventually return to the normoglycemic state. The animals of the latter group manifest significant B-cell proliferation in response to diabetes and the resulting increased number of functioning B-cells is usually associated with elevated levels of circulating IRI.

Diabetes-like syndromes can also be induced in laboratory rodents using a variety of chemical agents (e.g. streptozotocin and alloxan) and other experimental procedures such as pancreatectomy, lesions to the ventromedial nuclei of the hypothalamus, administration of contra-insulin hormones or anti-insulin serum, and induction by viruses (Mordes and Rossini, 1981)

Though no syndrome in animals corresponds exactly to any of the forms of diabetes in man, the advantages afforded by animal models continue to make them valuable research tools.

The following two sections will be focused on the two animal models that were used in my work : the spontaneously diabetic BB rat and the C57Bl/6J obese (ob/ob) mouse.

I. B. The BB Rat

I. B. 1. Characterization and etiology

The spontaneously diabetic Wistar rat (the "BB" rat) (sometimes called the "BB/W" rat) was first identified in 1974 at the Bio-Breeding Laboratories of Canada Ltd (Ottawa, Ontario). The BB rat is now recognized as an important animal model in the study of Type I- diabetes. It is characterized by an early age of onset (60-120 days), hyperglycemia (250-730 mg/100 ml), hypoinsulinemia (0-1 ng/ml), marked glycosuria, hyperketonemia, ketonuria, hyperglucagonemia, exaggerated plasma IRG

response to injected arginine, increased plasma levels of free fatty acids and branched-chain amino acids (Nakhooda et al, 1977, 1978a, 1978b). Within days of onset, many animals become severely ketoacidotic, dehydrated and moribund unless insulin treatment is instituted.

Unique among the spontaneously diabetic animals studied to date, the BB rat is the only rodent model, apart from the Chinese hamster, in which significant ketosis occurs without any sign of obesity. It is also the only animal model to display an intense pancreatic insulitis. At the time of onset of glycosuria (the earliest clinical manifestation of the syndrome), the BB rat has reduced B-cell numbers, small islets and marked infiltration of the islets with lymphocytes and macrophages (Nakhooda et al, 1977, 1978a, 1978b). In animals severely ill with acute diabetes, treated with insulin, and killed within several weeks of the onset of symptoms, the islets are small, reduced in number and contain only A, D and PP cells (Nakhooda et al, 1977, 1978a, 1978b).

The genetics of the BB rat are not yet defined. The syndrome occurs with equal frequency in both sexes and the incidence of spontaneous diabetes ranges from 30% to 50% in outbred strains but may be increased to 90% with selective inbreeding (Seemayer et al, 1980). Monogenic contributions to the etiology of the diabetes remain to be elucidated. Infection appears not to be a factor. BB rats have been raised in sterile gnotobiotic environments which exclude both viral and bacterial pathogens. Despite the absence of infectious organisms, diabetes was observed in the expected percentage of animals (Rossini et al, 1979). These experiments do not, however, exclude the possibility of a vertically transmitted, unidentified virus (Cahill and McDevitt, 1981).

Evidence for a cell-mediated immune pathogenesis in the BB rat was provided by: (a) the finding of an intense mononuclear cell infiltrate in

the islets during active B-cell loss (Nakhooda et al, 1977, 1978a, 1978b); (b) the demonstration of passive transfer of insulitis by lymphocytes from diabetic rats, injected into nude mice (Nakhooda et al, 1983); and (c) the presence of lymphopenia before and after development of diabetes (Jackson et al, 1981; Poussier et al, 1981). The syndrome could be modified and possibly prevented with anti-lymphocyte serum (Like et al, 1979), by neonatal thymectomy (Like et al, 1981) or by bone marrow transplantation from normal rats (Naji et al, 1981). An immune etiology is also suggested by the more recent findings of circulating autoantibodies reactive to islet cell surface and spleen lymphocytes (Dryberg et al, 1982), and to gastric parietal cells, smooth muscle and thyroid colloidal antigens (Elder et al, 1982).

I. B. 2. Comparison of the diabetic syndrome of the BB rat with human diabetes

The sudden onset of clinical disease, ketoacidosis, hyperglycemia, insulitis with B-cell degranulation and necrosis, insulinopenia and near absence of pancreatic insulin are strikingly similar to the characteristics of Type I diabetes mellitus. In contrast to human diabetes, however, plasma from BB rats was shown to contain islet cell surface antibodies but not islet cell cytoplasm antibodies (Dryberg et al, 1982). Both kinds of antibodies are present in the plasma of 60% of the human insulin-dependent diabetic population (Irvine et al, 1977; Lernmark et al, 1978). Also in contrast to reports in humans, thyroid microsomal or adrenocortical autoantibodies were not detected in the BB rat but autoantibodies to gastric parietal cells were found (Neufeld et al, 1980).

I. C. The Obese-Hyperglycemic Mouse

I. C. 1. Genetic development

In 1949 a few mice in the V strain at the Jackson Laboratory were

observed to be plump early in life and to become markedly obese (up to 90g) late in life. The obese mutation occurred in a noninbred stock and was first described by Ingalls et al in 1950 . Breeding experiments revealed that the obesity syndrome (gene symbol ob) was caused by a single autosomal recessive mutation on chromosome 6 (Ingalls et al, 1950). The ob mutation has been transferred into the C57Bl/6J and C57Bl/KsJ strains for studies involving the effect of inbred background. The mice used for the experiments described in this thesis are of the C57Bl/6J strain which is commercially available from the Jackson Laboratory, Bar Harbor, Maine.

Several other colonies of obese mice (ob/ob) exist in which the obese mutation is carried on different genetic backgrounds. The severity and the developmental pattern of the metabolic disturbances vary among the different strains. This variable expression can occasionally lead to great difficulty and confusion when trying to compare data reported in the literature. Most of the earlier studies on the ob/ob mouse islet cell metabolism were done using mice from the noninbred colony maintained at the Department of Histology, University of Uppsala, Sweden (Hellman, 1970). Those mice, although similar to the Jackson Laboratory C57Bl/6J obese mice in most respects, are characterized by massive islets of Langerhans. The obese mice bred at the University of Aston in Birmingham (Bailey et al, 1982) and those bred at the Imperial College, London, England (Beloff-Chain et al, 1975a, 1975b) are descended from mice carrying the original Jackson Laboratory mutation and introduced in local mixed colonies. Expression of the ob gene on these backgrounds results in a more severe form of diabetes-like syndrome than that produced on the C57Bl/6J background (Bailey et al, 1982). The obese mice used by Thurlby and Trayhurn (1978) were also derived from the Aston strain background.

Whatever the genetic background is, the homozygous obese mouse is

characterized by obesity, hyperphagia, non-ketotic hyperglycemia, hyperinsulinemia, hyperglucagonemia and hypertrophic, hyperplastic islets of Langerhans, but the severity and duration of the symptoms vary. The discussion in this thesis will be limited to the C57Bl/6J ob/ob mouse, unless specifically stated.

I. C. 2. Time course for the manifestation of the obese-hyperglycemic syndrome

The earliest demonstrable defect in the ob/ob mouse is a decreased thermogenesis detectable by a reduction in oxygen consumption (Boissoneault et al, 1976; Fried and Antapol, 1966; Kaplan and Leveille, 1973; Mayer et al, 1952), with a fall in core temperature at low environmental temperature demonstrable from 10 days of age (Trayhurn et al, 1977). This impaired thermogenesis may account for the increased fat cell size observed from day 14 to 20 and the increase in body weight (Joosten and van der Kroon, 1974a; Kaplan et al, 1976). Hyperinsulinemia is manifest from 15 days of age (Dubuc, 1976b; Godbole et al, 1980) together with increased lipogenesis (Godbole et al, 1980). While insulin levels rapidly increase, there is a gradual transition from hypoglycemia to hyperglycemia during the 17-28 day period (Dubuc, 1976b). Obesity as indicated either by increased body weight, carcass fat, or Lee Index (Bergen et al, 1975; Dubuc, 1976b; Godbole et al, 1980; Joosten and van der Kroon, 1974a) is detectable by days 17-21, although the animals are not visually detectable as "obese" until 25-28 days or even later.

After weaning (21 days), three phases in the development of the obese-hyperglycemic syndrome can be differentiated. In the first, dynamic phase (1-6 months of age), hyperinsulinemia and hyperphagia increase with the progressive appearance of glucose intolerance, fasting hyperglycemia and resistance to exogeneous insulin. Hyperglycemia reaches a peak level by 12

weeks of age (Garthwaite et al, 1980; Herberg et al, 1970; Mayer et al, 1953b; Westman, 1968), corresponding to a body weight of approximately 40-50g. Elevated levels of circulating insulin reach a maximum by 6 months (Herberg et al, 1970; Westman, 1968). The dynamic phase is also characterized by an increase in lipogenesis and fat cell hyperplasia, and by a decrease in protein deposition.

The intermediary or transitional phase, when the animals weigh about 55g, is characterized by rapidly changing glucose patterns i.e. an extremely high serum glucose level followed by an improving glucose tolerance, and decreasing insulin levels (Herberg et al, 1970).

In the third phase, serum glucose and insulin levels spontaneously decline and approach normal at about 16 months of age (Herberg et al, 1970; Westman, 1968). The body weight of the obese mice remains elevated until they are 13-17 months old then it starts to decrease. A more recent report, though in agreement with Westman (1968) and Herberg et al (1970) on the plasma glucose profile, claimed that the plasma insulin levels of the ob/ob mouse remain elevated until 15 months of age. The discrepancies on the plasma insulin profile between longitudinal studies (Garthwaite et al, 1980; Herberg et al, 1970; Westman, 1968) cannot be accounted for only by the dissimilarity in the background strain of obese mice since, on the one hand, Westman (1968) used the ob/ob mouse from the colony bred at the University of Uppsala, Sweden, but on the other hand, both Herberg et al (1970) and Garthwaite et al (1980) were using C57Bl/6J Jackson Laboratory ob/ob mice. Also, though Westman (1968) used the ob/ob mouse in the fasted state (12 h), Herberg et al (1970) and Garthwaite et al (1980) studied the obese mice in the fed state.

For all the studies reported in this thesis on the ob/ob mouse, the mice were used between 8-12 weeks of age i.e. in the dynamic phase of

obesity. At that age the ob/ob mouse has many metabolic and endocrine abnormalities in common with those of type II diabetes. These similarities include obesity, elevated food intake, altered basal insulin-glucose relationships and insulin resistance (Bray and York, 1979).

I. C. 3. Energy balance and thermoregulation

Obesity represents an imbalance between energy intake and energy expenditure (either via physical activity or heat loss). A number of investigators have reported an increase in food intake by ob/ob mice (Bailey et al, 1975; Bray and York, 1971; Fuller and Jacoby, 1955; Lin et al, 1979; Mayer et al, 1951). The hyperphagia alone cannot be entirely responsible for the excess energy deposition in ob/ob mice since neither energy restriction, exercise, nor the maintenance of a normal body weight through a combination of these two treatments, will prevent adiposity (Chlouverakis, 1972; Coleman, 1982; Dubuc, 1976a; Hollifield and Parson, 1958). All reports agree, however, that there is a spontaneous reduction in physical activity of ob/ob mice both during the dynamic postweaning phase of the obesity and during the more static adult phase (Clark and Gay, 1972; Joosten and van der Kroon, 1976; Yen and Acton, 1972).

Energy utilization (measured by oxygen consumption and expressed on the basis of surface-area) is decreased in adult ob/ob mice (Mayer et al, 1953b; McClintock and Lifson, 1957) and has been linked with an impairment in cold-induced thermogenesis (Davis and Mayer, 1954; Trayhurn and James, 1978). In small mammals, the main thermogenic organ is the brown adipose tissue (BAT) (Foster and Frydman, 1978, 1979; Thurlby and Trayhurn, 1980). BAT mitochondria have been shown to possess a unique proton conductance pathway that uncouples mitochondrial oxidation from phosphorylation resulting in heat production (Nicholls, 1979). In obese mice, defects in the BAT mitochondria (Himms-Hagen and Desautels, 1978; Hogan and Himms-

Hagen, 1980) and in the activation of BAT metabolism by the sympathetic nervous system (Knehans and Romsos, 1982; Seydoux et al, 1982) have been suggested to be involved in the impaired thermogenesis.

The defect in thermogenesis is, however, only partial. Under appropriate experimental conditions, obese mice can adapt to cold (Carlisle and Dubuc, 1982; Coleman, 1982; Hogan and Himms-Hagen, 1980; Knehans and Romsos, 1982; Seydoux et al, 1982; Trayhurn and James, 1978). Coleman (1982) recently suggested that a failure of thermogenesis in the mutant mice could not be solely responsible for their increased metabolic efficiency. He proposed that hyperinsulinemia, by increasing anabolic processes and decreasing degradation would spare energy normally used for tissue turnover and account for some of the increased metabolic efficiency.

I. C. 4. Lipid metabolism

In obese mice, the proportion of body fat to lean is higher when compared with lean controls and increases with age. Elevated fat content may account for more than 90% of the increased weight of obese mice (Bates et al, 1955). Enhanced fatty acid synthesis in the liver and adipose tissue has been demonstrated in both in vivo and in vitro experiments with a number of different isotopes (Assimacopoulos et al, 1974; Assimacopoulos-Jeannet and Jeanrenaud, 1976; Bégin-Heick and Heick, 1976; Elliot et al, 1976; Hems et al, 1975; Loten et al, 1974). This phenomenon has been related to the increased activity of all lipogenic enzymes (such as acetyl-CoA-carboxylase and fatty acid synthetase) in the adipose tissue and liver (Assimacopoulos-Jeannet and Jeanrenaud, 1976; Bray and York, 1971; Fried and Antapol, 1966; Martin et al, 1973; Salmon and Hems, 1973). The increase in hepatic fatty acid synthesis and esterification (due in part to an augmentation in glycerokinase activity) along with a decrease in oxidation of fatty acids to ketone bodies result in an intracellular accumulation of

lipids and an augmentation of the secretion of triglycerides as very low density lipoprotein (Assimacopoulos-Jeannet and Jeanrenaud, 1976). Despite this increased secretion, plasma triacylglycerol levels are not markedly increased in ob/ob mice (Salmon and Hems, 1973) since there is a compensatory increase in lipoprotein lipase activity in adipose tissue, heart and skeletal muscles (De Gasquet and Pequignot, 1972; Enser, 1972; Rath et al, 1974). The elevated levels of glycerokinase in adipose tissue of ob/ob mice (Koschinsky et al, 1971) along with the decreased response of that tissue to lipolytic agents (see Bégin-Heick, 1982 for review) further contribute to the elevated triglyceride deposition in the fat cells.

It has been suggested that the hyperlipogenesis of ob/ob mice could result from their hyperinsulinemia (Assimacopoulos-Jeannet and Jeanrenaud, 1976; Bray and York, 1979; Loten et al, 1974). Treatment of obese mice with either streptozotocin or anti-insulin serum decreases both hepatic and adipose tissue lipogenesis to near normal values (Loten et al, 1974, 1976) and increases ketone body production to values found in lean mice (Jeanrenaud et al, 1975). However, whether the plasma insulin was decreased by anti-insulin serum or streptozotocin (Loten et al, 1974, 1976) or by fasting (Bates et al, 1955), the percentage of fat remained approximately the same in treated and control obese mice. Thus the increased hepatic and adipose tissue lipogenesis cannot be due entirely to a secondary effect of the hyperinsulinemia (Bégin-Heick, 1982).

I. C. 5. Pathogenesis of the obese-hyperglycemic syndrome

I. C. 5a. The pancreas: morphology and insulin secretion

The following literature review on the endocrine pancreas and insulin secretion in the obese mouse will be restricted to reports on the C57Bl/6J Jackson Laboratory obese mouse as the islets of the obese mouse from the Swedish colony, used in the studies of Hellerstrom and Hellman (1964) and

from the Birmingham strain , used by Findlay et al (1973), have been shown to be markedly different from the islets of the C57Bl/6J ob/ob mouse.

The presence of a pancreatic abnormality in the obese mouse was first recognized in morphological studies. Light-microscopic examination revealed that the obese mouse pancreas is characterized by marked hypertrophy and hyperplasia of the islets of Langerhans (Bleisch et al, 1952; Gepts et al, 1960; Like, 1977; Wrenshall et al, 1955). Two month old obese mice are reported to have about six times as much islet tissue as nonobese mice, with the larger proportion of islet tissue being contributed by the large islets (Gepts et al, 1960). While hyperplasia of both A and B cells has been described in the pancreas of obese mice (Gepts et al, 1960), the greatest increase occurs in the number of B-cells (Baetens et al, 1976; Gepts et al, 1960).

In young obese mice (4-16 weeks of age), it was observed that the beta cells were degranulated, hypertrophied and contained numerous mitotic figures (Bleisch et al, 1952; Gepts et al, 1960; Like, 1977; Wrenshall et al, 1955). Hyalinization, fibrosis, hydropic degeneration or necrosis were not observed (Bleisch et al, 1952). In contrast, the B-cells of old ob/ob mice (more than 16 weeks of age), in which serum glucose and insulin levels were decreasing , showed B-cell regranulation (Herberg et al, 1970; Like, 1977). Measurement of pancreatic insulin confirmed these observations: pancreatic insulin content was found to be lower in young obese mice but higher in older mice when compared to age-matched lean control mice (Begin-Heick et al, 1979; Like, 1977; Mahler and Szabo, 1971; Patel et al, 1976; Stauffacher et al; 1967). Ultrastructural studies have confirmed the light-microscopic findings. Hence, B-cells of ob/ob mice with hyperglycemia and increased plasma insulin levels revealed secretory degranulation with increased rough endoplasmic reticulum and enlarged Golgi structures (Like,

1977).

As expected on the basis of morphological findings, insulin secretion in the ob/ob mouse differs markedly from that of the lean mice. In vivo studies revealed that obese mice, when administered a glucose load intraperitoneally (I.P.) (Begin-Heick et al, 1974; Cameron et al, 1972b; Dubuc, 1976b; Herberg et al, 1970), intravenously (I.V.) (Begin-Heick et al, 1979; Cameron et al, 1972b; Genuth et al, 1971) or orally (Begin-Heick et al, 1979; Genuth et al, 1971; Kreutner et al, 1975), show a much greater absolute and percent increases in plasma glucose, immediately after the glucose load, and a severe and sustained hyperglycemia during the remainder of the test, when compared to lean control mice. The glucose intolerance of obese mice has been shown to reflect not only a deficiency in their ability to dispose rapidly of added glucose, but also an abnormally large glycemic response to the control procedures (anesthesia, blood sampling, administration of saline instead of glucose) (Dubuc, 1976b; Genuth et al, 1971). When looking at corresponding plasma insulin levels during glucose tolerance tests, some researchers have reported a marked and persistent elevation of plasma insulin in response to glucose; no delay in the insulin secretory response was observed and the values of plasma insulin were much greater than those seen in the lean control mice (Begin-Heick et al, 1979; Cameron et al, 1972b; Dubuc, 1976b). On the other hand, a lack of increase of plasma insulin levels, from an already elevated basal plasma insulin value, was also reported after an I.V. glucose load (Genuth et al, 1971) and an I.P. glucose load (Herberg et al, 1970). The divergent results obtained by the various groups of researchers on the plasma insulin values cannot be readily explained on the basis of anesthesia versus no anesthesia, fed vs fasting state, or timing of blood sampling. Under all circumstances, however, the concentration of insulin in the plasma of the

obese mice was high in comparison with that in lean mice (Begin-Heick et al, 1979; Cameron et al, 1972b; Dubuc, 1976b; Genuth et al, 1971; Herberg et al, 1970).

Investigations have been done on insulin secretion *in vitro* using isolated pancreata or islet preparations. In 5 to 6 week old obese mice, insulin output by pancreatic pieces, in response to glucose or glucose and theophylline, was shown to be increased out of proportion to pancreatic insulin content when compared to lean control mice (Malaisse et al, 1968a). The increased responsiveness of the secretory process to the stimulant action of glucose was also demonstrated using collagenase-isolated islets studied in static tube incubation (Lavine et al, 1977; Loreti et al, 1974) and perifusion set-ups (Loreti et al, 1974; Rabinovitch et al, 1975).

Lavine et al (1977) reported that obese mouse islets release significant amounts of insulin at glucose concentrations that are nonstimulatory for islets of lean control mice. The heightened response to glucose of the islet insulin-secretory process could not be abolished by food deprivation (48 h to 7 days) or by chronic food restriction for a period of 5 weeks (Lavine et al, 1977). These findings confirmed the original observations of Mayer et al (1953b) that the altered islet composition could not be remedied by food restriction. Rabinovitch et al (1975) demonstrated that although the insulin response to 16.8 mM glucose in obese mouse islets was exaggerated when compared to islets from lean mice, it still showed the normal biphasic pattern.

The pancreas of the C57Bl/6J ob/ob mouse has also been shown to respond in a unique manner to agents, other than glucose, known to affect the islet function. Mannoheptulose, an inhibitor of insulin secretion, appeared to be ineffective in suppressing the insulin release (Hoshi and Shreeve, 1969). Caffeine, an insulin secretagogue, produced a significant

degranulation of B-cells in ob/ob mice but had no effect in lean mice (Kuftinec and Mayer, 1964).

I. C. 5b. Hyperinsulinemia and insulin resistance

In obese mice, plasma insulin-like activity and plasma immunoreactive insulin (IRI) are increased compared with the lean controls, both in the fed and the fasted states (Christophe et al, 1959; Stauffacher et al, 1967). Plasma IRI concentrations are about 8 times the normal value in young obese mice (5-6 week old) and increase to about 50 times the normal value in 6 month old obese mice (Herberg et al, 1970; Garthwaite et al, 1980; Genuth, 1969). Plasma insulin concentrations in obese mice markedly decrease with fasting (Begin-Heick, 1982; Christophe et al, 1959; Cuendet et al, 1973; Stauffacher et al, 1967) but approach normal values only after prolonged starvation (2 weeks) (Begin-Heick, 1982; Cuendet et al, 1973).

The high plasma insulin levels in obese mice might suggest decreased insulin degradation. However, calculations made from the rate of disappearance of radioactively labeled insulin suggest that the turnover of insulin is enhanced in ob/ob mice (Bray and York, 1979; Coore and Westman, 1970; Genuth, 1972).

The hypersecretion of insulin by the obese mouse is not a compensatory mechanism due to the production of an ineffective insulin since the excess hormone has been shown to be structurally normal (Genuth, 1969; Stauffacher et al, 1967): both pancreatic and plasma insulin of ob/ob and age matched normal mice are comparable with respect to the ratio of their biological potency and immunoreactivity.

In the obese mouse, hyperinsulinemia is accompanied by hyperglycemia, with blood glucose levels rising above 400 mg/dl in animals fed ad libitum (Westman, 1968). The presence of hyperinsulinemia, together with hyperglycemia, suggests that the obese mouse is insensitive to the

hypoglycemic effect of insulin. This has been demonstrated by the lack of response of the gluconeogenic and glycogenic pathway to insulin both in vivo (Kreutner et al, 1975) and in vitro (Seidman et al, 1970) and furthermore from results of glucose tolerance tests and from investigation of insulin sensitivity of muscle and adipose tissue (Bray and York, 1979; Assimacopoulos-Jeannet and Jeanrenaud, 1976). Stauffacher and Renold (1969) observed that, in the obese mouse, the muscle is more resistant than the adipose tissue. During the dynamic phase of obesity, the lipid metabolism of the liver remains sensitive to insulin stimulation (Assimacopoulos-Jeannet and Jeanrenaud, 1976; Loten et al, 1974), however, gluconeogenis and ketone body production loose their sensitivity to insulin suppression (Kreutner et al, 1975).

The basis of the insulin resistance in ob/ob mice is complex (Bray and York, 1979; Assimacopoulos-Jeannet and Jeanrenaud, 1976). The initial step in the cellular action of insulin involves binding to specific membrane receptors. Once insulin is bound to the receptors, the insulin-receptor complex becomes effective in modulating a cascade of processes beyond the cell surface that ultimately result in the hormonal effects (Freychet, 1976). Decreased binding of insulin in ob/ob mice has been demonstrated in membranes of all target tissues of insulin i.e. adipose tissue (Freychet et al, 1972), liver (Bégin-Heick et al, 1974; Kahn et al, 1973), and muscle (Le Marchand-Brustel et al, 1978). The diminished binding has been attributed to the loss of receptor sites rather than to any change in the affinity of the receptors (Freychet et al, 1972; Kahn et al, 1973; Le Marchand-Brustel et al, 1978).

The generalized decrease of insulin-receptor concentration in tissues from ob/ob mice accounts only partly for the observed decrease in insulin sensitivity of these animals. It was demonstrated that tissues from ob/ob

mice also show a decrease in responsiveness (Assimacopoulos-Jeannet and Jeanrenaud, 1976; Le Marchand-Brustel et al, 1978). It was thus hypothesized that the insulin resistance of the ob/ob mouse could be partly related to intracellular alterations that modify the normal message of the hormone at steps beyond the insulin-receptor interaction (Assimacopoulos-Jeannet and Jeanrenaud, 1976).

The insulin resistance of the ob/ob mouse is thought to be secondary to the hyperinsulinemia and obesity. The elevated plasma insulin levels of the ob/ob mouse were shown to be decreased by the following treatments: long-term fasting or caloric restriction (Chlouverakis, 1972; Le Marchand et al, 1977), alloxan (Mahler and Szabo, 1971; Solomon et al, 1974), streptozotocin (Batchelor et al, 1975; Le Marchand et al, 1977) and oxytetracycline treatment (Bégin-Heick and Heick, 1976; Bégin-Heick et al, 1974). However, fasting did not normalize the insulin-stimulated uptake of glucose by soleus muscle of ob/ob mice (Le Marchand-Brustel et al, 1978) nor did it improve the stimulatory effect of insulin on adipose tissue or liver metabolism (Le Marchand et al, 1977). Whereas alloxan treatment was shown to increase the insulin sensitivity of the diaphragm muscle of the ob/ob mouse (Mahler and Szabo, 1971), streptozotocin did not result in any increase in responsiveness of muscle or adipose tissue (Batchelor et al, 1975). Oxytetracycline, however, resulted in an increase in the insulin sensitivity of the diaphragm and in an increase in the binding of insulin to liver membranes (Bégin-Heick and Heick, 1976; Bégin-Heick et al, 1974).

I. C. 5c. Possible causes of hyperinsulinemia

As stated earlier, hyperinsulinemia develops from 15 days of age before any sign of hyperphagia (Dubuc, 1976b; Godbole et al, 1980). Moreover, food restriction (Dubuc, 1976a) of the obese mouse does not normalize plasma insulin levels. So the high rate of insulin secretion in

ob/ob mice is probably not due to hyperphagia. A primary decrease in peripheral insulin sensitivity is also probably not the reason for the hyperinsulinemia since increased insulin secretion develops before the insulin resistance.

Mayer et al suggested in 1953 that the high insulin secretion in obese mice could be secondary to an increased glucagon secretion. Plasma glucagon has been reported as normal (Cuendet et al, 1975) or slightly increased (Dubuc et al, 1977; Lavine et al 1975). Pancreatic glucagon content has also been found to be increased (Cuendet et al, 1975; Patel et al, 1976). Gepts and co-workers (1960) reported an increase in the number of A-cells in the obese mouse pancreas while Baetens et al (1976) reported a slight decrease of both somatostatin- and glucagon-containing cells. So, although an involvement of glucagon has been suggested in the pathogenesis of the obese-hyperglycemic syndrome, a specific disturbance of glucagon regulation could not account for the persistence of hyperinsulinemia and hyperglycemia.

Patel et al (1976) tried to link the hyperinsulinemia of the ob/ob mouse with their finding of a decreased pancreatic somatostatin concentration in 11-12 week old ob/ob mice. Somatostatin is known to have a potent inhibitory effect on insulin and glucagon secretion in human subjects and in animals (Reichlin et al, 1976). However, the hypothesis of Patel and co-workers (1976) is unlikely since it was shown later on that the total pancreatic content of somatostatin was increased in 2 month and 6 month old mice when compared to age-matched lean controls (Dolais-Kitabgi et al, 1979).

The other islet peptide studied in the ob/ob mouse is pancreatic polypeptide (PP). It was found by radioimmunoassay and immunocytochemical techniques that the pancreas of 5 month old ob/ob mice had a significantly

greater level of PP than control glands (Gingerich et al, 1978). The physiological importance of this observation has not been determined yet. A clear-cut delineation of the relationship between the four pancreatic polypeptides in the production of hyperinsulinemia in the ob/ob mouse cannot be obtained from the various reports published to date.

Hyperinsulinemia may arise in the ob/ob mouse from a progressive failure of the autoregulation of insulin secretion by insulin, as suggested by Loreti et al (1974). When rat insulin was added to the incubation medium, the secretory activity of the lean mouse islets was almost completely suppressed. The concentration of exogeneous insulin required to inhibit insulin secretion in the islets of obese mice was much higher than in the tissue of lean mice and increased with the age of the animals (Loreti et al, 1974).

The underlying mechanism of the insulin hypersecretion remains unclear. Assimacopoulos-Jeannet and Jeanrenaud (1976) proposed that hypothalamic disorders of ob/ob mouse could, via neural, humoral or other distorted control mechanisms, affect the normal regulation of the endocrine pancreas and alter the functional relationship between the A, B and D-cells. A primary etiology for the hypersecretion of insulin in ob/ob mice has also been proposed to reside in the endocrine pancreas per se. The original findings by Strautz (1970) that implantation of islets isolated from lean mice into obese mice could ameliorate the obese-hyperglycemic syndrome were taken to suggest that the absence of an islet factor is responsible for the development of the syndrome. However, more recently, Andersson et al (1981), using a different islet transplantation technique failed to confirm such a conclusion. It is noteworthy to mention that both Strautz (1970) and Andersson et al (1981) used obese mice at an age where both hyperglycemia and insulin resistance were already present. It was

thus unlikely, as discusssed by Andersson et al (1981) that one could cure the obese mouse by augmenting its capacity to produce insulin by islet transplantation since the ob/ob mice were already hyperinsulinemic.

I. C. 5d. Pituitary-thyroid system

Some degree of thyroid malfunction in ob/ob mice would be consistent with their low metabolic rate, decreased body temperature (Joosten and van der Kroon, 1974b; Ohtake et al, 1977), susceptibility to cold and hypoactivity (Joosten and van der Kroon, 1976). While earlier reports claimed that the uptake of [^{131}I] into the thyroid of ob/ob mice was normal (Goldberg and Mayer, 1952; Wykes et al, 1958), subsequent reports claimed a subnormal uptake (Joosten and van der Kroon, 1974b; Ohtake et al, 1977; York et al, 1978b). However, no abnormalities either in thyroid weight or histology have been detected in ob/ob mice (Goldberg and Mayer, 1952; Joosten and van der Kroon, 1974b; Wykes et al, 1958). Direct measurements of plasma thyroidal hormones (T_3 and T_4) by radioimmunoassay have shown normal or slightly increased concentrations (Herberg and Coleman, 1977; Ohtake et al, 1977; York et al, 1978b). Serum thyroid-stimulating hormone (TSH) is also normal and, increases in a manner similar to that seen in lean mice, after exposure to 4 °C (Ohtake et al, 1977). The hypothalamic concentration of thyrotropin-releasing hormone (TRH) is the same in ob/ob and lean mice (Nemeroff et al, 1978). These data, together with the demonstration that thyroid metabolism in the ob/ob mouse responds to injection of TSH and to inhibitory feedback from exogeneous triiodothyronine (Ohtake et al, 1977), suggest that the thyroid-pituitary axis of the ob/ob mouse is essentially normal. The hypometabolism and impaired thermogenesis of the ob/ob mouse is thus suggestive of some impairment in tissue response to thyroid hormone (Ohtake et al, 1977; York et al, 1978a, 1978b), which is borne out by the observation that prolonged

treatment with thyroid hormone increases heat production and reduces the obesity (Joosten and van der Kroon, 1974b; Mayer et al, 1952; Ohtake et al, 1977; van der Kroon et al, 1981; York et al, 1978a, 1978b).

Edelman and Ismail-Beigi (Edelman and Ismail-Beigi, 1974; Ismail-Beigi and Edelman, 1971) have presented data to suggest that much of the calorigenic response to thyroid hormones may result from the stimulation of the Na^+-K^+-ATPase activity in a number of tissues. The Na^+-K^+-ATPase concentration has been reported to be decreased in skeletal muscle (Lin et al, 1978), liver and kidney (York et al, 1978a) of ob/ob mice. It was thus suggested (Bray and York, 1979) that a defect in the Na^+ pump could explain the impaired thermogenesis of ob/ob mice (Joosten and van der Kroon, 1974b; Ohtake et al, 1977; Trayhurn et al, 1977) and could result in the observed increase in caloric efficiency because less energy was expanded on the maintenance of body temperature and of transmembrane ionic balance.

I. C. 5e. Role of adrenals

The adrenals of adult ob/ob mice are hypertrophied (Hellerstrom et al, 1962; Marshall et al, 1957). The hypertrophy is absent or minimal in young animals but increases with age. The increased adrenal weight results from a distinct hyperplasia of the cortex (Hellerstrom et al, 1962). There is also a direct relationship between body weight and adrenal cortex volume (Hellerstrom et al, 1962). The initial elevation in serum corticosterone is observed around day 17 (Dubuc, 1977) and increases with age (Garthwaite et al, 1980). The adrenal hyperactivity presumably results from excessive ACTH secretion (Edwardson and Hough, 1975) but it may also reflect an increased adrenal sensitivity to ACTH (Carstensen et al, 1961) as food-restriction of the obese mouse was able to normalize pituitary ACTH content (Edwardson and Hough, 1975) but did not result in any significant decrease in serum corticosterone levels (Dubuc, 1976a). The factors responsible for

the elevated pituitary content of immunoreactive ACTH (Edwardson and Hough, 1975) remain undetermined. Similar pituitary responses to crude corticotropin-releasing factor (CRF) as well as similar hypothalamic CRF activity have been found in obese and lean mice (Edwardson and Hough, 1975).

The hyperadrenocorticism of the ob/ob mouse is thought not to represent a primary genetic defect but to be secondary to the diabetes, hyperinsulinemia and insulin resistance (Bray and York, 1979). It would appear to aggravate the ob/ob syndrome. Adrenalectomy lowers blood glucose, restores insulin sensitivity and slows further weight gain in 2 month old obese mice (Solomon and Mayer, 1973; Yukimura and Bray, 1978). These effects may be related to the reduced food intake in adrenalectomized ob/ob mice (Yukimura and Bray, 1978).

I. C. 5f. The brain and hypothalamus

There are considerable data available to suggest that the genetic defect of obese mice may be expressed as abnormal hypothalamic function. The hyperphagia (Mayer, 1953a), decreased activity (Mayer, 1953b), hypogonadism (Batt et al, 1982; Ingalls et al, 1950; Swerdloff et al, 1976, 1978), increased energy efficiency (Bray and York, 1979), and hypothermia (Trayhurn et al, 1977) have been partly ascribed to altered functions of the hypothalamic area. Investigations on hyperphagia in obese mice suggest defects in satiety mechanisms. The ob/ob and lean mice differ in their response to manipulations of the palatibility of their diet. Whereas normal mice are able to compensate for the dilution of their food with cellulose, so that they continue to gain weight at a normal rate, obese mice do not increase their food intake sufficiently and their body weight levels off (Parson et al, 1954).

The hypothesis that defect(s) in hypothalamic regulation of food intake might be present in the ob/ob mouse also gains support from parabiosis experiments. When normal mice were parabiosed with ob/ob mice, obese mice ate less and gained weight less rapidly than control obese mice (Coleman, 1973). It was concluded by Coleman (1973) that the obese mouse was unable to produce sufficient "satiety factor" to regulate its food consumption. Candidates for the putative satiety factor include pancreatic polypeptide (Malaisse-Lagae et al, 1977) and cholecystokinin (Strauss and Yalow, 1979) among others but there are no convincing data as yet.

Abnormalities in the structure of the central nervous system have been described in obese mice (Bereiter and Jeanrenaud, 1979; van der Kroon and Speijers, 1979). Brain weight (Bereiter and Jeanrenaud, 1979; Garthwaite et al, 1980) and pituitary protein (Garthwaite et al, 1980) were reported to be lower in obese mice than in lean age-matched controls. The cross-sectional area of neurons in most parts of the brain of obese mice were reported to be significantly smaller than in lean mice (Bereiter and Jeanrenaud, 1979). Alterations in hypothalamic organization of obese mice have also been reported (Bereiter and Jeanrenaud, 1979). It was suggested that the generalized reduction in volume of neurons may be the anatomic basis for the hypothalamic defects (Bray and York, 1979).

The concentration of norepinephrine (NE), but not of dopamine, was reported to be increased in the hypothalamus, telencephalon, and brainstem of both ad libitum-fed (Lorden et al, 1975) and food-restricted (Oltmans et al, 1976) young ob/ob mice. No significant difference in NE turnover was observed, however, between the lean and the obese animals (Lorden et al, 1976). Using older animals (8 months old), Nemeroff et al (1978) could find no difference in hypothalamic levels of NE or dopamine. By 8 months

of age, the ob/ob mouse is no longer rapidly gaining weight, suggesting that the findings in the younger animals may be more important.

I. C. 5g. Growth hormone

Although growth hormone was suggested to be involved in the pathogenesis of the syndrome (Hellerstrom et al, 1970), this could not be confirmed by measurements of immunoreactive growth hormone levels, which were found to be identical in obese and lean mice (Roos et al, 1974). It is clear from the demonstration that the obese-hyperglycemic syndrome can develop in homozygous obese mice that are also homozygous for the dwarf gene and thus have no circulating growth hormone (Joosten et al, 1975), that the development of the obesity and insulin resistance is not dependent on growth hormone.

I. C. 5e. Reproductive system

The sterility of both male and female obese mice has been recognized since the appearance of the mutation (Ingalls et al, 1950). Earlier studies on the histological and anatomical appearance of the gonads suggested that a lack of gonadotropin stimulation could be the basis of the reproductive defects in ob/ob mice (Bray and York, 1971, 1979). This hypothesis has been investigated by Swerdloff et al (1976, 1978) and more recently by Batt et al (1982). Pituitary concentration of LH was shown to be normal, but that of FSH raised. Serum concentrations of LH and FSH were shown to be normal and low respectively, both in male (Batt et al, 1982; Swerdloff et al, 1976) and female (Batt et al, 1982) obese mice. These findings suggest impaired FSH release in both sexes.

The content and concentration of LH-RH in hypothalami of young adults of both sexes was found to be significantly higher in obese animals compared to their lean littermates and this LH-RH was biologically active (Batt et al, 1982). It was suggested that the impaired release of FSH in

the obese mouse could be caused by an inadequacy in the mechanism of release of LH-RH (Batt et al, 1982) probably associated with the animal's extreme sensitivity towards the negative feedback effect of gonadal steroids (Swerdloff et al, 1976). An insensitivity of the pituitary gland towards LH-RH (in its release of FSH) could not be excluded either (Batt et al, 1082).

The sterility of the obese mouse is thus a secondary aspect of the syndrome. The gonadal anomalies can be attributed to changes in pituitary function that are secondary to the obesity and/or to a defect or defects in the hypothalamus.

I.D. The Pancreatic Islets

In mammals, the islets of Langerhans are distributed throughout the exocrine pancreas, making up only 1 to 2% of the total volume of the pancreatic tissue. The efficiency with which the pancreatic islet organ maintains the blood sugar homeostasis is determined both by the total islet mass and the hormonal release of individual islet cells in response to changes in the blood sugar concentration. The islet organ is composed of endocrine cells, blood vessels, nervous elements and connective tissue (Boquist, 1977).

New formation of islet cells by mitotic division occurs in embryos and neonates whereas the mitotic activity is low in most adult species (Boquist, 1972). It was reported that 5-20 B-cells/1,000 B-cells may divide during 24 h in adult mice or rats. At this rate the doubling time would be 40-200 days (Hellerstrom, 1977; Hellman, 1959b; Logothetopoulos, 1972). However, mitotic activity may be observed readily among the islet endocrine cells in the adult period under pathological and experimental conditions. An example is the considerable proliferative capacity of the islet cells of the ob/ob mouse leading to B-cell hyperplasia (Bleisch et al, 1952; Gepts

et al, 1960; Like, 1977; Wrenshall et al, 1955).

I.D. 1. Vascular supply and innervation of the islets

The islets are markedly vascularized and possess a tortuous network of anastomosing capillaries that makes them ideally suited for a rapid delivery of their hormonal secretion into the bloodstream and also a prompt uptake of nutrients from the blood (Munger, 1977).

Pancreatic islets of all animals studied to date are well innervated with components of the autonomic nervous system (Munger, 1977). Stimulation of the parasympathetic nervous system (cholinergic) causes insulin secretion whereas stimulation of the sympathetic system (adrenergic) inhibits insulin secretion; the opposite responses are found for glucagon (Malaisse et al, 1967b).

I.D. 2. Endocrine cells

Histochemical, biochemical and immunofluorescence techniques have led to the distinction of several endocrine cell types, based mainly upon the structural appearance of their secretory granules.

It had been generally assumed, some years ago, that the four endocrine cell types (A,B,D and PP cells) were present throughout all the islets in the pancreas of most mammalian species, with the B-cells being the most numerous. Application of immunofluorescence techniques to more numerous samples revealed two populations of islets having distinct cellular content and topographical distribution (Orci et al, 1976). Islets from the body and tail of the pancreas (sometimes also refered to as dorsal or splenic part of the pancreas) were found to have a rim of glucagon-containing cells but few or no PP-containing cells. On the other hand, islets from the lower part of the head of the pancreas (also called ventral or duodenal pancreatic region) showed a well defined rim of PP-containing cells but very few or no glucagon-containing cells. Such preferential

location of PP-cells in the head of the pancreas was also found in the mouse (Orci et al, 1976). No difference in the distribution of B-cells or D-cells was found (Orci et al, 1976).

The differences in the hormones stored by the two kinds of rat islets have also been correlated with differences in secretion characteristics: dorsal islets were found to secrete more insulin and more glucagon in the presence of 16.7 mM glucose than did the ventral islets (Trimble and Renold, 1981).

I.D. 2a. The B-cell

The B-cell has long been known to manufacture insulin and is by far the best defined of the islet cell types (Hellerstrom, 1977). B-cells are characteristically stained by aldehyde fuchsin, hematoxyline, pseudoisocyanine, and toluidine blue O (Boquist, 1977). They constitute 60 to 80% of the normal mammalian pancreas (Hellman, 1959a) but may account for over 90% in some conditions (e.g. the ob/ob syndrome) (Hellerstrom, 1977). For this reason, the growth pattern of the pancreatic islets is to a large extent determined by the proliferation and loss of B-cells. The fine structural appearance of the B-cells varies somewhat in different species (Boquist, 1977). The main distinguishing structural feature of the B-cells is the secretory granule, which shows considerable species variation in morphological appearance. In rodent species, the secretory granules are characteristically rounded, whereas they are rectangular, square, hexagonal and irregular or rounded in humans (Like, 1967). The structural variations may be due to differences in the chemical or physical form of insulin (Like, 1967). Between the numerous secretory granules, the cytoplasm of B-cells contains the usual complement of organelles, including the Golgi apparatus, rough and smooth endoplasmic reticulum, scattered mitochondria, microtubules and cytoplasmic microfilaments.

I.D. 2b. The A-cell

The A cell is the source of pancreatic glucagon and these cells comprise 10 to 30% of the mammalian endocrine pancreas (Hellerstrom, 1977). In the rat, mouse and hamster the A-cells are typically located on the islet periphery whereas in man, they occur both on the islet periphery and dispersed among the other islet cell types (Hellerstrom, 1977).

I.D. 2c. The D-cell

The D-cell contains somatostatin, a tetradecapeptide. The occurence in these cells of a material cross-reacting with antibodies toward gastrin has been claimed by some authors but has been denied by others (Hellerstrom, 1977). D-cells usually comprise less than 10% of the islets of laboratory animals but seem to be considerably more prevalent in human islets (Hellerstrom, 1977). The D-cells are either diffusely distributed or in most species localized to the periphery of the islets, occasionally interposed between the peripheral A-cells and the central B-cells. In humans and rodents, somatostatin has been shown to exert an inhibitory effect on glucagon and insulin secretion (Reichlin et al, 1976).

I.D. 2d. The PP-cell

The PP-cell is the source of pancreatic polypeptide, a protein composed of 36 amino acid residues in a straight chain. In the islets, the PP-cells are relatively rare but they occur also in the acinar parenchyma. Although their precise biological significance remains to be clarified, there is much evidence to suggest that they are true hormone-producing cells (Hellerstrom, 1977).

I.D. 2e. Other islet cells

The agranular cells or nondifferentiated cells are regarded as precursors of granulated islet cells rather than true endocrine elements (Hellerstrom, 1977).

I.D. 3. Insulin biosynthesis

I.D. 3a. Mechanism of insulin biosynthesis

In 1967, Steiner and Oyer (1967) demonstrated with human insulinoma slices and isolated rat islets that radioactive amino acids were incorporated into a single polypeptide chain, proinsulin (MW 9,000), which was converted into insulin (MW 5,800). The two chains of insulin are synthesized from the amino terminus of the B chain through the so-called connecting peptide (C peptide) to the carboxyl terminus of the A chain as a single polypeptide chain.

More recently, mRNA isolated from islet tissue (Permutt, 1981; Yip et al, 1975) was translated in an mRNA-dependent, cell-free, protein-synthesizing system. The estimated molecular weight of the major protein synthesized was 12,000. This cell-free product has been called preproinsulin; it contains 23-25 amino acids at the amino terminus of proinsulin (Permutt, 1981).

Two different insulins are present in the pancreas of the rat (Clark and Steiner, 1969) and the mouse (Markussen, 1971). The initial translation products, preproinsulins I and II, differ by only three amino acids in the pre-region (Lomedico et al, 1979), two in the B chain (Clark and Steiner, 1969), and two in the C-peptide (Clark and Steiner, 1969). Insulins I and II of the mouse have amino acid composition identical with those of the rat (Markussen, 1971). In rats, insulin I/II was found to be present in a 60:40 ratio while in mice of the C57Bl/6J strain, the ratio varied from 65:35 in the lean mouse to 76:24 in the ob/ob mouse (Kakita et al, 1982). The significance of these observations is still not known.

The first step in the production of insulin is thus the synthesis of preproinsulin and proinsulin in the rough endoplasmic reticulum. From there, the proinsulin is transported by an energy-requiring mechanism to

the Golgi complex, either through direct intracisternal communications or through vesicles pinched off from the endoplasmic reticulum (Steiner et al, 1972; Yip et al, 1975).

The single-chain proinsulin molecule is converted to the double-chain insulin molecule by the enzymatic removal of the connecting C-peptide (Boquist, 1977). The enzymes responsible for the removal of the C-peptide are localized within the Golgi complex, and the conversion of proinsulin to insulin occurs in the Golgi area and continues in the B granules (Steiner et al, 1972). The half-time for the conversion of proinsulin to insulin is approximately 60 min (Steiner et al, 1972).

In the Golgi complex, the newly-formed insulin is packaged into secretory granules, and the individual granules pinch off from the Golgi membranes (Boquist, 1977). In addition to insulin, the secretory granules contain zinc and in most mammalian species insulin is stored as a zinc-insulin complex (Blundell et al, 1972)(see below, Zinc and insulin). The C-peptide from proinsulin is also stored in the islets and secreted in equivalent amounts to insulin (Steiner et al, 1972). The release of the stored hormone occurs through emiocytosis, which is a fusion of the granule membrane with the plasma membrane after which the contents of the granule are delivered extracellularly and dissolved. Before emiocytosis can occur, however, the secretory granules must be transported to the cell membrane, possibly through the interaction of the microtubular-microfilament system of the B-cells (Hedeskov, 1980; Lacy, 1975).

I.D. 3b. Regulation of insulin biosynthesis

Glucose is the major physiological stimulus for insulin biosynthesis and secretion in the B-cells but the mechanism of glucose stimulation of proinsulin synthesis is still uncertain. Studies on the mechanism of glucose stimulation of insulin biosynthesis were begun in 1970. Morris and

Korner (1970) were the first to report a specific effect of glucose on proinsulin synthesis relative to that of total islet protein. Permutt and Kipnis (1972) reported that the early effects of glucose on insulin biosynthesis were independent of new RNA synthesis. It was also found by the latter authors that exposure of islets to 20 mM glucose for 2 h, in the presence of actinomycin D, led to an inhibition of glucose-stimulated insulin biosynthesis. Using a protein-synthesizing wheat germ system to quantitate proinsulin mRNA, Itoh et al (1978) determined that during the first 60 min of exposure of isolated islets to high glucose concentration (36.7 mM), glucose regulates proinsulin synthesis by enhancing the availability of total proinsulin mRNA rather than by increasing the amount of proinsulin mRNA. Those findings were later confirmed by using a proinsulin cDNA hybridization method to quantitate the proinsulin mRNA (Itoh and Okamoto, 1980). Taken together, these data (Itoh and Okamoto, 1980; Itoh et al, 1978; Permutt and Kipnis, 1972) suggest that the early effects of glucose on preexisting mRNA is at a translational level and that there is a delayed effect of glucose on stimulation of new proinsulin mRNA.

In trying to find out how glucose initiates the insulin biosynthesis, the effects of compounds, other than glucose, were studied by a number of investigators. For example, the rates of oxidation of purine ribonucleosides and D-ribose, in mouse and rat islets, was correlated with their effectiveness as inducers of insulin secretion and proinsulin biosynthesis (Jain and Logothetopoulos, 1977, 1978). Mannoheptulose was shown to inhibit the glucose- and mannose-induced biosynthesis and release of insulin by interfering with the metabolism of these sugars (Ashcroft, 1980). Although there is general agreement between the rate of metabolism of various compounds and the rate of insulin biosynthesis and release which they support, it was shown that the threshold for stimulation of insulin

biosynthesis by glucose (Jain et al, 1975; Pipeleers et al, 1973) is marginally lower than that for glucose-stimulated insulin release (Malaisse et al, 1967a). Insulin release starts increasing when the glucose concentration is raised above 4mM (Malaisse et al, 1967a). Incorporation of [^{3}H]-leucine into proinsulin is maximally affected between Ø and 4.5 mM glucose (Jain et al, 1975; Pipeleers et al, 1973). Moreover, the biosynthesis of insulin can be dissociated from its secretion. For example, diazoxide, epinephrine and the absence of calcium ions were shown to inhibit insulin release but to have no effect on insulin biosynthesis (Lin and Haist, 1973).

I.D. 4. Insulin release

I.D. 4a. Kinetics of insulin release

The pancreatic B-cell responds to a rapid and sustained increase of the glucose concentration in the extracellular fluid with a biphasic pattern of insulin release, characterized by a short spike of release, followed by a nadir and a slowly rising second phase (Wollheim and Sharp, 1981). This biphasic response, first observed in the effluent of the perfused pancreas (Curry et al, 1968), is also seen in the portal vein in humans (Lund et al, 1975).

Studies on insulin release in perfused, isolated rat islets showed that the first phase of secretion reaches a maximum rate approximately 5 min after stimulation, and the second phase achieves a maximum level approximately 3Ø min after stimulation with glucose (Lacy et al, 1972). Data obtained by Grodsky (1972) with a perfused rat pancreas system showed that at high glucose concentration, the total amount of insulin released in the first phase is less than 1-2% of pancreatic insulin content while the second phase amounts to approximately 2Ø% of total insulin content.

Several suggestions about the mechanisms underlying the biphasic

nature of the insulin-secretory response have been proposed (cf Wollheim and Sharp, 1981). Grodsky (1972) proposed that insulin may be stored in compartments or pools with differing labilities to stimulating agents. That two-pool heterogeneous threshold model for beta cell response to glucose was rejected by Cerasi et al (1974) but subsequently supported and modified by Guyton et al (1978). It has also been suggested that beta granules aligned along microtubules may be responsible for the first phase of insulin secretion, whereas the late secretory phase might be due to release from granules initially free in the cytoplasm and which attach themselves to microtubules (Lacy, 1970). Another suggestion has been that beta granules located within the microfilamentous cell web were responsible for first-phase secretion and the later phase could correspond to mobilization of secretory granules along the microtubules (Malaisse et al, 1974). To date, however, no adequate direct evidence exists to account for morphological compartmentation within the B-cell. More recently, Wollheim and Sharp (1981) proposed a model for glucose-induced biphasic insulin release in which the rate of insulin release would be driven by changes in the concentration of cytosolic calcium.

Whatever the exact mechanism, a single primary action of glucose on the two phases seems indicated as the dose-response curve for both the first and the second phase of insulin release is the same sigmoidal function of the extracellular glucose concentration (Grodsky, 1972).

I.D. 4b. Factors influencing insulin secretion

It is now well established that a wide variety of agents, such as circulating nutrients (carbohydrates, amino acids, fat derived products), hormones, neurotransmitters and pharmacological substances may directly influence the release of insulin (Hedeskov, 1980; Lambert, 1976). In mammals, D-glucose seems to be the predominant factor controlling the B-

cell function and insulin secretion (Hedeskov, 1980; Lambert, 1976). Agents like glucose which are capable of promoting insulin release are designated initiators or primary stimuli. Agents that are ineffective alone but increase the secretory response to an initiator are referred to as potentiators or secondary stimuli. Examples of initiators and potentiators of insulin release are listed in Table 1.

The regulation of a single process of release by such a variety of factors implies that the B-cell is equipped with a number of distinct sensor systems able to identify each regulatory factor. In a few instances, it was proposed that the sensor system can be equated with receptor sites located at the plasma membrane e.g. the binding of glucagon to its receptor. However, the sensor system(s) involved in the recognition of nutrient secretagogues such as D-glucose of L-leucine, by the B-cell, have been less easy to define. For several years, two contrasting theories were considered (Ashcroft, 1980; Hedeskov, 1980; Malaisse et al, 1979a, 1979b). The first, often called the regulatory site hypothesis, postulated that nutrients such as glucose initiate the release of insulin by activation of a specific receptor, presumably a protein located in the B-cell membrane, to which glucose and other nutrients directly bind, thereby eliciting an undefined series of events leading to insulin release. The second theory, or the substrate site hypothesis, postulated that, in order to stimulate insulin release, glucose (or other nutrients) had to be metabolized in the islet cells, the secretory response being dependent on the availability of a metabolite or co-factor generated by the metabolism of glucose. This second or metabolic hypothesis was first described by Randle et al (1968) and has gained considerable support from a number of observations indicating that the secretory response to nutrients depends highly on their metabolism in the islet cells (Ashcroft, 1980; Malaisse et al, 1979a,

Table 1. Examples of initiators and potentiators of insulin release.

[adapted from Ashcroft, 1980; Hedeskov, 1980; Lambert, 1976].

Initiators	Potentiators
D-glucose	Fructose
Mannose	Pyruvate
N-acetylglucosamine	Lactate
Glyceraldehyde	Gastrointestinal hormones,
Dihydroxyacetone	(e.g. gastric inhibitory polypeptide
Inosine	secretin, gastrin)
Ribose (depends on species)	Glucagon
L-leucine	Methylxanthines
Arginine	Prostaglandins (in some cases)
Alpha-ketoisocaproic acid	cAMP

1979b). For instance, findings on the anomeric specificity of glucose metabolism, on the stimulatory effect of glycogenolysis on insulin release in glycogen-loaded islets, and on the stimulatory action of compounds some of which are related to glucose (glyceraldehyde, dihydroxyacetone, inosine) and some not (alpha-ketoisocaproate), all support the substrate-site hypothesis (Ashcroft, 1980; Hedeskov, 1980; Malaisse et al, 1979b).

In support of the substrate-site hypothesis, Malaisse and co-workers (1979b) proposed a model in which glucose or any other nutrient secretagogue would, via its metabolism, lead to an increased generation of H^+ ions which in turn could affect the ionophoretic transport of K^+ and Ca^{2+} across membranes in the B-cell. Another consequence of the metabolism of nutrients would be an increase in the concentration of reduced pyridine nucleotides which have been said to affect the affinity of the ionophoretic system for Ca^{2+} (Ashcroft, 1980; Malaisse et al, 1979b). The remodeling of cationic fluxes would thus lead to the accumulation of Ca^{2+} at a critical site of the B-cell. According to Malaisse et al (1979b), the process of glucose-induced insulin release can be viewed as a sequence of three major events: the recognition of glucose by the pancreatic B-cell, a subsequent remodeling of ionic fluxes (content, influx and efflux of Na^+, K^+, H^+ and Ca^{2+}), and the eventual activation of the effector system (microtubules/microfilaments) controlling the exocytosis of secretory granules.

More recently the stimulation of insulin release by the nonmetabolized analog of L-leucine (2-aminobicyclo-[2,2,1]heptane-2-carboxylic acid) was also reconciliated with the substrate-site hypothesis (Sener and Malaisse, 1980). It was found that the secretory response to that amino acid analog was primarily attributable to an activation of glutamate dehydrogenase, resulting in a stimulation of the oxidative deamination of endogeneous L-

glutamate and thus to an increase in catabolic fluxes (Sener and Malaisse, 1980). The latter finding, along with other data pointing to the fact that certain nutrient secretagogues (such as D-glucose and L-leucine) can act in the B-cell both as substrates and enzyme activators (the enzyme being considered the nutrient receptor), permitted the reconciliation of the substrate-site and the regulatory site hypotheses for insulin release (Malaisse et al, 1981).

I.D. 4c. Glucose metabolism and insulin release

Glucose is the major physiological initiator of insulin release. The transport of glucose through the B-cell membrane is a carrier-mediated process with a capacity equal to the highest detected in cell membranes (Hedeskov, 1980). The rate-limiting step in glucose metabolism is the phosphorylation of glucose to glucose-6-phosphate (Ashcroft, 1980; Hedeskov, 1980).

The major pathway for glucose metabolism in the islets is glycolysis (Sener and Malaisse, 1978). About 95% of the net uptake of glucose is accounted for by the formation of triose phosphates and more than 90% of the triose phosphates are formed via the glycolytic pathway (Sener and Malaisse, 1978).

Although islets are enzymatically equipped for the synthesis and degradation of glycogen, the incorporation of glucose into glycogen does not account for more than 0.2% of the total rate of glucose utilization in the presence of glucose 16.7mM (Hedeskov and Capito, 1974). Likewise, the flux of glucose carbon through the pentose phosphate cycle constitutes only 2-4% of total glucose utilization.

Approximately 25% of the glucose utilized by the islets is oxidized in the mitochondria via the tricarboxylic acid cycle but no detailed study of these metabolic events has yet been conducted in islet cells (Sener and

Malaisse, 1978).

The curves relating rates of insulin release and glucose utilization by B-cells to the extracellular glucose concentration are similar; both are sigmoidal, with a threshold of around 5mM glucose, a Km of about 8mM glucose and a plateau at approximately 20mM glucose (Ashcroft, 1980). In rat islets, the largest increase in insulin secretion occurs between 5 and 17 mM of glucose, which means that the B-cells are acutely sensitive to small changes in the glucose concentration within the physiological range (Hedeskov, 1980).

I.D. 4d. Role of cAMP in insulin release

Islets of Langerhans possess all the enzymes involved in the generation, hydrolysis, and mode of action of cyclic AMP which have been found in other mammalian cell types: adenylate cyclase, cyclic nucleotide phosphodiesterase, protein kinases (cyclic AMP-dependent) and phosphoprotein phosphatases (Hedeskov, 1980; Sharp, 1979; Wollheim and Sharp, 1981).

In 1965, the report that glucagon increased the rate of insulin secretion (Samols et al, 1965) suggested that cyclic AMP might be important in insulin release. Since that time, many reports have documented the stimulation of insulin release by agents such as glucagon, B-adrenergic agonists, phosphodiesterase inhibitors, cholera toxin and exogeneous cyclic AMP or dibutyryl cyclic AMP which raise intracellular cyclic AMP levels (cf Sharp, 1979; and Wollheim and Sharp, 1981, for review). It is recognized now that factors which increase the concentration of cyclic AMP in the B-cell enhance the rate of insulin release rate in response to glucose (Hedeskov, 1980; Sharp, 1979; Wollheim and Sharp, 1981). On the other hand, at nonstimulatory glucose concentrations (ie. less than 4-5mM), cyclic AMP levels can be raised in islets of Langerhans without necessarily

producing any effect on insulin release (Hedeskov, 1980; Sharp, 1979; Wollheim and Sharp, 1981). Cyclic AMP is thus a potentiator of glucose-induced insulin release. In addition, intracellular concentration of cyclic AMP in islets can be modestly increased by stimulatory level of glucose (Hedeskov, 1980; Sharp, 1980; Wollheim and Sharp, 1981). The mechanism by which glucose increases intracellular cyclic AMP is not understood (see below, involvement of Ca^{2+}-calmodulin).

I.D. 4e. Effect of cyclic AMP on calcium handling by islets

Experimental data accumulated to date seem to indicate that cyclic AMP stimulates insulin release by increasing the concentration of Ca^{2+} in the cytosol of the B-cells (Wollheim and Sharp, 1981). The action of cyclic AMP apparently does not depend on increased Ca^{2+} uptake nor on decreased Ca^{2+} efflux across the plasma membrane but rather is due to an action of cyclic AMP on intracellular storage sites (Wollheim and Sharp, 1981). It is still unclear whether the increase in cytosolic Ca^{2+} is a result of mobilization of Ca^{2+} from cellular stores (secretory granules, mitochondria, endoplasmic reticulum) or of inhibition of uptake into these stores. Phosphorylation of islet proteins mediated by cyclic AMP has been suggested to be important in the control of the movements of cellular calcium (Wollheim and Sharp, 1981). The glucose dependency of the action of cyclic AMP has been explained by Wollheim and Sharp (1981) as follows: cyclic AMP would have sufficient effect to raise cytosol Ca^{2+} and stimulate insulin release only when the rate of Ca^{2+} efflux across the plasma membrane is reduced by glucose.

Cyclic AMP, apart from its effect on the distribution of intracellular Ca^{2+} in the B-cells, has also been attributed other functions e.g. increase in synthesis of tubulin in rat islets, phosphorylation of contractile proteins of the microtubule-microfilamentous system and of other cell

components (Hedeskov, 1980).

I.D. 4f. Glucose-induced increase in cyclic AMP levels

It now seems generally accepted that glucose can stimulate accumulation of cyclic AMP in the islets (Hedeskov, 1980; Sharp, 1979; Wollheim and Sharp, 1981). This does not appear to be due to a direct stimulation of the adenylate cyclase since only one (Capito and Hedeskov, 1977) out of eight published reports (cf Sharp, 1979) claimed that glucose could stimulate adenylate cyclase in broken cell preparations of islets and the results of that single report have recently been questioned by its own authors (Thams et al, 1982).

The effect of glucose on cyclic AMP levels, like that on insulin release, depends on the metabolism of glucose and on the presence of extracellular calcium (Hedeskov, 1980; Sharp, 1979; Wollheim and Sharp, 1981). Calcium, by itself, is inhibitory to adenylate cyclase (Hedeskov, 1980; Sharp, 1979; Wollheim and Sharp, 1981) but it has been reported that Ca^{2+}-calmodulin stimulates rat islet adenylate cyclase activity (Sharp et al, 1980; Valverde et al, 1979). Consequently, it was suggested that the glucose-induced accumulation of cyclic AMP in islets is a secondary result of the rise in cytosolic Ca^{2+}, which, with calmodulin, causes an increase in the activity of adenylate cyclase (Sharp et al, 1980; Valverde et al, 1979). However, it has also been demonstrated, more recently, that Ca^{2+}-calmodulin does not affect mouse islet adenylate cyclase activity (Thams et al, 1982). Thus there is still no unequivocal explanation for the effect of glucose on islet cyclic AMP level.

I.D. 4g. Role of calcium in insulin release

It is now generally agreed that the common pathway through which all secretagogues eventually affect the process of insulin release involves the participation of Ca^{2+} (Hedeskov, 1980; Malaisse et al, 1981; Wollheim and

Sharp, 1981). By analogy with excitation-contraction coupling in muscle cells, it is thought that the accumulation of ionized calcium at a critical site of the B-cell triggers the exocytosis of secretory granules by activating a microtubular-microfilamentous system (Hedeskov, 1980; Malaisse et al, 1981; Wollheim and Sharp, 1981).

The cytosolic accumulation of Ca^{2+} may be due to a facilitation of Ca^{2+} entry into the B-cell, a decreased rate of Ca^{2+} outflow from the B-cell and/or an intracellular redistribution of Ca^{2+} ions between the cytosol and the other cellular pools (Hedeskov, 1980; Wollheim and Sharp, 1981). Distinct sensor systems may generate distinct messengers, which in turn may affect distinct Ca^{2+} movements. Glucose has been shown to increase the calcium content of islet cells by a combination of the three above mentioned mechanisms (Hedeskov, 1980; Malaisse et al, 1979b; Wollheim and Sharp, 1981). However, sustained insulin release is evoked by glucose only in the presence of sufficient amounts of extracellular Ca^{2+}, the threshold concentration in vitro being approximately 0.1 mM while the maximal effects of glucose occur with 1-5 mM Ca^{2+} (Hedeskov, 1980; Wollheim and Sharp, 1981).

The crucial role of Ca^{2+} in the mechanism of insulin release has further been demonstrated by the following findings: (a) high Ca^{2+} concentrations (2-10 mM), in the absence of any other secretagogue, can elicit a short-lived release of insulin in vitro; (b) certain calcium ionophores can cause insulin release when extracellular Ca^{2+} is present at normal concentrations even when glucose is absent from the medium (Hedeskov, 1980; Wollheim and Sharp, 1981).

The mechanism by which calcium stimulates insulin secretion is unknown. In other Ca^{2+}-regulated processes, Ca^{2+} exerts a controlling influence by binding to specific proteins e.g. troponin C in the initiation

of muscle contraction (Cheung, 1980; Wang and Waisman, 1979). Recently it has been suggested that the primary target for Ca^{2+} within the B-cell is the calcium-dependent modulator protein, calmodulin (CaM) (Means and Dedman, 1980b; Tomlinson et al, 1982).

I.D. 4h. Calmodulin

Calmodulin belongs to a family of homologous proteins that also includes parvalbumin, troponin C, myosin light chains, and mammalian intestinal Ca^{2+}-binding protein (Brostrom and Wolf, 1981; Cheung, 1980; Wang and Waisman, 1979). Calmodulin is a heat-stable Ca^{2+}-binding protein first discovered independently by Cheung (1970) and by Kakiuchi et al (1970), as an activator of brain cyclic nucleotide phosphodiesterase but later found to confer sensitivity to Ca^{2+} to a number of other proteins (cf Brostrom and Wolf, 1981; Cheung, 1980; Means and Dedman, 1980a, 1980b; Means et al, 1982; Wang and Waisman, 1979 for review). Within cells calmodulin is found predominantly in the soluble fraction although some is associated with membranes and organelles (Brostrom and Wolf, 1981; Cheung, 1980; Wang and Waisman, 1979).

The multiplicity of effects of calmodulin (see Table 2), its ubiquitous distribution and its highly conserved amino acid sequence among eukaryotic cells have led to the view that calmodulin may represent the major intracellular Ca^{2+}-receptor protein in non-muscle cells (Brostrom and Wolf, 1981; Cheung, 1980; Means and Dedman, 1980a, 1980b; Means et al, 1982; Wang and Waisman, 1979).

Some of the major physical and chemical properties of calmodulin are listed in Table 3.

The affinity constant of calmodulin for Ca^{2+} is approximately equal to the estimated intracellular free-Ca^{2+} concentration (ranging from a resting value of approximately 10^{-7} M for most cells to maximal concentrations of

about 10^{-5} M in highly stimulated cells) (Means and Dedman, 1980b).

The binding of Ca^{2+} alters the conformation of calmodulin, increasing its helical content and exposing hydrophobic regions. In this conformation, the calmodulin-Ca^{2+} complex can bind to target enzymes and through an unknown mechanism, alter their activities (Brostrom and Wolf, 1981; Cheung, 1980; Means and Dedman, 1980a; Wang and Waisman, 1979).

To date, no alternative function (i.e. enzymatic or structural) has been demonstrated for calmodulin except to bind Ca^{2+} (Means and Dedman, 1980b). The specificity of Ca^{2+} action in given cell types is not effected through calmodulin (since it is ubiquitous) but through the tissue-specific distribution of calmodulin regulated proteins (cf Table 2) (Brostrom and Wolf, 1981; Means and Dedman, 1980b; Wang and Waisman, 1979). The specificity of Ca^{2+} action can also result from the fact that various calcium-binding proteins found within any cell type can exist in one of several conformations depending on the number of Ca^{2+} ions bound per molecule (Brostrom and Wolf, 1981; Means and Dedman, 1980b; Wang and Waisman, 1979).

I.D. 4i. Determination of calmodulin levels

Because of its multifunctional roles, calmodulin can be assayed by the activation of a number of enzymes. For example, activation of Ca^{2+} - and calmodulin-dependent phosphodiesterase has been established as specific for calmodulin and can distinguish it from related proteins such as troponin C and parvalbumin (Klee et al, 1980; Means et al, 1982). Radioimmunoassays for calmodulin have also been developed (Chafouleas et al, 1979; MacManus et al, 1981; Wallace and Cheung, 1979).

The immunoactivity of calmodulin in all tissues and cells is considerably greater than the activity determined by the phosphodiesterase activation assay, but the differences in calmodulin levels as determined by

Table 2. Calmodulin-mediated processes.

[adapted from : Klee et al, 1980; Means and Dedman, 1980a; Means et al, 1982; Wang and Waisman, 1979]

Cyclic nucleotide metabolism:	adenylate cyclase
	: guanylate cyclase
	: phosphodiesterase
Protein phosphorylation	: calcium-dependent protein kinases
Contractile processes	: myosin light chain kinase
Microtubules/microfilaments functions	
Glycogen metabolism	: phosphorylase kinase (activation)
	: glycogen synthetase kinase (inactivation)
Calcium flux	: Ca^{2+} -Mg^{2+} ATPase
	: Ca^{2+} transport
Secretion	: intestinal ion secretion
	: neurotransmitter release
Other enzyme systems	: NAD^{+} kinase
	: tryptophan 5'-monooxygenase
	: phospholipase A_2
Regulation of DNA synthesis (?)	
Neoplastic transformation (?)	

Table 3. Physical and chemical properties of calmodulin.

[adapted from: Brostrom and Wolf, 1981; Cheung, 1980; Means and Dedman, 1980a; Means et al, 1982; Tomlinson et al, 1982].

- Resistance to denaturation by boiling ($t_{\frac{1}{2}}$ = 7 min at 100 °C), 8M urea, 1% SDS, high salt concentration, acidic pH.
- Straight chain polypeptide (148 amino acids)
- Molecular weight 16,700
- Monomer
- High ratio of acidic to basic residues (2.7)
- Isoelectric point, pH=4.0
- High ratio of phenylalanine to tyrosine (4:1)
- No cyteine or tryptophan content
- Characteristic U.V. absorption spectrum (peaks at 253, 259, 265, 268, 276 nm)
- Calcium binding, 4 sites; K = 1-100 μM
- Lysine at position 115 is trimethylated
- Hydrophobicity increased by Ca^{2+}
- Binds to phenothiazines and W compounds; calcium dependent

the two assays are not constant from tissue to tissue (Means and Dedman, 1980a; Means et al, 1982). This could be because calmodulin binding proteins [either other calmodulin-regulated enzymes or other calmodulin binding proteins with undetermined biological functions (cf Wang and Waisman, 1979)] in cell extracts compete with phosphodiesterase for Ca^{2+}-dependent binding whereas interference by calmodulin binding proteins is prevented in the RIA by the addition of EGTA. Alternatively, there may be structural modifications of calmodulin which prevent its interaction with some enzymes but allow association with others.

I.D. 4j. Regulation of calmodulin activity

Although the activity of calmodulin is generally controlled by changes in the intracellular free Ca^{2+} concentration, alterations in calmodulin levels can also represent an important regulatory mechanism that governs specific Ca^{2+}-mediated events e.g. (a) transient rise in the calmodulin content of eukaryotic cells is found before the initiation of DNA synthesis in vitro and in vivo; (b) a rise in intracellular content of calmodulin occurs upon transformation of cells to malignant cells (Means et al, 1982).

In other calmodulin dependent cellular events such as regulation of protein secretion, experimental evidence seems to indicate that total cell content of calmodulin might not have to change; a subcellular redistribution of calmodulin might be sufficient to bring about a change in cellular function (Means et al, 1982).

I.D. 4k. Interaction of drugs with calmodulin

Since the mid 1970's, a variety of pharmacological agents have been shown to bind calmodulin and inhibit its action (Weiss et al, 1982). The phenothiazine antipsychotics were the first drugs demonstrated to be calmodulin inhibitors (Weiss et al, 1982). Moreover, several other types of antipsychotics and other classes of pharmacological agents (e.g.

butyrophenones, naphthalenesulfonamides or W compounds) have now been shown to inhibit the actions of calmodulin (Weiss et al, 1982).

Among the most potent and better studied of these drugs is the phenothiazine tranquilizer trifluoperazine (TFP). The pK_a of trifluoperazine is 8.1 and the binding of TFP to calmodulin was found to be maximal between pH 5.0 and 8.0 (Weiss et al, 1982). Trifluoperazine is thought to bind specifically to the active conformation of calmodulin (i.e. Ca^{2+}-calmodulin), blocking the ability of calmodulin to modulate calmodulin-dependent enzymes (Weiss and Levin, 1978). The specificity of TFP has been inferred by: (a) its lack of Ca^{2+}-dependent binding to a variety of proteins; (b) the fact that its inhibitory effects are related to the presence of Ca^{2+} in concentrations that are required for activation of calmodulin-sensitive enzymes; and (c) the fact that its inhibitory effects and that of other phenothiazines are correlated with their binding affinities for calmodulin (Weiss and Levin, 1978).

TFP, at concentrations less than 100 μM, was found to have no effect on glucose utilization (Gagliardino et al, 1980; Henquin, 1981), glucose oxidation (Sugden et al, 1979; Valverde et al, 1981) nor on the incorporation of ^{3}H-leucine into proinsulin or total islet protein (Gagliardino et al, 1980). Trifluoperazine has also been shown to inhibit insulin release stimulated by glucose, leucine, alpha-ketoisocaproate, glibenclamide, tolbutamide and glyceraldehyde (Gagliardino et al, 1980; Henquin, 1981; Krausz et al, 1980). From these data and from studies on the effect of TFP on Ca^{2+} movements in islet cells, two different hypotheses have been formulated for the involvement of calmodulin in insulin release: (a) Schubart et al (1980) reported that the net Ca^{2+} retention by islet cells is not affected by TFP. They then suggested that TFP blocks insulin release at a site distal to the elevation of the calcium concentration.

Because glucagon-stimulated insulin release was not inhibited by TFP, they further suggested that cyclic AMP may regulate insulin release by a mechanism distinct from the increase in cytosolic calcium. The hypothesis was based mainly on the finding that various islet proteins are phosphorylated independently by both Ca^{2+} and cyclic AMP-dependent protein kinases (Gagliardino et al, 1980; Harrison and Ashcroft, 1982; Schubart et al, 1980);

(b) On the other hand, following the demonstration that TFP can interfere with the glucose-induced stimulation of Ca^{2+} uptake and mobilization from intracellular calcium strores, Wollheim and Sharp (1981) proposed that cyclic AMP and glucose could mobilize stored calcium by different mechanisms and that calmodulin might not be involved in the process of exocytosis per se. The latter hypothesis was also used to explain the lack of effect of TFP on the induction of insulin release by 3-isobutyl-1-methylxanthine (IBMX) (Janjic et al, 1981; Wollheim and Sharp, 1981).

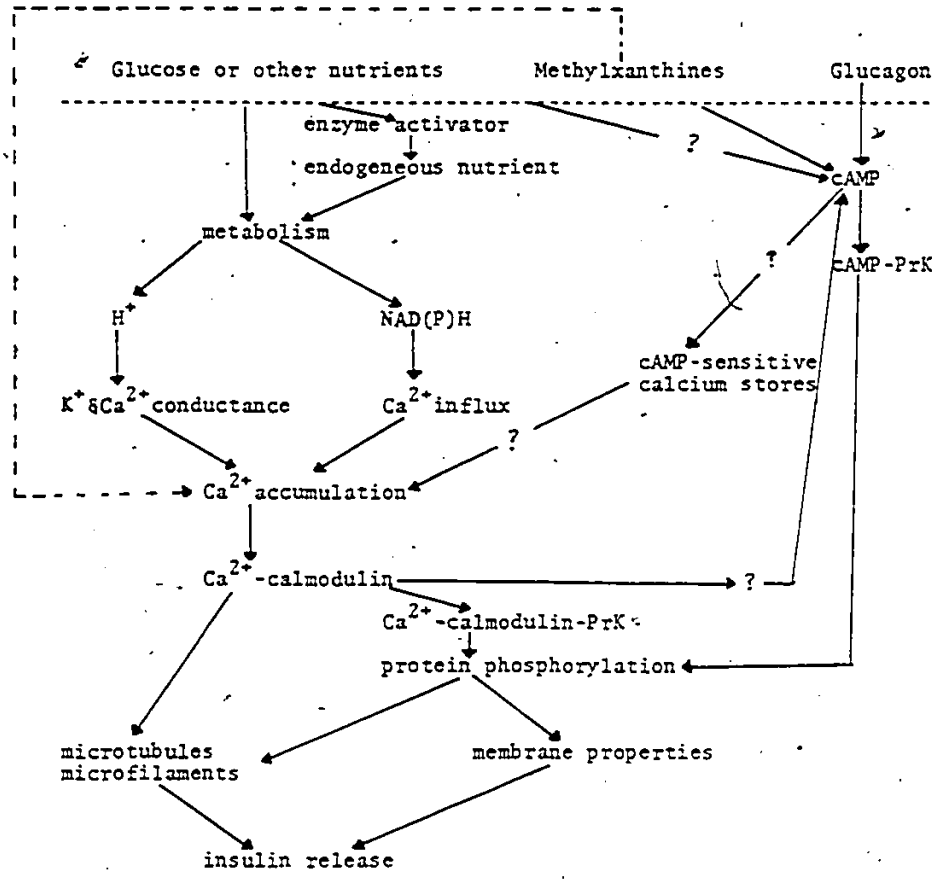
Therefore it is not clear how Ca^{2+}, calmodulin, adenylate cyclase and cyclic AMP interact in stimulus-secretion coupling in the B-cell. Figure 1 is a schematic representation of the possible mechanisms of insulin release based on experimental data cited in this literature review.

I.D. 5. Zinc and insulin

It was found in the 1930's that Zn^{2+} facilitates the crystallization of insulin in solution (Scott, 1934). In most mammalian species, histochemical, electron microscopic and X-ray diffraction techniques have shown zinc to be an integral part of the insulin crystal of the beta granule (Maske, 1957; Okamoto and Kawanishi, 1966; Wolters et al, 1979).

The content and localization of zinc in the B-cells correlate with the functional state of the islet. For instance, stimulation of insulin secretion by glucose or other secretagogues depletes the islets of most of

Fig. 1. Possible mechanisms in the control of insulin release. PrK: protein kinase. [adapted from Ashcroft, 1980; Hedeskov, 1980; Malaisse et al, 1979b, 1981; Schubart et al, 1980; Wollheim and Sharp, 1981].



their zinc content (Maske, 1957; Wolters et al, 1979); prolonged treatment with insulin causes a marked diminution of the number of B-cells with degranulation and depletion of zinc (Logothetopoulos et al, 1961); the content of zinc in rat islets decreases after tolbutamide administration (Wolters et al, 1979); the B-cells of zinc-deficient Chinese hamsters show decreased granulation (Boquist and Lernmark, 1969) and zinc-deficient animals have an impaired glucose tolerance (Boquist and Lernmark, 1969; Huber and Gershoff, 1973). The amounts of zinc in B-cell-rich pancreatic islets from the ob/ob mouse of the Swedish colony were also positively correlated to their content of immunoreactive insulin (Berglund and Hellman, 1976). In species where insulin is not crystallized or aggregated to hexamers in the presence of Zn^{2+}, islet zinc content is low (2-3mg/100g tissue) (Havu et al, 1977a; Peterson et al, 1974; Zimmerman and Yip, 1974) whereas in species where insulin has a specific zinc binding and coordinating capacity, the zinc content is higher (10-12 mg/100 g tissue) (Berglund and Hellman, 1976; Blundell et al, 1972; Havu et al, 1977b).

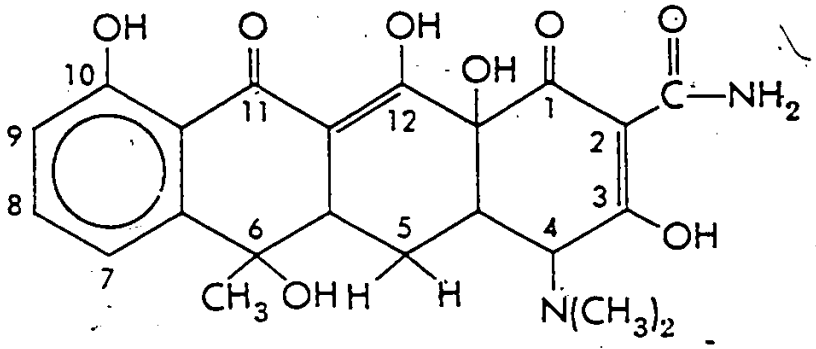
The mechanism whereby zinc plays a role in the synthesis, storage, and secretion of insulin is not yet well established. The generally accepted functions for zinc in the production of insulin by the zinc-rich B-cells have been listed by Emdin et al (1980) as: (a) assembly of the proinsulin and the derived insulin hexamers; (b) solubility of the zinc proinsulin hexamer; (c) precipitation and crystallization of the nascent insulin; and (d) crystal formation, which is presumed to reduce the rate of proteolysis. Moreover, a postsecretory role for a zinc-insulin complex has been suggested from the studies of Arquilla et al (1978): liver plasma membranes, when removed from mice pretreated with zinc or when treated with zinc <u>in vitro</u>, showed an enhanced binding and decreased degradation of insulin.

Zinc enters rat pancreatic islets by facilitated transport as well as by diffusion and it accumulates beyond the level needed to make two-zinc-insulin hexamers (Figlewicz et al, 1980; Ludvigsen et al, 1979); islets from rodents contain 3-4 times more zinc than is necessary for the zinc-insulin complex (Figlewicz et al, 1980; Havu et al, 1977b; Ludvigsen et al, 1979). Moreover, studies of the subcellular distribution of zinc in islet cells have revealed that a large part of the islet zinc is not associated with the secretory granules (Figlewicz et al, 1980). It was thus suggested that, within the pancreatic islets, zinc may be needed for metabolic processes other than insulin crystallization (Figlewicz et al, 1980; Ludvigsen et al, 1979).

I. E. Oxytetracycline

Oxytetracycline is an antibiotic elaborated by the actinomycete Streptomyces rimosus. Introduced in 1950, it belongs to a group of broad spectrum antibiotics, the tetracyclines. These drugs possess a wide range of bacteriostatic activity against Gram-positive and Gram-negative bacteria which overlaps that of many other antimicrobial drugs such as penicillin, streptomycin and chloramphenicol (Sande and Mandell, 1980). They have been in common use for the last 30 years for treatment of a variety of bacterial infections (Gale et al, 1972). The tetracyclines are derivatives of the polycyclic napthacene carboxamide and are very much alike in their chemical, antimicrobial, pharmacological and therapeutic properties, which permits their discussion as a class (Sande and Mandell, 1980). [See Table 4 for structural formulas of the tetracyclines]. The differences that exist between the tetracyclines are quantitative rather than qualitative and arise largely from the different rates of absorption and excretion and the different degrees of protein binding; for example, the extent of the diffusion of oxytetracycline throughout the body is suggested by the fact

Table 4. STRUCTURAL FORMULAS OF THE TETRACYCLINES



Tetracycline

CONGENER	SUBSTITUENT(S)	POSITION(S)
Chlortetracycline	—Cl	(7)
Oxytetracycline	—OH,—H	(5)
Demeclocycline	—OH,—H; —Cl	(6; 7)
Methacycline	—OH,—H; $=CH_2$	(5; 6)
Doxycycline	—OH,—H; $—CH_3$,—H	(5; 6)
Minocycline	—H,—H; $—N(CH_3)_2$	(6; 7)

that it has the highest mean relative volume of distribution of the tetracycline antibiotics (Sande and Mandell, 1980). All tetracyclines are concentrated in the liver and excreted by way of the bile into the intestine from which they are partially reabsorbed; they are excreted in the urine and the feces, the primary route for most being the kidneys. The tetracyclines are stored principally in the reticuloendothelial cells of the liver, spleen and bone marrow and in bone and in the dentine and the enamel of unerupted teeth (Sande and Mandell, 1980)..

The antimicrobial action of the tetracyclines is thought to result from their inhibitory action on bacterial protein synthesis. By specifically binding to the 30S subunit of the ribosome (Gale et al, 1972), they prevent the enzymatic binding of aminoacyl-tRNA into the ribosomal aminoacyl acceptor or "A" site, thereby preventing codon-anticodon interaction. The tetracyclines are also known to inhibit mitochondrial protein synthesis in rapidly growing tissues (De Vries and Kroon, 1970; Himms-Hagen, 1971) but have no direct effect on the enzymes already present (De Vries and Kroon, 1970). Inhibition of cytoplasmic protein synthesis has also been reported, but only at much higher concentrations (50 µg/ml plasma) (Bogert and Kroon, 1981; De Jonge, 1973). It is worth noting that mammalian cells lack the active transport system for the tetracyclines that is found in bacteria (Sande and Mandell, 1980).

I. E. 1. Chemistry

The tetracyclines are crystalline bases which are slightly soluble in water at pH 7 but form soluble sodium salts and hydrochlorides (Sande and Mandell, 1980). Four proton dissociations have been observed in the tetracycline molecule with pK_a's of 3.3, 7.7, 9.7 and 10.7 (Coibion and Laszlo, 1979). At neutral pH, tetracyclines are anions. The molecular structure of the tetracyclines (cf Table 4) reveals several enolic sites at

which chelation with divalent cations might be predicted to occur (Coibion and Laszlo, 1979).

Binding constants have been determined for a number of divalent cations (Cu^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+} etc), for some trivalent cations (Fe^{3+}, La^{3+}) (Celotti and Fazakerley, 1977; Gulbis et al, 1976) and also for monovalent cations such as Li^{+}, Na^{+}, K^{+}, Rb^{+} and Cs^{+} (Coibion and Laszlo, 1979). The interaction of divalent cations with tetracyclines was determined to be the strongest; consider the following dissociation constants: for Mg^{2+} and/or Ca^{2+}, 4×10^{-4}, for Zn^{2+}, 1.6×10^{-5} (Gulbis et al, 1976) while for Na^{+}, $K_d = 7 \times 10^{-2}$ and for K^{+}, $K_d = 90 \times 10^{-2}$ (Coibion and Laszlo, 1979). The affinity of tetracyclines for divalent cations is of the same order as those of the common amino acids (Albert and Rees, 1956).

Tetracyclines have been found to have fluorescence characteristics which are dependent not only on the level of divalent cations chelated to them, but also on the properties of the chemical microenvironment surrounding the tetracycline-divalent cation complex (Caswell and Hutchison, 1971b). Tetracyclines have been shown to bind preferentially to cations on membrane surfaces (Caswell and Hutchison, 1971a) and have been used as a fluorescent probe to study the association of divalent cations with mitochondria (Schuster and Olson, 1974), chloroplasts (Girault and Galmiche, 1978), membrane preparations (Caswell and Hutchison, 1971a; Franklin, 1971) and islet cells (Taljedal, 1978). Taljedal (1978) sugggested that in the B cells, the Ca^{2+} pools which can be identified as interacting with chlorotetracycline are those of importance for insulin secretion. Chlorotetracycline due to its hydrophobic nature has been shown to enter living cells as indicated by fluorescence from the mitochondria (Du Buy and Showacre, 1961). As far as the pancreas is concerned, in vitro

exposure to high concentrations of chlorotetracycline (50-100 μM) was shown to stain intracellular organelles in both endocrine (Taljedal, 1978) and exocrine (Chandler and Williams, 1978) pancreatic cells. On the other hand, at low concentration (10 μM) the plasma membrane was the predominant site for Ca^{2+}-chlorotetracycline deposition in islet cells (Taljedal, 1978).

I. E. 2. Oxytetracycline and the action of insulin

Oxytetracycline has been reported to have a variety of metabolic effects. It has been shown to influence carbohydrate metabolism and the action of insulin. In both humans (De Lollis and Privitera, 1954; Miller, 1966) and animals (Hiatt and Bonorris, 1970; Hiatt et al, 1966) which received either tolbutamide or exogeneous insulin, OTC potentiated the action of insulin. In particular, chronic oxytetracycline treatment of the ob/ob mouse has been shown by Begin-Heick et al (1974, 1976, 1979) to:

(1) produce significant decrease in body weight

(2) decrease the levels of serum IRI levels to within the range seen in lean animals within 5 days following the beginning of treatment

(3) increase the binding of insulin by the liver membranes after 5 days of treatment

(4) decrease blood glucose levels within 7 days of treatment

(5) normalize glucose and insulin tolerance

(6) decrease the weight and lipid content of the liver

(7) increase the insulin sensitivity in the diaphragm

(8) decrease the in vivo incorporation of ^{14}C-glucose and $^{3}H_2O$ into total liver lipids

(9) decrease the in vivo and the in vitro incorporation of ^{14}C-glucose and $^{3}H_2O$ into the adipose tissue

(10) result in a regranulation of the islet cells and an increase in the insulin content of the ob/ob pancreas.

These data were confirmed by the work of Dubuc and collaborators (Dubuc and Willis, 1978; Dubuc et al, 1978) who found in addition that OTC treatment produced a stimulation of longitudinal growth as well as a decrease in the adiposity of the ob/ob mouse. Following a study comparing the effect of food-restriction and OTC-treatment in the ob/ob mouse, Dubuc et al (1978) concluded that only OTC-treatment significantly reduced adiposity (i.e. the ratio of lean body mass to adipose tissue mass was increased by OTC but was not changed by food restriction).

The mechanism whereby oxytetracycline produces some of these effects is not known. The observations that OTC potentiates the effect of insulin in pancreatectomized dogs (Hiatt and Bonorris, 1970; Hiatt et al, 1966) and on various insulin target tissues of the ob/ob mouse (Begin-Heick and Heick, 1976; Begin-Heick et al, 1974) support the conclusion that the effects of OTC are extrapancreatic. On the other hand, the insulin potentiating effect of OTC in human diabetics treated with tolbutamide (Miller, 1966) and the regranulation of the obese mouse pancreas brought about by chronic treatment with OTC tend to support the idea that OTC may have an effect on the regulation of B-cell function.

II. STATEMENT OF THE RESEARCH PROBLEM

The obese mouse (ob/ob) has been used extensively as a model for the study of obesity, hyperglycemia, hyperinsulinemia and tissue resistance to insulin. In attempts to elucidate the nature of the abnormal metabolic processes which characterize this syndrome, pharmacological agents have been used to alter the metabolism of the obese animals. One of these agents is the antibiotic, oxytetracycline . Oxytetracycline was initially reported as having an insulin potentiating effect in pancreatectomized dogs receiving insulin replacement therapy, and in humans who received tolbutamide. It was then shown by two independent groups that administration of OTC tended to correct many of the metabolic abnormalities of the obese mouse.

It was of interest to find out whether the effects of OTC were only extrapancreatic (as could be inferred from its action in pancreatectomized animals) or whether it also had effects on the pancreatic B-cell (as suggested by the time course of its effect in the ob/ob mouse).

It was also of interest to study further the exaggerated insulin-secretory response in the ob/ob mouse in order to try to understand the nature of the defect.

In the spontaneously diabetic BB rat, B-cells are virtually absent. This animal thus offered a model in which the effects of OTC on the control of glycemia could be studied. Experiments were therefore designed to study the effect of OTC on the insulin requirements and on the handling of glucose by the BB rat.

Although the hyperinsulinemia and the exaggerated in vivo response of insulin secretion in the obese mouse have been well documented, little work has been done comparing the kinetics of insulin release in C57Bl/6J ob/ob mice and their lean controls. It was thus necessary at first to determine

if the in vivo and in vitro response of the insulin secretory mechanism to known secretagogues was quantitatively and/or qualitatively different from that in the lean mouse. The effect of OTC treatment on the response to secretagogues was then examined. These experiments were carried out in vivo to assess the response of the animals to the secretagogues and in vitro to examine the insulin secretory response of islets isolated from the insulin responsive tissues in order to dissect out effects of OTC which might be specific to the insulin-secreting tissue.

As beneficial effects of OTC were observed in vivo and in vitro, I sought to explain these effects. As the antibiotic is a strong chelating agent for divalent cations and since it has a great avidity for zinc, I hypothesized that OTC may have interacted with zinc ions and thus, that supplementation with zinc might reduce the insulin-secretory activity of the islets of the ob/ob mouse. This hypothesis was based on the known function of zinc in the formation of the insulin granule as well as on some evidence by others in our laboratory that the obese mouse may be relatively deficient in zinc.

Finally, in order to elucidate further if the exaggerated insulin secretory response seen in the untreated ob/ob mouse is due to specific defects in the beta cells, and considering the growing amount of evidence showing that calmodulin is important for stimulus-secretion coupling in pancreatic islets, the calmodulin system of the ob/ob mouse islets was evaluated by comparison with the calmodulin system of lean mouse islets and rat islets.

CHAPTER 2. MATERIALS AND METHODS

I. Chemicals

The following chemicals were obtained from J.T. Baker Chemical Co.: magnesium chloride, 2-mercaptoethanol, potassium chloride, sodium bicarbonate, sodium chloride, sodium hydroxide, Tris (Buffer), and zinc carbonate.

The following reagents were purchased from Fisher Scientific Co.: acetic acid, boric acid, calcium chloride, EDTA, hydrochloric acid, magnesium sulfate, potassium phosphate (monobasic), sodium phosphate (monobasic), and sodium phosphate (dibasic).

Sigma Chemical Co. (St-Louis, Mo) supplied the following: aminophylline, bovine serum albumin (fraction V RIA grade), calmodulin-deficient phosphodiesterase (lot# 12F-8500), cyclic AMP, EGTA, lidocaine, merthiolate (thimerosal), and snake venom (Ophiophagus hannah).

Collagenase and hyaluronidase were obtained from Worthington Biochemical Co. Ltd (Freehold, NJ); oxytetracycline (Terramycin and Liquamycin-LP) from Pfizer Co. (Montreal, Que.); trifluoperazine (Stelazine) from Smith, Kline and French Laboratories (Montreal, Que.); purified rat testis calmodulin (lot# 020381) from CAABCO Inc. (Houston, Texas); rat insulin standard (25 U/mg) from Novo Research Laboratories (Copenhagen, Denmark); protamine zinc insulin, crystalline zinc insulin and glucagon for the *in vivo* tolerance tests from Connaught Laboratories (Toronto, Ont.); Freund's complete adjuvant from Difco Laboratories (Detroit, Michigan); the purified guinea-pig y-globulin used to raise the anti-(guinea-pig y-globulin) (AGG) serum was from Cappel Laboratories (Cochranville, Pa); Tes-tape™ (glucose-enzymatic test strip) from Eli Lilly Co. (Toronto, Ont.); and Ketostix™ (ketones enzymatic test strip) from Ames (Toronto, Ont.).

Glucagon for the in vitro experiments (9 μU insulin/mg) (lot#:258-25J-120) and human insulin were gifts from Lilly Research Laboratories (Indianapolis, In). Guinea-pig anti-insulin serum (AIS) was kindly provided by Dr. J. Braaten (Department of Endocrinology and Metabolism, Civic Hospital, Ottawa, Ont.). The antiserum used for the calmodulin radioimmunoassays and [^{125}I]-calmodulin were provided by Dr. J.P. MacManus (Division of Biological Sciences, National Research Council of Canada, Ottawa, Ont.).

The 95% O_2/5% CO_2 gas mixture was supplied by Liquid Carbonic of Canada (Ottawa, Ont.).

Cyclic [8-^{3}H]AMP, [^{125}I]-labelled pork insulin (specific activity = 100 μCi/μg) and Aquasol were purchased from New England Nuclear Corporation (Lachine, Que.).

II. Animals

Male C57Bl/6J ob/ob mice and their lean controls (+/?) were obtained from the Jackson Laboratories, Bar Harbor, Maine, U.S.A., at 7-8 weeks of age. They were used in the various experiments at 9-12 weeks of age. Normal (nondiabetic) male Wistar rats were obtained from Bio-Breeding (Montreal, Que.). Spontaneously diabetic (BB) male rats were a gift from Drs I. Hynie and P. Thibert (Department of Health and Welfare, Ottawa, Ont.). They were between 4 and 5 mo of age. All had well established diabetes of at least 8-wk duration and all had been treated with insulin since the onset of the disease according to the protocol described in Chapter 4.

The New Zealand white rabbits used to raise the AGG-precipitating serum were purchased from D.J. Rabbit Ranch Ltd (Smith Falls, Ont.).

All animals were kept in a temperature controlled room at 24 ± 1 °C with 12 h light cycles and, unless otherwise specified, all animals had free access to food and water.

III. Treatments

The mice were housed in individual cages during the treatment periods. They were assigned at random to the following experimental groups; (1) lean control, (2) lean OTC-treated, (3) obese control, (4) obese OTC-treated, (5) food-restricted obese mice, (6) Zn-supplemented obese mice, and (7) obese OTC-treated and Zn supplemented.

III. A. Oxytetracycline treatment

Groups of lean and obese mice were treated for 7 days with a daily intramuscular injection of oxytetracycline (Terramycin) suspended in olive oil. All animals received the same daily dose of 100 mg/kg, in 0.2 ml, calculated on the basis of the average body weight of the untreated lean mice. The control animals received placebo injections which for most experiments consisted of saline. For the animals used for the glucose tolerance test (cf Chapter 5), half the obese control mice were injected with saline and the other half with lidocaine at the concentration present in the Terramycin preparation. There was no significant difference between the two sets of data, the results were therefore pooled. All injections were given between 16:00 and 16:30 h.

III. B. Food restriction

The food restriction schedule consisted of feeding groups of obese mice an amount of food equivalent to the average amount consumed by the OTC-treated obese mouse. This has previously been found to be equal to approximately 4.5 g/day (Bégin-Heick et al, 1974). The weighed food was placed in the appropriate cages at the same time as the OTC injections were given (16:00 h).

III. C. Zinc supplementation

Zinc carbonate was added to ground Purina chow to increase the zinc content from 30.3 ppm to 1,000 ppm. This level of zinc was chosen as it was

reported that laboratory animals can tolerate 1,000-2,000 ppm zinc in the diet, approximately 100 times the required dietary levels (National Research council, 1972) without showing any sign of toxicity (Smith and Larson, 1946). The diet was fed ad libitum for 4 weeks, previous experiments having shown the level of zinc in the plasma and some tissues of the ob/ob mouse to be increased within that time (Bégin-Heick et al, 1982).

III. D. Zinc supplementation + OTC-treatment

Oxytetracycline (Terramycin) was administered as described above for the last 7 days of the experiment.

IV. Glucose Determinations

Plasma glucose was measured by a glucose oxidase method, using a Beckman Glucose analyzer (Beckman Instruments, Fullerton, California). After separation of the plasma by centrifugation, a 5 µl portion of plasma was diluted with 10 µl of distilled water prior to analysis. Incubation media used for the isolated pancreatic islets were checked by the same method.

V. Preparation of the AGG-Precipitating Serum

AGG-precipitating serum was raised in rabbits according to a modification of the protocol of Hales and Randle (1963). A solution of 1mg/ml of purified guinea-pig y-globulin, in 0.9% NaCl, was emulsified in a 1:1 volume with complete Freund's adjuvant, using a Polytron P-10 homogenizer (Brinkman Instruments, Toronto, Ont.) (medium speed, 5s). Intramuscular injections into the hind legs of rabbits were given on a weekly basis for a period of a month, followed by booster injections once monthly. Antibody titer was maximal within 6 weeks following the beginning of the immunization.

VI. Insulin Determinations

Insulin was measured by a double antibody radioimmunoassay. As a new method was developed, it is described in detail in Chapter 3.

VII. In Vivo Response to Secretagogues

Twelve hours before each test, at 21:00 h, food was removed from the cages. At 09:00 h the following day, blood samples were taken from the tail vein into heparinized capillary tubes, (0 time sample), test compounds were injected intraperitonealy at the concentrations indicated in the legends to the Figures and blood samples were withdrawn at 5, 15, 30, 60 & 90 minutes following the injection of the test compound. The experiments were performed without anesthesia.

VIII. Islets

VIII. A. Preparation of islets

Fed mice were killed by decapitation between 9:00-10:00 h. Islets were prepared by a method based on the collagenase method of Lacy and Kostianovsky (1967). A mid-line incision was made and the pancreas rapidly covered with cold Krebs Ringer bicarbonate buffer pH 7.35, 4°C (cold KRB). The buffer contained 3mM glucose and had the following ionic composition (in mMol/l): Ca^{2+}, 2.1; Mg^{2+}, 0.98; Na^{+}, 141; K^{+}, 4.88; Cl^{-}, 105.2; SO_4^{2-}, 0.98; $H_2PO_4^{-}$, 0.98; HCO_3^{-}, 44] . It was then removed, freed from the spleen, fat and connective tissue, and minced into small pieces with scissors. The pancreas pieces were washed twice with fresh cold buffer and twice with buffer at 37°C . The washed pieces were then placed into the digestion buffer. Four to five pancreata were processed as a batch. For each pancreas, 10 mg of collagenase and 5 mg of hyaluronidase were added to 0.5-0.8 ml Krebs-Ringer bicarbonate buffer pH 7.35, 37°C (warm KRB) of the same composition as that described above, except that the concentration of HCO_3^{-} was 15 mMol/l. The slurry was shaken vigorously by hand at 37°C for

5-8 minutes. The reaction was stopped by the addition of 100 ml of cold KRB. Islets were isolated from the digest and placed in a common pool before selection for static tube incubation. Each tube received 4 or 5 well preserved islets. The tubes were selected at random for different treatments.

The isolation of rat islets was done by the same procedure except that 20 mg of collagenase and 10 mg of hyaluridonase were used for the digestion of each pancreas.

VIII. B. Validation of the method

Various preparations of the endocrine pancreas have been used for the in vitro study of the factors which regulate insulin secretion. These investigations were initiated by Anderson and Long in 1947, by means of the perfused rat pancreas and were followed by studies using small pieces of pancreas (Coore and Randle, 1964; Malaisse et al, 1967a). Although these preparations have proved useful to study the factors which may control insulin release, they are of limited value for elucidating the relationships between islet cell metabolism and release, because of the large excess of acinar tissue. Intact pancreatic islets devoid of surrounding exocrine cells were initially obtained by freehand microdissection (Hellerstrom, 1964). The collagenase technique, originally described by Moskalewsky (1965) was improved by Lacy and Kostianovsky (1967) and is used widely today. The advantage of the collagenase digestion procedure is that it is less laborious than the microdissection technique and thus constitutes a fairly rapid technique for the isolation of a large quantity of islets. As the absolute increase in insulin release in response to glucose was shown to be the same in collagenase-isolated islets and microdissected islets (Hahn and Michael, 1970; Lernmark, 1971), the

collagenase isolation method is in very wide use today.

Some of the difficulties associated with the isolation of islets of Langerhans by the collagenase technique and the effects of several experimental parameters on the insulin release from such islets have been reviewed at length (Lernmark, 1971; Scharp et al, 1980). The various steps of the method of isolation of the pancreatic islets used for the studies presented in this thesis, reflect consideration for factors that were shown to affect islet cell viability and IRI release.

(a) Since it was reported that the ability to isolate islets in animals is greatly reduced within minutes of the death of the animal (Scharp et al, 1980), the pancreas was bathed with cold KRB while still in situ in order to slow down all proteolytic reactions and removed with the spleen still attached so as to afford a "handle", thus avoiding direct injury or contamination of the pancreatic tissue.

(b) The primary problem with collagenase is that it is an enzyme complex produced by bacteria and the relative concentration of the components varies between batches. To provide uniformity, the same batch of enzyme was used for all the islet results presented in this thesis. The end-point of the digestion varies not only between batches of collagenase but also between strains, species and ages of donors (Scharp et al, 1980). The highest yield of islets free of acinar tissue, with the shortest time of collagenase/hyaluronidase digestion was obtained from the ob/ob mouse. This could be due to the connective tissue capsule that surrounds the ob/ob islets and which appears to be less prominent in rat islets or in islets of lean mice (Hellerstrom, 1967; Lernmark, 1971).

(c) The presence of exocrine tissue fragments could eventually lead to the proteolytic destruction of the released insulin. After the collagenase digestion of the pancreas, the liberated islets were hand picked twice to

insure complete removal of acinar tissue.

d) In order to avoid damage, the transfer of the individual islets was done under the stereomicroscope, with a Lang-Levy pipet with a larger opening than the biggest islet and the islets were suspended in oxygenated buffer at all times during the transfer procedure.

(e) BSA was added to the KRB buffer to protect insulin from proteolytic destruction and adsorption to glass.

(f) The islets were used immediately after isolation rather than after incubation in culture medium. Culturing of islets, even for relatively short periods, has been shown to produce differences in insulin content and release (Andersson and Hellerstrom, 1972; Jain et al, 1978; Kostianovsky et al, 1972; Rabinovitch et al, 1978), glucose oxidation (Andersson and Hellerstrom, 1972), glucose-induced increase in cAMP levels (Rabinovitch et al, 1978; Sharp , 1979), proinsulin biosynthesis (Jain et al, 1978) and adenylate cyclase activity (Lacy et al, 1976).

(g) The islets were preincubated at low glucose concentration (3 mM) in order to allow fading of possible insulin release in response to the variable glycemia of the animals (especially since the animals were used in the fed state) and also to restore the sensitivity of the B-cells to the secretagogues (Dahl and Henquin, 1978; Lernmark, 1971).

(h) Continuous gassing with O_2/CO_2 (95:5) during the preincubation and the incubation periods ensured availability of oxygen as islet respiration has been shown to influence insulin release (Hellerstrom, 1967; Hutton and Malaisse, 1980).

(i) The incubation vessels were shaken during the preincubation and the incubation periods so as to increase the diffusion of released insulin and counteract a possible feedback inhibition of insulin release due to high local concentrations of insulin (Lernmark, 1971).

(j) Every time a batch of islets was isolated, some islets were incubated at 3mM and others at 20mM glucose in order to check the functional viability of the islets. Shibata et al (1976) has reported that the characteristic response to a glucose challenge is considered a reliable index of islet viability.

In order to assess the validity of the islet isolation procedure, a comparison was established between the data obtained by myself and those already in the literature on the stimulation of insulin secretion by secretagogues. Rat islets were used for these experiments, because there are more data in the literature on rat than mouse islets and also because islets of normal mice have a very low insulin secretory response. Wistar rat islets were therefore isolated and incubated with the secretagogues used later with mouse islets. The results of those experiments are given in Fig. 2. There was a 12 fold increase in insulin release in going from 3 mM glucose to 20 mM glucose. Under similar experimental conditions, Rabinovitch et al (1978) had reported a 6 fold stimulation; Malaisse et al (1976), an 11 fold stimulation; and Jain et al (1975), an 18 fold stimulation. The addition of glucagon (5 µg/ml) or aminophylline (2.5 mM) resulted in a significant increase in insulin secretion at 10 and 20 mM glucose, the stimulatory effect being greater with aminophylline (as expected).

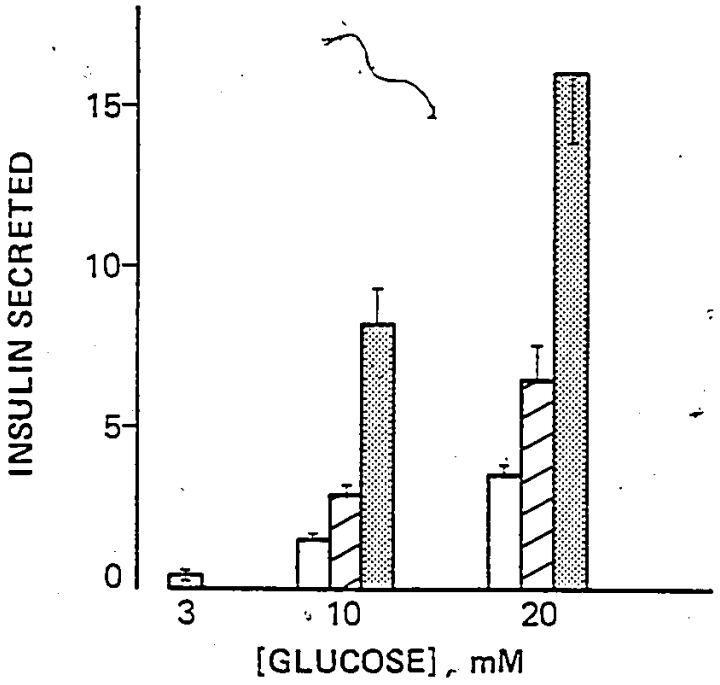
These data gave me confidence that the islet isolation procedure was valid and that the insulin secretion data obtained in mouse islets could be trusted.

Expression of results

In dealing with samples from lean and obese mice, one is confronted by difficulties in the selection of a method for the expression of results. The islet populations in the two groups of animals differ in size, shape

Fig. 2. The release of insulin by isolated rat islets.

The islets were preincubated in 3 mM glucose as described in the method section. They were incubated for 60 minutes at the glucose concentrations indicated on the abscissa, in the absence (open bars) or in the presence of glucagon (5 ug/ml) (hatched bars) or aminophylline (2.5 mM) (shaded bars). Insulin was measured as described in Chap. 3. The results were reported as percentage of insulin released per 60 minutes (i.e. secreted IRI X 100/ secreted IRI + islet IRI), as described on p.68. Results are given as the means ± SEM of 10-15 batches of islets (4 islets/batch).



and degree of granulation. In addition, whereas in the lean mice the islet cell population is fairly homogeneous, in the obese, within one pancreas, islets of different sizes and/or different degrees of granulation are found.

Secretory data have been expressed in the past on a per islet basis (Lacy and Kostianovsky, 1967), as a function of islet DNA (Parman, 1975), as a function of islet protein content (Sehlin, 1976), as a function of islet dry weight (Gagerman, 1980; Hellman, 1975; Wolters and Konnijnendijk, 1980) or as a function of islet size, expressed as volume (Reaven et al, 1981) or surface area (Jahr et al, 1978). Measurements of islet DNA, protein content or islet dry weight require large amounts of islet tissue, which is at a premium, and are associated with large random errors (Gagerman, 1980). A correlation could not always be established between islet volume and islet insulin secretion (Hayek and Woodside, 1979; Reaven et al, 1981). However, a significant correlation was reported to exist between islet insulin content and insulin release (Steinke et al, 1972). Such a correlation had previously been observed by Malaisse et al (1968b) while working with pieces of pancreas. Insulin release from isolated islets was also reported as a function of insulin content by Garcia et al (1976), Jain et al (1975) and by Gold et al (1982). It was therefore decided to express the insulin secretion results as a percentage of insulin released per 60 minutes, that is (secreted IRI X 100)/ (secreted IRI + islet IRI), wherever comparisons between control and treated mice were required.

VIII. C. In vitro response to secretagogues

The tubes containing the islets were preincubated at 37 °C for 45 minutes in a medium consisting of warm KRB containing 1 mg/ml bovine serum albumin and 3mM glucose. After replacement of the preincubation medium with fresh medium, the islets were incubated at the desired concentration

of the secretagogues for a period of 60 minutes. Throughout the preincubation and incubation, the islets were maintained in an atmosphere of 95% O_2 / 5% CO_2 at 37°C. At the end of the incubation period, the medium was removed and frozen for subsequent determination of insulin. After washing three times with fresh buffer, the islets were extracted with 1.0 ml of cold acid/alcohol (64% ethanol, 31% water, 2% HCl) for 48h at 4°C (Davoren, 1962). After appropriate dilution, these extracts were used for the determination of insulin.

VIII. D. In vitro response to trifluoperazine

Batches of 4 islets/tube were preincubated for 45 min in warm KRB containing 1 mg/ml bovine serum albumin, 3 mM glucose and where indicated 20 μM trifluoperazine. The islets were then incubated with either 3 or 20mM glucose with TFP added at the concentrations indicated. Other experimental details were as in section VIII. C.

VIII. E. Sizing of islets

Islets were sized on a dissecting microscope with the help of an eyepiece micrometer. The volume of the islets was estimated as in Jain and Logothetopoulos (1977), by measuring the two transverse diameters and the average height of the islet and assuming a disk-like shape [$V = 4/3 \pi abc$, where a, b, and c represent the measurement of each diameter divided by two]. The rat islets were divided into three groups on the basis of the size of the longest diameter: (A) large size islets: .30mm and over, (B) medium size islets: .19-.27mm, (C) small size islets: .15mm or less.

VIII. F. Preparation of islet extracts for calmodulin assay

The islet sonicates were prepared according to a modification of the method of Sugden et al (1979). The KRB buffer used for the isolation of islets was removed by 5 washes with 10mM Tris-HCl (pH 8.0), followed by 1.0 min spins at 100xg and removal of the supernatant. The islets were

finally suspended in 0.5 ml of the Tris-HCl buffer and disrupted by three 5s pulses of sonication at position 60 on a Sonic Dismembrator (Quigley Rochester Inc., Rochester, N.Y.). The islet sonicates were then placed in a boiling water bath for 3 min. The boiled samples were centrifuged for 15s in an Eppendorf 3200 centrifuge, frozen in ethanol/dry ice and stored at -20°C until calmodulin determination was done (within one month). Before assaying for calmodulin, the islet sonicates were diluted so that each islet corresponded to a final volume of 10 µl.

IX. Calmodulin Assay

IX. A. Phosphodiesterase assay of calmodulin

Phosphodiesterase (PDE) assays were performed according to the two-step assay of Thompson et al (1974) with the exception that the pH of the anion exchange resin was adjusted at 3.0 with acetic acid as described by Boudreau and Drummond (1975). Essentially PDE assays were done in a final volume of 400µl containing 40mM Tris-HCl (pH 8.0), 5mM MgCl , 3.75 mM 2-mercaptoethanol, 0.1mg bovine serum albumin/ml, cyclic [^{23}H]AMP (38.1 Ci/mmol, 200,000 cpm) and unlabeled cyclic AMP at a final concentration of 50µM. Basal activity was measured in presence of 1mM EGTA. Blank values were determined either by substituting the assay buffer for the enzyme or with a boiled enzyme preparation. In either case the blanks were always less than 2% of cyclic [^{3}H]AMP added. Tubes were incubated for 10min at 30°C and the reaction was terminated by placing the tubes in a boiling water bath for 2.5min. After cooling on ice for 10min, 100µl of a snake venom suspension (2mg/ml) were added to each tube. The tubes were then incubated for 10min at 30°C and the reaction was terminated by adding 1.0ml of a 1:3 slurry of AG 1X8 resin, 200-400 mesh (Bio-Rad , Mississagua, Ont.). After equilibration for 15min at 4°C, the tubes were centrifuged at 100xg for 10min and 0.5ml aliquots of the supernatants were added to 10ml

Aquasol and counted.

The activator-dependent phosphodiesterase activity was measured in the presence of 0.05mM $CaCl_2$ and appropriate amounts of purified calmodulin or islet extract. In all experiments the amount of enzyme caused hydrolysis of less than 25% of the substrate.

IX. B. Validation of the method

For the measurements of PDE activity, conditions were chosen such that the rate of [^{3}H]-adenosine formation was proportional to the concentration of PDE. The activity of a fixed amount of calmodulin-free PDE (1.65×10^{-3} units) was stimulated by the addition of amounts of rat testis calmodulin ranging from 0-50 ng. A typical activation curve is shown in Fig. 3. Half-maximal activation required 5 ng calmodulin. The mean maximal activation was approximately 3 to 3 1/2 fold. In the absence of added Ca^{2+} and with EGTA present to chelate traces of Ca^{2+} in the reagents, calmodulin did not stimulate PDE.

The effects of increasing concentrations of trifluoperazine on the activation of PDE by 50 ng calmodulin are shown in Fig. 4. 100 µM TFP lowered PDE activity to values seen in the absence of calmodulin.

A curve of PDE activation by sonicates from rat islets is shown in Fig. 5. Activation occurred to a maximum extent of approximately 3 fold which is equivalent to the maximal activation achieved with authentic calmodulin (cf Fig.3). Similar curves were obtained with all categories of islet sonicates under study. The activity of PDE stimulated by islet extracts was maximally inhibited by 100 µM TFP. Islet calmodulin content was quantitated by comparison with standard curves for PDE activation constructed using authentic calmodulin which were included in each experiment. Islet calmodulin levels were calculated from the amount of

Fig. 3. The Ca^{2+}-dependent activation of PDE by calmodulin.

PDE activity was measured in the presence of varying amounts of calmodulin. Results are expressed as % stimulation of the PDE activity in controls with no added calmodulin (30 pmole/min). The closed circles refer to assays conducted in the presence of Ca^{2+} (0.05 mM) and the open circles are values obtained in the absence of added Ca^{2+} and the presence of EGTA (1 mM).

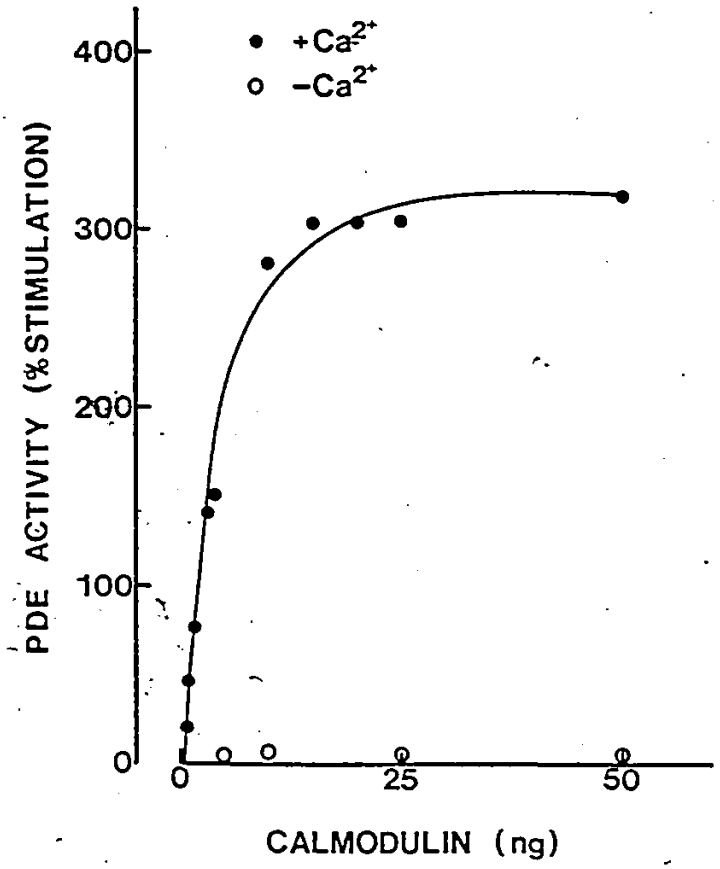


Fig. 4. Effect of trifluoperazine on calmodulin-activated PDE.

The activity of PDE stimulated by 50 ng calmodulin was measured in the presence of Ca^{2+} (0.05 mM) and of varying concentrations of TFP. Results are expressed as % stimulation of the PDE activity in controls with no added calmodulin (28 pmole/min).

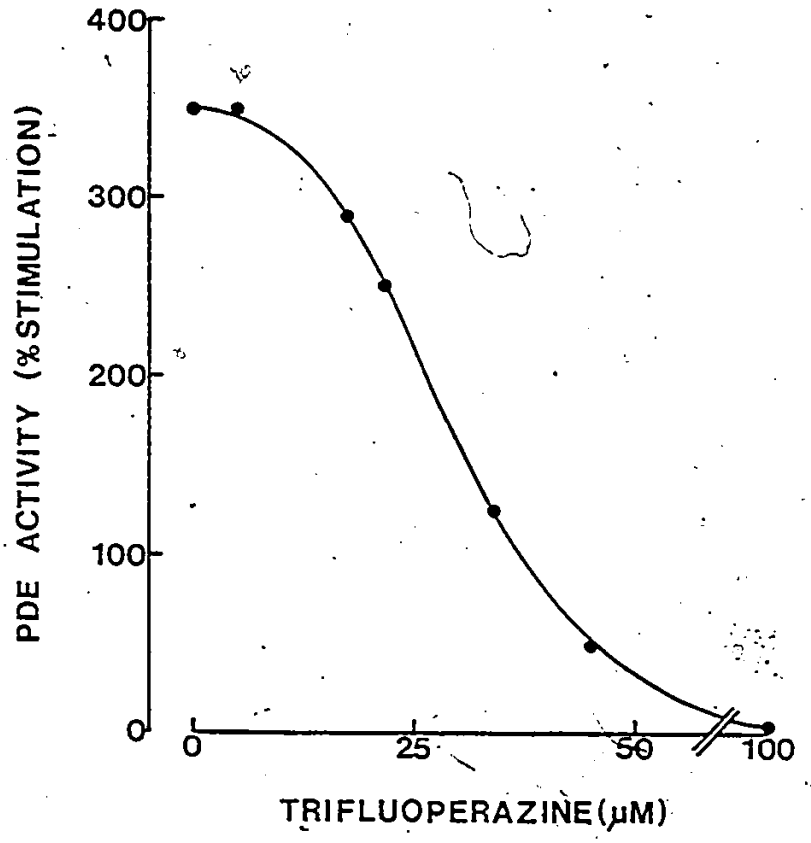
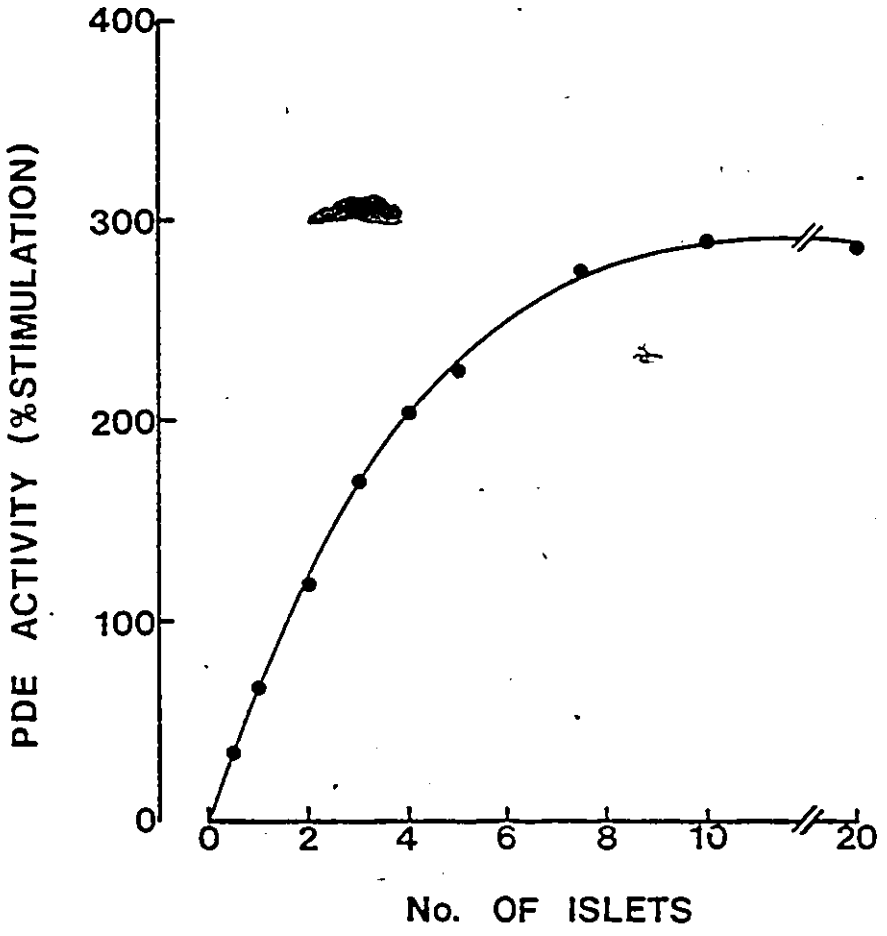


Fig. 5. Activation of PDE by islet extracts.

The effect of varying amounts of islet extract, equivalent to the number of islets shown, was tested on the activity of PDE. Results are expressed as % stimulation of the PDE activity in presence of Ca^{2+} and in absence of added extract (35 pmole/min). The results are the mean values for 2 separate preparations of extract from rat islets.



islet extract required to stimulate PDE in the range where activation was proportional to the amount of islet extract used.

IX. C. Radioimmunoassay of calmodulin

The radioimmunoassay of calmodulin was performed as described by MacManus et al (1981) using an antiserum raised in sheep against performic acid oxidized (Van Eldik and Watterson, 1981) rat testis calmodulin (MacManus, 1979) and [^{125}I]-calmodulin iodinated by the Bolton-Hunter procedure (Chafouleas et al, 1982). The purified calmodulin used as standard in the phosphodiesterase assay was also used as the standard for the RIA. Essentially the radioimmunoassay was performed in a final volume of 500 µl RIA buffer [75 mM NaCl, 1 mM EGTA, 125 mM Tris, 20 µg/ml bovine serum albumin (pH 8.4)] containing 40,000 cpm ^{125}I-labeled CaM, 2 µg anti-CaM, and standard amounts of CaM, or tissue extracts. The tubes were incubated at room temperature for 2 h, and then at 4°C for 20 h before precipitation of the immune complex with 1 ml Pluronic F-38 (BASF, Rexdale, Ont.) at a final concentration of 14% (w/v) following centrifugation at 6000 X g for 30 min. The precipitates were counted in a Beckman Biogamma counter.

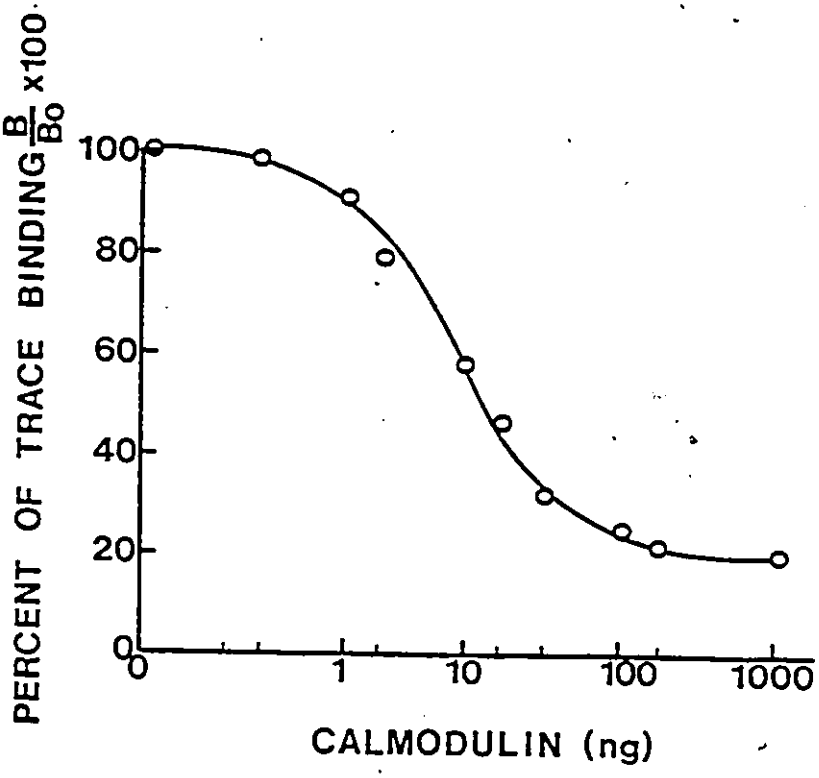
A typical standard curve is illustrated in Fig.6. Half maximal displacement of the ^{125}I-calmodulin occurred at 10 ng calmodulin.

X. Statistical Analyses

Results are expressed as mean ± SEM. For the *in vivo* experiments n=number of animals, while for the *in vitro* experiments, n=number of batches of islets. The day-to-day variation in the total amount of insulin secreted by different batches of islets studied under the same conditions was assessed by calculation of the coefficient of variation. The day-to-day variation was found to be less than 5%; the results could therefore be grouped. Other statistical tests were used where applicable: Student's t

Fig. 6. Calmodulin radioimmunoassay standard curve.

The assay was conducted as described in the method section. B_o represents the radioactivity (cpm) precipitated when no standard calmodulin was added to the antibody mixture. B represents the amount of radioactivity precipitated when known amounts of standard calmodulin were added.



tests for paired and unpaired samples, linear regression analysis, analysis of variance, and the Kolmogorov-Smirnov two-sample one-tailed test, as indicated in the legends to Tables and Figures.

CHAPTER 3. AN IMPROVED DOUBLE ANTIBODY RADIOIMMUNOASSAY FOR THE DETERMINATION OF INSULIN.

Since the pioneering work of Berson and Yalow, a number of immunoassays for insulin have been developed (Yalow, 1980). In spite of these developments, there is no general agreement in the literature on insulin immunoassay procedures which would give "real" values nor on the problem in obtaining the values (Ashby and McKechnie, 1980; Grant, 1968, 1972; Henderson, 1970, 1971; Malvano et al, 1974; Orosz et al, 1971; Spellacy and Buhi, 1971; Thorell and Lanner, 1973; Yalow, 1980) . Problems encountered with methods currently in use include the inhibition of the action of the precipitating antibody by human serum, leading to falsely elevated results (Hales and Randle, 1963; Morgan and Lazarow, 1963). As well, falsely lowered values may occur if the precipitation of the insulin antiinsulin complex by anti-y-globulin is too rapid (Grant, 1972).

Interaction of antibodies with y-globulin (Grant, 1968; Kuzuya and Samols, 1964) or complement (Morgan et al, 1964a, 1964b) have been blamed for these discrepancies. The addition of heparin (Soeldner and Slone, 1965) or EDTA (Morgan et al, 1964a, 1964b) was, at one time, promoted as a solution to the problem. Both substances were subsequently shown to contribute to the error (Grant, 1972; Henderson, 1970). There is general agreement that extended incubation periods minimize the inhibitory interactions (Soeldner and Slone, 1965) but these extended times are inconvenient and inefficient. In the clinical laboratory, methods other than double antibody techniques are now widely used (Parker, 1981; Yoshioka et al, 1979). They have the disadvantage of being very costly for use as a routine assay. Because of this, the method of Morgan and Lazarow (1963) is still widely used in research (two hundred and forty citations between 1977 and 1980). The method developed for this work allows the processing of

numerous samples at minimum cost and in relatively short time. This method involves a technical modification which improves the precision and eliminates the "inhibitory" effect of serum and of anticoagulants in the samples tested.

A. Methods

Guinea-pig AIS was diluted so as to give 50% precipitation of radioactivity when no standard insulin was added. The batch used for the present study required a final dilution of 1:1,200,000. A 1:90 final dilution of the AGG-precipitating serum was used in our method while it was found that, to obtain the same degree of precipitation, a 1:50 final dilution was required in the method of Morgan and Lazarow (1963). Normal guinea-pig serum was used at a final dilution of 1:800. All solutions and final dilutions were prepared using 0.04 M sodium borate buffer (pH 8.0) containing 1% BSA (borate-BSA buffer).

Samples used to verify the technique

After an overnight fast, venous blood samples (a.c. samples) were taken from 14 healthy volunteers in 3-cm^3 Vacutainer tubes containing either potassium EDTA (1.5 mg/ml blood), lithium heparin (47.6 USP/ml blood), or no anticoagulant. Similar samples were obtained from the same subjects 1.5h after a meal containing approximately 100 g carbohydrate (p.c. samples).

Mice were killed by decapitation and the blood was collected into heparinized test tubes. Alternately, in some cases, blood was collected from a tail vein into a heparinized capillary tube (85 ul). The plasma was used for the determination of insulin.

Media consisting of Krebs-Ringer bicarbonate buffer containing 0.1% BSA and the compounds used to modulate insulin secretion (glucose, glucagon, aminophylline, and TFP) were also assayed.

Islet cells were extracted using a standard method (Davoren, 1962). These extracts were diluted 1:10 with the borate-BSA buffer before being analyzed for their insulin content.

For recovery experiments, 50 µl of plasma and serum or 10 µl of incubation and extraction media were assayed in the presence and in the absence of the appropriate standard insulin in amounts varying between 100 and 800 pg. The volumes of the unknown samples chosen for these assays represented upper limits of volumes used experimentally.

Assay procedures

All assays were carried out in triplicate, care being taken to ensure that the blood samples were not hemolyzed. The two assay methods used were the method of Morgan and Lazarow (1963) taken as the reference method (method R) and our method which combines features of the methods of Hales and Randle (1963) and Morgan and Lazarow (1963) (method A). Table 5 gives a comparison between the procedures of Hales and Randle (1963), Morgan and Lazarow (1963) (method R), and method A.

B. Results

For method A, all assays were done at 4 °C in borate buffer (pH 8.0), containing 1% BSA. This buffer was chosen over the sodium phosphate buffer (pH 7.4) used by Hales and Randle (1963) since preliminary experiments had demonstrated that the phosphate buffer did not produce optimal precipitation and thus the insulin bound to antibody could not be separated by centrifugation, whereas with the use of borate buffer, centrifugation could be used. Similar results have been reported by Soeldner and Slone (1965).

For method A, the antibodies were preprecipitated as suggested by Hales and Randle (1963). In addition, normal GPS was added to absorb nonspecific antibodies, thus obviating some of the problems encountered by

Hales and Randle (1963) and others (Grant, 1968; Kuzuya and Samols, 1964; Morgan et al, 1964a, 1964b). As can be seen in Figs 7 and 8, with both human and rat insulin standards the standard curve for method A had a steeper slope than that of the method R, allowing more precise measurements. The whole range of the standard curve (Ø to 3.2 ng) could be used, while the curve obtained with method R was acceptable only in the much narrower range of Ø and Ø.4 ng.

Because there exists much confusion in the literature about the appropriate type of blood sample to use for insulin determination (Grant, 1968, 1972; Henderson, 1970, 1971; Orosz et al, 1971; Spellacy and Buhi, 1971; Thorell and Lanner, 1973), a study was conducted to determine if any differences could be found between groups of samples treated in different ways. The results of these analyses are given in Table 6. Using method R, no precipitation of the insulin-antibody complex was found when EDTA was used as the anticoagulant. This was true at low and high insulin levels. With method A, no such effect of EDTA was encountered and both plasma samples (lithium heparin and EDTA) behaved in exactly the same way. There were no significant differences between the treatments. With paired data analysis, there was a significant difference ($P<Ø.Ø2$) between serum and plasma samples, serum samples giving consistently higher results than plasma. The heparin plasma values in both the a.c. and p.c. samples were correlated with the serum values. The correlation coefficient was 96% and the regression line was: plasma = 1.Ølserum - Ø.2.

The addition of EDTA (Ø.Ø1 M) has been suggested as a means of improving the precipitation of the insulin-antibody complex (Morgan et al, 1964a, 1964b). The results presented in Table 6 indicate, however, that in method R, the use of EDTA actually decreased the values at low insulin levels, whereas there was no significant difference at high insulin values.

Using method A, the addition of EDTA did not produce significant differences in the insulin levels obtained in any of the groups.

Studies were also conducted using method A to determine the recovery of insulin when standard insulin plus a known volume of insulin-containing plasma or serum were added. These experiments were done using samples of human blood. The results presented in Fig. 9 show a highly significant correlation ($P<0.001$) between the theoretical and the experimental results. Similar results were obtained using mouse plasma, islet cell extracts, and islet incubation media ($r=0.999$, $P< 0.001$).

TABLE 5. Comparison of insulin assay techniques

Hales and Randle (1963)	Method A	Morgan and Lazarow (1963)
Mix: AIS (50 μL) AGG (50 μL)	Mix[a]: AIS (300 μL) AGG (100 μL) GPS (100 μL)	Mix: Standard insulin or sample (300 μL) [^{125}I]Insulin (100 μL) AIS (400 μL)
↓ Incubate 16–24 h	↓ Incubate 24 h	↓ Incubate 48 h
Add: Standard insulin or sample (100 μL)	Add: Standard insulin or sample[b] (300 μL)	Add: AGG (100 μL) GPS (100 μL)
↓ Incubate 6 h	↓ Incubate 6 h	↓ Incubate 72 h
Add: [^{125}I]Insulin (100 μL)	Add: [^{125}I]Insulin (100 μL)	
↓ Incubate 16 h	↓ Incubate 16–18 h	
Filter (cellulose acetate) Wash tube and membrane Count	Centrifuge at 2000 × *g* Decant, wipe tubes Count precipitate	Centrifuge at 2000 × *g* Decant, wipe tubes Count precipitate
Buffer: BSA (0.9%) – $NaPO_4$, pH 7.4	BSA (1%) – sodium borate, pH 8.0	BSA (1%) – sodium borate, pH 8.0

[a]The mixture of AIS, AGG, and GPS was freshly prepared in bulk in the amount necessary for each assay and 500 μL of the mixture was added to individual tubes before the first incubation.

[b]The volumes of unknown serum or plasma samples used varied between 10 and 50 μL. The remainder was made up with buffer. Other details were as described in Materials and methods.

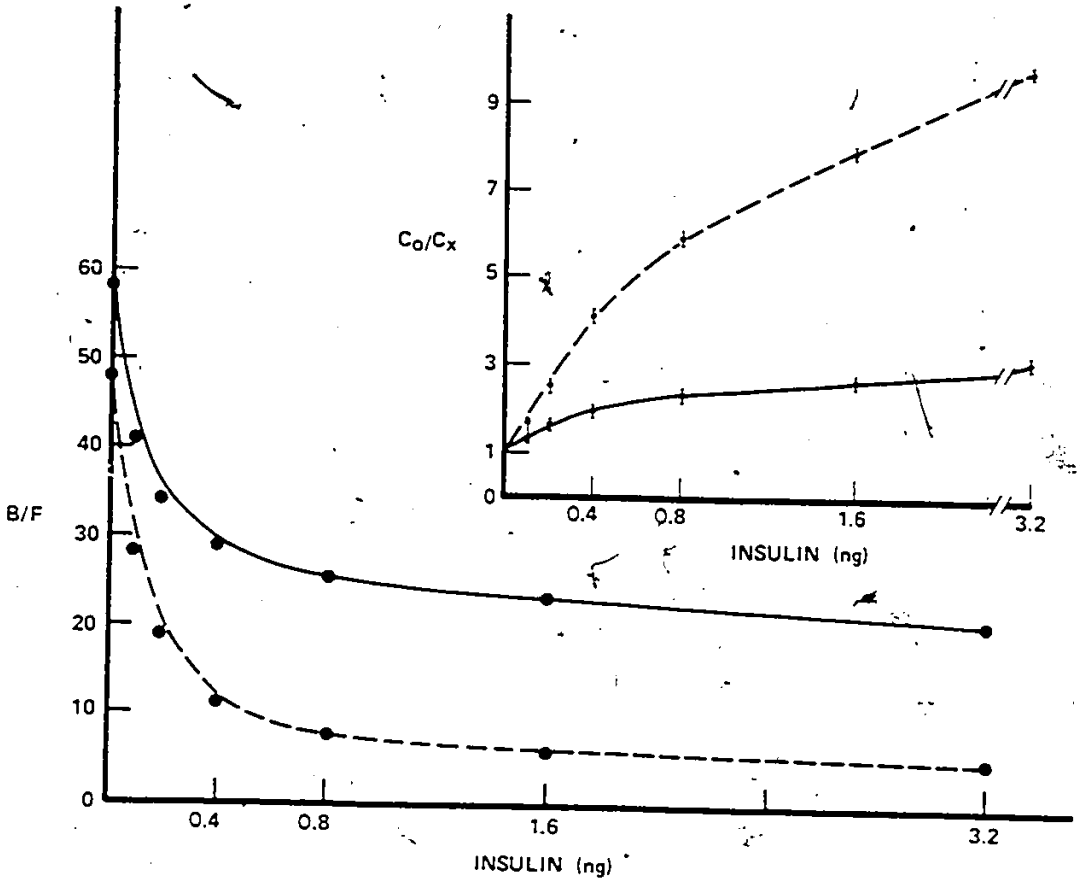


FIG. 7 . Comparison of insulin assay curves using a human insulin standard. The assays were conducted as described in Materials and methods and in Table 5 . C_o represents the radioactivity (cpm) precipitated when no standard insulin is added to the mixture of antibodies. C_x represents the amount of radioactivity (cpm) precipitated when known amounts of standard insulin are added to the antibody mixture. B represents cpm bound to antibody and F represents total counts. ●———●, method R; ●– – –●, method A.

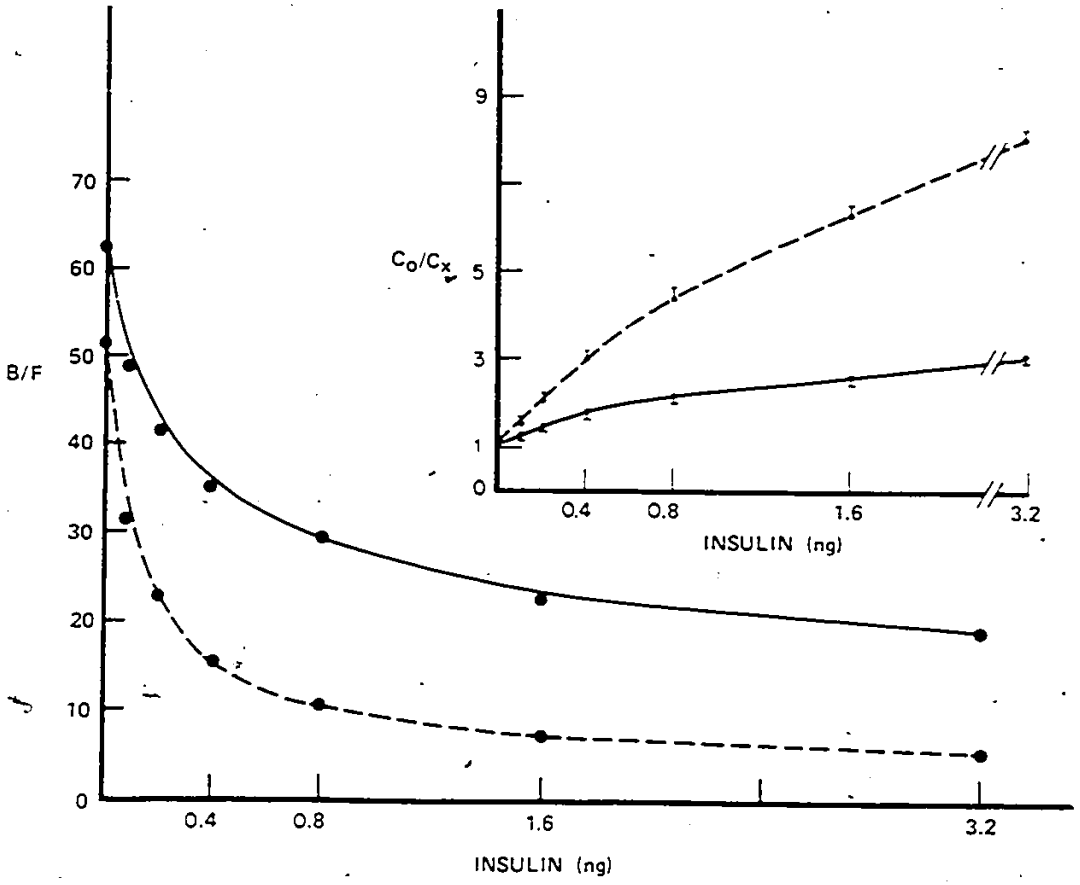


FIG. 8. Comparison of insulin assay curves using a rat insulin standard. The assays were conducted as described in Materials and methods, and in Table 5. Symbols are as for Fig. 7.

TABLE 6. Insulin levels measured by different methods in human subjects

Sample	Insulin (ng/mL) Lithium-heparin plasma	EDTA plasma	Serum
	a.c. levels		
Method			
R	0.51±0.07	*	0.57±0.06
R + EDTA	0.38±0.07	*	0.36±0.07
A	0.49±0.05	0.43±0.05	0.70±0.07†
A + EDTA	0.54±0.03	0.58±0.05	0.68±0.05
	p.c. levels		
R	1.93±0.18	*	1.92±0.16
R + EDTA	1.70±0.26	*	1.75±0.23
A	1.49±0.18	1.49±0.15	1.65±0.16†
A + EDTA	1.51±0.17	1.63±0.15	1.69±0.17

NOTE: Blood samples were obtained from the same 14 human subjects after a 12-h fast and 1.5 h following a meal containing at least 100 g of carbohydrate. The samples were obtained and treated as described in Materials and methods. Method R refers to the method of Morgan and Lazarow (1963). Method A is the method under study. EDTA when added to the assay mixture was present at a final concentration of 0.01 $mol \cdot L^{-1}$ and was added at the same time as the standard or sample. Results are expressed as means ± SEM.

*No radioactive precipitate was recovered under the conditions used.

†A significant difference ($P < 0.02$) between serum and plasma samples on the basis of paired *t*-tests.

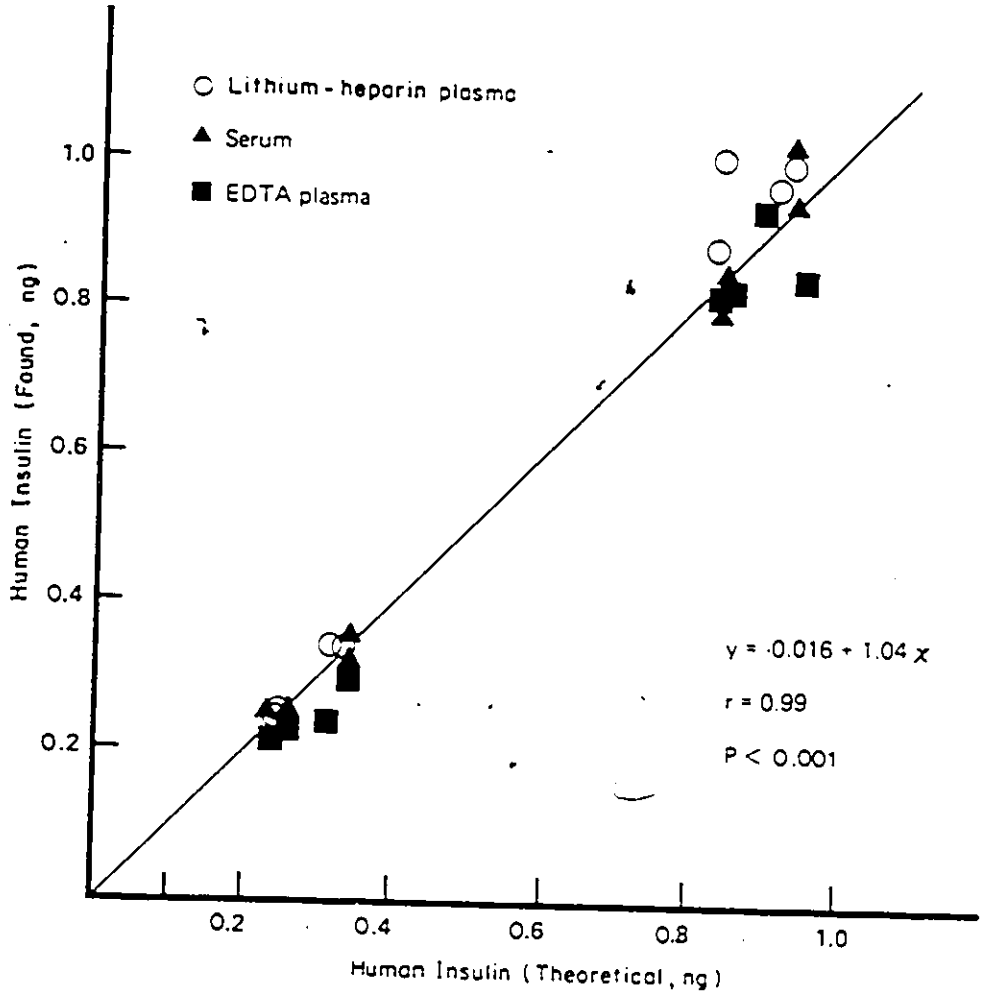


FIG. 9. Recovery of standard insulin added to serum or plasma samples. ○, lithium-heparin plasma; ■, EDTA plasma; ▲, serum. Known amounts of standard human insulin were added to serum or plasma samples which were analyzed alone in parallel. The assays were done using method A. The results were analyzed by linear regression. $y = 0.016 + 1.04x$, $r = 0.99$, $P < 0.001$.

C. Discussion

It has been shown previously (Rodbard et al, 1971; Yalow, 1980) that nonequilibrium methods of radioimmunoassay tend to improve the sensitivity of these assays. This principle was used in the insulin assay described in this chapter (method A) which also offers several advantages over methods widely used in research (Hales and Randle, 1963; Morgan and Lazarow, 1963). The incubation time is relatively short and the insulin-antibody precipitate can be easily separated by centrifugation at low speed, as long as borate buffer rather than phosphate is used as the assay buffer. Insulin levels can be measured precisely and reliably from serum and also from plasma samples obtained by the addition of either heparin or EDTA as anticoagulants. The use of additional EDTA, which had previously been recommended (Morgan et al, 1964a, 1964b), was found to be unnecessary. While serum samples gave higher values, the relationship between the types of samples is constant so that the use of either type of sample will give consistent results. This is important, as the ability to use plasma is of value when only small volumes of blood are available. From the recovery experiments, it is not possible to decide that one type of sample is superior to the other in giving the "true value". Because of its high degree of precision, this assay is particular well suited for limited sample sizes as in infants or in small laboratory animals.

CHAPTER 4. EFFECT OF OXYTETRACYCLINE TREATMENT ON THE RESPONSE TO INSULIN IN THE SPONTANEOUSLY DIABETIC BB RAT.

In an efffort to study some of the factors contributing to the syndrome of diabetes of the BB rat, I attempted to find out whether chronic treatment of genetically diabetic rats with the antibiotic oxytetracycline would result in improvement of their diabetic state. Oxytetracycline has been found to affect glucose metabolism and the action of insulin. In both humans and animals that received either tolbutamide or exogeneous insulin, oxytetracycline potentiated the action of insulin (Hiatt and Bonorris, 1970; Hiatt et al, 1966; Miller, 1966). It was also found in other studies (Begin-Heick and Heick, 1976; Begin-Heick et al, 1974, 1979; Dubuc and Willis, 1978) that OTC administration resulted in a reduction of the severity of many of the metabolic abnormalities shown by the ob/ob mouse. In view of these findings, experiments were designed to further elucidate the extrapancreatic action of oxytetracycline by studying its effect in genetically diabetic rats.

A. Methods

Care and maintenance of the diabetic animals

This was done in accordance with a protocol designed by the Animal Resources Division, Health and Welfare, Canada. Each day between 8:30 and 11:00 h, the diabetic animals were weighed and a fresh urine sample was tested for urinary ketones (Ketostix™) and glucose (Tes-Tape™) levels. Protamine-zinc insulin was then administered subcutaneously. The diabetes in these animals is extremely brittle. The dosage of insulin is therefore adjusted daily to achieve three goals: maintain body weight, keep urine free of ketones, and allow some glycosuria to prevent hypoglycemic reactions. The rats were kept under observation for at least 1 wk to stabilize them prior to the experiments (pretreatment period). At the

beginning of the pretreatment period, the rats were assigned randomly to each group.

Experiment I

Diabetic and normal rats were divided into treated and control groups. The treated groups received 100 mg/kg oxytetracycline (Terramycin) intramuscularly. The treatments were given daily between 8:30 and 11:00 h.

On day 8 of treatment, a glucose tolerance test was done. The animals were fasted 12 h overnight from 21:00 h on day 7. The following day, blood samples were withdrawn from the tail vein (0 time) and the animals then received glucose (1 g/kg body wt.) by intraperitoneal injection. Blood samples were withdrawn at 30, 60, and 120 min following the injection of glucose.

Experiment II

This was done using Liquamycin-LP (Rogar/STB) a preparation of oxytetracycline for veterinary use. This allowed the injection of much smaller volumes of the drug and caused less discomfort to the animals. Other conditions were as in experiment I.

On day 7 of treatment, the diabetic rats received only one-half of the insulin dose that they had received the previous day. Between 8:30 h and 9:00 h the following day, they were given crystalline zinc insulin (0.5U/kg body wt.) by subcutaneous injection after a blood sample had been withdrawn from the tail vein (0 time sample). Samples were withdrawn at 15, 30, 60, and 120 min following the administration of insulin.

This protocol was adopted as prior experiments had shown that injection of the full dose of Protamine-zinc insulin, followed by a 12-h overnight fast and the injection of the test dose of crystalline insulin led to very rapid induction of hypoglycemia and convulsions particularly in the oxytetracycline-treated animals.

Experiment III

This was designed to test whether the effects of oxytetracycline might not in part be due to the effect of the drug on food consumption. Oxytetracycline was administered as Liquamycin, as in experiment II. The saline-treated group (controls) were fed preweighed amounts of food. The cages were checked every morning to ensure that all the food had been consumed. The amounts of food given each day were based on food consumption patterns of treated animals in experiments I and II. Analysis of variance did not reveal any significant differences in food intake between the treated animals and the food restricted controls (cf. Table 7). On day 7 of treatment, the animals were fasted 12 h overnight and a glucose tolerance test was done the next morning as described for experiment I. No groups of nondiabetic animals were included in this experiment.

In all three experiments, after the test performed on day 8 (glucose and insulin tolerance) the animals were fed and the various treatments continued. On day 10, the animals were killed and the blood was collected.

Other methods were as described in Chapter 2.

B. Results

B. 1. Experimental groups and drugs

The experiments described in this paper were done over a 2-yr period, using different groups of diabetic rats as they were made available to me. Two different forms of the drug were also used for treatment. To allow comparisons of the data, analyses of variance were performed to assess the possible influence of these two variables of time and drug: (1) Variations between groups: On the last day of the pretreatment period, no differences were found among the three different experiments as to food consumption (Table 7), body weight (Table 8), and insulin dosage (Table 9). This indicated that the groups of rats were comparable one with the other before

any treatment was administered. (2) Effect of oxytetracycline as Terramycin vs. Liquamycin: In experiments I and II, there are no significant differences in food consumption (Table 7), body weights (Table 8), or dose of insulin (Table 9) at the end of the treatment between the animals treated with Terramycin (experiment I) and those treated with Liquamycin (experiment II). In fact, of the three parameters studied in these two groups, only the amount of food consumed by the treated animals was significantly different from the control and pretreatment values. The drug treatment had similar effect on the food consumption of the normal rats.

B. 2. Insulin dosage

These were administered according to the protocol described in Methods. In experiments I and II, comparable insulin dosages were given to the control and treated groups. This resulted in lower urinary glucose output in the treated animals (Ø to +1) than in the control animals (+2 to +4). In experiment III, consequent to the restriction of food, the control animals required less insulin to maintain the urinary glucose output between +2 and +4. To keep the urinary glucose within approximately the same range in the treated rats, the doses of insulin had to be decreased even further in that group.

B. 3. Glycemia

In the diabetic animals, both forms of oxytetracycline decreased the plasma glucose levels in the fasted and fed states (Table 1Ø). The effect of Liquamycin (experiments II and III) was greater than of Terramycin in the fed animals. In the normal fed rats, there was a small but significant decrease in plasma glucose levels with the drug treatment. To differentiate between the effect of the drug itself and its effect on food consumption, a food-restricted control group was studied (experiment III).

Food restriction alone did not ameliorate the diabetic state to the

same degree as the oxytetracycline treatment. Thus, the glycemia was higher in the control group in both the fed and fasted state (Table 10). Figure 10 summarizes the relationship between plasma glucose and amounts of insulin administered in the animals used for experiment III. On the last day of the preexperimental period (Fig. 10A), the mean plasma glucose values of the two groups were not significantly different from each other. The mean dose of insulin given to the two groups was also not significantly different. On the last day of the experimental period, the insulin dosage was reduced in both groups but significantly more in the treated group. In spite of smaller insulin doses, the treated group had a significantly reduced glycemia, whereas the glycemia in the control group was at the same level as in the pretreatment period. In the fasted state (Fig. 10B), insulin doses that were not significantly different in the two groups produced a significantly lower glycemia in the treated group. Only when the treated rats were given no insulin were the mean plasma glucose levels equal in the control and the treated groups (Fig. 10C).

B. 4. Effect of oxytetracycline treatment on glucose tolerance

The results of glucose tolerance are given in Figures 11 and 12. The results given in Fig. 11 show a striking difference in the response to glucose of the control and oxytetracycline-treated diabetic rats. In the control diabetic rats, fasting glucose levels were predictably high and rose to values around 400 mg/dl 30 min after the administration of glucose. There was no significant decrease from that peak value during the 2-h of the experiment.

In the case of the oxytetracycline-treated diabetic rats, there was also a substantial increase provoked by the glucose load; however, the decline of plasma glucose value was rapid. The rate constant from peak glucose value at 30 min to minimal glucose level at 120 min was 3.61 s^{-1}

for the oxytetracycline-treated, as opposed to 1.88 s^{-1} for the control diabetic animals (see Table 11) and the levels had returned to normal at 2h. It should be noted that there was a large difference between the plasma glucose levels of the control and treated diabetic rats at all the times studied. By comparison, both groups of normal rats responded similarly to the glucose load. In this experiment (experiment I), Terramycin was used as the source of oxytetracycline and the dosage of insulin throughout the experimental period had been maintained at levels close to those given during the pretreatment period. As a result of the vigorous insulin treatment, the fasted plasma glucose of the treated group was significantly lower than that of the controls.

In experiment III, in spite of the drastic reduction in insulin dosage and the reduction in food intake of the control group, the 0-time plasma glucose levels were still significantly different in the control and the treated group (Fig. 12). In the control rats, the plasma glucose at 120 min was not significantly different from that at peak glucose concentration. In the treated rats, the glucose concentrations at 120 min were significantly lower ($P<0.05$) than the peak concentrations.

Both groups of diabetic rats weighed less at the end of experiment III, although the food-restricted rats lost significantly ($P<0.05$) more weight than the drug-treated rats (41.4 ± 3.5 g vs 28.2 ± 4.5 g, respectively). This decrease in body weight may be the result of the lower amounts of insulin administered.

B. 5. Effect of oxytetracycline treatment on insulin tolerance

The results of the insulin tolerance test are given in Fig. 13. The oxytetracycline treatment produced little change in the response of the normal rats to insulin with the exception of the sample obtained at 120 min. Whereas at that time, plasma glucose was already going back to

starting values in the normal controls, it remained similar to the 60-min value in the normal rats that had been treated with oxytetracycline. In the diabetic rats, the glucose values were significantly lower in the oxytetracycline-treated group than in the controls, at all the times studied. The absolute values of plasma glucose were markedly different at 0 time between the control and treated diabetic rats and between both groups of diabetic rats and normal rats. To obviate these differences, the rate constants for disappearance of glucose between times 0 and 30 min were calculated. These were found to be significantly different in the control than in the treated groups for both the normal and diabetic animals (Table 11). These data indicate that previous oxytetracycline treatment increased the rate at which glucose was cleared from the circulation following a single injection of a small dose of insulin.

TABLE 7
Food consumption in control and treated diabetic and normal rats

	Food consumed (g)			
	Diabetic		Normal	
	Control	Treated	Control	Treated
Experiment I				
Before	31 ± 1.8*	32 ± 1.4*	—	—
After	27 ± 2.7* (5)	18 ± 2.3† (5)	27 ± 0.8* (5)	17 ± 1.9† (5)
Experiment II				
Before	32 ± 2.5*	26 ± 1*	27 ± 0.2*	27 ± 0.3*
After	26 ± 1.5* (5)	17 ± 1.8† (5)	24 ± 0.9* (5)	14 ± 1.2† (5)
Experiment III				
Before	29.4 ± 1.3*	29 ± 2.5*	—	—
After	18 ± 0.5† (5)	15 ± 1.7† (6)	—	—

The experiments were as described in METHODS. The terms "before" and "after" refer to the food intake on the day immediately preceeding the beginning of the treatment and on the last (10th) day of the treatment period. Each value represents mean ± SEM for the number of animals given in parentheses. Values not followed by the same superscript are significantly different ($P < 0.05$).

TABLE 8
Body weight of control and treated diabetic and normal rats

	Diabetic		Normal	
	Control	Treated	Control	Treated
Experiment I				
Before	418 ± 10.3	392 ± 18	395 ± 6	374 ± 1
After	418 ± 9.5	381 ± 15	395 ± 7	365 ± 13
	(10)	(12)	(5)	(5)
Experiment II				
Before	453 ± 8.5	445 ± 12	483 ± 8	476 ± 7
After	459 ± 8.4	439 ± 12	491 ± 8	463 ± 7
	(8)	(10)	(5)	(5)
Experiment III				
Before	429 ± 15	435 ± 19.3	—	—
After	387 ± 14†	405 ± 19.3*	—	—
	(5)	(6)		

The experiments are described in detail in METHODS. The terms "before" and "after" refer to body weights on the day immediately preceding the beginning of the treatment period and on the last day (10th) of the treatment period. Analysis of variance revealed no difference in the pretreatment weights of animals in any given experiment. There were no significant differences between the pretreatment weights of any of the diabetic groups. Based on paired *t* tests, there were no significant differences between pre- and posttreatment weights in experiments I and II.

* $P < 0.005$.

† $P < 0.001$.

Values are means ± SEM for the numbers of animals given in parentheses.

TABLE 9
Insulin dosage administered to control and treated diabetic rats

	Insulin (U/100 g body wt.)	
	Control	Treated
Experiment I		
Before	0.66 ± 0.06*	0.83 ± 0.04*
After	0.72 ± 0.05*	0.79 ± 0.08*
	(10)	(12)
Experiment II		
Before	0.74 ± 0.08*	0.85 ± 0.09*
After	0.66 ± 0.08*	0.82 ± 0.11*
	(8)	(10)
Experiment III		
Before	0.88 ± 0.06*	0.74 ± 0.04*
After	0.38 ± 0.05†	0.23 ± 0.05‡
	(5)	(6)

The experiments were as described in METHODS. The terms "before" and "after" represent the values obtained on the day immediately prior to the beginning of the treatment period and on the last (10th) day of the treatment period, respectively. Values given are means ± SEM for the numbers indicated in brackets. Values not followed by the same superscript are significantly different ($P < 0.05$).

TABLE 10
Glucose levels in fed and fasted control and treated rats

	Glucose (mg/dl)					
	Diabetic			Normal		
	Fasting		Fed	Fasting		Fed
Controls (Exp. I & II)	200 ± 37*	(13)	391 ± 28*	110 ± 3*	(10)	118 ± 5*
Food restricted controls (Exp. III)	322 ± 62*	(5)	402 ± 70*	—		—
Treated						
Exp. I	82 ± 10†	(12)	252 ± 42†	106 ± 3*	(5)	107 ± 4†
Exp. II	90 ± 15†	(5)	120 ± 12‡	—	(5)	95 ± 2†
Exp. III	117 ± 24†	(6)	152 ± 30‡	—		—

The experiments were as described in METHODS. The fasting glucose levels were obtained on day 7 after the beginning of the treatment in experiments I and II and on day 8 in experiment III. The glucose levels in the fed animals were obtained at 9:00 h on day 10 of treatment. Values are means ± SEM for the number of animals given in parentheses. Values in the same column not followed by the same superscript are significantly different ($P < 0.05$).

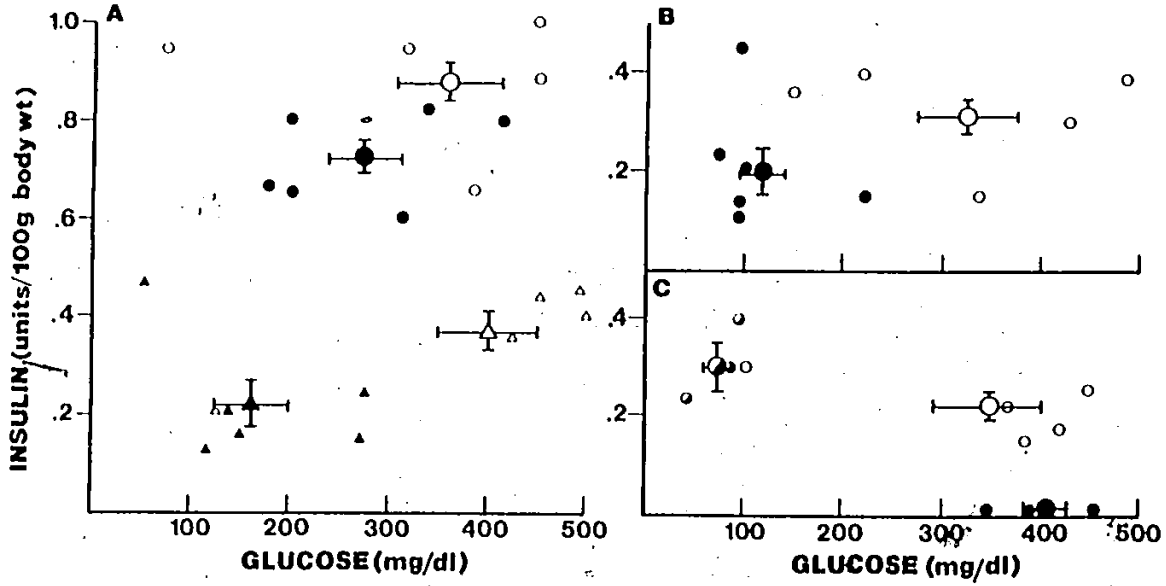


FIGURE 10. The relationship between insulin dosage and plasma glucose levels in food restricted control and in oxytetracycline-treated rats. Glucose samples were withdrawn from the tail vein and analyzed for glucose as described in CHAP.e. Open symbols represent food-restricted controls and closed symbols oxytetracycline-treated rats. Panel A: ○, ●, levels on the day immediately preceeding the treatment period; △, ▲, levels on the last (10th) day of treatment. Panel B: levels following a 12-h fast on day 8. Panel C: levels on day 7; ○, control rats; ● and ◑, treated animals. Large symbols represent mean values ± SEM for each of the groups. The insulin values shown are those given 24 h before samples were taken for glucose determinations.

Fig. 11. Change in glucose tolerance of diabetic rats following oxytetracycline treatment.

Rats were treated with OTC for 7 days; they were then fasted overnight from 21:00 h to 9:00 h, as described in the method section under experiment I. After removal of a 0-time sample, glucose (1 g/kg body wt.) was injected intraperitoneally, and blood samples were removed at the times indicated. The plasma was separated by centrifugation and the glucose was measured. Each point represents the mean ± SEM for the number of animals given on the figure. Significant differences ($P<0.05$) between control and treated groups are indicated by an asterisk.

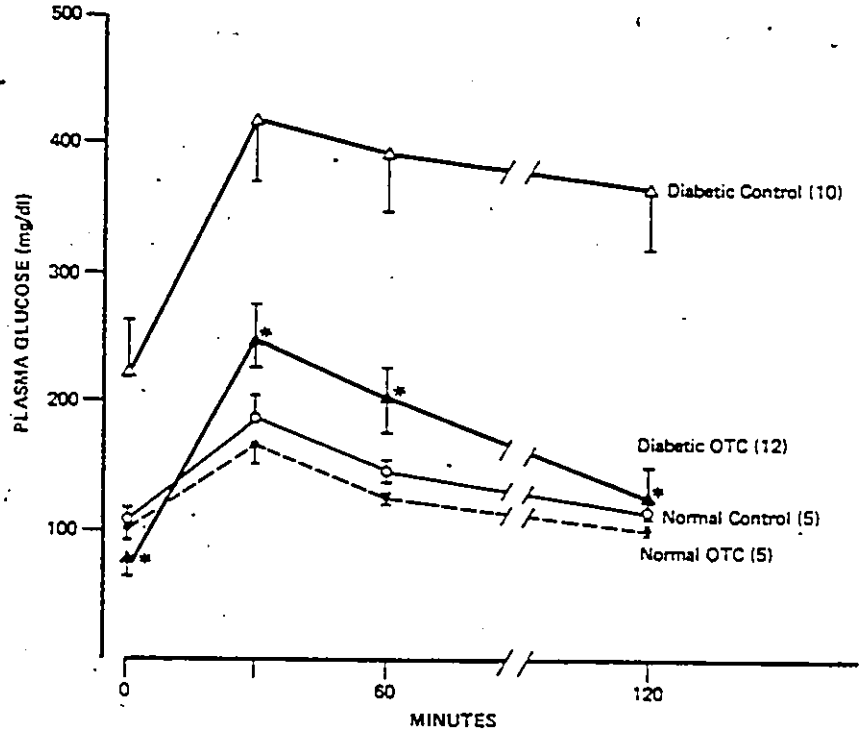


Fig. 12. Comparison of glucose tolerance in control, food-restricted control, and oxytetracycline-treated diabetic rats. Circles represent the result of glucose tolerance tests from experiment III. Triangles represent the values for the glucose tolerance test of experiment I. Open symbols represent the values obtained in control rats; closed symbols represent the values obtained in treated rats.

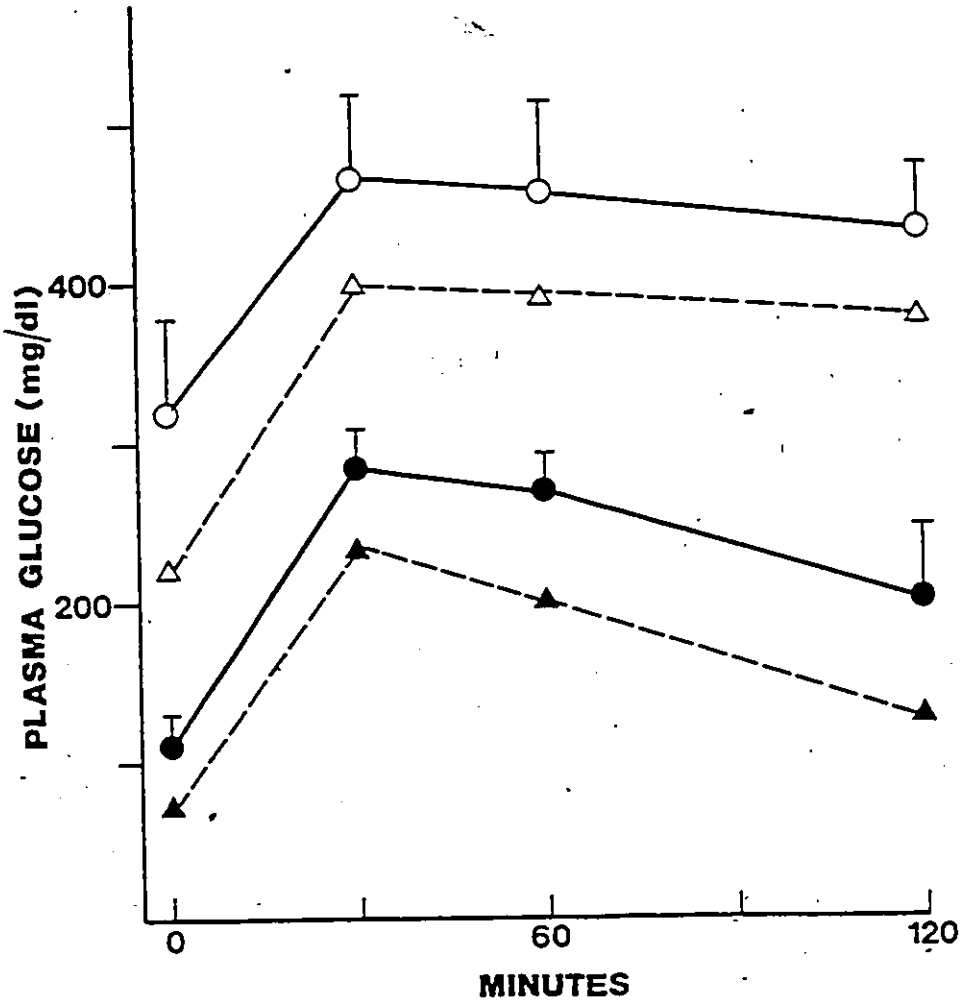


Fig. 13. Change in response to insulin in normal and diabetic rats following oxytetracycline treatment.

Rats were injected with OTC or saline for 7 days. On day 7, the diabetic rats were given one-half their normal insulin dose and they were given food ad libitum. On day 8, the insulin tolerance test was performed as described in the method section under experiment II. A significant difference ($P<0.05$ or less) between control and treated groups is indicated by an asterisk. Each point represents the mean ± SEM for five animals in all groups with the exception of the diabetic control group, which included four animals.

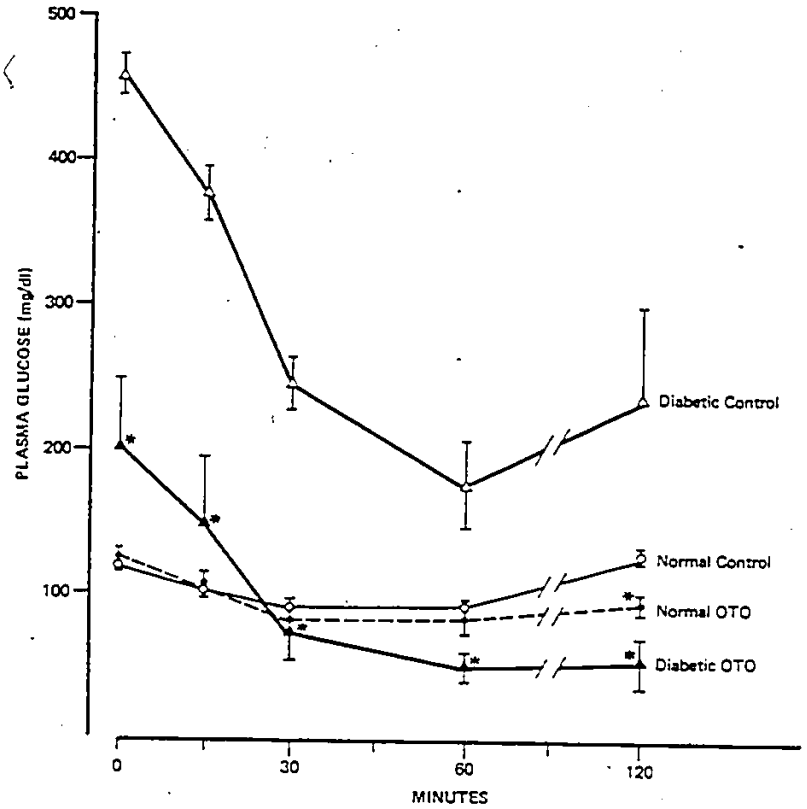


TABLE 11
Rate constants for the disappearance of glucose in control and OTC-treated rats following the injection of glucose (A) or insulin (B)

	Rate of glucose disappearance (K, s^{-1})			
	(A) 30–120 min post-glucose		(B) 0–30 min post-insulin	
Group	Normal	Diabetic	Normal	Diabetic
Control	2.51 ± .13	1.88 ± 0.21	0.53 ± 0.05	1.30 ± 0.19
OTC-treated	2.56 ± .05	3.61 ± 0.62	0.82 ± 0.08	2.00 ± 0.14
P	>0.05	<0.02	<0.05	<0.025

First order rate constants were calculated from the data in Figure 11(A) and Figure 13(B), respectively. Numbers given represent means ± SEM for the numbers of animals indicated in the legends to the figures.

C. Discussion

Many lines of evidence indicate that the antibiotic oxytetracycline potentiates the action of insulin. Previous studies (Bégin-Heick and Heick, 1976; Bégin-Heick et al, 1974, 1979) on the ob/ob mouse indicate that in that animal model, chronic treatment with oxytetracycline is able to reduce glycemia and insulinemia towards more normal values and also to decrease body fat and incorporation of $^{3}H_2O$ into lipids (a measure of the capacity to synthesize fatty acids de novo) in the liver and adipose tissue. The sensitivity of muscle to insulin and the number of insulin receptors in the liver were found to be largely restored by the treatment. Oxytetracycline treatment was also found to lead to regranulation of the B-cells and to normalization of the insulin secretory response (Bégin-Heick et al, 1979). Some of these results have since been confirmed by Dubuc and Willis (1978).

Because the ob/ob mouse has functional, albeit abnormal, islets of Langerhans, it is difficult in this model to assess whether the effects of oxytetracycline are pancreatic, extrapancreatic, or both. In vitro systems that involve the external addition of oxytetracycline are ruled out because tetracyclines are strong chelating agents toward the divalent cations that are necessary for many cellular functions (Albert and Rees, 1956; Weinberg, 1954).

The BB rat was used in this study because the syndrome is associated with very small and rare islets containing virtually no B-cells due to widespread insulitis leading to B-cell destruction (Nakhooda et al, 1977, 1978). In this model, oxytetracycline treatment also appeared to potentiate the effect of insulin, indicating that the antibiotic exerts some of its effects by acting on the insulin responsive tissues at the periphery. Plasma glucose levels were reduced significantly in both the fed and the fasted state as a result of the treatment. This was so, even when the

insulin dosage was reduced considerably and, as in experiment III, was significantly lower than in the control group.

Although oxytetracycline treatment reduced food intake significantly in the treated animals, reduction of food intake alone can not be responsible for the improvement of the diabetic status, since a food restricted group did not show the same improvement as the oxytetracycline-treated group. In fact, only by withholding insulin completely from the oxytetracycline-treated rats was it possible to produce comparable plasma glucose levels in the food-restricted control and treated groups.

In experiments I and II, similar doses of insulin were administered to all animals on the basis of their body weight. This led to differences in urinary glucose output between the control and the treated groups, the former always having higher level. In experiment III, the insulin dosages were adjusted so as to produce comparable glycosuria. This led to the administration of lower dosages of insulin to the oxytetracycline-treated rats.

The standard protocol for maintenance of the diabetic animals (see Methods) is based on daily assessments of glycosuria. Comparisons of glycosuria and glycemia indicated a poor correlation between the two measurements, which accounts for some of the intragroup variation (cf. Fig. 10).

Ideally, the glucose and insulin tolerance tests should have been performed in groups of animals with the same starting glycemia. This could have been achieved by either of three methods: (1) withholding insulin completely from the oxytetracycline-treated rats (cf. Fig. 10), (2) increasing the dose of insulin in the control rats, or (3) infusing glucose into the oxytetracycline-treated rats. Each of these methods in its own way would have caused further problems. If option (1) were used, the treated

animals would have been in a catabolic state compared with the control. The control animals are extremely prone to hypoglycemic shock; option (2) was therefore ruled out because the food-restricted animals lacked the means to compensate for too much insulin. In addition to being technically difficult, option (3) would have imposed an additional stress on the animals.

The calculation of rate constants is a method routinely used to assess rates of disappearance of substrate, irrespective of starting concentrations. Calculation of such rate constants for the disappearance of glucose (cf. Table 11) indicated a highly significant difference between the control and treated diabetic groups, a further indication that oxytetracycline treatment improves the handling of glucose and potentiates the action of insulin.

The exact mechanism whereby oxytetracycline acts is not known. Hiatt and Bonorris (1966, 1970), who are responsible for the first observation on the insulin-potentiating effect of oxytetracycline, suggested that it may act by blocking the glucose mobilizing effect of catecholamines. In this regard, it is interesting to note that whereas the insulin tolerance curves in the untreated normal and diabetic rats showed a tendency toward returning to starting levels between 60 and 120 min (cf. Fig. 13), in the oxytetracycline-treated animals, the blood glucose values remained constant. In fact, in the normal animals the only significant difference ($P<0.05$) between control and treated groups occurred at 120 min due to the return of the control animals to the starting value (Fig. 13). It is now widely accepted that the glucose mobilizing effects of catecholamines in the liver are mediated by alpha-adrenergic receptors, which in turn promote the uptake of Ca^{2+} by liver cells (Exton, 1980). One of the possibilities that thus presents itself is that oxytetracycline may prevent this movement

of Ca^{2+}, precluding the mobilization of glycogen via alpha-adrenergic stimulation.

Tetracyclines have been used as fluorescent probes to characterize the association of divalent cations with biological membranes (Caswell and Hutchison, 1971a; Girault and Galmiche, 1978; Schuster and Olson, 1974). In islet cells, fluorescence probing with chlortetracycline has been used to study the interaction of Ca^{2+} with the membrane (Taljedal, 1978). The changes in fluorescence are thought to be brought about by migration of the tetracycline–divalent cation complexes within the membrane. A possibility, which has already been discussed (Begin-Heick et al, 1979), is that oxytetracycline may be able to form complexes with Zn^{2+} and insulin, and insulin present in such complexes may have increased affinity for its receptor. The formation of stoiochiometric chlortetracycline, Zn^{2+}, and insulin complexes has been shown to occur in vitro, and there is some evidence that it may also occur in vivo (Prochazka et al, 1965). Thus, the interaction of oxytetracycline and divalent cations at the membrane level could form a basis for the insulin potentiating effect of the drug.

CHAPTER 5. INSULIN SECRETION IN THE ob/ob MOUSE.

EFFECT OF OXYTETRACYCLINE TREATMENT.

The purpose of this study was to determine whether the insulin secretory-response to known secretagogues could be quantitatively or/and qualitatively affected by oxytetracycline treatment. The effect of glucose, glucagon and aminophylline was studied both *in vivo* and *in vitro*. I also sought to determine whether the secretory response of islet cells *in vitro* could be modified by previous treatment of the animal with oxytetracycline.

Methods: These are described in detail in Chapters 2 and 3.

A. Results

A. 1. *In vivo* experiments

Effect of glucose

Fig. 14 represents the response of lean and obese mice to the intraperitoneal injection of glucose and serves as a landmark for evaluating the action of other secretagogues. As observed previously under somewhat different experimental conditions (Bégin-Heick, 1974, 1979), OTC treatment partially restored the ability of the obese mouse to handle a glucose load. At all times studied, the plasma glucose levels were significantly lower (P at least <.05) in the OTC-treated than in the control obese mice. The plasma glucose levels at 0 time and at 5 minutes were similar in the OTC-treated obese and in both groups of lean mice, however, the levels continued to rise until the 30 minute point in the OTC-treated obese, whereas the peak glucose levels were obtained at 15 minutes in the lean. The OTC treatment decreased the levels of insulin in the obese mouse to the levels observed in the lean controls. The insulin levels in the OTC-treated lean mouse were also decreased as compared to the control. Furthermore, the insulin curves in both lean and obese OTC-treated mice did not show a peak of insulin secretion such as was found in the lean

controls. The results in Fig. 14 indicate clearly that OTC did not produce its effects by decreasing the food consumption of the obese mouse. Food restriction did not produce a significant decrease in insulin production. Marked and sustained hyperglycemia was produced in response to a glucose load in the food-restricted animals.

Effect of glucagon

In the obese mouse, the effect of previous OTC-treatment on the response to an acute injection of glucagon was striking: the glucose production in response to glucagon was not significantly different from that which was found for the lean mouse. In contrast, in the obese control and obese food-restricted groups, the glucagon injection elicited an exaggerated elevation of the blood glucose levels. In the OTC-treated lean mice, there was no significant change in the blood glucose levels throughout the experiment, i.e. no glucose peak was observed, so that the plasma glucose concentration was significantly different from the levels observed in the lean control at 5 minutes (Fig. 15).

The effects on insulin levels were varied (Fig. 15). Both the lean and obese control groups showed a peak value around 5 minutes and a return towards Ø time levels within the 9Ø minutes of the experiment. The return to the Ø time level was, however, much more rapid in the lean mice. It should be noted also that, although the changes were qualitatively similar in the lean and obese mouse, the levels were at least ten times greater in the obese mouse.

There were two distinct effects of OTC-treatment on insulin secretion. Firstly , the peak of secretion seen at 5 min in the controls was virtually absent in the OTC-treated animals both lean and obese. Secondly, in the early part of the experiment, both the lean- and the obese- treated mice had insulin levels which were significantly lower than

their respective controls. Food restriction although (or perhaps because) it diminished insulin secretion led to increased blood glucose levels throughout the duration of the test.

Effect of aminophylline

The responses to aminophylline are shown on Fig. 16. In both control and treated obese mice, an acute aminophylline injection produced a rapid and sustained elevation of the blood glucose levels. The blood glucose levels were, however, significantly lower in the OTC-treated than in the control obese mice at all times with the exception of the sample taken at 90 minutes. In the obese control mouse this sustained hyperglycemia occurred in spite of sustained hyperinsulinemia, whereas in the OTC-treated obese mouse, the insulin levels, while still elevated compared to the values seen in the lean mouse, were at least 5 times less than those seen in the obese control mouse. In the lean mouse, the levels of glucose and insulin were in proportion to each other, both being higher in the controls than in the OTC-treated mice. In both the latter groups, the increases were relatively modest in comparison to those seen in the obese mice. Again in this case, food restriction of the obese diminished the secretion of insulin but not the production of glucose.

A. 2. Islet cell distribution

Fig. 17 represents a profile of the numerical distribution, on the basis of the largest diameter, of islets from different groups of mice. In accordance with previous reports on rat and mouse islets (Gepts et al, 1960; Tejning, 1947), the islets were found to be asymmetrically distributed with an increase in the islet number with decreasing size. The largest proportion of islets in lean mice (control and OTC-treated) was contributed by small islets (diameter 76-152 um) while islets of intermediary size (diameter 152-229 um) were the most numerous in control

obese mice. Due to the asymmetrical distribution of the islets, a non-parametric statistical test [the Kolmogorov-Smirnov test, (Siegel, 1956)] was used to evaluate if islets from one population were significantly larger or smaller than islets from another population. The results of the statistical analysis are given in Table 12.

Treatment of the obese mouse with OTC resulted in a distribution profile of the islets which resembled that seen with islets of lean mice. Only 25% of the islets of OTC-treated obese mice had a diameter exceeding 152 µm as opposed to 50% in islets of control obese mice. The islets were, however, still significantly larger, on the average, than the islets of lean mice (Table 12).

Food restriction of the obese mouse resulted in a slight increase in the number of smaller size islets. However, taken as a whole the islet population was not significantly different from that of the obese control mouse (Table 12).

A. 3. In vitro experiments

Effect of glucose on insulin secretion

The results of these experiments are given in Fig. 18. Compared to the tissue from the lean mice, the islets from the control obese mice secreted exaggerated amounts of insulin at all the glucose concentrations studied. Treatment of the obese mouse with OTC led to a significant decrease of insulin secretion. The insulin secretory activity of the OTC-treated obese mouse islets was still significantly greater than that of the lean mouse. Restricting the food intake of the obese mouse to the levels consumed by the OTC-treated obese mouse did not decrease insulin secretion as much as did the OTC treatment.

Effect of glucagon

The addition of glucagon to the incubation medium at various glucose

concentrations produced a modest stimulatory effect on the islets from the control lean mice. At glucose concentrations of 10 mM and below, glucose alone or glucose + glucagon had little effect on the islets from lean mice previously treated with OTC. The stimulation produced by both secretagogues was significantly higher than that found in the control mouse when the glucose concentration of the medium was 20 mM (Fig. 19). In the control obese mouse, the already exaggerated insulin secretion due to glucose was boosted even further by the addition of glucagon. In the OTC-treated obese mouse, there was a modest increase caused by glucagon. Food restriction of the obese mouse did not lead to any significant improvement in the pattern of insulin release when glucagon was combined with 20 mM glucose.

Effect of aminophylline

There were no significant differences in the response to aminophylline of the islets of lean control or lean OTC-treated mice. (Fig.20). In the islets of the obese (control, food-restricted and OTC-treated) aminophylline stimulated the secretion of insulin maximally at 10 mM glucose. While the addition of aminophylline significantly stimulated insulin secretion in the islets from the OTC-treated obese mouse, the magnitude of the stimulation was much less in this group than in the obese controls or food-restricted obese controls.

Fig.14. Plasma glucose and IRI following a glucose load (1g/kg).

The mice were prepared and injected as described in Chapter 2. Top panel, plasma glucose, bottom panel, plasma IRI. Results are given as means ±S.E.M. n=8 in each group. Abbreviations: ln-CTL=lean control; ln-OTC=lean OTC-treated; ob-CTL=obese control; ob-OTC=obese OTC-treated; ob-FR=obese food-restricted.

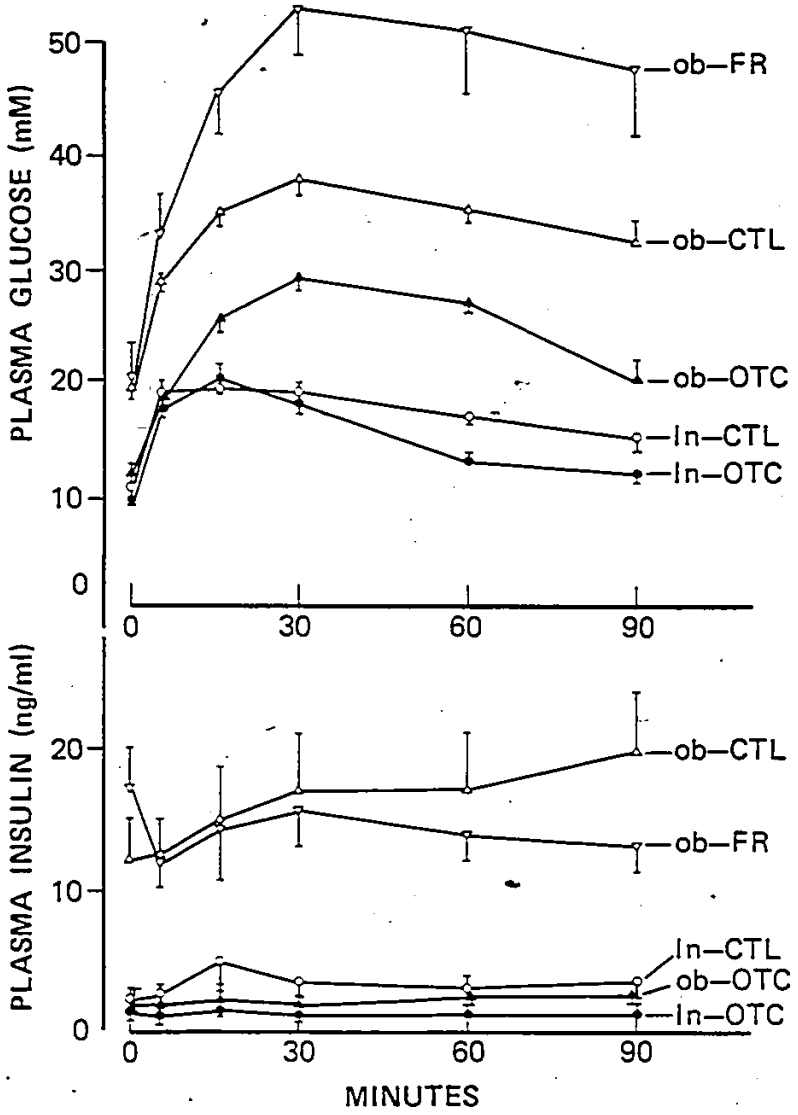


Fig. 15. Plasma glucose and IRI following a glucagon injection.

All animals received 0.025mg of glucagon. Other details are as given in the legend to Fig.14.

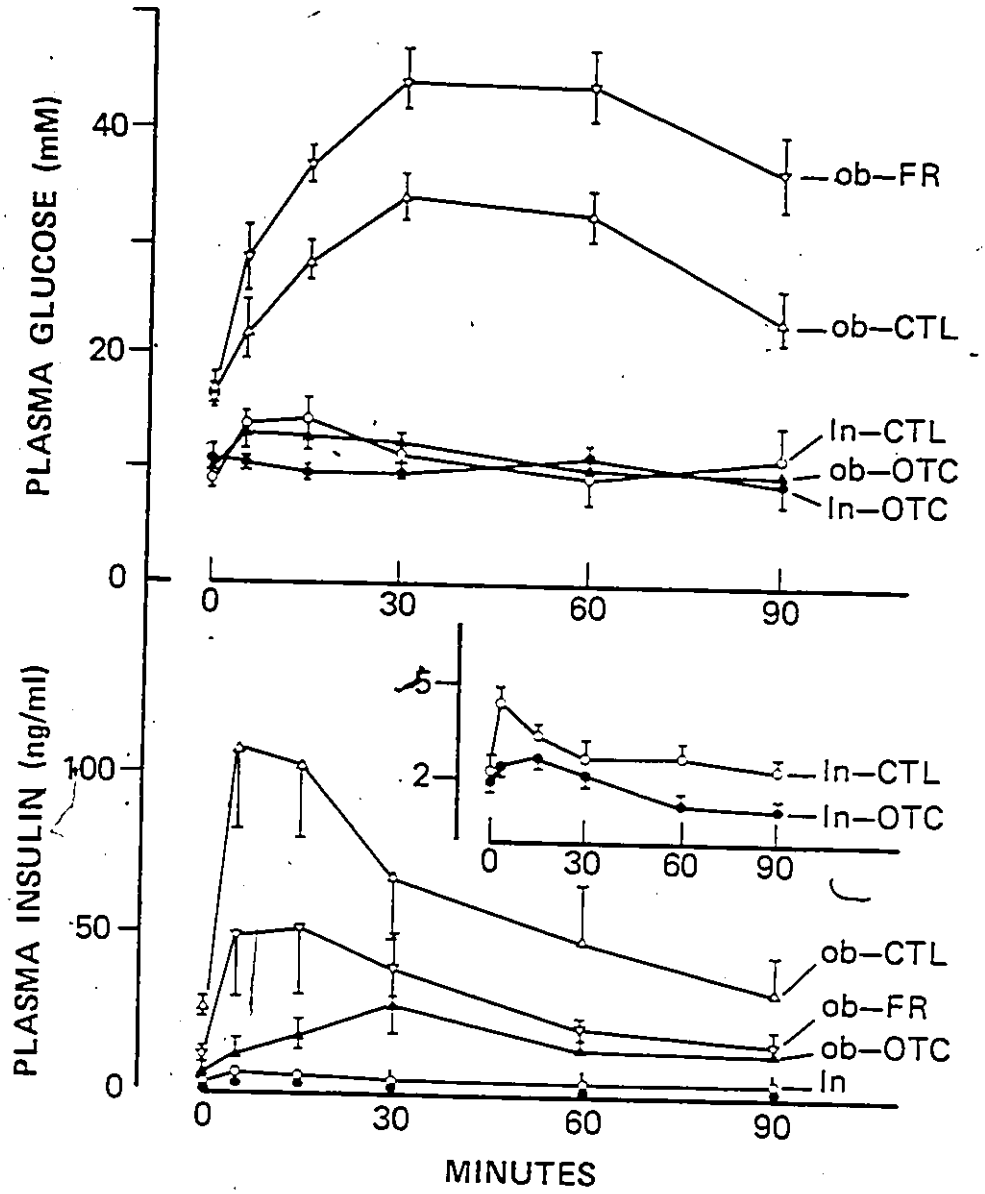


Fig. 16. Plasma glucose and IRI following an injection of aminophylline.

All animals received 3mg. of aminophylline. Other details are as given in the legend to Fig. 14.

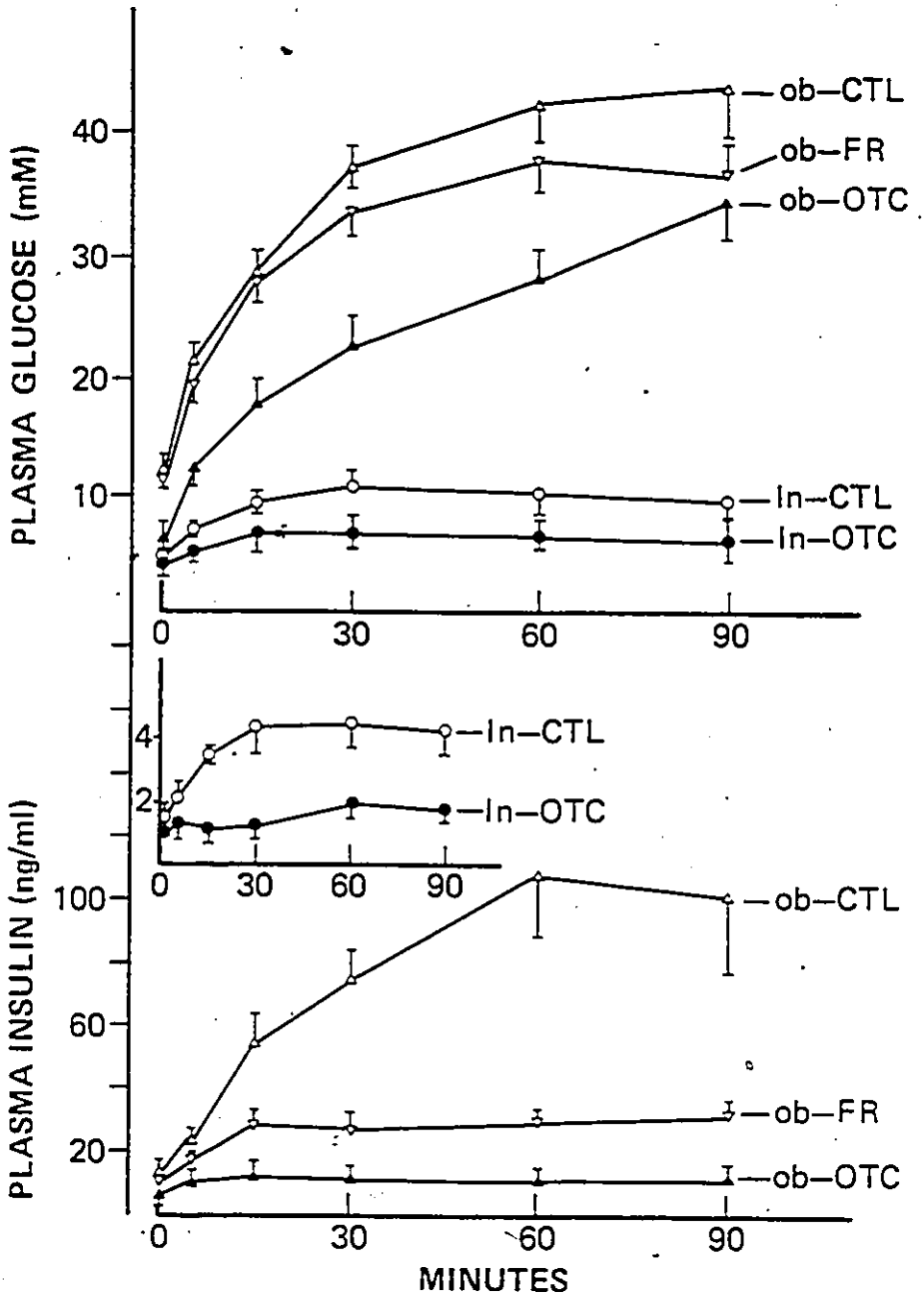


Fig. 17. Effect of oxytetracycline treatment on the islet cell distribution in lean and obese mice.

The value given in ordinate is the percentage of the total number of islets of the size indicated by the abscissa. The data were obtained by measuring the largest diameter of a minimum of 100 islets of each kind. The islets were sized as described in Chapter 2. Abbreviations are as given in the legend to Fig. 14.

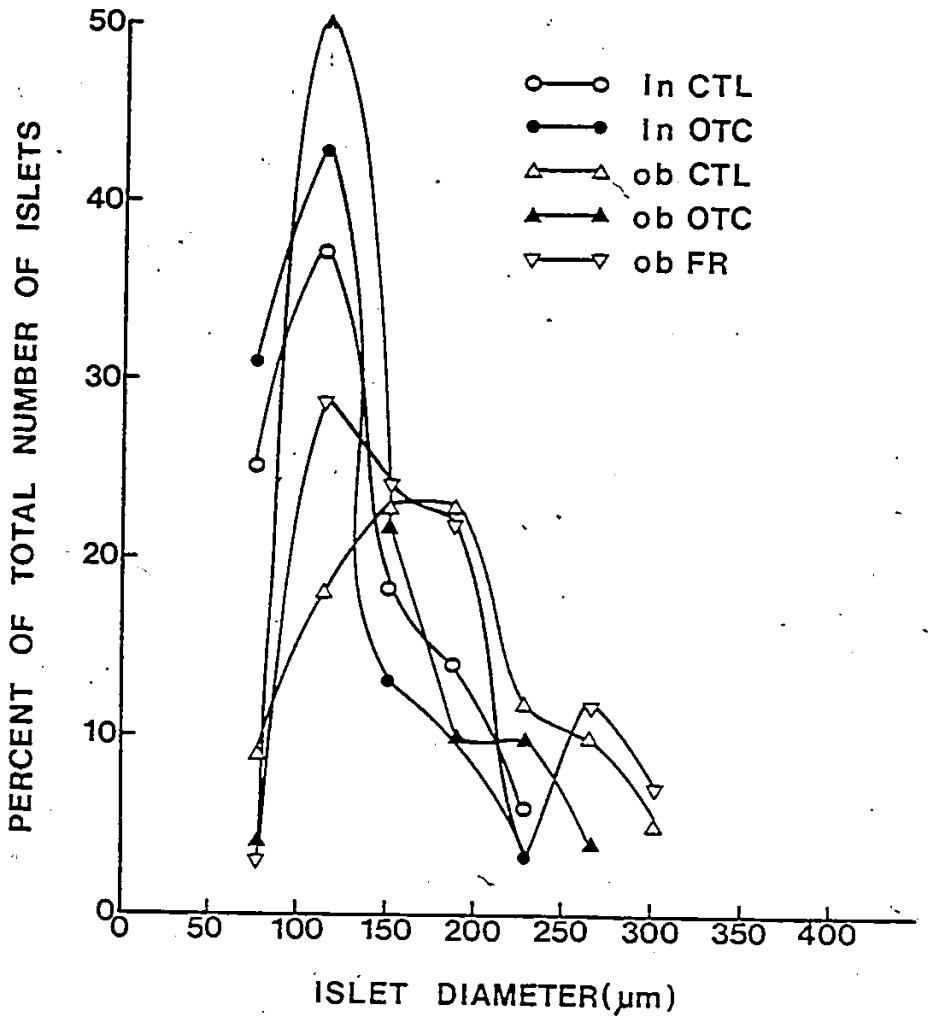


Table 12. Statistical analysis of the numerical distribution of the islets (effect of OTC treatment).

Islet populations under study			P
ob CTL	vs	ln CTL	< 0.001
ln CTL	vs	ln OTC	NS
ob CTL	vs	ob OTC	< 0.001
ob CTL	vs	ob FR	NS
ob OTC	vs	ln CTL	< 0.02

The numerical distributions of the islets on the basis of their largest diameter (Fig. 17) were compared by the Kolmogorov-Smirnov two-sample one-tailed test. The islets from the population in the left column are either not significantly different (NS) or larger than the islets from the corresponding population in the right column. Abbreviations are as given in the legend to Fig. 14.

Fig. 18. The release of insulin

by isolated islets of Langerhans.

The islets were preincubated in 3mM glucose as described in Chapter 2. They were incubated for 60 minutes at the glucose concentrations indicated on the abscissa. Insulin was measured as described in Chapter 3. The results are expressed as percentage of insulin released per 60 minutes (i.e. secreted IRI X 100/ secreted IRI + islet IRI), as described in Chapter 2. Results are given as the means ± S.E.M. of 15-20 batches of islets (5 islets/batch).

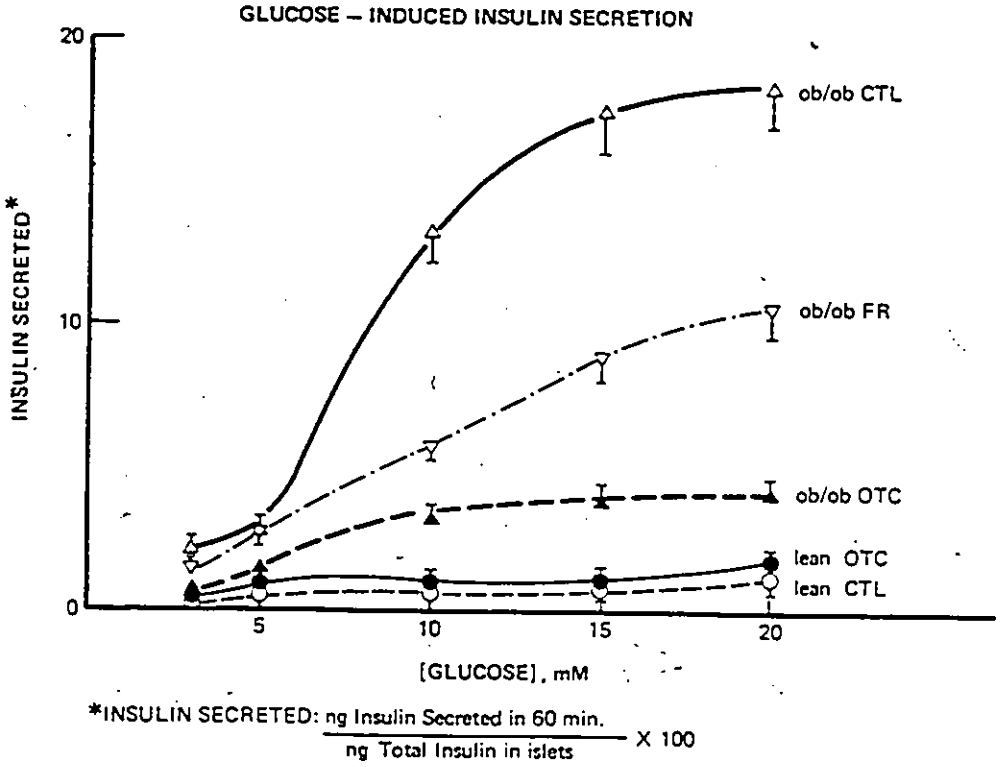


Fig. 19. The effect of glucagon

on insulin secretion by isolated islets.

The release of insulin was measured in the presence (hatched bars) and in the absence (open bars) of glucagon (5µg/ml). Other details are as given in the legend to Fig. 18. *P< .05, **P<.005, between glucose alone and glucose + glucagon.

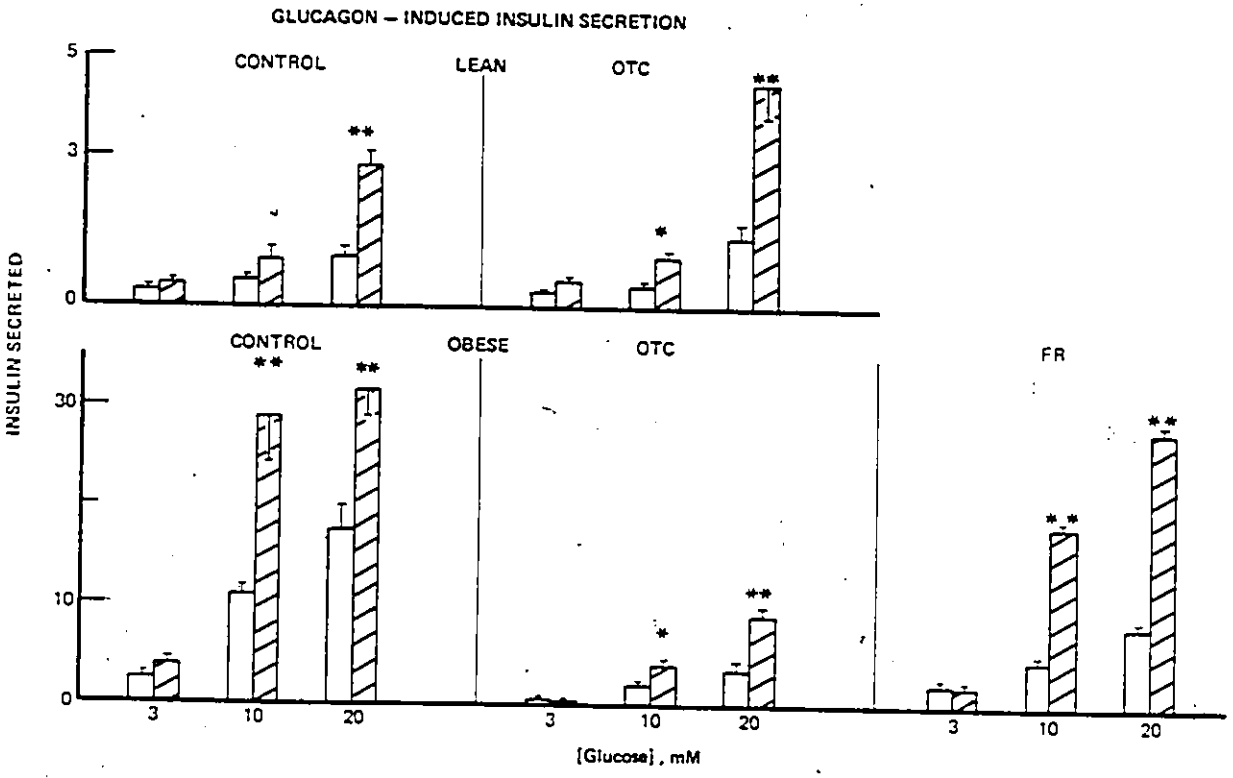
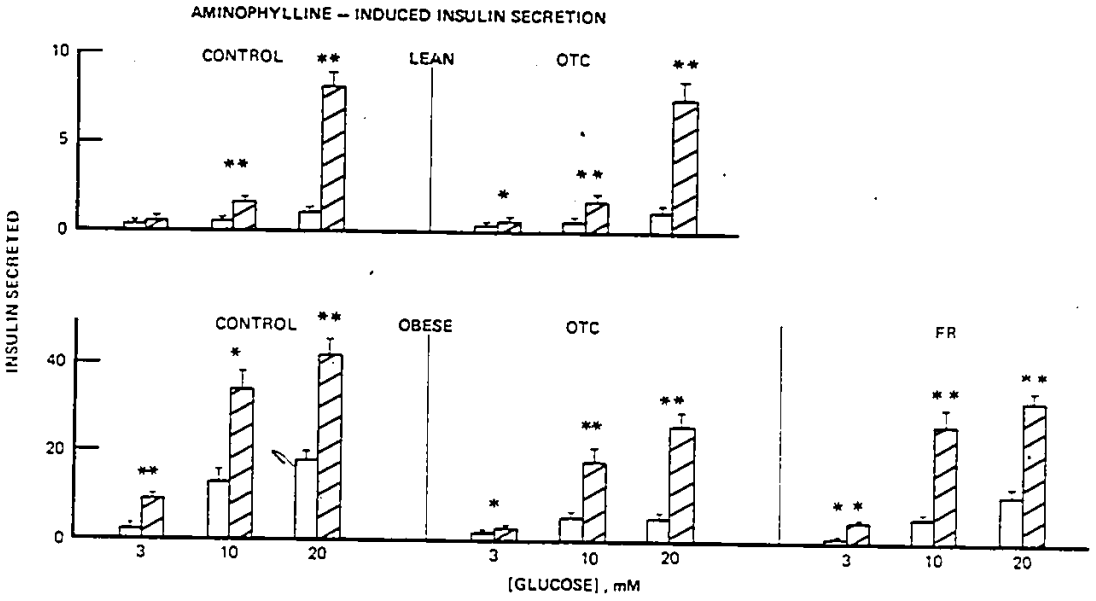


Fig. 20. The effect of aminophylline on insulin release by isolated islets.

The release of insulin was measured in the presence (hatched bars) and in the absence (open bars) of aminophylline (2.5 mM). Other details are as given in the legend to Fig. 18 and Fig. 19. * P< .05, ** P< .005, between glucose alone and glucose + aminophylline.



B. Discussion

The data presented in this Chapter support previous data (Bégin-Heick et al, 1974, 1979) on the effect of oxytetracycline on glucose tolerance in the obese mouse. The slightly more elevated plasma glucose values obtained for the obese OTC-treated mouse in this study could be accounted for by the fact that, in the previous studies (Bégin-Heick et al, 1974, 1979), the mice had been treated for 10 days as opposed to 7 days in the present one. There was a remarkable correlation between the in vivo and in vitro pattern of insulin secretion in response to glucose. The lack of increase in plasma insulin levels beyond the 0 time value which I report here was not surprising as the respective fasting plasma glucose levels (10 mM for the obese OTC-treated and 20 mM for the obese control) were already beyond the concentration capable of evoking maximal responses in vitro (compare Fig.14 with Fig.18). The in vitro observations could also explain, at least in part, the difference in the plasma insulin response to the I.P. glucose load that was obtained by Cameron et al (1972b) and Herberg et al (1970) for the C57Bl/6J ob/ob mouse. The ob/ob mice used by Cameron et al had a plasma glucose of less than 10 mM at time 0 of the tolerance test and a significant increase in insulin secretion was observed after the glucose load. The ob/ob mice used by Herberg et al [of corresponding age to the ones used by Cameron et al] had a plasma glucose of 17 mM at the beginning of the test and no increase in plasma insulin levels was observed after the glucose load. The results obtained by Herberg et al are thus more comparable to those which I show here.

In the light of the results obtained with glucose, which is the major physiological initiator of insulin release (Hedeskov, 1980), it was important to see if OTC had a general effect on the insulin secretory mechanism of the obese mouse. Two other secretagogues, glucagon and

aminophylline, which are well known potentiators of insulin release but differ in their mechanism of action, were also used.

Glucagon has been shown to potentiate insulin release through its effect on the adenylate cyclase-cAMP system (Malaisse, 1973; Montague and Howell, 1975) and not by a direct effect on Ca^{2+} translocation. In turn, the insulinotropic action of cAMP produced by glucagon or other factors is thought to be due to a glucose independent translocation of calcium within the B-cell from an organelle-bound pool to a cytoplasmic pool (Siegel et al, 1980). It was shown that this cAMP-induced translocation of calcium is only able to promote sustained secretion of insulin when the B-cell is simultaneously exposed to a suitable insulinotropic substrate (e.g. glucose, leucine) which itself favors calcium acccumulation in the cytosol by inhibiting calcium efflux (Wollheim and Sharp, 1981). Thus the in vitro studies with glucagon were done in presence of glucose. Correlations between the in vivo and the in vitro findings could again be observed. In the lean treated animals, the data from Fig.15 show that following an injection of glucagon the plasma glucose remained at about 10mM; the in vitro data of Fig.19 show that in the lean animals, glucose + glucagon had little effect on insulin release at glucose concentrations of 10 mM and below. The lack of significant change in plasma glucose following the glucagon injection, as seen in obese OTC-treated and lean OTC-treated mice, may be due to an increased sensitivity of these animals to insulin.

Aminophylline, like other methylxanthines, is an inhibitor of cyclic nucleotide phosphodiesterase and thus its administration should result in increased intracellular cAMP levels (Robison et al, 1971). However the xanthines are not restricted to a single mechanism of action (Robison et al, 1971; Wells and Kramer, 1981). In the case of the pancreas, direct action on calcium uptake by an islet microsomal fraction has been described

(Sugden and Ashcroft, 1978). Caffeine and 3-isobutyl-1-methyl-xanthine (IBMX) have been shown to result in an inhibition of islet mitochondrial calcium accumulation which could not be duplicated by cAMP (Sugden and Ashcroft, 1978).

Intraperitoneal injection of aminophylline into the obese control mouse led to the highest level of plasma glucose observed in these studies, without any sign of decrease even after 90 minutes. These findings are in keeping with reports on the extreme sensitivity of the obese mouse to caffeine, a single injection of which was reported to cause prolonged elevation of blood glucose (Kuftinec and Mayer, 1964). The dose of aminophylline used in the present studies (120 mg/kg), administered on the basis of lean body weight, was lower than that previously used by Cameron et al (1972c) in Swiss mice (240mg/kg). When used in the ob/ob mouse, the higher dose led to the death of all the mice during the test. Even with the reduced dose, approximately 50% of the obese mice died within 48 hr after the single injection. No experimental findings could be found in the literature to explain the long term glucotropic effect of caffeine and aminophylline in the obese mouse.

The aminophylline tolerance test demonstrated that the decrease in insulin release from the islets of the obese OTC-treated mice was not merely due to a lack of priming of the islets with high enough glucose concentration: Fig.16 shows that 90 minutes after injection, the plasma insulin level of obese OTC-treated mice had not changed significantly from basal value while the plasma glucose was then as high as in the obese control mice. It seems rather that the islet cells of the obese mice reached their maximum secretory capacity at much lower glucose concentration. These *in vivo* insulin results were in keeping with the *in vitro* findings (compare Fig.16 and Fig.20): in the ob/ob mouse, maximal

insulin release in vitro was seen with a combination of 10 mM glucose + aminophylline. Increasing the glucose concentration in the medium further did not potentiate the insulin secretory activity of aminophylline in the islets of the obese mouse as it did in the islets of the lean mouse.

OTC treatment of the obese mouse therefore resulted principally in a more appropriate response of the insulin secretory mechanism to stimuli (in vivo and in vitro) as well as a more appropriate ratio of glucose to insulin (in vivo). These effects of OTC-treatment cannot be related to the decrease in food intake produced by OTC (Bégin-Heick and Heick, 1976; Bégin-Heick et al, 1974, 1979; Dubuc and Willis, 1978) as food-restricted obese mice had glycemic responses at least as high as those of untreated obese mice, although their insulin secretion was somewhat diminished. It has been shown that oxytetracycline increases the sensitivity to insulin in a number of different systems (Bégin-Heick and Heick, 1976; Bégin-Heick et al, 1974; Dubuc and Willis, 1978; Hiatt and Bonorris, 1970; Hiatt et al, 1966; Miller, 1966). This study supports the previous findings that OTC treatment of the ob/ob mouse increases the insulin sensitivity of peripheral tissues (Bégin-Heick and Heick, 1976; Bégin-Heick et al, 1974, 1979; Dubuc and Willis, 1978). The glucose curves obtained in vivo, for all three secretagogues, was significantly lower (except at time 90 minutes during the aminophylline tolerance test) in the obese OTC-treated mouse than in the obese control in spite of significantly decreased plasma insulin levels.

Autoregulation of insulin secretion exists in the islets of the lean mouse but this mechanism appears to be impaired in the obese mouse islets (Loreti et al, 1974). Thus any agent capable of making the islets more sensitive to the feedback action of insulin on insulin secretion would produce a diminished insulin secretory response. OTC has already been

shown to increase insulin-receptor activity in liver membranes of obese mice (Bégin-Heick et al, 1974). There is already evidence for the presence of insulin receptors on rat pancreatic islets and their cells (Verspohl and Ammon, 1980; Verspohl et al, 1982). It is proposed that the greatly reduced in vitro insulin release by the islets of the obese OTC-treated mouse could result from a restoration of the sensitivity to insulin of the obese mouse islets brought about by OTC. The effect of OTC on islet cell sensitivity to insulin could also explain the flat insulin curves obtained in vivo for the lean OTC-treated mouse.

It has already been proposed that the OTC effect appears to be related to membrane function (Bégin-Heick et al, 1979). The effects of OTC could be due to its metal chelating properties (Albert and Rees, 1956; Weinberg, 1954). In studies on islet cells (Taljedal, 1978) as well as of other structures [chloroplast (Girault and Galmiche, 1978), mitochondria (Schuster and Olson, 1974), membrane preparations (Caswell and Hutchison, 1971; Franklin, 1971)] it has been reported that tetracyclines interact strongly with membranes and divalent cations. It has also been suggested that in the B-cell, the Ca^{2+} pools which can be identified as interacting with chlortetracycline are those of importance for insulin secretion (Taljedal, 1978). In the light of the knowledge accumulated to date on the mechanism of insulin secretion evoked by glucose, glucagon and aminophylline (see above) and their relationship with calcium ions, tetracyclines could alter the movement and/or the distribution of Ca^{2+}. OTC could also be interacting with Zn^{2+} in the islet cell. Zinc is known to play a prominent role in the structure and function of insulin as well as being important for the formation of granules within the B-cell (Emdin et al, 1980).

CHAPTER 6. EFFECT OF ZINC ON INSULIN RELEASE IN THE ob/ob MOUSE.

A relative or absolute zinc deficiency has been suggested to play a role in the pathogenesis of diabetes mellitus in humans (Pidduck et al, 1970; Tarui, 1963). Zinc is known to enhance the binding of insulin to hepatocyte membranes (Arquilla et al, 1978) and to have an additive effect to that of insulin on lipogenesis in rat adipocytes (Coulston and Dandona, 1980). Furthermore, zinc-deficient animals are less sensitive to insulin (Quaterman et al, 1966), have an impaired glucose tolerance (Boquist and Lernmark, 1969; Huber and Gershoff, 1973) and have degranulated islets (Boquist and Lernmark, 1969). Insulin resistance, impaired tolerance to glucose and degranulated islets are also characteristics of the ob/ob mouse.

There were already indications in the literature that zinc metabolism may be abnormal in the obese mouse. Thus, the immune system, which is known to require an adequate zinc status (Prasad, 1978), has been reported to be defective in this animal model (Chandra, 1980). Significantly lower zinc levels were also found in the plasma and femur of the obese mouse (Bégin-Heick et al, 1982).

OTC is known to interact very strongly with divalent cations, particularly zinc (Gulbis et al, 1976; Weinberg, 1954), and it has been shown to form stable complexes with zinc and insulin (Prochazka et al, 1965). In attempting to find an explanation for the mechanism of action of OTC on the islet cell, I hypothesized that it may be interacting with zinc within the islet cells.

Because of the role played by zinc in the process of insulin storage and secretion, the effect of dietary zinc supplementation on insulin secretion by isolated islets was assessed. In view of the findings reported

in Chapter 5, I also determined the effect of combining zinc supplementation with OTC-treatment.

Methods: These are described in detail in Chapters 2 and 3.

A. Results

A. 1. Insulin content and size of islets

The insulin content of islets is given in Table 13. Data on islets from lean mice (control and OTC-treated) and from food-restricted obese mice are included for comparative purposes. Zinc supplementation and food restriction both led to a significant increase in the insulin content of the obese mouse islets. Oxytetracycline treatment of the obese mouse, alone, or in combination with zinc supplementation resulted in a significant increase in insulin content when compared to control values; the highest islet insulin content resulted from a combination of the two treatments.

The numerical distribution of the islets on the basis of their largest diameter is shown in Fig. 21 and the statistical analyses of these data are given in Table 14. Zinc treatment of the obese mouse did not lead to any significant difference in the size distribution of the islets. The combination of OTC-treatment and zinc supplementation resulted in a distribution profile which was not significantly different from that resulting from OTC-treatment alone.

A. 2. <u>In vitro</u> glucose-induced insulin secretion

The results of these experiments are shown in Fig. 22. Zinc supplementation alone significantly diminished the abnormally high insulin secretory response to glucose of the ob/ob mouse islets [at 10 mM, $P < 0.001$; at 15 mM, $P < 0.025$; and at 20 mM, $P < 0.05$]. The secretion of insulin was maximally stimulated at 15 mM glucose in the islets of the zinc-fed obese mouse as was the case in the obese controls. When zinc-supplemented

obese mice were also treated with oxytetracycline (ob Zn-OTC group), the effect of the two treatments together was greater than that of either alone in correcting the exaggerated secretion of insulin. The results obtained with the islets from the obese OTC-Zn group were the closest to those obtained with the islets of lean mice (compare Fig. 18 with Fig. 22), although the insulin secretory activity was still significantly greater at all the glucose concentrations studied.

Table 13. Insulin content of isolated islets.

Group	Insulin (ng/5 islets)	
ln CTL	160 ± 10.7	a
ln OTC	158 ± 8.2	a
ob CTL	79 ± 8.5	b
ob OTC	430 ± 34	c
ob Zn	219 ± 13	d
ob Zn-OTC	660 ± 55	e
ob FR	218 ± 15	d

Insulin was measured as described in Chapter 3. The data are averages ± SEM from a minimum of 25 determinations. Values followed by different letters are significantly different from each other ($P < 0.05$). Abbreviations: ln CTL=lean control; ln OTC=lean OTC-treated; ob CTL=obese control; ob OTC=obese OTC-treated; ob Zn=obese zinc-supplemented; ob Zn-OTC=obese zinc-supplemented and OTC-treated; ob FR=obese food-restricted.

Fig. 21. Effect of zinc and oxytetracycline treatment on the islet cell distribution in ob/ob mice.

The value given in ordinate is the percentage of the total number of islets of the size indicated by the abscissa. The data were obtained by measuring the largest diameter of a minimum of 100 islets of each kind. The islets were sized as described in Chapter 2. Abbreviations are as given in the legend to Table 13.

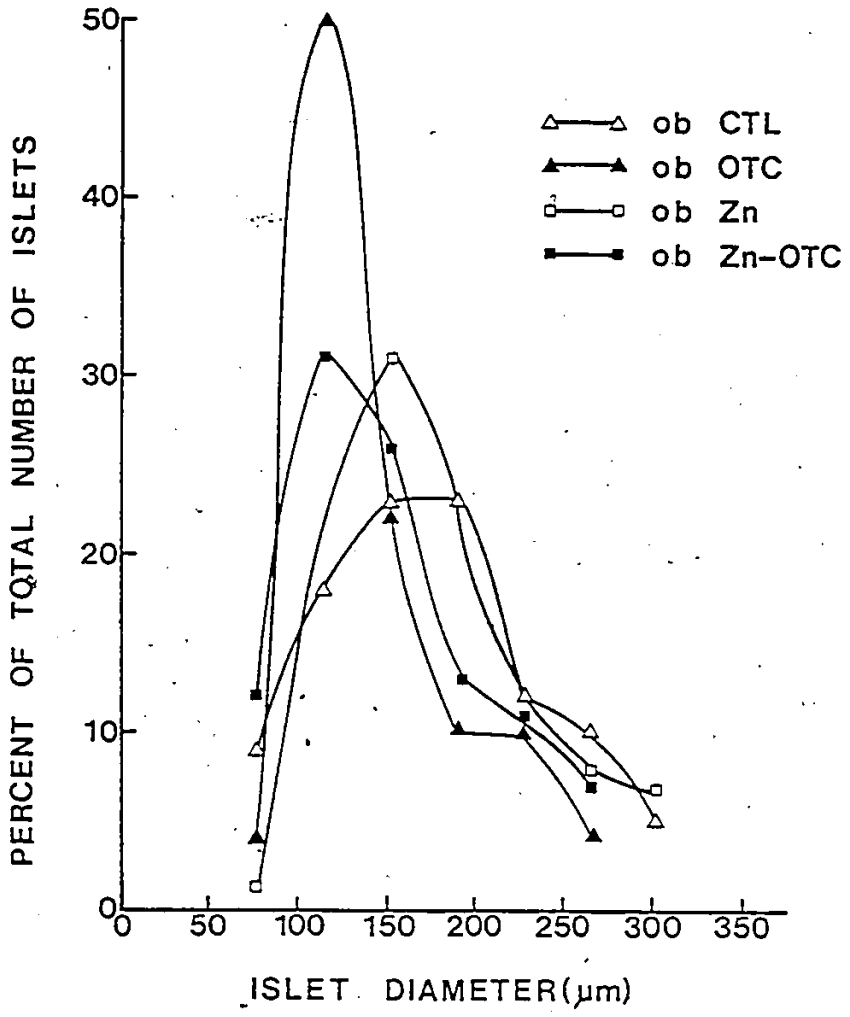
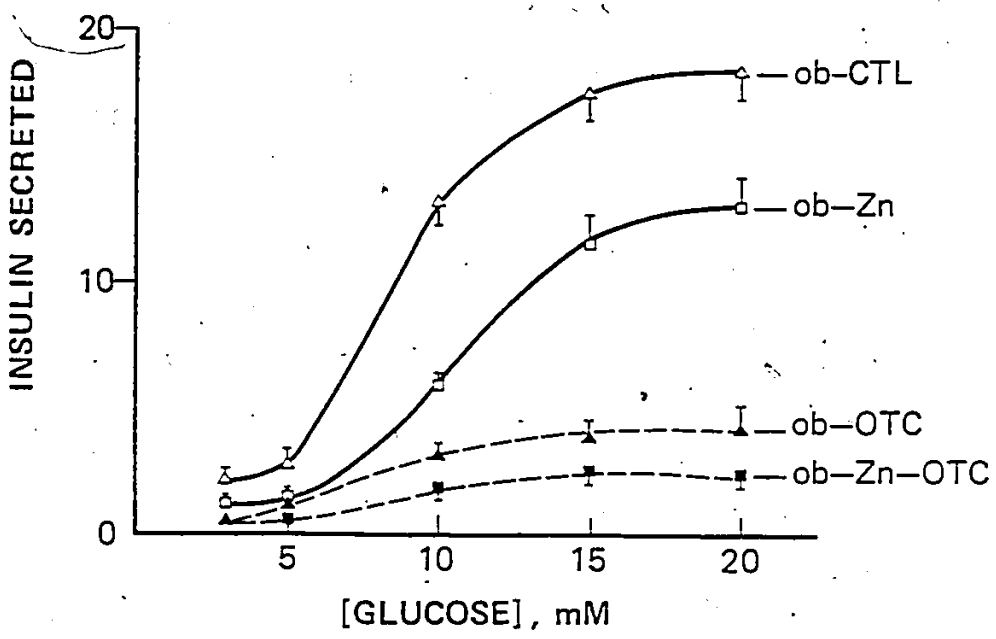


Table 14. Statistical analysis of the numerical distribution of the islets (effect of zinc and OTC treatment).

Islet populations under study			P
ob CTL	vs	ob Zn	NS
ob CTL	vs	ob OTC	<0.001
ob CTL	vs	ob Zn-OTC	<0.05
ob OTC	vs	ob Zn-OTC	NS
ob Zn-OTC	vs	ln CTL	<0.05

The numerical distribution of the islets on the basis of their largest diameter (Fig. 21) was compared by the Kolmogorov-Smirnov two-sample one-tailed test. The islets from the population in the left column are either not significantly different (NS) or larger than the islets from the corresponding population in the right column. Abbreviations are as given in the legend to Table 13.

Fig. 22. Effect of zinc and oxytetracycline treatment on the glucose-induced insulin release by isolated islets of ob/ob mice. The islets were preincubated in 3 mM glucose as described in Chapter 2. They were incubated for 60 minutes at the glucose concentrations indicated on the abscissa. Insulin was measured as described in Chapter 3. The results are reported as percentage of insulin released per 60 minutes (i.e. secreted IRI X 100/ secreted IRI + islet IRI) as described in Chapter 2. Results are expressed as the means ± SEM of 10-15 batches of islets (5 islets/batch). Abbreviations are as given in the legend to Table 13.



B. Discussion

In the studies described in this Chapter, it was demonstrated that dietary zinc supplementation of the ob/ob mouse resulted in a significant diminution of the abnormally high insulin secretory response of the islets to glucose. Zinc has been implicated as a regulator of insulin secretion. The evidence in favor of this hypothesis is that zinc added in vitro inhibits the release of insulin from isolated islets (Figlewicz et al, 1981; Ghafghazi et al, 1979, 1981) and this inhibition can be reversed by increasing the calcium concentration of the incubation medium (Gahfghazi et al, 1981). The physiological significance of these phenomena has been questioned (Figlewicz et al, 1981), as the zinc concentration (60 μM) required to achieve significant inhibition is far greater than the levels found in plasma (10-20 μM) (Larsson et al, 1976). However, as rightly pointed out by Ghafghazi et al (1981), the intracellular zinc concentration is not known and may be greater than plasma concentration. Zinc uptake by islets in vitro is a very slow process but it results in zinc concentrations which are 30 times greater than those in the external medium (Ludvigsen et al, 1979). High extracellular concentrations of zinc may therefore be needed to produce significant changes in the intracelular concentration over short incubations.

Zinc inhibition of calmodulin has been proposed as a molecular mechanism to explain the diverse cellular inhibitory effects of zinc and the antagonism between zinc and calcium in a variety of cell types (Brewer et al, 1979). Other effects, such as stabilization of membrane proteins (Bettger and O'Dell, 1980; Chvapil, 1973) or effects on the microtubule system (Larsson et al, 1976) could also be envisaged.

In some experiments, the 4 wk dietary zinc supplementation program of the ob/ob mouse was coupled with a daily injection of OTC during the last 7

days of the feeding period. It resulted in an even greater decrease in the glucose-stimulated insulin release than that observed after zinc supplementation alone (cf Fig. 22). It was proposed in Chapter 5 that OTC could improve the sensitivity to insulin of the ob/ob mouse islets. OTC has already been shown to increase insulin-receptor activity in liver membranes of obese mice (Begin-Heick et al, 1974). An enhancement of the action of insulin on peripheral tissues has also been demonstrated to be brought about by zinc (Arquilla et al, 1978; Couston and Dandona, 1980). It is, therefore, postulated that zinc and OTC could have additive effects in decreasing the exaggerated insulin release of ob/ob mouse islets possibly via an improvement of insulin sensitivity of the tissues, including the B-cell.

It had been proposed by Ghafghazi and co-workers (1979) that the inhibitory effect of zinc on glucose-induced insulin secretion may be a regulatory mechanism. It is therefore conceivable that a defect in zinc metabolism could contribute to the defective regulation of insulin release in the islets of the obese mouse.

CHAPTER 7. CALMODULIN IN ob/ob MOUSE ISLETS.

The obese mouse has hyperplastic islet cells which secrete insulin in an exaggerated manner when challenged either *in vivo* or *in vitro*. Insulin secretion induced by many secretagogues, including glucose, glucagon and aminophylline, is thought to be ultimately due to an increase in the cytosolic concentration of ionized calcium (Wollheim and Sharp, 1981). It has been suggested that the primary intracellular target for Ca^{2+} within the B-cell is the Ca^{2+}-dependent regulator protein calmodulin (Tomlinson et al, 1982).

The presence of calmodulin has been demonstrated in rat islets (Landt et al, 1982; Sugden et al, 1979; Valverde et al, 1979) and more recently in mouse islets (Thams et al, 1982). In rat islets, calmodulin modulates the activity of cAMP phosphodiesterase (Sugden and Ashcroft, 1981), adenylate cyclase (Sharp et al, 1980; Valverde et al, 1979), protein kinase (Landt et al, 1982) and ATP dependent calcium transport via the $Ca^{2+}+Mg^{2+}$-ATPase of islet cell membranes (Kotagal et al, 1982). There is also some evidence that calmodulin has a role in the process of assembly and disassembly of microfilaments via the calcium-calmodulin dependent activation of myosin light chain kinase (MacDonald and Kowluru, 1982). The inhibition of insulin release from isolated islets by phenothiazines (Janjic et al, 1981; Krausz et al, 1980; Schubart et al, 1980; Sugden et al, 1979; Valverde et al, 1981) which are considered to be specific inhibitors of Ca^{2+}-calmodulin, is also an indication that calmodulin is important in stimulus-secretion coupling. In the present study I set out to determine if the exaggerated glucose-stimulated insulin release in the ob/ob mouse is related to the islet calmodulin system. Firstly, I studied the effect of trifluoperazine on glucose-induced insulin secretion. Secondly I examined the calmodulin levels of the obese mouse islets as determined by bioassay

with cyclic nucleotide phosphodiesterase. The findings obtained with islets isolated from the ob/ob mouse pancreas were compared with corresponding data obtained from lean mice and Wistar rats.

Methods: These are as described in detail in Chapters 2 and 3.

A. Results

A. 1. Effect of trifluoperazine on glucose-induced insulin release

Fig. 23 shows the effect of 20 uM TFP on insulin release in islets of lean and obese mice and rats. At low glucose concentration (3mM), TFP, when present only during the incubation period, caused a stimulation. ($P<.01$) of the release of insulin in the islets of the ob/ob mouse but not in the rat or lean mouse islets. The effect of TFP on glucose-stimulated insulin secretion was evaluated by subtracting the insulin released in the presence of 3 mM glucose from that released at 20 mM glucose, giving the "net" response to high glucose (cf Krausz et al, 1980). The data are given as the right hand sets of bars in Fig. 23. The net response to high glucose was inhibited by 72% in rat islets and by 49% in obese mouse islets when TFP (20 uM) was present in the incubation medium only. In the lean mouse islets, however, there was a small but significant ($P<0.05$) stimulation of insulin release under this condition.

Since the lean mouse islets appeared more resistant than rat islets or obese mouse islets to the inhibitory effect of 20 uM TFP on glucose-induced insulin release, it was of interest to study the sensitivity of the lean mouse islets to other concentrations of TFP. The data of Fig. 24 show that adding 10 uM TFP to the incubation medium of lean mouse islets resulted in no significant change in the basal insulin release but in a significant increase in the net response to high glucose, similar to the results that were obtained with 20 uM TFP. At 50 uM TFP, however, there was a significant inhibitory effect on the net response to 20 mM glucose. This

was due to the fact that a significant increase in the basal insulin release was also observed.

When TFP (20 μM) was present both in the preincubation and incubation media, the basal insulin release was significantly increased in islets of lean and obese mice as well as in rat islets. Under such experimental condition, the net response to high glucose was maximally inhibited in all three categories of islets.

From the data of Figs. 23 and 24, it appeared that lean mouse islets are less sensitive than obese mouse islets or rat islets to the inhibitory action of TFP on glucose-induced insulin release. Since TFP binds to Ca^{2+} - calmodulin, a lesser effect of TFP could be explained by greater levels of calmodulin in the islets of lean versus obese mice. In order to verify this point, the calmodulin level of islets of lean and obese mice and of rats was determined.

A. 2. Calmodulin content of mouse and rat islets

The calmodulin levels of rat islets were determined by PDE assay and by radioimmunoassay. The results are presented in Table 15. The levels of calmodulin obtained by RIA were on the average 4 times greater than those found with the PDE assay. These observations on the relationship between the results obtained with both assays are in full agreement with reports of calmodulin levels from various other tissues (Chafouleas et al, 1979; MacManus et al, 1981; Wallace and Cheung, 1979). Because all previously reported results on islet calmodulin were obtained with the PDE assay, it was decided to compare the calmodulin levels of lean mouse islets with those of ob/ob mouse islets by making use of the PDE assay. The results are found in Table 16. The calmodulin level per islet was significantly higher ($P<.05$) in the obese mouse than in the lean mouse and corresponded approximately to the average value found in medium size rat islets. As the

obese mouse islets and medium size rat islets had similar volumes (Table 16), I verified if there was any relationship between calmodulin level and islet volume, using rat islets as a benchmark. There was no significant difference between the two mouse islet groups and the medium size rat islet group when the calmodulin results were expressed on the basis of mean islet volume (Table 16). On the same basis, the large rat islets then appeared to have a lower calmodulin level while the small rat islets appeared to have very high calmodulin levels. When the calmodulin results were expressed as a function of the average insulin content of the islets (Table 16), the physiologically normal islets (i.e. lean mouse and rat islets) had similar ratio while the islets from obese mice had a significantly more elevated ratio, as would be expected, in view of the degranulation of the islets in this group.

Fig. 23. Effect of 20 uM trifluoperazine on glucose-induced insulin release in rat and mouse islets.

The islets were preincubated in 3 mM glucose as described in Chapter 2. They were incubated for 60 minutes in 3 or 20 mM glucose. Trifluoperazine was either absent at all times (shaded bars), present during the incubation period only (open bars), or present during both the preincubation and the incubation periods (hatched bars). The net response to high glucose with and without trifluoperazine (right-hand bars) was obtained by subtraction of the insulin response in the presence of 3 mM glucose. Insulin was measured as described in Chapter 3. Results are given as the means $\pm$ SEM of 10-15 batches of islets (4 islets/batch). In each group, bars which are labelled with different letters are significantly different ($P<.05$) from each other.

Fig. 23. Effect of 20 uM trifluoperazine on glucose-induced insulin release in rat and mouse islets.

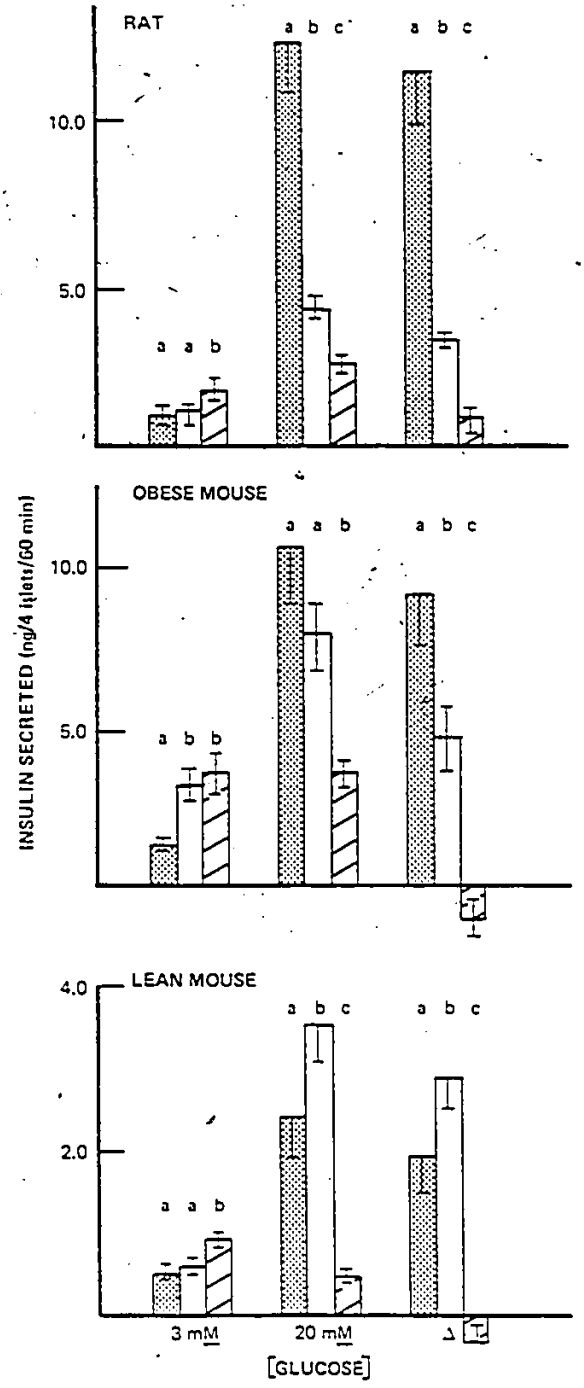


Fig. 24. Effect of different concentrations of trifluoperazine on the insulin secretory process in lean mice.

The islets were preincubated in 3 mM glucose as described in Chapter 2. They were incubated for 60 min in 3 (o—o) or 20 mM glucose (●—●). TFP, at concentrations indicated on the abscissa was present during the incubation period only. The net response to high glucose with or without TFP was obtained by subtraction of the insulin response in the presence of 3 mM glucose (△—△). Insulin was measured as described in Chapter 3. Results are given as the means ± SEM of 10-15 batches of islets (4 islets/batch). Asterisks denote a significant effect of TFP.

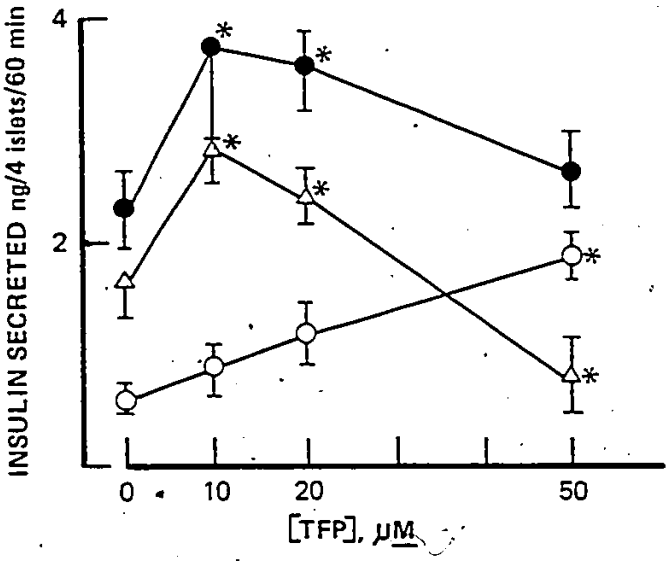


Table 15. Comparison of phosphodiesterase and radioimmunoassay for calmodulin determination in rat islets.

Size of islets	PDE Assay (ng calmodulin / islet)	RIA
A	3.82 ± 0.34	18
B	1.54 ± 0.29	7.0
C	1.13 ± 0.09	3.5

Rat islets were separated according to the size of the longest diameter as described in Chapter 2. A: .30mm and over; B: .19-.27 mm; C: .15mm or less. The results of the PDE assay are expressed as means ± SEM for 2-3 different islet preparations. Each preparation was assayed at several dilutions. The RIA values are the average of two different dilutions of one islet preparation.

Table 16. Relationship between calmodulin levels (as determined by PDE assay), islet volume and islet insulin content.

Source of islets	Insulin content	Volume	Calmodulin		
	ng/islet	$10^3 mm^3$	ng/islet	ng/mm^3	pg/ng insulin
Mice					
Lean	32 ± 2.1	0.70	1.05 ± 0.11	1,500	33
Obese	16 ± 1.7*	1.15	1.85 ± 0.08*	1,233	120
Rats					
A	223 ± 12	6.16	3.82 ± 0.34	620	17
B	103 ± 12	1.27	1.54 ± 0.29	1,213	15
C	33 ± 4	0.24	1.13 ± 0.09	4,708	34

The insulin content and the volume of the islets were determined as described in Chapters 3 and 2, respectively. The results are expressed as means ± SEM from a minimum of 25 determinations in the case of insulin. The volume results represent the average of 100 measurements but no SEM is given since parametric analysis cannot be used in this case (cf Chapter 5, section A.2). Rat islets were separated according to size (A, B and C) as described in Chapter 2. The asterisk denotes a significant difference ($P<0.05$) between lean and obese mice. Other details are as given in the legend to Table 15.

B. Discussion

As so much work had been done with rat islets (Janjic et al, 1981; Kotagal et al, 1982; Krausz et al, 1980; Mac Donald and Kowluru, 1982; Schubart et al, 1980; Sharp et al, 1980; Sugden and Ashcroft, 1981; Sugden et al, 1979; Valverde et al, 1979, 1981), I initially decided to use rat islets as a benchmark to study the calmodulin system of lean and obese mouse islets. I was able to reproduce the data reported in the literature for the effect of TFP on insulin secretion and for calmodulin levels in rat islets. This therefore gave me confidence that the differences which I found between lean and obese mouse islets and also between mouse islets and rat islets were due to physiological aberrations and species variations, respectively.

As reported in the literature for rat islets, TFP can result in an inhibitory (Janjic et al, 1981; Krausz et al, 1980; Schubart et al, 1980; Sugden et al, 1979; Valverde et al, 1981) or a stimulatory (Krausz et al, 1980; Sugden et al, 1979; Valverde et al, 1981) effect on glucose-induced insulin secretion. Both effects of TFP were noted in mouse and rat islets. As reviewed by Krausz et al. (1980), Ca^{2+}-calmodulin activates enzyme systems in islet cells which, individually, can stimulate or inhibit insulin secretion (Krausz et al, 1980). When TFP is present, an inhibition or a stimulation of insulin release could result depending on which systems are normally most affected by Ca^{2+}-calmodulin. TFP could inhibit insulin secretion by preventing the Ca^{2+}-calmodulin stimulation of adenylate cyclase and/or protein kinase. On the other hand, inhibition of phosphodiesterase and/or Ca^{2+}-ATPase by TFP could lead to a stimulation of insulin release.

In the present study, there were marked differences between the data obtained with lean mouse islets and those found with rat islets. The islets

from lean mice were found to be much more resistant than rat islets to the inhibitory effect of TFP on insulin secretion (cf Figs. 23 and 24). Variations have been reported by others in the behavior of mouse and rat islets. For instance, certain metabolizable insulin secretagogues produce different responses in the two species (Jain and Logothetopoulos, 1978). It was also reported more recently that Ca^{2+}-calmodulin does not stimulate the adenylate cyclase activity of albino mouse islets (Thams et al, 1982) while it does so in rat islets (Sharp et al, 1980; Thams et al, 1982; Valverde et al, 1979).

In comparing the results obtained with lean mouse islets versus obese mouse islets, the former were found to be less sensitive than the latter to the inhibitory effect of TFP on glucose-induced insulin secretion. The lesser inhibitory effect of TFP was not due to higher levels of calmodulin in lean versus obese mouse islets, as the calmodulin levels were actually higher in the obese than in the lean (1.85 vs 1.05 ng/islet). The observation of higher calmodulin levels in islets of obese mice gains support from the fact that elevated calmodulin levels have been observed in several tissues of streptozotocin diabetic mice and in tissues of C57Bl/Ks diabetic mice (Morley et al, 1982). The pathophysiological significance of these findings remains to be elucidated.

The calmodulin levels of rat islets which I found are in agreement with values previously reported. For example, a value of 1.54 ng/islet was found for the medium (B) size rat islets (Table 15); assuming an intracellular volume of 2 nl/islet (Sener and Malaisse, 1978) and a molecular weight for calmodulin of 16,700 (Cohen et al, 1978), this corresponds to approximately 45μM calmodulin as compared to reported values of 36 μM (Sugden et al, 1979), 50 μM (Valverde et al, 1979) and 16 μM (Landt et al, 1982). The calmodulin level which I found for the lean mouse

islets is higher than that recently reported by Thams et al (1982) for albino mouse islets (1.Ø5 ng/islet versus Ø.6 ng/islet).

It is not known whether it is the concentration of calmodulin or its intracellular distribution which is important in terms of metabolic functions (Tomlinson et al, 1982). It should also be noted that the use of a pharmacological agent like TFP to study the calcium-calmodulin system in pancreatic islets has its limitations. Whether the effects of TFP on insulin secretion can be entirely attributed to its binding to intracellular calmodulin has been questioned recently. TFP has been reported to inhibit Ca^{2+}-phospholipid-dependent protein kinase in islets (Yaney and Sharp, 1981). Moreover, phenothiazines have lipid solubilities and detergent properties which provide them with a wide range of effects on membranes and membrane-associated processes (Brostrom and Wolf, 1981; Means et al, 1982).

The results presented in this Chapter indicate major species differences in the interplay of the various factors which regulate insulin secretion and also qualitative as well as quantitative differences in the insulin secretory process in lean and obese mice. Whether the exaggerated insulin release of the obese mouse islets is directly or indirectly related to the calmodulin levels remains to be demonstrated.

CHAPTER 8. CONCLUSION

Previous to the studies presented in this thesis, it was known that OTC increases the sensitivity of humans and animals to insulin. This was confirmed here using the BB rat which, unlike pancreatectomized dogs, has a spontaneous diabetes resulting from the destruction of the islets, similar to human Type I diabetes mellitus (cf Chap. 4).

I used the ob/ob mouse to establish if OTC also had an effect on the endocrine pancreas. First, it was necessary to find out if there were differences in the insulin secretory response of obese vs lean mice. It had been demonstrated previously by others that the insulin secretory response to glucose was enhanced but that the biphasic nature of the response was preserved in this animal. With the studies presented in Chap. 5, however, I was able to show that the response to various secretagogues differed between lean and obese mice. Thus, in vitro, the insulin secretory response to secretagogues, including glucose, was maximally stimulated at much lower concentrations in islets from the obese than from the lean mouse. I have also provided evidence that calmodulin levels are higher in the islets of obese mice and that the characteristics of the inhibition of insulin secretion by TFP are different in the two groups (cf Chap. 7). These data show that the insulin secretory response differs not only in magnitude but also in quality in ob/ob vs lean control mice.

Experiments done on OTC-treated animals challenged with aminophylline in vivo showed that the insulin secretory response in these animals could be dissociated from the glycemia. Isolated islets from OTC-treated obese mice, incubated in vitro, had a secretory response to stimuli which was significantly lower than that found in untreated obese mice. These data led me to postulate that OTC does act on the endocrine pancreas as well as on peripheral tissues, possibly via similar mechanism(s). It is envisaged

that OTC treatment may improve the autoregulation of insulin secretion by making the B-cell more sensitive to insulin.

In Chap. 6, I reported that supplementation of the diet with zinc is able to reduce the exaggerated insulin secretory response to glucose and that a combination of OTC treatment and zinc supplementation was more effective than either treatment alone. This lends credence to my original hypothesis (p. 59) that OTC may be interacting with zinc in cells and thus influence insulin secretion.

Further experiments could be directed at:

(1) testing the hypothesis that OTC helps restore an effective autoregulation of insulin secretion in ob/ob mouse islets. This could be done by comparing the concentration of exogeneous insulin required to inhibit the insulin secretion in islets of untreated lean mice with that required in islets from obese control and OTC-treated mice, and by studying the effect of OTC treatment on islet insulin receptor activity.

(2) elucidating the abnormal insulin secretory response of the ob/ob mouse islets further. This could include: (a) a comparison of the calcium dependency of the insulin secretory process in islets of lean and obese mice. Marked differences were found between the calmodulin system of those two types of islets (cf Chap. 7) and the activation of enzyme systems by calmodulin is known to be dependent on the calcium concentration. (b) a study of the effect of <u>in vitro</u> exposure of ob/ob mouse islets to various zinc concentrations since it had been proposed by Ghafghazi et al (1979) that the inhibitory effect of zinc on glucose-induced insulin secretion in rat islets may be a regulatory mechanism.

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