FURTHER STUDIES ON THE ISOLATED ISLETS OF LANGERHANS OF OBESE HYPERGLYCAEMIC MICE AND THEIR NORMAL LEAN LITTERMATES.

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SUMMARY

Investigation of mouse islet respiratory and secretory activity has shown microdissected islets to be more suitable for *in vitro* studies than collagenase isolated islets. The hypersecretory activity of obese islets was verified and estimations of LDH isoenzyme patterns have indicated substantial anaerobic metabolism despite an increased capacity for oxygen uptake.

In vitro studies with normal and obese mouse islets have not demonstrated a ^{c}AMP or dbc-AMP stimulation of glucose induced insulin secretion. A functional adenyl cyclase/^{c}AMP-phosphodiesterase system has been demonstrated; operating at a higher level in obese islets than in normal. The effects of pharmacological and insulinogenic agents on membrane bound cyclase activity was investigated. Only limited correlation existed between the stimulation of islet cyclase or inhibition of islet phosphodiesterase and insulin secretion. The stimulation of islet adenyl cyclase was a β-adrenergic receptor function and its reduction an α-adrenergic receptor function. Glucose does not initiate insulin secretion by the direct stimulation of β-cell adenyl cyclase. An alternative path via glucagon has been suggested. The magnitude of cyclase and phosphodiesterase activity was similar in normal and in obese islets.

The ionic requirements for insulin secretion were present in normal and obese islet tissue.

Besides influencing directly the total biosynthesis and secretion of newly synthesized insulin and proinsulin in normal and obese islets, dbc-AMP may exert its effects on insulin secretion via islet glucose metabolism. Dbc-AMP increased the activity of islet G6PDH and islet glycogenolysis. Products of the latter may be utilised for the resynthesis of ATP from ADP.

SUMMARY

Cont'd....

A correlation was made between the serum I.R.I. profile and the morphological/biochemical constitution of obese islets during the manifestation of the obese hyperglycaemic syndrome with age.

The role of the adenyl cyclase system in insulin biosynthesis/secretion; islet glucose metabolism and the aetiology of the obese hyperglycaemic syndrome in mice has been discussed.

ACKNOWLEDGMENTS

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STATISTICAL ANALYSIS

All analyses were carried out on an Olivetti Programma 101 desk computor. Mean values $\pm$ S.E. and coefficients of Variation were computed for groups of data.

The significance of the differences between groups of data was evaluated by student 't' test and probability levels of $p < 0.05$ were taken as statistically significant.

Best straight lines were computed by the method of 'least squares'.

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INTRODUCTION

The normal physiological stimulus for insulin release and synthesis is believed to be the concentration of glucose perfusing the islets of Langerhans (1-3). Studies with 2-deoxyglucose, a glucose analogue which was phosphorylated but not further metabolised and which inhibited intracellular glucose metabolism, have indicated glucose metabolism by the islets to be essential for the regulation of insulin secretion and synthesis (4). However, Grodsky and coworkers observed that 2-deoxyglucose did not inhibit the ability of glucose to increase insulin release by the isolated perfused rat pancreas and mannose was found to be as effective as glucose in stimulating insulin secretion (5).

Substances other than sugars have been implicated in the process of insulin secretion. The oral sulphonylurea drugs increase the pancreatic secretion of insulin (1). Seltzer suggested this effect was due to solubilization of pre-existing insulin rather than to any increase in insulin synthesis. Increased insulin release from pancreatic tissue as a consequence of *in vitro* addition of the sulphonylureas has also been reported (6).

Under certain circumstances, the amino acids leucine and arginine have been found to initiate insulin secretion from pancreatic islets (7). Children with idiopathic hypoglycaemia show a depression in their blood glucose following the administration of leucine (8). This leucine induced hypoglycaemia was usually associated with increased plasma insulin activity and suggested an effect on insulin secretion by islets (9) (10). However, a primary action on the

liver to reduce hepatic glucose output has been suggested as the cause of leucine-induced hypoglycaemia (11).

R-Candela and coworkers reported that ATP stimulated insulin secretion both *in vivo* (12) and *in vitro* (13), and this stimulation plus that induced by glucose was partially inhibited by pre-treatment of the pancreatic slices with n-ethylmaleimide (14).

Many studies have been performed in an attempt to correlate changes in β-cell function with alterations in the histological appearance of the β-cells. Increased insulin secretion has been produced by the administration of glucose (15), adrenal steroids (16), growth hormone (17), glucagon (18), oral antidiabetic drugs (19) and many other pharmacological and physiological agents. Indeed the diversity and increasing number of potentially insulinogenic substances have made the insulin secretion response to stimulation almost non-specific. Histologically, stimulation of secretion was found to be consonant with a degranulation of the β-cells which was not usually complete until after 48 hours and appeared to correlate with the prevailing degree of hyperglycaemia (20). The degranulation of the β-cells was associated with decreased extractable insulin from the pancreas (21), and an increased amount of insulin-like activity in the plasma (22). Subsequently there was an enlargement of the β-cell nucleus and golgi apparatus. Increased β-cell mitotic activity was also present if the hyperglycaemic stimulus was prolonged (20) and was generally followed by the deposition of glycogen. This polysaccharide has been observed by electron

microscopy and estimated by microchemical analysis in islet tissue (23) but its importance has yet to be determined.

Much work has been carried out with the obese hyperglycaemic mouse since Christophe and colleagues first presented evidence to show that the pancreas of the animal secreted excess insulin (24). This was associated with considerable hyperplasia and hypertrophy of the islets and their volume was estimated to be some 10x that of their normal lean littermates (25). Pronounced degranulation of β-cells was accompanied by enlarged nuclei and nucleoli (26). The golgi apparatus was fragmented and its hypertrophy and increased complexity has been described in association with increased insulin secretion (27).

The electron microscope permitted a more detailed inspection of the anotomical changes associated with the processes of insulin biosynthesis and secretion. Lacy formulated the process of emiocytosis to describe the latter (28) and such a sequence was reported to occur in the β-cells of the obese hyperglycaemic mouse (29). With only minor modification the basic concept of emiocytosis would seem adequate to explain the physical process of β-granule secretion. However, despite the many detailed descriptions of the process of insulin biosynthesis and secretion, little is known or understood of the biochemical and biophysical mechanisms involved. Studies in the past were hampered by the fact that mammalian islets were diffusely spread throughout the exocrine tissue and comprised only a very small percentage of the total pancreatic mass (30).

Nevertheless various approaches have been used in an attempt to study β-cell metabolism and its relationship to insulin biosynthesis and secretion. With the advent of the genetically obese mouse (31), came the opportunity to use and obtain larger quantities of islet material. Obese mice and the recently introduced genetically diabetic(db,db)mice (32) have been used to obtain a greater understanding of theaetiology of human obesity and diabetes (33-37).

In the last decade two principle methods emerged for the harvesting of mammalian islet tissue in sufficient quantities for biochemical analysis. They were, freehand microdissection (38) and collagenase digestion (39). Attempts have been made to exclude all cells other than β-cells either by utilizing only the central portion of microdissected islet tissue or by gently rupturing isolated islets and separating the component cells, after prior histochemical and microscopic identification (40). The method of collagenase digestion has become very popular because of the ease that it lends itself to technical assistance and the grand scale on which it can be carried out. Many of the observations made in the past concerning the metabolism of the β-cells were made using only one of these experimental approaches and occasionally the results obtained by one technique could not be confirmed with the other preparation.

It was on this basis, that a comparison of the metabolic viability of microdissected and collagenase isolated islets using respiratory and secretory parameters was made the preliminary feature of the work presented in this thesis.

The more suitable technique was then used to separate islet tissue from obese hyperglycaemic mice and their normal lean litter mates. An assessment of the physiological viability of islets taken from these animals was made by investigating the possibility of a regulatory feedback inhibition of previously released insulin on insulin secretion and an investigation of islet LDH - isoenzyme patterns.

The results of a great deal of research in recent years have suggested an adenyl cyclase system to be involved at some point in the process of insulin secretion (41). Indeed both adenosine 3', 5'-cyclic monophosphoric acid (^{c}AMP) and the substrate from which it is derived (ATP) have been shown to stimulate insulin secretion in vitro (42). Cerasi & Luft have recently suggested the adenyl cyclase system to be an integral part of a stimulatory chain of events leading to insulin secretion and that the diabetic syndrome may result from the failure of this adenyl cyclase system or at least the malfunction of some part of it (43). It was important, therefore, to locate and determine the adenyl cyclase and ^{c}AMP-phosphodiesterase activity in the islet tissue of normal and obese hyperglycaemic mice. An investigation of the effects of pharmacological, physiological and insulinogenic agents on the system might provide information not only concerning the stimulus for insulin secretion or the regulation of the latter but also on the aetiology of the obese hyperglycaemic in mice and diabetes mellitus. The obese mouse itself shows certain characteristics of maturity onset diabetes. In addition, determination of ^{c}AMP-phosphodiesterase activity might provide information on the turnover of ^{c}AMP in islet cells. The demonstration of a functional adenyl cyclase system

demanded an evaluation of the role of ^{c}AMP in islet tissue. Since ^{c}AMP did not enter cells easily, in vitro studies were carried out with dbc-AMP ($N^{6},O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid). The latter had the advantage of greater membrane solubility and was virtually resistant to attack by ^{c}AMP-phosphodiesterase. Investigation of the effect of dbc-AMP on insulin secretion; oxygen uptake; LDH-isoenzyme pattern; total RNA and DNA and insulin and proinsulin biosynthesis in normal and obese islet tissue was supplemented by investigation of the role of ^{c}AMP in islet glucose metabolism. The latter was carried out by evaluating the effect of ^{c}AMP on certain enzymes associated with the major metabolic pathways. Also since islet ATP was the substrate for islet adenyl cyclase and the influx of Ca^{2+} was believed to be essential for the process of insulin secretion (44,45). The levels of both were established in normal and obese islet tissue.

Finally the obese hyperglycaemic syndrome in mice has been shown to develop in severity with age (31). The primary metabolic lesion responsible for the syndrome might reside in the obese islet per se. Comparison of the serum IRI profile and islet morphology with certain aspects of islet metabolism after 10, 20 and 40 weeks of age was made to elaborate more concisely the situation in the obese mouse islet and contribute to a deeper understanding of the aetiology of the obese hyperglycaemic syndrome.

The obese hyperglycaemic syndrome in mice, genotype (ob.ob).

Renold and coworkers (33,34,35,36,46) and more recently Bray (47) have extensively reviewed the information available on syndromes associated with hyperglycaemia and hyperinsulinism in laboratory

rodents. Studies on obese mice have shown their inappropriate hyperglycaemia to be a genetically controlled abnormality. The obese syndrome is inherited as an autosomal recessive gene with complete penetrance showing a maturity-onset type of diabetes and obesity (33,36,48,49,31). The obese mouse is genetically independent of other diabetic mice and the obese is located on linkage group XI. No heterozygous effects have been observed in the obese and the latter are almost always infertile (50). There is an elevated serum IRI and hypersecretion of insulin, together with resistance to insulin and hypercholesterolaemia. Affected animals are usually non-ketotic. Obese mice placed on a restricted diet become sensitive to insulin injection, their islets become normal in appearance and their life span resembles that of the lean litter-mate (50). It is probable that the syndrome in mice begins with a spontaneous decreased responsiveness to insulin as the primary event. The decreased effectiveness of insulin is generally associated with a rather long lasting hyperinsulinaemia. This insulin resistance is most marked in adult or middle-aged animals and may decrease later, as Westman observed in the Swedish obese mouse colony (51). Both hypertrophy and hyperplasia of the β-cells are frequently present at least at some point during the life history of the syndrome (52). Hellman has emphasised that in the obese mouse there are no true abnormalities in the β-cells but only such changes that one would expect to occur in normal β-cells exposed to persistently high serum glucose levels (53).

Westman has accumulated data concerning the earliest manifestations of the obese syndrome (54). The homozygous mice showing the syndrome can be recognised by their greater accumulation

of adipose tissue at about 10 weeks (55). Alonzo & Maren have shown that the increase in body weight continues until 8-14 months of age attaining a weight of about 70g.(56). The obese animals of this size display a total amount of body fat about 20x that of their normal lean littermates (57). Moderate hyperphagia of the obese mice together with increased alimentary efficiency and relative immobility may be responsible for the gross overweight (58). The shorter life span of obese mice compared to their lean littermates (59) can be remedied by food restriction (56,60). Blood sugar levels start to increase after the appearance of overweight at about two months and glycosuria is found with increasing age (55). The serum concentration of insulin also increases with age and this increase coincides approximately with the period of rapid gain in body weight (54). Maximal hyperglycaemia, with a mean value above 300 mg/100 ml developes after 3-4 months and then gradually returns to normal levels (54,61). The hyperglycaemia of obese mice,which declines after about 7 months of age, has been reported to be very sensitive to fasting and to almost disappear after 17 hours starvation (55,61). The serum insulin remains high for several months and then, like blood sugar, declines to concentrations in the same range as those of lean litter-mates (54). The peak mean value for obese mouse serum insulin has been shown to occur later than the peak of the serum glucose level (66). The combination of high insulin and glucose concentrations in the serum has been regarded as indicative of peripheral insulin resistance in the obese mouse (61,62). The finding of a marked hyperplasia of obese islets (63,64) also supports the concept of an increased insulin demand. Gepts (63) reported that the islets of adult obese mice consisted of more than 90% β-cells which display cytological signs of hyperactivity (26). In young obese

animals showing raised serum insulin levels, the total pancreatic content of extractable insulin was found to be lower than that of the lean littermates (62). On the other hand, in adult obese mice the same authors reported large stores of insulin in the pancreas. In old obese mice the serum insulin like activity was shown to decrease, indicating an exhaustion of the endocrine pancreas (61). Stauffacher and coworkers have demonstrated a 90% reduction in biological activity of serum insulin from both obese and lean mice using insulin antibodies (62). Mayer et. al. provided evidence to indicate an increased tolerance against exogenous insulin (55), yet Shull & Mayer (65) also noted that the administration of a low dose of insulin (1 U/mouse), brought about a fall in the blood sugar level equivalent to that in the lean littermates. The increased tolerance to the administration of exogenous insulin appeared before both overweight and hyperglycaemia in the obese mouse and did not seem to be due to a more rapid disappearance of hormone from the serum (66). Indeed obese mice were found to have a considerably greater turnover of the hormone compared to the lean littermates (67). The absolute disappearance rate of insulin from the sera of obese animals was considerably higher than in normal animals and this observation was consonant with the available morphological evidence for a high rate of insulin secretion from the β-cells of obese mice (63,26). Westman suggested that the pattern of development of the obese hyperglycaemic syndrome conformed with the view that diabetogenic factors were active during a limited period in the life of the obese animal (54). The appearance of insulin resistance in young mice, which later become obese, could induce an increased insulin secretion with a hyperplasia of islet β-cells in an attempt

to overcome the insulin insensitivity and thus counteract hyperglycaemia by a compensatory hyperinsulinaemia. Westman has shown (54) that only when the obese mice reached a constant body weight, did the increased serum insulin concentration seem adequate to keep the blood sugar at the same level as in lean littermate controls. Any subsequent falling off of the serum insulin level would probably reflect either reduced diabetogenic influences or some morphlogical degeneration of the islet tissue. Christophe and coworkers (68) investigating the obese syndrome in Bar Harbour mice found the total islet tissue in the obese pancreas to be 6x that of the pancreas in the lean littermate. Also the multiplication of the α-cells appeared to be greater than that of the β-cells. In this mouse it was suggested that the diabetes was due to the development of insulin insensitivity in peripheral tissue (perhaps muscle). Stauffacher et. al. found that the muscle tissue of the obese mouse (ob,ob) was insensitive to insulin and this insensitivity was causally related to the development of the syndrome (62). Chlouverakis & White suggested that the insensitivity of the 'Birmingham' obese mouse to insulin was the product of a tissue resistance and this insensitivity was secondary to obesity or to a factor associated with it, since it disappeared following a reduction in body weight (69). Obese mice have been shown to be capable of eliminating their blood glucose at a near normal rate (70). A finding which was puzzling in view of their marked insulin resistance. Also Chlouverakis has demonstrated an increased absolute rate of tissue glucose uptake in obese mice which was probably due to the expanded mass of adipose tissue in these animals (71). The adiposity of the obese mouse was assumed to precede the occurance of hyperinsulinaemia and hyperglycaemia (75).

Genuth and coworkers indicated that insulin resistance was not likely to be the basic genetic abnormality in obese mice and suggested that hyperinsulinaemia and hyperphagia, either causally related or both due to the same genetic hypothalamic disturbance, were more likely to initiate the obesity seen post weaning. This obesity would then in turn further augment insulin resistance and hyperinsulinaemia, with diabetes the ultimate outcome (72). It has been suggested that the pathogenesis of the syndrome of obesity and hyperglycaemia in the mouse differs from that of hypothalamic obesity. The basic difference being that, in the latter, the regulation of food is deranged, resulting in hyperphagia and obesity (regulatory obesity), whereas in the obese mouse (ob,ob) hyperphagia is secondary to a primary metabolic defect (metabolic obesity) (73,74). The primary metabolic defect has yet to be found and evaluated. Chlouverakis has shown that obese mice could become obese even in the absence of excessive food intake and this suggested that the hyperphagia of obese mice was not totally responsible for their obesity (76). The hyperphagia of the obese mouse was perhaps a means by which the animal could achieve the genetically determined mass of adipose tissue.

Genuth has suggested that the hyperinsulinaemia of obese mice was due to a primary abnormality in islet cell function rather than to insulin resistance alone (77). An elegant study by Strautz using obese mice implanted with millipore diffusion chambers containing islets separated from normal lean littermates, demonstrated that the spontaneous obesity of the hereditary obese mouse was the result of a missing or defective pancreatic islet factor(s). This factor was present in the islets of normal non-obese mice and its presence was necessary for normal glucose and lipid metabolism and insulin sensitivity (78). The factor was assumed to exert its influence

either by a direct action on peripheral tissue (79) or indirectly by its control of insulin response to blood glucose loads (80). The pancreatic source of this factor was suspected after the observation that obese mouse islets were deficient in silver positive (α^1) cells (81). Hellman & Lernmark after using water extracts of pigeon α^1-cells to inhibit insulin secretion (82) suggested that the α^1-cell was the source of a third pancreatic factor, gastrin. The results of Lernmark and coworkers were consonant with the hypothesis of a local regulation of the endocrine activity in β-cells by adjacent α^1-cells (83).

Strautz's work (80) did not support Clarke's early theory that the obese syndrome was caused by an islet hyperglycaemic factor possibly glucagon (84). However, evidence was presented to suggest that the primary action of the factor was on lipid metabolism. Indeed Mayer proposed that the factor was directly involved in lipogenesis in adipose tissue (79). He suggested that this factor repressed glycerokinase activity in adipose and other extrapancreatic tissues. The glycerokinase allowed cells to reutilise glycerol released by lipolysis and facilitated the esterification of fatty acids and hence fat deposition. The results of Koschinsky and coworkers were consonant with Mayer's hypothesis (85). Glycerol kinase activity was higher in obese fat cells than normal and in both cases regulated by the serum insulin levels. In consequence the hyperinsulinaemia of the obese mouse was assumed to be an important factor which encouraged fat deposition by inducing glycerol kinase activity. Many of the other pathophysiological characteristics of obese mice, described earlier, would be secondary responses to the abnormal lipid and glucose metabolism in adipose tissue.

Work on the spontaneous diabetes in laboratory animals suggested that human diabetes mellitus was a disorder of which the heritable component was polygenic rather than the product of a somatic recessive gene with irregularities accounted for by 'variable penetrance' (37,86). In human diabetes, however, there was the additional complicating feature that the phenotypic expression of one or several of the genes involved was likely to be conditioned by environmental factors.

CHAPTER 1

SECTION 1

Comparison of the glucose induced insulin secretory activity and total insulin content of freehand microdissected and collagenase isolated islets of normal and obese mice.

Synopsis of the methods available for the isolation of islet tissue:

With a total volume of only a few percent of the whole gland, the pancreatic endocrine organ has been found to be dispersed throughout the exocrine parenchyma in the form of small islets, the islet of Langerhans (87). Laguesse was the first to suggest that the islets were the site of an internal secretion (88). However, significant progress in islet research was not achieved until Lowry's microtechniques (89-91) were applied to the pancreatic islet tissue of mammals by Lacy (92). Here samples of islet tissue (0.02-0.2 µg) were microdissected from unstained frozen-dried sections of pancreas and either assayed for insulin content (93) or used for quantitative studies of several oxidative enzymes (94). The islets were identified by their characteristic grey-brown colouration and great vascularization. Lazarow used the same techniques in an attempt

to characterise the metabolic pathways of rat islet tissue (95). The potential of the methods available for dissecting islet tissue from frozen sections was limited in view of the rather small amounts of tissue obtained. Also freezing and drying of material, prior to metabolic studies requiring surviving cells was not recommended (96).

In response to a requirement for greater versatility Hellerström introduced a microdissection procedure for the isolation of pancreatic islets (38). It consisted of a refinement of Bensley's mechanical technique (97) without the injection of neutral red and had the advantage of providing a good yield of fresh islet tissue in a relatively short time. Mice, especially those showing the obese hyperglycaemic syndrome were better suited to Hellerström's technique than rats or guinea pigs because of their very thin pancreas. Tissue viability was ensured by using a bicarbonate buffer dissecting medium rather than 0.25 mol/l. sucrose at 2-5°C. Viability in this type of medium has been confirmed (98) and microdissected islets have been shown to be metabolically active (99).

A variation of microdissection was demonstrated by Keen and coworkers (100-102) and consisted of ligating one of the main pancreatic ducts. The subsequent degeneration of the exocrine tissue after 2-4 weeks left the islets to be dissected out of the atrophied organ. The pathological material obtained did show metabolic viability since Jarrett, Keen & Track have used this preparation to demonstrate glucose induced rat islet RNA synthesis (103).

The microdissection of islet tissue did not lend itself to bulk automated biochemical analysis and it was probably no surprise that Moskalewski introduced an enzymatic method for the separation of guinea pig islet tissue from the surrounding exocrine tissue using collagenase digestion (39). Partially digested tissue was allowed

to sediment and islets were separated by dilution with buffer and a little dissection. Lacy modified Moskalewski's method and obtained islet tissue from the collagenase digested rat pancreas by both sedimentation and the use of sucrose gradient centrifugation (104). By light microscopy the islets isolated by this technique appeared intact, but failed to give a uniform insulin response to high and low glucose. A modification of the collagenase method was used for the separation of islets from rabbit pancreas by Howell & Taylor (105,106). In this case islets were separated from the diluted enzyme solution by gentle centrifugation. A sequence of washings in low glucose buffer followed by centrifugation resulted in a crop of islets free of exocrine fragments. Gerner recently applied what must be a final modification of the collagenase technique. Islets were separated from the collagenase brei by ficoll gradient centrifugation (107).

The isolation techniques of microdissection and collagenase digestion were the ones of choice in more recent islet research. The metabolic viability of both microdissected (99) and collagenase (109) isolated islets has been established, although Lacy and coworkers were unable to demonstrate a tolbutamide stimulation of insulin secretion from isolated rat islets (108).

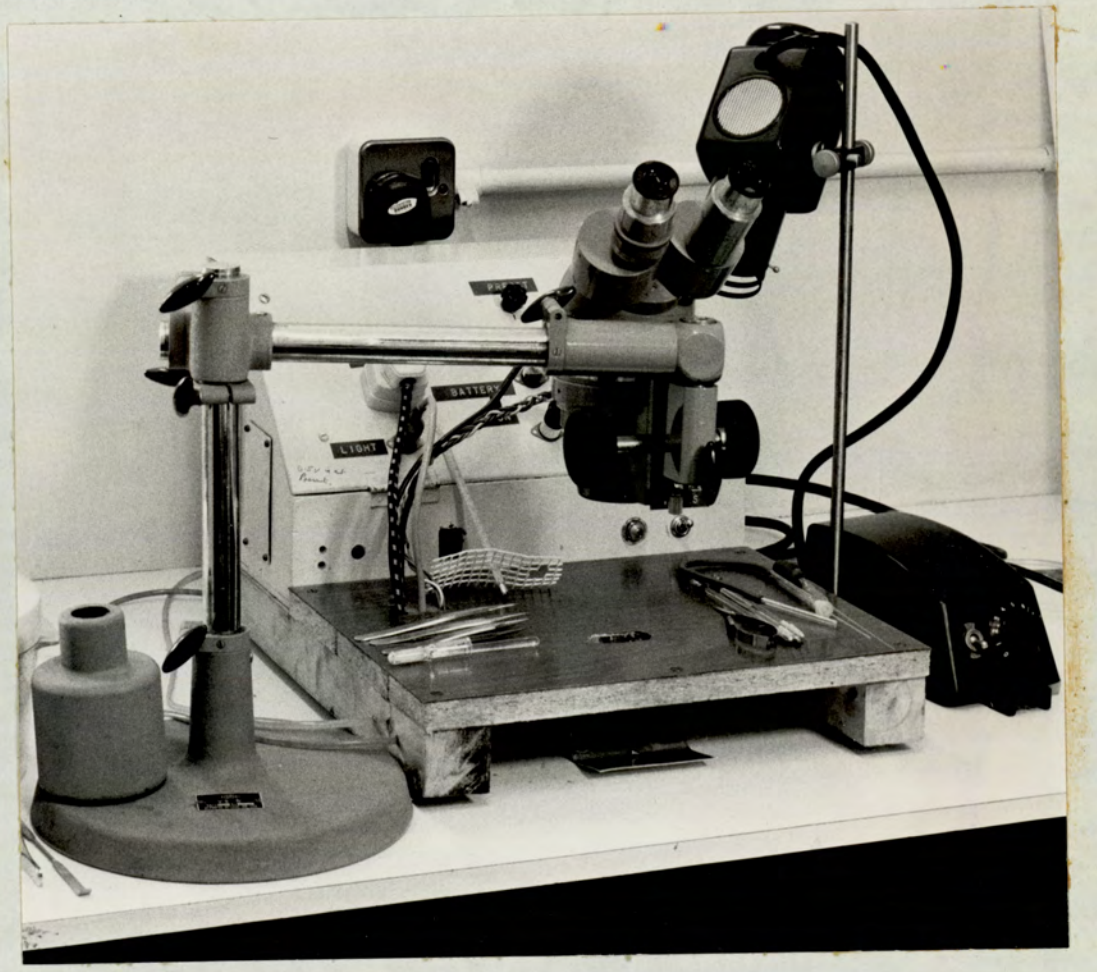
The following study was undertaken to establish which of the two isolation techniques, microdissection or collagenase digestion, provided the more viable and suitable preparation for subsequent biochemical estimations.

MATERIALS AND METHODS

Microdissection

Islets were microdissected from the freshly excised pancreas of mice by a modification of Hellerström's method (38). A photograph of the apparatus used is shown below.

Apparatus for the microdissection of islet tissue.
PLATE (1).

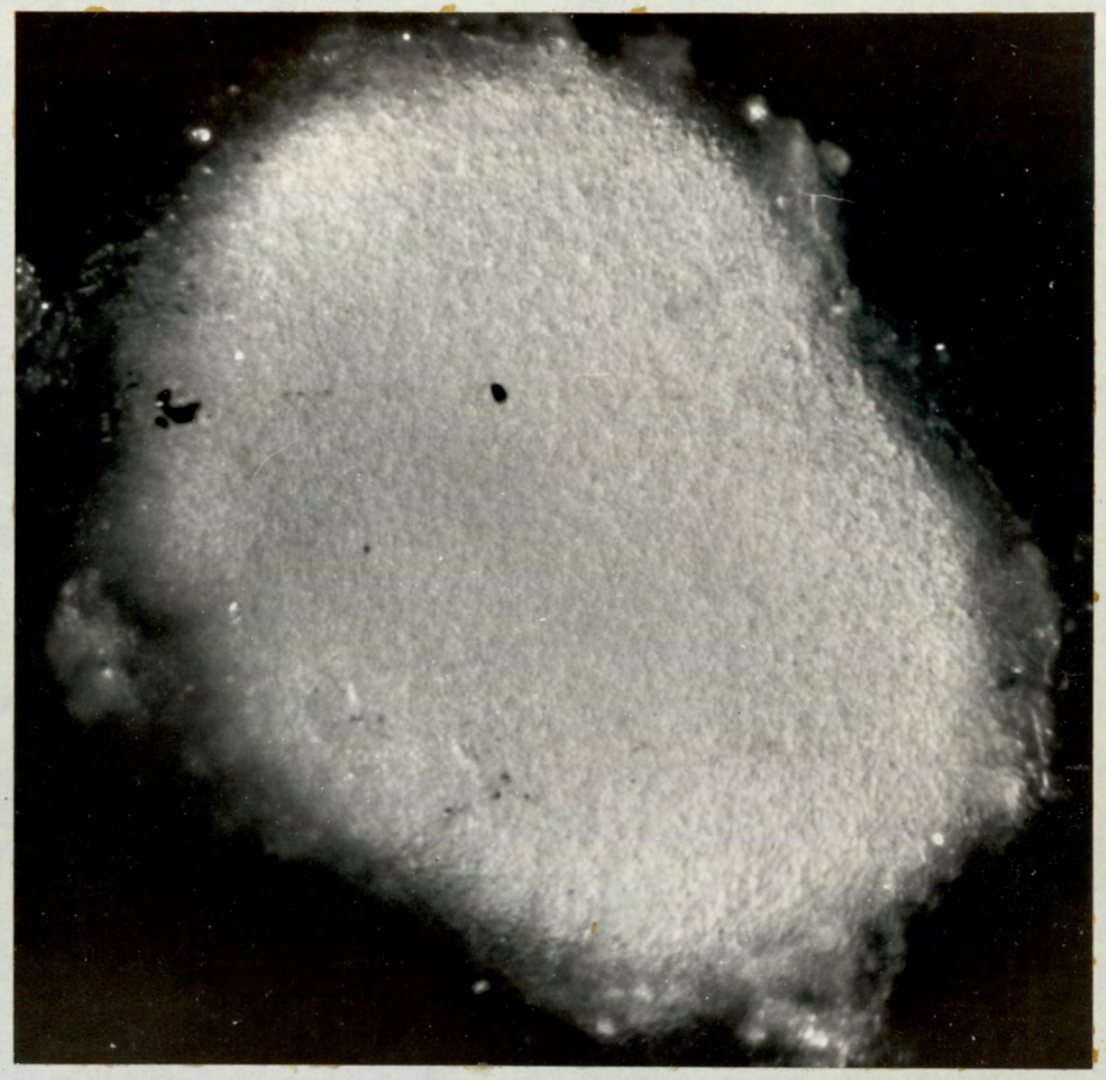


Details of the operating instructions and circuitry are given in the appendix.

The excised pancreas was placed in the small black plastic dissecting well containing Gey & Gey bicarbonate buffer (110) supplemented with glucose (0.6g/l) and maintained at 2-5°C by a water cooled thermo-electric element (Frigistor). Temperature

increases in the dissecting medium were detected and compensated for by a pair of thermistors in one corner of the dissecting well. The dissecting area was viewed through an olympus stereo microscope (x7.5 - 30). Oblique illumination was supplied by a single side lamp with adjustable intensity. The dissecting medium was changed regularly to avoid clouding by fat globules and tryptic attack on isolated islets by enzymes liberated from cut surfaces of exocrine tissue. The pancreas was first cut into manageable portions with fine scissors and all fat removed. Islets identified against the black background were dissected and teased freehand from the pieces of pancreas with 20 gauge hypodermic needles attached to glass rods and fine watchmaker's forceps. In all mice used, there was a marked accumulation of islet tissue along the large blood vessels of the more central parts of the pancreas. With some practise the microdissection of an islet was completed within 5 minutes. The large islets of obese hyperglycaemic mice were easily identified in the fresh pancreas by their bright creamy colour and pink vascularisation. Obese islets were easily detached from surrounding exocrine, but were often closely applied to each other. Hence the difficulty with obese islet microdissection was the separation of islets from each other without rupturing, rather than the separation from adjacent exocrine tissue. Microdissected islets were transferred, with the aid of a finely drawn-out glass pipette, from the dissecting well to a black solid watchglass containing ice cold oxygenated Gey & Gey buffer (110). The finely drawn-out pipette was found to be more convenient than a braking pipette, wire loop or glass needle since several islets could be transferred in a single operation. Microdissection was usually carried out from 9.00-11.00 hours each morning and during this period sufficient fresh tissue was obtained for any subsequent biochemical analyses.

PLATE 2 A typical islet obtained from a normal mouse by microdissection (x160)



Collagenase Digestion

The collagenase isolation of islet tissue was carried out by a modification of the methods of Lacy & Kostianovsky (104) and Howell & Taylor (105,106). Pancreatic tissue was taken from mice killed by hyperextension of the neck and emersed in ice cold oxygenated ($95\%O_2/5\%CO_2$) Gey & Gey buffer (110) containing low glucose (0.6g/L). Buffer was injected into the organ with an 18 gauge needle until the connective tissue planes of the organ were grossly distended. This procedure was essential since it ensured

subsequent uniform enzyme activity and presented a maximum tissue surface area to the enzyme. Excess blood vessels and fat were removed. The tissue was then cut into small pieces with scissors and washed twice in cold buffer before being poured into a small glass weighing bottle containing 2ml. of a collagenase low glucose buffer solution. The bottle was securely stoppered and shaken rapidly ('Mickle, electric shaking incubator', Gomshall, Surrey) for an appropriate period of time at 37°C.

The amount of collagenase required for digestion varied among the manufacturer's lots and the duration of the incubation period was found to be critical. Insufficient incubation resulted in incomplete separation of islets from exocrine tissue, whereas over incubation caused the erosion and often complete destruction of islets. Even separated islets had to be carefully scrutinised since many were found to be nothing more than emptyspheres of tissue. The following optimal incubation times were established for the different collagenase lots used.

Collagenase form II (Koch-light),	5mg/ml,	12-20 minutes
Collagenase I (Sigma),	5mg/ml,	20-30 minutes
Collagenase III (Sigma),	3mg/ml,	15-20 minutes

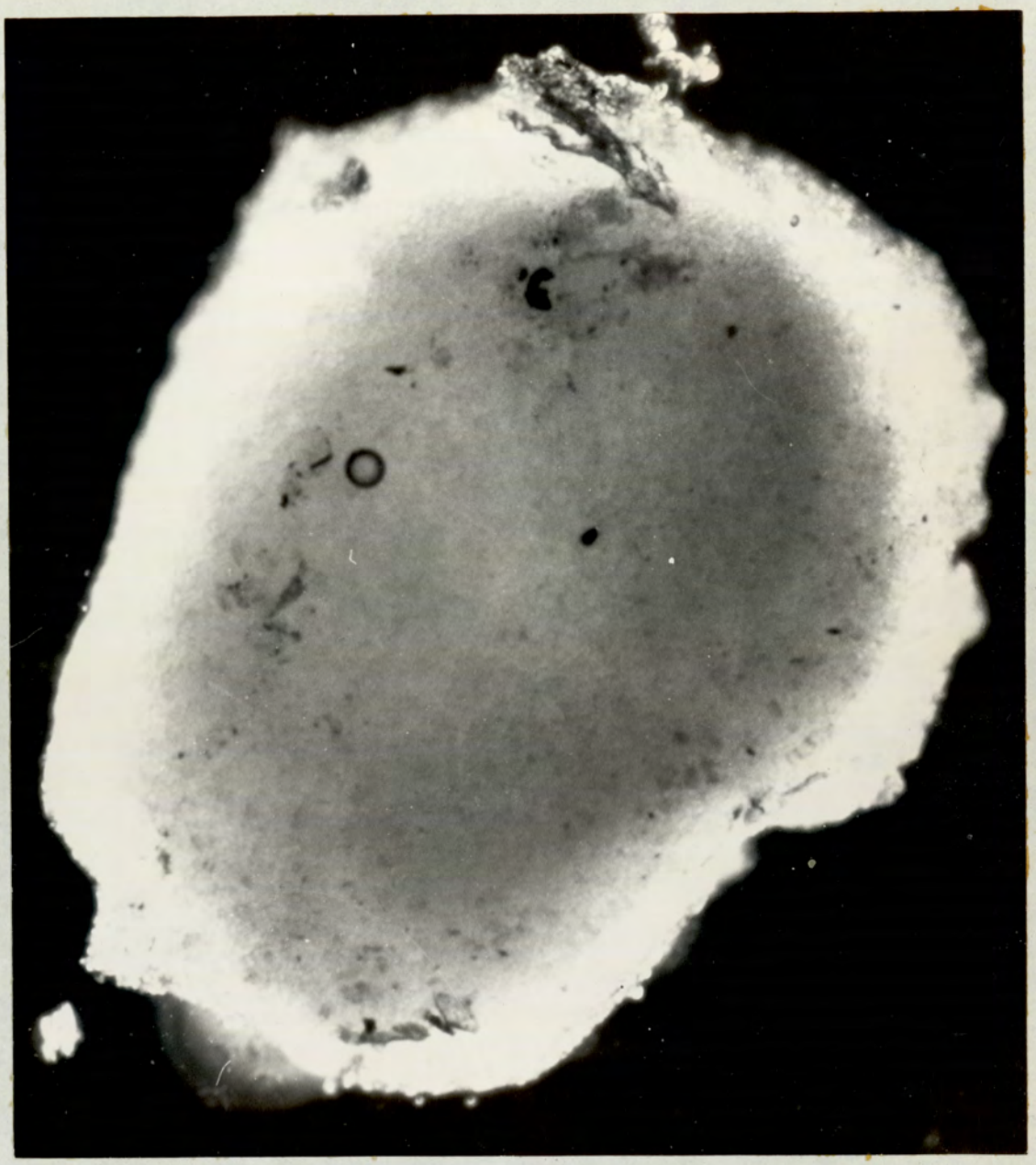
Collagenase form II (Koch-light) was the preparation of choice. The approximate duration of incubation was determined in preliminary experiments by sampling the contents of the incubation bottle at 2 minute intervals and continuing the incubation until the islet tissue appeared to be predominantly free of adhering exocrine fragments. With practise the incubation time was assessed fairly accurately by the size of the fragments and general appearance of the digest.

After the appropriate period of incubation, the digest was poured into 10 ml. of low glucose buffer (0.6g/L) and the whole lightly centrifuged for 5 minutes. The supernatant was discarded. The fragments were resuspended in a further 10 ml. of buffer, centrifuged and the supernatant again discarded. This procedure was repeated a third time. The precipitate from the last centrifugation was dispersed in 10 ml. of buffer and the whole transferred to a petri-dish with a black painted bottom. The dispersed precipitate was observed under a dissecting microscope and all remnants of adhering exocrine tissue were detached from islets by gently sucking up and down in a pasteur pipette. Islets still connected to resistant blood vessels after digestion were either cut free with fine scissors or with needles similar to those used in the microdissection procedure. A preliminary separation of islet tissue was made with a finely drawn-out pasteur pipette. Islets separated in this way were then diluted with buffer and transferred again until a crop was obtained free from exocrine tissue. Islets so isolated were checked under the microscope and maintained in oxygenated (95% O_2/5% CO_2), low glucose buffer (0.6g/L), in a black solid watchglass over ice (2-4^{o}C) until required. A typical normal mouse islet obtained by collagenase digestion is shown overpage.

ANIMALS

Obese hyperglycaemic mice (ob,ob) and their normal lean litter mates (NLL) together with Tyler's original strain of white mice (TO) were fed rat and mouse research diet 41B (Pilsbury's Ltd., Birmingham). All mice were 20-25 weeks old and allowed free access to food and water until death by hyperextension of the neck. Animals were culled at 9.00 hours each morning in order to eliminate any

PLATE 3. A typical islet obtained from a normal mouse by collagenase digestion. (x160).



circadian variation of islet activity (111). Animals were not fasted over night prior to use in experiments since starvation has been shown to reduce *in vivo* and *in vitro* glucose stimulated insulin secretion and the total extractable insulin of isolated rat islets (112-114). Grey and coworkers have suggested that the impairment of

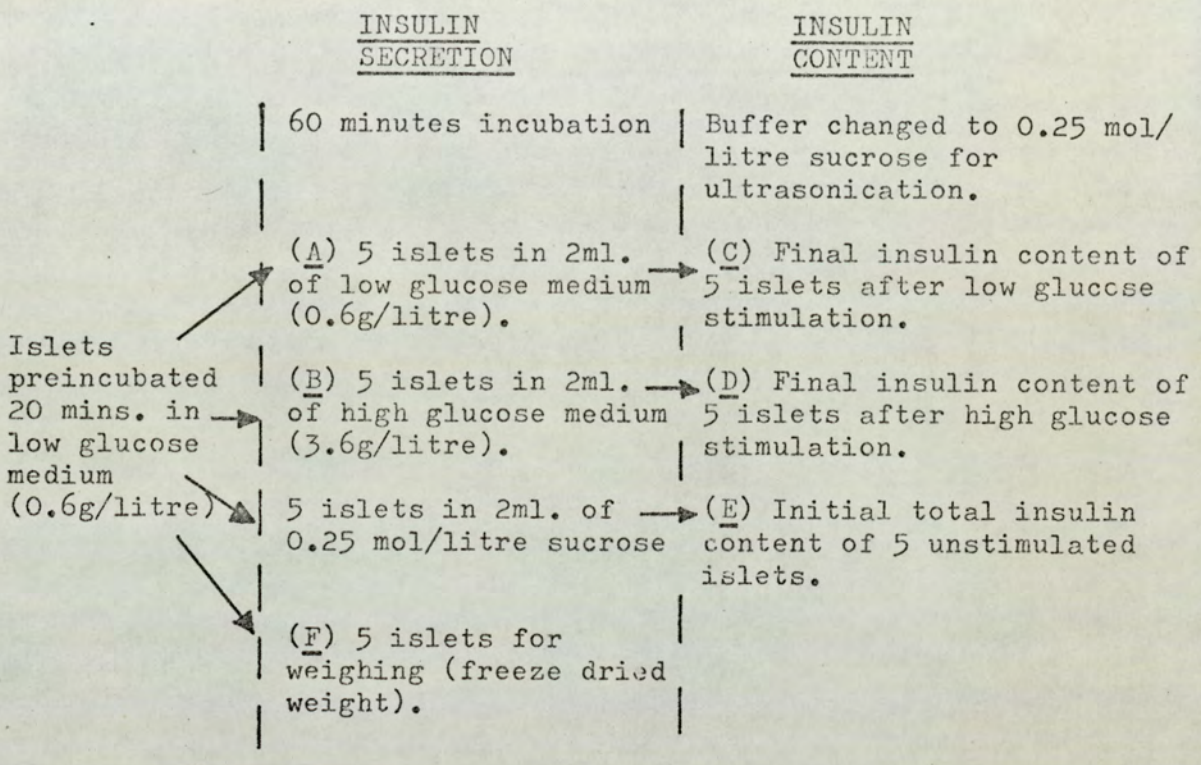
glucose stimulated insulin secretion during fasting and its restoration by refeeding were regulated by changes in a glucose-inducible enzyme system in the pancreatic β-cell (114).

Incubation of islets and preparation of samples for insulin immunoassay.

Oxygenated (95% O_2/5% CO_2) Gey & Gey buffer (110) was the basic incubation medium used, supplemented with the appropriate amount of glucose. In preliminary experiments bovine serum albumin V 1g/litre (Armour Pharmaceuticals Ltd., Eastbourne, Sussex.), was added to the incubation medium. In subsequent work, however, this practise was discontinued because of the excessive frothing produced during the continuous oxygenation of incubated islets. Instead, incubation vials, although precoated with silicone ('Siliclad' or 'Sigmacote (T)') were rinsed with a solution of bovine serum albumin V in Gey & Gey buffer (2g/litre) prior to the addition of incubation medium and islets.

Animals and their excised pancreata were weighed prior to the extraction of islets by either freehand microdissection or collagenase digestion. All islets were used within one hour of isolation and those of sound appearance preincubated for 20 minutes at 37°C in 5 ml. of continuously oxygenated low glucose buffer (0.6g/litre). After preincubation islets were quickly washed with fresh buffer at 37°C and divided into groups of five islets according to the programme below. The islets taken from a single mouse pancreas were generally sufficient to cover this programme. In order to investigate the effect of glucose, islets were

Incubation programme for microdissected and collagenase isolated islets.



transferred with a finely drawn-out pipette into 1 dram vials (Johnsen & Jorgenson Ltd., London) each containing 2ml. of oxygenated buffer at 37°C and a predetermined amount of glucose (A) & (B). Each 5 islet lot contained a range of islet sizes and were made equivalent by eye. In order to measure the initial total immunoreactive insulin content (E), 5 islets were placed in 2ml. of 0.25 mol./litre sucrose and retained at 2-4°C until required for homogenisation. A further 5 islets (F)(judged by eye to be about the same size as those used for insulin secretion and content experiments) were processed to give their dry weight in mg. by the technique described below. This procedure of weighing 5 islets separately had to be adopted since islets used in secretory studies were also required for measurement of final insulin content after glucose stimulation and were consequently homogenized.

Estimation of the freeze-dried weight of islet tissue.

A small aluminium foil planchet was prepared with its containing surface pierced several times with the point of a needle. 5 islets were transferred to the planchet and the latter was placed on a filter paper. All buffer was subsequently drained from the planchet to the filter paper by capillary action. The planchet and contents were then quenched in liquid nitrogen cooled isopentane and freeze-dried over night at $\leq$ 0.001 mmHg and -70°C ('Speedivac' - Pearse tissue dryer, Model I, Edwards High Vacuum Ltd., Crawley, Sussex). Planchet and freeze-dried contents and then planchet alone were weighed on a ultra-microbalance with sensitivity of 1µg (Mettler UM6, capacity 10 mg). The weight of freeze-dried islet tissue was found by difference.

The incubation and homogenisation of islet tissue.

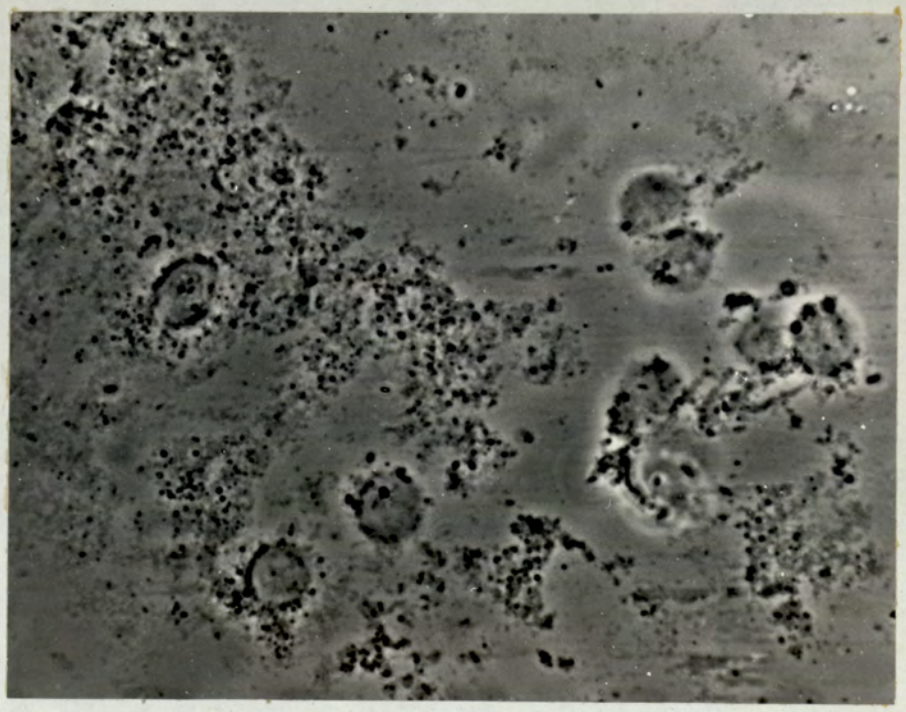
Vials containing 5 islets and 2 ml. of low or high glucose buffer were incubated for 1 hour at 37°C and continuously oxygenated, shaking was effected by placing the vials on the moving tray of the water bath. Lernmark & Hellman (115) have shown that shaking the incubation medium considerably enhances the rate of insulin secretion from islet tissue, possibly by continually presenting fresh medium to the β-cells.

After incubation 0.1 ml. aliquots of the incubation medium of (A) & (B) were transferred with an eppendorf micropipette (Marburg, Eppendorf (R), Hamburg) to immunoassay tubes containing 0.1ml. of phosphate buffer B_1 (an isotonic buffer used in the insulin immunoassay technique). The tubes so prepared were capped, quick frozen in liquid nitrogen and stored at -20°C until required for estimation of insulin content by immunoassay. Sufficient quantities

of the sample were taken to allow immunoassay in triplicate. After sampling all supernatant buffer was removed from the incubation vials and replaced with two successive 2ml. lots of 0.25 mol/litre sucrose. The first acted as a wash and the second as a sonication medium. The contents of vials (C) (D) and (E) were then each subjected to 4 minutes ultrasonication (Ultrasonic disintegrator, 60 watt, $\frac{1}{2}$" titanium probe M.S.E., London), during which time the temperature of the contents was maintained below 10°C using an ice bath. The complete ultrasonic disruption of islet tissue was monitored by phase contrast microscopy on drops of sonicate taken at half-minute intervals.

PLATE 4

Disrupted normal mouse islet tissue after ultrasonication (x820).



After ultrasonication the sonicates were transferred to larger plastic vials and each made up to 30ml. with buffer B_1. Vials so prepared were stored at -20°C until required for immunoassay.

The Insulin Immunoassay.

Insulin assays based on the reaction between ^{131}I - labelled insulin and insulin antibody have been described by Yalow & Berson (116) and Grodsky & Forsham (117). Their assays depended upon the use of insulin antibody to obtain samples of insulin from mixtures prepared by adding standard or unknown solutions of unlabelled insulin to a fixed amount of ^{131}I - labelled insulin and subsequently comparing the specific activities of the mixtures (which diminished as the amount of unlabelled insulin increased). The principle was one of isotope dilution. Many other procedures based on this principle have been described. They differ in minor details and in the method used for separating insulin bound to antibody from insulin remaining free (118-120). Most insulin-antibody complexes are soluble and methods had to be devised for the separation of antibody bound insulin from free insulin. Early attempts employed electrophoresis (116) and fractional precipitation of salts (117). The assay was achieved empirically by comparing the activities of standard and unknown solutions of hormone. Hales & Randle (121) made use of the fact that insulin antibody or the complex it formed with insulin could be precipitated with anti-γ-globulin serum, as first described by Skom & Talmage (122). This precipitation reaction was used initially to separate the antibody-bound insulin from the free insulin. However, it was found that insulin antibody that had been precipitated with anti-γ-globulin serum was still capable of reacting with insulin. In consequence new assay methods were developed with pre-precipitated insulin antibody and the ^{125}I label. These assays form the basis of the insulin immunoassay system in use today.

The measurement of insulin by radioimmunoassay.

The insulin contents of incubation media and sonicates of islet tissue were determined by the double antibody radioimmunoassay of Hales & Randle (121) as modified by Quabbe (123). Solutions of crystalline ox insulin and later human insulin were used as standards. Insulin assays were performed with a radioimmunoassay kit (Code IM 39, The Radiochemical Centre, Amersham, Bucks.)

Principle of the method.

The method was based on the competition of insulin in the sample to be assayed and of radioactive insulin for reaction with an antibody which was specific to insulin. The amount of radioactive insulin bound to the antibody therefore varied inversely with the concentration of insulin in the assay sample. In the method the complex of insulin and anti-insulin was rendered insoluble by a second antibody. The precipitate was separated from free insulin by centrifugation (123) and its radioactivity measured. The concentration of insulin in a given sample was found by referring to a calibration curve prepared from measurements on pure insulin.

Method

All glassware was cleaned with 4% pyroneg (Diversey (U.K.) Ltd., London, W.1.) rinsed in distilled water and then in deionised water. All pipettes and syringes were rinsed with buffer A1 or B1 as appropriate and then washed out with the solution to be used. All solutions were prepared with double glass distilled, deionised water of constant quality (Elgastat). Variations in the quality of ordinary distilled water used for the preparation of buffers has been reported to give rise to considerable variations in the amount of insulin bound to the antibody precipitate (121).

Preparation of Buffers.

Phosphate buffer A1.

Consisted of 6.2g NaH_2PO_4-$2H_2O$, 0.25g. of sodium ethyl mercurithiosalicylate (thiomersalate) and 17ml. of 30% bovine albumin in 1 litre of deionised water. The pH. was adjusted to 7.4 with 2N NaOH. The adjustment of the pH was important. The ionic strength of the medium has been found to affect the recovery of radioactivity in the precipitate, the recovery being diminished at higher ionic strengths (121). This buffer was used for the dilution of anti-sera and iodinated insulin.

Isotonic buffer B_1.

Consisted of 9.0g. of NaCl made up to 1 litre with Buffer A_1. This buffer was used for the dilution of samples.

Wash buffer C_1.

Consisted of 500ml. of horse serum (No.2 Wellcome) + 500 ml. of Buffer A_1. This buffer of high protein content was used for washing antibody precipitates and rarely required filtering.

Reconstitution of the insulin binding reagent.

To one bottle of binding reagent (a dessicate of anti-insulin precipitated by a second antibody) was added 8ml. of deionised water and the contents carefully mixed to avoid foaming. 0.1ml aliquots were dispensed (eppendorf pipette) into polysyrene immunoassay tubes (Hopkins & Williams). One bottle of reagent was sufficient for up to 80 tubes. Tubes were set up for i) zero tubes, to which no unlabelled insulin was added; ii) the standard insulin solutions; iii) the test samples and finally, iv) in initial experiments, washing "blanks". All assays were carried out in triplicate. One or two tubes containing buffer A_1 only, without insulin binding agent were included in each run as controls of the washing procedure.

The addition of unlabelled insulin (standards or unknown).

0.1ml. of the insulin containing solution (standard or unknown or 0.1ml. of buffer $\underline{B}_1$ for zero values) was added to the assay tubes. The same applicator was used throughout and rinsed carefully before each new solution was dispensed. When the insulin content of the unknown was expected to exceed that of the highest standard it was first diluted with a known proportion of buffer $\underline{B}_1$ and 0.1ml. of the diluted solution was then used. The contents of each tube were mixed thoroughly on a "whirli-mixer" (Fisons) and stored at 2-4°C for 6 hours to improve the sensitivity of the assay.

The addition of labelled insulin (minimum specific activity 50 μCi/mg).

Labelled insulin was supplied in vials containing 0.1 μg of iodinated insulin dissolved in 5ml. of buffer $\underline{A}_1$. A working solution was prepared by adding 1.0ml of this solution to 7ml of buffer $\underline{A}_1$. The diluted mixture contained 250 pg. of iodinated insulin per 0.1 ml. 0.1 ml. of this working solution of iodinated insulin (250 pg.) was added to each assay tube and the contents of the latter mixed and stored at 4°C for 18 hours. 0.1 ml. samples of the working solution of iodinated insulin were taken at this stage for determination of the total radioactivity added to each tube.

Quabbe's modification for the collection of the antibody precipitate (123).

After 18 hours the tubes were centrifuged at 4°C in a'Mistral 4L'refrigerated centrifuge (M.S.E.) at 1500 rev/min. for 60 minutes. After this period of time the unbound insulin, labelled and unlabelled was carefully decanted from each tube. The remaining precipitates were washed with buffer $\underline{C}_1$ (0.5ml) and the contents of each tube shaken and centrifuged as before. The same procedure was repeated a

third time. It was important not to leave the assay tubes too long after the finish of each centrifugation stage since there was a real possibility of the precipitate dissolving back into the buffer $\underline{C}_1$. Precipitates from the final centrifugation were air-dried over night, placed into white capped glass scintillation vials and their ^{125}I radioactivity counted on a gamma-matic scintillation spectrophotometer (NE 8651, MK II Nuclear Enterprises (GB) Ltd., Scotland). Tubes were included for the measurement of background activity at the beginning and end of each counting batch. Tubes were counted for 400 or 1000 seconds and their activities in counts per minute were corrected for background. A mean count rate was computed for each triplicate and obviously erroneous count rates were omitted. A good agreement between replicates was indicative of a satisfactory assay. Also the washing procedure was judged to be satisfactory when controls containing no insulin binding agent contributed less than 5% to the total counts. The zero value (iodinated insulin bound to antibody precipitate in the absence of added unlabelled insulin) was never less than 20% of the total count and was governed by the titre and dilution of the anti-insulin serum used. Where a comparison was being made between two types of test samples, the same immunoassay kit was used for both. This minimised any variation that might have been caused by using the zero values of a new immunoassay kit.

Preparation of insulin standards.

First (200 µg/ml.) and second (2 µg/ml.) stock insulin standard solutions were made up as per technical bulletin 86/6 provided with the assay kit. An initial working standard solution of bovine insulin was prepared by diluting 0.5 ml. of the second stock solution to 50 ml. with buffer B_1. From this initial standard (500 µU bovine insulin/ml., 1 mg. $\triangleq$ 25 Units of insulin) a range of standards

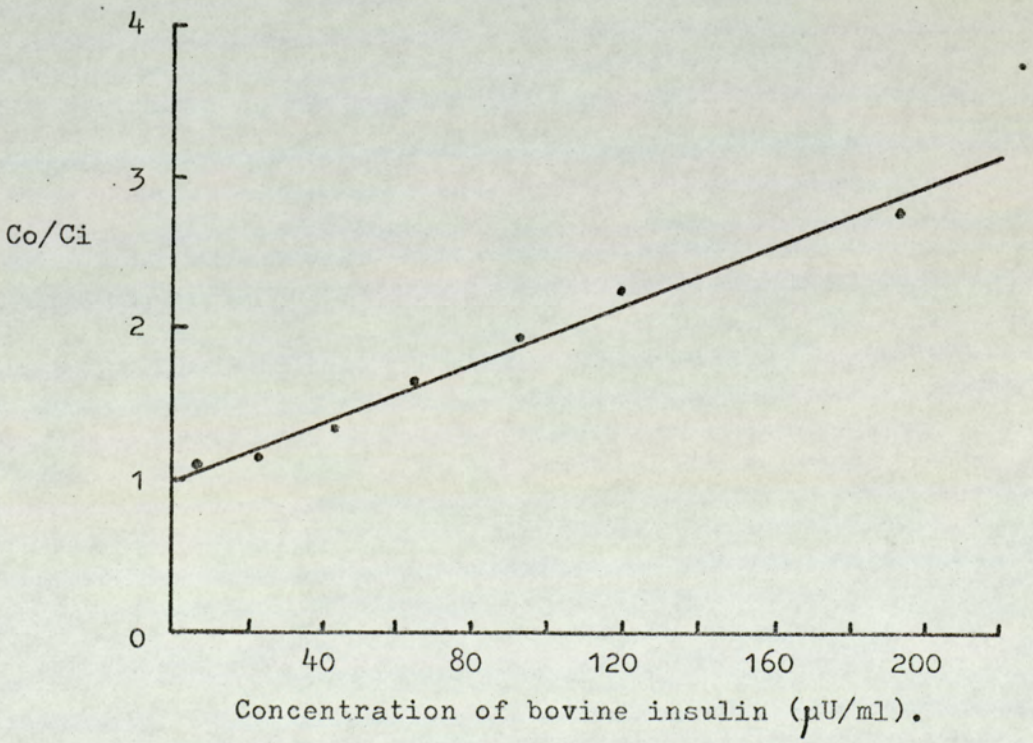
(12.5 - 200μU/ml) was prepared by dilution with buffer B_1.

Calculation.

The $\frac{Co}{Ci}$ ratio of Hales & Randle (121) (the radioactivity of the insulin antibody complex in the absence of unlabelled insulin/the radioactivity of insulin antibody complex when the concentration of unlabelled insulin is i) was represented by the ratio of Zero values/ Test values (samples and standards) in the assay used. All values were corrected for background. A standard graph was obtained by plotting the Co/Ci values of standards against their known insulin content in μU/ml. The Co/Ci value has been shown to be linearly related to i (for concentrations of unlabelled insulin up to 320 μU/ml.) and to have a value of unity when i = zero (121). Co/Ci values were computed for test samples and the corresponding insulin concentrations in μU/ml. read from the standard graph.

Fig. 1

A typical standard graph for insulin immunoassay.



The concentration of insulin in each of the samples was expressed in terms of the particular species of insulin standard used i.e. bovine or human. Pure mouse insulin was not available for the preparation of standards. Because of the chemical differences between insulins derived from different species, the anti-insulin serum to pig insulin used in the assay might have shown partial species specificity. Under the conditions of the assay the anti-serum to pig insulin has been shown to react with similar sensitivity to ox, human and pig insulins. Other insulin species were not investigated and might not have been equivalent to ox or human insulin. For this reason an absolute error could have been present in the values obtained. However this error as long as it remained constant would not affect the significance of any comparison carried out. Values of insulin concentration obtained from insulin secretion and insulin content samples were adjusted for dilution (x8 for secretion and x30 for content) and expressed in terms of μU/5 islets/hr or μU/mg. freeze-dried islet tissue/hr. for secretion and μU/5 islets or μU/mg. freeze-dried islet tissue for content. The percentage of the final total insulin content secreted was evaluated as follows,

$$\frac{\text{Insulin secreted from 5 islets in one hour}}{\text{Final total insulin content of 5 islets}} \times 100\%$$

The final total insulin content was the sum of the insulin remaining in 5 islets after 60 minutes incubation and the amount of insulin secreted from the 5 islets during that hour.

Results.

Substantial amounts of immunoreactive insulin were secreted from both microdissected and collagenase isolated TO. mouse islets in the presence of low glucose (0.6g/litre). Increasing the glucose concentration of the incubation medium from 0.6g/litre to 3.6g/litre

stimulated the rate of insulin secretion from both microdissected and collagenase isolated islets by 7.4% and 1.6% respectively (Table 1.). At the same time the increase in exogenous glucose concentration brought about a reduction in the insulin content of both types of islets presumably by stimulating their rate of insulin secretion. Similarly for both microdissected and collagenase isolated islets the percentage of the final total insulin content secreted, increased with the increase in glucose concentration of the incubation medium.

Microdissected islets showed a significantly greater rate of insulin secretion than did collagenase isolated islets at both high and low glucose concentration. Also the insulin content (insulin content of 5 islets after one hour of incubation) was significantly higher in microdissected islets than in collagenase isolated islets in the presence of low glucose medium, although no significant difference was observed in the presence of high glucose medium. The percentage of the final total insulin content secreted was greater for microdissected than collagenase isolated islets in the presence of both low and high glucose medium. The percentage increase in the rate of insulin secretion resulting from the increased glucose concentration of the incubation medium was computed as the difference between the percentage of the final total insulin content secreted in the presence of low glucose and the percentage of the final total insulin content secreted in the presence of high glucose. This value (Table 1.) represented the stimulant effect of increased glucose on the rate of insulin secretion and was found to be significantly higher for microdissected than for collagenase isolated islets. On this basis the insulin secretory mechanism of microdissected islets appeared more sensitive to the stimulant effect of glucose than the same mechanism in collagenase isolated islets.

TABLE 1.

The effect of glucose on the insulin secretory activity and insulin content of of TO. mouse islets obtained by freehand microdissection and collagenase digestion.

(Mean Values ± S.E.)

		MICRODISSECTED ISLETS		COLLAGENASE ISOLATED ISLETS	
Insulin Secretion.	N (no. of mice)	Low glucose (0.6g/Litre)	High glucose (3.6g/Litre)	Low glucose (0.6g/Litre)	High glucose (3.6g/Litre)
µU/5 islets/hr.	10	615 ± 13	883 ± 11 *	473 ± 10 †	531 ± 9 * †
µU/mg.freeze dried islets/hr.	10	18090 ± 726	25952 ± 873 *	13264 ± 543 †	14890 ± 596 †
Insulin Content. (after 1 hr. of glucose induced stimulation).					
µU/5 islets.	10	3860 ± 52	3309 ± 63 *	3470 ± 26 †	3372 ± 35 *
µU/mg. freeze dried islets.	10	113251 ± 3357	96980 ± 2814 *	97110 ± 3050 †	94370 ± 3102
% of the final total insulin content secreted.	10	13.8 ± 0.25	21.1 ± 0.39 *	12.0 ± 0.18 †	13.6 ± 0.19 * †
% Increase in insulin secretion as a result of increased glucose concentration.	10	-	7.4 ± 0.5	-	1.6 ± 0.1 †

* Denotes a significant difference ($P < 0.05$) between low and high glucose values for both microdissected and collagenase isolated islets.

† Denotes collagenase and microdissected islet values compared, for both low and high glucose, P values < 0.05.

TABLE 2.

The initial total insulin content and mean final total insulin content of collagenase isolated and microdissected islets of TO. mice expressed in terms of μU/mg. freeze dried islets or μU/5 islets.

(Mean values ± S.E.)

	N (no. of mice)	Microdissected islets	Collagenase isolated islets
Initial total insulin content (E).			
μU/mg. freeze dried islets.	10	144210 ± 4034 *	120474 ± 3932 †
Mean final total insulin content (C & D).			
μU/mg. freeze dried islets.	10	127137 ± 3666	109817 ± 3603 †
% difference between initial total insulin content and mean final total insulin content (lost insulin).		11.8 ± 0.9	8.9 ± 0.2 †
Initial total insulin content (E).			
μU/5 islets.	10	4915 ± 46 *	4304 ± 39 * †
Mean final total insulin content (C & D).			
μU/5 islets.	10	4333 ± 54	3923 ± 37 †
% difference between initial total insulin content and mean final total insulin content (lost insulin).		12.5 ± 0.8	10.0 ± 0.2

* Denotes significant difference ($P < 0.05$) between the initial and mean final, total insulin contents of both collagenase isolated and microdissected TO. islets.

† Denotes collagenase and microdissected islet values compared, P values < 0.05.

The initial total insulin content (E) of 5 microdissected islets was found to be significantly higher than the same for 5 collagenase isolated islets (Table 2.). Similarly the mean final total insulin content (the sum of the final total insulin content at low glucose and the final total insulin content at high glucose, divided by 2) was significantly higher in microdissected islets than in collagenase isolated islets. The percentage difference between the initial total insulin content and the mean final total insulin content (lost insulin) was found to be higher for microdissected than for collagenase isolated islets. Collagenase isolation may have limited the capacity of secreted insulin to adhere to glass surfaces resulting in a lower percentage of 'lost insulin'.

A significant correlation ($P<0.05$) was established between the weight of 5 islets (µg.) and the rate of glucose induced insulin secretion (mU/mg. freeze dried islets/hr.) for both microdissected and collagenase isolated islets (Fig.2). Within the limits of the experimental data available an increase in islet tissue weight was consonant with a reduction in the rate of insulin secretion for both microdissected and collagenase isolated islets in the presence of low and high glucose concentration. A similar relationship was observed for the weight of 5 islets (µg.) and the insulin content of 5 islets (mU/mg.) after low and high glucose stimulated insulin secretion. (Fig.3). The negative correlations shown by these data were difficult to interpret since one might normally assume the capacity for glucose induced secretion and the insulin content of islet tissue to increase with increasing islet weight. No significant correlation was demonstrated between the weight of 5 islets and the final total insulin content of islet tissue subjected to low or high glucose, whether

FIG.2. Relationship between the weight of 5 islets (µg) and the low and high glucose induced rate of insulin secretion (mU/mg/hr) from microdissected and collagenase isolated islets of TO. mice.

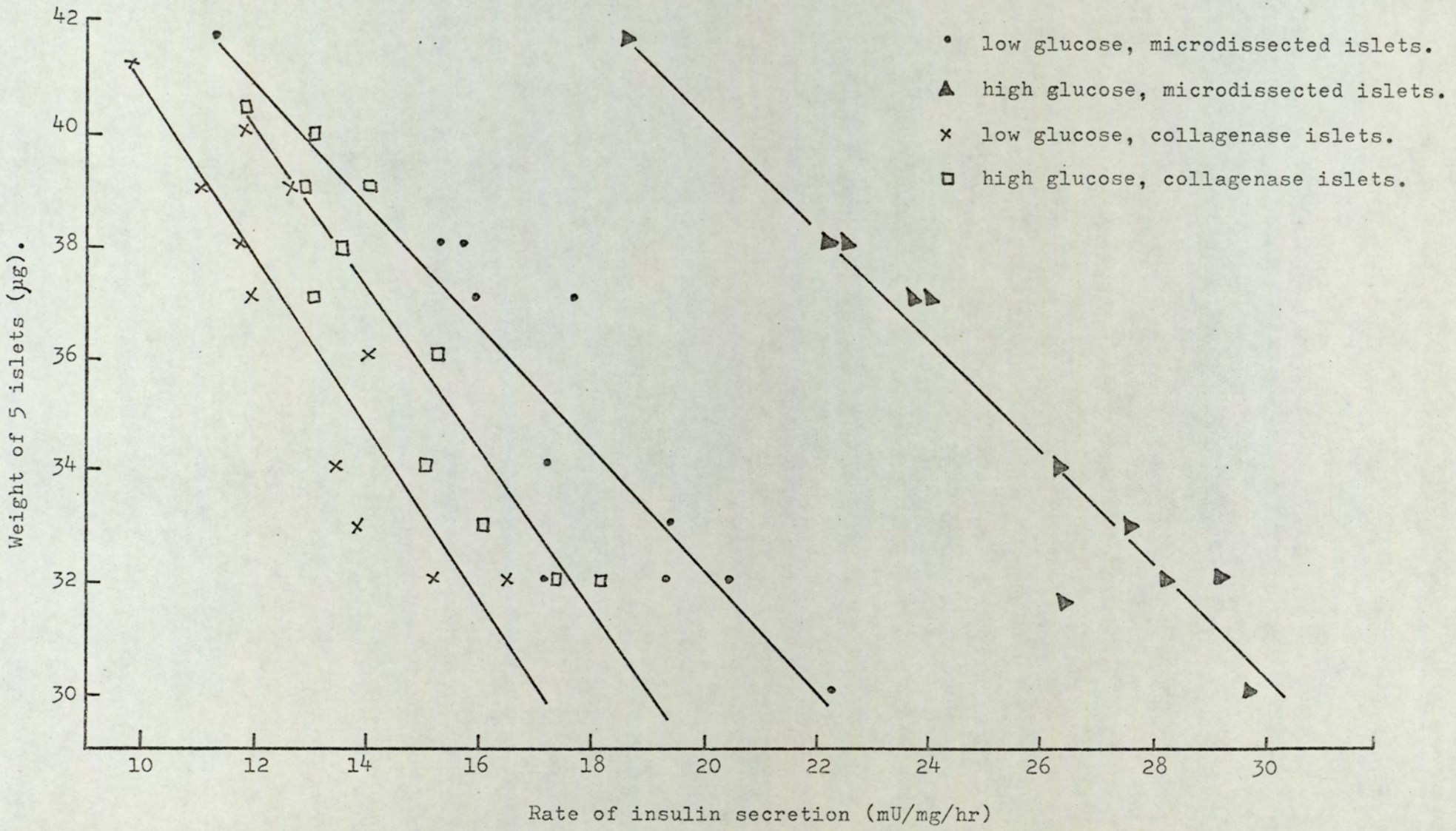


FIG.3. Relationship between the weight of 5 islets (µg) and the insulin content (mU/mg.) after low and high glucose stimulated insulin secretion from microdissected and collagenase isolated islets of TO. mice.

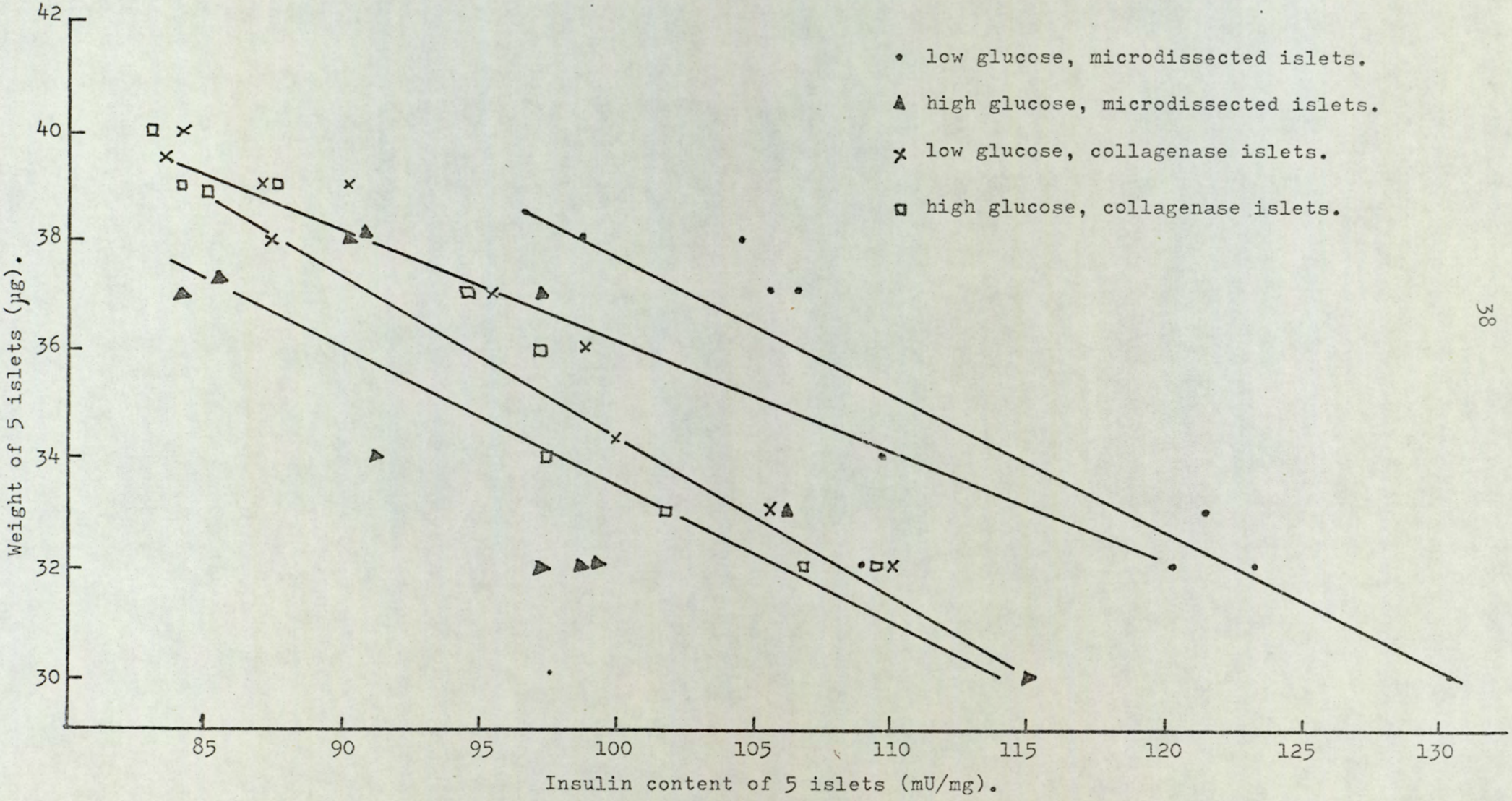
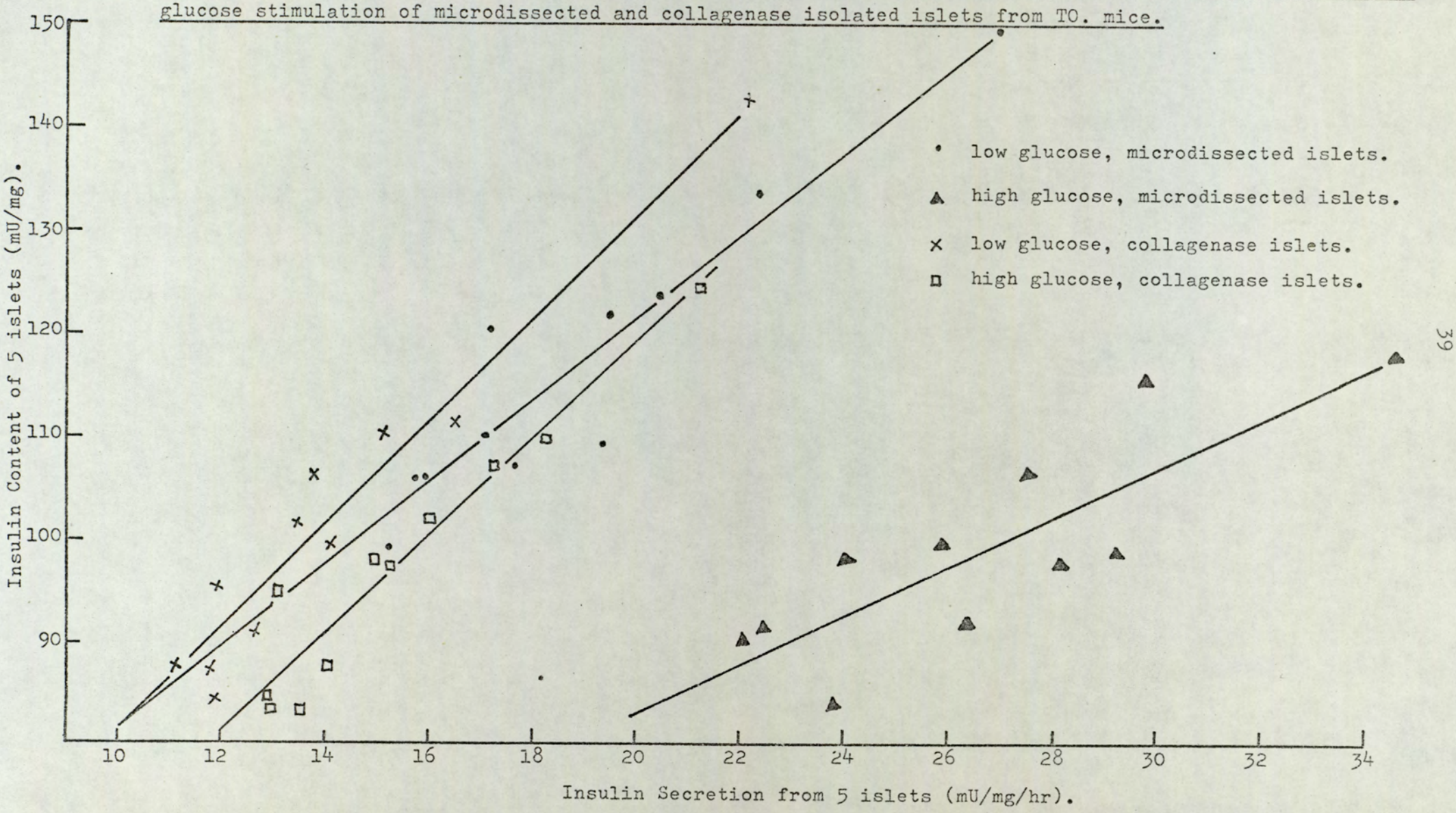


FIG.4. Relationship between glucose induced insulin secretion (mU/mg/hr.) and the insulin content (mU/mg.) after glucose stimulation of microdissected and collagenase isolated islets from TO. mice.



obtained by microdissection or collagenase digestion. The presence of large and small islets in the 5 islet lots used for the experiments described here, might have contributed to the negative correlations observed; in so far as large islets may contain no greater insulin content than medium sized islets. The increased size of large islets being due solely to increased islet tissue mass. Indeed the large islets of normal mice have been shown to have no greater glucose induced insulin secretory capacity than medium sized islets (124) while the very large islets of AO mice have been found to secrete less insulin than the normally sized ones (125). Different immunoreactive specific activities for both secreted and contained insulin of the different sized islets, and the presence of proinsulin for which the insulin immunoassay used showed no discrimination, might also have contributed to the anomaly observed.

Examination of the relationship between glucose induced insulin secretion and the insulin content of islet tissue after glucose stimulation (Fig.4) showed that a certain minimum insulin content (760 mU/mg. freeze dried islet tissue) was required for subsequent glucose induced insulin secretion from both microdissected and collagenase isolated islets. At islet insulin concentrations above 60 mU/mg. freeze dried islet tissue, the islet insulin content was found to be linearly related to the rate of insulin secretion from both microdissected and collagenase isolated islets in the presence of low and high glucose concentration.

A significant negative correlation was observed between the whole pancreas weight (in g.) and the remaining insulin content of collagenase isolated islets, after high glucose stimulation, when expressed as mU/5 islets and after low glucose stimulation, when expressed as mU/mg. freeze dried islets. Also a significant negative correlation

existed between the whole pancreas weight and the initial total insulin content (E) of collagenase isolated islets. No other correlative relationship was found for any of the other parameters plotted within these co-ordinates. Regression lines indicated that mice with pancreas weights in excess of 0.16 g. showed a reduction in both the initial total insulin content and the insulin content after low and high glucose stimulation of collagenase isolated islets. This suggested that the pancreas might contain a certain optimal amount of islet tissue and insulin. Any pancreas in excess of 0.16 g. would contain increased amounts of exocrine but not endocrine tissue resulting in a proportionately lower amount of islet tissue and hence lower insulin content.

Discussion.

Microdissected islets were shown to have a greater glucose induced insulin secretory rate and insulin content (initial and final) than collagenase isolated islets. However, Ashcroft and coworkers (126) have demonstrated that the method of islet extraction, be it collagenase or microdissection did not impair the capacity of the islets to secrete insulin or to oxidise ^{14}C-glucose to $^{14}CO_2$ at a rate proportional to the glucose concentration of the incubation medium. Like Weinges (109) these workers demonstrated a stimulation of insulin release by tolbutamide from collagenase isolated islets. Yet Lacy, Young & Fink (108) were unable to show such a stimulation from collagenase isolated rat islets. Hahn & Michael (127) were unable to demonstrate any difference in the rate of glucose induced insulin secretion from microdissected and collagenase isolated mouse islets. But provided evidence to suggest that steel needles were potentially capable of reducing the secretory properties of microdissected islets.

Microdissection was a time consuming technique and although islets were kept in oxygenated buffer over ice at 2°C, a given yield was assumed to show a spectrum of viability, increasing from the first to the last islet isolated. Collagenase extraction was found no quicker to execute, but provided a greater yield of constant viability. Preincubation ironed out the viability differences in a given experimental group of islets, but did not iron out the viability difference, that existed prior to preincubation, between microdissected and collagenase isolated islets. In the future the extraction of islet tissue might well be carried out by the method described by Hahn and colleagues (128), who suggested a short collagenase digestion followed by a rapid final separation by microdissection.

In the experiments carried out, results were expressed both in terms of islet number (5) and islet freeze dried weight (mg.). Data expressed in terms of freeze dried weight were found to be the more informative and consonant with the conclusions of Hellman's synopsis on the methodology of islet studies (129). The viability of β-cells was found to be best preserved by dissecting in a chilled medium devoid of collagenase. Microscopical observations of microdissected islets, as well as more sensitive metabolic criteria such as high ATP content (130,131) and a constant rate of oxygen consumption (132) supported this belief. However, Hellman (129) used the weight of islets after they had secreted insulin, and prior to the measurement of insulin content, for evaluation of the insulin secretory rate. In consequence all these islet weights were low, since no account was made of the weight lost due to secreted insulin. The use of separate groups of islets for weighing, similar in every way to those used in experimental analyses, has been suggested as a way of getting round this difficulty.

Representative in vitro insulin secretory rate studies have been shown to require at least 10 islets in each test group (128). This facilitated accurate measurement of islet weight and accommodated variation in islet size, a factor important in the provision of representative secretory data (124, 125). However, the use of 5 islet lots was valid as long as insulin secretion and content were expressed in terms of freeze dried islet weight (mg.).

Values for the freeze dried weight of 5 islets, used to evaluate insulin secretory and content data were on average, some x6 greater than the values obtained by Hellerstrom (38), and Lernmark & Hellman (125) using the same method. The discrepancy must have arisen from either inefficient freeze drying equipment and adherent saline or incomplete microdissection. The measurements of insulin secretion and content defined the activities of β-cells and not whole islets. The insulin content of β-cells reflected a balance between the synthesis of insulin and its utilisation and transport. Many workers have expressed their results in terms of secretion/minute, even though secreted insulin was collected over the space of 30 minutes or one hour (133). The unification of secretory data in this way was not particularly accurate since Grodsky and coworkers (5) have shown glucose induced insulin secretion to be a dual phasic release and not a constant one. Hahn and colleagues devised a means of expressing insulin secretion in terms of the protein content of islets and this method was used to eliminate any controversy over the use of islet numbers or islet weight (128). However, albumin adsorbed onto islets from the incubation medium would invalidate the estimation of islet protein content.

Although the glass incubation vials used in insulin secretion and content experiments were silicone treated and rinsed out with bovine serum albumin $\overline{V}$ prior to use. A discrepancy existed between the values for the final total insulin content and initial total insulin content,

defined as 'lost insulin'. Presumably some secreted insulin was lost by adsorbtion onto glass surfaces, but reduced immunoreactivity of insulin secreted in response to glucose stimulation, might also have contributed to this lost insulin.

Hahn & Michael have suggested that measurements of insulin secretion should only be made under conditions of depressed 'basal insulin release' (134). Hahn has shown the basal release (low level release of insulin in the absence of glucose) to be increased by certain collagenase concentrations and decreased by maintaining the temperature as low as possible ($2^{o}C$); washing islets in ice cold buffer and transferring islets to new medium halfway through the incubation period. Creutzfeldt and coworkers (135) have suggested the basal insulin release from isolated islets to be due to β-cells disintegrating and emptying their contents into the incubation medium regardless of added substances. Compared with isolated islets, the basic insulin release of incubated pancreatic pieces was found to be less.

Hahn & Michael demonstrated the possibility of a feedback inhibition of insulin secretion by previously secreted insulin, during both the islet preparation and preincubation periods(134). Feedback inhibition might be an integral part of the control process for normal insulin secretion. In which case any insulin lost in the preparation or preincubation period might affect this feedback control. The altered response might then manifest itself during the test incubation period. Careful washing with buffer helped to remove insulin leaked from islets during the extraction procedures. Creutzfeldt and colleagues demonstrated that insulin secretion decreased during the incubation of rat pancreatic pieces and isolated islets (135). This decrease was not due to insulin destruction by proteolytic enzymes as

suggested by Malaisse and coworkers (136). Although the insulin secretion was decreased during incubation, when the incubation medium was changed every 30 minutes, the amount of insulin released was found to be much higher. Similarly, practically no insulin secretion was observed from fresh pancreatic tissue after incubation in a medium that had been previously used for incubation, while fresh pieces of the same pancreas incubated in a new medium showed a normal glucose induced stimulation. These results suggested that studies on insulin secretion *in vitro* should be done only if the incubation medium was constantly renewed or used very briefly.

The *in vitro* rate of insulin secretion was significantly enhanced by shaking the incubation medium. Hellman suggested that shaking imitated the effects of the blood circulation through islets and acted to depress feedback inhibition, by reducing high levels of insulin in the immediate surroundings of the β-cells (137). Mouse islets have been observed to secrete less than 1% of their original insulin content during 30 minutes incubation (129) and rat islets, even in the presence of high glucose, less than 5% of their total stored insulin after 60 minutes incubation (138,139). Experiments with microdissected and collagenase isolated islets have shown the percentage of the final total insulin content secreted from 5 islets to be greater than this, indeed as much as 21% from microdissected islets in the presence of high glucose (Table 1). Significantly perhaps, the percentage of the final total insulin content secreted in the presence of either low or high glucose was found to be greater from microdissected than collagenase isolated islets. In fact, broadly speaking, results from experimental work have suggested collagenase isolated islets to be inferior to microdissected islets in terms of glucose induced secretion and insulin content.

The process of islet extraction by collagenase digestion has been shown to have a number of serious drawbacks. Moskalewski was the first to suggest that trypsin released during the collagenase isolation procedure might cause the detachment of cells from islets and thus reduce their size, insulin content and total secretory capacity (39). He observed injured cells around the periphery of collagenase isolated islets and assumed these to be the result of either mechanical stirring or the transfer procedure. Damage to cells in the islet periphery might lead to a leakage of insulin in amounts inversely related to the islet size. Leclercq-Meyer, Brisson & Malaisse observed erratic glucagon secretion from collagenase isolated rat islets and suggested erosion damage to peripherally located α cells to be the cause of this (140). Commercially available preparations of collagenase differed in their purity, but all contained some protease and peptidase activity which could lead to reduced glucose induced insulin secretion and insulin content of islets isolated by collagenase digestion. By perfusing isolated islet capillaries, collagenase solutions could also interfere with the process of insulin secretion at the β-cell/capillary junction. However, in favour of the collagenase technique, Gerner and coworkers have demonstrated collagenase isolated islets to be free from exocrine and capable of glucose induced insulin secretion (107). Similar evidence has been presented by Weinges (109) and many others (105,106).

Critics of the microdissection technique have suggested that the surrounding connective tissue and a minimum of exocrine tissue must be left adhering to islets for fear of rupture. Consequently the microdissected islet was not as 'clean' as the collagenase isolated islet. Even with the collagenase technique, the connective tissue capsule was never allowed to digest completely away, because this invariably lead to the loss of cells, leakage of contents and difficulty

in transfer. The final separation of islets was carried out with a minimum of microdissection as suggested by Hahn and colleagues (128).

Malaisse and coworkers have demonstrated that in normal rats of comparable age, the secretory activity of pieces of pancreas *in vitro* appeared to be related proportionally to their insulin content (138). A significant correlation has been demonstrated between the rate of glucose induced insulin secretion and the insulin content after glucose stimulation, for microdissected and collagenase isolated islets (fig.4). Malaisse and coworkers also observed in normal rats that the total pancreatic output and content of insulin was proportional to the body weight (138). No such relationships were established for either microdissected or collagenase isolated islets of TO. mice.

Negative correlations observed between the weight of 5 microdissected or collagenase isolated TO. mouse islets and their rate of glucose induced insulin secretion (fig.2) were only partially explained on the basis of a reduced insulin secretion from the largest members of the 5 islet lots. This reduced secretion may have been due to a longer insulin diffusion path in the absence of any marked increase in vascularisation. However, this hypothesis would not hold true for the reduced glucose induced secretion shown by large obese mouse islets (125), since their increased size was associated with a pronounced intensification of the blood supply. In addition, Malaisse and colleagues have shown the secretion of insulin from pieces of rat pancreas to be directly related to the weight and size of incubated tissue (136).

Negative correlations observed between the weight of 5 microdissected or collagenase isolated islets and their insulin content after glucose stimulation were equally perplexing. The presence of large islets in each 5 islet lot might again have contributed to this anomaly, in so far as their large size may have been the result of an increased number of non-insulin containing cells

i.e. connective tissue cells and ∝-cells. But again these details were not considered sufficient to adequately explain the anomalies observed.

The proteolytic breakdown of insulin was considered a significant source of error when investigating insulin secretion from the mammalian pancreas in vitro (98,136). This lysis was believed to be due to a substance, probably trypsin, released from exocrine tissue. The lytic factor has been observed in the presence of exocrine tissue (or their exudate) but not in the presence of 'clean' isolated islets (125,141). The kallikrein inhibitor Trasylol(R) (Bayer) has been used by several workers to 'suppress' the activity of this lytic factor in in vitro studies of insulin secretion from pieces of pancreas (138,142) and its value in studies directed towards the measurement of glucagon secretion from isolated islets has been substantiated (143-145).

SECTION 2

Comparison of the in vitro basal insulin secretion from microdissected and collagenase isolated islets of TO. strain mice.

Sussman, Vaughan & Stjernholm noticed that the isolated perfused rat pancreas constantly secreted a low concentration of insulin. Adrenaline at concentrations known to inhibit normal glucose induced insulin secretion had no effect on this basal insulin output (42). Malaisse and coworkers demonstrated that more than 70% of the basal secretion of insulin observed in the absence of glucose was abolished by 2 mmol./litre cyanide. This suggested that the basal secretion corresponded to an active secretion rather than a passive diffusion of insulin (146). It seemed probable that the basal secretion of insulin was not controlled by the circulating glucose level, but possibly by

some other circulating agent such as glucagon or amino acids (147). It was suggested that because of the juxtaposition of α and β-cells within the islet, intra islet glucagon (or even gastrin) might modulate or control basal insulin secretion.

In view of the importance attached to the basal secretion of insulin from islet tissue in vitro (134,135) and because basal secretion may also be the result of leaky or damaged β-cells (42), measurements of this parameter were made from microdissected and collagenase isolated TO. mouse islets.

Materials and Methods.

Islets were isolated by microdissection and collagenase digestion. Basal insulin secretion (the secretion that occurred in the absence of added glucose) and insulin content were estimated by immunoassay (121) as previously described (Chapt. I, Section I). The in vitro basal secretion of insulin and the initial total insulin content of microdissected and collagenase isolated islets were compared. Also the percentage of the final total insulin content basally secreted was computed for both microdissected and collagenase isolated islets.

Results and Discussion.

Results of studies on basal insulin secretion and the insulin content of microdissected and collagenase isolated islets after 1 hour of incubation in the absence of glucose have been summarised in Table 3.

No significant difference was observed between the in vitro basal insulin secretion from microdissected and collagenase isolated islets. Similarly no significant difference was found between microdissected and collagenase isolated islets when either the percentage of the final total insulin content basally secreted after one hour, or the mean weight of 5 islets was considered. Hence both types of islet secreted a similar proportion of their final total insulin content as a basal secretion during one hour of incubation. However, the final total insulin content of microdissected islets was significantly higher than

TABLE 3.

The in vitro basal insulin secretion and the final total insulin content of microdissected and collagenase isolated TO. mouse islets after one hour of incubation in the absence of glucose.

(Mean Values ± S.E.)

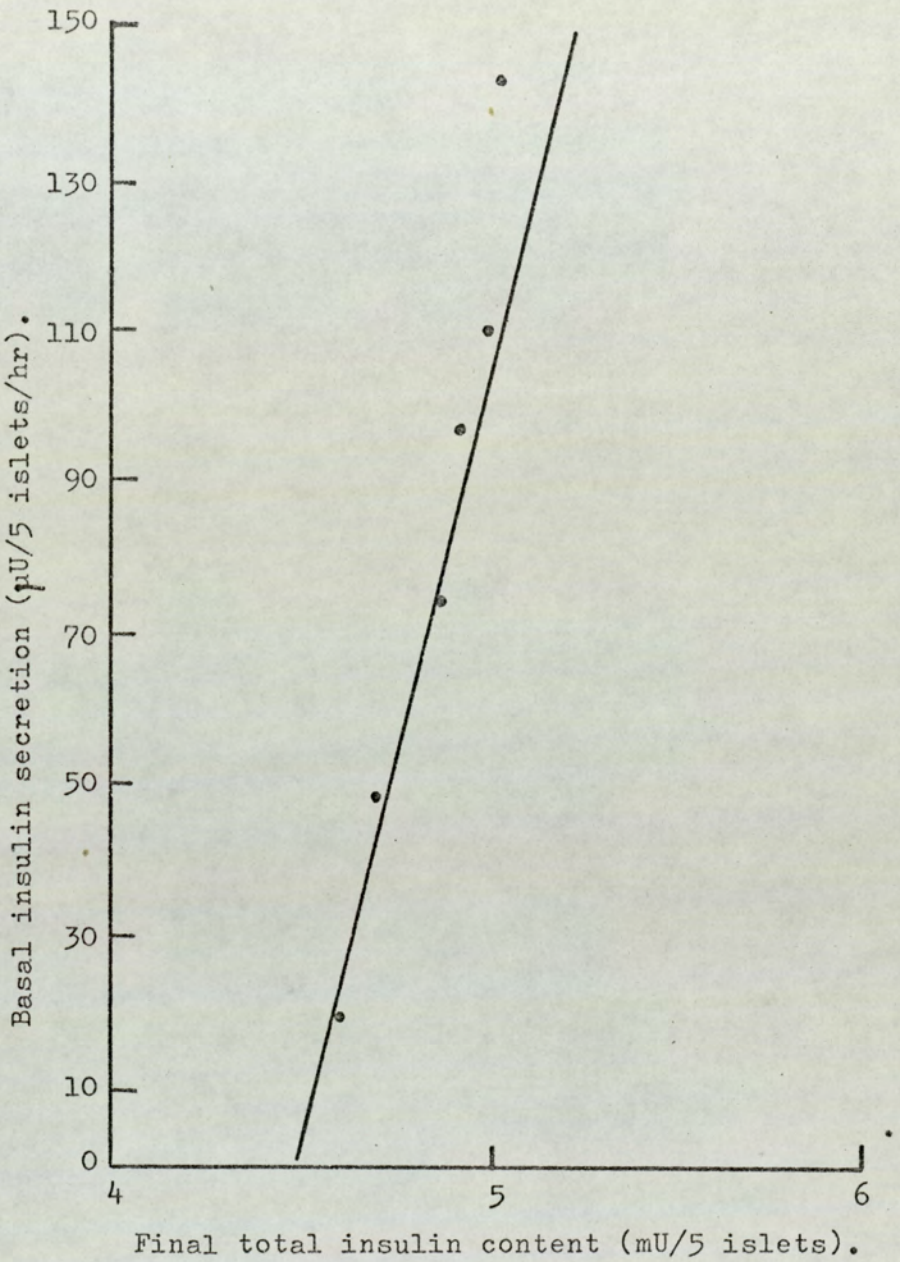
Basal insulin Secretion	N (no. of mice)	Microdissected islets	Collagenase isolated islets
μU/5 islets/hr.	5	94 ± 16	123 ± 11
mU/mg/hr.		2.8 ± 0.4	3.5 ± 0.3
Final total † insulin content			
μU/5 islets	5	4902 ± 55	4223 ± 106 *
mU/mg.		149.5 ± 5.4	121.2 ± 5.8 *
% of the final total insulin content basally secreted	5	1.86 ± 0.3	2.86 ± 0.3
Mean weight of 5 islets (μg)	5	33 ± 1.5	35 ± 0.8

* Denotes final total insulin content of microdissected and collagenase isolated islets compared, P value < 0.05.

† Denotes the sum of the basal insulin secretion and remaining insulin content after 1 hr. of incubation in the absence of glucose.

that of collagenase isolated islets. A significant positive correlation ($P < 0.05$) was demonstrated between the in vitro basal insulin secretion and the final total insulin content of microdissected islets but not collagenase isolated islets (fig.5). This relationship suggested that a certain minimum insulin content was required within microdissected islets (4.5 mU/5 islets) for the basal secretion of insulin to take place. Above this threshold value any further increase in islet insulin content facilitated a proportional increase in the rate of basal insulin secretion. This proportional relationship suggested that the basal secretion was something more than just a

FIG.5. The relationship between the rate of in vitro basal insulin secretion and the final total insulin content of microdissected TO. mouse islets.



leakage and perhaps an active process, which occurred only when a predetermined islet insulin content was attained. Malaisse and colleagues have indicated that the absolute rates of insulin secretion by pancreatic tissue in vitro depended upon the actual stores of insulin present (146). Since microdissected and collagenase isolated islets were found to have a similar in vitro rate of basal insulin secretion, it seemed unlikely that the reduced response of collagenase isolated islets to glucose stimulation compared to microdissected islets

(seen in Chapter I, Section I), would be the result of collagenase digestion or erosion of peripheral islet cells. On the other hand, the reduced final total insulin content of collagenase isolated islets compared to microdissected, would lend support to this thesis.

SECTION 3

The effect of exogenous insulin on the glucose induced secretion of insulin from the microdissected islets of TO. mice, obese hyperglycaemic mice and their normal lean littermates.

An assessment of the versatility and sensitivity of microdissected islets was made by investigating the influence previously secreted insulin might have on its own release. Frerichs and coworkers using glucose stimulated pancreatic slices found evidence for an inhibition of insulin secretion by endogenous insulin allowed to accumulate in the incubation medium and by exogenous insulin added to the incubation medium (148). Loubatieres and colleagues demonstrated an inhibition of insulin release from the perfused rat pancreas when the glucose containing perfusate was enriched with exogenous insulin at a concentration of 1.5 - 2 mU/ml. (149). Generally the concentrations of exogenous insulin used to inhibit insulin secretion were very much higher than the usual circulating hormone levels found in normal mice and very often higher than the levels found in obese hyperglycaemic mice i.e. 72.4 ± 9.5 μU/ml. and 741.21 ± 196.83 μU/ml. respectively (150). Feedback inhibition of insulin secretion by insulin has been described *in vitro* by Hahn & Michael using normal mice (134), by Sodoyez and coworkers using hamsters (151) and obese mice (152) and by Hellman & Lernmark using the lean littermates of obese mice (115). Also Iverson has demonstrated a feedback inhibition by insulin on insulin secretion from the isolated perfused dog pancreas (153).

Contrary to these observations, Grodsky and coworkers using the isolated perfused rat pancreas were unable to detect any inhibition of insulin release by high concentrations of fish insulin (154). Similarly, Malaisse and coworkers showed that neither insulin nor guinea pig anti-insulin serum (GPAIS) modified the rate at which insulin was secreted by isolated rat islets in response to glucose (141). Also, Sodoyez and colleagues were able to show that insulin secretion by golden hamster islets was not affected by the presence of exogenous insulin when the glucose concentration of the medium was 100 mg% (151). However, at 200 mg% glucose, exogenous insulin at a concentration of 250 μU/ml. significantly inhibited insulin secretion. Islets stimulated maximally by glucose at 300 mg% were insensitive to exogenous insulin at the concentration of 250 μU/ml., barely inhibited by exogenous insulin at 500 μU/ml. but were strongly inhibited when the concentration of exogenous insulin was 1000 μU/ml. Their results suggested that exogenous insulin inhibited further insulin release and that the insulin level at which this feedback mechanism operated was determined by the intensity of the insulinogenic stimulus (151). Hahn & Michael using male AB mice and a glucose concentration of 200 mg% were only able to show an inhibition of *in vitro* insulin secretion by levels of exogenous insulin above 2000 μU/ml.(155). Similar results were obtained by Malaisse and coworkers (141), who were unable to show an *in vitro* inhibition of insulin release below 1000 μU/ml of exogenous insulin.

An attempt has been made to ascertain whether the glucose induced insulin secretory rates of islet tissue, observed in earlier experiments, were subject to inhibition by previously secreted insulin. The effect of exogenous insulin was investigated on the rate of insulin secretion from microdissected islets of TO. mice, obese hyperglycaemic mice and their lean littermates. A study of this nature provided information on the occurrence of the feedback phenomenon in three types of mice

with different metabolic profiles. Initially, however, the relative 'normality' of the obese mouse lean littermate (NLL) had to be established.

The colony of obese hyperglycaemic mice and their lean littermates originated from stock animals obtained from the Institute of Animal Genetics, Edinburgh. The breeding of lean heterozygotes (ob/+) gave rise to homozygotes (ob/ob) that showed the obese syndrome, lean homozygotes (+/+) and more heterozygotes (ob/+). Only heterozygotes were suitable for continuation of the colony. Among the normal lean littermates were on average 2/3 heterozygotes (ob/+) and 1/3 homozygous normal (+/+) animals with indistinguishable phenotypes. Attempts were made to distinguish between heterozygotes and normal homozygotes by test mating them with a known homozygote. Homozygous normal animals (+/+) provided no (ob/ob) progeny when mated with the known homozygote, while test mating with a heterozygote (ob/+) produced one in four (ob/ob) among the progeny. This test mating proved to be a very tedious and time consuming process and as a consequence no further attempts were made to identify the heterozygous and normal homozygous components of the lean littermates used in experimental work. However, the gross morphological appearance of the pancreata, the blood glucose and serum insulin levels of TO., NLL. and obob mice were investigated in order to establish the 'normality' of the obese mouse lean litter mate (NLL).

MATERIALS & METHODS

A) Verification of the 'normality' of the obese mouse lean littermate.

Gross morphological examination of the excised pancreata of TO., NLL and obob mice was carried out to ascertain the size and distribution of islet tissue. 20-25 week old animals were allowed free access to food and water prior to blood sampling from the tail under pressure by the method of Atkins & Thornburn (156). Blood glucose was estimated in the three types of mice irrespective of sex by the glucose oxidase

method (Boehringer) using a Unicam SP 600 spectrophotometer (436nm). Serum insulin values were estimated by the double antibody radioimmunoassay of Hales & Randle (121).

B) Feedback inhibition of glucose induced insulin secretion by exogenous insulin.

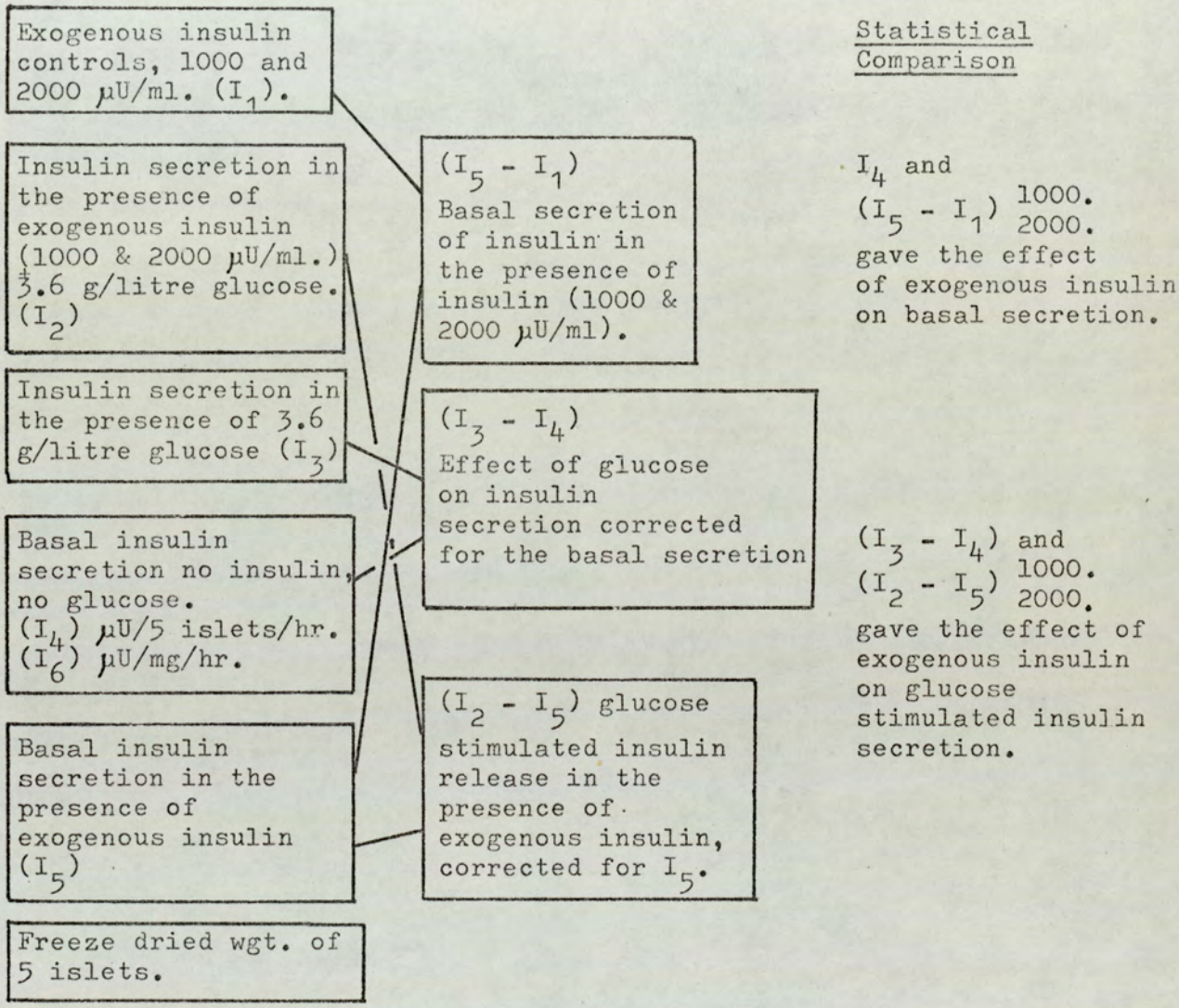
20-25 week old TO., NLL and obob mice were allowed free access to food and water until killed by hyperextension of the neck. All islets were isolated by microdissection and no attempt was made to distinguish between the heterozygous and homozygous normal components of NLL mice. No account was made of the weight of experimental animals or their excised pancreata.

Preparation of insulin controls.

Exogenous insulin concentrations of 1000 and 2000 μU/ml. were prepared from crystalline bovine insulin (24.5 U/mg, BDH.). The insulin was dissolved in a minimum of 0.01 N HCl and made up to volume with Gey & Gey buffer after adjustment of the pH to 7.4 with 0.01N NaOH. All glass ware used was coated with inert silicone compounds (Siliclad or Sigmacote). A single glucose concentration of 3.6 g/litre was added to the incubation medium, designed to stimulate the secretion of insulin from all three types of microdissected mouse islets *in vitro*. No use was made of the largest obese mouse islets and no estimations were made of the total insulin content (initial or final) of any of the islet types used in these experiments. Insulin concentrations were measured by the radioimmunoassay of Hales & Randle as before (121), but samples were diluted by a factor of x20 with buffer B_1 prior to assay. After 20 minutes preincubation groups of 5 islets were incubated for 60 minutes at 37°C with continual shaking in 2 ml. of Gey & Gey buffer containing the appropriate amounts of glucose and exogenous insulin. A summary of the test incubation programme is shown

below. Separate groups of 5 representative islets were used for weighing.

Incubation programme for TO., NLL and obob mouse islets.



Glucose induced insulin secretion was corrected for basal insulin secretion in the presence and absence of exogenous insulin.

RESULTS

A) Verification of the 'normality' of the obese mouse lean littermate.

Low power examination of the excised pancreata of TO., NLL and obob mice showed a certain characteristic distribution of islet tissue. Islets in all three types of pancreas were situated in greatest number and size along the main duct and major blood vessels, nearer

the duodenum than the spleen. The islets of TO. mice were very small, but plainly visible because of the very thin exocrine layers. In lean littermates some pancreata showed a TO. mouse type of islet size and distribution, while others showed a massive increase in the amount of islet tissue, with some islets showing distinct hyperplasia. Obese mice showed numerous large islets with hyperplasia, hypertrophy and intensive vascularisation. In consequence, there seemed to be a component of the lean littermates that showed an intermediate morphological picture between TO. and obob. mice as regards islet distribution and size. However, blood glucose and serum insulin values of NLL mice were not found to differ significantly from the values recorded for TO. mice (Table 4), although obese mice showed significant hyperglycaemia and hyperinsulinaemia.

TABLE 4. Blood glucose and serum insulin levels† of 20-25 week old TO. strain white mice, obese hyperglycaemic mice (obob) and their normal lean littermates (NLL). (Mean values ± S.E.)

	N (no. of mice)	TO	NLL	obob
Blood glucose. mg%.	10	88 ± 6	92 ± 8	237 ± 48 *
Serum insulin. µU/ml.	20	65 ± 12	72 ± 10	741 ± 197 *
Insulin/glucose ratio. µU/mg.		74	78	313

* Denotes significantly different ($p < 0.05$) from TO. and NLL values.

† Serum insulin values were kindly supplied by Miss D. Aryanyagam of this university.

B) Feedback inhibition of glucose induced insulin secretion by exogenous insulin.

The effects of exogenous insulin on the *in vitro* glucose induced insulin secretion and the basal insulin secretion from TO., NLL and obob mouse islets have been summarised in Table 5. Exogenous insulin

controls gave only slightly lower values than theoretically calculated. The islet weights of TO., NLL and obob mice were all significantly different, even though the largest obese islets were not used. Obese islets showed the greatest rate of basal insulin secretion when expressed in terms of μU/5 islets/hr. However, when expressed as μU/mg. freeze dried islets/hr., no significant difference was observed between TO. and obob islets, and NLL islets showed the lowest rate of basal insulin secretion. Glucose (3.6 g./litre) significantly stimulated the rate of insulin secretion from islets of TO., NLL and obob mice (the difference between I_6 and $I_3 - I_4$).

In certain cases, exogenous insulin was found to significantly influence the <u>in vitro</u> rate of basal insulin secretion. 2000 μU/ml. of exogenous insulin significantly increased the rate of <u>in vitro</u> basal insulin secretion from TO. islets (##, <u>Table 5</u>). While 1000 μU/ml. significantly increased the rate of <u>in vitro</u> basal insulin secretion from obese islets (ø, <u>Table 5</u>). For both TO. and NLL islets, the rate of <u>in vitro</u> basal insulin secretion in the presence of 2000 μU/ml. exogenous insulin was significantly greater than that observed in the presence of 1000 μU/ml. exogenous insulin. Also in the presence of 1000 μU/ml. of exogenous insulin, the rate of basal insulin secretion was significantly higher from obese islets than from TO. or NLL islets. In the presence of 2000 μU/ml. exogenous insulin the <u>in vitro</u> rate of basal insulin secretion was significantly higher from TO. and obob mouse islets than from NLL. In only one case (**, <u>Table 5</u>), was there a significant inhibition of glucose stimulated insulin release by exogenous insulin. This inhibition (30%) was observed for TO. mouse islets in the presence of 2000 μU/ml. of exogenous insulin.

The rate of glucose stimulated insulin secretion <u>per se.</u> ($I_3 - I_4$, corrected for the rate of basal secretion) was significantly greater from TO. and obob mouse islets than from NLL. While no significant

TABLE 5. The effect of exogenous bovine insulin on the basal and glucose stimulated insulin secretion from microdissected islets of TO. white mice, obese mice and their normal lean littermates. (Mean values ± S.E., n = number of determinations from pooled islets).

Mice	N	Wgt. 5 islets (µg)	I_1 Exogenous insulin controls µU/ml. 1000	I_1 2000	I_2 (µU/5 islets /hr.) Secretion in presence of exogenous insulin and glucose 3.6g/L. 1000	I_2 2000	I_3 (µU/5 islets /hr.) Secretion in presence of glucose only 3.6g/L.	$I_5 - I_1$ (µU/5 islets /hr.) Basal insulin secretion due to exogenous insulin corrected for I_1 1000	$I_5 - I_1$ 2000	Basal Insulin Secretion (I_4) µU/5 islets /hr.	Basal Insulin Secretion (I_6) µU/mg/hr.	$I_3 - I_4$ Effect of glucose 3.6g/L on insulin secretion corrected for basal secretion µU/mg/hr.	$I_2 - I_5$ Glucose stimulated insulin released in the presence of exogenous insulin corrected for I_5 µU/mg/hr. 1000	$I_2 - I_5$ 2000
TO.	5	36 ± 1.4	956 ± 9.4	1913 ± 11	1772 ± 28	2688 ± 44	832 ± 13	159 ± 30	250 ± 26 *$	89 ± 6	2508 ± 241 δ	20934 ± 1089 **	18656 ± 2153	14665 ± 908
NLL	5	43 ± 1.8 †			1480 ± 23	2412 ± 35	492 ± 11	77 ± 28	110 ± 25 *†	72 ± 6	1671 ± 156 †δ	9839 ± 622 †	10473 ± 899 †	9806 ± 661 †
Ob	5	63 ± 17 α‡			2744 ± 21	3556 ± 51	1637 ± 28	378 ± 38 α‡ø	260 ± 59 α	181 ± 17 α‡	2911 ± 327 αδ	23312 ± 628 α	22602 ± 835 α	22177 ± 688 α‡

* Denotes a significant difference between vials containing 1000 and 2000 µU/ml. of exogenous insulin ($P<0.05$).
† Denotes significant difference ($P<0.05$) between NLL. and TO.
α Denotes significant difference ($P<0.05$) between Obob and NLL.
‡ Denotes significant difference ($P<0.05$) between Obob and TO.
δ Denotes significant difference ($P<0.05$) between I_6 and I_3-I_4.
$ Denotes significant difference ($P<0.05$) between I_5-I_1 2000 and TO. I_4.
ø Denotes significant difference ($P<0.05$) between I_5-I_1 1000 and Obob. I_4.
** Denotes significant difference ($P<0.05$) between TO., I_3-I_4 and I_2-I_5 2000.

difference was observed between the rate of glucose induced insulin secretion from TO. and obob mouse islets. These results indicated that hypersecretory obese islets were capable of normal glucose induced secretory activity, when isolated from the prevailing in vivo insulin resistance. In the presence of both 1000 μU/ml. and 2000 μU/ml. of exogenous insulin, obese islets showed the highest rate of glucose induced insulin secretion and NLL islets the lowest. However, in the presence of 1000 μU/ml. of exogenous insulin, the rates of glucose induced insulin secretion from TO. and obob islets were not significantly different.

Discussion

Malaisse and coworkers suggested that levels of circulating insulin should be related to the level of blood glucose stimulating the secretion of insulin and that the serum insulin/blood glucose ratio in μU/mg. was an indication of the functional state of the β-cells (157,158). In spite of the morphological differences observed between the pancreata of TO. and certain NLL mice, the insulin/glucose ratios for the two were found to be virtually the same (Table 4). A high ratio, such as that observed for obese mice, would correspond to an increased secretory responsiveness of the pancreas to glucose, whereas any subsequent lowering of the usual ratio observed for normal mice would suggest an evolution towards impairment of β-cell function. However, Duprey and colleagues have suggested their 'insulin secretion coefficient' (the ratio of blood insulin measured at a given level of blood sugar to an average normal blood insulin for the same blood sugar) to be a better estimation of β-cell function (159).

Westman was also unable to show any abnormalities of carbohydrate metabolism in the lean littermates of homozygous obese mice, and the heterozygous components of the lean littermates were only distinguished through progeny of those litters which lacked the gene (66). The establishment of 'normality' in the lean littermate allowed future work

to concentrate on the difference between itself and the obese mouse, and to dispense with the TO. individual as a control.

Little evidence was found for an inhibition of glucose induced insulin secretion by exogenous insulin. Although observed for TO. mouse islets in the presence of 2000 μU/ml. of exogenous insulin, the phenomenon was not observed with the islets of either obese hyperglycaemic mice or their normal lean littermates. Since the glucose induced secretion from TO., NLL and obob mouse islets never exceeded 2000 μU/ml. (I_3, Table 5), the possibility of a feedback inhibition of insulin secretion by previously secreted insulin was unlikely in the type of incubation system currently used. It was interesting to note the higher rate of basal insulin secretion from obese islets compared to NLL islets. Although, the observed similarity between the rates of basal insulin secretion from TO. and obob mouse islets, might question the 'normality' of the lean littermate. There was evidence also to suggest (#, ∅, Table 5) that certain concentrations of exogenous insulin were capable of significantly stimulating the rate of basal insulin secretion from TO. and obob mouse islet tissue.

The results of the work presented here were consonant with those observed by Malaisse and coworkers (141) and Hahn & Michael (155), who were unable to show an inhibition of insulin secretion below 1000 μU/ml. and 2000 μU/ml. of exogenous insulin respectively. The interpretation of experimental results required the assumption that significant amounts of either added or secreted insulin were not lost by proteolytic destruction or adsorption to glass during incubation. This assumption was reasonable on the basis that only clean islets were used for incubation and their volume was small in relation to the volume of the incubation mixture. Also the incubation vials were coated with a layer of inert silicone compound and the amounts of insulin

recovered in control vials were reasonably close to the theoretical, even though mouse insulin standards were not used. Sodoyez and coworkers suggested that the concentration of insulin required for the inhibition of insulin secretion increased in proportion to the metabolic stimulus (151). A variable adjustment such as this would permit a continuous minimal output of insulin under basal conditions in vivo, while allowing it to reach successively higher cut off levels as the glucose concentration in the serum increased. However, this hypothesis has yet to be confirmed. A possible mechanism for the feedback inhibition of insulin secretion by previously secreted insulin may be tentatively mentioned at this point. Insulin might suppress the activity of β-cell adenyl cyclase and hence the formation of cyclic AMP or stimulate the activity of cyclic AMP-phosphodiesterase, thus decreasing further cyclic AMP initiated insulin synthesis and secretion. In support of this hypothesis, cyclic AMP has been suggested to mediate the stimulatory effect of glucose, glucagon and theophylline or insulin secretion (41) and insulin has been shown to lower the level of cyclic AMP in adipose tissue (160,161) and the activity of adenyl cyclase in rat liver plasma membranes (162,163).

More accurate in vitro work on insulin secretion requires the complete separation of secreted insulin from its secretory site. The changing of incubation media after 30 minutes and the use of isolated islets instead of pieces of pancreas in perifusion systems such as that of Burr and coworkers (142) might go some way to achieving this.

CHAPTER 2.

SECTION I.

The rate of oxygen uptake by microdissected and collagenase isolated islets and by exocrine tissue from TO. strain white mice, obese hyperglycaemic mice and their normal lean littermates.

Both manometric and polarographic techniques have been used for the measurement of the rate of oxygen consumption ($-Q_{O_2}$). The manometric techniques, the capillary, Cartesian diver and Warburg respirometers facilitated the accurate measurement of gas pressure changes (O_2 or CO_2) that resulted from respiring substrates (164-166). The Cartesian divers, first described by Linderstrøm - Lang (167) and later by Holter (168) replaced the capillary respirometers and worked on the principle that if for some reason the quantity of gas in a glass diver decreased or increased, the pressure change that resulted was a measure of the change in quantity of the gas. When reactions took place within the diver that involved the liberation or adsorption of gas, the rate of these processes was estimated quantitatively by measuring at different times the pressures at which the diver was in unstable equilibrium at a fixed level. Pressure changes were measured very accurately and Cartesian divers proved useful tooks for the measurement of total respiration on a microscale (169). The Warburg method of measuring gas exchanges involved the use of a constant-volume manometer operated in a constant temperature bath. As the reaction proceeded, changes were observed as a decrease or increase in the height of a column of Brodie's fluid in a manometer connected to the substrate vessel. The essential principle involved was that at constant temperature and constant gas volume, any changes in the amount of a gas could be measured by changes in its pressure (170).

Respiratory measurements on isolated islet tissue have been limited to those performed by Hellerström (171) and Friz (172) using the Cartesian diver microgasometer technique. Hellerström's preliminary data were used to confirm the viability of islets obtained by freehand microdissection (38) and his more recent work has investigated the rate of oxygen consumption ($-Q_{O_2}$) of normal and obese mouse islets (173) in the presence of various carbohydrates (174) and hypoglycaemic sulphonylureas (175-177).

In the work described below, an oxygen electrode was used to determine the rate of oxygen consumption of mouse islet and exocrine tissue. In techniques using Warburg Manometry and the 'two-compartment Cartesian diver' of Hamberger (178) substrate(s) were added directly to a respiring tissue after a predetermined period of time, and the effect evaluated quantitatively. In the case of oxygen electrode respiratory chambers, it was found almost impossible to introduce test materials during the respiratory period without the formation of air bubbles, unless a great deal of sophistication was called upon (179). Although sophisticated prototypes were constructed, none proved to be reliably leakproof. In consequence oxygen consumption studies were carried out with islets and exocrine tissue in both the presence and absence of test materials, using a system that proved to be virtually leakproof.

The Oxygen Electrode.

The use of an oxygen electrode (a polarographic device for measuring oxygen tensions) had certain advantages over the various manometric techniques available. Manometric techniques could not be used for following rapid changes in metabolic rate, because of the time taken for the oxygen of the gas phase to come into equilibrium with the dissolved oxygen in the tissue. The polarographic method was more sensitive because the electrode responded to the amount of oxygen dissolved in an aqueous solution at atmospheric pressure, rather than to the much larger amount in the gas phase over it. The sensitivity of

the oxygen electrode was increased by reducing the volume of the aqueous solution in contact with it. The polarographic technique *per se.* was less tedious and time consuming to use than manometry. Also because the oxygen electrode depended upon the electrolysis of dissolved oxygen at a cathode immersed directly in the metabolising medium, diffusion lags were reduced to a minimum.

The Principle of the oxygen cathode.

The principle of the oxygen electrode has been summarised in a number of recent reviews (180-182). When a platinum electrode was made a few tenths of a volt negative with respect to a suitable reference electrode in a solution which contained dissolved oxygen, the oxygen at the cathodal surface was electrolytically reduced and a current flowed (183). When the cathode was made sufficiently negative, usually between -0.6 and -0.9 volts relative to a calomel half-cell, the velocity of the reaction was limited chiefly by the maximum rate at which oxygen could diffuse to the electrode surface, and was affected to a lesser extent or not at all by changes in potential. At these potentials, at least, the current was found to be proportional to oxygen concentration in the body of the solution.

Nature of the electrode reaction.

The chemical reactions at the cathode are not known in detail. Evidence has been presented to suggest that the following reactions take place and describe theoverall process (182).

$$O_2 + 2H_2O + 2e^- \longrightarrow H_2O_2 + 2OH^-$$

$$H_2O_2 + 2e^- \longrightarrow 2OH^-$$

The sequence was insensitive to pH., since no hydrogen ions were utilised. Small amounts of H_2O_2 were produced if the second stage did not proceed at the maximum velocity possible, and the medium became alkaline.

Characteristics of the oxygen cathode.

a) The speed of response.

For the evaluation of the behaviour of an oxygen electrode when first turned on or when rapid changes in oxygen concentration were taking place in the medium, it was necessary to know the speed of response of the electrode reaction. If the electrochemical reaction was not initially rapid and there was no reason to suppose that it was, there would be an upper limit to the rapidity with which the oxygen cathode could follow changes in oxygen concentration in the medium. By reducing diffusion lags to a minimum (182) an estimate of the response time of the electrode reaction was made. The electrode was found to have a response time of no greater than 0-5 ms.

b) Electrode ageing.

Oxygen cathodes have been observed to age (181). This was observed as a slow downward trend of the electrode response over a period of time, even though the oxygen tension of the medium had been maintained constant by equilibrium with a gas mixture. Ageing was found to be slight in simple dilute salt solutions and generally approached a steady value. However, appreciable ageing occurred in solutions containing phosphate, especially if divalent cations were present with a high percentage of protein i.e. boving serum albumin $\overline{V}$. The cause of ageing was not known, beyond the fact that something became attached to the metallic cathode surface, by precipitation either in the form of insoluble salts of Ca^{2+}, Mg^{2+}, CO_3^{-}, $PO_4^{=}$ or perhaps proteinate (181). Ageing was eliminated by covering the electrode with a polythene membrane (181,184) and this required the reference electrode to be in contact with the electrolyte solution inside the membrane.

c) Diffusion of oxygen into sample.

A problem encountered when measuring low rates of oxygen uptake was the diffusion of oxygen from the oxygen rich medium into the sample (185). When tissue pieces were large, the diffusional loss of oxygen from the

medium into the tissue appeared as a high rate of consumption at the beginning of each period of measurement. This initial high rate was the diffusion artifact. This artifact was not so important when isolated islets were used since the diffusional equilibrium was reached in a very short period of time.

d) Storage effects and type of gas used for standardisation.

Lloyd & Seaton observed that the response of an oxygen electrode to a change from oxygen to nitrogen was a compound function of an expected fast component, partly due to electrode wash out, combined with a slow component that suggested some form of oxygen storage in the electrode (186). Catalase was found to reduce this store which indicated that hydrogen peroxide, a known by-product in the electrolytic reduction of oxygen was found to be chiefly responsible (182).

Oxygen has been shown to be toxic for some tissues (187) and evidence has been presented to favour the use of air instead of oxygen for electrode standardisation (185). However, high oxygen concentrations were required by rapidly respiring tissue to prevent anoxia. In general, tissues were kept as nearly saturated as possible with oxygen at the tension of the ambient medium and the delivery to cells by diffusion had to keep pace with the rate of consumption, for the record of oxygen concentration in the external medium to be a true measure of the oxygen consumption by the tissues.

e) The membrane covered electrode.

The use of a polythene membrane prevented electrode ageing and the production of a movement artifact. In the Clark type of electrode (184) the electrolysis medium was completely separated from the medium in which oxygen was to be measured, by a non-conducting membrane permeable to oxygen. Hence no ionized contaminants were likely to migrate to the surface of the oxygen cathode. However, a possible source of instability of the Clark type oxygen cathode was the slight variation in the thickness of the layer of solution between the membrane and cathode,

which occurred if the membrane was not sufficiently taut. Such variations were capable of causing changes in the diffusion distance through this layer and fluctuations in electrode response.

In the work described below an oxygen cathode has been used to estimate the rate of oxygen consumption ($-Q_{O_2}$) of exocrine tissue, microdissected and collagenase isolated TO. mouse islets and microdissected islets of obese hyperglycaemic mice and their normal lean littermates.

Materials & Methods.

The Oxygen cathode assembly and circuit.

The principal component of the circuit was a vibron electrometer (Model 33B-2, EIL. Richmond, Surrey), plate 5. The instrument had an input stray current of less than 10^{-15}A and an input resistance of $10^{15}\Omega$. The zero stability was within 0.1 mV over a 12 hr. period. The high resistance side of the potential to be measured (all measurements were obtained in terms of potential) was connected to the instrument by means of a coaxial plug. During experimental work the instrument was left on continuously to eliminate zero drift. The vibron was connected to the oxygen cathode via a pO_2 accessory unit (potentiometer modification, Model D 48A (1), E.I.L., Richmond, Surrey). The polarisation voltage was obtained from a $1\frac{1}{2}$ V. dry cell and adjusted to 700 mV. The output from the oxygen cathode was passed through a $10^6\Omega$ shunt resistence before passing into the vibron. The vibron scale was set to give a full scale deflection of 1000mV. (x100). The oxygen cathode assembly sat on a magnetic stirrer (MS 3. Grant Instruments, Cambridge) set at 550 rev/min. The response of the oxygen cathode was sensitive to the rate of stirring when tissue was present in the respiratory chamber. A constant value of 550 rev/min adequately stirred islet tissue without rupture. The whole apparatus was maintained at $37 \pm 1^{\circ}C$ in a thermostatically controlled constant temperature room.

PLATE 5. Apparatus for the measurement of the rate of islet oxygen consumption.

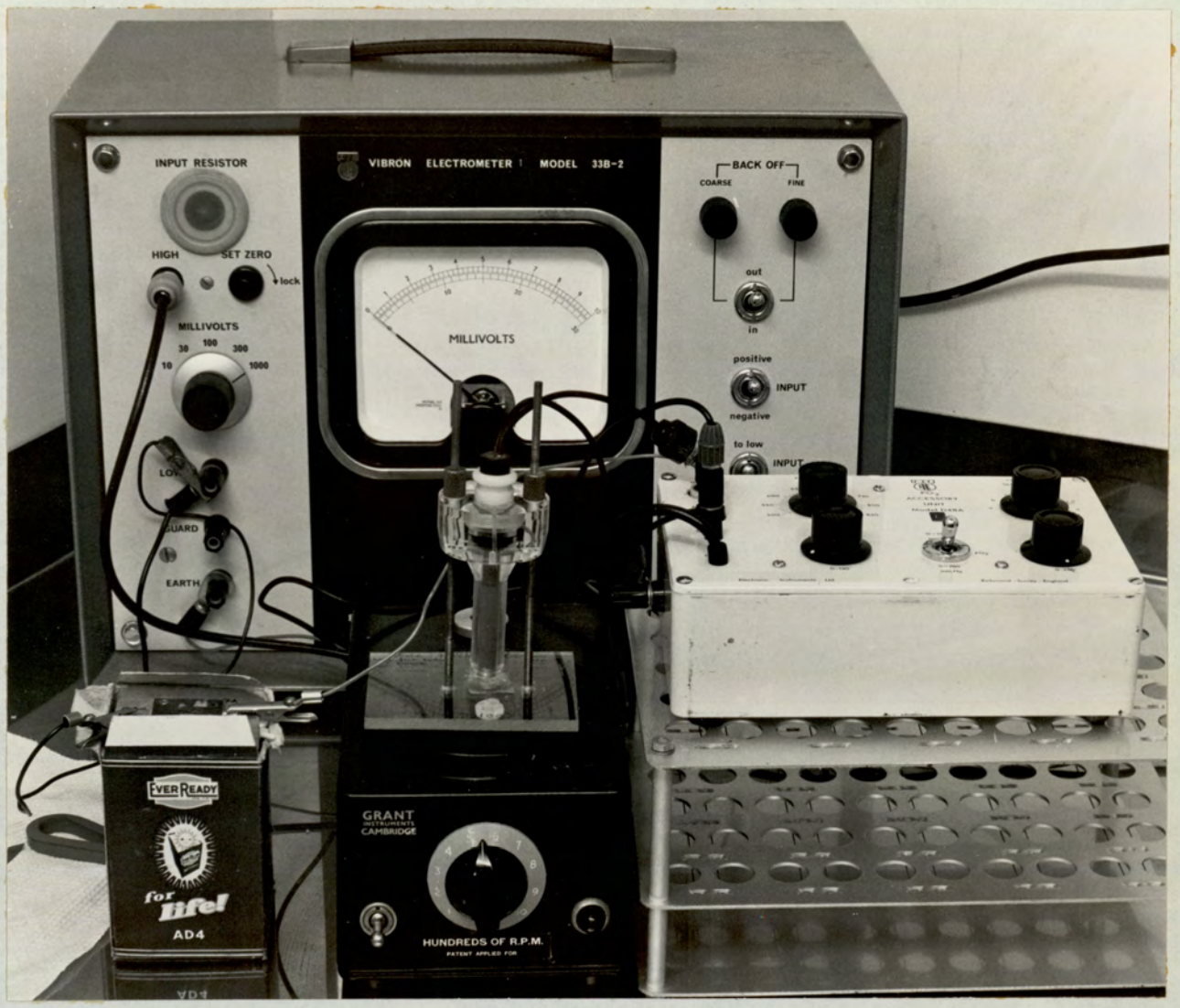
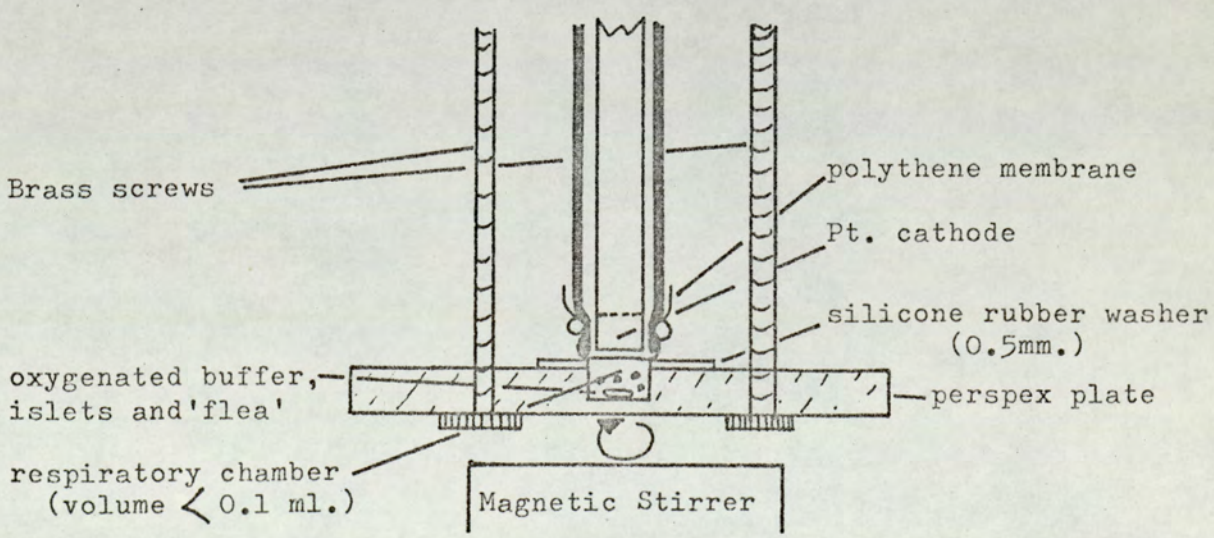


FIG. 6. The electrode and respiratory chamber assembly.



The electrode was bolted flush onto a 0.5 mm. silicone rubber washer around the mouth of the perspex respiratory chamber, efficiently trapping 0.1 ml. of oxygenaged buffer (Fig.6). This joint was found to be perfectly airtight for periods of at least 3 hours or more. The respiratory chamber was checked for air bubbles by examining the underside of the perspex plate with a magnifying glass. Air bubbles were avoided since they caused large errors in the measurement of the rate of islet oxygen consumption. The magnetic stirrer agitated a metal "flea" in the respiratory chamber (small piece of hypodermic syringe needle set in araldite) and facilitated the stirring of respiring islet tissue in the oxygenated buffer. This stirring prevented stagnation at the electrode membrane surface and ensured that the readings of oxygen concentration obtained were representative of the whole respiratory chamber and not just of the portion nearest the membrane. Clogged "fleas" were freed with a robust horse-shoe magnet.

The non-conducting but oxygen permeable membrane separated the oxygen containing sample from the electrolysis system. The membrane served to maintain a constant diffusion zone for oxygen around the cathode and its nature and thickness determined the speed of response of the electrode. The membranes used were 12 μm in thickness and made of polythene (Precision Scientific Co. Ltd., Scientific Techniques Ltd., Bletchworth, Surrey). The outer casing of the oxygen electrode served as a reservoir for the electrolyte solution (3.8g. Na_2HPO_4; 14.5g. KH_2PO_4, 7g. KCl in 1 litre of distilled water) and an overflow tube in the electrode housing allowed the escape of excess buffer during assembly. The electro-induction of oxygen diffusing through the membrane from the sample took place in the thin layer of electrolyte between cathode and membrane. This layer also served to conduct the current which flowed when the cathode was depolarized by oxygen and in this way no current flowed through the sample. The electrolysis system was thus completely independent of the composition of the sample.

The perspex interior surfaces of the respiratory chamber were treated with silicone "Repel -Cote" (a 2% v/v solution of dimethyldichloro-silane in carbon tetrachloride, Hopkins & Williams) to prevent damage to respiring islet tissue.

For short term storage the oxygen electrode was immersed in 5% sodium sulphite, a solution virtually free of dissolved oxygen, to prevent cathodic reduction and to remove air bubbles from the contained electrolyte by short circuit.

Calibration of the oxygen electrode.

The current output from an oxygen electrode has been reported to be a measure of the activity of the oxygen in the solution in which it is immersed, not the concentration (188). The activity of a gas in solution has been shown to be the product of its concentration and activity coefficient. The activity coefficient (α) was related to the ionic strength (I) of the solution by the expression, $\text{Log}\,\alpha = KI$. Where K was a constant which depended on the nature of the electrolyte (189). The presence of both electrolytes and non-electrolytes has been shown to influence the solubility and the activity coefficient of oxygen in aqueous solution (190). In water the activity coefficient was unity and so the activity of oxygen was equivalent to the concentration of dissolved oxygen. The latter was obtained from solubility tables, so the current output from an electrode could be calibrated directly with respect to the concentration of dissolved oxygen. However, in order to express the oxygen uptake of islet tissue in terms of absolute amounts of oxygen it was first necessary to calibrate the vibron scale with respect to the islet containing medium i.e. a buffered saline. As neither the solubility or the activity coefficient of oxygen in this medium were known, calibration could not be effected by reference to these. Hence a more direct measurement of oxygen content was necessary for the islet containing buffer solutions.

Several other methods of calibration have recently been introduced. Robinson & Cooper (191) modified the earlier method of Estabrook & Mackler (192) and demonstrated a calibration in which oxygen was consumed in the stoichiometric oxidation of a specified amount of NADH by N - methylphenazonium methosulphate and the change in electrode current was equivalent to a known uptake of oxygen from the solution. Le Fevre has described a simple method for the preparation of oxygen standard solutions for use with the oxygen electrode (193). The method involved the mixing of two solutions, one equilibrated with 100% oxygen and the other with 100% nitrogen. The electrode response in water equilibrated with known oxygen/nitrogen gas mixtures was found to be indistinguishable from the response obtained in mixed solutions calculated to give the same percent oxygen saturation.

Calibration method.

A bicarbonate buffered saline (Gey & Gey(110)) containing no glucose or bovine serum albumin $\overline{V}$ was used as the oxygenation medium. The use of albumin was avoided because of its possible effect on oxygen diffusion at the polythene membrane and the excessive frothing produced during the oxygenation procedure.

25 ml. of basic saline (+ low or high glucose if used under test) was gassed with 95% O_2/5% CO_2 for 15 minutes at 37°C. The electrode, separated from the respiratory chamber was washed with distilled water to remove all traces of sodium sulphite. The zero of the vibron electrometer was preset and after 15 minutes exactly, the oxygen electrode was inserted into the oxygenated saline and a O_2 saturated saline value obtained in mvolts ($\triangleq$ 500 mV). The response time of the electrode was less than 1 s. and the saline was stirred continuously during measurement at 550 rev/min. with a teflon coated magnet. Immediately after vibron measurement, the oxygenated saline was poured into a small glass weighing bottle, of predetermined volume and a ground glass

stopper rapidly inserted to prevent the loss of oxygen. The stopper was then removed and 0.1 ml. of manganese sulphate (2 mol./litre) and 0.1 ml. of alkaline sodium azide KI reagent were quickly added below the surface of the contents of the weighing bottle. The stopper was quickly refitted with the exclusion of all air bubbles. The contents were mixed by inversion and the brown precipitate of manganic hydroxide allowed to settle at 37°C. After use the oxygen electrode was cleaned, dried and retained for use in double distilled water at 37°C.

The same procedure was carried out for 25 ml. of saline that had been bubbled with nitrogen for 15 minutes at 37°C. An O_2 depleted saline value was obtained from the vibron and an air tight sample prepared for estimation of oxygen content as before.

Determination of the dissolved oxygen in the saline samples.

This was effected by the method of Winkler (194) using the sodium azide modification (195). The manganic hydroxide precipitate in the sample was dissolvedby the addition of 50% sulphuric acid. The manganic sulphate produced was used to liberate iodine quantitatively from potassium iodide and the iodine was titrated with $N/_{80}$ sodium thiosulphate to a colourless end point using starch as an indicator. The dissolved oxygen content in mg/litre was computed from,

$$\text{Dissolved oxygen (mg/litre)} = \frac{\text{Volume of } N/_{80} \text{ Thiosulphate (ml)} \times 100}{\text{Volume of sample titrated (ml.)}}$$

The dissolved oxygen values of the O_2 saturated and O_2 depleted saline samples in mg./litre provided the upper and lower calibration points on the vibron scale in terms of mvolts. In preliminary work the oxygen content of 50/50 mixtures of 15 minutes oxygenated saline and 15 minutes nitrogen bubbled (oxygen depleted) saline were estimated to ensure the linearity of the vibron scale. Calibration of the scale in the manner described was carried out each day prior to experimental runs, in order to compensate for changes in humidity and barometric pressure. The latter was obtained from a Fortin's barometer maintained at $37 \pm 1°C$.

Measurement of the oxygen consumption of isolated islet and exocrine tissue.

Normal TO. strain white mice, obese hyperglycaemic mice and their normal lean littermates were allowed free access to water and 41B rat and mouse research diet (Pilsbury's Ltd., Birmingham.) until weighing and subsequent death by hyperextension of the neck. Islets were isolated from TO. mice pancreata by microdissection and collagenase isolation, and from NLL and obob mice pancreata by microdissection. Small exocrine fragments and isolated islets were retained in low glucose buffer (0.6 g./litre) at 2°C over ice until required.

With the vibron circuitry connected up and calibrated as described earlier, 25 ml. of saline containing no glucose, low glucose (0.6g./litre) or high glucose (3.6g./litre) was oxygenated for 15 minutes at 37°C. During the oxygenation, the base plate of the electrode, containing the respiratory chamber and stirrer "flea" was placed on the stage of a dissecting microscope. During the last few minutes of the oxygenation, a number of islets with well preserved connective tissue capsules (generally pooled from more than one animal) were rapidly transferred to the respiratory chamber. All supernatant saline was removed from the vicinity of the islets. A portion of freshly oxygenated buffered saline (0.2 ml, $\pm$ glucose) was added to the respiratory chamber as quickly as possible taking care not to flush out the contained islets. Sufficient buffer was added to create a high meniscus over the respiratory chamber. The oxygen electrode was then quickly tightened down onto the silicone rubber washer completely sealing off the respiratory chamber. Excess saline was removed from the surface of the base plate and the stirrer "flea" loosened with an horse shoe magnet. The whole electrode assembly was positioned on top of the magnetic stirrer and connected into circuit. An initial reading was taken from the vibron at zero time and the difference between this value and the O_2 saturated saline value was an indication of the oxygen lost as a result of islet manipulation and electrode assembly. There was always sufficient oxygen remaining in the

saline to effect a measurement of the rate of oxygen consumption. The atmospheric pressure was recorded at 37°C. Further vibron readings were taken at 10 minute intervals or less depending on the rate of oxygen uptake, until the saline oxygen content was reduced to the vicinity of the nitrogen depletion value or the time exceeded two hours. Values were plotted as they were obtained, on a graph of mVolts/Time in minutes (Fig.7). After use the electrode was carefully unscrewed from the base plate, washed in distilled water and stored in 5% sodium sulphite until the next determination. The stirrer "flea" was recovered from the respiratory chamber and islets transferred to foil "boats", freed of excess buffer, freeze dried and weighed as previously described (p. 24).

Testing for leakage from the respiratory chamber.

Test runs employing the same procedure as described above were carried out without islet tissue, in an attempt to detect any loss of oxygen from the respiratory chamber. These test runs assumed that the electrode itself used no oxygen. The result of a typical leakage test run is shown in Fig. 8.

Estimation of Respiratory chamber volume.

This was carried out gravimetrically, by first weighing the whole electrode assembly dry and then weighing with the respiratory chamber filled with distilled water. The stirrer "flea" was present on both occasions. The mean volume of the respiratory chamber was found to be 0.082 ml.

Calculation of results.

The curve obtained by plotting mvolts/time (Fig.7) was sigmoidal showing an initial slow acclimatisation, followed by a period of rapid oxygen uptake. The subsequent reduction in oxygen consumption possibly reflected a fall in the rate of tissue respiration as the supply of dissolved oxygen became limiting. The gradient of the curve was used to compute the rate of oxygen uptake by the tissue in terms of µl. of

FIG. 7. Oxygen uptake profile for obese mouse exocrine and islet tissue in the presence of low glucose (0.6 g/litre.).

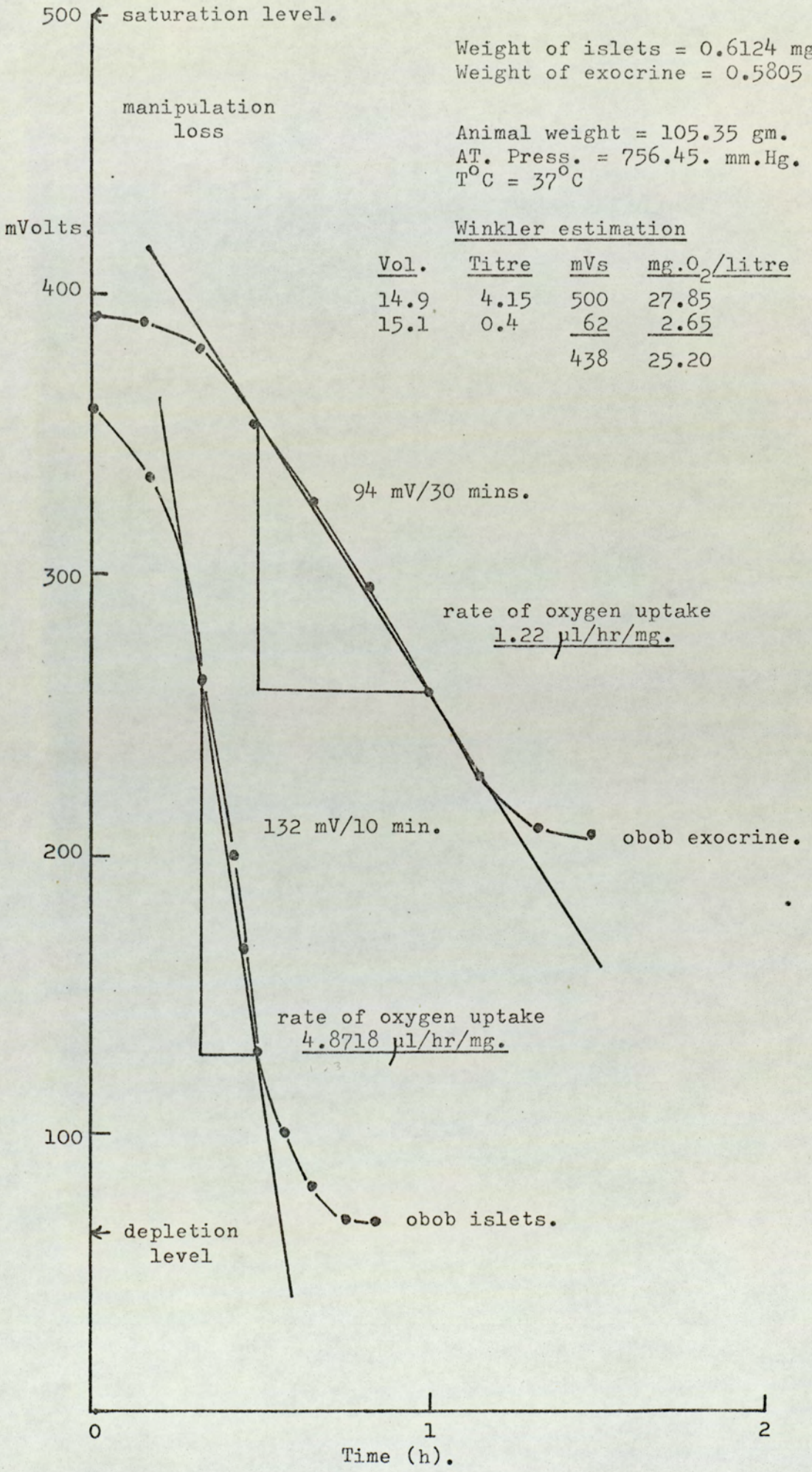
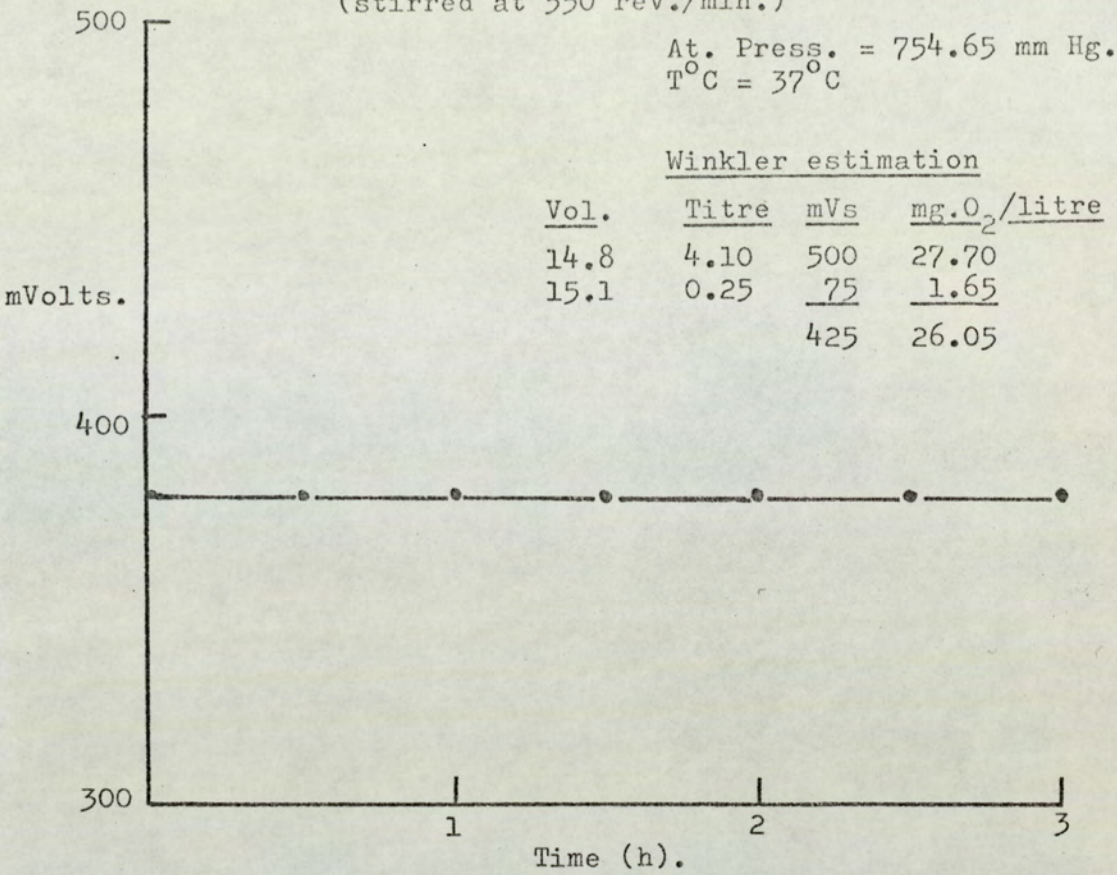


FIG.8. Leakage test on respiratory chamber.
(stirred at 550 rev./min.)



At. Press. = 754.65 mm Hg.
$T^{o}C = 37^{o}C$

Winkler estimation

Vol.	Titre	mVs	mg.O_2/litre
14.8	4.10	500	27.70
15.1	0.25	75	1.65
		425	26.05

O_2 used/h/mg. freeze dried tissue at $37^{o}C$. Respiratory periods never exceeded 2 h.

Example calculation for Figure 7.

Winkler estimations of oxygen saturated and oxygen depleted saline values gave a deflection of 500 --> 62 mV. The difference, 438 mVolts corresponded to 25.2 mg. of O_2/litre.

So 438 mV $\equiv$ 25.2 mg/litre of O_2 at 756.45 mm Hg. & $37^{o}C$.

32 gm. of O_2 $\equiv$ 224000ml. at s.t.p.

32 mg. $\equiv$ 22.4 ml. at s.t.p.

1 mg. of O_2 $\equiv$ 0.7 ml. of O_2 at $273^{o}C$ & 760 mm Hg.

1 mg. of O_2 will occupy the following volume at 756.45 mm Hg. & $37^{o}C$.

$$\text{i.e. } \frac{P_1V_1}{T_1} = \frac{P_2V_2}{T_2}$$

$$\frac{0.7 \times 760}{273} = \frac{756.45 \times V_2}{310}$$

$V_2 = 0.7986$ ml.

Hence 1 mg. of O_2 will occupy 0.7986 ml. at 756.45 mm Hg. & $37^{o}C$.

Hence 438 mV $\equiv$ 25.2 x 0.7986 ml. of O_2/litre

1 mV $\equiv$ $\frac{25.2 \times 0.7986}{438}$ ml. of O_2/litre

Now the gradient for the exocrine tissue of the obese mouse (Fig.7)

$$= 94 \text{ mV}/30 \text{ min.}$$

$$\therefore \quad 94 \text{ mV} = \frac{25.2 \times 0.7986}{438} \times 94 \text{ ml. of } O_2/L/ 30 \text{ minutes.}$$

$$= \frac{25.2 \times 0.7986 \times 94.2}{438 \times 1000} \text{ ml. of } O_2/\text{ml/h.}$$

The volume of the respiratory chamber = 0.082 ml.

$$= \frac{25.2 \times 0.7986 \times 94 \times 2 \times 0.082 \times 1000}{438 \times 1000} \mu\text{l of } O_2/\text{h.}$$

Hence the rate of oxygen uptake by exocrine tissue = 0.7081 µl of O_2/hr.

Wgt. of exocrine tissue = 0.5805 mg.

0.5805 mg. uses 0.7081 µl of O_2/h.

1 mg. uses 1.2198 µl/h.

Hence the rate of oxygen uptake ($-Q_{O_2}$) of obob exocrine tissue = 1.2198 µl/h/mg freeze dried tissue at 37°C.

Similarly the gradient for obob islet tissue (Fig.7) was 132 mV/10 minutes

The rate of O_2 uptake was computed as 2.984 µl of O_2/h.

0.6124 mg. of obob islets were used

$\therefore$ 0.6124 mg. of obob islets used 2.984 µl of O_2/h.

1 mg. used 4.872 µl of O_2/h.

The rate of oxygen uptake ($-Q_{O_2}$) of obob islets was 4.872 µl of O_2/h/mg. freeze dried tissue, 37°C.

Using the system so described, a comparison was made between the rates of oxygen uptake of microdissected and collagenase isolated islets of TO. strain white mice in the absence and presence of low (0.6g/litre) and high (3.6g/litre) glucose. Also the rates of oxygen uptake of islets and exocrine tissue from obese mice and their normal lean littermates were measured in the absence and presence of low and high glucose. The possibility of relationships existing between islet or exocrine tissue weights and their rates of oxygen uptake were investigated under similar conditions of glucose concentration.

RESULTS:

Leakage tests on the electrode assembly (Fig. 8) showed the respiratory chamber to be leakproof for up to 3h., after which time a loss of only 0.0061 µl of O_2/h. was observed for a subsequent 4h. period. Measurements of oxygen uptake never lasted for longer than 2h.

The rates of oxygen uptake by microdissected and collagenase isolated TO. islets, together with the rates of oxygen uptake by microdissected islets of obese mice and their normal lean littermates have been summarised in Table 6. Coefficients of variation for all groups of data were within 5%.

Islets isolated from TO. mice by microdissection showed significantly higher absolute rates of oxygen uptake than islets isolated by collagenase digestion, in the absence and in the presence of low and high glucose. However, both low and high glucose induced a greater percentage stimulation of the rate of oxygen uptake by collagenase isolated TO. islets than by microdissected, which suggested that collagenase isolated TO. islets were more sensitive to glucose than microdissected TO. islets. In the absence of glucose, microdissected obob islets showed the greatest rate of oxygen uptake and microdissected NLL islets the lowest. Low glucose (0.6g/litre) significantly stimulated the rate of oxygen uptake by microdissected islets of NLL and obob mice but not the microdissected islets of TO. mice. High glucose (3.6g/litre) significantly stimulated the rate of oxygen uptake by TO., NLL and obob mouse islets. The rate of oxygen uptake by obob microdissected islets was the most responsive of the three to low glucose stimulation but the least responsive to high glucose (Table 6). This latter observation may have been the result of a continual exposure of obese islets to hyperglycaemia while in vivo. Generally speaking, increasing the glucose concentration, correspondingly increased the rate of oxygen uptake by all three types

TABLE 6. The effect of glucose on the rates of oxygen uptake by microdissected and collagenase isolated islets of TO. white mice and the rates of oxygen uptake by microdissected islets of obese hyperglycaemic mice and their normal lean littermates. (Mean Values ($-Q_{O_2}$) ± S.E., n = number of determinations from pooled islets).

		Collagenase isolated islets $\mu l\ O_2/h/mg.$ f.d. islets.		Microdissected islets $\mu l\ O_2/h/mg.$ f.d. islets.	
Glucose concentration.	N	TO.	TO.	NLL.	obob.
No glucose	3	0.744 ± 0.014	1.71 ± 0.024 *	1.43 ± 0.014 δ	4.09 ± 0.061 ‡ **
Low glucose (0.6g/litre)	3	1.11 ± 0.020 (50% †)	1.78 ± 0.120 * (4.1%)	1.51 ± 0.017 (6% †)	4.70 ± 0.085 ‡ ** (15% †)
High glucose (3.6g/litre)	3	1.79 ± 0.022 (142% ϕ)	3.17 ± 0.118 * (85% ϕ)	3.20 ± 0.102 (124% ϕ)	6.56 ± 0.296 ‡ ** (60% ϕ)

* Denotes microdissected and collagenase isolated TO. mouse islets compared, in the presence of the appropriate amount of glucose ($P < 0.05$)

† Denotes significant % stimulation ($P < 0.05$) by low glucose.

ϕ Denotes significant % stimulation ($P < 0.05$) by high glucose.

δ Denotes TO. and NLL. microdissected islets compared, in the absence of glucose ($P < 0.05$).

‡ Denotes NLL. and obob microdissected islets compared in the presence of the appropriate amount of glucose ($P < 0.05$).

** Denotes obob and TO. microdissected islets compared, in the presence of the appropriate amount of glucose ($P < 0.05$).

TABLE 7. The effect of glucose on the rate of oxygen uptake by exocrine tissue from TO. white mice, obese hyperglycaemic mice and their normal lean littermates. (Mean values ± S.E., N = number of mice).

Exocrine tissue.

(μl O_2/h/mg. freeze dried exocrine tissue).

Glucose concentration.	N	TO.	NLL.	obob.
No glucose.	3	0.96 ± 0.017	0.91 ± 0.007 δ	0.81 ± 0.041 ✱✱
Low glucose. (0.6g/litre)	3	1.14 ± 0.035 (19% †)	1.27 ± 0.014 δ (40% †)	1.27 ± 0.041 (57% †)
High glucose. (3.6g/litre)	3	1.39 ± 0.010 (45% ø)	1.05 ± 0.007 δ (15%)	1.20 ± 0.049 ‡✱✱ (48% ø)

† Denotes significant % stimulation by low glucose ($P < 0.05$).

ø Denotes significant % stimulation by high glucose ($P < 0.05$).

δ Denotes TO. and NLL. exocrine compared, in the presence of the appropriate amount of glucose ($P < 0.05$).

‡ Denotes NLL. and obob. exocrine compared in the presence of the appropriate amount of glucose ($P < 0.05$).

✱✱ Denotes obob. and TO. exocrine compared in the presence of the appropriate amount of glucose ($P < 0.05$).

of microdissected islets.

The effects of glucose on the rates of oxygen uptake by exocrine tissue from TO. white mice, obese mice and their normal lean littermates have been summarised in Table 7. Coefficients of variation for all groups of data were within 4%. The rates of oxygen uptake shown by microdissected islets of TO., NLL and obob mice in the presence and absence of glucose were significantly higher ($P < 0.05$) than the corresponding values for exocrine tissue subjected to the same treatment. In the absence of glucose TO. mouse exocrine showed the highest rate of oxygen uptake, and obese mouse exocrine the lowest. All differences were significant ($P < 0.05$). Low glucose significantly stimulated the rate of oxygen uptake from TO., NLL and obob mouse exocrine tissue, while high glucose stimulated the rate of oxygen uptake from TO. and obob mouse exocrine only. Of the three types of exocrine, that from obese mice demonstrated the greatest percentage stimulation of the rate of oxygen uptake in the presence of both low and high glucose. Results would also suggest that the rates of oxygen uptake by NLL and obob exocrine were less responsive to high glucose than low glucose, since values for the percentage stimulation of the rate of oxygen uptake by NLL and obob exocrine were lower in the presence of high glucose than low glucose (Table 7). Indeed low glucose may be the saturation threshold for NLL and obob exocrine tissue. Only in the case of TO. mouse exocrine did increasing glucose concentration correspondingly increase the rate of oxygen uptake. On the whole, the low glucose percentage stimulation of the rate of oxygen uptake by exocrine tissue was found to be greater than that shown by islet tissue for TO., NLL and obob mice, while investigation of the high glucose stimulation of the rate of oxygen uptake found the reverse to be true.

Although the number of determinations were limited, directly proportional relationships between the weight of tissue and the oxygen uptake in terms of µl/hr were observed for collagenase isolated TO. islets, microdissected TO., NLL and obob islets and the corresponding exocrine tissue, in the absence and presence of glucose.

Discussion.

The oxygen electrode assembly has been shown to be a reliable instrument for the measurement of the rate of oxygen uptake by exocrine and islet tissue of mice. Microdissected TO. mouse islets demonstrated a higher mean absolute rate of oxygen uptake than collagenase isolated TO. islets in the absence and presence of low and high glucose. However, collagenase isolated islets showed a greater percentage stimulation of their rates of oxygen uptake by low and high glucose than did microdissected islets. In consequence collagenase isolated islets were more sensitive to increasing glucose concentrations than microdissected islets. Hellerström and coworkers have shown glucose to be the dominant, but not the only respiratory substrate for islet tissue (196) and results presented here would suggest that collagenase digestion does not interfere with the aerobic metabolism of glucose by islet tissue. The previous findings of reduced insulin secretion and content for collagenase isolated islets (Chapter I, Section I) were valid, since the concept of insulin release as an energy dependent process (197) did not necessarily suggest that the secretory function of islet β-cells would be impaired by moderate increases or reductions in their capacity to take up oxygen. Indeed, the rate of islet oxygen uptake represented the product of the rates of oxygen uptake of all the constituent cells of an islet (i.e. α cells, connective tissue cells; blood cells; capillary endothelium) and not just β-cells. Collagenase might influence insulin secretion by acting detrimentally on the

emiocytotic mechanism. However, the high rates of oxygen uptake shown by obese islets compared to TO. and NLL, might reflect increased islet metabolic activity and increased insulin secretory activity in response to hyperglycaemia.

A high rate of oxygen uptake might be no more an indication of tissue viability than a low rate and one can only conjecture as to whether islets were respiring normally under the conditions of oxygen supersaturation imposed upon them, during the course of these experiments.

Results reported for exocrine tissue were likely to be greatly influenced by the digestive state of the animal. The active resynthesis of digestive enzymes in the exocrine pancreas of an animal that had just fed would be consonant with an increased rate of oxygen uptake by that tissue. Results also indicated that a normal proportional relationship existed between the weight of tissue and the rate of oxygen uptake for islets and exocrine, irrespective of the method of islet isolation, in the presence and absence of glucose. There was also evidence of a low glucose (0.6g/litre) saturation of the rate of oxygen uptake by NLL and obob mice exocrine tissue.

Deep within isolated islets, even in the presence of excessive vascularisation, the rate of oxygen uptake from the surrounding medium may have been diffusion controlled i.e. the rate of oxygen uptake by the more centrally situated islet cells being independent of the oxygen concentration in the respiratory chamber. If this was the case, a more accurate assessment might have been made by taking into account isolated islet surface area and the length across which there was diffusion, together with the diffusion coefficient of oxygen in islet tissue. However, values of the rate of oxygen uptake by obese islets, obtained with an oxygen electrode, compared favourably with those obtained by Hellerström (171,174,196) using the Cartesian diver. Literature on the rate of oxygen uptake by normal mouse islet tissue was limited, although

Hellerström, cited in work presented by Hellman (173), has shown islets from obese mouse lean littermates to exhibit a linear rate of oxygen uptake for at least 4h. Hellerström, in an attempt to increase his yield of islet tissue for metabolic studies, recently modified the microdissection technique to include a period of collagenase digestion (196). He found that irrespective of whether the islets had been isolated with or without preliminary collagenase treatment, they were all found capable of maintaining a constant rate of oxygen uptake for at least 2½h. However, in contrast to the work presented here, Hellerström observed a tendency towards higher endogenous respiratory rates with greater variation between individual observations, for collagenase isolated islets rather than microdissected islets. The difference observed might be inherent in the method used for measurement of the rate of oxygen uptake.

The secretion of proteins from a cell was believed to require energy. The intracellular transport of protein and the secretory process itself have been shown to involve complex sequences of intracellular events, each of which may be more or less dependent on the supply of metabolic energy (198). Present knowledge concerning the energy requirements for the various steps in the synthesis and secretion of insulin by the β-cell is fragmentary. Insulin secretion was abolished or inhibited when the β-cell was exposed to anoxia or uncouplers of oxidative metabolism (98). Glucose was regarded as the most important physiological regulator of insulin secretion and the most important substrate for oxidative energy production in the β-cell. The regulation of oxidative metabolism and its significance for the specific function of the β-cell remains poorly understood. Work by Stork and coworkers (175,177) has indicated that the endogenous oxygen uptake by islets could be enhanced by the addition of hypoglycaemic sulphonylureas. Their observations suggested the presence in the normal β-cell of an endogenous substrate supply sufficient to

maintain the oxidative metabolism for several hours (199). This endogenous substrate was thought to be glycogen and the latter has been demonstrated in the islet β-cells of obese mice (200,201). Although the rate of degradation of glycogen has been shown to be of the same order as the exogenous respiratory rate, the amount of available glycogen present in islet tissue would be used in less than 1 hour. Hence a mixed endogenous substrate supply in the β-cell has been suggested (199). However, the increased rate of islet oxygen uptake shown by sulphonylureas in the absence of glucose (175,177) might, at lease in part, be due to stimulation of glycogenolysis, since Hellman and colleagues have demonstrated a marked depletion of the glycogen content of islets exposed to sulphonylureas (200,202). Hellerström & Gunnarsson have shown that a variety of carbohydrate and amino acid insulin secretagogues were capable of serving as substrates in the oxidative metabolism of the β-cell (199). While Lin & Haist using a radiorespirometric apparatus have shown the CO_2 production from labelled glucose and other carbohydrates to correlate well with the amount of newly synthesised insulin and proinsulin produced by isolated rat islets (203). The presence of glucose was found to induce the greatest CO_2 production and produce the largest quantity of proinsulin and insulin. From evidence of this sort it seemed reasonable to assume, that the energy made available to the β-cell during the breakdown of these substrates would be utilised to drive the specific secretory and synthetic processes. This view was supported by the similarity between curves describing insulin release and glucose oxidation (199) in relation to the extracellular glucose concentration (204) and by the observation that certain well known inhibitors of insulin secretion, mannoheptulose, glucosamine and adrenaline (98), were found to lower the substrate utilization by the β-cell (196). The rapidity with which insulin is liberated across the β-cell membrane in response to glucose or sulphonyluneas might suggest the

involvement of highly specific transport systems, the energy requirements of which are so far poorly understood.

SECTION 2

The role of lactate dehydrogenase isoenzymes in the microdissected islets of obese hyperglycaemic mice and their normal lean littermates.

Evidence has been presented to suggest that the oxygen tension and the capacity for aerobic or anaerobic glucose metabolism in a particular tissue was reflected in its content of the different molecular varieties of lactate dehydrogenase (205,206).

LDH (L - lactate : nicotinamide adenine dinucleotide oxidoreductase. E.C. 1. 1. 1. 27) has been shown to consist of several different factions or isoenzymes that may be separated by their electrophoretic mobility under appropriate standardised conditions (207,209). The principal factors involved and the general theory underlying the electrophoretic separation of proteins have been extensively reviewed by Stern (210).

The basis of LDH heterogeneity was explained by the tetramer hypothesis, which suggested that a cell produced two types of monomer, the polypeptide H (expressed by its prevalence in heart muscle) and the polypeptide M (produced by skeletal muscle) (211,212). The synthesis of the two different polypeptide sub-units, H and M was regarded as being under the control of separate genes and the isoenzyme patterns found in different tissues, which depended upon the relative amounts of H and M polypeptides, were controlled by the relative genetic activities (213). Hence two separate genes controlled the synthesis of five distinct isoenzymes.

Monomers were thought to combine by random association and generate tetrameric molecules of two pure tetramers H_4 (LDH_1) and M_4(LDH_5) and of three hybrid molecules H_3M_1 (LDH_2); H_2M_2(LDH_3); H_1M_3(LDH_4) in proportions that depended on the relative amounts of monomers H and M

(Monomer H : M ratio). The proportional (or percentile) distribution of individual isoenzymes could be calculated for any monomer H : M ratio and the corresponding H : M ratio could be calculated from any experimentally obtained LDH isoenzyme pattern. Although the isoenzymes described catalyze the same reaction, LDH isoenzymes have been found to differ from each other in a number of physical, chemical and kinetic properties. These have been reviewed by Wilkinson (214) and Latner & Skillen (215). The difference in kinetic properties of LDH isoenzymes has been correlated with the type of cellular metabolism (216). LDH_1 (the electrophoretically most rapid anodic isoenzyme) represented the aerobic type of tissue, which produced energy mostly by oxidative cycles (TCA.cycle) whereas LDH_5, (the electrophoretically slowest cathodic isoenzyme) was typical of the anaerobic type of tissue, which obtained energy mainly by glycolysis. LDH_5, because of its relative resistance to inhibition by high pyruvate concentrations (212,217) was assumed to be geared for the production of lactate, while LDH_1, which did not allow the rapid production of lactate, favoured the complete oxidation of pyruvate via the TCA. cycle.

The aim of this study was to investigate the LDH isoenzyme patterns of islet tissue from obese hyperglycaemic mice and their normal lean littermates and to establish the prevailing type of islet glucose metabolism. The total LDH activity of a variety of normal and obese mouse tissues have been compared for reference.

Materials & Methods.

Microdissected islets, exocrine tissue; subcutaneous fat; adrenal and thyroid glands were taken from 20 - 25 week old normal and obese mice. All tissues were weighed wet, but free of adherent buffer or blood and homogenised in 0.05 mol./litre barbital buffer, pH. 8.4 (17g. barbitone Na, 23.5 ml. 1 mol./litre HCl, double glass distilled water to 1 litre, ionic strength 0.1, diluted to ionic strength 0.05 with distilled water)

using a tissue : volume ratio of 1 : 9. All homogenates were subsequently centrifuged for 30 minutes at 12,500 rev/min., 4°C (M.S.E. Mistral 4L) and the clear supernatants produced, immediately used for estimation of either protein content and total LDH or LDH isoenzyme distribution.

Estimation of supernatant protein concentration.

The colourimetric estimation of protein was effected by the micromethod of Lowry and coworkers (218) using folin - ciocalteu phenol reagent (BDH, Poole, Dorset). The following reagents were made up,

(A) 2% Na_2CO_3 and 0.02% Na.K Tartrate in $N/_{10}$ NaOH.

(B) 0.5% $Cu\,SC_4 . 5\,H_2O$.

(C) 5 ml. of (A) added to 0.1 ml. of (B) made up fresh each day.

(D) Folin Ciocalteu phenol reagent.

Preparation of protein standards.

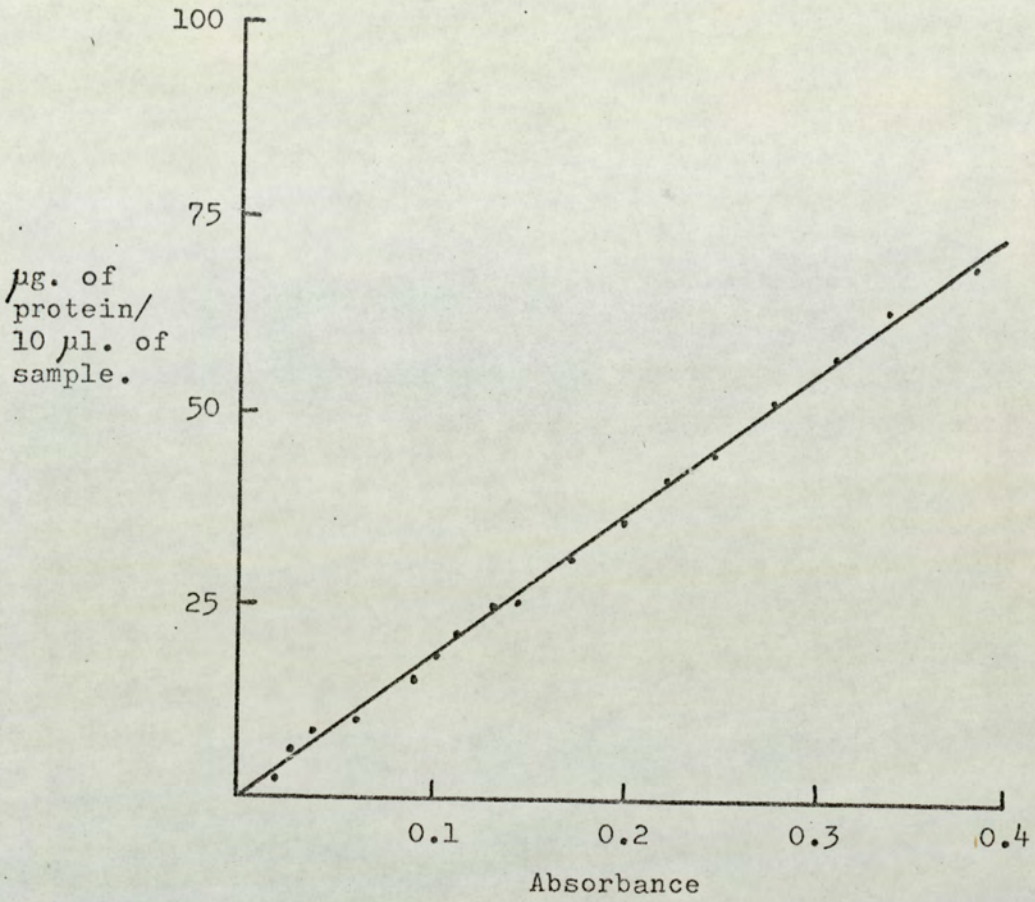
A range of fresh protein standards (0 - 60 µg/10µl) in barbital buffer were prepared from crystalline bovine albumin $\overline{V}$ (Armour Pharmaceuticals, Eastbourne, Kent). 10 µl. samples of the protein standard solutions were used to construct a standard curve, figure 9. The use of fresh protein standard solutions reduced the risk of surface denaturation and facilitated a representative standard curve.

The measurement of protein concentration.

To 10 µl. of homogenate supernatant, protein standard solution or barbital buffer (for reagent blank) in a plastic immunoassay tube was added 100 µl. of a mixture prepared from 1250 µl. of (A) and 25 µl. of (B). After 10 minutes, 10 µl. of (D) was added and the contents mixed immediately. A blue colouration developed on standing and after 30 minutes, the contents were transferred with a 50 µl. micropipette to quartz microcuvettes (Unicam, 1 cm. light path) and the absorbance read on a Beckman D B spectrophotometer at 750 nm. Barbital buffer was used to standardise the instrument and a 10 µl. barbital buffer reagent blank

FIG. 9. Standard curve for Lowry protein determination.

(Bovine Albumin $\overline{V}$ equivalents, in barbital buffer, pH. 8.4)



was processed with the unknown protein solutions. The absorbance obtained from protein samples was corrected for the absorbance shown by blank tubes containing barbital buffer only. The protein content of the sample was obtained from the standard curve in terms of µg. of bovine albumin $\overline{V}$ equivalents/10 µl. of sample. Barbital buffer did not affect the colour development in the microdetermination of protein content (219).

Determination of Total Lactate dehydrogenase activity.

Total LDH activity was estimated in the barbital buffer supernatant of tissue homogenates, using an LDH enzyme kit (Boehringer, U/V method, TC - G - 1, Cat. No. 15977 TLAB). The enzyme reaction was represented by,

$$\underset{\text{pyruvate.}}{CH_3 . CO . COO^-} + NADH + H^+ \xrightleftharpoons{\text{LDH 1-5}} \underset{\text{lactate.}}{CH_3 . CHOH . COO^-} + NAD^+$$

Because of the limited volume of islet homogenate supernatants, 25 µl. samples were used for the determination of total LDH activity.

The rate of oxidation of $NADH_2$ at 25°C was followed by measuring the fall in absorbance, at 1 minute intervals on a SP.800 U/V spectrophotometer at 340 nm (Slit width setting 0.02). The time interval was preset with a programming accessory. Absorbance measurements were recorded for at least 4 minutes and the mean absorbance change/minute evaluated, ΔE/min. The total LDH activity was calculated from,

$$\Delta \text{E/min.}_{340\text{ nm}} \times 5053 \times 4 \text{ (25µl. Sample)} = \text{mU/ml.}$$

(mU (serum equivalent)/ml. of supernatant).

The unit was defined as the amount of enzyme which converted 1 µ mole of substrate/minute under highly optimum conditions. Measurements related to 1 ml. of body fluid.

$$1 \text{ U/ml.} = 1 \mu \frac{\text{mole of substrate converted}}{1 \text{ min.} \times 1 \text{ ml.}}$$

Example calculation for pooled normal islet tissue.

Wet weight of islet tissue = 7.8 mg.

1 : 9 homogenisation yielded 70.2 µl. of homogenate.

25 µl. of supernatant taken for total LDH estimation.

10 µl. of supernatant taken for protein content.

Protein determination (750 nm).

	Absorbance
Barbital buffer blank	0.015
10 µl. of protein soln.	0.208

Absorbance corrected for blank = 0.193

31.7 µg. protein/10 µl. or 3.17 mg/ml. protein from standard curve fig. 9.

Total LDH determination (340 nm).

Δ E/min = 0.0119.

X5053 x 4 = 970.176 mU/ml.

317 mg. of protein ≡ 970.176 mU LDH activity.

1 mg. ≡ 306.05 mU.

Total LDH activity = 306.05 mU LDH/mg. islet protein or 306.05 U/gm. at 25°C pH. 8.4.

Histochemical control.

8 µm unfixed cryostat sections of fresh pancreas were stained for total LDH activity by the method of Nachlas, Walker & Seligman (220,225). The incubation medium was applied as a paraffin wax sided reservoir over the section for 30 - 40 minutes at 37°C in air. Sections were finally fixed in 10% formol saline and mounted in glycerine jelly.

The separation and quantitisation of islet LDH isoenzymes.

The LDH isoenzymes of islet tissue taken from obese hyperglycaemic mice and their normal lean littermates were separated by small scale zone electrophoresis in Difco special agar - noble gel/barbital buffer, pH. 8.4 (0.05 ionic strength) cooled by petroleum spirit, according to Prochazka and colleagues' modification (221) of Wieme's original method (222). The use of agar-gel electrophoresis had several advantages over paper as a support medium for zone electrophoresis. Because it did not absorb proteins, it could be prepared in a homogenous state and its transparency allowed the direct photometric determination of the separated protein fractions. Agar-gel electrophoresis had a high resolving power and during isoenzyme separation passive diffusion was less than with paper or cellulose acetate.

Preparation of the electrophoresis tank and agar coated slides.

Agar gel blocks were prepared from 1% agar-noble in barbital buffer pH. 8.4 (0.05 ionic strength), and 200 ml. of barbital buffer was poured into each end of the electrophoresis tank covering the diffusion holes and platinum electrodes (plate 6).

3 ml. of 1% agar noble in barbital buffer was pipetted onto 3" x 1" microscope slides. The former was allowed to set and the prepared slides retained on moist filter paper until required.

Homogenisation of islet tissue.

All tissues were homogenised in barbital buffer, pH. 8.4 using a tissue : volume ratio of 1 : 19. Homogenates were centrifuged at

12,500 rev/min. for 45 minutes at 0°C in a refrigerated centrifuge (M.S.E. Superspeed 50) and the resulting supernatants used for the estimation of isoenzyme distribution.

Addition of supernatant to agar layer on slide.

Small vertical slots were made in the prepared agar slides with 0.5 cm. square pieces of stiff filter paper. 3 - 4 µl. of homogenate were carefully inserted into the slots with a drawn out micropippette and the slots sealed over with a drop of almost set, 1% agar noble in barbital buffer pH. 8.4. 6 slides so prepared, were placed agar side downmost across the agar blocks in the electrophoresis tank, plate 6. Slides were labelled at the cathodic end to facilitate identification after electrophoresis. Protein fractions were separated for 50 minutes at 130 volts, all slides were switcned on simultaneously and cooled with petroleum spirit during electrophoresis. After the latter, slides were retained for development and the electrodes reversed, so that the agar blocks could be re-used.

Preparation of the agar substrate sandwich and the development and evaluation of the LDH isoenzyme pattern.

An agar gel substrate layer was used instead of a liquid substrate and consisted of equal volumes (1 ml.) of the substrate medium and of 2% agar in 0.05 M phosphate buffer, pH. 7.2, allowed to set on a 3" x 1" microscope slide.

The method for the colourimetric estimation of the individual isoenzymes was based on the reduction of NAD., coupled with the reduction of INT. with phenazine methosulphate as an intermediate electron carrier. The reduced tetrazolium salt provided an insoluble purple formazan band at the site of enzyme activity. The composition of the substrate medium was as follows. All reagents were analytical grade.

Solution (A) p-iodonitrotetrazolium violet (INT), 10 mg; dimethylforamide 0.5 ml; DGD. H_2O 4.5 ml. (5ml.)
A yellow, light sensitive solution retained in the dark until required.

(B) 0.2 M phosphate buffer pH. 7.2.

(C) NaCN, 20 mg; $MgSO_4 \cdot 7H_2O$, 40 mg; in 3 ml. DGD.H_2O pH. adjusted to 7.2 (3ml.).

(D) 16 g. 70% Na lactate (Calibochem), DGD.H_2O to 100 ml, pH. adjusted to 7.2 (100 ml.)

(E) 70% Nicotinamide adenine dinucleotide (NAD) 20 mg. in 1 ml. DGD H_2O (1 ml.).

(F) Phenazine methosulphate (Sigma) 0.8 mg. dissolved in 1 ml. DGD. H_2O. (1 ml.)

Solutions (A), (E) and (F) were freshly prepared. A mixture was made of 2.5 ml. of (A); 2.5 ml. of (B); 1.5 ml. of (C); 2.0 ml. of (D) and 1 ml. of (E). 0.5 ml. of (F) was added to bring the volume to 10 ml. The substrate agar layer was positioned on top of the supernatant agar layer like a sandwich and the latter was incubated on a moist filter paper in a closed petri dish for 2 h. at 37°C. After development purple zones appeared at sites of LDH activity and fixation was carried out in 5% acetic acid for 3 h. Fixed agar layers containing the isoenzyme zones were dried at room temperature under filter paper strips. Typical isoenzyme patterns for muscle and islet tissue are shown in plate 7.

Evaluation of the isoenzyme pattern was made using a Kipp & Zonen densitometer with automatic integration. A recorder gave the isoenzyme pattern in the form of density curves fig. 10, while the areas under the peaks were estimated with the integrator. The activity of the individual isoenzymes was expressed as the percentage of the total LDH activity recorded on the integrator. This percentage was used to indicate the relative amounts of each LDH isoenzyme in islet tissue and to calculate the ratio of monomer H to monomer M. The total isoenzyme counts obtained from the integrator could not be equated with Boehringer kit measurements of total LDH, because the former was a colourimetric estimation and the latter a U/V estimation. The U/V method

PLATE 6. Electrophoresis tank for the separation of LDH isoenzymes.

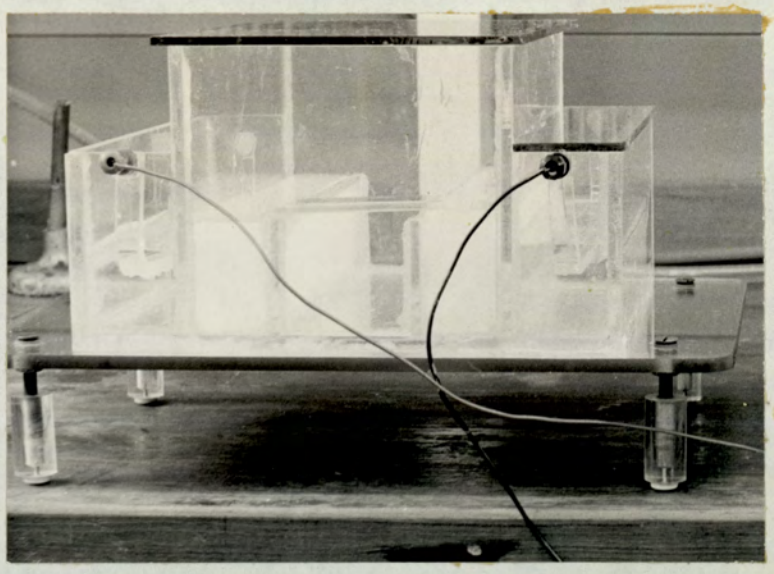
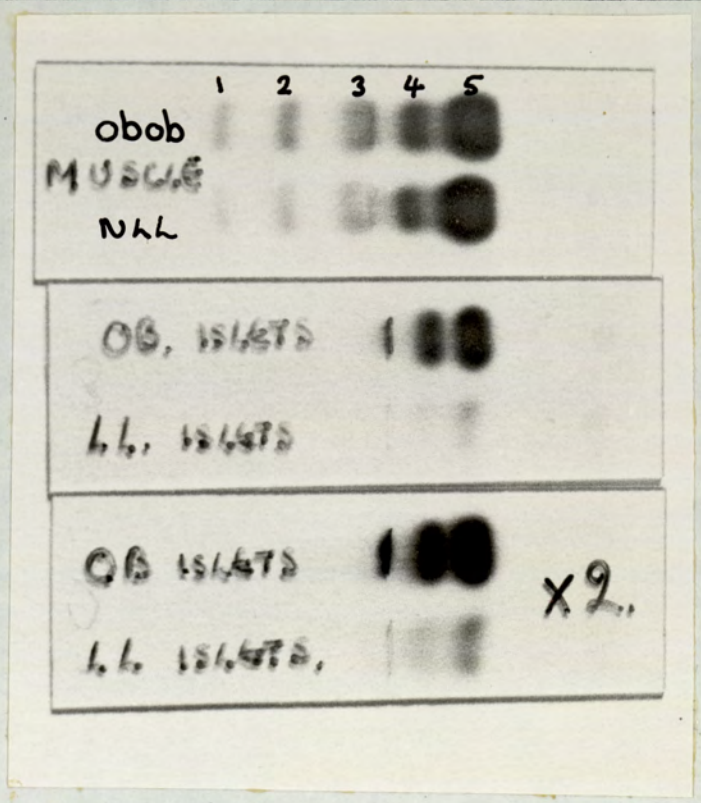


PLATE 7. Typical isoenzyme patterns for muscle and islet tissue of obese hyperglycaemic mice and their normal lean littermates.

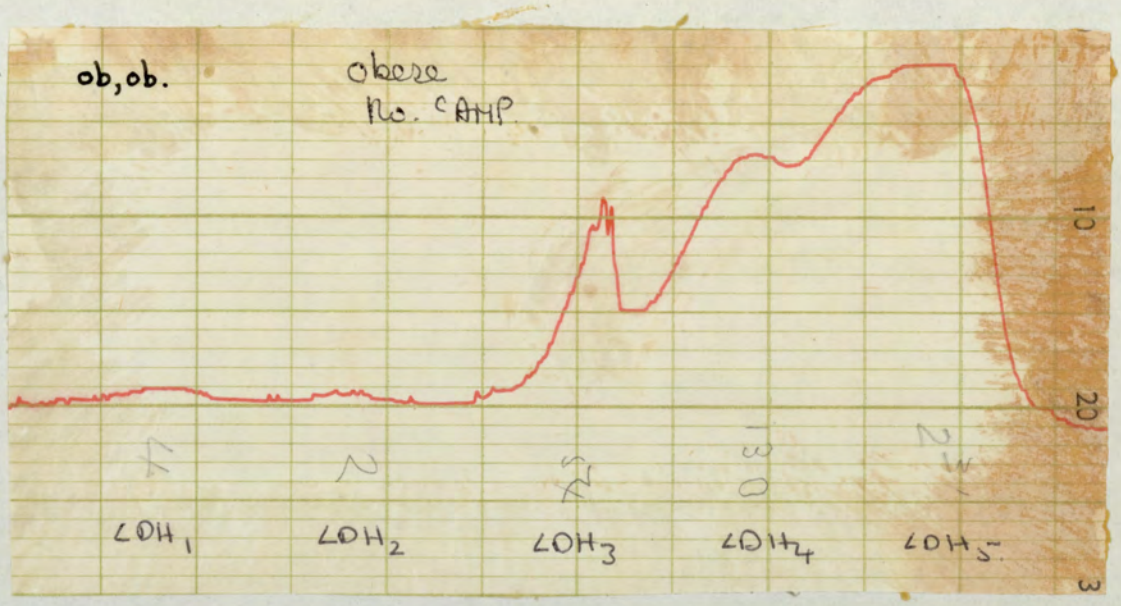


has been shown to have about 5x the sensitivity of the tetrazolium salt method (223).

Results.

Both islets and exocrine tissue exhibited a fine granular, histochemical staining for LDH in the cytoplasm. The nuclei appeared

Fig. 10. Density profile for electrophoretically separated islet LDH isoenzymes.



as unstained spots. Islet tissue displayed a somewhat weaker LDH activity than did exocrine tissue and appeared as pale round areas in a darker blue background under low power, plate 8. Control sections, processed in the absence of substrate (sodium lactate) remained relatively unstained. Norikoff & Masek (224) have shown that in fresh frozen sections the product of incubation with nitro. B.T. (220) was not a mitochondrial formazan deposit but a crystalline artifact lying at another level outside the mitochondria. Gelatin has been shown to reduce the production of crystalline artifacts by nitro B.T. (226,227).

The total LDH activity of various tissues from obese hyperglycaemic mice and their normal lean littermates have been presented in table 8. Coefficients of variation for groups of data were within 20%. In obese mice the total LDH activity of islet; exocrine; fat and thyroid tissue was significantly higher than the corresponding values for lean littermate tissues. No significant difference was observed between the total LDH activities of normal and obese mouse liver. But the adrenal tissue of normal mice demonstrated a significantly higher total LDH

TABLE 8. The total lactate dehydrogenase activity in tissues from 20 - 25 week old obese hyperglycaemic mice and their normal lean littermates. (Mean values ± S.E., n = no. of determinations).

Total LDH activity in U/g. tissue protein.

	N†	Islets	N	Exocrine	Liver	Fat	Adrenal	Thyroid
NLL mice	5	305 ± 6	3	1232 ± 10	4577 ± 176	3696 ± 54	2326 ± 269	2987 ± 219
obob mice	5	458 ± 41*	3	1920 ± 158*	5972 ± 1813	5478 ± 372*	1106 ± 125*	5534 ± 307*

* Denotes significant difference ($P < 0.05$) between values for NLL. and obob. mice.

† The islets of several mice were pooled for each determination.

TABLE 9. The lactate dehydrogenase isoenzyme pattern of islet tissue from obese hyperglycaemic mice and their normal lean littermates, expressed as percentages of the total activity recorded by densitometer integration. (Mean values ± S.E.).

n = no. of determinations, islets pooled from several mice for each determination.

	N	% distribution of each isoenzyme					
		LDH_1	LDH_2	LDH_3	LDH_4	LDH_5	H : M Ratio
NLL islets	6	-	1.88 ± 0.8	3.43 ± 0.5	30.30 ± 0.4	65.0 ± 0.8	10 : 90
obob islets	6	-	2.60 ± 0.5	4.13 ± 0.8	14.58 ± 1.4*	77.95 ± 2.2*	8 : 92

* Denotes significant difference ($P < 0.05$) between NLL. and obob islet values.

PLATE 8. Total LDH activity in 20 week old obese mouse islet tissue.

(x 40)

activity than obese adrenal tissue. Consonant with the histochemical findings was the observation of a significantly higher total LDH activity in exocrine tissue than in islets, for both normal and obese mice. The percentage distribution of individual isoenzymes of islet tissue, expressed as percentages of the total activity measured by densitometer integration has been summarised in table 9. These percentages indicated the relative amounts of each LDH isoenzyme in normal and obese islet tissue. Significantly lower percentages of LDH_4 and higher percentages of LDH_5 were found for obese islets compared with normal. Obese islets showed the greater predominance of LDH_5.

Evaluation of the H : M ratio of normal and obese islet LDH isoenzymes was carried out according to the tetramer hypothesis as follows.

	M_4	Tetramers	H_4	
	↓		↓	
Cathodic				Anodic
M_4	M_3H	M_2H_2	MH_3	H_4
(LDH_5)	(LDH_4)	(LDH_3)	(LDH_2)	(LDH_1)

Anaerobic metabolism. Aerobic metabolism.

$$M - LDH\ \% = (M_4 + 0.75\ HM_3 + 0.5\ H_2M_2 + 0.25\ H_2M)$$

$$L - LDH\ \% = 100 - (M - LDH\%)$$

The amount of M - LD was calculated by multiplying the percentage of

each isoenzyme by the proportion of M - LDH present in it.

The proportion of M monomer was much greater than the proportion of H monomer in the islets of both normal and obese mice. In addition the proportion of M - LDH monomer was marginally greater in obese islets than in normal and would suggest a greater LDH_5 activity in obese islet tissue.

Discussion.

Histochemical and microchemical analyses have demonstrated higher total LDH activity in exocrine than in islet tissue of both obese hyperglycaemic mice and their normal lean littermates. Results were consonant with the work by Procházka and colleagues (228) and Hellman & Täljedal (226) on obese mice. The higher total LDH activity of obese mouse islet, exocrine, fat and thyroid tissues and the lower total LDH activity of obese mouse adrenal tissue, compared to the corresponding values for normal lean littermate tissues, might be characteristic of the obese syndrome per se. (228). The higher obese thyroid total LDH activity was consonant with the increased metabolic load of these animals. Of the six tissues investigated islets of both NLL. and obob mice showed the lowest total LDH activity and the reason for this was not merely one of substantial vascularisation.

One major source of error associated with the measurement of total LDH activity manifested itself when the amounts of LDH_1 and LDH_5 differed greatly in a single tissue, since under these conditions the use of a single substrate concentration did not accommodate the different substrate optima of the individual isoenzymes. However, the conclusions derived from measurements of islet tissue total LDH were valid because the LDH_1 and LDH_5 isoenzymes were not present in vastly differing amounts.

The LDH isoenzyme pattern of normal and obese islet tissue, Table 9 has suggested the presence of substantial anaerobic metabolism (energy gained mainly by glycolysis). Obese islets demonstrated a higher

proportion of LDH_5 activity than normal islets despite showing a greater rate of oxygen consumption in vitro, (Chapter II, Section I). The increased tendency for anaerobic metabolism shown by normal and obese islets might have been the result of their isolation. The conditions of their in vitro storage, short as it was, may have been inconsistent with the maintenance of normal in vivo aerobic metabolism.

Pfleiderer & Wachsmuth (205) were the first to point out that LDH_5 was more predominant in anaerobically metabolising tissues such as human liver, whereas LDH_1 was more predominant in aerobically metabolising tissues such as brain and heart. LDH_1 and LDH_5 have been shown to play different roles in the relation between the T.C.A. cycle (tricarboxylic cycle) and glycolysis. LDH_1 (due to its kinetic properties) regulated glycolysis at a rate sufficient to maintain the T.C.A. cycle by pyruvate supply (212). Increaseddemands on energy supply could not be provided by glycolysis, because LDH_1 acted as a limiting factor of glycolysis. Increased glycolysis resulted in LDH_1 inhibition (due to an increased concentration of pyruvate), and less NAD created by the reaction pyruvate to lactate was available, thus the rate of glycolysis was diminished. When the increased demands on energy supply were provided in the presence of LDH_1 by the intensification of the TCA cycle, it was apparent that glucose could not be the main fuel and alternative fuels (fatty acids) were used. LDH_5 was not inhibited by high concentrations of pyruvate and increased demands on energy supply in the presence of LDH_5 could not be provided by intensification of the TCA. cycle. But LDH_5 has been shown to be activated by TCA. cycle intermediates (229). Increased concentration of these metabolites accelerated the reaction pyruvate to lactate and less pyruvate was then available to maintain the TCA. cycle.

Markert suggested that the fundamental role of lactate dehydrogenase was the regulation of the NAD/NADH ratio (230). A similar role has been proposed for the cytoplasmic and mitochondrial forms of α-glycerophosphat

dehydrogenase. It was suggested that these isoenzymes might aid the build up of mitochondrial NADH if dihydroxyacetone phosphate was reduced in the cytoplasm and α-glycerophosphate was oxidised in the mitochondria (231). In glycolysis lactate dehydrogenase and Lα glycerophosphate dehydrogenase have been shown to compete for NADH produced as a result of the conversation of glyceraldehyde-3-PO_4 to 1, 3 diphospho-glyceric acid in the presence of D - glyceraldehyde - 3 - PO_4 dehydrogenase. The reaction in this direction depended upon the removal of the product, 1, 3 diphosphoglyceric acid, by the activity of phospho-glycerate kinase, which converted the high phosphate transfer potential of 1, 3 diphosphoglycerate to a more negotiable form as ATP. Hellman has proposed that the sequence phosphoglyceradehyde dehydrogenase/ phospho-glycerate-kinase might represent a control site for insulin secretion by acting as a rate limiting step for glucose degradation in the β-cells (129). The demonstration of a strong product inhibition by 1, 3 phosphoglycerate would explain why phosphoglyceraldehyde dehydrogenase was strongly dependant upon the subsequent kinase reaction, even when phosphoglyceraldehyde dehydrogenase was present in high concentrations. On the basis of the kinetic characteristics of the two enzyme sequence, Hellman suggested a regulation of insulin secretion in terms of the 'phosphate potential' of the β-cells (129). Any conversion of β-cell ATP to ADP, such as that observed in the presence of sulphonylureas (130,131), would lead to increased amounts of ADP and inorganic phosphate being available for the stimulation of the phosphoglyceraldehyde dehydrogenase, phosphoglycerate kinase step. Under certain conditions LDH and Lα glycerophosphate dehydrogenase might modify the activity of glyceraldehyde - 3 - PO_4 dehydrogenase and glucose metabolism by limiting the supply of NAD. On the basis of Hellman's hypothesis (129), LDH activity might influence the proposed control site for glucose stimulated insulin secretion. Also, any modification of the further metabolism of glyceraldehyde-3-PO_4 by LDH might influence

the rate of glucose metabolism via the pentose phosphate pathway. Histochemical investigations (232,233) and quantitative microchemical enzyme assays (94) on pancreatic tissue from various mammalian species, as well as studies on isolated rat islets (101,133), have suggested that the metabolism of glucose, to some extent, proceeded via the pentose phosphate pathway.

Watkins and coworkers have shown NADH and NADPH to stimulate insulin secretion from toadfish islet tissue *in vitro* (234,235) and their work has suggested that NADPH and NADH do not enter islet cells, but stimulate insulin secretion from toadfish islet at lease, by acting at the cell membrane.

Goldstein & Davis have suggested from their work on the 3-D architecture of islets, that the islet *per se* may be conceived of as a network of anastomosing capillaries with closely apposed islet cells filling the spaces between them (236). In consequence it was surprising to find an islet LDH isoenzyme pattern characteristic of a low oxygen tension and anaerobic metabolism. But this observation did not necessarily mean that oxygen was not required for the normal secretory activity of β-cells. Indeed, Coore & Randle demonstrated that ischaemia prevented the glucose stimulated insulin secretion from pieces of rabbit pancreas (98). Work on the perfused rat pancreas suggested that the vascular supply to islets probably existed in parallel to the rest of the pancreas and that insulin secretion may be modified by the shunting of blood away from the islets (237). When extra insulin was required the 'parallel circulation', offered the means for rapidly increasing the blood supply to the islets independently of any increase in the general blood flow to the whole pancreas. This hypothesis was consonant with the isoenzyme pattern observed for islet tissue and would facilitate transitions from anaerobic to aerobic metabolism and *vice versa* in islet tissue under appropriate conditions.

CHAPTER 3.

Evaluation of the role of the adenyl cyclase system in islet cell metabolism and the process of insulin secretion.

SECTION I (A).

The effect of ^{c}AMP and db^{c}AMP on the basal secretion and the glucose stimulated secretion of insulin from isolated islets of Langerhans of obese hyperglycaemic mice and their normal lean littermates.

In recent years an adenyl cyclase system has been suggested to be involved either in the mechanism of insulin secretion or in the metabolism of glucose within the β-cell (41). Cyclic AMP (Adenosine 3', 5'-cyclic monophosphoric acid, ^{c}AMP) the product of adenyl cyclase activity, was first synthesised by Cook, Lipkin & Markham (238) and later observed in tissue particles by Sutherland and colleagues (239,240). Cyclic AMP has recently been the subject of a very comprehensive review (244). The cyclic nucleotide has been shown to be a key regulatory factor in a number of metabolic processes (241,242) and its presence has been established in a variety of mammalian tissues (243). Sutherland, Rall & Menon appointed the name adenyl cyclase to a particulate membrane fraction that produced ^{c}AMP from ATP, pyrophosphate being the other product (245). The reaction was essentially not reversible (246). Membrane bound adenyl cyclase was suggested as the site of hormone interaction with the effector cell (247). The activation of this cyclase by a hormone or effector agent (1st messenger) resulted in the production of ^{c}AMP, the intracellular second messenger. This was the basis of Sutherland's second messenger hypothesis (241,247) in which adenyl cyclase and ^{c}AMP were viewed as a means for transmitting extracellular information provided by the hormone to the interior of the cell via changes in ^{c}AMP levels. As an intracellular second messenger ^{c}AMP not only mediated the actions of a number of hormones, but was also implicated in the secretion of certain peptide

hormones (248). ^{c}AMP in the cell was quickly broken down to 5' AMP by a specific ^{c}AMP-phosphodiesterase (PDE). The methylxanthines were found to be potent inhibitors of this phosphodiesterase and there was much evidence to suggest that the pharmacological effects of methylxanthines were produced through this inhibition (240,249). The intracellular concentration of ^{c}AMP at any given time, depended for the most part upon the relative activities of adenyl cyclase and ^{c}AMP phosphodiesterase (^{c}AMP-PDE).

Our knowledge of the enzymic steps in the production of most physiological responses remains limited. It was necessary, therefore, to resort to certain criteria in order to determine whether ^{c}AMP was or was not involved in the particular physiological response (247). The criteria involved looking for positive, qualitative and quantitative and temporal correlations between the effects of the hormone or effector agents on adenyl cyclase activity/^{c}AMP levels and the physiological response. However, correlations of this sort would not prove a role for adenyl cyclase and ^{c}AMP in the physiological response, because of the possibility of coincidence rather than cause and effect. In consequence parallel studies were made in an attempt to investigate the effect of exogenous ^{c}AMP or its dibutyryl derivative on intracellular events. Because ^{c}AMP itself did not enter cells very well, comprehensive use was made of the more membrane soluble dibutyryl derivative ($N^{6},O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid, db-^{c}AMP) (250,251). Although this derivative was initially designed to enter cells more easily by reducing the polar nature of ^{c}AMP, this has not been proved, and it probably owed its greater activity to the fact that it was not hydrolysed to any great extent by ^{c}AMP-PDE.(252).

A considerable body of evidence has accumulated in recent years to suggest that ^{c}AMP might be involved in the regulation of insulin secretion from the pancreas and may even be essential for this process to occur (253-255). Initial work interpreted as evidence against such a role was

the finding that whereas glucagon stimulated insulin release (256), adrenaline inhibited it (98) and both glucagon and adrenaline were known to be stimulators of adenyl cyclase activity (257). However, catecholamines were capable of interacting with at least two different types of receptors and Robison and colleagues had shown that those effects of catecholamines which were either known or thought to be mediated by ^{c}AMP all involved the participation of adrenergic β-receptors (242). Porte showed that the inhibiting effect of adrenaline on insulin secretion was mediated via α- adrenergic receptors and β-adrenergic stimulation increased insulin secretion from the pancreatic islet (258-260). In consequence various agents (β-adrenergic stimulants, theophylline, caffeine, glucagon and corticotrophin), the majority of which were thought to act through the adenyl cyclase systems of liver, adipose (261) and adrenal cortex (269) respectively were also found to stimulate insulin secretion in vivo (258,262-265) and in vitro (41,266-268,270). Evidence for the direct stimulation of insulin secretion by ^{c}AMP or its dibutyryl derivative has come from in vitro studies by Malaisse and coworkers (41) and Ashmore (271) using pieces of rat pancreas and Sussman & Vaughan (42,272) using the perfused rat pancreas. Similar results have been obtained in vivo by Bressler, Cordon & Brendel (273,274) using normal white mice and by Hertelendy using sheep (275).

Work by Turtle & Kipnis has demonstrated the presence of ^{c}AMP in the pancreatic islets of rats (276). Theophylline, which enhanced insulin secretion in vivo (265) and in vitro (41), also increased the ^{c}AMP level in pancreatic islets. Glucagon in the presence of theophylline, further enhanced the level of ^{c}AMP. Adrenaline in the presence of theophylline, prevented the accumulation of ^{c}AMP, but when phentolamine was added accumulation of the nucleotide occurred normally. β-adrenergic blockade with propranolol did not antagonise the action of adrenaline and this fact suggested that the production of ^{c}AMP was decreased to a very low level when an α-adrenergic stimulation was applied (276). These observations, taken in conjunction with the observed parallel effects

upon insulin secretion *in vivo* and *in vitro*, suggested that the β-cell level of ^{c}AMP might modify the rate of insulin secretion.

The general observation with adult pancreas was that ^{c}AMP and the effectors which brought about an increase in its endogenous accumulation did not stimulate insulin secretion unless a certain minimum concentration of glucose (30 mg% (253); or ≏ 100 mg% (41)) was present. It was suggested on the other hand, that glucose did not under these conditions need the addition of ^{c}AMP in order to stimulate insulin secretion (253). Although it would be difficult to investigate the effect of glucose on insulin secretion in the total absence of ^{c}AMP, because the latter has been shown to be present in islet tissue (276). It seemed possible that ^{c}AMP might act either by increasing the rate at which glucose was converted into an active metabolite (or transported into a special compartment) or else by promoting the action of the active metabolite, without actually being required for the process to occur. Equally so, ^{c}AMP might influence the emiocytotic secretion process (278) or be associated with β-granule movement within β-cells (277).

On the basis of evidence presented above and the established suitability and viability of microdissected islet tissue, experiments were directed towards confirmation of the effect of ^{c}AMP and db-^{c}AMP on the rate of *in vitro* glucose induced insulin secretion.

Materials & Methods.

Islets were obtained by microdissection from the excised pancreata of 20-25 week old obese hyperglycaemic mice and their normal lean littermates. Insulin secretion was measured by the double antibody technique of Hales & Randle (121) as modified by Quabbe (123) and the effects of 3.6 g./litre glucose, 5mmol./litre ^{c}AMP and db-^{c}AMP were investigated. After preincubation, islets were incubated for 30 minutes in 1ml. of oxygenated buffer (Gey & Gey (110)) supplemented with or without the appropriate concentrations of test materials at 37°C.

After 30 minutes this buffer was replaced with a fresh 1 ml. of supplemented buffer at 37°C and incubated for a further 30 minutes. The two 1 ml. incubation volumes were combined and the insulin concentration assayed. A dilution factor of x20 was used (Buffer B_1) and 0.1 ml. aliquots of the diluted sample were taken in triplicate. The double incubation was carried out to reduce the possibility of a feedback inhibition of insulin secretion by previously secreted insulin (135,139). Groups of 5 islets were weighed as described previously (Chapter 1, Section 1). Use of the very largest obese islets was avoided and no measurement was made of the initial or final insulin content. The incubation programme is shown over page, (page 110).

The effects of ^{c}AMP and db-^{c}AMP on the rate of basal insulin secretion and glucose induced insulin stimulation from the islets of obese hyperglycaemic mice and their normal lean littermates were investigated and the results summarised in Table 10.

Results:

Although a significant difference was observed between the rates of basal insulin secretion from NLL. and obob. mouse islets when expressed in terms of islet number (I_4), no significant difference was observed when the rate was expressed in terms of μU/mg. f.d.islets/hr. (I_5). Also no significant difference was observed between the rates of basal insulin secretion (μU/mg./h) shown for both NLL. and obob islets in Tables 5 (I_6) and 10 (I_5). 3.6 g./litre glucose significantly stimulated ($P < 0.05$) the rate of insulin secretion from both NLL. and obob islets, but to a much greater extent in the latter.

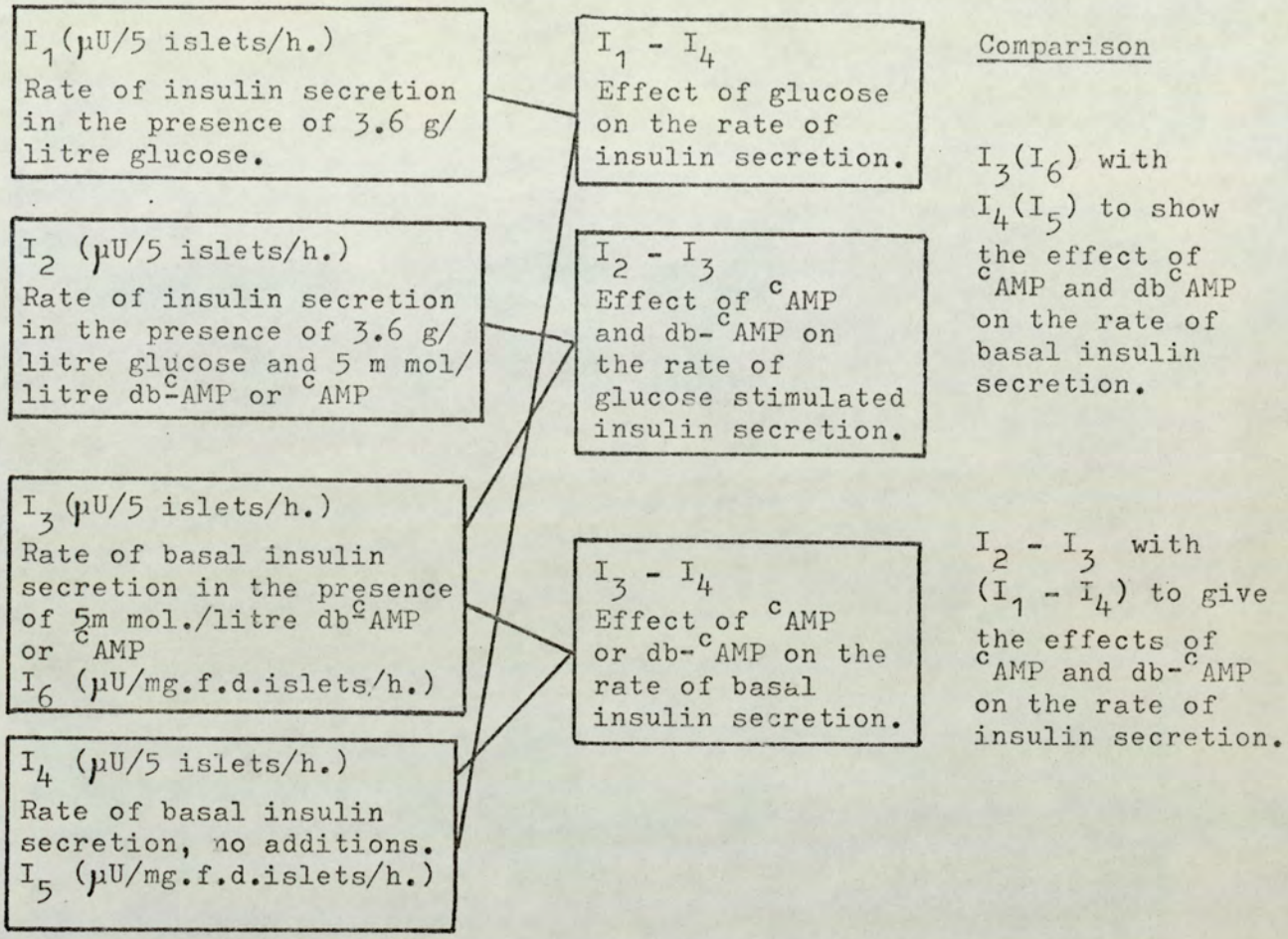
Comparison of I_5 and I_6 indicated that neither ^{c}AMP nor db-^{c}AMP had any significant effect on the rate of basal insulin secretion from

TABLE 10. The effect of ^{c}AMP and db-^{c}AMP on the in vitro basal insulin secretion and glucose stimulated insulin secretion from isolated islets of Langerhans of 20-25 week old obese hyperglycaemic mice and their normal lean littermates. (Mean values ± S.E., glucose 3.6g/litre, n = number of mice).

Mouse Type	N	I_1 Insulin secretion in the presence of glucose µU/5islets/h.	I_2 Insulin secretion in presence of glucose and cyclic nucleotides µU/5islets/h.		I_3 Basal secretion in presence of cyclic nucleotides µU/5islets/h.		I_4 Basal Secretion µU/5islets/h,	I_5 Basal Secretion µU/mg/h.	I_6 Basal Secretion in presence of cyclic nucleotides µU/mg/h.		$I_1 - I_4$ glucose stimulated insulin secretion µU/mg/h.	$I_2 - I_3$ Effect of db-^{c}AMP and ^{c}AMP on glucose stimulated insulin secretion µU/mg/h.	
			db-^{c}AMP	^{c}AMP	db-^{c}AMP	^{c}AMP			db-^{c}AMP	^{c}AMP		db-^{c}AMP	^{c}AMP
NLL.	5	491 ± 18	566 ± 28	493 ± 6	83 ± 7	85 ± 5	88 ± 9	2060 ± 281	1899 ± 167	1946 ± 120	9228 ± 338	11155 ± 819	9433 ± 664
obob.	5	1667 ± 27 *	1812 ± 44 *	1664 ± 49 *	201 ± 15 *	189 ± 16 *	157 ± 13 *	2449 ± 251	3084 ± 129 *	2991 ± 242 *	23430 ± 1137 *	25061 ± 1476 *	22924 ± 1305 *

* Denotes obob. value significantly greater ($P < 0.05$) than corresponding NLL. value.

Incubation Programme.



normal or obese mouse islets. Similarly, comparison of $(I_1 - I_4)$ and $(I_2 - I_3)$ indicated that neither ^{c}AMP nor db-^{c}AMP had any significant effect on the rate of glucose stimulated insulin secretion from normal and obese mouse islets. However, in the presence of both cyclic nucleotides, the rates of basal insulin secretion and glucose stimulated insulin secretion from obese islets were significantly higher than the corresponding rates from normal islets.

Discussion.

The fact that neither 5mmol/litre ^{c}AMP nor 5mmol/litre db-^{c}AMP had any significant effect on either the rate of basal insulin secretion or the rate of glucose stimulated insulin secretion from normal and obese mouse islet tissue contrasted sharply with a great deal of the current literature. Yet Lacy, Young & Fink (108) and Vance, Buchanan & Williams (279) were also unable to demonstrate a stimulation of insulin secretion from isolated rat islets using ^{c}AMP. That this lack of

stimulation was due to the absence of theophylline (a ^{c}AMP-PDE inhibitor) seemed unlikely, since Malaisse and colleagues were able to show a stimulation with db-^{c}AMP in the absence of theophylline at very much lower (44) and higher (41) concentrations of db-^{c}AMP, than were used in the present experiment. However, one would only expect ^{c}AMP to be affected by this lack of theophylline, since db-^{c}AMP has been shown to be resistant to the action of ^{c}AMP-PDE(249,250) and in addition, Sodoyez and colleagues have suggested that db-^{c}AMP was not hydrolysed by ^{c}AMP-PDE in islet β-cells (280). Also the lack of stimulation was not due to any reduction in the physiological responsiveness of the islets, since islets of both normal and obese mice, similarly isolated, treated and assayed were shown to increase their rate of insulin secretion in response to increased glucose, Table 5 (page 59).

Mention might be made here of the recent work by Swislocki on the stability of db-^{c}AMP in aqueous bicarbonate and phosphate buffers (281). Db-^{c}AMP was found to decompose to the N^{6}-monobutyryl derivative (mb-^{c}AMP), ^{c}AMP and butyrate, in several buffers regularly used for the study of intact cell systems *in vitro*. Buffers used to incubate biological material at pH. 7.4 were found capable of converting as much as 25% of db-^{c}AMP to mb-^{c}AMP in 1 hour. Although Posternak and colleagues reported that db-^{c}AMP had to be activated by removal of the butyryl group from the ribosyl residue before it became effective (250), this was not always the case. In adipocytes the removal of butyryl groups from db-^{c}AMP did not appear to be a prerequisite for biological activity (282). Butyrate itself has been shown to stimulate insulin secretion *in vivo* (283) and *in vitro* (284). Consequently in experiments designed to demonstrate a stimulation of insulin secretion by db-^{c}AMP from incubated pancreatic tissue, the stimulation obtained might be due to butyrate rather than db-^{c}AMP. Also the higher the concentration of db-^{c}AMP used, and the longer the period of incubation, the greater

will be the tendency for this to occur. However, because no stimulation was observed with either ^{c}AMP or db-^{c}AMP in the work presented here, any possible effect by released butyrate was clearly absent.

SECTION I (B).

The effect of ^{c}AMP and db-^{c}AMP on the rate of oxygen uptake by microdissected islets from obese hyperglycaemic mice and their normal lean littermates.

Positive correlations obtained by Hellerström, between substrates that stimulate insulin secretion and the rate of oxygen consumption by isolated islets (196,199), prompted an investigation of the effect of exogenous db-^{c}AMP on the rate of oxygen uptake by normal and obese islet tissue.

Materials & Methods.

5mmol/litre db-^{c}AMP (Sigma) was dissolved in Gey & Gey buffer (110) in the presence of low (0.6g/litre) or high (3.6g/litre) glucose. Measurements of the rates of oxygen uptake by microdissected islets of normal and obese mice in these media followed the procedure described previously, (Chapter 2, Section 1) and values were expressed in terms of µl. of O_2 consumed/h/mg. freeze dried islet tissue, 37°C.

Results

The effects of db-^{c}AMP on the rate of oxygen uptake by microdissected islets of normal and obese mice in the presence of low and high glucose have been summarised in Table 11.

A higher rate of oxygen uptake was observed for obese islets compared to normal, in the presence of low glucose/db-^{c}AMP and high glucose/db-^{c}AMP. Also, in the presence of the same db-^{c}AMP concentration elevation of the glucose concentration brought about a significant increase in the rate of oxygen uptake by both NLL. (117%) and obob islets (37%). NLL. islets appeared to be more responsive to high glucose

TABLE 11. The effect of db-^{c}AMP (5mmol/litre) on the rate of glucose induced oxygen uptake by microdissected islets of obese hyperglycaemic mice and their normal lean littermates.

(Mean values ± S.E., n = number of determinations, islets were pooled from several mice for each determination.)

Substrate	N	NLL. islets	obob islets
		(µl. of O_2/h./mg.f.d.islet tissue)	
Low glucose (0.6g/litre) + 5mmol/litre db-^{c}AMP.	4	1.95 ± 0.06 (‡, 30%)	5.22 ± 0.10 * (‡, 11%)
High glucose (3.6g/litre) + 5mmol/litre db-^{c}AMP.	4	4.23 ± 0.09 † (117% ø)	7.11 ± 0.08 *† (37% ø) (8%)

* Denotes significant difference ($P < 0.05$) between NLL. and obob islets.

† Denotes significant difference ($P < 0.05$) between low and high glucose values.

ø Denotes the percentage increase due to the presence of high glucose.

‡ Denotes value significantly different ($P < 0.05$) from value obtained in presence of glucose alone (Table 6)

(The % represents the % stimulation of the rate of oxygen uptake by 5mmol/litre db-^{c}AMP.)

than obob islets.

Comparison of the rate of oxygen uptake by NLL. islets in the presence of low glucose and high glucose, with and without 5mmol/litre db-^{c}AMP, using results from Table 6 (Chapter 2, Section 1), have shown db-^{c}AMP to produce a significant 30% stimulation of the low glucose induced rate of oxygen uptake and a significant 31% stimulation of the high glucose induced rate of oxygen uptake, Table 11. In the case of obob islets, however, a significant stimulation of the rate of oxygen uptake by db-^{c}AMP was observed only in the presence of low glucose and was of the order of 11%. A major contribution to the statistical significance observed in these experiments was the low standard errors of the mean values and the high degree of repeatability shown for the technique. Coefficients of variation for groups of data were within 4%.

Discussion.

Db-^{c}AMP (5mmol/litre) was found capable of increasing the rate of low glucose induced oxygen uptake by islets from normal (NLL) and obob mice and the rate of high glucose induced oxygen uptake by islets from normal mice. Normal islets were more responsive to the stimulant effect of db-^{c}AMP than obese. This might have been the result of the higher adenyl cyclase activity (285) and presumably ^{c}AMP levels in obese islets, presensitising the latter to the effect of exogenous db-^{c}AMP.

The stimulation of the rate of glucose induced oxygen uptake by db-^{c}AMP was consonant with the suggestion that islet glycogen might be the endogenous substrate for islet respiration (200,201) and Idahl & Hellman have suggested a regulation of β-cell glycogen via ^{c}AMP (286).

The concentration of exogenous db-^{c}AMP to which islet tissue was exposed in the current experiments was vastly in excess of the physiological amounts shown to be normally present (287). Hence the stimulation shown by 5mmol/litre db-^{c}AMP may not have been a normal

physiological response.

In contrast to the work presented here, Hellerström and colleagues have shown very recently, that the insulin secretagogues glucagon, adrenaline and db-^{c}AMP (1mmol/litre), in the presence of glucose (2g/litre) greatly reduced the rate of obese mouse islet oxygen consumption (196,199). This anomaly might have resulted from the different concentrations of db-^{c}AMP and glucose used by these workers.

Butyrate produced from the in vitro decomposition of db-^{c}AMP (281) might have been used by islet tissue under test, as a respiratory substrate. However, that it would have been utilised in preference to the glucose already present seemed doubtful, although the tendency might have been greater in the presence of low glucose.

SECTION 1 (C).

The effect of db-^{c}AMP (5mmol/litre) on the total lactate dehydrogenase activity and isoenzyme pattern of islet tissue from obese hyperglycaemic mice and their normal lean littermates.

Cyclic AMP has been suggested to mediate the actions of a number of peptide hormones on their respective target tissues (248). Also exogenous ^{c}AMP has been used to mimic the effects of certain steroid hormones i.e. oestradiol, on the assumption that ^{c}AMP may be involved as a mediator in the action of this hormone (288). Goodfriend & Kaplan demonstrated that the administration of oestradiol to immature female rats and rabbits resulted in a selective increase in the slower moving isoenzymes ($LDH_{4\ \&\ 5}$) of the uterus (289). The findings after the administration of oestradiol have indicated that the hormone increased the synthesis of both M and H sub-units in most tissues, but that in certain tissues, such as the uterus, there was preferential synthesis of the M sub-unit. This would indicate that the hormonal control of

selective sub-unit synthesis occurred only in specific target tissues. On the other hand thyroxine and tri-iodothyronine have been shown to activate directly the adenyl cyclase activity of monkey spermatazoa (290) and alter the LDH isoenzyme pattern in liver (291). The latter change involved the loss of LDH_5 activity and a decrease in LDH_{2-4} activities, or in other words a repression of M sub-unit synthesis. The effect of ^{c}AMP on LDH isoenzyme patterns was of interest, because of the possible hormonal control of sub-unit synthesis mediated via ^{c}AMP and the possible control by the hormone of the type of metabolism prevailing in the tissue.

In consequence the effect of db-^{c}AMP was investigated on the total LDH and LDH isoenzyme pattern of islet tissue from normal and obese mice. Db-^{c}AMP was used to mimic the potential action of hormone on islet adenyl cyclase activity.

Materials & Methods.

Microdissected islets from 20-25 week old normal and obese mice were preincubated for 15 minutes in oxygenated Gey and Gey buffer (110) supplemented with 1g/litre and 3g/litre glucose respectively. After preincubation islets were incubated for a further 30 minutes at 37°C in 2ml. of buffer pH. 7.4, supplemented with glucose (1g/litre for normal mouse islets and 3g/litre for obese mouse islets) in the presence and absence of db-^{c}AMP (5mmol/litre). During incubation islet tissue was aerated with a 95% air/5% CO_2 gas mixture.

Islets were transferred to glass homogenizers after incubation, washed with fresh buffer and then used for the estimation of total LDH activity and LDH isoenzyme pattern according to the methods previously described, (Chapter 2, Section 2).

Results:

Aeration of the incubation medium was effected with an air/CO_2 mixture instead of an O_2/CO_2 mixture since both Cahn (292) and Goodfriend & Kaplan (293) demonstrated that increased oxygen tension enhanced the

synthesis of H sub-units or retarded the synthesis of M sub-units. Similarly Hellung-Larson & Anderson (294) demonstrated that aeration with high oxygen concentrations increased the proportion of H containing isoenzymes. However, since heavy metal ions were shown to regulate the repressive effect of the high oxygen tension, the latter was clearly not the sole regulator of sub-unit synthesis.

Total LDH activities of normal and obese islet tissue, incubated in the presence and absence of 5 mmol/litre db-^{c}AMP have been summarised in Table 12 below.

TABLE 12. The effect of db-^{c}AMP (5mmol/litre) on the total LDH activity of normal and obese mouse islet tissue after 30 minutes incubation in vitro at 37°C.

(Mean values ± S.E., n = number of determinations, islets pooled from several mice for each determination).

	N	Total LDH activity in U/g. islet protein. db-^{c}AMP Absent	db-^{c}AMP Present
NLL. islets (1g/litre glucose)	4	208 ± 9	166 ± 4 †
obob. islets (3g/litre glucose)	4	349 ± 8 *	324 ± 14 *

* Denotes significant difference ($P < 0.05$) between NLL. and obob. values.

† Denotes significant difference ($P < 0.05$) between db-^{c}AMP present and db-^{c}AMP absent values.

Incubation alone, in the presence of the appropriate amount of glucose, reduced the total LDH activity of both normal and obese islets by 32% and 24% respectively, when compared with fresh tissue values, Table 8. The higher level of total LDH activity in obese islets was maintained in both the presence and absence of db-^{c}AMP. 5mmol/litre db-^{c}AMP significantly reduced the total LDH activity of NLL. islet tissue only.

TABLE 13. The lactate dehydrogenase isoenzyme pattern of 30 minutes incubated islet tissue from obese mice and their normal lean littermates in the presence and absence of db-^{c}AMP (5mmol/litre), expressed as percentages of the total activity recorded by densitometer integration.

(Mean values ± S.E., n = no. of determinations, islets pooled from several mice for each determination.)

	N	% distribution of each isoenzyme					H : M Ratio
		LDH_1	LDH_2	LDH_3	LDH_4	LDH_5	
NLL. islets (1g/litre glucose)	3	-	1.25 ± 1.0	8.8 ± 2.5	15.68 ± 4.6*	74.15 ± 6.8*	9 : 91
NLL. islets (1g/litre glucose + 5mmol/litre db-^{c}AMP).	5	-	2.08 ± 0.9	11.04 ± 1.1	29.16 ± 0.7	57.54 ± 1.7	15 : 85
obob. islets (3g/litre glucose + 5mmol/litre db-^{c}AMP).	9	0.46 ± 0.2	0.61 ± 0.1†	9.1 ± 1.2	24.86 ± 1.2†	64.74 ± 2.2†	12 : 88
obob. islets (3g/litre glucose	9	0.6 ± 0.4	1.37 ± 0.6	9.57 ± 0.8	25.49 ± 2.0	62.98 ± 2.7	13 : 87

* Denotes significant difference ($P < 0.05$) between % values for NLL. (1g/litre glucose) and NLL. (1g/litre glucose + db-^{c}AMP).

† Denotes significant difference ($P < 0.05$) between % values for NLL. (1g/litre glucose + db-^{c}AMP) and obob.(3g/litre glucose + db-^{c}AMP).

Investigation of the effect of 5mmol/litre db-^{c}AMP on the LDH isoenzyme pattern of incubated NLL. islet tissue, Table 13, has demonstrated a significant reduction in the proportion of LDH_5 and a significant increase in the proportion of LDH_4. 5mmol/litre db-^{c}AMP had no significant effect on the LDH isoenzyme distribution of incubated obese islets. In the case of normal (NLL) islets, incubation in the presence of db-^{c}AMP brought about a repression of the M type sub-unit, which was reflected by a reduced proportion of LDH_5 and a change in the H : M ratio from 9 : 91 to 15 : 85.

After incubation in the presence of glucose only, the proportions of the constituent LDH isoenzymes in normal and obese islets were found to be about the same. In the case of obese islets, incubation in the presence of glucose alone brought about a repression of the M type sub-unit, which was reflected by the change in the H : M ratio from 8 : 92 (Table 9) to 13 : 87.

Finally in the presence of db-^{c}AMP and the appropriate amount of glucose, incubated normal islets showed elevated proportions of LDH_2 and LDH_4 compared to incubated obese islets (Table 13).

Discussion

In normal islets at least, evidence has been presented for an M-LDH sub-unit repression by 5mmol/litre db-^{c}AMP, suggesting that the anaerobic portion of glucose metabolism in these islets might possibly be converted to a more aerobic form by the action of db-^{c}AMP. In consequence, insulinogenic peptide hormones and adenyl cyclase activators, such as adrenaline and possibly sulphonylureas, might effect M - LDH sub-unit repression, via the production of ^{c}AMP in NLL. islet cells and conceivably, by this action, alter or modify the contribution of the various metabolic pathways to the overall process of islet glucose metabolism and perhaps influence the mechanism of insulin secretion.

The stimulus for insulin secretion traditionally inferred a glycolytic intermediate (98). If the metabolism of glucose via glycolysis and insulin secretion was to some extent regulated by the sequence phosphoglyceraldehyde dehydrogenase/phosphoglyceratekinase (129) which depended on a supply of NAD possibly contributed by LDH activity, then any effect ^{c}AMP might exert on the LDH isoenzyme pattern (M sub-unit repression) could influence the above sequence and possibly insulin secretion.

However, too much emphasis should not be placed on this hypothesis. Since incubation itself, in the presence of glucose reversed the original observation of a higher proportion of LDH_5 in obese islets, Table 9. Also, db-^{c}AMP induced M sub-unit repression was not demonstrate for obese islets, Table 13 and their greater tendency towards anaerobic metabolism and the fact that they were probably presensitised to higher levels of endogenous ^{c}AMP, might have been the reason for this. There was no evidence in the literature to suggest that butyrate per se, derived from the decomposition of db-^{c}AMP (281), was capable of influencing total LDH activity or the distribution of LDH isoenzymes. However, 2-oxobutyrate and 2-hydroxybutyrate have been shown to be potential substrates for LDH (295).

SECTION 2.

The measurement of adenyl cyclase activity in isolated islets of Langerhans from obese hyperglycaemic mice and their normal lean littermates.

The response of adenyl cyclase to hormone and non-hormone effectors has been observed in purified membrane systems, homogenates and intact cells (244). Islets, like many other tissues contain several different cell types, each of which may contain discrete adenyl cyclase systems. Studies on the cyclase activity of islet tissue homogenates, provided information on the total adenyl cyclase activity of the component cell

types, because methods for the separation of α and β cells from each other, in quantities suitable for biochemical analysis, were not available. However, the fact that β-cells comprised some 80% of normal mouse islets (30) and 90% of obese mouse islets (63) justified the assumption that the adenyl cyclase activity measured was representative of the β-cell population.

The following criteria were observed for the measurement of adenyl cyclase activity (296). The substrate ATP concentration was maintained in excess to provide maximum rates of ^{c}AMP formation under conditions of zero order reaction. Low, limiting levels of substrate complicated the reaction kinetics and tended to increase the effect exogenous unlabelled substrate might have on the reaction i.e. by reducing the specific activity of the substrate. The activity of ^{c}AMP-phosphodiesterase was inhibited without interfering with ^{c}AMP formation and ^{c}AMP was separated effectively from other related nucleotides produced as a result of ATP hydrolysis.

Maintenance of the substrate ATP concentration.

Mammalian cell membrane preparations and tissue homogenates have been shown to contain high ATPase activity. In consequence, means had to be employed to maintain the concentration of substrate ATP for maximal rates of adenyl cyclase activity, particularly since most cyclase systems investigated have been found to have Km values in the range 0.1 - 1.0mmol/litre (297,298) and utilise only a small part of the substrate ATP. One way was to use high concentration of ATP (3mmol/litre or higher). But high concentrations of this nucleotide have been shown to have effects on the system other than as a substrate. Rodbell and colleagues suggested that ATP at these high concentrations might be capable of dissociating the bound effector (hormone) from the adenyl cyclase receptor (discriminator site) (299). Also high ATP concentrations were found capable of altering the free Mg^{2+} concentration sufficiently enough to alter the hormone response and Drummond and

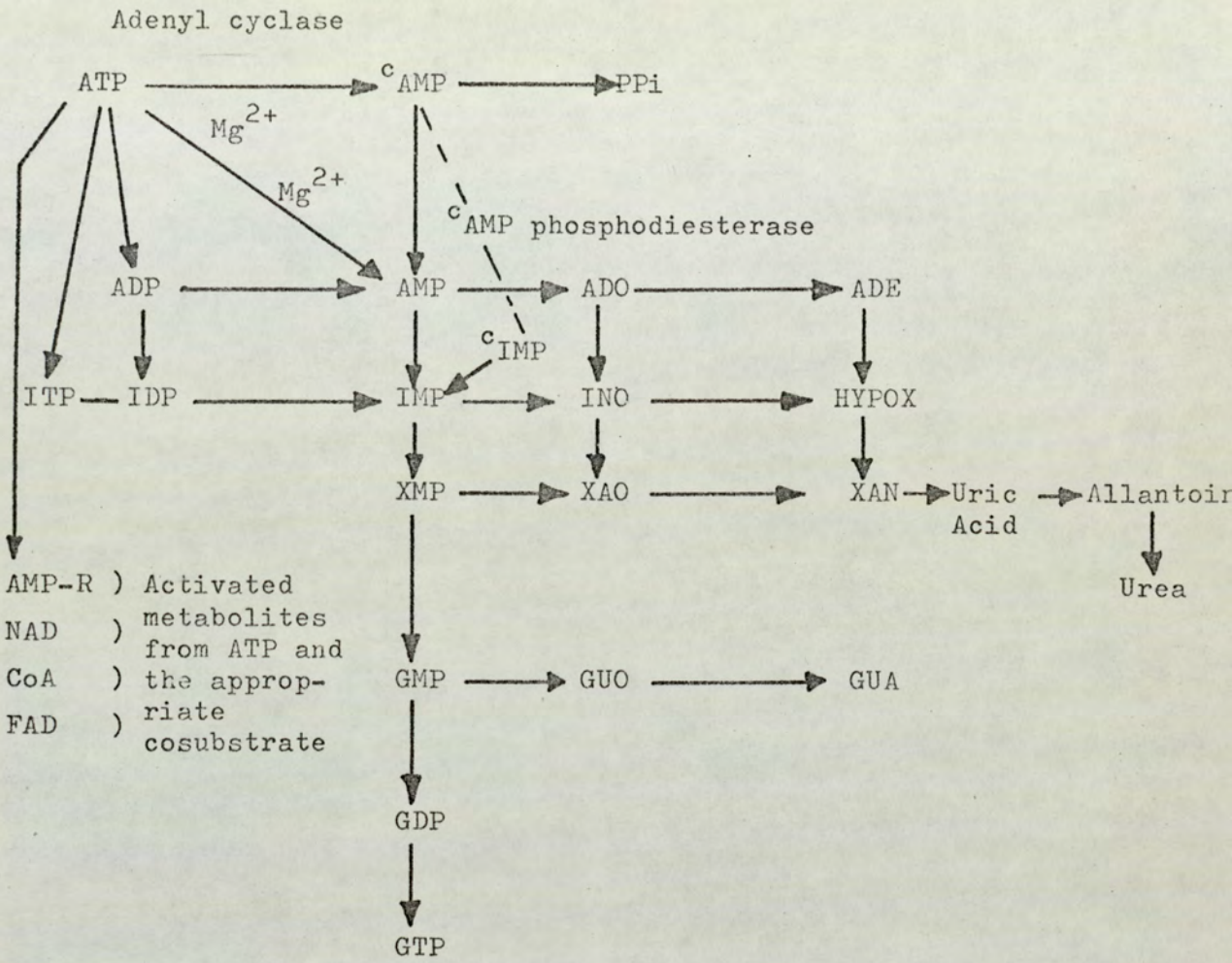
coworkers have shown ATP concentrations in excess of the Mg^{2+} concentration to inhibit cardiac muscle adenyl cyclase (300).

The presence of contaminating ATPase complicated the assay of adenyl cyclase by necessitating the use of an enzymic ATP regenerating system and both creatine phosphate/creatine kinase and phosphoenolpyruvate/pyruvate kinase systems have been widely used. However, these enzymatic systems were not particularly efficient when concentrations of ATP below 0.5mmol/litre were employed. Also they have been shown to require Mg^{2+} in the same concentration range as that required for adenyl cyclase and alterations in the apparent activity of the latter with changes in the Mg^{2+} concentration might merely have reflected changes in the activity of the regenerating system (301). Recently Rodbell and colleagues solved the problem of maintaining ATP concentrations even at initial concentrations of 0.1mmol/litre by using the di-phosphoimide analogue (AMP-PNP) of ATP. This compound was only slowly hydrolysed by nucleotidepyrophosphohydrolases in membranes, was not hydrolysed by ATPases and proved a suitable substrate for adenyl cyclase (302).

The prevention of ^{c}AMP hydrolysis.

Theophylline has been widely employed for the inhibition of ^{c}AMP-phosphodiesterase (249). Also cold ^{c}AMP has been added to adenyl cyclase incubations as a means of impeding the destruction of the labelled product (297). ^{c}AMP *per se.* was without effect on adenyl cyclase activity, even at high concentrations (303). Drummond and coworkers have demonstrated that the addition of a combination of theophylline and cold ^{c}AMP was the most effective way of preserving labelled ^{c}AMP.

Fig. 11. Structurally related metabolites of ATP and ^{c}AMP.



The separation of labelled ^{c}AMP from related labelled nucleotides.

The adenyl cyclase assay depended upon the complete separation of ^{c}AMP from a mixture contaminated with large quantities of structurally similar compounds. Figure 11 shows most of the products of ATP and ^{c}AMP metabolism that are potentially capable of appearing in an adenyl cyclase incubation medium, if only in very small quantities. The complete chromatographic separation of these intermediates could not be effected without numerous lengthy chromatographic sequences (304). However, by using ATP - α - ^{32}P as the substrate in adenyl cyclase assays, interference from the bulk of the derived nucleotides and bases could be avoided (297). But the ATP - α - ^{32}P was expensive and its short half-life required skilled handling. A sensitive, but not simple method for determining adenyl cyclase activity was reported by

Krishna, Weiss & Brodie (305) and Krishna & Birnbaumer (306). The procedure was based on the observation that with the exception of ^{c}AMP, all other nucleotides were completely precipitated with zinc sulphate/barium hydroxide solutions. A similar separation of ^{c}AMP has been demonstrated with aluminium oxide columns(307,308).

Because of the poor permeability of cells to ATP and the loss of this substrate due to breakdown and binding to other materials in intact tissues, it has not been feasible to measure adenyl cyclase activity in whole tissue or isolated cells. One approach that circumvented the problem of adding ATP, was to allow the cells to synthesise labelled ATP from labelled adenosine or adenine. This method has been carried out on fat cells (309) and brain slices (310). After the addition of hormones or other agents that stimulated ^{c}AMP production, labelled ^{c}AMP formed from the intracellular pools of ATP was isolated by Krishna's method (305). However, this technique did not give the quantity of ^{c}AMP formed, because the labelled ^{c}AMP might not have been produced from the total pool of ATP. Indeed, Shimizu and coworkers demonstrated that only a fraction of the intracellular ATP was used in the production of ^{c}AMP (310).

Evidence has been presented to suggest that the substrate for adenyl cyclase was a Mg^{2+}ATP complex, rather than ATP per se. (298,300). Little or no cyclase activity was observed in the absence of Mg^{2+} and different tissues required substrates containing various $[Mg^{2+}]$: [ATP] ratios. Adenyl cyclase activity was increased as long as the Mg^{2+} concentration remained higher than the concentration of ATP (300), although very high $[Mg^{2+}]$ reduced cyclase activity. The latter observation in adrenal tissue suggested that free ATP rather than Mg^{2+}ATP was the substrate for adenyl cyclase in this tissue (311). The optimal Mg^{2+}ATP ratios observed for adenyl cyclase activity were in the region of 1 : 1.5 (311), 1 : 2 (312) and tended to be tissue specific. Work on fat cell ghost (313) and amphibian erythrocyte

ghost adenyl cyclases(314) has shown them to be base specific and capable of using dATP (deoxyadenosine triphosphate) as substrate as well as ATP.

The purpose of the work presented below was to demonstrate the presence of an adenyl cyclase system in the islet tissue of obese mice and their normal lean littermates and to investigate the effect of glucose; pharmacologically active agents; cell membrane active transport inhibitors; amino acids and certain protein hormones on islet adenyl cyclase activity. This work was performed using whole islet homogenates and until a suitable method becomes available for the separation of component islet cells, enzyme activity can only be expressed in terms of whole islet protein.

Biochemical studies of broken cell systems from pigeon erythrocytes (318) and rat liver (319) have shown adenyl cyclase to be associated with the plasma membrane. In an attempt to determine more precisely the cytochemical localisation of adenyl cyclase activity in islet tissue use has been made of the recently introduced technique of Reik and colleagues (317).

Materials and Methods.

Cytochemical localisation of adenyl cyclase.

The basis of the cytochemical reaction was the precipitation of a heavy metal salt of pyrophosphate (PPi), produced by the action of adenyl cyclase on ATP. Lead ions (Pb^{2+}) precipitated pyrophosphate from the medium and the final product was visualised under the electron microscope.

Microdissected islets of normal and obese mice were dropped into a fixative consisting of 1% gluteraldehyde in 0.05mol/litre sodium cacodylate buffer, pH. 7.4, containing 15% sucrose. Islets were then washed overnight in a buffer solution containing a 1 : 1 mixture of sodium cacodylate buffer and 15% sucrose. Reik and coworkers have shown that fixation in 1% gluteraldehyde, preserved both ultrastructural

morphology and adenyl cyclase activity (317).

Islets were washed for 4, one hour periods in 1 : 1 sodium cacodylate/15% sucrose buffer prior to incubation for 30 minutes at 30°C in the following standard medium (10 islets/ml.), 80mmol/litre tris-maleate buffer, pH. 7.4; 8% sucrose; 10mmol/litre theophylline; 4mmol/litre anhydrous magnesium sulphate; 4.8mmol/litre lead nitrate (fresh); 0.5mmol/litre ATP (Disodium salt) and 10mmol/litre sodium fluoride (for the inhibition of ATPase and stimulation of adenyl cyclase). Control incubations contained no ATP. After incubation, islets were washed in tris-maleate buffer, pH. 7.4, containing 8% sucrose and then post-fixed with 1% osmium tetroxide in 0.05 mol/litre veronal acetate buffer, pH. 7.4, containing 8% sucrose for 3h. at 4°C. The fixative was poured off and islets washed for 5 minutes in veronal acetate buffer, pH. 7.4. After removal of the buffer, islets were dehydrated for 5 minutes in 50%, 70% and 90% ethanol and finally for 30 minutes in two changes of absolute ethanol. Dehydration was followed by submersion in epoxy-propane for 30 minutes (50/50 epoxy resin/epoxy propane) followed by 2, 30 minute changes of epoxy resin (7 parts epoxy A, 3 parts epoxy B and 1.5% araldite accelerator, dy 062). Islets were then transferred to the bottom of gelatin capsules (2 islets per capsule) and the latter topped up with epoxy resin and an identification tag. Capsules and their contents were cured for 48 h. at 60°C, after which time the gelatin capsules were removed by emersion in hot water and the blocks returned to the oven to harden. Thin sections (grey gold interference colour) were cut on an LKB ultramicrotome using glass knives and mounted on carbon coated EM. grids. Unstained sections were examined under an AEI electron microscope. Photomicrographs were taken of areas of enzyme activity. The experiment was repeated twice and islet blocks were sampled at random from both experiments.

The measurement of adenyl cyclase activity.

20 - 25 week old normal and obese mice of both sexes were starved overnight (water was available ad libitum) before removal of islets by microdissection. Mean blood sugar values (tail samples taken from a representative number of animals (156)) were 85 mg% for normal mice and 298 mg% for obese mice. Isolated islets were weighed wet and then homogenised over ice at 2°C in 1 mmol/litre potassium bicarbonate, pH. 7.0 (tissue = volume ratio, 1 : 9) and the protein content of the homogenate determined without centrifugation as described previously (218). A standard protein curve was prepared using bovine albumin V in 1mmol/litre potassium bicarbonate, pH. 7.0 and the islet protein content of samples was adjusted to 2µg/10µl with potassium bicarbonate and rechecked before use in the adenyl cyclase assays. The standardised protein solution was retained over ice (2°C) in a silicone treated glass tube sealed with 'parafilm' and mixed thoroughly prior to use ("Whirlimixer", Fisons). Tris buffer was not used for homogenisation because there was some evidence to suggest that it might interfere with the colour development in the Lowry protein determination (315). Potassium ions have also been shown to interfere with the protein determination, but only at concentrations of 12mmol/litre and above (316)

Adenyl cyclase assay.

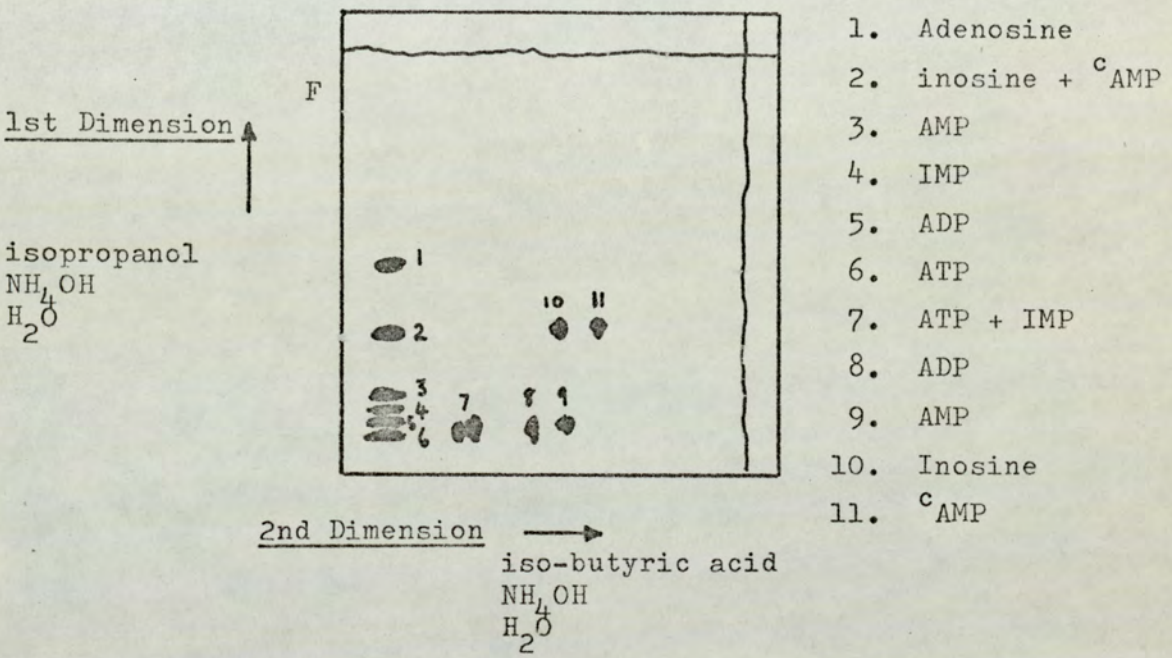
Adenyl cyclase activity was measured radiometrically by the method of Bär & Hechter with some modifications described below (297).

Assays were carried out in 0.5 x 5 cm. silicone coated glass tubes at 37°C and performed on fresh islet homogenates within 1 hour of isolation. Reactants were dispensed from Hamilton syringes over ice to reduce evaporation. The final concentration of reaction components in a total of 50 µl. were, 40mmol/litre tris-HCl, pH.7.0 at 37°C, 5mmol/litre $MgCl_2$; 0.1% crystalline bovine albumin V; 10mmol/litre theophylline; 1mmol/litre ^{c}AMP (Sigma); pyruvate kinase II, 0.05mg/ml. (Sigma); phosphoenolpyruvate tri-sodium salt, 8mmol/litre (Sigma);

1.8mmol/litre (^{3}H-2) ATP (final specific activity 0.8mCi/mmol., The Radiochemical Centre, Amersham). To the reactants was added 2 µg of islet protein sample in a volume of 10 µl. Blank assays contained 10 µl. of 1mmol/litre portassium bicarbonate instead of protein solution. Test substances were added to the tris-HCl buffer of the incubation medium at the appropriate concentration.

Incubations were carried out in 'parafilm' sealed tubes for 10 minutes at 37°C. The reaction was stopped by immersing the lower part of the assay tube in boiling water for 2 minutes. A 100 µl. carrier containing the following fresh reagents, 5mmol/litre ATP; ADP; AMP; ^{c}AMP; 2mmol/litre adenosine; inosine and inosine monophosphate (IMP) was added to the incubation mixture and thoroughly mixed. After centrifugation for 5 minutes at 3000 rev./min. the contents of the supernatant were separated by two dimensional ascending thin layer chromatography at room temperature using precoated cellulose F.254 plates (20 x 20 cm) MERCK (Anderman & Co. Ltd., London). The thin layer plates were dried at 60°C for 2h. and allowed to cool over silica-gel before use to remove all traces of moisture. Small quantities of supernatant were applied as a single thin 1 cm. line in the left hand corner of a thin layer plate with a silicone treated, drawn out capillary tube. After each application the line spot was dried (hair dryer) prior to further addition. Although several solvent systems were investigated, the following sequence gave the most efficient separation of nucleotides and nucleosides (analar reagents were used throughout). Separation in th first dimension was effected with isopropanol: ammonium hydroxide: distilled water (7 : 1 : 2), 4 - 6 h. In this system adenosine had the greatest Rf value (0.47), followed by inosine + ^{c}AMP (0.38) and ATP, ADP, IMP + AMP (0.03-0.07). The latter nucleotides were not well separated and remained near the origin. Adenosine was removed from the plate at this point and the remainder subjected to the second dimension system of isobutyric acid: ammonium hydroxide : H_2O (66 : 1 : 33), 5 - 7

In this system ^{c}AMP had the greatest Rf value (0.67), followed by inosine (0.53); ATP and IMP (0.3); ADP (0.45) and AMP (0.57). IMP co-chromatographed with ATP in the second system. A solvent system capable of separating IMP from ATP, as well as effecting a reasonable separation of the other nucleotides and nucleosides was not found.



Authentication of the various nucleotides and nucleosides on the thin layer plates was effected by using commercially available compounds subjected to the same solvent systems. Solvent tanks were allowed one day to equilibrate before use. Test materials and theophylline added to the adenyl cyclase incubation, co-chromatographed with the solvent front in the first dimension. After development, spots were identified under u.v. light, ringed with pencil and scraped off the glass plate with a spatula, as efficiently as possible, into borosilicate scintillation vials. 0.2 - 0.3 ml. of a solubilising agent, hyamine 10x' hydroxide (Kock light, donated by Birmingham University Medical School) was added and the contents of each maintained at 50°C for 30 minutes. After treatment 12 ml. of toluene scintillation fluid was added to each vial (3g. 2.5 diphenyloxazole (PPO) and 200 mg. 1, 4 - bis, 2 - (4 methyl-5-phenyloxazolyl) benzene (POPOP), (Kock light) were dissolved in 1 litre of analar toluene. The newly made up scintillant

was bubbled with N_2 to remove oxygen and stored at 0°C). Batches of prepared vials were stored in the dark prior to counting on a Packard liquid scintillation counter for 5 or 10 minutes. Each sample was counted twice. The counting efficiency was determined by reference to an internal standard. A tritium correction factor for the internal standard (multiplication factor, MF) was applied to the counts/minute. The efficiency was corrected to 100% and given by,

$$\text{Efficiency } \% = \frac{1}{MF} \times 100$$

Actual counting efficiencies ranged from 32 - 38% and no improvement was observed after adding 12 ml. of a thixotropic gelling agent (4% Cab-o-sil in toluene scintillation fluid, G.L. Cabot, Inc. U.S.A., courtesy of Birmingham University Medical School).

Attempts were also made to elute the developed spots with ethanol from the thin layer plates into scintillation vials. The ethanol was allowed to evaporate prior to the addition of scintillation fluid. The elution however was not found to be quantitative and the counts obtained were irregular.

Calculation of results.

The amount of ^{c}AMP was calculated from the specific activity of the ATP used as substrate. Counts/10 minutes were converted to counts/minute and the multiplication factor correction for the internal standard applied to give a value in disintegrations/minute. The disintegrations/minute were corrected for scintillation fluid blank values (100-180 d/min.) and used to evaluate adenyl cyclase activity as follows, d/min → d/sec. per 50µl sample.

d/sec. x 20 x 1000 d/litre/10 minutes.

d/sec. x 20 x 1000 x 500 d/mg.islet protein/10mi

$$\text{d/sec.} \times \frac{20 \times 1000 \times 500}{3.7 \times 10^{7}}$$ mCi/mg.islet protein/10 mi

Specific activity of ^{3}H - ATP = 0.8159 mCi/mmol.

$$\frac{d/sec. \times 20 \times 1000 \times 500}{3.7 \times 10^{7} \times 0.8159}$$ (mmol.product/mg.islet protein/10 minutes)

$$\frac{d/sec. \times 1000}{3.7 \times 0.8159}$$ μmol/mg.islet protein/10 minutes.

$$d/sec. \times 331.2574$$ μmol. of product/mg.islet protein/10 minutes 37°C, pH.7.0.

Since 1 mol. ATP ⟹ 1 mol ^{c}AMP, adenyl cyclase activity was expressed in terms of μmol ^{c}AMP produced/mg.islet protein/10 minutes, at 37°C and pH. 7.0.

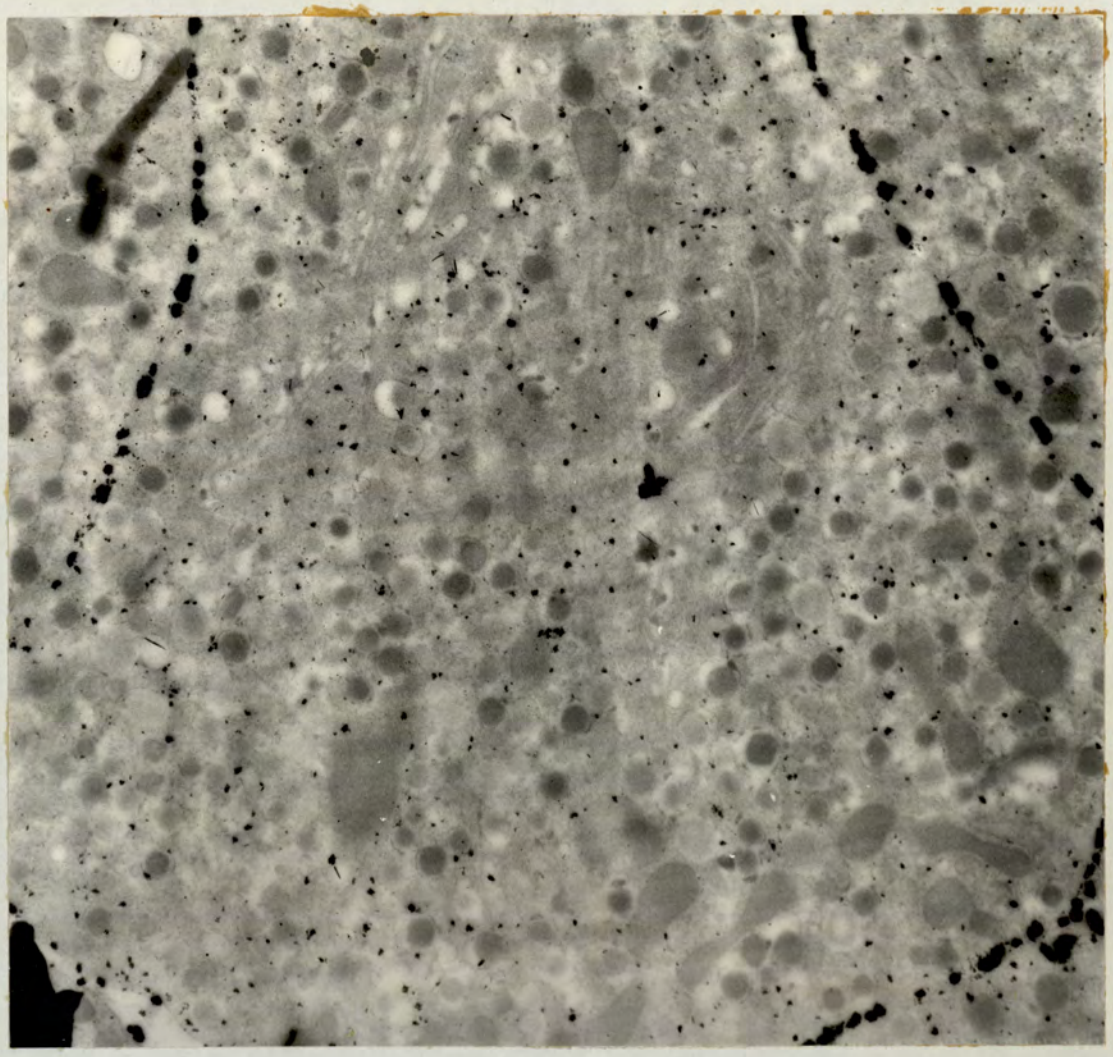
The effects of incubation time, pH. and protein concentration were investigated on islet adenyl cyclase activity and the cyclase activities of normal and obese islets were compared in the presence of various insulinogenic and pharmacological agents.

Results

The cytochemical localisation of adenyl cyclase activity.

The deposition of very electron dense reaction product indicated areas of adenyl cyclase activity. The reaction product was deposited as a coarse granular precipitate and assumed to be the lead salt of pyrophosphate (317). In sections of both normal and obese islet tissue, sites of adenyl cyclase activity were observed on the outer surface of α and β-cell plasma membranes. These electron dense deposits were seen wedged between opposing cell membranes and at the junctions of α and β-cells with capillaries (Plates 9-11). In addition, Plate 10 illustrated the deposition of electron dense material in the pars fibrosa region of the nucleolus. This region has been suggested to be the site of ribosomal RNA synthesis and to be involved in the mechanism of protein synthesis (320,321). There was little evidence of any adenyl cyclase activity in β-granule membranes and most sections contained a certain amount of background staining due to the lead content of the incubation medium. A much more coarse deposition of

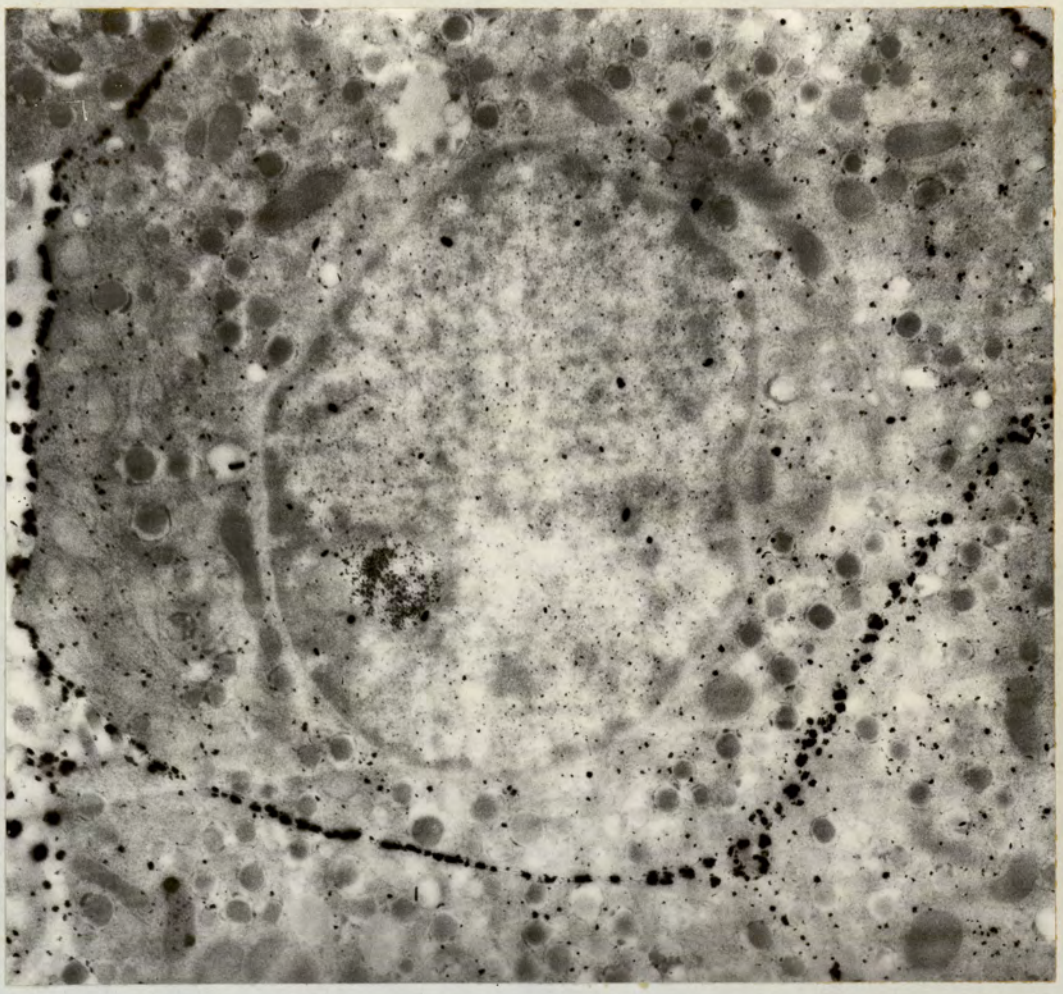
PLATE 9. Adenyl cyclase activity associated with the plasma membrane of a β-cell. (obese mouse islet, unstained, x 20,000).



electron dense material was observed at the junction of ∝ and β-cells with adjacent capillaries, Plates 12 & 13; regions where a most rapid and intense secretion of hormone might be expected to occur in response to a blood borne stimulus. Little or no deposition was observed in the lumen of islet capillaries, except where it was associated with the outer surface of erythrocyte plasma membranes, Plate 14. This activity shown by erythrocyte plasma membranes re-emphasised the variety of cells in islets potentially capable of contributing to the total adenyl cyclase activity of an islet tissue homogenate and was of special importance in the case of obese islets wit their high degree of vascularity. The higher adenyl cyclase activity recorded for obese mouse islet tissue (285), might have resulted from

PLATE 10. Adenyl cyclase activity associated with the β-cell plasma membrane and pars fibrosa of the nucleolus.

(Obese mouse islet, unstained, x 15,000).

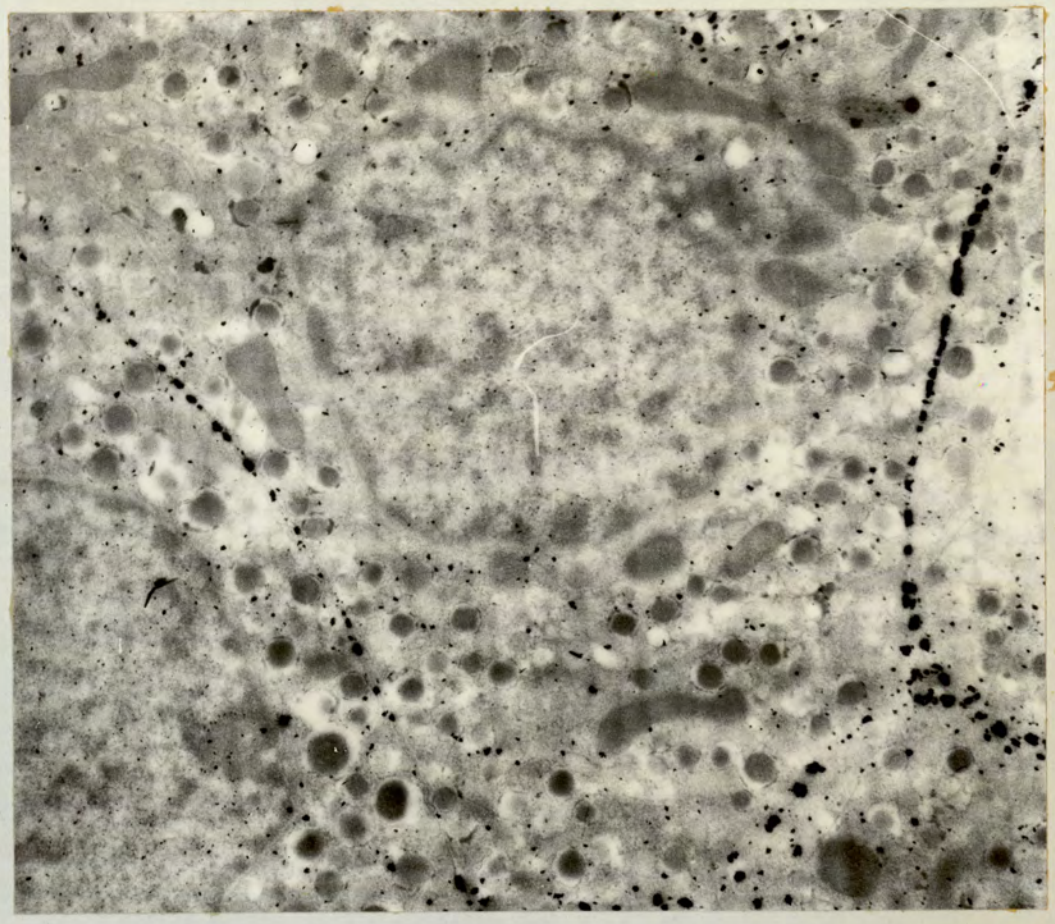


the higher erythrocyte content. Sheppard & Burghardt have demonstrated adenyl cyclase activity in mammalian erythrocytes and their ghosts (322,323) and Ziene & Greenough have measured adenyl cyclase in blood platelets (324). Sections of normal and obese islet tissue controls (incubation in the absence of ATP) showed no deposition of electron dense material at islet cell plasma membranes or within nucleoli, Plates 15 & 16.

The optimal conditions for the assay of adenyl cyclase activity in normal and obese islet tissue.

The counts/minute values recorded for no protein blanks were very high (in the region of 0.4% of the total activity) and it was possible that

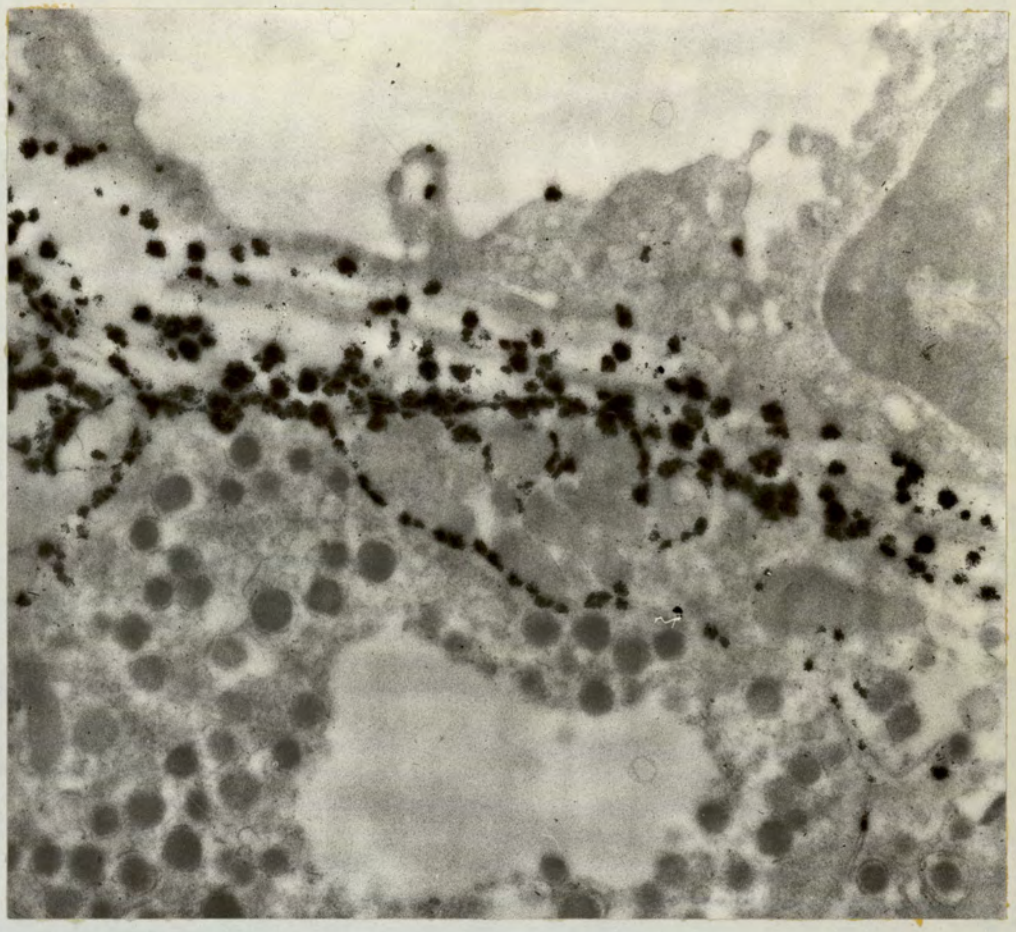
PLATE 11. β-cell plasma membrane localised adenyl cyclase activity. (Normal mouse islet, unstained, x 15,000).



these high blank values markedly reduced the sensitivity of the assay for adenyl cyclase in islet extracts containing very low enzymatic activity. Bär & Hechter (297) indicated that the blank count could be reduced from 0.2-0.4% of the total count, to something in the region of 0.005% by using different solvent systems in the second dimension i.e. n-propanol/ammonium sulphate/H_2O, (2ml : 60g : 100ml.) or ethanol/1mol/litre ammonium acetate (7 : 3v/v). However, investigations showed that a satisfactory separation of nucleotides could not be effected using these solvent systems in the second dimension. Oldam suggested that these high blank readings were caused by the presence of impurities in the labelled substrate, by concomitant enzyme reactions or by analytical procedures which did not result in a complete separation of the substrate and product (325). The high blank suggested that some impurity in the substrate had been

PLATE 12. Adenyl cyclase activity localised at the α-cell/capillary endothelium junction.

(Obese mouse islet tissue, unstained, x 20,000).



included with the product in the separation procedure. The additive effects of radiation decomposition during storage and the use in the assay of a low specific activity substrate possibly contaminated with high specific activity impurities, would tend to magnify the effect of the impurities.

Concomitant non-enzymatic reactions if present would have caused enzymatic activity to be over-estimated and trace metal ions; the presence of oxygen; boiling of the incubation contents and pH. have all been shown to be contributory (325).

a) The effect of incubation time.

Mammalian tissues have been shown to contain ATPase activity several orders of magnitude greater than adenyl cyclase (297).

PLATE 13. Adenyl cyclase activity localised at α-cell and β-cell capillary junctions. (Obese mouse islet tissue, unstained, x 20,000)

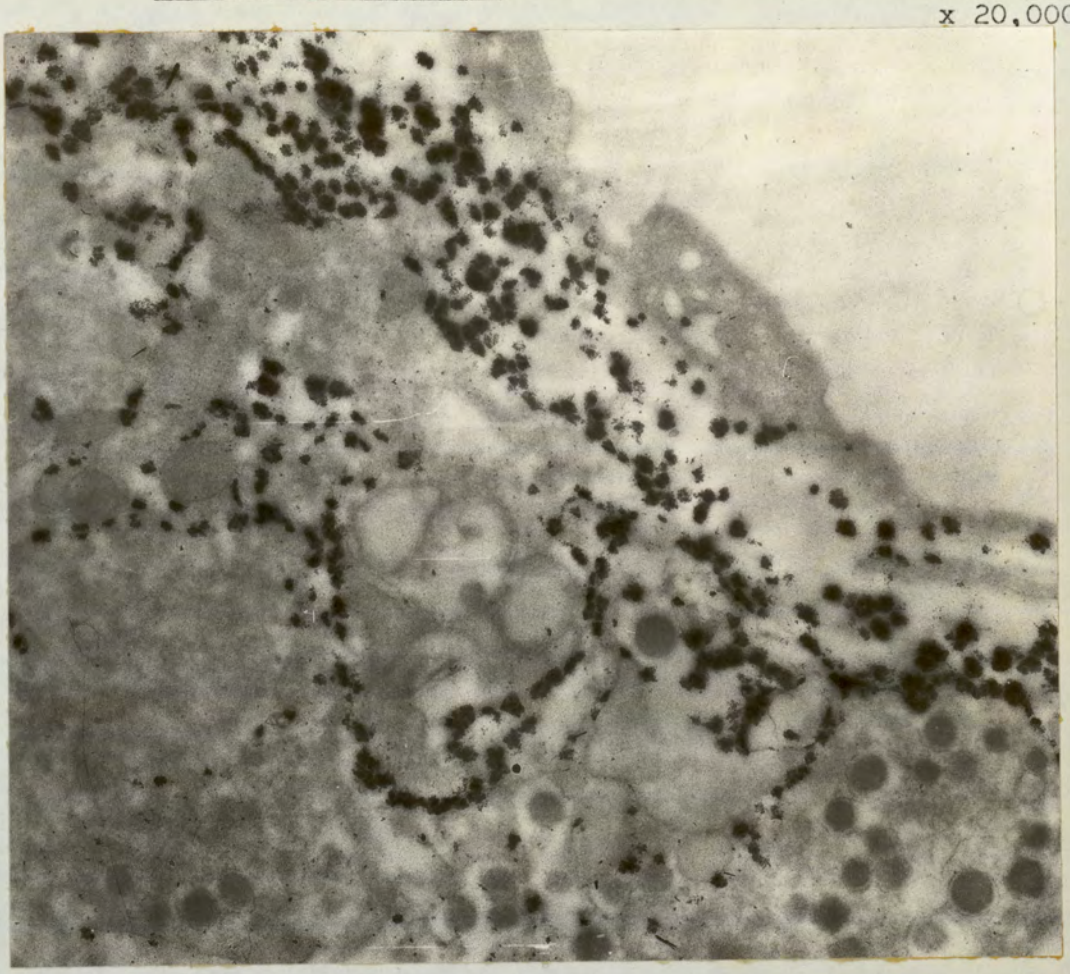


PLATE 14. Adenyl cyclase activity associated with the plasma membrane of an erythrocyte. (Normal mouse islet tissue, unstained, x 30,000).

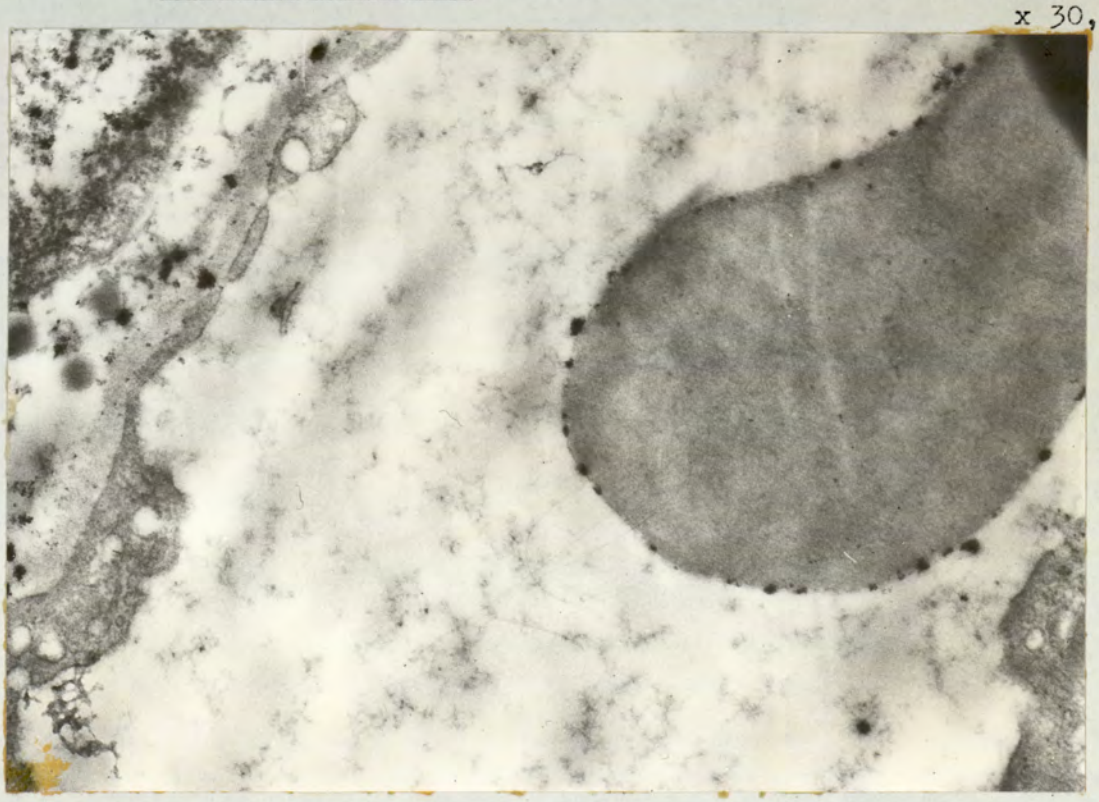
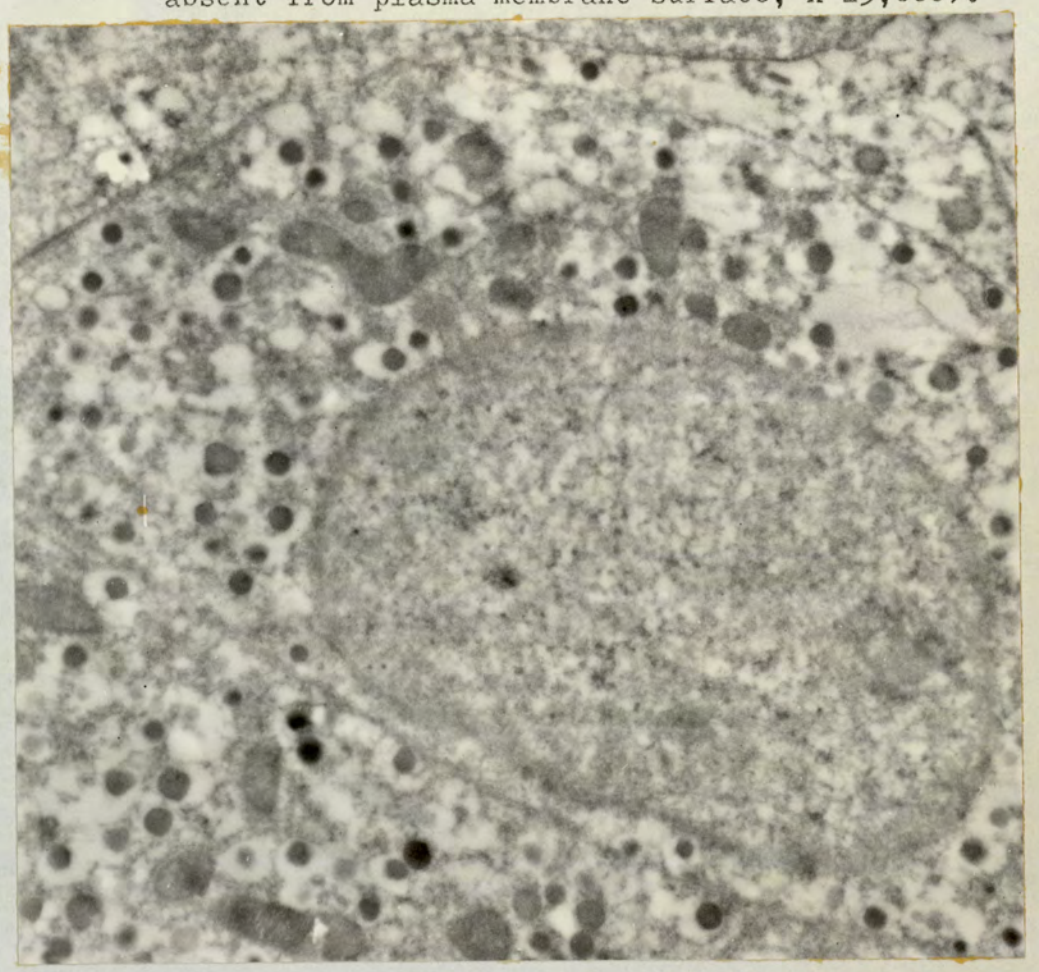


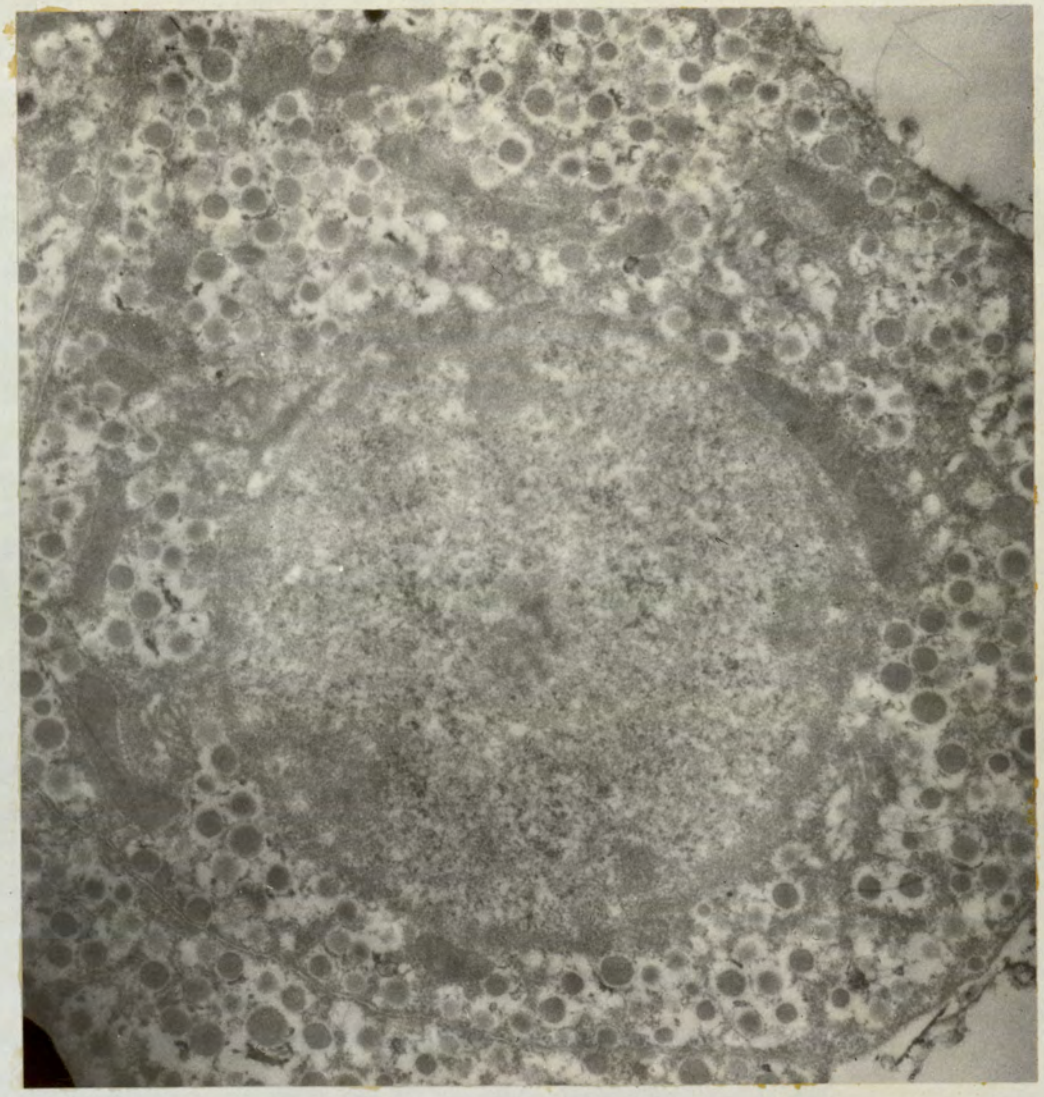
PLATE 15. Control section, showing β-cell from obese mouse islet. (No ATP in incubation medium. Adenyl cyclase activity absent from plasma membrane surface, x 15,000).

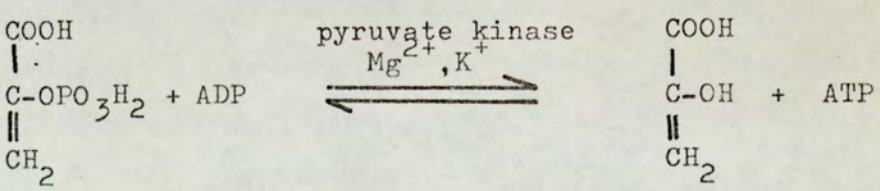


Total ATPase activity in normal and obese islets.

Total ATPase activity was demonstrated histochemically in ice cold acetone fixed cryostat sections of islet tissue from 20-25 week old normal and obese mice by Pearse's modification (327) of the lead method of Wachstein & Meisel (326). Brownish-black deposits indicated the sites of ATPase activity, Plates 17 & 18, and seemed to be limited to the connective tissue capsules and capillary networks of islet tissue. Only a very weak reaction was observed within islet cells and similar results have been obtained by Hellerstrom and coworkers (328). These observations suggested that appreciable ATPase activity would exist in whole islet homogenates. In the adenyl cyclase assay therefore, non specific ATP breakdown during incubations was reduced with an enzymic ATP regenerating system consisting of phosphoenolpyruvate and pyruvate kinase as shown over page.

PLATE 16. Control section, showing ∝-cell from a normal mouse islet. (No ATP in incubation medium. Adenyl cyclase activity absent from plasma membrane surface, x 15,000).





phosphoenolpyruvate. pyruvate.

Both Mg^{2+} and K^{+} were required for pyruvate kinase activity. In the adenyl cyclase assay the K^{+} was supplied via the homogenising solution.

Non-specific ATP breakdown by the islet homogenate was monitored in the adenyl cyclase assay in order to determine the optimal incubation time and the effectiveness of the ATP regenerating system.

PLATE 17. ATPase activity in islet and exocrine tissue of a 25 week old normal (NLL) mouse (x40).

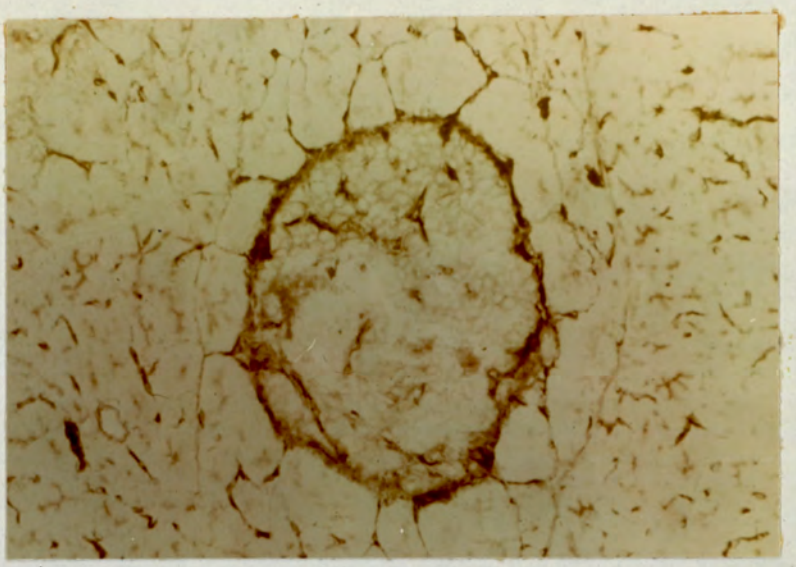
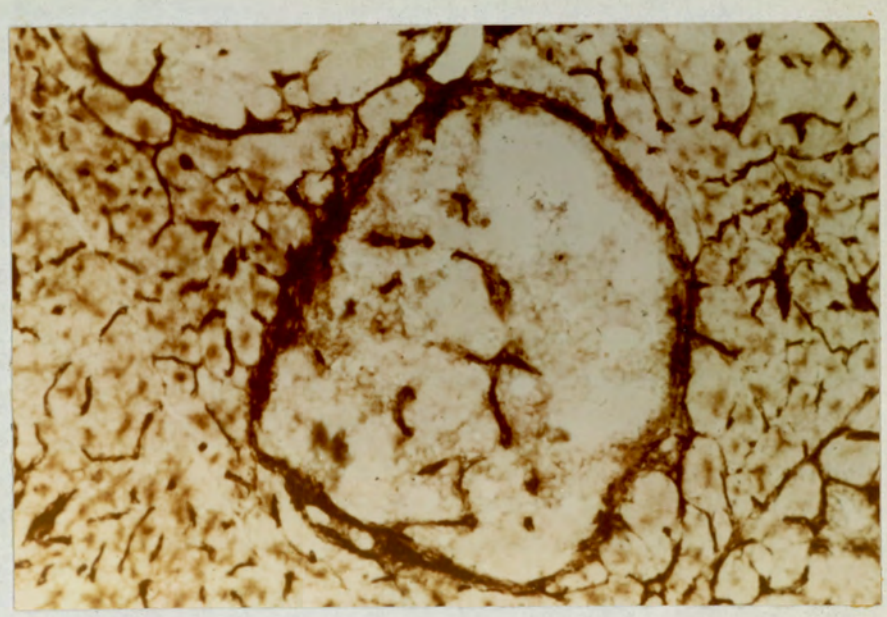


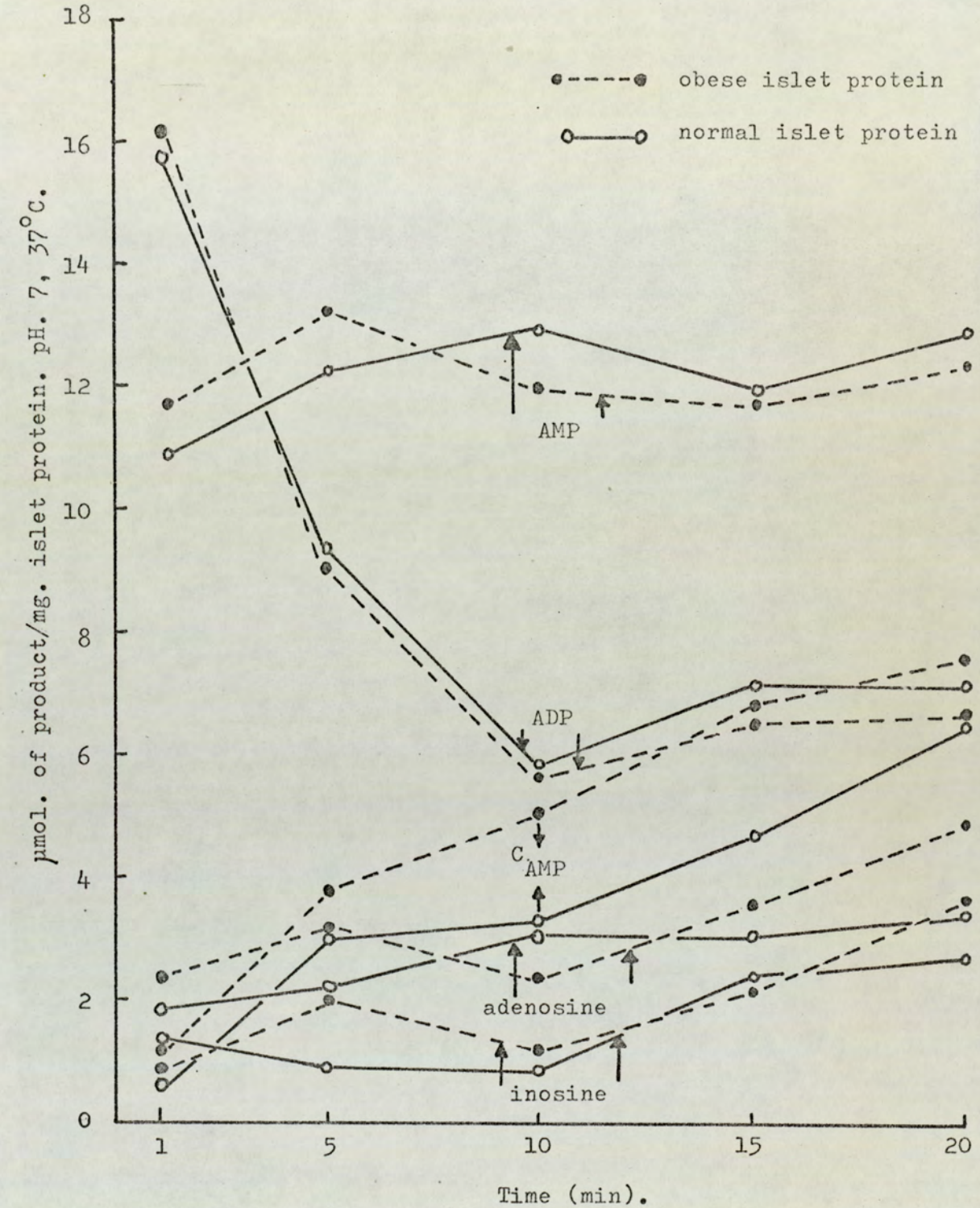
PLATE 18. ATPase activity in the islet and exocrine tissue of a 25 week old obese mouse (x40).



Adenyl cyclase incubations were carried out for 1-20 minutes using normal and obese islet protein, and after 1; 5; 10; 15 and 20 minutes, the amounts of labelled ADP; AMP; ^{c}AMP; IMP; adenosine and inosine produced were counted. A typical time course of ATP hydrolysis by normal and obese mouse islet adenyl cyclase at 37°C and pH. 7 is shown in Fig. 12.

The formation of ^{c}AMP had the basic characteristics of an enzyme reaction. The first part of the graph was somewhat non-linear and

FIG. 12. Time course of ATP hydrolysis by normal and obese mouse islet adenyl cyclase activity at 37°C and pH. 7.0.



probably the result of rapid ATP hydrolysis by ATPases competing for cyclase substrate in the first few minutes of the reaction (304). As soon as the ATP level in the reaction mixture stabilised in response to the ATP regenerating system, ^{c}AMP formation became almost linear. With both normal and obese islet tissue ^{c}AMP production increased almost linearly with time over a period of 20 minutes, while the increases in adenosine, inosine and AMP were more gradual. High ADP levels

resulting from high initial ATPase activity were reduced by the ATP regenerating system to a stable level after 10 minutes. 10 minutes was chosen as the optimum incubation time for subsequent adenyl cyclase assays, a point in time prior to the appearance of significant amounts of adenosine and inosine in the incubation medium. However, even with the preservation of ATP by the regenerating system 5'AMP; ADP; adenosine and inosine were always formed.

The amount of ATP hydrolysed during incubations, found by summing the activities of the respective metabolites was found to be independent of the initial substrate ATP concentration for up to 20 minutes of incubation. Consequently in the presence of the ATP regenerating system only a very small part of the substrate ATP was being utilised to produce ^{c}AMP and the rest of the metabolites. By summing the activities (d/sec) of ATP; ADP; AMP; ^{c}AMP adenosine and inosine (IMP co-chromatographed with ATP) at the different time intervals shown on Fig. 12, for both normal and obese islet tissue and then comparing these values with the value (d/sec) obtained by counting substrate ^{3}H-ATP in 50µl. of incubation medium added directly into a scintillation vial without incubation, a measure of the total % radioactive yield (or conversely a measure of the % radioactivity lost during incubation and chromatography) was evaluated. The average radioactive yield after chromatography was found to be 66%, or 34% of the added radioactivity was lost during the incubation and chromatographic procedures.

b) The effect of pH. on adenyl cyclase activity.

Adenyl cyclase assays using obese islet protein only were carried out with tris-HCl buffers generating pH. values of 6; 7; 8 and 9 at 37°C. The pH. of the potassium bicarbonate used for islet homogenisation was adjusted to the appropriate pH. under test with N/10 HCl or NaOH. Incubations were carried out for 10 minutes and blanks without protein were used for each pH. The adenyl cyclase activity of obese islet

FIG.13. The effect of pH. on obese islet adenyl cyclase activity.

(each point represents the mean of 3 determinations, ± S.E.)

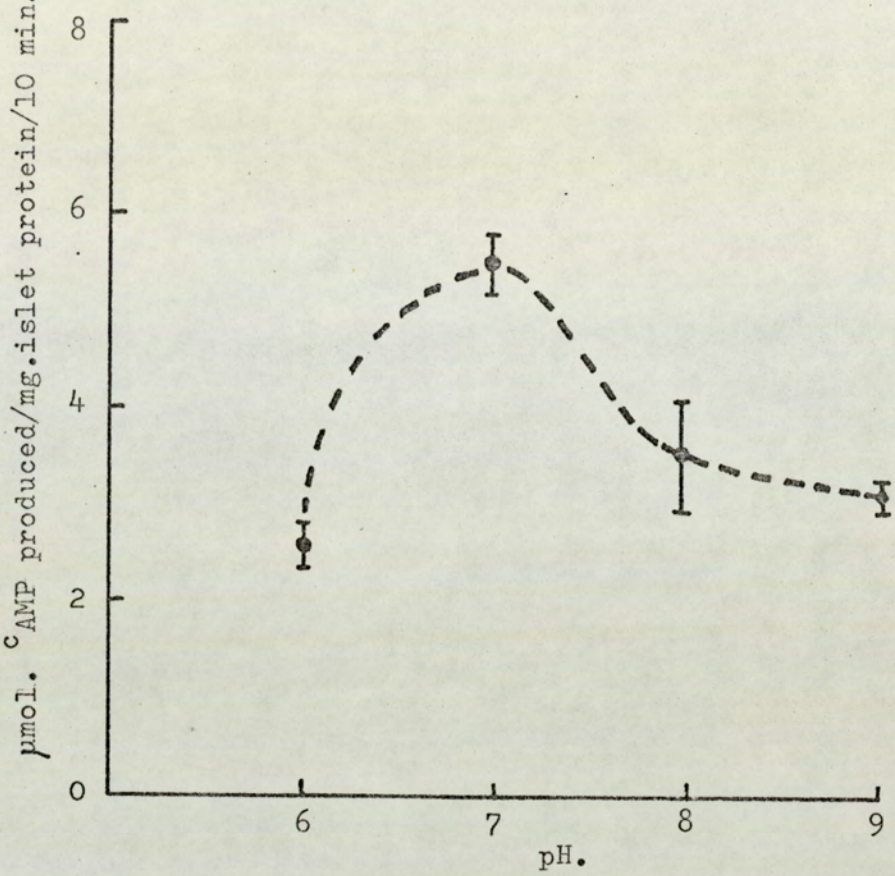
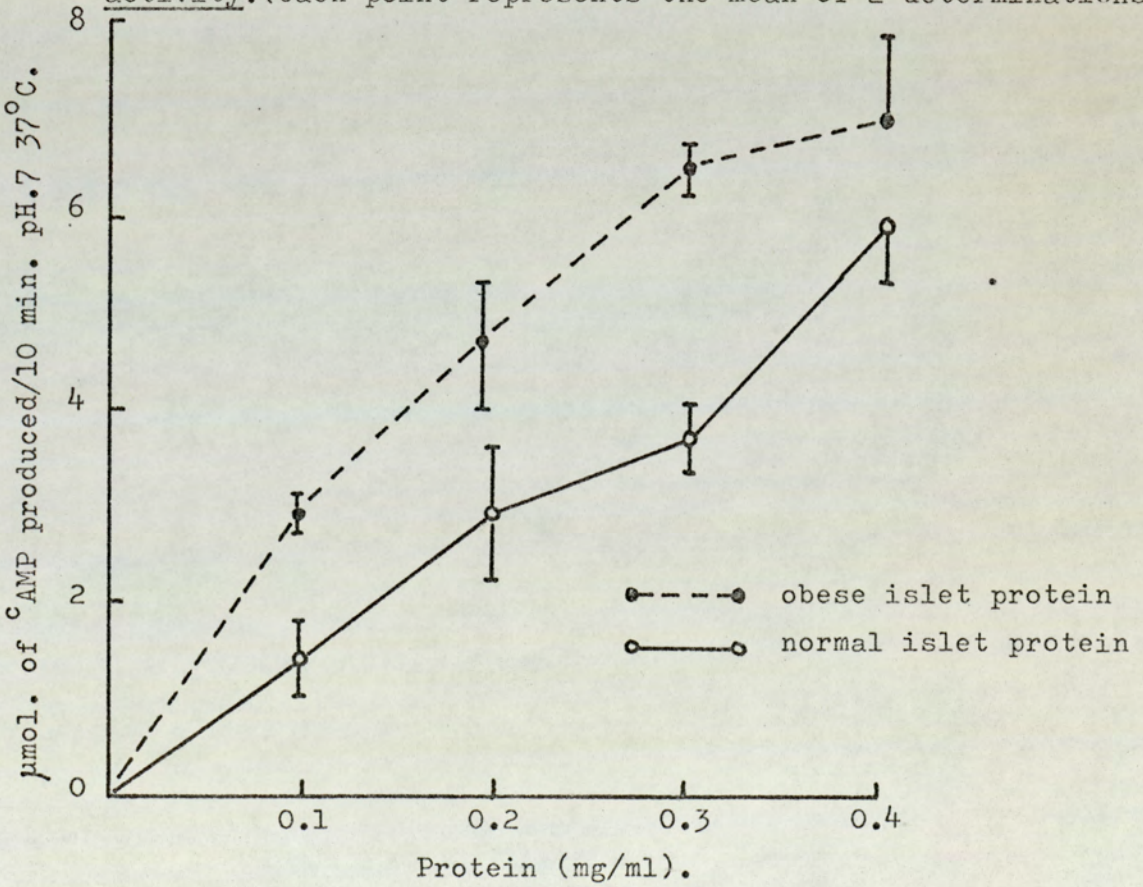


FIG.14. The effect of islet protein concentration on adenyl cyclase activity.(each point represents the mean of 2 determinations, ± S.



protein at the respective pH. values is shown in Fig. 13. Obese islet adenyl cyclase activity had a pH. optimum of about 7 at 37°C.

c) The effect of islet protein concentration on adenyl cyclase activity.

At the optimum pH. and incubation time adenyl cyclase activity was found to be dependent on islet protein concentration. The tissue : volume ratio (1 : 9) used in homogenisation gave an adequate working volume from which initial protein concentrations could be prepared. The effect of islet protein concentration on adenyl cyclase activity at pH. 7.0 and 37°C is shown in Fig. 14. Adenyl cyclase assays were carried out as previously described using an incubation time of 10 minutes. The protein concentrations used were estimated and rechecked by Lowry's method as described previously (218). Blank assays contained no protein. Over the range used adenyl cyclase activity increased almost linearly with protein concentration. However, at any one concentration the activity of obese islet protein was greater than that of normal islet protein.

d) Comparison of normal and obese islet adenyl cyclase activity.

Using the enzyme optima previously determined a comparison was made between the adenyl cyclase activity of normal and obese islets. Also the effect of sodium fluoride (NaF) was investigated on the adenyl cyclase activity of obese mouse islet protein. The results of these investigations are shown in Table 14. Adenyl cyclase activity was found to be significantly higher in islets from obese mice than in islets from normal mice. NaF (10mmol/litre) significantly stimulated obese islet adenyl cyclase activity (+33.4%), while higher concentrations (20mmol/litre) had no effect.

e) The effects of glucose and pharmacological agents on normal and obese islet adenyl cyclase activity.

The results of these investigations have been summarised in Table 14. The number of animals used (n) for each determination was very low. It was considered more important at the time of

investigation to use the limited number of animals available, to investigate the effect of as large a range of materials as possible on islet adenyl cyclase activity rather than to obtain more satisfactory statistical analyses and by doing so only investigate the effect of a few.

i. Glucose (1-4g/litre).

Glucose (2g/litre) significantly reduced the islet adenyl cyclase activity of both normal and obese mice, but had no significant effect at a concentration of 1 or 4g/litre.

ii. Glibenclamide (HB419). (Hoechst, U.K., Ltd.)

This potent oral antidiabetic sulphonylurea was found to be totally insoluble in tris-HCl buffer, and all other commonly used physiological media. It was found to be soluble, however, in N/50 sodium hydroxide and the pH. could be lowered to 8.7 before precipitation of the sodium salt. Consequently, when HB419 was dissolved in a minimum of N/50 sodium hydroxide and then made up to volume with tris-HCl buffer to give concentrations of 3 and 30 µg/ml., the resultant solution had a pH. of about 9, well above the optimum pH. for islet adenyl cyclase. Indeed, the adenyl cyclase activity at pH.9 was only 50% of the usual activity recorded at the optimum pH. value (Fig.13). However, islet adenyl cyclase activity was assayed under these conditions and blanks without islet protein, contained the appropriate concentration of HB419.

Even under conditions of elevated pH., HB419 (30µg/ml.) significantl elevated normal and obese islet adenyl cyclase activity above the usual control values, Table 14. Normal islets demonstrated a greater percentage stimulation of their adenyl cyclase activity than did obese islets and in consequence normal islet cyclase was assumed to be the more sensitive to this concentration of HB419.

iii. Adrenaline (10^{-7} & 10^{-5}mol/litre).

Fresh adrenaline solutions were used in the absence of ascorbic acid. Adrenaline significantly increased adenyl cyclase activity in islets from both normal and obese animals. The greater percentage stimulatio

occurred at 10^{-5} mol/litre in both cases. Normal islet adenyl cyclase appeared more sensitive to adrenaline stimulation than obese in so far as both 10^{-7} and 10^{-5} mol/litre concentrations elevated the normal islet adenyl cyclase activity above the usual values, Table 14. In the presence of both concentrations of adrenaline, values for obese islet adenyl cyclase activity were significantly greater than the corresponding normal values.

iv. Phentolamine (10^{-4} & 10^{-2}mol/litre) in the presence of 10^{-5} mol/litre adrenaline.

Phentolamine (an α-adrenergic receptor blocking agent) allowed adrenaline to stimulate β-adrenergic receptors exclusively. Phentolamine (10^{-2}mol/litre) in the presence of adrenaline significantly increased cyclase activity in the islets of both normal and obese mice. The greater percentage stimulation was shown at 10^{-2}mol/litre phentolamine and was about x3 greater in islets from normal animals. The stimulatory effect of phentolamine (10^{-2}mol/litre) in the presence of adrenaline (10^{-5}mol/litre) exceeded the effect of adrenaline (10^{-5}mol/litre) alone in both types of islets. Again, normal islet adenyl cyclase seemed to be more sensitive to phentolamine stimulation than obese, as both 10^{-4} & 10^{-2}mol/litre concentrations in the presence of adrenaline significantly elevated normal islet cyclase activity above the usual levels. In the presence of 10^{-4}mol/litre phentolamine obese islet adenyl cyclase values were still significantly higher than normal islet values. However, no significant difference was observed at the higher phentolamine concentration.

v. Propranolol (10^{-4}& 10^{-2}mol/litre) in the presence of 10^{-5}mol/litre adrenaline.

L-propranolol (a β-adrenergic receptor blocking agent) allowed adrenaline to stimulate α-adrenergic receptors exclusively. Studies on several β-adrenergic receptor blocking agents have shown the adenyl cyclase blocking effect to be exerted by the levo-form only, the

dextro-form having little or no effect (273). L-propranolol in the presence of adrenaline reduced islet cyclase activity in both types of islets. The greater percentage reduction occurred at the higher propranolol concentration. The percentage reduction in normal islet adenyl cyclase activity was some x2 greater than that observed for obese islet adenyl cyclase. However, in the presence of both concentrations of propranolol, the cyclase activity of obese islets remained significantly higher than that of normal islets.

vi. Isoprenaline (10^{-6} & 10^{-4}mol/litre) in the presence of phentolamine (10^{-2}mol/litre).

Fresh isoprenaline solutions were used in the absence of ascorbic acid. This combination of drugs was designed to selectively stimulate β-adrenergic receptors. Isoprenaline (10^{-4}mol/litre) in the presence of phentolamine stimulated the adenyl cyclase activity of islets from both normal and obese mice. The greater percentage stimulation occurred at the greater isoprenaline concentration. The percentage stimulation was twice as great in islets from normal animals and exceeded the effect of adrenaline alone in both types of islets. In the presence of 10^{-4}mol/litre isoprenaline, but not 10^{-6} mol/litre, values for obese islet adenyl cyclase activity were significantly higher than the corresponding values for normal islets.

vii. Phenylephrine (10^{-6} & 10^{-4}mol/litre) in the presence of propranolol (10^{-2}mol/litre).

This drug combination was designed to selectively stimulate α-adrenergic receptors and effected a reduction of adenyl cyclase activity in both types of islets. The greater percentage reduction occurred at 10^{-4}mol/litre phenylephrine with islets from both normal and obese animals. At both phenylephrine concentrations, values for obese islet cyclase activity were significantly higher than the corresponding values for normal islets.

In summary, results suggested that the stimulation of islet

TABLE 14. Effect of metabolic and pharmacological agents on the adenyl cyclase activity of islet tissue from obese hyperglycaemic mice and their normal lean littermates.

(Mean values ± S.E., one determination per mouse from homogenised islets).

	N	Normal islets (μmol cAMP produced/mg islet protein/10min, pH.7, 37°C)	Obese islets	% deviation from usual values	
				Normal	Obese
Usual Value	7	2.98 ± 0.17 ✝	4.65 ± 0.25		
NaF					
10mmol/litre	3	-	6.21 ± 0.42 *		+33.4
20mmol/litre	3	-	4.56 ± 0.46		- 1.96
HB419					
3μg/ml.	3	3.59 ± 0.11 ‡	4.25 ± 0.22 ‡	+20.56	- 8.61
30μg/ml.	3	4.90 ± 0.05 * ✝	6.08 ± 0.12 *	+64.70	+30.79
Glucose					
1g/litre	3	2.73 ± 0.38	4.13 ± 0.39	- 8.39	-11.22
2g/litre	3	1.91 ± 0.08 *	3.31 ± 0.01 *	-35.82	-28.78
4g/litre	3	2.71 ± 0.48	4.09 ± 0.44	- 9.11	-12.17
Adrenaline					
10^{-7}mol/litre	3	3.82 ± 0.08 * ✝	5.64 ± 0.42	+28.35	+21.29
10^{-5}mol/litre	3	3.94 ± 0.27 * ✝	5.70 ± 0.10 *	+32.43	+22.48
Phentolamine + Adrenaline (10^{-5}mol/litre)					
10^{-4}mol/litre	3	3.79 ± 0.12 * ✝	4.96 ± 0.24	+27.24	+ 6.57
10^{-2}mol/litre	3	5.43 ± 0.07 * ‡	5.89 ± 0.41 *	+82.51	+26.51
Propranolol + Adrenaline (10^{-5}mol/litre)					
10^{-4}mol/litre	3	2.66 ± 0.41 ✝	4.37 ± 0.27	-10.59	- 6.00
10^{-2}mol/litre	3	2.00 ± 0.01 * ✝	3.48 ± 0.30 *	-32.85	-15.25
Isoprenaline + Phentolamine (10^{-2}mol/litre)					
10^{-6}mol/litre	3	3.53 ± 0.47	4.73 ± 0.02 ‡	+18.70	+ 1.59
10^{-4}mol/litre	3	4.42 ± 0.11 * ✝	5.94 ± 0.42 *	+48.38	+29.78
Phenylephrine + Propranolol (10^{-2}mol/litre)					
10^{-6}mol/litre	3	2.79 ± 0.21 ✝ ‡	3.82 ± 0.18	- 6.14	-17.87
10^{-4}mol/litre	3	1.99 ± 0.20 * ✝	3.36 ± 0.33 *	-33.23	-27.83

* Denotes significant difference ($P<0.05$) between treated values and usual values for both normal and obese islets.

✝ Denotes significant difference ($P<0.05$) between normal and obese islet values subjected to the same treatment.

‡ Denotes significant difference ($P<0.05$) between the effects of different concentrations of a single test material on cyclase activity, for both normal and obese values.

Coefficients of variation for sets of results ranged between 5%and 15%.

α- adrenergic receptor(s) reduced adenyl cyclase activity, while stimulation of islet β-adrenergic receptor(s) increased adenyl cyclase activity.

f). The effect of membrane active agents on the adenyl cyclase activity of normal and obese mouse islets.

Adenyl cyclase has been shown to be a membrane bound enzyme in a variety of tissues (245), including normal and obese mouse islets. Because of the limited amount of tissue available, it has so far proved impossible to obtain an homogenous islet cell membrane preparation in sufficient concentration to facilitate biochemical analysis. In consequence, investigation of the effects of membrane active agents on islet adenyl cyclase activity was carried out using whole islet homogenates. The effects of calcium, alloxan, ouabain and phloridzin on normal and obese mouse islet adenyl cyclase activity have been summarised in Table 15.

1) Calcium (0.1, 1.0 and 5mmol/litre).

Calcium was added in the form of $CaCl_2$(anhydrous) to the tris HCl. of the cyclase incubation medium. The calcium concentrations used, supplemented, but were greatly in excess of the normal levels of tissue calcium already present in the islet homogenates. The effect of calcium added inadvertently to incubations via the many assay reagents was accounted for by the blank vials.

1.0 and 5.0mmol/litre calcium significantly reduced both normal and obese mouse islet adenyl cyclase activity, when compared with the usual values, Table 14, while 0.1mmol/litre calcium had no significant effect. In the case of both normal and obese islets, the greatest percentage reduction of adenyl cyclase activity occurred at the highest calcium concentration, but the percentage reductions observed in the presence of all three calcium concentrations were marginally more pronounced in obese islets than in normal.

TABLE 15. Effect of membrane active agents on the adenyl cyclase activity of islet tissue from obese hyperglycaemic mice and their normal lean littermates. (Mean values ± S.E; one determination per mouse from homogenised islets).

	N	Normal islets (μmol. of cAMP produced/mg islet protein/10min, pH 7, 37°C	obese islets	% deviation from usual values Table 14. Normal	Obese
Calcium.					
0.1mmol/litre	3	3.08 ± 0.37ø	4.60 ± 0.58ø	+3.49	-1.12
1.0mmol/litre	3	2.04 ± 0.29*	2.81 ± 0.43*	-31.37	-39.70
5.0mmol/litre	3	1.67 ± 0.41*	1.93 ± 0.48*	-43.98	-58.46
Alloxan (in vivo).				(% deviation from untreated alloxan values	
Untreated	3	2.66 ± 0.43†	4.23 ± 0.26		
Treated	3	1.91 ± 0.39†	3.59 ± 0.12	-28.21	-15.14
Alloxan (in vitro).					
10^{-4}mol/litre	3	1.34 ± 0.46*	1.35 ± 0.11*	-55.11	-71.04
10^{-3}mol/litre	3	0.73 ± 0.19*	1.49 ± 0.25*	-71.51	-67.95
Ouabain.					
10^{-5}mol/litre	3	2.03 ± 0.37*	3.24 ± 0.39*	-31.74	-30.45
10^{-3}mol/litre	3	1.47 ± 0.36*	2.47 ± 0.32*	-50.66	-46.83
Phloridzin.					
2 x 10^{-5}mol/lit.	3	2.88 ± 0.23‡	3.56 ± 0.18*	- 3.18	-23.56
10^{-3}mol/litre	3	1.65 ± 0.25*†	3.17 ± 0.21*	-44.72	-31.87

* Denotes significant difference ($P<0.05$) between treated values and usual values (Table 14) for both normal and obese islets.

† Denotes significant difference ($P<0.05$) between normal and obese islet values subjected to the same treatment.

‡ Denotes significant difference ($P<0.05$) between the effects of different concentrations of a single test material on cyclase activity, for both normal and obese values.

ø Denotes significant difference ($P<0.05$) between calcium concentrations of 0.1 and 5 mmol/litre for both normal and obese islets.

Coefficients of variation for sets of results ranged between 15% and 30%.

Calcium (K_i = 0.14mmol/L) has been shown capable of inhibiting pyruvate kinase activity (329,330) and consequently of reducing the effectiveness of an ATP regenerating system. However, the calcium concentrations used in the cyclase assays did not noticeably effect values obtained for blank vials containing no protein.

ii) Adenyl cyclase activity of islet tissue from alloxanised normal and obese mice.

After fasting for 24h. (water was given ad libitum), 20-25 week old mice were alloxanised according to the method of Mayer (331). An i.p. injection of alloxan monohydrate (5mg. per animal) was given in 0.2ml. of citric acid buffer, pH.4 (McIlvaine). Control animals were injected with buffer only. Blood sugar samples were taken from the tail (156) of control and alloxanised animals on the 5th day after injection. Test and control animals were culled on the 6th day, and adenyl cyclase activity measured in microdissected islet tissue by the usual procedure. Different animals were alloxanised over a period of several weeks.

TABLE 16A. Mean blood sugar values of control and alloxanised normal and obese mice. (Mean values ± S.E.)

(Blood sugar was measured by Boehringer glucose oxidase metho

	(No. N of mice)	Normal mice (mg/100ml.)	Obese mice (mg/100ml.)
Control Animals	3	85 ± 7	299 ± 20
Alloxan treated animals. (5mg/animal)	3	175 ± 12	204 ± 6

The differences between control and alloxanised normal and obese mice were significant ($P<0.05$). The reduced blood sugar values (compared to control values) shown by the alloxanised obese animals, suggested some sort of resistance to alloxan, probably by absorption into fat depots. A similar lowering of the blood sugar in alloxanised obese animals has been observed by Mayer (331).

Adenyl cyclase activity was found to be significantly higher in islets of obese mice than in islets of normal mice, whether untreated or alloxanised. In vivo alloxan administration had no significant effect on the adenyl cyclase activity of either normal or obese mouse islet tissue.

iii) The effect of alloxan in vitro on the adenyl cyclase activity of normal and obese mouse islet tissue.

Alloxan monohydrate was added to the tris-HCl of the incubation medium to give final concentrations of 10^{-4} and 10^{-3} mol/litre. These concentrations of alloxan significantly reduced the adenyl cyclase activity of both normal and obese islet tissue when compared with the usual values given in Table 14. The percentage reductions were similarly high for both normal and obese islets.

iv) The effect of ouabain on the adenyl cyclase activity of normal and obese mouse islet tissue.

Ouabain octahydrate, a crystalline glycoside has been shown to be irregularly and poorly absorbed in vivo, but to be a specific inhibitor of cation transport at low concentrations in vitro (332,333). This effect was used to distinguishbetween the active and passive transport of water and cations across the cell membrane. Ouabain has been shown to inhibit the activity of the Na^+/K^+ ATPase system in a variety of tissues (334). This inhibition was reversed by an increase in the K^+ concentration (335). However, ouabain has been shown to have little effect on the active transport of cations when they are added to the inside of the cell membrane (336).

The effect of ouabain on the islet adenyl cyclase activity of both normal and obese mice was investigated in the presence of low K^+ concentration. The latter originated from the islet homogenising solution. A very slight turbidity was observed with the higher concentration of ouabain (10^{-3}mol/litre) when added to the tris-HCl buffer of the incubation medium at 4^{o}C. Ouabain at both concentrations

significantly reduced the adenyl cyclase activity of normal and obese islets, when compared with the usual values Table 14. The greater percentage reduction of cyclase activity was observed at the higher ouabain concentration for both normal and obese islets.

v) The effect of phloridzin on the adenyl cyclase activity of normal and obese islets.

Phloridzin has been shown to inhibit the transport of hexoses and other simple carbohydrates across renal tubules (337) and pieces of hamster small intestine (338). Phloridzin, like ouabain, was shown to inhibit cell membrane Na^+- K^+ requiring ATPase activity and this observation suggested that the inhibition of sugar transport by phloridzin might be mediated in part via an effect on Na^+ dependent aspects of that transport system (339,340). Keller & Lotspeich have shown that high concentrations of phloridzin influence the energetics of tissue metabolism (341). While low concentrations of phloridzin (2×10^{-5}mol/litre) proved sufficient to inhibit glucose transport without affecting the endogenous metabolism of rat intestinal mucosa, higher concentrations also inhibited endogenous metabolism (342). Phloridzin was assumed to inhibit glucose absorption by acting on a mechanism responsible for the passage of glucose across the membrane on the luminal side of the mucosal cell.

The effect of phloridzin (BDH, Poole, Dorset) at concentrations of 2×10^{-5}mol/litre and 10^{-3}mol/litre was investigated on the adenyl cyclase activity of normal and obese islets. The higher concentration produced a slight turbidity in the tris-HCl buffer at 4°C.

Results showed that at concentrations of phloridzin sufficient to inhibit glucose transport (2×10^{-5}mol/litre), only obese islet adenyl cyclase activity was significantly reduced below the usual values shown in Table 14. However, at the higher concentration of phloridzin, known to inhibit endogenous metabolism as well as glucose transport, the adenyl cyclase activity of both normal and obese islets was significantly

reduced. In the presence of 10^{-3}mol/litre phloridzin the adenyl cyclase activity of obese islets was still significantly higher than the corresponding value recorded for normal islets, although no such difference was observed at the lower phloridzin concentration.

Blank values in phloridzin assays were a little higher than normal and it seemed possible that phloridzin might have inhibited some of the non-specific ATPase activity of the islet homogenates, better preserving substrate ATP for conversion to ^{c}AMP. By doing this phloridzin would have supplemented the effect of the ATP regenerating system in the cyclase assay. However, this action of phloridzin would not necessarily have provided increased ^{c}AMP levels, since islet adenyl cyclase had already been shown to be saturated by the substrate ATP concentration used in the cyclase assay and previous work had demonstrated ^{c}AMP production to be independent of the substrate ATP concentration. On this basis the reduction in islet cyclase activity produced by phloridzin was probably due to a direct effect upon islet adenyl cyclase per se.

The assumption that islet homogenate adenyl cyclase activity could be equated with islet cell membrane adenyl cyclase activity and that any change in the former reflected a change in the latter, was not strictly accurate. Recent work has shown that adenyl cyclase activity was not limited to the plasma membrane only, but could also be assayed in nuclei (343,344) and in mitochondrial and microsomal fractions of cells (345).

g) The effect of certain insulinogenic amino acids and protein hormones on the adenyl cyclase activity of normal and obese islet tissue (Table 16B).

i) L-Leucine and L-Arginine (0.5 & 5.0mmol/litre).

The respective amino acid was added to the tris-HCl buffer of the cyclase incubation medium. Blank controls contained the appropriate concentration of amino acid but no islet protein.

Neither leucine nor arginine had any significant effect on the adenyl cyclase activity of either normal or obese islets. In the presence of 0.5mmol/litre leucine and of 5mmol/litre arginine, obese islet adenyl cyclase activity was significantly higher than the corresponding value for normal islets (Table 16B).

ii) Glucagon (10-20µg/ml; Sigma)

Glucagon had a history of rapid degradation *in vitro* by tissue homogenates. Its integrity could be preserved *in vitro*, but only with great expense, by using the Kallikreen inhibitor Trasylol® (Bayer U.K.). Economics did not permit the use of this material in adenyl cyclase incubations and in an attempt to offset *in vitro* degradation, relatively high concentrations of glucagon were employed. These concentrations of exogenous glucagon, supplemented, but were greatly in excess of the normal islet tissue level of the hormone. The appropriate quantity of glucagon was first dissolved in a minimum of N/10 HCl and then made up to concentration with Tris-HCl buffer. The pH. of the glucagon solution was readjusted to 7 with Tris buffer (Fison

10µg/ml. glucagon significantly increased normal islet adenyl cyclase activity compared to the usual values Table 14, but had little effect on obese islet activity. However, 20µg/ml. glucagon significantl increased obese islet adenyl cyclase activity, but had little effect on normal islet activity (Table 16B). A greater percentage stimulation of normal islet adenyl cyclase activity was observed in the presence of 10µg/ml. glucagon, than was observed for obese islet adenyl cyclase in the presence of 20µg/ml glucagon. Normal islet adenyl cyclase was assumed to be the more sensitive and to have a lower threshold of stimulation to glucagon than obese islet adenyl cyclase. Only in the presence of 20µg/ml. glucagon were values for obese islet adenyl cyclase activity significantly greater than the corresponding normal islet values.

TABLE 16B. The effect of insulinogenic amino acids, protein hormones and animal age on the adenyl cyclase activity of normal and obese mouse islets. (Mean values ± S.E., one determinatio per mouse from homogenised islets).

	N	Normal islets (µmol. of ^{c}AMP produced/mg. islet protein/10 min. pH.7.0 37°C).	Obese islets	% deviation from usua values table 14. Normal	Obese
L-Leucine					
0.5mmol/litre	3	2.99 ± 0.20†	4.17 ± 0.29	+0.53	-10.27
5mmol/litre	3	3.10 ± 0.38	4.01 ± 0.34	+4.23	-13.83
L-Arginine					
0.5mmol/litre	3	2.95 ± 0.38	4.72 ± 0.23	-0.95	+1.35
5mmol/litre	3	2.88 ± 0.20†	4.61 ± 0.41	-3.17	-1.01
Glucagon					
10 µg/ml.	3	4.27 ± 0.38*	5.62 ± 0.77	+43.56	+20.82
20 µg/ml.	3	3.41 ± 0.28†	6.18 ± 0.34*	+14.62	+32.92
Crude Secretin					
1 Crick unit	3	3.53 ± 0.47†	5.55 ± 0.21	+18.70	+19.39
10 Crick units	3	3.49 ± 0.27†	5.63 ± 0.37	+17.22	+21.05
Age					
10-15 weeks	3	1.72 ± 0.10†‡	4.01 ± 0.20	-42.13	-13.83
20.25 weeks	7	2.98 ± 0.17†ø	4.65 ± 0.25	Usual Values	
40-45 weeks	3	3.10 ± 0.34	3.95 ± 0.36	+4.23	-15.02

* Denotes significant difference ($P<0.05$) between treated values and usual values (Table 14) for both normal and obese islets.

† Denotes significant difference ($P<0.05$) between normal and obese islet values subjected to the same treatment.

‡ Denotes significant difference ($P<0.05$) between normal islet 10-15 week values and 40-45 week values.

ø Denotes significant difference ($P<0.05$) between normal islet 10-15 week values and 20-25 week values.

Coefficients of variation for sets of results ranged between 13% and 16%.

iii) The effect of a crude secretin preparation on the adenyl cyclase activity of normal and obese islets.

The effects of 1 and 10 crick units/ml. of crude secretin (346) (sterile powder, 20 crick units/mg, Grade III, Sigma) in tris-HCl buffer were investigated on the adenyl cyclase activity of normal and obese islet tissue. The crude secretin preparation contained between 25% and 30% pancreozymin.

Neither 1 nor 10 crick units/ml. of crude secretin had any significant effect on the adenyl cyclase activity of either normal or obese islet tissue, when compared to the usual values Table 14. However, the absolute values recorded for obese islet adenyl cyclase activity were significantly higher than the corresponding values recorded for normal islets in the presence of both concentrations of crude secretin (Table 16B).

Discussion

Cytochemical studies have shown adenyl cyclase activity to be associated with the plasma membrane and pars fibrosa (nucleolus) of β-cells and the plasma membrane of α-cells. The random sampling of tissue with regard to both the tissue blocks examined and the micrographs taken was assumed sufficient to eliminate sampling errors. Adenyl cyclase activity was limited to the outer cell layers of both normal and obese islets. This might have been due to inadequate penetration of islet tissue by the incubation medium after gluteraldehyde fixation. Reik and colleagues have shown that 1% gluteraldehyde itself did allow th survival of a fair proportion of adenyl cyclase activity (317). The demonstration of membrane bound adenyl cyclase activity in islet β-cells was consonant with its suggested role as an integral part of the β-adreno-receptor (242).

The reaction product was found on the outer surface of α and β-cell plasma membranes. As this deposition probably represented the site of pyrophosphate release and since the bulk of biochemical evidence

has indicated ^{c}AMP to be released on the inside of the cell membrane (242), it seemed possible that adenyl cyclase might release its two products on opposite sides of the plasma membrane. The rate of ^{c}AMP efflux into the external environment of the cell, however, may be a factor in determining the intracellular level of the nucleotide (347).

It was interesting to note the deposition of electron dense material in the pars fibrosae of the nucleoli of certain β-cells. The presence of adenyl cyclase activity in this region was consonant with the suggested role for ^{c}AMP in ribosomal RNA synthesis (320,321), enzyme induction (348) and possibly protein synthesis.

There were, however, a number of anomalies associated with the method used for the cytochemical localisation of adenyl cyclase activity. Adenyl cyclase was not the only α and β-cell membrane bound enzyme with the capacity to breakdown ATP and produce pyrophosphate. Most cell membranes contain ATPase, with a far greater capacity than adenyl cyclase to breakdown ATP. Indeed, micrographs have shown that the sites of adenyl cyclase activity were also potential sites of ATPase activity.

NaF (10mmol/litre) was added to incubations both to stimulate adenyl cyclase activity (245) and to inhibit the activity of all other Mg^{2+} dependent enzyme systems, including ATPases (298), by forming a complex with the metal (349). Adenyl cyclase has been reported to be the only Mg^{2+} dependent enzyme that is stimulated by NaF (245). However, this stimulation has been observed only with the adenyl cyclase of broken cell preparations (homogenates) (296). In fact the fluoride ion has not been shown to increase the levels of ^{c}AMP in intact avian erythrocytes (350) and like ATP did not traverse or penetrate cell membranes easily. In consequence the functional use of ATP and NaF in the cytochemical localisation of adenyl cyclase activity in intact tissue has yet to be justified. It may be that 1% gluteraldehyde treatment permitted the access of ATP and NaF to the cyclase system.

Plasma membrane bound adenyl cyclase activity was limited to cells of the islet periphery. Regions where one might expect the non-specific, non-enzymatic precipitation of lead salts during incubation. This precipitation could have resulted from chemical reactions between impurities and substrate at the elevated temperature. However, non-specific precipitation would have led to the appearance of large deposits of electron dense material in islet capillaries. This was never observed, indeed the deposition in capillaries was specifically localised to erythrocyte plasma membranes (Plate 14).

In support of the cytochemical evidence, radiometric analyses have also demonstrated the presence of an adenyl cyclase system in normal and obese mouse islet tissue, operating at a pH. similar to that found in other tissues (297,304,351). Enzyme activity was proportional to both incubation time and protein concentration and found to be higher in obese islets than in normal. Obese islet cyclase activity was significantly increased in the presence of fluoride. Although cyclase activation by fluoride has been demonstrated with a variety of tissues (239,297,304,352), the actual mechanism has yet to be elucidated. Dousa & Rychlik (304) suggested that fluoride in addition to stimulating adenyl cyclase directly, without affecting the apparent K_m (297,298), maintained the level of substrate for adenyl cyclase by blocking the breakdown of both ATP and ADP. These two nucleotides have been shown to inhibit ^{c}AMP-phosphodiesterase (^{c}AMP-PDE) activity and hence contribute to the accumulation of ^{c}AMP (353), although fluoride itself did not inhibit ^{c}AMP-PDE activity (354). Fluoride might also act by the direct stimulation of adenyl cyclase. This was suggested by the fact that when an ATP regenerating system was used under conditions which preserved ATP to the same degree as fluoride, ^{c}AMP formation was less (304). The fluoride ion has been shown to inhibit ATPase activity and in this way might help to conserve the substrate concentration (298). The work of Drummond and colleagues

(298,300) on cardiac tissue adenyl cyclase has shown that the main effect of fluoride addition was to elevate the V_{max} of the reaction without effecting the K_m for the substrate (Mg^{2+}ATP). The elevation of the V_{max} was suggested to be a common feature of both the hormonal and fluoride stimulation of adenyl cyclase activity, perhaps reflecting an increased reactivity of the catalytic site with the substrate. Whether fluoride has a separate binding site or whether it binds as a magnesium fluoride complex has not yet been determined. In the case of islet tissue high concentrations of NaF (> 10mmol/litre) were without significant effect on adenyl cyclase activity. A similar observation was made by Drummond & Duncan (298) with high concentrations of fluoride. NaF would be a very acceptable alternative to the enzymatic ATP regenerating system used in the cyclase assay. However, it could only be used for assays investigating non-hormonal activation, since fluoride has been shown to prevent hormonal stimulation of cyclase activity (355).

Theophylline and other closely related methylxanthines have been widely used to inhibit ^{c}AMP-PDE activity in adenyl cyclase assays. However, both Bär & Hechter (297) and Drummond & Duncan (298) have shown that the addition of cold ^{c}AMP to adenyl cyclase assays was a more effective way of preserving the labelled product. When cold ^{c}AMP and theophylline were added together the recovery of labelled ^{c}AMP was almost 100% (298). On this basis both materials were included in the standard assay medium used for the measurement of islet adenyl cyclase activity.

The chromatographic separation of metabolites in the assay for adenyl cyclase, although thought comprehensive enough by some workers (297,298), was clearly limited. IMP could not be separated from ATP in the solvent systems used and no account was made of the vast number of other metabolites capable of being produced from the enzymatic degradation of ATP (Figure 11). For a more comprehensive separation,

lengthy chromatographic procedures were required involving the sewing of paper chromatograms or quantitative elution (304). These techniques were not easily mastered and radioactive yields were often irregular and always reduced by the process.

Investigation of the effects of metabolic and pharmacological agents on islet adenyl cyclase activity demonstrated that glucose, believed to be the physiological stimulus for insulin biosynthesis and release both *in vivo* and *in vitro*, did not stimulate normal or obese islet adenyl cyclase activity. Indeed, there was evidence for a significant reduction in cyclase activity in the presence of 2g/litre glucose for both normal and obese islets. This reduced adenyl cyclase activity may have been the result of an increased osmolarity of the incubation medium, since marked changes in osmolarity due to increased glucose concentration have been shown to significantly influence cell membrane permeability (356) and perhaps membrane bound adenyl cyclase activity.

Glucose, believed to be effective in regulating insulin secretion as a consequence of its metabolism in the β-cell (126,133) may provide both the signal or signals that trigger the secretion mechanism and some of the factors necessary for the process to occur.

Turtle & Kipnis demonstrated that the concentration of ^{c}AMP in islet cells paralleled the effects of adrenaline and glucagon on insulin secretion and this suggested that ^{c}AMP may play a role in regulating this process (276). There has been much discussion as to the site of action of ^{c}AMP in the series of events that lead from the stimulation to the secretion of insulin from the β-cell. Several authors suggested that ^{c}AMP was an essential component of the secretion mechanism and that the concentration of ^{c}AMP in the β-cell might directly regulate the rate of insulin secretion *in vivo* (357) and *in vitro* (266). However, work by Montague & Cook has indicated that the maintenance of an increased concentration of ^{c}AMP in islet cells was

not necessary for the secretion of insulin in response to glucose from rat islets (287,358). Their results strongly suggested that the effect of glucose on the insulin secretion mechanism was not mediated by an increase in the concentration of ^{c}AMP in the β-cell, and so an increase in the concentration of ^{c}AMP could not be regarded as an essential component of the secretion mechanism. Further, since caffeine and theophylline produced significant increases in the concentration of ^{c}AMP but had less effect on insulin secretion than did 20mmol/litre glucose, which itself did not alter the ^{c}AMP concentration, it was assumed unlikely that small changes in the concentrations of ^{c}AMP were important in the regulation of insulin secretion. It has been suggested that ^{c}AMP might act indirectly on the secretory process by potentiating the effect of glucose (359) and Malaisse and coworkers were unable to obtain effects for glucagon and theophylline on insulin secretion unless glucose ($>$5mmol/litre) was also present in the incubation medium (41). However, it was thought unlikely that ^{c}AMP would mediate the effect of glucose on the secretion mechanism because the ^{c}AMP-PDE stimulator imidazole did not prevent the secretory response of the islet to glucose, even though it was thought to lower the intracellular concentration of ^{c}AMP (360). The work of Montague & Cook (287,358) and results from experiments with normal and obese mouse islets have suggested that there was no glucose effect on insulin secretion mediated via β-cell adenyl cyclase (285). However, results from work using islet homogenates did not rule out the possibility of glucagon, released in response to exogenous glucose, acting on the β-cell adenyl cyclase system and initiating insulin secretion. Glucagon released during homogenisation might have maximally stimulated islet β-cell adenyl cyclase activity, and as a consequence, exogenous glucose could not then be expected to further increase cyclase activity. However, if maximum cyclase stimulation did occur in response to released glucagon, then agents such as adrenaline would not have been expected to

stimulate islet adenyl cyclase activity (285). The whole picture is again complicated by the presence of free insulin in islet homogenates. This hormone has been reported to inhibit adenyl cyclase activity in rat liver plasma membranes (162). Also recent work by Basabe and colleagues using the perfused rat pancreas suggested that glucagon, ^{c}AMP and theophylline stimulated insulin secretion by acting on mechanisms different from the ones stimulated by glucose (361).

The role of adenyl cyclase, and the ^{c}AMP it produces may be to influence or even regulate the way in which glucose is metabolised in the β-cell, Although Montague & Cook have suggested that ^{c}AMP may modulate the secretion of insulin in response to glucose by an effect on the movement of granules in the β-cell (287).

The potent sulphonylurea HB 419 (30μg/ml) besides stimulating adenyl cyclase activity in both normal and obese mouse islets, has also been shown to be a potent stimulator of insulin secretion in vivo and in vitro (109,363). Like other sulphonylureas, HB 419 had beneficial effects on the numerical and functional deficiency of β-cells and was believed to act via the mobilisation of endogenous insulin. The hypoglycaemic action of sulphonylureas was believed to be produced by at least two mechanisms; stimulation of insulin secretion from the islets and less clearly defined extra-pancreatic effects (254). Evidence has suggested the action of HB 419 to be a direct effect upon insulin secretion and not a stimulation of the glucose metabolism in the β-cells. Mannoheptulose has been shown to inhibit the stimulation of insulin secretion obtained with glucose but not that obtained with the sulphonylurea tolbutamide and this has been considered to reflect a fundamental difference in the mechanisms by which these two compounds affect β-cell function (98). Sulphonylureas like several other insulin secretagogues have been shown to mobilise glycogen (130). The sulphonylurea stimulation of insulin secretion might be mediated by an increased degradation of glucose-6-phosphate and this would be

corroborated by the sulphonylurea depletion of islet β-cell glycogen stores.

A great deal of conflicting evidence has been reported concerning the possible effect of sulphonylureas on the β-cells adenyl cyclase syste Sirek and coworkers using dogs, demonstrated that HB419 stimulated the secretion of insulin from the pancreas by a mechanism which involved β-adrenergic receptor stimulation (362). In 1967, Robison, Butcher & Sutherland forwarded the hypothesis that the β-adrenergic receptor was likely to be identical with that part of the adenyl cyclasesystem that converted ATP to ^{c}AMP (242). Using this assumption Sirek and collaborators suggested that HB419 stimulated the secretion of insulin at least in part by activating the synthesis of ^{c}AMP through the adenyl cyclase system (362). In addition, Pozza and colleagues, while investigating tolbutamide induced insulin secretion in man recently suggested that this sulphonylurea induced the secretion of insulin by a mechanism involving the adenyl cyclase/^{c}AMP-phosphodiesterase system (364). However, the direct stimulation of adenyl cyclase by sulphonylureas might not be their only course of action. The integrity and physiological activity of ^{c}AMP in islet tissue would be preserved by sulphonylureas inhibiting islet ^{c}AMP-phosphodiesterase activity. Indeed Brooker & Fichman have shown chlorpropamide and tolbutamide to inhibit ^{c}AMP-phosphodiesterase activity in rat kidney homogenates (365). From initial work Malaisse and colleagues concluded that the intimate mode of action of sulphonylureas upon the β-cell was different from that of glucose (366). However, after comparing the effects of glycodiazine and HB419 on insulin secretion they observed that the stimulation of insulin secretion by sulphonylureas was more marked in the presence than in the absence of glucose (363). As a consequence of their work Malaisse and colleagues suggested that HB419 did not act upon the β-cell through the adenyl cyclase/^{c}AMP-phosphodiesterase system because the insulinotropic effect of sulphonylureas did not correspond to a

potentiation of glucose induced secretion comparable to that observed with ^{c}AMP and those agents which stimulated its synthesis or inhibited its breakdown.

Adrenaline (10^{-5}mol/litre), as in other tissues (257,367,368), increased the adenyl cyclase activity of islet tissue from both normal and obese mice. Normal islet adenyl cyclase was also significantly stimulated by a concentration of 10^{-7}mol/litre adrenaline and appeared to be more sensitive to this catecholamine than the cyclase of obese mouse islets. Turtle & Hagon have recently shown adrenaline to stimulate the adenyl cyclase activity of teleost pancreatic islet tissue (369). As a result of the adrenaline induced stimulation of adenyl cyclase activity one might have expected to find increased levels of ^{c}AMP in islet tissue. However, both Turtle & Kipnis (276) and Montague & Cook (287) have shown similar concentrations of adrenaline to markedly reduced rat islet ^{c}AMP levels. Adrenaline, besides stimulating islet adenyl cyclase activity, might have increased the capacity of islet cells to utilise or degrade ^{c}AMP and this would account for the low ^{c}AMP concentration found in rat islets after adrenaline treatment. Adrenaline might have induced an overall increase in the turnover of ^{c}AMP by stimulating both adenyl cyclase and ^{c}AMP-phosphodiesterase activity.

Investigationsof the effects of α and β-adrenergic receptor blocking agents on the adenyl cyclase activity of normal and obese islet tissue, together with the effectsof drug combinations specifically designed to stimulate discrete α and β adrenergic receptors, have shown the increase in islet adenyl cyclase activity to be a β-receptor function and the reduction an α-receptor function. A similar conclusion was drawn by Turtle & Hagan (369), when the β-blocker propranolol was shown to completely inhibit the adrenaline stimulation of teleost pancreatic islet adenyl cyclase activity. Normal mouse islet adenyl cyclase showed the greater responsiveness to adrenergic receptor

stimulation. The reduced pharmacological sensitivity of obese mouse islet adenyl cyclase might have been a characteristic of the obese mouse hyperglycaemic syndrome *per se.*

Turtle & Kipnis have also shown the effects of adrenaline on ^{c}AMP accumulation to be mediated via α and β-adrenergic receptor sites. Stimulation of the α-adrenergic receptor inhibited, and stimulation of the β-adrenergic receptor increased ^{c}AMP synthesis. Also, in rat islet tissue the effects of ^{c}AMP reproduced the effects of adrenaline when α-adrenergic receptor activity was blocked (276). Recent work by Burges & Blackburn (370), investigating the differential effects of β-adrenoreceptor agonists and antagonists on adenyl cyclase from rat heart and lung, has shown that the enzymes have the characteristics of β-1 and β-2 type adrenoreceptors, respectively. The presence of such adrenoreceptors in islet tissue has yet to be established.

The normal physiological effect of adrenaline on insulin secretion has been shown to be predominantly one of inhibition (the α-adrenergic receptor function) and this has been shown to occur in dogs *in vivo* (371) and in rabbit (98) and rat pancreas (267) *in vitro*. Adrenaline effectively inhibited both the initial transient secretion and the second more persistent phase of insulin secretion from islets of normal and obese mice *in vitro* (372). However, under certain conditions with the use of adrenergic blocking agents, Porte has shown that catecholamines can exert different effects on insulin secretion (258-260,373). α-Adrenergic receptor stimulation inhibited insulin secretion and β-adrenergic receptor stimulation promoted insulin secretion. Similar conclusions were drawn by Loubatières and coworkers while attempting to characterise the islet β-adrenergic receptor (374,375).

The parallel effect of α and β adrenergic receptor stimulation on islet adenyl cyclase activity, ^{c}AMP accumulation and insulin secretion *in vivo* and *in vitro* might suggest β-cell ^{c}AMP to be essential for the

process of insulin secretion and perhaps capable of modifying the rate of insulin release. However, work using diazoxide has indicated that this is by no means the whole picture. Diazoxide, while inhibiting both phases of insulin secretion (361,376,377) has been shown to inhibit hepatic ^{c}AMP-phosphodiesterase activity (378) and preserve ^{c}AMP levels. It was suggested by Levine (379) and substantiated by Creutzfeldt and coworkers (380) that diazoxide acted beyond the ^{c}AMP stage, actually on the β-granules, blocking their release by stimulating both the rate of formation of multigranular sacs and the intracellular digestion of β-granules. Diazoxide only inhibited the secretion of stored insulin and not the secretion of newly synthesised insulin (135). Montague & Cook showed that diazoxide like adrenaline reduced the concentration of ^{c}AMP in islet cells and inhibited the insulin secretory response to glucose (287). This was interpreted as indicating that a certain concentration of ^{c}AMP in the β-cell might be necessary for the secretion mechanism to function. However, it was possible that the effects of adrenaline and diazoxide on ^{c}AMP concentrations were secondary to other effects on β-cell metabolism and that the inhibition of insulin release was related to alterations in β-cell metabolism rather than to changes in the concentration of ^{c}AMP.

In vivo, adrenaline has been shown to inhibit selectively the rapid insulin response to glucose, but not to influence insulin output stimulated by prolonged hyperglycaemia (381). These observations provided further evidence for a model of insulin secretion which included a small storage pool available for immediate secretion in response to a glucose challenge and a more slowly responding pool regulating insulin secretion in the basal and steady state. Studies by Feldman & Lebovitz have suggested that amines and catecholamines which inhibit insulin release, do so by both an indirect action (release of endogenous pancreatic noradrenaline) and a direct effect of the agents themselves on the β-cell (382). They have recently shown

NH_4^+ ions,at concentrations normally found in blood,to inhibit insulin secretion from golden hamster pancreas stimulated by high glucose but not that stimulated by sulphonylureas (383). It has been suggested that the NH_4^+ ion modulates insulin secretion in physiological and pathological states probably by interfering with the metabolism of glucose in the islets.

Cytochemical evidence has shown islet adenyl cyclase activity to be limited to the plasma membranes of α and β cells and the pars fibrosae of the nucleoli of certain β-cells. In the pars fibrosa, ^cAMP, the product of adenyl cyclase activity, may be involved in the production of ribosomal RNA, enzyme induction and possibly protein synthesis. In support of the membrane localisation of adenyl cyclase, agents known to inhibit membrane bound enzyme systems or to interfere with the physical make up of cell membranes and consequently the materials that are transported across them, have been shown to reduce by similar proportions, the adenyl cyclase activity of both normal and obese islet tissue.

Extracellular Ca^{2+} ions have been shown to be essential for the glucose induced stimulation of insulin secretion from the perfused rat pancreas (384,385), incubated pieces of rabbit pancreas (386,387) and both foetal (388) and adult rat pancreas (389). Also, the glucose mediated action potentials demonstrated in islet cells by Dean & Matthews (390), were thought to be due to calcium influx. Hales & Milner demonstrated that the optimal calcium concentration for insulin secretion was in the region of 2.5 mmol/litre and that concentrations above this tended to reduce glucose stimulated insulin release (386). However, the optimal extracellular calcium concentration for secretion might have depended as importantly on experimental conditions as on the cell studied.

O.1mmol/litre calcium did not significantly effect islet adenyl

cyclase activity. But concentrations of calcium known to be capable of increasing glucose stimulated insulin secretion (1 and 5mmol/litre) in vitro (385-386), significantly reduced the adenyl cyclase activity of both normal and obese islets. Results obtained by many authors investigating the effect of calcium on adenyl cyclase activity have shown appreciable variability. Kipnis suggested that calcium was not essential for activation of the adenyl cyclase system but was required for the stimulation of hormone release (391). Burke, while investigating the cyclase activity of thyroid tissue, showed calcium (1 and 5mmol/litre) to produce a marked reduction in basal and stimulated adenyl cyclase activity. 1mmol/litre calcium induced a 40-60% reduction in cyclase activity and was without effect on the ATP regenerating system of the assay and ^{c}AMP-phosphodiesterase (392). Bär & Hechter, however, have demonstrated that trace amounts of calcium were required for the ACTH stimulation of adenyl cyclase in bovine adrenal membrane fractions as well as rat fat cell ghosts (345). Results from the work of other authors have shown that in the main, there may be a requirement for low calcium, but high concentrations inhibit (393). Any stimulatory effect of Ca^{2+}, irrespective of concentration, on adenyl cyclase activity, would have functional significance. The evidence for this has been reviewed by Rasmussen & Tenenhouse, who proposed that the function of adenyl cyclase was to either alter the permeability of the plasma membrane to Ca^{2+} or to effect its intracellular release from the membrane and subsequent translocation (394). The stimulation of membrane bound adenyl cyclase by protein hormones or suitable effectors might facilitate the entry of calcium into the cell, where it might act as a type of third messenger (395). Hales & Milner have suggested that a rise in the intracellular concentration of calcium may provide the signal for the release of insulin from β-cell granules (387). On the evidence presented above, agents capable of stimulating adenyl cyclase activity might be expected to effect Ca^{2+} transport through cell membranes.

However, Malaisse-Lagae and coworkers have shown the cyclase stimulator adrenaline, to inhibit the uptake of labelled calcium into isolated rat islets (396). At first sight and on the basis that adrenaline has been shown to inhibit insulin.secretion, this evidence would seem to dissociate adenyl cyclase from the mechanism of Ca^{2+} influx, but the latter might be a β-adrenergic receptor function of adenyl cyclase.

Kakiuchi & Yamazaki have demonstrated the stimulation of rat brain ^{c}AMP-PDE activity by Ca^{2+} and suggest that physiological concentrations of Ca^{2+} may regulate the activity of ^{c}AMP in the cell by regulating its concentration (397). The presence of a similar control system in islet tissue has not been substantiated, (Chapter 3, section 3).

Adenyl cyclase has been shown to have a high requirement for Mg^{2+} and to utilise Mg^{2+}ATP as its substrate (298). Assuming the influx of calcium to occur across the β-cell membrane independently of adenyl cyclase but in response to the physiological event occurring in the cell i.e. insulin secretion. This transport of calcium might considerably effect cyclase activity by acting competitively with Mg^{2+} for chelation to substrate ATP. On this basis insulin secretion could be associated with a reduction in adenyl cyclase activity. However, assuming the passage of calcium across the β-cell membrane to be dependent on ^{c}AMP induced membrane permeability; the competitive inhibition of Mg^{2+} dependent adenyl cyclase activity by calcium, would result in calcium restricting its own entry into the β-cell as a function of its own concentration. Insulin secretion would not then be a function of increased intracellular calcium, but the theory would accommodate the low calcium requirement of insulin secretion and islet adenyl cyclase activity.

The effects of alloxan on the adenyl cyclase activity of normal and obese mouse islet tissue were a little more conclusive. Alloxan in vivo, had no significant effect on the adenyl cyclase activity of islet tissue microdissected from the pancreata of normal and obese

animals 6 days after injection. Normal mice showed sub-chronic hyperglycaemia but the blood sugar levels of obese mice were somewhat reduced. A similar phenomenon had been observed by Mayer (331), suggesting that the obese animals were not alloxan diabetic. A large proportion of the i.p. injected alloxan may have been rapidly absorbed into the fat of the obese animals. Lukens demonstrated that the half-life of alloxan in the blood was very short, and the metabolic products not diabetogenic (298). The reduced potential of the injected alloxan might also have been the subject of an elevated circulating resistance to the drug. Glucose has been shown to protect against alloxan diabetes in mice, probably by inducing a conformational change in the β-cell membrane (399). The diabetic condition following alloxan poisoning has been shown to be due to the lack of insulin (400). The mode of action of alloxan in producing diabetes and the mechanism by which it selectivel kills β-cells remains unclear. Many hypotheses have been put forward and these have formed the subject of countless reviews (401,402). That diabetogenic doses of alloxan act at intracellular sites as well as at the cell membrane has been suggested by Dixit & Lazarow, who have shown increased activities of pentose phosphate and T.C.A. cycle enzymes in islet tissue from alloxanised rats (403). Similarly, Hellerström & Gunnarsson have suggested that an early cytotoxic action of alloxan was to inhibit the glucose metabolism of the β-cell, possibly by interfering with intra-mitochondrial oxidative processes (199). Besides glucose and other sugars (399,404), glutathione and cysteine (405,406) and adrenaline (407) and caffeine (331) have also been found to antagonize the alloxan effect in vivo. However, as yet no unifying concept has been postulated which adequately explains the chemical heterogeneity of these alloxan antagonists. Caffeine and theophylline, which prolong the intracellular life of ^{c}AMP by competitive inhibition of ^{c}AMP-phosphodiesterase (249), have also been shown to stimulate the secretion on insulin (265,408). The ability of caffeine to protect against

alloxan diabetes suggested the possibility of an alloxan action on islet tissue adenyl cyclase activity and this was found to be the case. Experiments *in vitro*, where alloxan was added to the incubation medium of the cyclase assay, have shown a significant reduction in the adenyl cyclase activity of both normal and obese mouse islet tissue. Similar results were obtained by Cohen & Bitensky using a variety of tissues including hamster islet cell adenoma (409). They found that alloxan did not stimulate ^{c}AMP-phosphodiesterase, affect pyruvate kinase activity in the ATP regenerating system or prevent the competitive inhibition of ^{c}AMP-phosphodiesterase by the concentration of theophylline used in the cyclase assay. Their work showed that the inhibitory effect of alloxan was not an indirect consequence of effects on substrate availability or product survival, but resulted from the direct interaction of alloxan with adenyl cyclase. With the exception of the β-adrenergic receptor blocking agents, which prevent the stimulation of adenyl cyclase by catecholamines (247), no other selective inhibitors of mammalian adenyl cyclase have been described. Alloxan and methylxanthines would tend to exert opposite effects on intracellular ^{c}AMP levels and this might in part account for the alloxan/caffeine antagonism shown *in vivo* by Mayer (331). However, in spite of the fact that alloxan inhibited the adenyl cyclase activity of normal and obese mouse islet tissue, one could not assign to this inhibition the responsibility for the diabetogenic effect of alloxan *in vivo*.

Recent work by Malaisse and coworkers has shown ouabain to increase the rate of insulin secretion from incubated pieces of rat pancreas (410). A significant effect, however, was only observed when ouabain (50μmol/litre) was added to an incubation medium containing glucose at a concentration of 150 mg/100 ml. The stimulation of insulin secretion by ouabain has been observed both *in vivo* (411,412) and *in vitro* (413-415). Burr and colleagues using a perifusion system have demonstrated that ouabain inhibits the primary, but stimulates the secondary phase of

glucose induced insulin secretion from pieces of rat pancreas (416). Their work supported the postulate that the two phases of glucose-induced insulin secretion might be mediated through at least partly differing release mechanisms and that the primary component may be dependent on the integrity of the Na^+, K^+ pump. That ouabain does in fact inhibit the β-cell sodium pump has been demonstrated by Howell & Taylor (106) who found that ouabain inhibited the uptake of ^{42}K by isolated rat islets. The stimulation of insulin secretion by ouabain has been shown to require both sodium and Ca^{2+} in the extracellular medium (387,388,415) while the presence of high glucose was not essential (417).

Ouabain has been shown to inhibit Na^+, K^+ activated ATPase systems in a variety of tissues (334,418,419) and to inhibit the adenyl cyclase activity of both normal and obese islets. The site of Na^+, K^+ ATPase inhibition by ouabain has been suggested to be limited to the outside of the cell membrane (419). It would seem logical for ouabain to inhibit adenyl cyclase activity, since the latter enzyme is no more than a Mg^{2+} dependent ATPase. Indeed, adenyl cyclase may well prove to be some part of a Na^+, K^+, Mg^{2+} activated ATPase system in cell membranes. The inhibition of an islet β-cell membrane bound Na^+, K^+ activated ATPase system by ouabain would result in the intracellular accumulation of Na^+ (420) and the possible stimulation of insulin secretion as described by Hales & Milner (417). If the role of adenyl cyclase proves to be one of increasing the permeability of the β-cell membrane to calcium via ^{c}AMP, as suggested by Rasmussen & Tenenhouse (394); then the ouabain inhibition of adenyl cyclase would lead to a reduction in the influx of Ca^{2+}. Low concentrations of calcium have been shown to strongly inhibit Na^+, K^+ activated ATPase (421), possibly by competitive inhibition with Mg^{2+}, and Ca^{2+} influx into the β-cell has been suggested to be a prerequisite for insulin secretion (415). The fact that ouabain inhibited the primary insulin response to glucose but

not the secondary, and reduced both normal and obese mouse islet tissue adenyl cyclase activity, might have suggested a role for adenyl cyclase in the primary insulin secretory response and the possibility of a contribution to the secondary response via a stimulation of insulin biosynthesis. The mechanism of the ouabain induced reduction of islet adenyl cyclase activity remains to be investigated. Jeanrenaud and coworkers have shown ouabain to inhibit the formation of ^{c}AMP in adipose tissue, but to have no effect on ^{c}AMP once formed (421) and Burke has demonstrated the ouabain inhibition of TSH activated thyroid gland adenyl cyclase activity (422). On the other hand Shimizu and colleagues have shown ouabain, in the presence of Ca^{2+}, to increase ^{c}AMP in brain slices. Ouabain did not inhibit ^{c}AMP-phosphodiesterase, hence the inhibition of the latter was not the reason for the stimulated formation of ^{c}AMP (423). The absence of an ouabain induced stimulation of islet adenyl cyclase activity might have resulted from the lack of Ca^{2+} in the cyclase incubation medium. The low Ca^{2+} levels added as impurities in the incubation reagents and in the islet homogenate itself may not have been sufficient to elicit a response. Also, by using an homogenate, ouabain was given the opportunity to influence islet membrane bound adenyl cyclase intracellularly. This possibly unphysiological situation might well have induced the observed reduction in cyclase activity.

In various tissues, phloridzin has been shown to inhibit the active transport of glucose across cell membranes (337,338,424). Also, phloridzin like ouabain has been shown to inhibit cell membrane Na^{+}, K^{+} activated ATPase activity (337,339). Phloridzin, at a concentration known to inhibit endogenous cell metabolism and glucose transfer in rat intestine (342), significantly inhibited the adenyl cyclase activity of both normal and obese islet tissue. Low concentrations of phloridzin, reported to adequately inhibit glucose transfer without affecting endogenous metabolism (342), reduced the cyclase activity of obese islets only. The cyclase of obese islets was found to be more sensitive

to phloridzin than that of normal islets.

The phloridzin inhibition of islet adenyl cyclase activity might suggest the latter's involvement in the active transport of glucose across β, α_2 and α_1 islet cell membranes. The failure of phloridzin to inhibit insulin secretion suggested that β-cells were freely permeable to glucose (98,425) and Matschinsky & Ellerman showed the intracellular concentration of glucose in pancreatic islets of obese mice to closely parallel the blood glucose concentration over a wide range (201). In addition, phloridzin did not influence the rate of glucose oxidation in normal mice (426) nor did it inhibit the stimulatory effect of glucose upon insulin secretion (425). However, recent work by Matschinsky & Ellerman has indicated that the penetration of glucose into the β-cell, although very rapid, was carrier mediated; since under certain conditions both mannoheptulose and xylitol had produced a counter-transport of glucose from the β-cells. Hellman, Sehlin & Täljedal suggested that the glucose uptake by the mammalian β-cell depended on the stereochemical configuration of the molecule, and indicated an inter-action between D-glucose and the β-cell membrane (427,428). They concluded that the uptake of D-glucose by the β-cell was effected by a stereospecific transport system similar to that suggested for a number of other cells (429,430), on the basis that at low temperatures (8°C) D-glucose was more readily taken up by islets than L-glucose and that the D-glucose uptake was saturable (concentration dependent) and inhibited by phloridzin. The clear implication of these results was that D-glucose did interact with the cell membrane, possibly with some sort of glucoreceptor. Similar conclusions have recently been described by Matschinsky and colleagues (431). Glucose, however, did not stimulate adenyl cyclase activity or the levels of ^{c}AMP in islet tissue (287,358). But adenyl cyclase may be associated with the transport of glucose across the islet cell membranes, since islet adenyl cyclase activity was inhibited by phloridzin, which has been shown to

inhibit the uptake of glucose into β-cells (428). On the other hand if islet tissue adenyl cyclase does prove to be part of a Na^+, K^+, (Mg^{2+}) activated ATPase system, then inhibition of this system by phloridzin would automatically reduce adenyl cyclase activity. Work by Téllez de Iñón & Burgos using rat kidney cortex suggested that phloridzin and ouabain inhibited the same Na^+, K^+ ATPase (432). Also of course, like ouabain, phloridzin may have induced the inhibition of islet homogenate adenyl cyclase by acting on the 'wrong' or normally inaccessible side of islet cell plasma membranes. Recent work by Orci and colleagues examining the ultrastructure of β-cell membranes by freeze etching electron microscopy has established the possibility of membrane bound globular enzymes or multi enzyme complexes in β-cell membranes (433). These globular enzymes may prove to be glucoreceptors and/or adenyl cyclase systems.

Although glucose may be the major physiological stimulus for the secretion of insulin, alternative physiological mechanisms have been shown to be present in the mammalian islet. In ruminants, fatty acids provided the major metabolic fuel and have been shown to exert marked effects on insulin secretion (283). In other species notably fish, amino acids have been suggested as the most important stimulus for insulin secretion (434). Evidence has been presented to suggest that both leucine and arginine stimulate insulin secretion <u>in vivo</u> (435,436) and <u>in vitro</u> (7,437), although some discrepancy remains as to whether the stimulation required the presence of glucose or not. Hellman, Sehlin & Täljedal demonstrated that amino acids did not act as insulin secretogogues by serving as metabolic fuels for the β-cells. Their results were consistent with the existence of mechanisms auxillary to glucose metabolism for the control of insulin secretion (438). Milner has shown leucine to be the only essential amino acid to stimulate insulin secretion in the absence of glucose (439). Arginine was the most potent of the essential amino acids in stimulating insulin secretion

in vivo (436) and the most effective of the glucose dependent amino acids *in vitro* (439). It seemed probable that the stimulatory action of arginine and glucose was channelled through one single mechanism. Efendic, Cerasi & Luft suggested that arginine stimulated insulin secretion *in vivo* by modulating the insulinogenic signal produced by glucose in the β-cell, and not by its own primary action on the insulin secretory machinery (440). They suggested that a possible point of action was the site at which ^{c}AMP was perceived as a specific inducer of the insulin secretion mechanism, where arginine might act as some sort of sensitiser.

Neither leucine nor arginine, at concentrations known to stimulate insulin secretion *in vitro* (439) had any significant effect on the adenyl cyclase activity of either normal or obese islets. The effect of these amino acids in the presence of an appropriate amount of glucose was not investigated.

Measurements of adenyl cyclase activity in whole islet homogenates reflected the sum total activity of all the intrinsic cell types. Since islets have been shown to be a heterogeneous collection of at least three cell types with different physiological activities, the stimulation of the adenyl cyclase activity of one cell type could have been compensated by a reduction in cyclase activity of another. In consequence it was difficult to define with any certainty, the effect an agent had produced on the cyclase activity of a single islet cell type i.e. the β-cells. This anomaly would only be settled conclusively by the direct study of pure α and β-cell preparations from both normal and obese mouse islet tissue. It could be argued, therefore, that while arginine did not stimulate β-cell adenyl cyclase and might even have caused a reduction in β-cell cyclase activity, it could at the same time have stimulated the cyclase of α_1 and/or α_2 cells. However, it seemed unlikely that arginine *per se*. was stimulating insulin secretion via the stimulation of glucagon secretion and the subsequent

action of the latter on the β-cell adenyl cyclase, since this mechanism also, would ultimately require an increase in islet adenyl cyclase activity as a result of the original arginine treatment. The result of using arginine in the presence of glucose might have been quite different, although glucose itself could not be expected to work by such a mechanism, since it also did not stimulate islet adenyl cyclase activity. If one assumed that in the islet homogenate endogenous glucagon was rapidly degraded, actions of arginine and glucose on β-cell adenyl cyclase activity via glucagon would not then be expected to occur and this might well have been the situation in the experimental work carried out. On the basis of this assumption the effect of arginine and glucose on insulin secretion could well have been mediated via the glucagon stimulated β-cell adenyl cyclase system. Kaneto & Kosaka have demonstrated the stimulation of glucagon secretion by arginine (441).

Because leucine has been shown to stimulate insulin secretion in the absence of glucose (7,439) its site of action might be directly on the β-cell. Leucine transport across the β-cell membrane may be linked to ionic transport and the movement of leucine across this membrane could be the signal for insulin secretion (439). Extracellular Na^+ has been shown to be a prerequisite for insulin secretion stimulated by leucine (417). Hellman, Sehlin & Täljedal have shown the pancreatic β-cells of obese mice to contain a mechanism for the transport of neutral amino acids, similar to the A system of many other cells (442). The stimulating effect of glucose on insulin biosynthesis did not seem to be mediated by this system and it was concluded that the co-transport of amino acid and Na^+ was not enough to elicit the discharge of insulin from mature β-cells. Matthews & Dean have shown high concentrations of leucine to initiate action potential discharge in islets, and a positive correlation appeared to exist between the ability of agents to initiate action potential discharge in islet cells and their ability to cause insulin secretion (443). Equally well of course, leucine might act

directly on the β-cell membrane at some sort of membrane receptor locus as recently suggested by Hellman and colleagues (428). If leucine has to enter the β-cell in order to exert its effect on insulin secretion, its transport might be effected via ^{c}AMP. Tews, Woodcock & Harper have shown ^{c}AMP to stimulate amino acid transport into rat liver slices *in vitro* (445). That leucine does enter β-cells was established by experiments demonstrating the incorporation of labelled leucine into insulin (446).

Glucagon has been shown to stimulate insulin secretion both *in vivo* (262,322) and *in vitro* (41,447). Turner & McIntyre, using rabbit pancreas *in vitro* suggested that glucagon had a greater stimulant effect at higher glucose concentrations (448). Malaisse and colleagues (41) and Ohneda and coworkers (449) have suggested that glucose was necessary for the stimulation of insulin secretion by glucagon, while other studies have shown glucagon to initiate the *in vitro* secretion of insulin in the absence of glucose (272,408,444,447). Simultaneous measurement of glucagon and insulin secretion, in the presence of glucose, from the perfused rat pancreas established an inverse relationship between glucagon and insulin output (450). The dynamics of secretion from the perfused rat pancreas showed a primary secretion of insulin and a secondary secretion of glucagon (451). The presence or absence of glucose in the perfusion medium influenced this pattern inversely: glucose concentrations of about 100 mg% potentiated insulin secretion and decreased glucagon secretion, whereas the absence of glucose was accompanied by augmentation of glucagon, and depression of insulin secretion.

As in a variety of other tissues (257,453), glucagon stimulated the adenyl cyclase activity of both normal and obese mouse islets, but normal mouse islet cyclase appeared to be more sensitive to the stimulan- effect of glucagon than obese. In support of this observation, glucagon has been shown to initiate a prompt elevation of the plasma insulin

levels in normal humans, but a reduced response in the maturity onset diabetic (452). In certain metabolic aspects, the human maturity onset diabetic syndrome can be compared with the obese hyperglycaemic syndrome in mice.

The addition of glucagon to the islet homogenate would only in theory have supplemented the existing physiological concentration, since glucagon released by homogenisation would have been rapidly degraded.

Bearing in mind the effect of glucagon on the adenyl cyclase activity of normal and obese mouse islet tissue, one might speculate as to the mechanism of glucose induced insulin secretion. Glucagon may be released from the α_2 cells of pancreatic islets by stimuli such as glucose (454); arginine (441,454,455); alanine (456); pancreozymin (451,457); theophylline (455); ouabain (455) and adrenaline (140). The appropriate substrates in the diet would induced the secretion of gut or jejunal glucagon (458,459). Glucagon, acting with greater intensity in the presence of high glucose, might then stimulate the adenyl cyclase system of the β-cell membrane. The reversible binding of glucagon to β-cell membranes would be facilitated by guanosine tri and di phosphates, since these nucleotides have been shown to stimulate the turnover of glucagon at the adenyl cyclase discriminator site and increase the rate of activation of adenyl cyclase by the hormone (460). Some association between Ca^{2+}, and ^{c}AMP released intracellularly, would then stimulate insulin secretion. In support of this hypothesis Allan & Teppermann, suggested that glucagon stimulated the secretion of insulin from the pancreatic β-cell through the mediation of ^{c}AMP (461). Also, Turtle & Kipnis have shown glucagon to elevate ^{c}AMP levels in rat islets (276) and Turtle & Hagon have shown glucagon to stimulate the adenyl cyclase activity of teleost pancreatic islet tissue (369). Malaisse & Malaisse-Lagae have suggested 'entero-glucagon' to be the physiological activator of the β-cell adenyl cyclase system (462).

This factor was found to act upon insulin secretion in the same manner as pancreatic glucagon: it enhanced the stimulant action of glucose and leucine, but had no effect either in the absence of substrate or in the simultaneous presence of glucose and mannoheptulose. Because of the close proximity of α_2 and β-cells, pancreatic glucagon might be expected to initiate insulin secretion more rapidly and with greater intensity, than 'entero-glucagon'. Curry has pointed out that the major action of glucagon was probably to increase the secretion of prestored insulin rather than to stimulate insulin synthesis and/or the secretion of newly synthesised hormone (463). This suggestion was consonant with the work of Taylor, who found that glucagon did not stimulate the incorporation of labelled leucine into insulin (464).

However, certain aspects of the glucagon mechanism proposed above proved difficult to explain. Edwards & Taylor have shown β-hydroxybutyrate to inhibit the release of glucagon (454,465), while this compound has been reported to stimulate insulin secretion both *in vivo* (466) and *in vitro* (467). Also, high concentrations of glucose have been shown to inhibit, although not always significantly, glucagon secretion both *in vivo* (455,457) and *in vitro* (454,465,468). Although glucagon secretion was reduced in the presence of high glucose, the physiological potential of the glucagon actually released may have been increased, since *in vitro* studies by Turner & McIntyre have shown glucagon to have a greater stimulant effect at higher glucose concentrations (448). It may be that both insulin and glucose exert regulatory control over glucagon release, insulin deficiency abolishing the usual suppression of glucagon release at high glucose concentrations and insulin excess increasing the need for glucagon at low glucose concentrations. Fussgänger and coworkers have demonstrated that the dynamics of pancreozymin stimulated insulin secretion from the isolated perfused rat pancreas consisted of a primary secretion of insulin and a secondary secretion of glucagon (451). In this situation it would

appear therefore that glucagon was not stimulating the secretion of insulin. Malaisse and colleagues have indicated that the glucagon secretagogue neutral red, did not modify the rate of insulin secretion induced by glucose from pieces of rat pancreas (360). Also glucose was found to stimulate insulin secretion under conditions where, imidazole, a ^{c}AMP-phosphodiesterase activator, completely abolished the stimulatory effect of exogenous glucagon upon insulin secretion. Although any differences between the physiological requirements of the two phases of insulin secretion were ignored, Malaisse and colleagues concluded that glucagon was not necessarily involved in the stimulation of insulin by glucose and the effect of locally released glucagon if any, was only to enhance the stimulant action of glucose.

Leucine, which has been shown to stimulate the secretion of insulin in the presence and absence of glucose (439), did not stimulate the secretion of pancreatic glucagon (455) or the adenyl cyclase activity of normal and obese mouse islet tissue. The leucine induced stimulation of insulin secretion was clearly not mediated via adenyl cyclase. Leucine might act via islet β-cell glucose metabolism and the production of some sort of intracellular signal, or a specific membrane bound leucine receptor, or perhaps by influencing a membrane bound sodium-calcium system leading to an increased level of membrane bound or intra-β-cell calcium.

Adrenaline has also been shown to stimulate glucagon release in the presence of glucose (140) and inhibit glucose induced insulin secretion in vitro. Although these observations have important physiological consequences i.e. the simultaneous stimulation of glucagon and inhibition of insulin secretion by endogenously released catecholamines would ensure the maximal mobilisation of glucose from the liver and free fatty acids from the adipocyte, they rather complicate the hypothesis of glucagon stimulated insulin secretion via β-cell adenyl cyclase. The adenyl cyclase system does not appear to be involved in the release of glucagon

(279). Also, glucagon does not appear to stimulate insulin secretion through a β-receptor, as adrenaline does, since Malaisse and colleagues have shown the addition of propranolol *in vitro* to have no effect on the insulin secretory capacity of glucagon (41). However, glucagon and adrenaline might act through separate adenyl cyclase systems. Separate adenyl cyclase systems have been demonstrated for glucagon and adrenaline in rat liver (257,469,470).

Dupré has demonstrated crude secretin containing extracts of duodenal mucosa, in man, to accelerate the rate of disappearance of i.v. glucose and increase the plasma insulin like activity (471,472). Purified preparations of secretin have been shown to increase insulin secretion from pieces of dog and rabbit pancreas (473,474). Both pancreozymin (475) and secretin (476) were shown to increase the peripheral insulin levels in man. This effect was exaggerated during hyperglycaemia and was associated with the accelerated disposal of glucose. Buchanan and colleagues using isolated rat islets have shown secretin and pancreozymin to have no significant effect on the secretion of insulin or glucagon (477) and similar results have been obtained by Hinz and colleagues using rat islets in the presence and absence of glucose (478). *In vivo* work by the same group, has shown the stimulation of insulin secretion by secretin, but not pancreozymin or glucose, to be dependent upon the presence of intact exocrine pancreas (479). Similar results have been obtained in man using subjects suffering from chronic insufficiency of the exocrine pancreas (480). On the other hand, Turner & Jarrett were unable to show a secretin or pancreozymin stimulation of glucose induced insulin secretion from pieces of rabbit pancreas (481). Lerner & Porte have demonstrated a uniphasic insulin response to secretin in man. They suggested that insulin secretion occurred only from a stored, readily available insulin pool, which was relatively small and could be discharged faster than it could be refilled (482).

Allan & Tepperman suggested that secretin, like glucagon and pancreozymin stimulated the secretion of insulin from the pancreatic β-cell via ^{c}AMP (461). But secretin was shown to have no significant effect on the adenyl cyclase activity of normal and obese mouse islet tissue. Turtle & Hagan, however, have been able to demonstrate a stimulation of teleost pancreatic islet adenyl cyclase by secretin, while pancreozymin/cholecystokinin was without effect (369). Rodbell, Birnbaumer & Pohl have demonstrated a stimulation of fat cell ghost adenyl cyclase, but not rat liver plasma membrane adenyl cyclase, by purified secretin (483). While Butcher & Carlson have shown secretin to increase the concentration of ^{c}AMP in isolated fat cells and increase the formation of ^{c}AMP from ATP in homogenates of isolated fat cells (484). It may be that the pancreczymin present in the crude secretin preparation, used for islet adenyl cyclase experiments, masked the potential effect secretin might have had on cyclase activity. Or, perhaps intact mouse exocrine pancreas was also required for the stimulant effect of secretin on islet adenyl cyclase. Raptis and coworkers have indicated that the direct and unaffected action of glucose in stimulating insulin secretion from β-cells suggested that a "gluco-receptor" on the β-cell surface was working more or less independently of both the exocrine pancreas and the intestinal hormones. While an "entero-receptor" of the β-cell, that responded to the insulin stimulating action of intestinal hormones was likely to be dependent on the presence of intact exocrine tissue (485). This type of receptor may be entirely limited to the exocrine tissue, mediating the gastrointestinal control of insulin secretion solely by special intrapancreatic exocrine - endocrine connections as yet undiscovered.

Secretin alone, has been shown to effect a remarkable increment in plasma insulin concentration in maturity - onset diabetics (485). This observation supported the view that an insufficient release of intestinal hormones following food ingestion could contribute to the

pathogenesis of maturity-onset diabetes (486). Deckert demonstrated that secretin was a much more potent stimulus for insulin secretion than i.v. glucose in diabetics, which suggested that the defect in the diabetic might have manifested itself either as a blockade of normal glucose transport into the β-cells or as anabnormal metabolism of glucose by these cells (487). The elevation of plasma insulin levels in diabetics after secretin suggested that their islet cells, though still containing apparently adequate insulin reserves, were hyporesponsi to rising blood glucose concentrations (452).

The sequence of events leading to the augmentation of insulin secretion in response to the oral ingestion of glucose has been the subject of a great deal of investigation. Chisholm, Young & Lazarus suggested that secretin participated in the augmentation of insulin secretion after oral stimuli by a rapid sequence of humoral events (488). Gastrin released secretin and the latter stimulated the secretion of insulin. Secretin release was assumed to be augmented by a local stimulus in the duodenum and insulin secretion by the rising level of blood glucose or amino acids. It seemed unlikely that the entire gastrointestinal contribution to insulin secretion would be mediated by secretin, and Dupré and colleagues indicated that both secretin and pancreozymin released in response to physiological stimuli might contribute to the stimulation of insulin secretion after the ingestion of food (489). Kraegen and colleagues have suggested secretin to have a dual role in insulin secretion, an early direct stimulation followed by a prolonged potentiation of the glycaemic stimulus. Secretin was suggested as the dominant factor in the enteric component of insulin secretion after an oral glucose load (492). Gastrin per se. may be capable of a far more direct effect on β-cell activity, since this hormone has been reported to be a normal constituent of α_1 cells (490) and a stimulator of insulin secretion by direct β-cytotropic action (491).

Because of the difficulty in obtaining highly purified preparations of adenyl cyclase with the retention of hormonal sensitivity, it has been impossible to define the molecular constitution of the adenyl cyclase, 'hormone receptor' system. To date no real evidence dissociating the receptor and adenyl cyclase has been presented. A model of the adenyl cyclase system which at least provided a useful working hypothesis was formulated by Rodbell and associates (460). The cyclase system was described as a cell membrane bound, semi crystalline state information transfer unit consisting of three basic but separate molecular components, one or more discriminator(s) situated on the outer surface of the cell membrane, a similar number of phospho-lipid plasma membrane transducers and on amplifier unit on the inner surface of the cell membrane. This system was believed to translate the input signal provided by the hormone or effector agent into a final output signal, ^{c}AMP, with a chemical structure completely unrelated to the input signal. The discriminator was assumed to be a type of selective sensing device, specific for one type of peptide hormone and many discriminators were assumed to be coupled to a single amplifier. The hormone was believed to bind non-covalently and reversibly to the discriminator and the sequence of events suggested to occur at the discriminator was consisten with the rate theory of drug action proposed by Paton (493). The rate of turnover of the hormone at its discriminator determined the quantum efficiency or potency of the hormone on the activation process and the rate of dissociation of hormone from the discriminator, particularly at high concentrations of the hormone, was the rate limiting factor in the excitation process. Rodbell and colleagues found GTP and/or GDP to stimulate the turnover of glucagon at its discriminator and hence increase the rate of activation of adenyl cyclase by glucagon. The guanosine nucleotides by stimulating the rate of release of bound hormone from its discriminator, maintained a constant supply of

unoccupied discriminators for the excitation process. The amplifier or catalytic component amplified the input signal and embraced the site at which ATP was converted to ^{c}AMP in the presence of Mg^{2+}. The transducer was seen as a means of coupling the discriminated input signal to the amplifier, which then generated the output signal. The transducer relayed or coupled the information imparted to the cyclase system by a hormone induced configurational change in the discriminator and was believed to have properties that allowed it to communicate both with the differently structured discriminators and either directly or indirectly with the amplifier. Kuehl and colleagues suggested that prostaglandins were integral components of the adenyl cyclase complex in the cell membrane (494). Exogenous prostaglandins, penetrating the cell membrane, would supplement the transducing component of the adenyl cyclase complex, which would then increase cyclase activity by modifying the basal volume of signal reaching the catalytic site from the discriminators. Information was limited as regards where, how and at what point on the information transfer unit agents capable of modulating cyclase activity exerted their influence. The fluoride ion was believed not to activate adenyl cyclase through the same process as hormones (460). F^{-} was thought to act at some point beyond the discriminator, probably at the level of the amplifier. EDTA and mono-valent cations were believed to act at the level of the discriminator and/or transducer components, while Ca^{2+} and sulphydryl agents acted at the level of the amplifier catalytic site. Ca^{2+} ions were thought to inhibit adenyl cyclase activity, possibly by competing with Mg^{2+} for the catalytic site (298). Magnesium could act at two sites, at the interaction of ATP with the catalytic site and also at the level of the amplifier. The amplifier and discriminator were expected to somehow interact in a way that would produce a change in the affinity of the information transfer unit for Mg^{2+} with the result that the

catalytic activity would be enhanced. The catalytic sub-unit would be expected to be basically similar in all tissues, while the structure of the discriminators would vary and accommodate hormonal specificity. Because the specificity of hormone action was expressed through components that were separate from the enzyme or catalytic component, adenyl cyclase per se. was probably not the receptor or initial site of action of hormones. However, although adenyl cyclase systems have been postulated with a variety of specific discriminators, there still remained the possibility of hormones interacting with entirely separate cyclase systems, at lease in some tissues, as suggested by Bitensky and colleagues for rat liver (469).

Maddaiah has suggested a membrane model that might explain the activation of adenyl cyclase by polypeptide hormones and adrenaline (495). Certain functional amino acid residues of proteins were found to be analogous to the functional groups of adrenaline. The regulatory site for stereospecific interaction, imposed on polypeptide hormones certain structural requirements similar to those of adrenaline. Maddaiah proposed that the receptor sites of tissues specific for a peptide hormone were part of or were situated in close proximity to the regulatory site of the cyclase. Polypeptide hormones were thought to first bind to the receptor inducing a conformational change in the polypeptide such that tyrosyl and amino groups were brought into a spatial configuration similar to that of adrenaline. The hormone in the new conformational state or "active" form was then assumed to interact at the regulatory site to increase the activity at the catalytic site of the cyclase. Specificity of a polypeptide hormone for adenyl cyclase activation occurred at the stage of binding to the receptor and also when this binding induced a conformational change. Maddaiah's model, however, left certain observations unexplained. For example,

ACTH has been shown to be a potent activator of the cyclase in adrenal cortex (496), whereas adrenaline was inactive and β-adrenergic blocking agents prevented the stimulation of cyclase by catecholamines but not by polypeptide hormones.

An adenyl cyclase system consisting of receptor, regulator and catalytic units would probably require an intact membrane structure for a complete response. Homogenisation therefore, might have seriously reduced the magnitude of adenyl cyclase activity recorded in experiments using normal and obese mouse islet tissue.

Cerasi & Luft have demonstrated that in genetically potential diabetes (prediabetes) and diabetes the initial discharge of insulin in response to glucose stimulation was impaired even though the overall insulin secretion remained unaltered (497). Low doses of theophylline restored the impaired glucose induced discharge of insulin in genetically potential diabetics, which implied that a reduced rate of ^{c}AMP production was occurring in the islets of the latter (498). Cerasi & Luft suggested that glucose or one of its early metabolites might be looked upon as a signal by a receptor of the β-adrenergic type, and that this signal was mediated via an adenyl cyclase, ^{c}AMP system in order to elicit insulin secretion (497). Because the inhibition of glucose metabolism in the β-cell was always accompanied by an inhibition of insulin secretion (254), Cerasi & Luft combined glucose metabolism and the adenyl cyclase system in an attempt to explain the sequence of events in the β-cell concerned with the glucose induced stimulation. of insulin secretion (43,499). Their hypothesis stressed the dual actior of glucose as a metabolite and as an effector in relation to insulin secretion. It implied that glucose or one of its early metabolites acted along two systems. On one hand glucose acted as a substrate and was metabolised via glycolysis as in other cells, and on the other hand glucose might act on a specific receptor which in turn activated adenyl cyclase. The glucose receptor or a unit linking the receptor to the

adenyl cyclase was assumed to be a β-receptor, or these two were assumed to be controlled by a β-receptor. The increased level of ^{c}AMP produced by adenyl cyclase activation initiated insulin secretion by acting on a specific insulin releasing unit possible the microtubular system of Lacy and coworkers (500). The functional specialisation of the β-cell was assumed to reside at two levels. Firstly in the perception of glucose as a signal for the activation of adenyl cyclase and secondly in the perception of ^{c}AMP as a signal for insulin discharge. Glucose as the signal was assumed to induce the discharge of insulin, the function of which was to regulate glucose metabolism. The β-cell could use its own glucose metabolism as a parameter of glucose metabolism in the body in general and modulate the signal system accordingly. It was suggested that modulation of the signal chain by the metabolic chain might take place at many levels i.e. by controlling the sensitivity of the glucose receptor through a metabolite; by influencing the transport of calcium to the receptor site; by increasing the availability of substrate ATP for adenyl cyclase; by increasing the sensitivity of the insulin secreting system to ^{c}AMP by way of a metabolite and/or by influencing the transport of calcium to this site and finally by making energy available for any of the above mechanisms.

Insulin secretion was known to be stimulated by a variety of compounds besides glucose i.e. sulphonylureas, amino acids and several hormones. Tolbutamide was assumed to enhance the metabolism of glucose, and its modulating action on the signal chain, rather than a direct effect on the signal system per se. A similar role was assumed for arginine (440) and the vast number of insulin secretogogues regarded as modulators of glucose induced insulin secretion. Although a membrane bound glucose receptor has been postulated recently by Hellman and coworkers (428), the fact that glucose, arginine, leucine and Ca^{2+} were without effect on normal and obese mouse islet adenyl cyclase suggested that the transfer unit postulated by Rodbell and

colleagues (460), stimulated principally by glucagon, might be more consonant with facts presently known. On the other hand, glucoreceptors and adenyl cyclase receptors may be located separately on the β-cell membrane, but subsequent intracellular events initiated by their stimulation may be closely related and perhaps even dependent upon each other.

In diabetes as well as genetically potential diabetes (prediabetes), glucose fails to induce the normal discharge of insulin (501). Both Cerasi & Luft (43) and Kipnis (391) have suggested that the defective insulin discharge in diabetes and genetically potential diabetes might be caused by a selective impairment of the β-cell glucoreceptor leading to an inability of the cell to recognise hyperglycaemia as a stimulator of adenyl cyclase activity. In diabetes and genetically potential diabetes, tolbutamide was assumed to act synergistically with glucose by increasing the sensitivity of the insulin secreting mechanisms to ^{c}AMP, and thus compensating for the reduced activation of adenyl cyclase by the defective glucoreceptor. A defective adenyl cyclase system in diabetes was rather unlikely becaus glucagon, which stimulated islet adenyl cyclase activity, was shown by Simpson and colleagues (502) to induce almost normal insulin discharge in diabetic subjects, suggesting that the adenyl cyclase in β-cells of diabetics was more or less intact. Yet Cerasi & Luft described three types of possible defect in the β-cell adenyl cyclase system that could result in impaired insulin secretion (503). These were: a decreased adenyl cyclase activity leading to a decreased intracellular concentration of ^{c}AMP; an increased ^{c}AMP-phosphodiesteras activity resulting in reduced ^{c}AMP levels and finally a normal level of ^{c}AMP in the β-cell, but a decreased sensitivity of the third messenger, insulin (504) to ^{c}AMP, which was responsible for the stimulation of secretion. Their studies with theophylline, propranolol and human growth hormone in subjects believed to be genetically

potential diabetics led them to the conclusion that a defective insulin secretion in genetically potential diabetics was due primarily to a decreased adenyl cyclase activity of their pancreatic β-cells. The validity of this hypothesis, however, has yet to be established.

SECTION 3.

The ^{c}AMP-phosphodiesterase activity of isolated islets of Langerhans from obese hyperglycaemic mice and their normal lean littermates.

Sutherland & Rall first described a cyclic 3',5' nucleotide phosphodiesterase from beef heart. This enzyme hydrolysed ^{c}AMP to 5' AMP (240). Studies have shown ^{c}AMP-phosphodiesterase (^{c}AMP-PDE) to be widely distributed, having its richest source in the brain cortex (249). Since ^{c}AMP may occupy a unique position in cellular metabolism, and ^{c}AMP-PDE may play a major role in regulating its tissue level, a great deal of interest has recently been shown in this enzyme. Evidence has been presented to suggest that more than one type of ^{c}AMP-phosphodiesterase may exist in a single tissue. Jard & Bernard have demonstrated two phosphodiesterases in rat kidney extracts and frog bladder epithelium (505) while Kakiuchi and colleagues have demonstrated two phosphodiesterases in rat brain with different properties (506). Monn & Christianson have detected seven different molecular forms of phosphodiesterase by electrophoresis, but not more than four occur in any one tissue (507). Whole pancreas was found to contain one phosphodiesterase having a molecular weight in the region of 150,000. Cheung has shown the phosphodiesterase of ox brain to require a protein activator for activity (508). This activator was present in crude homogenates, but absent from purified preparations. He suggested that phosphodiesterase acted via this activator *in vivo*.

Evidence presented by Turtle & Kipnis, demonstrating a stimulation of insulin secretion by theophylline, a recognised ^{c}AMP-PDE inhibitor (265), assigned special significance to the possible regulatory role of ^{c}AMP-PDE in islet β-cells. Indeed, a feedback inhibition of ^{c}AMP mediated insulin secretion, by previously secreted insulin stimulating ^{c}AMP-PDE activity in β-cells, has been suggested (280).

On the basis of these observations, histochemical and radiometric estimations of ^{c}AMP-phosphodiesterase activity have been made using the isolated islet time of normal and obese mice. The hyperinsulinism observed in obese hyperglycaemic mice might be the consequence of a very low genetically predetermined islet β-cell phosphodiesterase activity.

^{c}AMP-phosphodiesterase activity was originally determined in more or less purified organ extracts by a two or more step enzymic procedure, involving the estimation of inorganic phosphate release (249) The main disadvantage of this method was the low sensitivity and/or the interference by drugs with the colourimetric determination of inorganic phosphate. More recent methods for the measurement of ^{c}AMP-phosphodiesterase activity employed radiometric analysis, in which the amounts of labelled 5'AMP and other reaction products formed from the hydrolysis of labelled substrate ^{c}AMP were estimated after chromatographic separation. The radiometric estimation of islet ^{c}AMP-phosphodiesterase activity obeyed the same criteria as described previously for adenyl cyclase (325). Similarly ^{c}AMP-PDE activity could only be expressed in terms of the whole islet.

MATERIALS & METHODS.

a) Histochemical demonstration of ^{c}AMP-phosphodiesterase activity in the islet tissue of 20-25 week old normal and obese mice.†

Enzyme activity was demonstrated in 8μm, air dried cryostat sections, by the multistep procedure of Shanta, Woods, Waitzman & Bourne

† (A cytochemical method has recently been reported (676)).

(327,509). In thismethod, substrate ^{c}AMP was split by tissue ^{c}AMP-phosphodiesterase activity to 5'AMP. The latter was then broken down by endogenous 5'-nucleotidase, and by exogenous 5'-nucleotidase (Crotalus atrox venom, Sigma) added to the incubation medium. Free phosphate groups were captured by Pb^{2+} groups at pH. 7.5.

b) Radiometric estimation of ^{c}AMP-phosphodiesterase activity in the islet tissue of 20-25 week old normal and obese mice.

Microdissected islets of normal and obese mice were homogenised and their protein concentration estimated as described previously using a standard protein curve prepared from bovine albumin standard solutions in 1mmol/litre potassium bicarbonate, pH. 8.

^{c}AMP-phosphodiesterase assay.

Enzyme activity was estimated radiometrically by a procedure derived from the work of several authors but originally according to the method of Bär & Hechter (297). All assays were carried out in 0.5 x 5 cm. silicone treated glass tubes at 37°C and performed on fresh islet homogenates within one hour of isolation. The final concentration of reaction components in a total of 50µl. dispensed from hamilton syringes over ice to prevent evaporation were: 40mmol/litre tris HCl, pH. 8, at 37°C; 5mmol/litre $MgCl_2$; 0.1% crystalline bovine albumin V and 1.79 mmol/litre(^{3}H-8) ^{c}AMP (final specific activity 0.77 mCi/mmole, Radiochemical Centre, Amersham, Bucks.) and 2µg of islet protein in 10µl of 1mmol/litre potassium bicarbonate, pH. 8.0. Blank assays contained 10 µl of 1mmol/litre potassium bicarbonate instead of protein solution. Agents to be investigated were added to the tris-HCl of the incubation medium of both test and blank tubes to give the appropriate concentrations in a total volume of 50µl. Incubation was carried out in parafilm sealed tubes for 10 minutes at 37°C. The reaction was stopped by immersion in boiling water for two minutes. A 100µl carrier containing 5 mmol/litre, 5'AMP, ^{c}AMP, IMP and 2mmol/litre adenosine and inosine was added to the incubation mixture. After centrifugation at

3000 rev/min for 5 minutes the contents of the supernatant were separated by a single descending chromatography (4-6 hrs.) on Whatman G.F. 81 glass fibre paper. The supernatant was applied to the paper as per the adenyl cyclase assay, but over a longer period of time in order to concentrate the spot (the spots tended to increase in size on glass fibre paper during chromatography). The most efficient separation of nucleotides was effected with a solvent system consisting of n-Butanol: isopropanol: concentrated ammonium hydroxide (7:2:1). In this system adenosine had the greatest Rf (0.93) followed by ^{c}AMP (0.81) and inosine (0.4) which tended to streak. IMP co-chromatographed with 5'AMP, which remained at the starting line during chromatography. The distribution of the nucleotides on the chromatogram had previously been verified using commercially available authentic compounds subjected to the same solvent system. Test materials added to the ^{c}AMP-PDE incubation medium ran with the solvent front. After chromatography papers were dried and the separated spots identified under u.v. light, circled with pencil and cut out with scissors. Radioactive spots were positioned upright, but flat against the inner side of borosilicate glass scintillation vials. This process has been reported to increase the efficiency of the counting (510). 12ml. of toluene scintillation fluid (as per the adenyl cyclase assay) was added to each scintillation vial and the tritium content measured on a Phillips liquid scintillation counter. Sample vials were counted for 10 minutes each after being retained in the dark for at least 12 hours. A tritium correction factor was applied and counting efficiency corrected to 100% automatically. The counting efficiency varied between 28-34%.

Towards the end of the ^{c}AMP-PDE studies a report by Therriault & Winters indicated that a more comprehensive separation of a greater number of incubation products i.e. xanthine, hypoxanthine and adenine could be effected by running chromatograms in a second dimension with n-butanol: iso-propanol: water (52:25:25)(681). However, separation in

this solvent system was not found to be adequate, xanthine could not be separated from ^{c}AMP, and adenosine, hyproxanthine and adenine could not be separated from each other.

Calculation of results.

Counts/10 minutes were converted to ct/min and the multiplication factor correction for the internal standard applied to give disintegrations/min at 100% counting efficiency. In the ^{c}AMP-PDE assay, enzyme activity was calculated as the sum of the counts for 5'AMP, inosine and adenosine after 10 minutes incubation. The radioactivity of samples in d/sec. adjusted for scintillation fluid and protein free blanks were used as follows to evaluate the amount of substrate ^{c}AMP hydrolysed. Intermediate stages of the calculation were as per the adenyl cyclase assay.

$$\frac{\text{d/sec. x 20 x 1000 x 500 x 1000}}{3.7 \times 10^{7} \times 0.7743}$$ μmole ^{c}AMP hydrolysed /mg. islet protein/10 minutes

$$\frac{\text{d/sec x 349.0523}}{}$$ μmoles ^{c}AMP hydrolysed/mg. islet protein /10 minutes, pH. 8, 37°C.

Scintillation fluid blank values were subtracted at the d/min. stage after being corrected for counting efficiency (values ranged from 110 - 200 d/min).

^{c}AMP-phosphodiesterase activity was expressed in terms of μmoles ^{c}AMP hydrolysed/mg. islet protein/10 minutes, pH. 8, 37°C. The influence of incubation time, pH. and protein concentration was investigated on ^{c}AMP-PDE activity, prior to comparing the enzyme activity of normal and obese islet tissue. In addition the effects of theophylline in the presence of ^{c}AMP, calcium; adrenaline; glucose and imidazole were investigated on normal and obese islet ^{c}AMP-PDE activity.

RESULTS

Histochemical localisation of islet ^{c}AMP-phosphodiesterase.

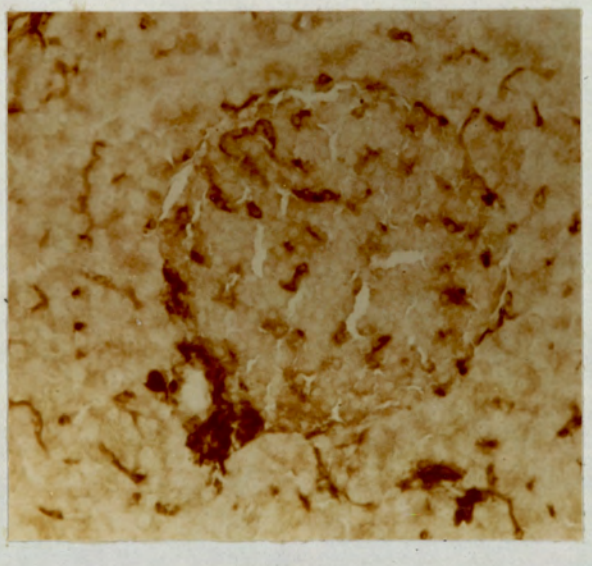
The distribution in both normal and obese mouse islet tissue, Plate 19, a & b respectively, closely resembled that observed for islet

ATPase activity. Black/brown areas of enzyme activity were limited to the connective tissue capsules and capillary networks of islets. The intensity of the weak intracellular reaction seemed to be about the same for islet and exocrine tissue of both normal and obese mice.

PLATE 19.

Histochemical localisation of islet ^{c}AMP-phosphodiesterase activity.

a) Normal Mouse (x50).



b) Obese Mouse (x40).

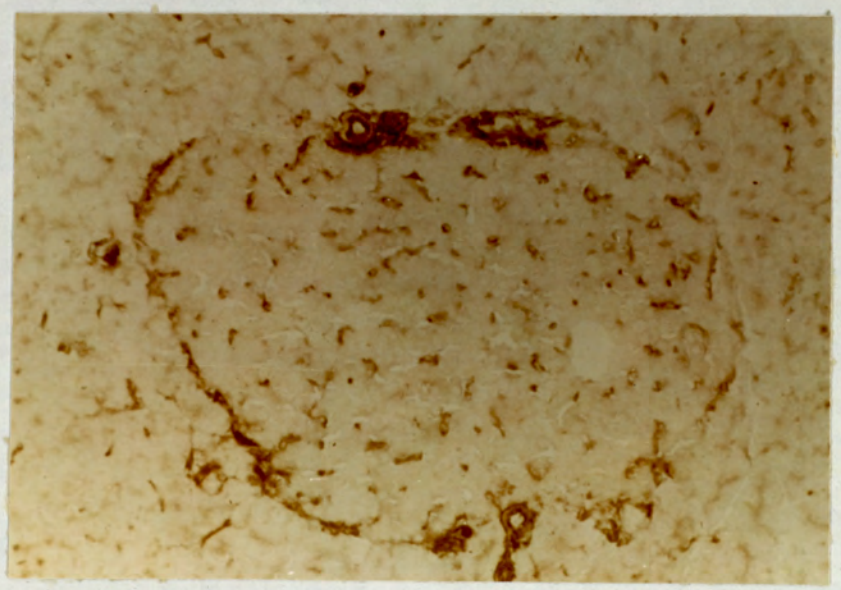
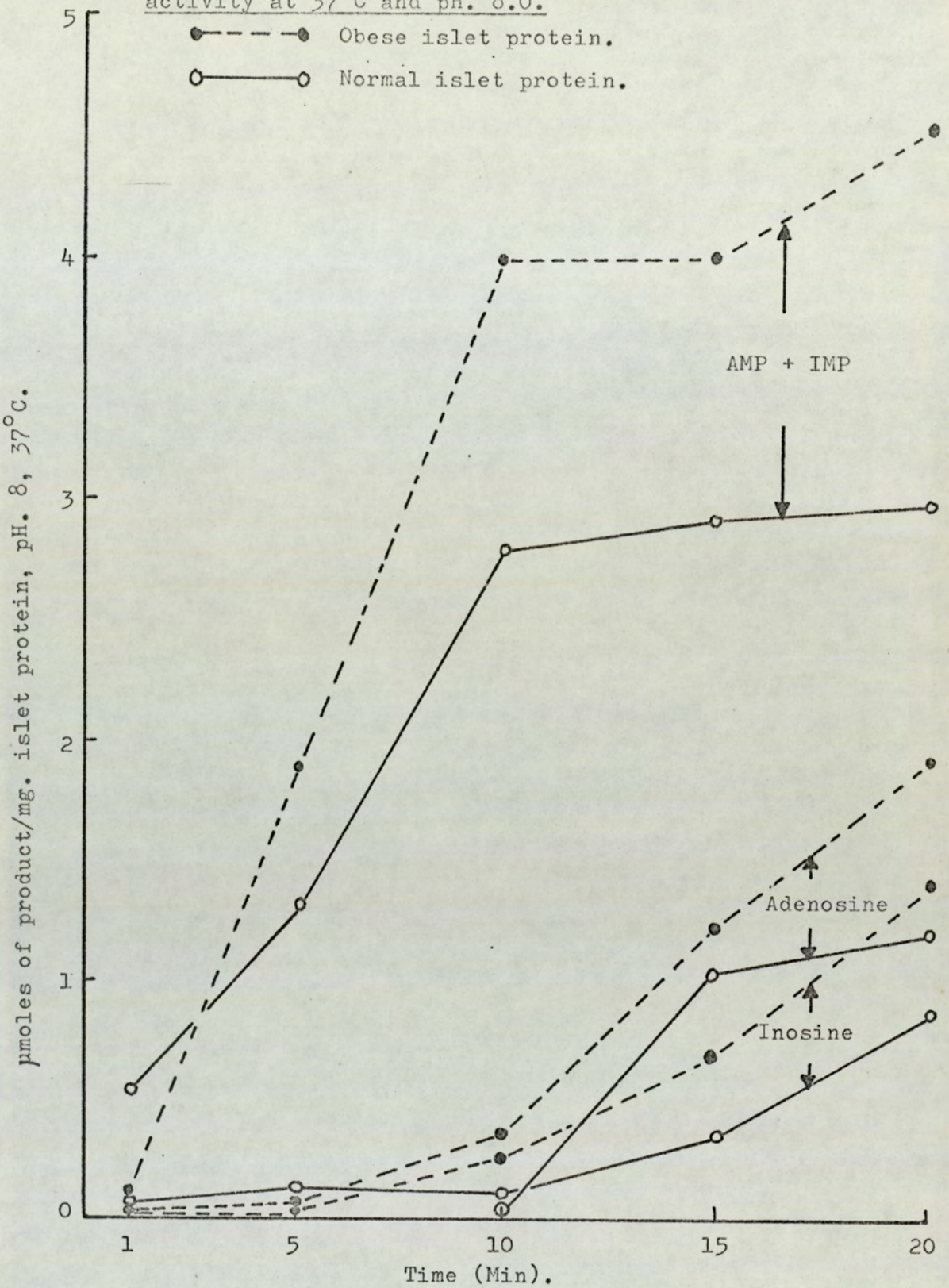


FIGURE 15. Time course of ^{c}AMP hydrolysis by ^{c}AMP-phosphodiesterase activity at 37°C and pH. 8.0.



Determination of the optimal conditions for islet ^{c}AMP-phosphodiesterase activity.

As in the assay for adenyl cyclase, protein free blank counts for 5' AMP, inosine and adenosine were very high (in the region of 0.7% of the total activity). It was clear that the ^{c}AMP-phosphodiesterase assay suffered the same imperfections as the assay for adenyl cyclase (325).

FIG. 16. Time course of ^{c}AMP hydrolysis (^{c}AMP-phosphodiesterase activity). Expressed as the sum of the activities of 5'AMP, IMP, adenosine and inosine at pH.8 and 37°C.

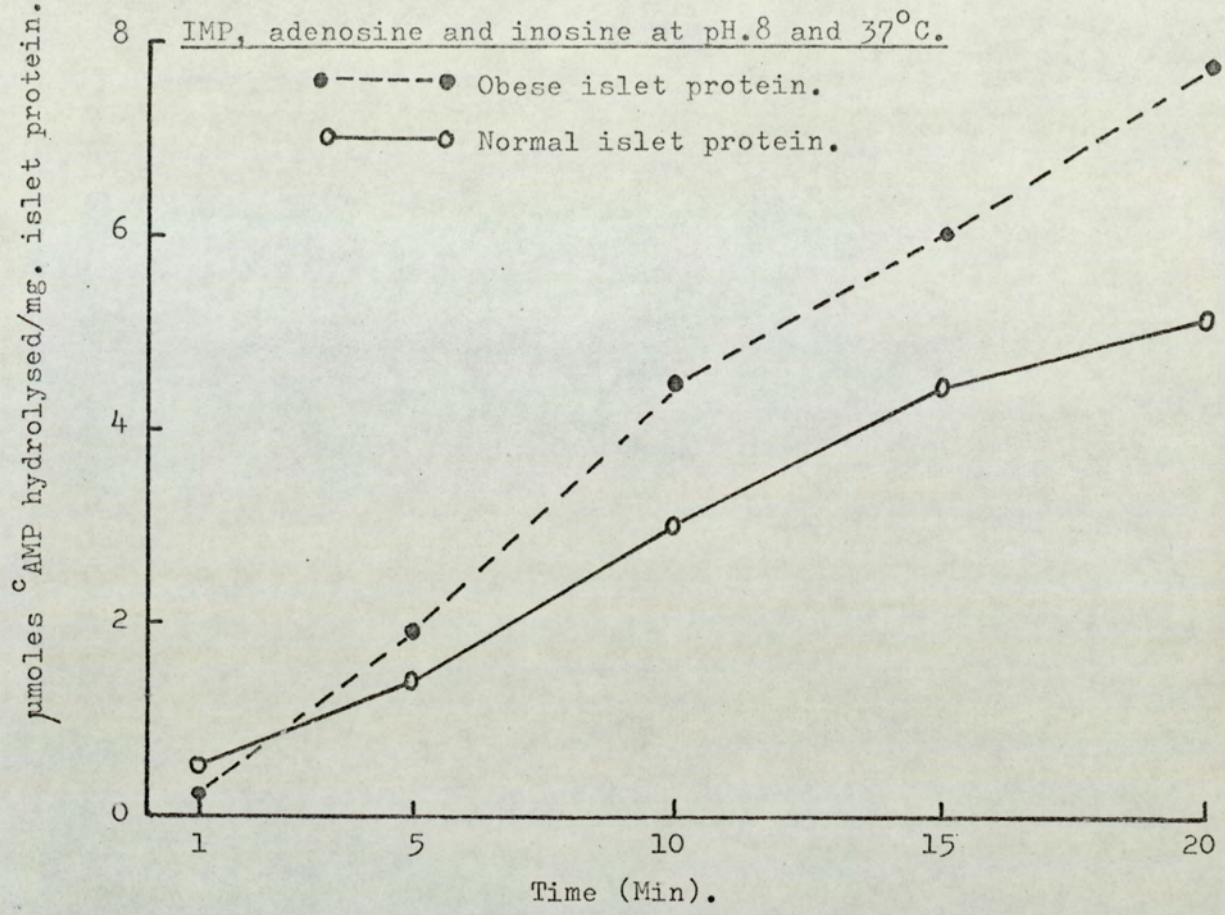


FIG. 17. The effect of pH. on obese islet ^{c}AMP-phosphodiesterase activity. (each point represents the mean of 3 determinations ± S.E.).

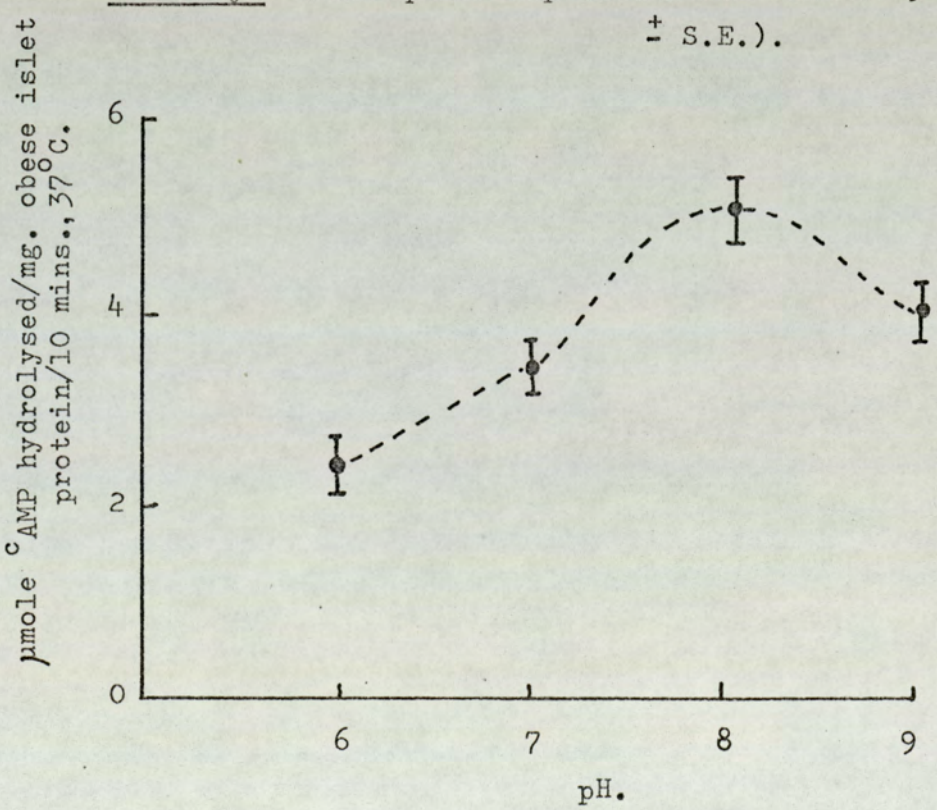
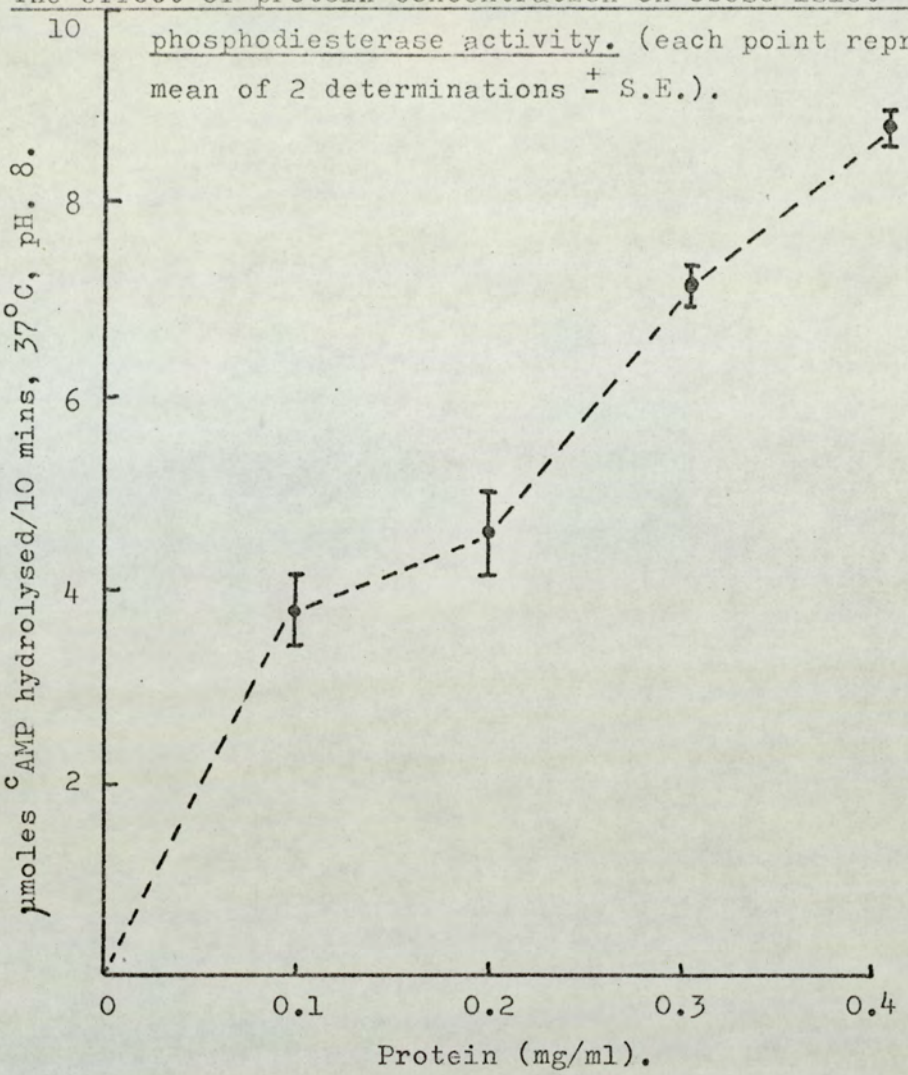


FIG. 18. The effect of protein concentration on obese islet ^{c}AMP-phosphodiesterase activity. (each point represents the mean of 2 determinations ± S.E.).



a) The effect of incubation time.

^{c}AMP-PDE incubations were carried out for 1-20 minutes using normal and obese islet protein, and after 1; 5; 10; 15 and 20 minutes the amounts of labelled 5'AMP (+IMP); adenosine and inosine were counted. A typical time course of ^{c}AMP hydrolysis by normal and obese mouse islet ^{c}AMP-phosphodiesterase at 37°C and pH. 8 is shown in Fig. 15. 10 minutes was chosen as the optimum incubation time prior to the build up of excessive amounts of adenosine and inosine in the incubation medium. Total ^{c}AMP-PDE activity was taken as the sum of the activities of 5'AMP (+IMP); adenosine and inosine. ^{c}AMP hydrolysis was found to increase almost linearly over the time period used, Fig. 16. The amount of ^{c}AMP hydrolysed during incubations, found by summing the activities of the respective metabolites, was found to be independent of the initial substrate ^{c}AMP concentration for up to 20 minutes of incubation. Only a very small part of the substrate ^{c}AMP was utilised to produce 5' AMP and the rest of the metabolites

during this time period. The use of substrate concentrations in excess of the apparent K_m (0.1-0.5mmol/litre) ensured zero order kinetics and cancelled out any effect small amounts of endogenous ^cAMP, known to be present in islet tissue, might have had on enzyme activity. By summing the activities (d/sec) of ^cAMP; 5'AMP; adenosine and inosine (IMP co-chromatographed with 5'AMP) at the different time intervals shown in Fig. 15, for both normal and obese islet tissue and then comparing these values with the value (d/sec) obtained by counting substrate ^3H-^cAMP in 50µl. of incubation medium added directly into a scintillation vial without incubation, a measure of the total % radioactive yield (or conversely a measure of the % radioactivity lost during incubation and chromatography) was evaluated. The average radioactive yield after chromatography was found to be 81% or 19% of the added radioactivity was lost during the incubation and chromatographi procedures.

b) The effect of pH. on ^cAMP-phosphodiesterase activity.

^cAMP-phosphodiesterase assays, using obese islet protein only were carried out with tris-HCl buffers generating pH. values of 6; 7; 8 and 9 at 37°C. The pH. of the potassium bicarbonate used for islet homogenisation was adjusted to the appropriate pH. under test with N/10 HCl or NaOH. Incubations were carried out for 10 minutes and protein free blanks were employed for each pH. value. The ^cAMP-PDE activity of obese islet protein at the respective pH. values is shown in Fig. 17. Obese islet ^cAMP-phosphodiesterase activity had a pH. optimum of about 8 at 37°C.

c) The effect of islet protein concentration on ^cAMP-phosphodiesterase activity.

At the optimum pH. and incubation time, ^cAMP-PDE activity was found to be dependent on islet protein concentration. The effect of obese islet protein concentration on ^cAMP-PDE activity at pH. 8 and 37°C is shown in Fig. 18. ^cAMP-phosphodiesterase assays were carried

out as described previously using protein concentrations estimated and rechecked by Lowry's procedure (218). Blank assays contained no protein. Over the range used ^{c}AMP-PDE activity increased almost linearly with protein concentration. Normal islet ^{c}AMP-PDE activity was assumed to show the same relationship with respect to normal islet protein concentration.

d) Comparison of normal and obese islet ^{c}AMP-phosphodiesterase activity.

Using the enzyme optima so determined a comparison was made between the ^{c}AMP-phosphodiesterase activity of normal and obese islets. Also the effect of theophylline (10mmol/litre) in the presence of ^{c}AMP (unlabelled, 1mmol/litre) was investigated on the ^{c}AMP-PDE activity of obese islet protein in order to justify the use of this combination in the assay for adenyl cyclase.

^{c}AMP-phosphodiesterase activity was found to be significantly higher in obese islets than in normal, Table 17. The combination of theophylline (10mmol/litre) in the presence of ^{c}AMP (1mmol/litre) reduced obese islet ^{c}AMP-PDE activity by some 94% justifying its inclusion in the adenyl cyclase assay. Similar results have been obtained by Drummond & Duncan (298). Although both adenyl cyclase and ^{c}AMP-PDE activities have been found to be higher in islets from obese mice, the ratio of cyclase to phosphodiesterase in islet tissue from both normal and obese animals was found to be about the same i.e. 1:1.

e) The effects of calcium, adrenaline, glucose and imidazole on normal and obese islet ^{c}AMP-phosphodiesterase activity.

The results of these investigations have been summarised in Table 17.

i) Calcium (0.1 and 1.0mmol/litre).

The calcium, added as anhyd. calcium chloride, was greatly in excess of the endogenous concentration naturally present in islet homogenates. Neither concentration of exogenous calcium significantly

TABLE 17. The effect of theophylline in the presence of ^{c}AMP; Ca^{2+}; adrenaline; glucose; imidazole and age on the ^{c}AMP-phosphodiesterase activity of islet tissue from obese hyperglycaemic mice and their normal lean littermates. (Mean values ± S.E., one determination per mouse from homogenised islets).

	N	Normal islets	Obese islets	% deviation from usual values	
		μmoles ^{c}AMP hydrolysed/mg. islet protein/10 min., pH. 8, 37°C.		Normal	Obese
Usual Value					
(20-25 week old mice).	6	3.08 ± 0.25†	4.49 ± 0.22		
Theophylline (10mmol/litre + ^{c}AMP (1mmol/ litre.	6	-	0.28 ± 0.13*		-94
Calcium					
0.1 mmol/litre	4	3.31 ± 0.32†	5.18 ± 0.28	+7.5	+15.4
1.0 mmol/litre	4	3.22 ± 0.49†	4.59 ± 0.19	+4.6	+2.2
Adrenaline					
10^{-7} mol/litre	4	3.37 ± 0.33†	4.90 ± 0.29	+9.5	+9.0
10^{-5} mol/litre	4	3.52 ± 0.48	4.76 ± 0.30	+14.3	+5.9
Glucose					
1g/litre	3	2.92 ± 0.30	4.13 ± 0.58	-5.1	-8.0
2g/litre	3	3.57 ± 0.67	4.61 ± 0.39	+16.1	+2.6
4g/litre	3	2.91 ± 0.42	3.44 ± 0.34*	-5.5	-23.3
Imidazole					
3mg/litre	4	3.30 ± 0.53†	5.30 ± 3.4	+7.2	+17.9
4mg/litre	4	3.70 ± 0.44†	6.72 ± 0.61*	+20.2	+49.5
Age					
10-15 weeks	3	3.13 ± 0.53	3.86 ± 0.45	+1.7	-14.0
40-45 weeks	3	1.81 ± 0.21*†	4.02 ± 0.27	-41.0	-10.6

* Denotes significant difference ($P<0.05$) between treated values and usual values for both normal and obese islets.

† Denotes significant difference ($P<0.05$) between normal and obese islet values subjected to the same treatment.

Coefficients of variation for sets of results ranged between 16% and 22%.

influenced the usual values of normal and obese islet ^{c}AMP-phosphodiesterase activity. In the presence of both concentrations of calcium, obese islet ^{c}AMP-phosphodiesterase activity was always significantly higher than the corresponding value obtained for normal islets.

ii) Adrenaline (10^{-7} and 10^{-5}mol/litre).

Fresh adrenaline was used in the absence of ascorbic acid. Neither concentration of adrenaline significantly influenced the usual values of normal and obese islet ^{c}AMP-phosphodiesterase activity. Only in the presence of 10^{-7}mol/litre adrenaline was obese islet ^{c}AMP-phosphodiesterase activity significantly higher than the corresponding normal islet value.

iii) Glucose (1, 2 and 4g/litre).

No significant difference was observed between the usual normal and obese islet ^{c}AMP-phosphodiesterase values (Table 17) and values obtained for normal and obese islets in the presence of 1 or 2g/litre glucose. However, 4g/litre glucose significantly reduced obese islet ^{c}AMP-PDE activity, compared to the usual obese value. No significant reduction was observed for normal islet ^{c}AMP-PDE activity at this glucose concentration.

iv) Imidazole (3 and 4mg/litre, GIII, low fluorescent blank, Sigma).

3mg/litre imidazole had no significant effect on the usual normal and obese islet ^{c}AMP-PDE activities (Table 17). However, a significant increase in obese islet ^{c}AMP-PDE activity was observed in the presence of 4mg/litre imidazole. No significant increase was observed for normal islet ^{c}AMP-PDE activity at this imidazole concentration. In the presence of both concentrations of imidazole, obese islet ^{c}AMP-PDE activity was always significantly higher than the corresponding normal islet value.

Discussion

^{c}AMP phosphodiesterase has been demonstrated in the isolated

islets of both normal and obese mice operating at a pH. similar to that found in other tissues (511). Under the incubation conditions imposed, islet ^{c}AMP-PDE activity was found to be linearly related to both incubation time and islet protein concentration. ^{c}AMP-phosphodiesterase activity like adenyl cyclase was found to be higher in islet tissue from obese mice, but the ratio of cyclase to phosphodiesterase in both normal and obese islets was about the same, 1 : 1. The in vivo balance of this enzyme system, which has the capacity to control the β-cell concentration of ^{c}AMP (in the absence of a diffusion of ^{c}AMP from the cell) remains to be clarified, especially since many metabolites produced naturally as a result of ATP metabolism have been shown capable of inhibiting ^{c}AMP-PDE activity. Dousa & Rychlik have shown ADP, ATP and pyro-phosphate to inhibit rat kidney ^{c}AMP-PDE and contribute to the accumulation of ^{c}AMP (511). The inhibition of ^{c}AMP-PDE activity by such a variety of compounds (including methylxanthines) suggested that it was highly susceptible to regulation and might prove to be a useful in vivo control mechanism. Cheung expressed the view that ATP and other metabolites maintained ^{c}AMP-PDE in an inhibited state in the cell, so that the rate of ^{c}AMP breakdown was related to the concentration of ATP and other metabolites in the cell (512). The active form of ^{c}AMP-phosphodiesterase was probably a metal-enzyme complex with Mg^{2+} or Mn^{2+} and the mechanism of inhibition by pyrophosphate, nucleoside/triphosphates and citrate was via chelating the metal ion in the enzyme. Fluoride has not been shown to inhibit ^{c}AMP-PDE activity and contribute to increased intracellular levels of ^{c}AMP by this manner. The greater proportion of microsomal ^{c}AMP-phosphodiesterase activity (513) in the cell has been shown to remain latent. This extra activity was unmasked by the addition of a nonionic detergent triton X-100 (512), although this compound was not shown to activate the enzyme per se. As a consequence of this latency, the potential islet ^{c}AMP-PDE activity was probably

much greater than the islet adenyl cyclase activity recorded. Indeed Butcher & Sutherland pointed out that the tissue levels of phosphodiesterase were far higher than those of adenyl cyclase (249). This potential excess of ^{c}AMP-phosphodiesterase might be an insurance against the prolonged intracellular activity of ^{c}AMP as a function of its elevated concentration.

The incubation of ^{c}AMP with islet homogenate resulted in the formation of a number of nucleoside metabolites of 5'-AMP. The occurence in islet tissue of 5' nucleotidase, adenosine deaminase, inosinase and probably xanthine oxidase was expected, although their relative activities were not known. Pöch has recently suggested the addition of unlabelled 5' AMP(1mmol/litre) to ^{c}AMP-phosphodiesterase assays to prevent the breakdown of labelled 5' AMP during incubations (514).

Theophylline and many xanthine derivatives (515) have been shown to increase the ^{c}AMP level in tissues by inhibiting ^{c}AMP-phosphodiesterase. In the present study, theophylline in the presence of ^{c}AMP produced a 94% inhibition of islet ^{c}AMP-PDE activity. The type of inhibition produced by theophylline has been the subject of a great deal of controversy. The literature has provided sufficient evidence to support both competitive and non-competitive inhibition, although the differences observed were probably tissue specific.

Calcium, adrenaline and glucose did not significantly influence the ^{c}AMP-phosphodiesterase activity of either normal or obese mouse islet tissue. The marginally significant reduction observed at the highest glucose concentration might possibly have been the result of an osmotic effect on enzyme activity. Cheung found 2mmol/litre calcium to be inhibitory on rat brain ^{c}AMP-phosphodiesterase (512) but found low calcium to be a requirement for bovine brain ^{c}AMP-phosphodiesterase (516). Kakiuchi & Yamazaki have demonstrated the presence of both calcium dependent and calcium independent ^{c}AMP-PDE activity in rat

brain (397). The calcium dependent enzyme was controlled by physiological concentrations of calcium in the region of 5×10^{-6} - 2×10^{-5} mol/litre. It would seem therefore that the calcium concentrations used in the normal and obese islet ^{c}AMP-phosphodiesterase assays were too high. Low physiological concentrations of calcium were probably required for activity, whereas higher concentrations inhibited. As yet, however, little evidence has been presented to substantiate this in islet tissue and calcium uptake probably did not initiate ^{c}AMP mediated insulin secretion via an inhibition of ^{c}AMP-phosphodiesterase.

Imidazole has been shown to stimulate ^{c}AMP-PDE activity of brain (516,517), liver (518) and adipose tissue (519). In the present work however, only obese mouse islet ^{c}AMP-PDE activity was stimulated significantly by imidazole. In obese islet tissue therefore, imidazole would be expected to reduce the intracellular levels of ^{c}AMP. This observation was consonant with the work of Malaisse & colleagues, who have shown imidazole to inhibit the stimulant effect of glucose, glucagon and theophylline on insulin secretion from pieces of rat pancreas (360) and was suggestive of a role for ^{c}AMP in insulin secretion. Also the increase in insulin secretion induced by glucagon appeared to be more sensitive to imidazole than that induced by theophylline or glucose and might suggest the normal course of events to be a glucagon stimulation of insulin secretion via an increase in the ^{c}AMP levels of β-cells.

The majority of mammalian ^{c}AMP-phosphodiesterases have been shown to be stimulated by imidazole and inhibited by methylxanthines. One possible explanation for the effects of these compounds suggested that they were structural analogs of regulatory substances native to the organism for which the ^{c}AMP-PDE was derived (520). Recent findings have indicated that insulin might have an effect on both the formation and the activity of ^{c}AMP-PDE, although the effect on activity appeared to require an unphysiologically high concentration of the hormone.

Some authors have been unable to demonstrate a stimulation of ^{c}AMP-PDE by insulin and have suggested the hormone to exert its effects at the level of adenyl cyclase (521,162), but Loten & Sneyd have recently shown insulin to stimulate the ^{c}AMP-PDE activity of adipose tissue (522,523). Work by Sodoyez and colleagues using isolated hamster islets in the presence of 100mg/ glucose has suggested a role for ^{c}AMP in the feedback regulation of insulin secretion by previously secreted insulin (151,280). From indirect evidence using db-^{c}AMP, they suggested that exogenous insulin inhibited insulin secretion by stimulating β-cell ^{c}AMP-PDE and reducing the intracellular level of ^{c}AMP, rather than interfering with the action of ^{c}AMP *per se.* on insulin secretion. These workers also found that insulin inhibited insulin secretion induced by glucagon more markedly than that induced by ^{c}AMP, suggesting that insulin, in addition to its action on ^{c}AMP-PDE, might be inhibiting the stimulatory action of glucagon on β-cell adenyl cyclase. Insulin has been shown to inhibit the effect of glucagon and adrenaline on the adenyl cyclase activity of liver (163). The situation in the β-cell may be that ^{c}AMP-PDE activity is continuously stimulated by secreted insulin. This hypothesi would ensure a rapid turnover of ^{c}AMP in islet tissue and at the same time accommodate the spectrum of insulin secretory activity shown by the variety of known insulinogenic agents i.e. the greater the rate of activation of adenyl cyclase the greater the secretion of insulin and the more insulin secreted the greater the stimulation of ^{c}AMP-phosphodiesterase activity.

The measurement of islet adenyl cyclase and ^{c}AMP-phosphodiesterase activity was complicated by the presence of a relatively high endogenous concentration of insulin in the tissue. In the islet adenyl cyclase assay, agents stimulating cyclase activity, did so despite the capacity of insulin to induce inhibition. Any insulin stimulation of ^{c}AMP-phosphodiesterase in the adenyl cyclase assay was effectively blocked

by theophylline. Also, the capacity of adrenaline, HB419 and glucagon to stimulate islet adenyl cyclase activity must have exceeded the capacity of insulin in the homogenate to reduce it. In vivo, the situation might have been quite different and insulin might have had the capacity to decrease the level of β-cell ^{c}AMP by inhibiting adenyl cyclase and stimulating ^{c}AMP-PDE activity. In the ^{c}AMP-PDE assay the presence of insulin in the islet homogenate could have stimulated the activity of islet ^{c}AMP-phosphodiesterase and provided unphysiologically high values of enzyme activity.

Although HB.419 has been shown to stimulate normal and obese mouse islet adenyl cyclase activity, recent evidence has suggested that sulphonylureas might also be capable of mediating the ^{c}AMP induced secretion of insulin via an inhibition of islet ^{c}AMP-phosphodiesterase (524).

The hypersecretion of insulin has been shown to be a characteristi of the obese-hyperglycaemic syndrome in mice. (54). For ^{c}AMP to be directly implicated in the mechanism of insulin secretion, the level of ^{c}AMP at any one time must be greater in obese islets than in normal. Consonant with this assumption, adenyl cyclase and ^{c}AMP phosphodiestera activities were found to be higher in obese islets than in normal. The adenyl cyclase/^{c}AMP-phosphodiesterase system was therefore working at a higher level in obese islet tissue. The adenyl cyclase/^{c}AMP-PDE ratio 1 : 1, evaluated for both normal and obese islets simply indicated that the phosphodiesterase was capable of breaking down ^{c}AMP at approximately the same rate as the cyclase was capable of producing it. The increased level of adenyl cyclase and ^{c}AMP-PDE activity could have been consonant with an increased rate of glucose metabolism in obese islets. In this context, Malaisse and colleagues have suggested that ^{c}AMP might enhance the effect of glucose upon insulin secretion by activating glucose metabolism within the β-cell (41). Previous discussion has suggested the glucagon stimulation of insulin secretion to occur via a stimulation of β

membrane adenyl cyclase. The hypersecretion of insulin from obese islets was probably not due to increased glucagon secretion since Gepts and colleagues (63)and Hellman (525) have shown α cells to occur with decreased frequency in obese islets, the hyperplasia being restricted to β-cells. However, the hypersecretory behaviour of obese islets might have been the result of the same amount of glucagon acting at the cell membranes of an increased number of β-cells. The resulting hyperinsulinaemia being maintained by a tissue resistance to circulating insulin.

CHAPTER 4.

The Monovalent (Na^+,K^+) and divalent (Ca^{2+},Mg^{2+}) ion content of islets, exocrine tissue and blood plasma of normal and obese mice.

Hales & Milner have shown extracellular Na^+ and Ca^{2+} to be essential for the glucose induced secretion of insulin (386,387,414,417). A sodium pump was suggested, the inhibition of which was thought to be associated with the stimulation of insulin secretion via an increase in the intracellular concentration of sodium. Their work led them to postulate the existence in the β-cell of a sodium-dependent calcium uptake process and that a late event in the stimulation of insulin secretion involved Ca^{2+} uptake by the β-cell through this process (415,526). Malaisse and Malaisse-Lagae have also suggested the influx of Ca^{2+} to be involved in the process of insulin secretion (44,410).

With this evidence in mind and the possibility of a regulation of islet adenyl cyclase activity by Ca^{2+} acting competitively with Mg^{2+} for the catalytic site of the enzyme (298,460), an evaluation was made of the levels of Na^+; K^+; Ca^{2+} and Mg^{2+} in the islets, exocrine tissue and blood plasma of obese hyperglycaemic mice and their normal lean littermates.

MATERIALS & METHODS.

All glassware was thoroughly washed with double distilled deionised water and all reagents were analar grade. Exocrine and islet tissue of 15 h. fasted 20-25 week old, male normal and obese mice were dissected from excised pancreata in 0.25 mol/litre sucrose at $4^{o}C$ and transferred to preweighed watchglasses. Islets were pooled from many mice. The tissues were freed of excess dissecting medium by careful mopping with lens tissue and dried at $105^{o}C$ for 48 hours until constant weight. The dried tissue was then processed according to the procedure of MacIntyre & Davidsson (527). After being ground in an earthenware motar with a teflon pestle, the tissue powder was washed with one change of ether/ petroleum-ether mixture (50/50,v/v) to remove fat. Excess wash medium was poured off and the remainder carefully evaporated with an air-drier. The remaining tissue was again dried at $105^{o}C$ for 12 hours and then stored in a dessicator at room temperature.

20 mg. of fat free dried tissue powder (or, in the case of islets, as much as was available) was transferred to preweighed nickel crucibles. The contents of which were weighed at room temperature and then ashed ($550^{o}C$) until white, in an electric furnace. Ashed tissue samples were cooled in a dessicator prior to ion determination.

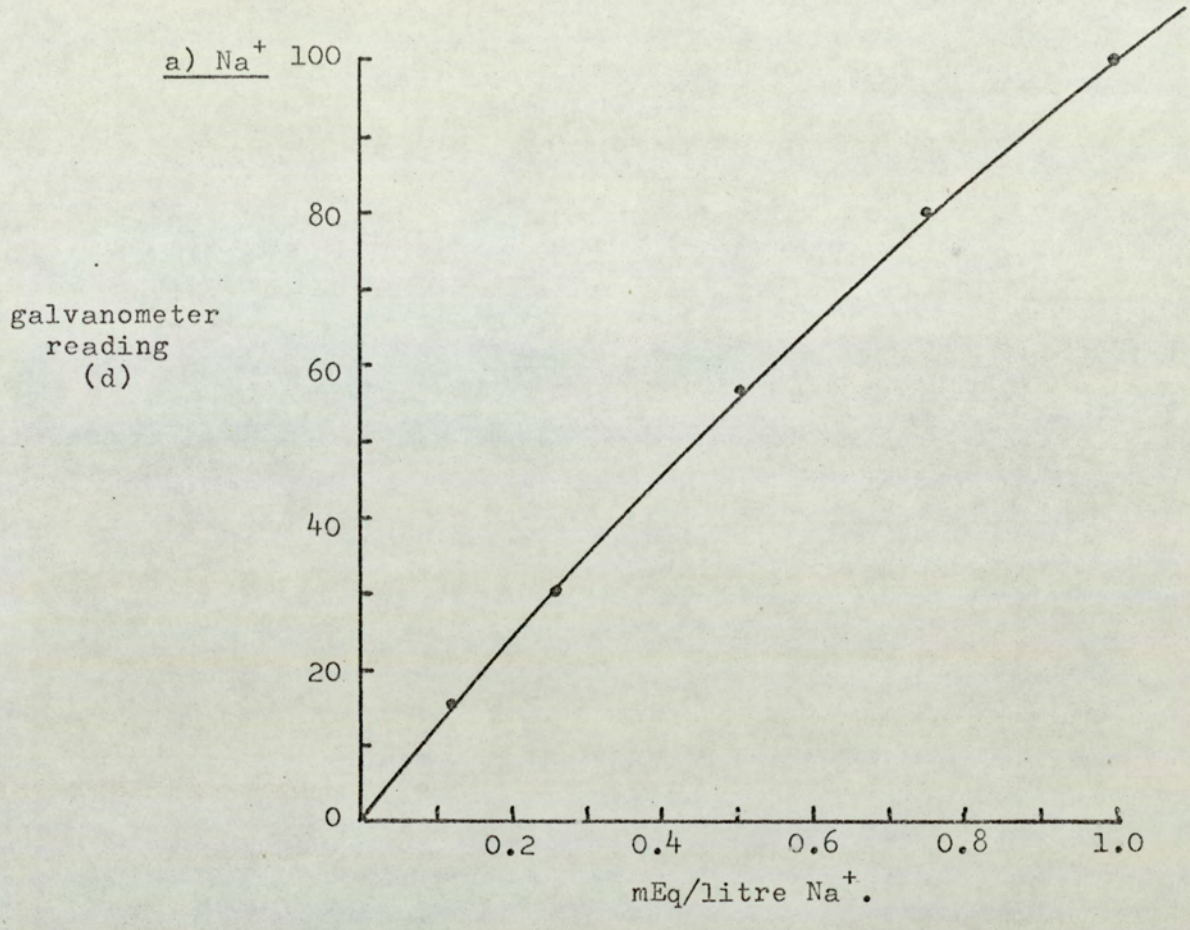
Na^{+} and K^{+} were estimated by emission using an E.E.L. flame photometer and the appropriate filters, while Ca^{2+} and Mg^{2+} were estimated by absorption using an SP 900 A, Unicam atomic absorption spectrophotometer.

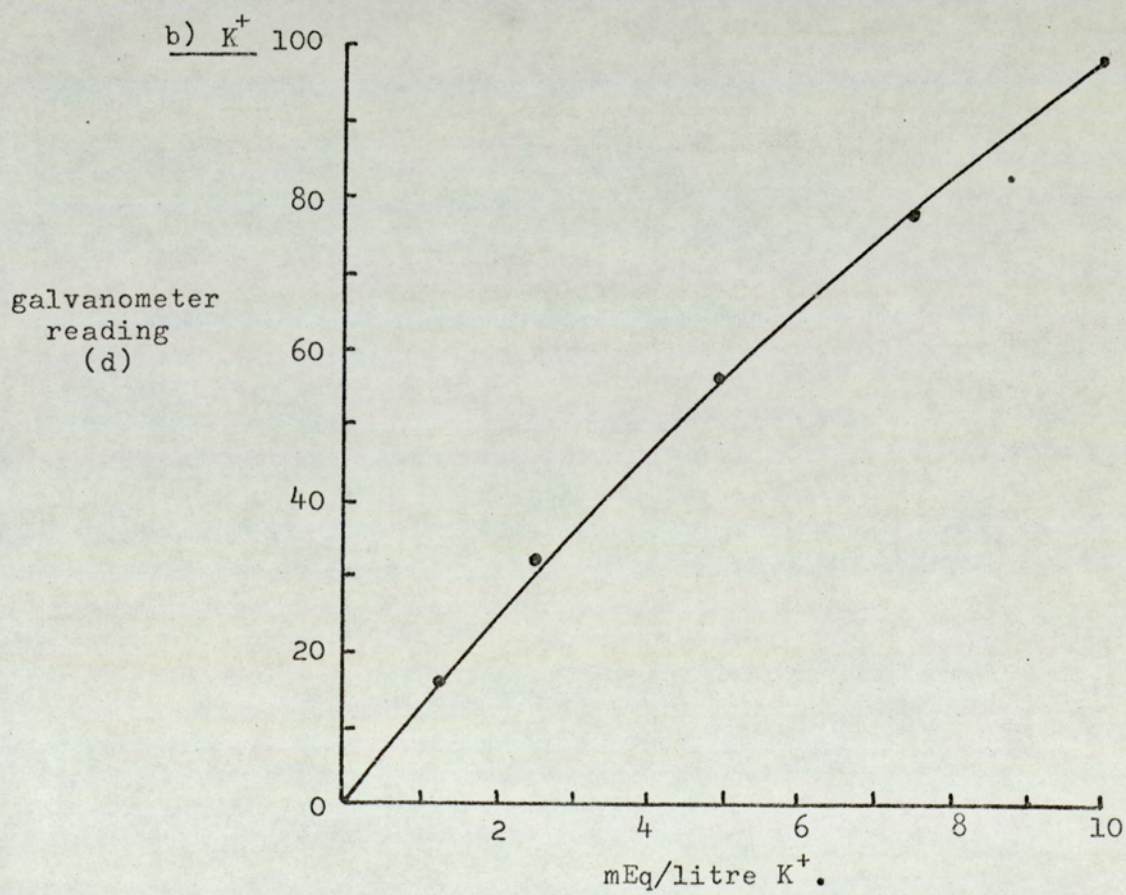
Determination of Na^{+} and K^{+}.

Quantities of NaCl and KCl were dried on foil for 48 hrs. and used to prepare stock solutions of 1 mEq/litre,Na^{+} and 10 mEq/litre,K^{+}. 0.1 mEq/litre of Na^{+} was added to the K^{+} stock solution to prevent spectral interference by Na^{+} in the K^{+} estimation. Standard solutions were prepared over the range 0.1 - 1.0 mEq/litre Na^{+} and 1 - 10 mEq/litre K^{+}.

4 ml. of HCl (100ml. of ^{c}HCl/litre of double distilled deionised

FIG. 19. Standard curves for Na^+ and K^+ estimation by emission.





water) was added to the ash of each tissue sample and the contents stirred with a teflon paddle. Four estimations were rapidly carried out on each sample, and the mean taken. The instrument was flushed out with deionised double distilled water between estimations. Standard curves for Na^+ and K^+ estimations are shown in Fig. 19 a & b.

Calculation:

The weight of dry tissue powder/4ml. was converted to dry wgt./litre. The galvanometer deflection 'd' was read off the standard curve to give the ion concentration in terms of mEq/litre. Results were then expressed in terms of mEq/Kg fat free dried tissue.

Determination of Ca^{2+} and Mg^{2+}.

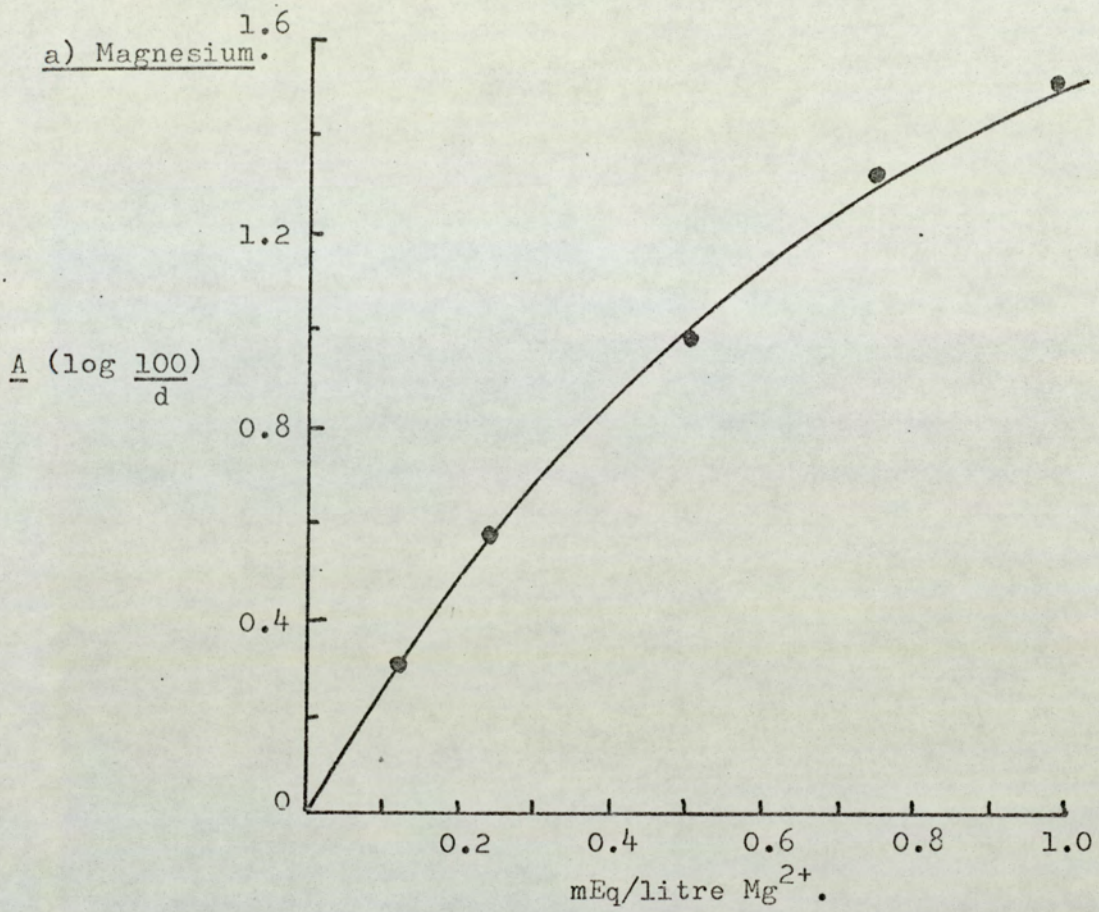
Tissues were taken to the ash stage as per the determination of Na^+ and K^+.

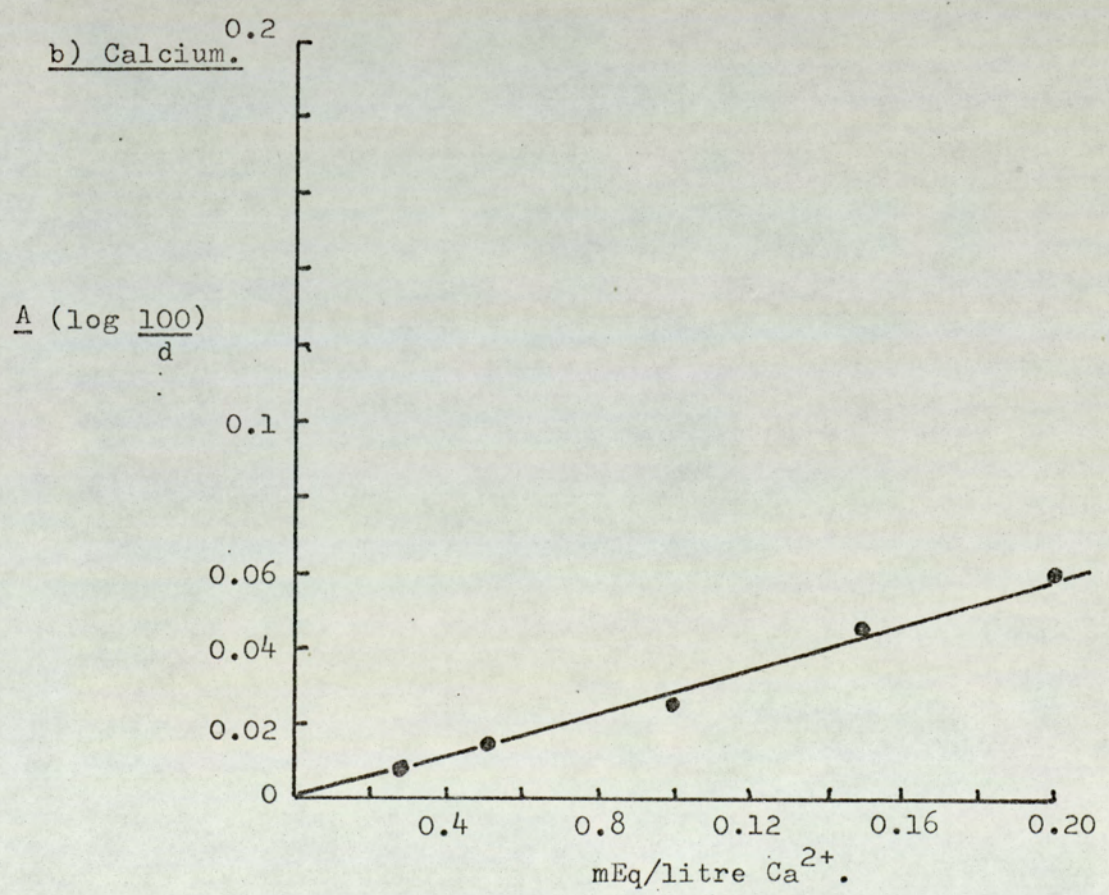
Tissue Magnesium.

A range of standard solutions was prepared from a 10 mEq/litre stock solution of $MgCl_2.6H_2O$ (dried at 105°C for 48 h.) in HCl/ lanthanum chloride (10g. lanthanium chloride/litre of HCl soln: 100ml. of conc. acid/litre double distilled deionised water). Standards ranged from 0.1 - 1.0 mEq Mg^{2+}/litre and were prepared from the stock solution by dilution with lanthanum chloride/HCl. solution. The stock solution was used to peak the wavelength for Mg^{2+})285.2 nm., slit width 0.1 and filter 3). An air/acetylene flame was used with a combined Ca^{2+}/Mg^{2+} lamp operating at 8 mA. The amount of absorption was a function of the concentration of the absorbing atoms. Over a wide concentration range Beer's law tended to be followed although in most cases the plot of concentration against degree of absorption was slightly curved, giving lower sensitivities at higher concentrations. The absorbance (A) was calculated from $A = \log \frac{100}{d}$,

where 'd' was the galvanometer deflection given by the sample or standard, when the galvanometer had been set to give a reading of zero with the

FIG. 20. Standard curves for Ca^{2+} and Mg^{2+} estimation by absorption.





shutter closed and ≙ 100 when the unabsorbed lamp radiation was being detected. Standard curves of absorbance (A)/Mg^{2+} concentration of standard solutions in mEq/litre were prepared. A typical standard curve is shown in Fig. 20a.

4ml. of HCl/lanthanum chloride solution was added to ashed tissue samples and the contents of the crucibles stirred. The galvanometer deflection observed for each sample was converted to absorbance (A) and the corresponding value in mEq/litre read from the standard curve. Two or occasionally three determinations could be made on a 4ml. sample. The mean value of these was used to calculate the tissue concentration of magnesium.

Tissue Calcium

Standard solutions of Ca^{2+} were prepared in a similar way to magnesium, using $CaCl_2.2H_2O$ dried at 105°C for 48 h. The wave length was peaked at 422.7 nm. and estimations were carried out using a slit width of 0.08, filter position 3, and an air/acetylene flame. A typical standard curve for Ca^{2+} is shown in Fig. 20,b. The estimation of Ca^{2+} was very near the detection limit of the instrument and a great deal of scale expansion and electrical damping had to be used.

There were certain precautions to be observed for the measurement of Ca^{2+} and Mg^{2+} by absorption. Phosphate interference has been shown to be the main source of error, producing falsly low estimations of Ca^{2+} and Mg^{2+} concentration. Iida and colleagues used half saturated oxine to remove phosphate interference (528). The oxine competed with the phosphate, so that magnesium oxinate was precipitated as the sample dehydrated in the flame. Iida and colleagues pointed out that phosphate interference did not occur when the measuring instrument was fitted with a rotating nozzle atomiser burner (as in the Unicam SP900A). This burner permitted only a fine mist to enter the flame. The large coarse droplets, which caused the phosphate interference seen with total consumption burners, were thrown away from the flame by centrifugal

force. Willis has shown that serum Mg^{2+} could be determined by atomic absorption, without interference, when dilution with water was the only sample treatment. In serum the high protein concentration largely compensated for the chemical interference by phosphate (529). However, in tissues, phosphate interference was always a possibility and Willis demonstrated 10g/litre lanthanum chloride to be an effective material in its elimination.

Calculation for Ca^{2+} and Mg^{2+} in tissues.

The dry wgt. of tissue/4ml. was converted to dry wgt./litre. The galvanometer deflection was converted to absorbance $A = \log \frac{100}{d}$. This value was obtained in terms of mEq Ca^{2+} or Mg^{2+}/litre from the appropriate standard curve and results were then expressed in terms of

mEq Ca^{2+} or Mg^{2+}/Kg fat free dried tissue.

The measurement of plasma Ca^{2+} and Mg^{2+} concentration.

Plasma Magnesium

All glassware and instruments were treated with heparinised physiological saline prior to use. Normal and obese mice were quickly stunned, decapitated with large scissors and their bodies thrust into the top of small filter funnels. Blood was collected in heparinised immunoassay tubes, centrifuged at 3000rev/min for 10 minutes and the plasma separated and stored at -15°C until required. Just over 0.5 ml. of plasma could be obtained from each mouse by this technique. After thawing 0.5 ml. of plasma was transferred to a 5 ml. graduated flask and made up to the mark with HCl/lanthanum chloride solution. The samples so obtained were often a little cloudy and their condition was not improved by centrifugation. Although it was originally intended to take both blood and islets from the same mouse, this was not always found to be possible. The Mg^{2+} stock solution and standard curves were similar to those used for the measurement of tissue Mg^{2+}. Three estimations were made on each standard solution and the mean value used to plot the standard curve. The mean coefficient of variation for estimations on

standard solutions was 7%.

Plasma Calcium

Plasma samples were prepared as for magnesium. A stock solution of 10 mEq Ca^{2+}/litre ($CaCl_2.2H_2O$) was used to prepare standard solutions ranging from 0.125 to 1.0 mEq Ca^{2+}/litre. Two estimations were made on each standard solution and the mean value used to plot the standard curve. The mean coefficient of variation for estimations on standard solutions was 4%.

Calculation for plasma Mg^{2+} and Ca^{2+} values.

0.5 ml. of plasma was diluted to 5 ml.(dilution x10). The sample deflection 'd' was converted to absorbance $A = \log \frac{100}{d}$
The Absorbance value was read off the standard curve in terms of mEq Mg^{2+} or Ca^{2+}/litre and corrected for dilution (x10). Plasma Mg^{2+} and Ca^{2+} concentrations were expressed as mEq/litre of plasma. Plasma Ca^{2+} levels are sometimes expressed in terms of mg%. This value can be obtained by multiplying the mEq/litre value by 2.

Results:

The concentrations of certain monovalent and divalent ions in 20-25 week old normal and obese mouse islet and exocrine tissue have been summarised in Table 18. Obese islets contained significantly higher concentrations of Na^+; K^+; Ca^{2+} and Mg^{2+} than normal islets and obese exocrine tissue. The Na^+ and K^+ concentrations in normal islets were significantly higher than the corresponding values for normal exocrine. The calcium levels in normal mouse islet and exocrine tissue were about the same, while the Mg^{2+} concentration in normal exocrine tissue was significantly higher than the Mg^{2+} concentration in normal islets. Normal and obese exocrine tissue contained similar concentration of Na^+; K^+ and Ca^{2+}; but normal exocrine tissue contained significantly higher concentration of Mg^{2+} than obese.

The ratios of the different ions to each other in islet and exocrine tissue have been summarised in Table 19. The Na^+/K^+ ratio was similar

TABLE 18. Monovalent and divalent ion concentrations in the pancreata of male mice. (20-25 week old mice; mean values ± S.E;)

(n = number of determinations, islets pooled from many mice for single determination).

	Islets (mEq/Kg fat free dried tissue)			Exocrine (mEq/Kg fat free dried tissue)			+ significance, $p<0.05$.			
	N		*CV	N		*CV	N/Ob islets	N/Ob exocrine	N.islet /N exo.	Ob isl /Ob exo
Na^+										
normal	5	101.53 ± 3.0	7%	3	73.07 ± 3.2	8%	+	-	+	+
obese	5	130.82 ± 2.3	4%	3	72.6 ± 4.6	11%				
K^+										
normal	5	503.48 ±12.1	5%	3	417.24±13.6	6%	+	-	+	+
obese	5	645.04 ±12.3	4%	3	447.89± 6.1	3%				
Mg^{2+}										
normal	4	80.83 ± 1.3	3%	3	116.65± 3.0	5%	+	+	+	+
obese	4	112.11 ± 2.4	4%	3	59.31± 3.4	10%				
Ca^{2+}										
normal	5	7.89 ± 0.8	23%	3	7.73± 2.7	61%	+	-	-	+
obese	5	12.77 ± 0.9	16%	3	9.23± 0.7	14%				

* Coefficient of Variation.

TABLE 19. Ion ratios in the pancreata of male normal and obese mice.

(ratios of Mean values).

	Na^+/K^+	Mg^{2+}/Na^+	Mg^{2+}/K^+	Ca^{2+}/Na^+	Ca^{2+}/K^+	Ca^{2+}/Mg^{2+}
normal islets	1 : 5	1 : 1.3	1 : 6.2	1 : 13	1 : 64	1 : 10
obese islets	1 : 5	1 : 1.2	1 : 5.8	1 : 10	1 : 51	1 : 9
normal exocrine	1 : 6	1 : 0.6	1 : 3.6	1 : 10	1 : 54	1 : 15
obese exocrine	1 : 6	1 : 1.2	1 : 7.7	1 : 8	1 : 49	1 : 7

Cont'd.....

to that found in other tissues (527).

In plasma the situation was a little different. Whereas in islet tissue, the mg^{2+} concentration was several orders of magnitude greater

than Ca^{2+}, in plasma the reverse was true, for both normal and obese mice (Table 20).

TABLE 20. Plasma Mg^{2+} and Ca^{2+} concentrations of 20 - 25 week old normal and obese mice. ($\underline{n}$ = number of mice, Mean Values $\pm$ S.E.).

	N	Obese Mice (mEq/litre, plasma)	CV†	N	Normal Mice (mEq/litre, plasma)	CV†
Mg^{2+}	3	2.11 $\pm$ 0.05	4%	3	1.87 $\pm$ 0.05*	5%
Ca^{2+}	6	4.30 $\pm$ 0.51	29%	8	3.82 $\pm$ 0.28	20%

The Ca^{2+}/Mg^{2+} ratio in plasma from 20 - 25 week old normal and obese mice

1 : 0.49 1 : 0.49

* Denotes significant difference ($P < 0.05$) between normal and obese mouse plasma Mg^{2+} concentrations.

† CV = coefficients of variation.

While there was no significant difference between the plasma Ca^{2+} levels of normal and obese mice, the plasma Mg^{2+} concentration of obese mice was significantly higher than that of normal mice. Both normal and obese mice had a similar Ca^{2+}/Mg^{2+} plasma ratio. Clearly, the levels of Ca^{2+} and Mg^{2+} in normal and obese mouse plasma would accommodate the competitive inhibition of a β-cell membrane bound Mg^{2+} dependent enzyme system by calcium, as long as the active site of the enzyme was on the outside of the cell membrane. Such a situation has not been suggested for adenyl cyclase, the catalytic site of the latter was believed to be in contact with the intracellular environment (460).

Coefficients of variation for calcium determinations were high, because estimations were made near the detection limit of the instrument.

Discussion:

A loss of ions invariably occurred during the microdissection of islets and exocrine tissue. Compared to normal, the higher levels of all four ions in obese islets probably reflected their higher metabolic activity. The higher concentrations of Na^{+} and Ca^{2+} in obese islets, compared to normal, were consonant with the presence of an active sodium dependent calcium uptake system in obese islet β-cells (415) and might have accounted for their hypersecretion of insulin.

From the Ca^{2+}/Mg^{2+} ratios of both normal and obese islets it was difficult to see how Ca^{2+} might competitively inhibit Mg^{2+} dependen enzyme systems such as adenyl cyclase when Mg^{2+} was in so great an excess, although the specific intracellular translocation of Ca^{2+} would be a way of achieving this.

Besides confirming the presence and proportion of the four ions in islet tissueand plasma, these total icn estimations have not contributed a great deal towards an understanding of the role played by ions, especially calcium and sodium in the mechanism of insulin secretion. When measurements were made of the total Mg^{2+} or Ca^{2+} content in a tissue, the concentration value obtained was an expression of the sum of the bound and free or ionised forms of the metal. In consequence little physiological importance could be attached to the measurement of total Ca^{2+} concentration either of islet tissue or plasma, especially since it was probably the influx of ionised calcium that was associated with the secretion of insulin.

Hellman and colleagues have estimated the islet ATP content of 6 month old obese mice to be 6.9 mmoles of ATP/Kg. dry weight of islets (131). Both Mg^{2+}ATP and Ca^{2+}ATP may be potentially capable of acting as substrates for β-cell adenyl cyclase. Using Hellman's ATP concentration and the known concentrations of Mg^{2+} and Ca^{2+} in obese islet tissue (Table 18), theoretical Mg^{2+}/ATP and Ca^{2+}/ATP ratios have been calculated. The Ca^{2+}/ATP and Mg^{2+}/ATP ratios in obese islets were

found to be 1 : 14 and 1 : 3 respectively. The greater abundance of Mg^{2+}/ATP would support its postulated role as the substrate for islet adenyl cyclase and its ratio of 1 : 3 was in reasonable agreement with the optimal ratios of 1 : 1.5 and 1 : 1 reported for adrenal tumour (530) and cardiac tissue adenyl cyclase (298) respectively.

At this point it seemed relevant to briefly summarise the current ideas and hypotheses concerning the possible role of ions, especially Ca^{2+} and Na^{+} in the mechanism of glucose induced insulin secretion. Douglas & Rubin first used the phrase stimulus - secretion coupling to describe the events occurring in a secretory cell exposed to its immediate stimulus (531). These events led to the extracellular release of the secretory product. Douglas demonstrated the process of stimulus-secretion coupling in different systems to have a number of common characteristics (532). The more important of these were membrane depolarisation, a requirement for extracellular calcium and energy dependence. Extracellular calcium and sodium have been shown to be required for the stimulation of insulin secretion from rabbit pancreas <u>in vitro</u> by a whole range of diverse stimuli (387,533). Insulin secretion was stimulated by raising the extracellular K^{+} concentration (417,534). Indeed, many excitable tissues could be depolarised by raising the K^{+} concentration and this effect probably resulted from an increase in the sodium permeability of the cells. Glucose induced insulin secretion was stimulated by agents known to inhibit the sodium pump in other tissues and increase intracellular sodium levels i.e. ouabain (417). The stimulation of insulin secretion by a raised extracellular potassium concentration or ouabain required the presence of extracellular calcium and sodium (387). Howell & Taylor using isolated rabbit islets concluded, that though unphysiological concentrations of K^{+} may influence the secretion of insulin, fluxes of K^{+} in the islets did not appear to be important in the initiation of insulin secretion (106). Insulin secretion was also inhibited by high extracellular calcium or high extracellular

Mg^{2+} (387). Hales & Milner suggested that the situation found in insulin secretion might be similar to the calcium uptake system described in crab nerve by Baker & Blaunstein (535). This system had properties similar to those defined by Hales & Milner for insulin secretion i.e. the calcium uptake varied with the intracellular sodium content (being increased by a raised concentration) and was inhibited by high extracellular Mg^{2+} and Na^{+} concentrations. Hales & Milner suggested that insulin secretion was likely to be triggered by an increased uptake of calcium into the β-cell by a similar system (45). The glucose stimulation of insulin secretion was suggested to be composed of three stages.

i) The metabolism of glucose by the β-cell leading to the production of a signal; ii) the action of the signal on the sodium-calcium system leading to an increased calcium uptake by the β-cell, iii) the secretion of insulin triggered by calcium. The most specific inhibitors were expected to act at stage i) and the least specific at stage iii) i.e. inhibitors which blocked all known stimuli of insulin secretion were likely to act late in the chain of events in stimulus secretion coupling. Since all stages of the mechanism were likely to be energy requiring, anoxia and dinitrophenol probably acted late in the sequence i.e. at the calcium uptake or insulin secretion stage or both. Diazoxide probably acted by blocking both calcium movements across β-cell membranes (45) and the physical secretion of β-granules from the β-cell (380). Adrenaline has been suggested to act late in the secretion mechanism (45). A criticism of the work of Hales & Milner was their use of stabilised pieces of whole rabbit pancreas for ion studies in vitro. The exocrine pancreas has been shown to contain substantial concentrations of Na^{+}; K^{+}; Ca^{2+} and Mg^{2+} (Table 18). The diffusion of ions from portions of exocrine pancreas into both the incubation medium and the microenvironment of islet cells, could have significantly influenced their insulin secretory activity, especially

when pieces of whole pancreas were incubated in media containing controlled exogenous concentrations of ions. However, the greater part of the work of Hales & Milner has been verified and expanded by Malaisse and colleagues (44,410). The inhibitory effect of adrenaline on glucose induced insulin secretion, acting via α-adrenergic receptors was suggested by Malaisse and colleagues to be mediated through a blockade of the influx of alkaline earth cations into the β-cell (44). Adrenaline was shown to inhibit the uptake of ^{45}Ca by isolated rat islets incubated in the presence of glucose (396). Glucose on the other hand was found to stimulate ^{45}Ca uptake by isolated rat islets and changes in uptake were found to parallel changes in insulin secretion by the islets. Malaisse and colleagues suggested that the insulinotropic effect of glucose; mannose; leucine; arginine (536,537) and HB419 (538) was mediated via an accumulation of calcium in the β-cell. However, the rate of calcium uptake was suggested to be not merely a function of the rate of insulin secretion, rather their results suggested that glucose, when metabolised in the β-cell stimulated calcium uptake which in turn triggered the secretion of insulin. In 1968, Lacy and his colleagues suggested that a microtubular-microfilamentous cytoskeleton could provid internal pathways for the controlled movement of insulin secretory granules in the pancreatic β-cell during the process of emiocytosis (500). The results of recent work by Malaisse and coworkers have suggested that the influx of Ca^{2+} and its redistribution could trigger the secretory process by causing the contraction of the microtubular-microfilamentous system, that may be composed of actomyosin like material (539), favouring the migration of secretory granules and their extrusion by the process of emiocytosis. The involvement of this system was suggested by the inhibition of glucose induced insulin secretion from pieces of pancreas preincubated in the presence of colchicine, a compound known to interfere with the structure and function of microtubules in a number of cells (540). That ^{c}AMP may be associated

with calcium dependent glucose stimulated insulin secretion has also been suggested recently by Malaisee and colleagues (541). They indicated that the primary site of ^{c}AMP action in the β-cell corresponde to either a facilitation of glucose metabolism, a modification of calciu distribution or an interaction with the microtubular system. An effect of ^{c}AMP upon calcium distribution in the β-cell was suggested by the fact that, whereas glucose was unable to promote insulin secretion in the absence of extracellular calcium, the addition of db-^{c}AMP or theophylline to the calcium depleted medium restored the insulinotropic action of glucose. Also theophylline produced a dramatic increase in ^{45}Ca efflux from perfused islets, in the presence or absence of glucose. The insulinotropic action of ^{c}AMP has been suggested to be due to a glucose-independent translocation of calcium within the β-cell, from an organelle-bound pool to a cytoplasmic pool of ionised calcium readily available for transport across the cell membrane. However, the net uptake of calcium by the β-cell depended on the relative rates of Ca^{2+} efflux and influx at the β-cell membrane and Malaisse and Brisson concluded that the stimulant effect of glucose on Ca^{2+} uptake was due in part to a decreased efflux of Ca^{2+} from the β-cell. Changes in calcium metabolism were expected to account for both the rapidity and sensitivity of the insulin secretory response to glucose (542). Recent work by Sehlin, Hellman and Täljedal on the ^{45}Ca uptake by microdissected islets of obese mice has demonstrated that whereas glucose enhanced the initial uptake of calcium, the equilibrium level of ^{45}Ca was not found to depend on the glucose concentration. The results of their work failed to demonstrate a clear correlation between insulin secretion and the rate of calcium uptake by β-cells, but suggested that secretion might depend on mechanisms directing the allocation of calcium to less mobile compartments of these cells such as mitochondria (543).

The observation that the glucose induced secretion of insulin was Na^{+} and Ca^{2+} dependent (387) suggested to Dean & Matthews that the

depolarisation of the β-cell membrane might initiate insulin secretion and that stimulants of insulin secretion might first affect the biophysical properties of the β-cell membrane leading to a transmembranal flux of Na^+ and Ca^{2+} ions. The ionic flux might then accelerate the mechanism responsible for insulin secretion (390). The membrane potential of islet cells was found to be glucose dependent (544) and increasing glucose concentration caused the appearance of small action potentials to be superimposed on the membrane potential. A positive correlation appeared to exist between the ability of glucose to initiate action potential discharge in islet cells and its ability to initiate insulin secretion (544). Alloxan was shown to initiate a rapid irreversible depolarisation of mouse islets, without the appearance of action potentials (545). However, islet cells could be protected against the depolarising effect of alloxan by pretreatment with high glucose and this suggested that alloxan might act via glucoreceptors located possible at the β-cell membrane. Matthews & Dean suggested that the triggering mechanism for action potentials activated by carbohydrates shared a similar but not necessarily identical, stereospecificity with the mechanism responsible for insulin secretion (546). They described a possible scheme by which action potential discharge might be correlated with insulin secretion. The process was triggered by glucose or a metabolite acting on a membrane receptor. Leucine and tolbutamide presumably acted with different membrane sites but the result was a membrane conformational transition resulting in an increased selective membrane permeability; ionic flux; the production of an action potential and the secretion of insulin. The maintenance of a normal membrane potential was dependent on the calcium ion (547) and Dean & Matthews indicated that the action potential induced by glucose in islet β-cells was due predominantly to calcium entry and that Na^+ tended to repress this calcium influx (548).

CHAPTER 5.

The role of ^{c}AMP in the β-cell.

Malaisse, Malaisse-Lagae & Mayhew suggested that the β-cell contained an adenyl cyclase system through which phosphorylase and possibly phosphofructokinase might be activated (41). Methylxanthines and ^{c}AMP were found to stimulate insulin secretion only in the presence of glucose and Malaisse and colleagues speculated that these agents stimulated insulin secretion mainly by enhancing the glycolytic pathway (462). Their findings prompted them to suggest that the intracellular accumulation of ^{c}AMP did not initiate the process of insulin secretion, but rather that it potentiated the stimulant effect of glucose upon the β-cell. Sutherland and coworkers indicated that a variety of enzyme processes were affected by ^{c}AMP and that the latter did the real work of the hormone by changing enzyme activities, permeability and other cellular processes (244,247,504). Enzyme patterns were known to vary in different tissues, thus allowing for variations in physiological responses when a single component such as ^{c}AMP was altered. ^{c}AMP was found to have the highest enthalpy of hydrolysis of most of the known cyclic nucleotides (549) and it seemed possible that the energetic properties of the cyclic phosphate moiety of ^{c}AMP were utilised in its biological activity.

Although ^{c}AMP has been shown to stimulate insulin secretion and to be a normal constituent of islet cells, its role in the β-cell has yet to be defined. On this basis the effect of ^{c}AMP or its dibutyryl derivative was investigated on a number of normal and obese mouse islet cell perameters: DNA and RNA content; the incorporation of ^{3}H-leucine into newly synthesised proinsulin and insulin; glucose-6-phosphate dehydrogenase activity and finally total glycogen content.

SECTION 1.

The effect of db-^{c}AMP on the total RNA and total DNA content of normal and obese islets.

Protein synthesis in animal tissues is controlled by the nucleus (550), the specificity of the template being determined by the nucleotide sequence of mRNA (551) and the protein itself being released from the mRNA template. Insulin synthesis in islet tissue appeared to be similar to protein synthesis in other tissues (552). Sandritter in 1964 using quantitative histochemical methods to study the mechanism of insulin synthesis/secretion suggested that the activity of the β-cell nucleus was directly linked to the insulin concentration in islets (553). The role of the cell nucleus in various stages of endocrine function, the correlation between nucleus and cytoplasm and the role of RNA in secretional processes was stressed, and a working hypothesis that the nucleus and cytoplasm of islet cells formed a functional unit and acted jointly during the process of insulin synthesis was postulated (553). It was shown conclusively that sites of active protein synthesis were also rich in RNA and that increased numbers of RNA particles could be attributed to an increased synthetic activity of the cell.

Clearly measurements of total RNA and total DNA, and changes in their concentration caused by exogenous materials could not accurately be correlated with changes in insulin biosynthesis or secretion. Indeed protein synthesis itself tended to be reflected more in the activity of the mRNA component (550). However, preliminary investigation of the effect of db-^{c}AMP on total RNA and total DNA in islet tissue facilitated a subsequent and more elaborate study of the effect of db-^{c}AMP on insulin and proinsulin biosynthesis.

Adenyl cyclase has been demonstrated in the nuclei of several different cell types (344,554) and the product of its activity, ^{c}AMP, has been shown to activate existing enzymes and induce the synthesis of

new enzymes (248,348). Perlman & Pastan were the first to suggest that ^{c}AMP regulated transcription (555). ^{c}AMP was believed to stimulate either the transcription of the specific messenger or its transmission from nucleus to cytoplasm. The mechanism by which ^{c}AMP stimulated the synthesis of functional messenger was and remains unknown. It has been suggested that the phosphorylation of histones and other nuclear proteins mediates the differential transcription of genes (556). A possible explanation for the effects of ^{c}AMP on transcription was reported by Langan who observed that the phosphorylation of histones was stimulated by ^{c}AMP (557). Because histones were thought to usually inhibit gene transcription, it was postulated that the ^{c}AMP mediated phosphorylation of histones caused them to be displaced from the DNA allowing genes to be transcribed into RNA. A causal link between the phosphorylation of nuclear protein and the activation of genes remains to be established, as does the anticipated precursor relationship between RNA synthesised in response to stimulation by ^{c}AMP and specific mRNA. Dokas & Kleinsmith demonstrated that within one hour after the administration of ^{c}AMP a dramatic increase occurred in the ability of liver cell nuclei to synthesise RNA (554). This effect on RNA synthesis was highly specific for ^{c}AMP. However, there was evidence to suggest that many of the effects of ^{c}AMP on the induction of enzyme and protein synthesis occurred at levels other than transcription (348,557). MacManus & Whitfield have shown ^{c}AMP at physiological levels to stimulate DNA synthesis in rat thymic lymphocytes (558). Also Grand & Gross have demonstrated db-^{c}AMP to stimulate protein synthesis directly and independently of secretory activity in rat parotid gland slices (559).

The total RNA and total DNA content of normal and obese islet tissue was estimated using fresh tissue and incubated tissue treated with db-^{c}AMP.

MATERIALS & METHODS.

Islets were microdissected from the excised pancreata of normal and obese mice and pooled for experimental procedures. In incubation experiments islets were washed with fresh low glucose Gey & Gey buffer (110) and then incubated for the appropriate length of time in 2ml. of continuously oxygenated ($95\%O_2/5\%CO_2$) Gey & Gey buffer at 37°C, supplemented with 300mg/100ml and 100mg/100ml glucose for obese and normal islets respectively in the presence and absence of db-^{c}AMP (5mmol/litre).

Estimation of total DNA and total RNA concentration.

Both fresh and incubated islets were weighed wet in glass micro-homogenisers (all excess buffer removed with lens tissue) and then homogenised thoroughly in ice cold double distilled water, tissue/volume ratio, 1 : 9. The total RNA and DNA content of the homogenate so prepared was estimated by a modification of the method of Wannemacher, Banks & Wunner (560). To the homogenate was added 0.25ml. of ice cold 0.6 N perchloric acid. The contents were mixed, allowed to stand 10 minutes in ice and then centrifuged (10 mins, 1500 rev/min) and the supernatant discarded. The precipitate was washed twice with ice cold 0.2N perchloric acid. After washing 0.4 ml. of 0.3N KOH was added to the precipitate, and the contents mixed and incubated for 1 hour at 37°C with the tube sealed to prevent evaporation. 0.1ml. of ice cold 60% perchloric acid was added to the cooled alkaline digest and the mixture allowed to stand 10 minutes in ice. After centrifugation (10 minutes; 1500 rev/min) the supernatant was carefully removed with a drawn out pipette and retained for the estimation of total RNA. The precipitate was washed twice with 5ml. of 5% ice cold perchloric acid and finally stored in 95% ethanol for the estimation of total DNA.

RNA estimation.

The RNA supernatant was diluted to 3ml. with 0.2N perchloric acid, stirred thoroughly, and its absorbance read on a Beckman DB

spectrophotometer at 260 nm.

DNA estimation.

0.2 ml. of 0.5N perchloric acid was added to the DNA precipitate, and the latter hydrolysed at 96 ± 1°C for 45 minutes. After centrifugation (1500 rev/min, 10 minutes) the supernatant was removed and the remaining precipitate washed twice with 1.4 ml. of 0.5N perchloric acid. The supernatant and the two washes were combined and absorbance measurements obtained at 265 nm and 290 nm.

Preparation of standard curves for RNA and DNA estimations.

A range of calf liver RNA standards (Type IV, Sigma, absorbance peak at 259 nm.) 0 - 50 µg/3ml. were prepared. The different standard amounts of RNA were dissolved in the smallest volume of 0.3N KOH and subsequently made up to a working volume with 0.2N perchloric acid (usually 300ml.). A typical standard curve for calf liver RNA is shown in Fig. 21.

DNA standard curves were prepared in a similar manner using a calf thymus preparation (Na salt, Type I, Sigma) in 0.5N perchloric acid. Absorbance measurements were made at 265 and 290 nm, Fig. 22. The absorbance difference for samples read at these wavelengths was specific for the DNA moiety (561) and its value was obtained in terms of µg DNA from the standard curves.

Calculation:

RNA.

X mg. of islets was ultimately incorporated into 0.5 ml. of supernatant. This was diluted to 3 ml. (dilution factor x6). The absorbance was measured and its value obtained in terms of µg RNA (Y) from the standard curve.

Hence total RNA content = $\frac{Y \times 6}{X}$ µgRNA/mg. wet islet tissue.

FIG.21. Standard curve for the estimation of islet total RNA content.

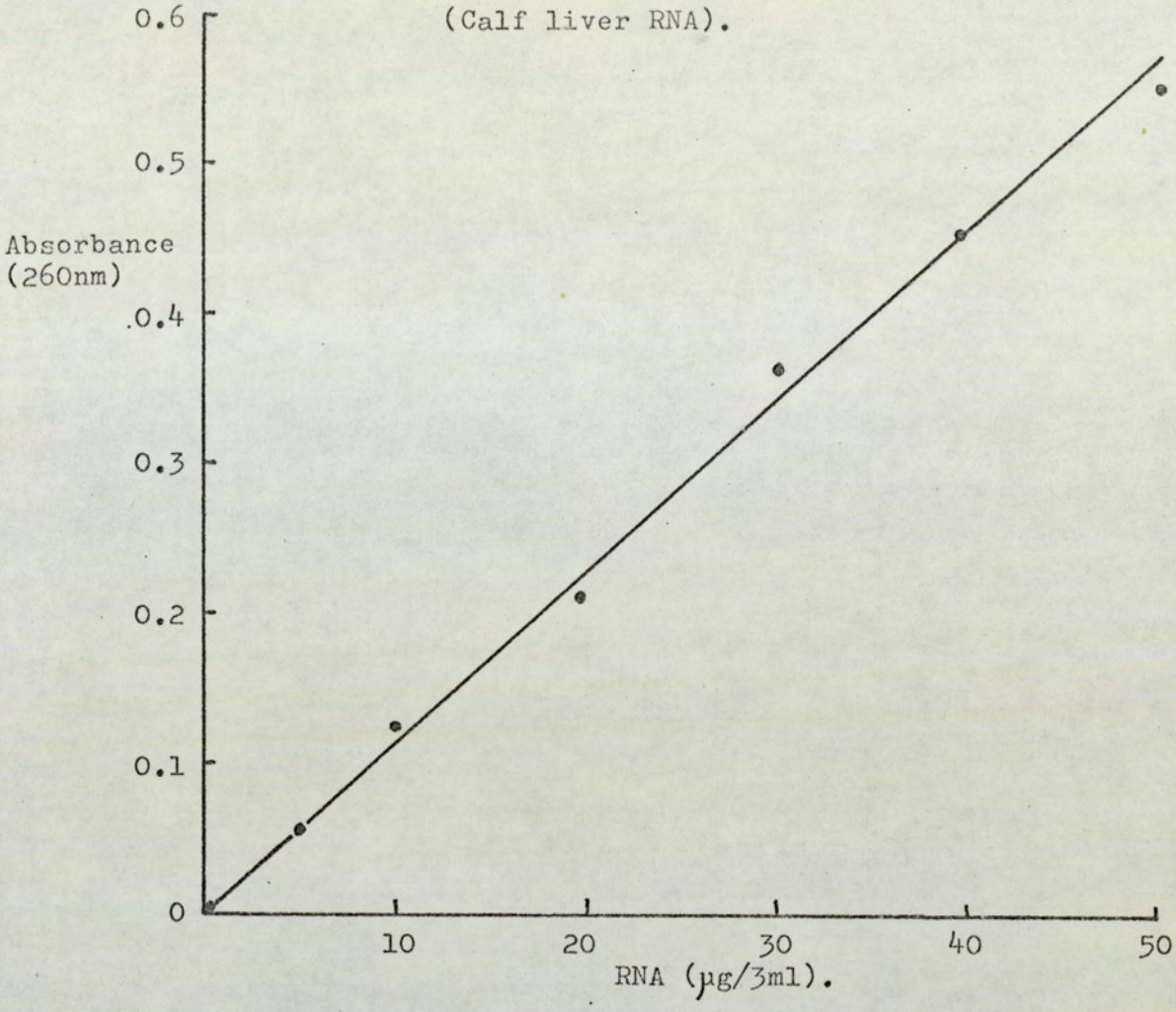
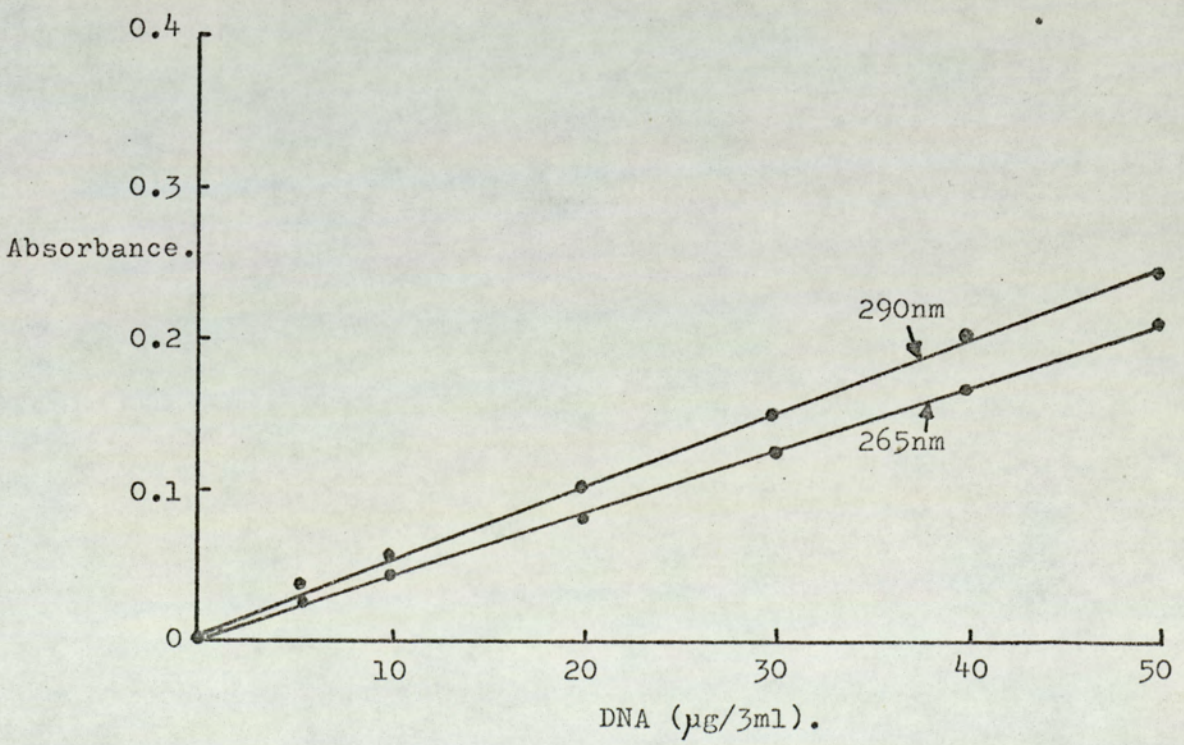


FIG.22. Standard curve for the estimation of islet total DNA content.

(Calf thymus DNA).



DNA.

x mg. of islets ultimately provided a 3ml. solution. Its absorbance gave a value of Z µg. of DNA from standard curves i.e. difference between absorbance at 265 nm and 290 nm in terms of µg. DNA. Hence total DNA content = $\frac{Z}{x}$ µg DNA/mg. wet islet tissue.

The total RNA and total DNA content of normal and obese mouse islet tissæwas estimated after 15 and 180 minutes incubation in the presence and absence of db-^{c}AMP (5mmol/litre).

Results:

While no significant difference was observed between the total DNA contents of normal and obese islets, the latter contained the higher content of total RNA (Table 21). The incubation of obese islets, for up to 20 minutes, gradually reduced their total RNA content but had no serious effect upon their content of total DNA, Fig. 23. 180 minutes of incubation exaggerated the reduction in total RNA but had no significant effect on the total DNA content of obese islets. A 15 minute incubation period was chosen in order to demonstrate the effect of db-^{c}AMP (5mmol/litre) on the total DNA and total RNA content of normal and obese islet tissue.

Db-^{c}AMP significantly increased the total RNA content of both normal and obese islets after 15 minutes incubation, by 31% and 17% respectively. Normal islets appeared to be more responsive to db-^{c}AMP than obese in this respect. On the other hand db-^{c}AMP had no significant effect on the total DNA content of normal and obese islets after 15 minutes incubation. Similarly, db-^{c}AMP had no significant effect on the total RNA content of normal islets subjected to 180 minutes incubation, but significantly increased the total RNA of obese islets by 41% after the same period of incubation. Rather surprisingly, whereas db-^{c}AMP had no significant effect upon obese islet total DNA content, this compound induced a 49% increase in the total DNA content of normal islets after 180 minutes incubation.

TABLE 21. The total RNA and total DNA of fresh, incubated and db-^{c}AMP treated islets of 20 - 25 week old normal and obese mice. (Mean values ± S.E., n = number of determinations, islets pooled from several mice for each RNA/DNA determination).

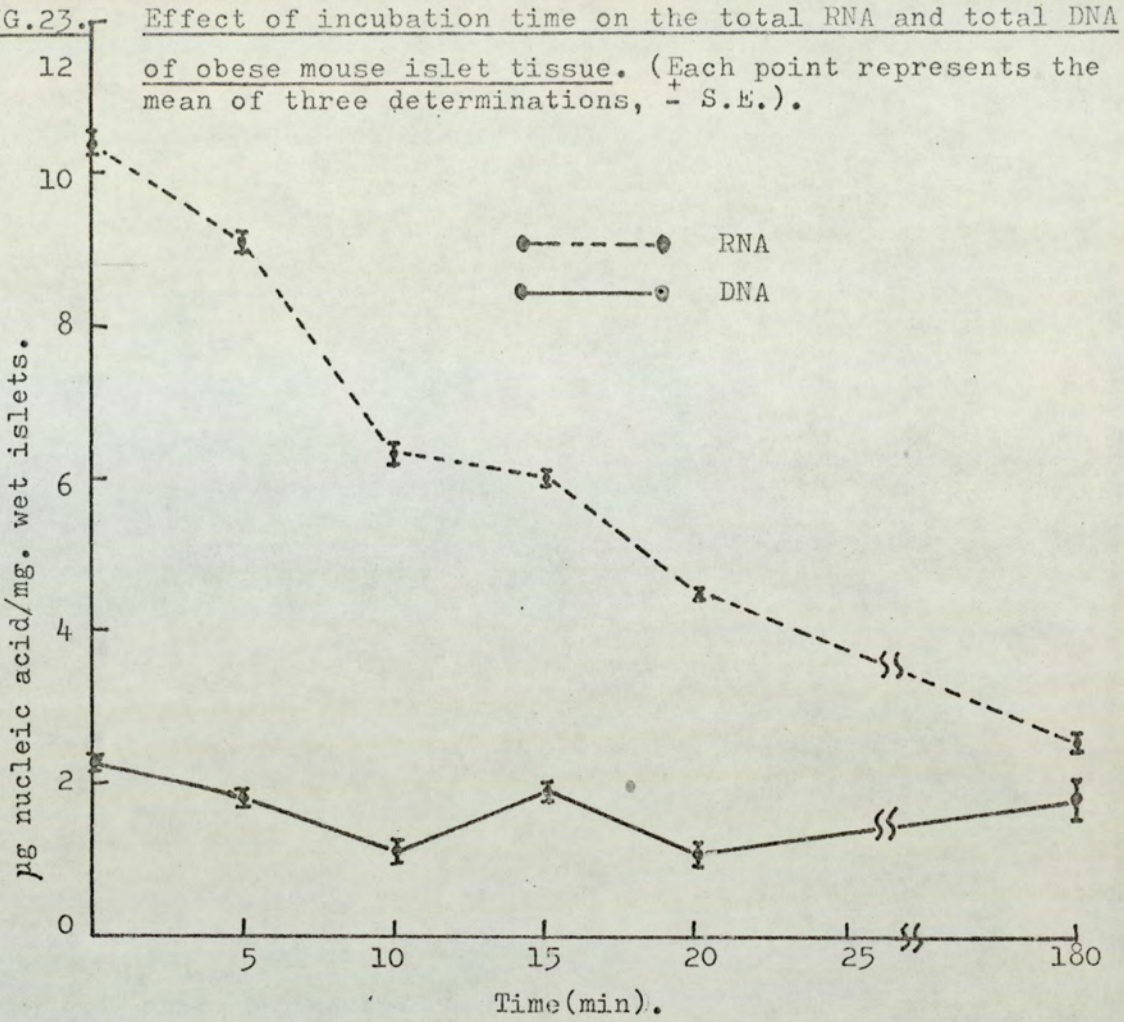
		Total RNA(µg/mg wet islets)				Total DNA(µg/mg wet islets)			
	N	Normal islets	CV	Obese islets	CV	Normal islets	CV	Obese islets	CV
Usual fresh tissue values	6	4.37 ± 0.66	3%	10.38 ± 0.07*	2%	1.80 ± 0.24	34%	2.29 ± 0.21	22%
Effect of db-^{c}AMP									
15 minutes of incubation									
No db-^{c}AMP	4	3.73 ± 0.08	4%	6.55 ± 0.24*	7%	1.93 ± 0.38	39%	1.70 ± 0.16	19%
db-^{c}AMP (5mmol/litre)	8	4.88 ± 0.22†	13%	7.64 ± 0.04*†	2%	1.97 ± 0.20	29%	2.04 ± 0.17	23%
180 minutes incubated									
No db-^{c}AMP	3	2.65 ± 0.09	6%	2.52 ± 0.05	3%	1.23 ± 0.09	13%	1.86 ± 0.25	24%
db-^{c}AMP (5mmol/litre)	3	2.20 ± 0.30	24%	3.56 ± 0.05*†	3%	1.83 ± 0.08†	7%	2.01 ± 0.19	16%

* Denotes significant difference ($P < 0.05$) between normal and obese islet values subjected to same treatment.

† Denotes significant difference ($P < 0.05$) between values obtained in the presence and absence of db-^{c}AMP for both normal and obese mice.

CV = Coefficient of Variation.

FIG.23. Effect of incubation time on the total RNA and total DNA of obese mouse islet tissue. (Each point represents the mean of three determinations, ± S.E.).

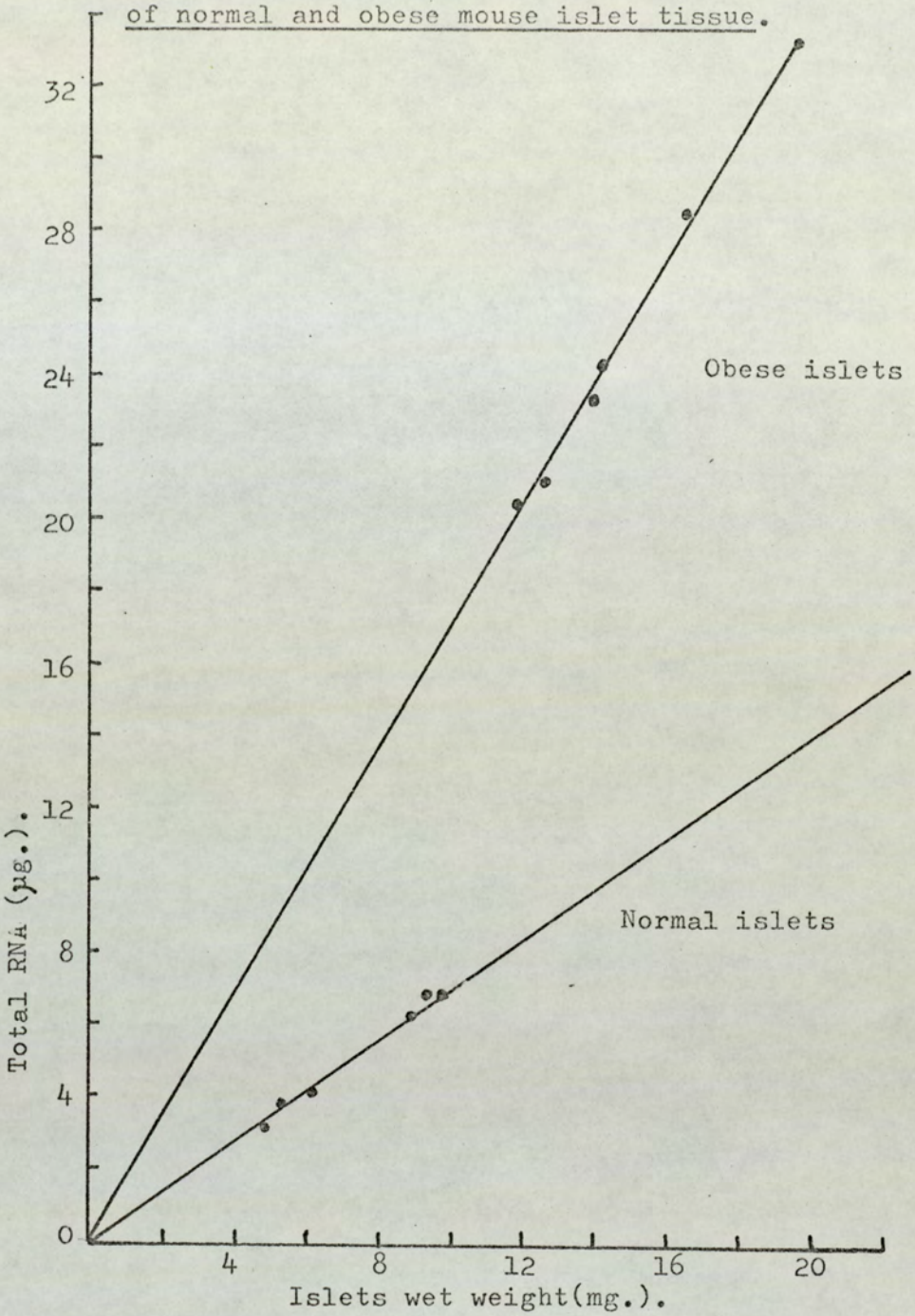


Coefficients of variation for groups of results have shown the estimation of total RNA to have a better degree of repeatability than the total DNA estimation (Table 21). In addition the total RNA content of both normal and obese islets was proportionally related to the weight of tissue used Fig. 24. No such relationship could be demonstrated for the total DNA content of normal and obese islet tissue.

Discussion.

Compared to normal the higher total RNA content of obese islets was consonant with their insulin hypersecretory activity. Also, the similar normal and obese islet total DNA levels were not entirely unexpected. The elevation of the total RNA levels but not total DNA levels, of both normal and obese islets after 15 minutes incubation with db-^{c}AMP suggested the possibility of a role for db-^{c}AMP in the transcription process and possibily protein synthesis. However, a db-^{c}AMP induced increase in the total islet RNA content could not be

FIG.24. The relationship between the wet weight and total RNA content of normal and obese mouse islet tissue.



accurately equated with an increased insulin biosynthesis. Db-^{c}AMP might exert both translational and transcriptional effects. The action of ^{c}AMP at the transcriptional level has already been described (554,557) and the phosphorylation of histone was probably stimulated by ^{c}AMP via a ^{c}AMP dependent histone kinase (562). ^{c}AMP dependent protein kinases have been demonstrated in a variety of animal tissues and catalyse the phosphorylation of casein, protamine or histone by ATP (563). ^{c}AMP might exert its effect by dissociating the kinase into an inhibitory and a catalytically active subunit; the inhibitory component binding ^{c}AMP (565). Also, ^{c}AMP complexed to protein kinase has been shown to be resistant to ^{c}AMP-phosphodiesterase hydrolysis (567). Such protein kinases almost

certainly occur in islet tissue and may be intimately associated with insulin biosynthesis and perhaps even the mechanism of insulin secretion per se. Indeed Kuo & Greengard have suggested that the wide variety of effects elicited by ^{c}AMP were mediated via the stimulation of protein kinases (564). The particularly attractive feature of this hypothesis was that it provided a single reaction mechanism by which ^{c}AMP could bring about its diverse effects, with the specificity of the action of ^{c}AMP residing in the nature of the particular protein kinase and/or its substrates.

Evidence for an effect of ^{c}AMP on translation in protein synthesis has been indirect and tentative. Although Pastan & Perlman investigating the synthesis of tryptophanase in E. Coli have provided evidence of an effect for ^{c}AMP at the polysome (566).

SECTION 2.

The effect of glucose, db-^{c}AMP and NADPH on the incorporation of [^{3}H]-leucine into newly synthesised insulin and proinsulin of normal and obese mouse islets in vitro.

Taylor, Smith & Gardner showed that when isolated rat pancreas was incubated in a bicarbonate-buffered medium containing [^{3}H]- leucine as well as glucose, a substantial incorporation of tritium appeared to take place into rat insulin and into material with the properties of A chain (568). The isolation of labelled insulin, produced by incubating pieces of whole pancreas with labelled amino acids in vitro, was complicated by the contamination caused by the large excess of other pancreatic proteins and the fact that certain animals possessed two chromatographically separable types of insulin i.e. rat (569). An immunological method for the isolation of radioactive insulin produced from ox pancreatic slices has been described in which insulin was precipitated specifically by antiserum made against ox insulin (570). Using this method, the effect of various factors on the incorporation of [^{3}H]- leucine into insulin were

studied (571). 2,4-dinitrophenol was found to abolish the incorporation of tritium into an acidic ethanol soluble fraction of pancreatic proteins and ox insulin *in vitro* (572) while alloxan had no effect. This suggested that alloxan was without any primary effect on insulin biosynthesis. The effect of 2,4-dinitrophenol suggested that thelabelling of insulin or at least its A chain was carried out via a biosynthetic mechanism that was energy dependent (573) and that incorporation was by the synthesis of protein. The incorporation of labelled leucine into insulin was found to be enhanced by increasing glucose concentrations and it was suggested that a high concentration of glucose in the incubation medium was important in achieving a rapid rate of incorporation into insulin (574). This effect appeared to be specific for insulin, and alterations in the glucose content of the medium had no effect on the incorporation into acid-ethanol extracts of other pancreatic proteins. Though it had long been known that high concentrations of glucose could promote the secretion of insulin from islet tissue, there was less evidence of a direct effect of sugars on insulin biosynthesis. However, both glucose and mannose were shown to increase leucine incorporation into slices of ox and rabbit pancreas (575) and isolated rabbit islets (576) *in vitro*. The synthesis of insulin was thought to be dependent on the provision of energy from the oxidation of sugars, although in other tissues such as muscle, the incorporation of labelled amino acids into protein seemed to be independent of glucose concentration (577). The glucose induced increase in the rate of insulin biosynthesis was of physiological importance in preventing an excessive depletion of β-cell insulin under conditions of maximal stimulation.

Using slices of rabbit pancreas Howell & Taylor investigated the time taken for the secretion of newly synthesised insulin *in vitro*, previously labelled by incubation of the slices with [^{3}H]-leucine (578). They found a marked increase in the specific radioactivity of insulin

secreted from slices in response to a high concentration of glucose in the second and third hours after labelling. After this period the secretion of labelled insulin was again diminished i.e. 4 h. after labelling. During the time taken between synthesis and secretion it waspresumed that newly synthesised insulin became associated with the β-granules. This was believed to involve the movement of the newly synthesised protein from the rough-surfaced endoplasmic reticulum to the golgi-complex, where granule formation probably occurred. It was not known accurately, what further time was necessary for the β-granules to move to the periphery of the β-cells in order to allow insulin to be secreted.

In 1967, Steiner & Oyer discovered a precursor to insulin which they named proinsulin (579). The existence of proinsulin as the precursor molecule for insulin has now been established in fish (580) and mammals (581,582) and has recently been the subject of a comprehensive review (583). The amino acid sequence of porcine proinsulin has been determined (584,585) and the C-peptide from bovine pancreas isolated and characterised (586). Proinsulin has been found in the serum of human subjects, suggesting that under certain circumstances, proinsulin was released from pancreatic islets (587). Clark & Steiner have shown that in the <u>in vitro</u> incubation of rat islets, increasing the glucose concentration resulted in the selective release of an increased proportion of newly synthesised proinsulin relative to insulin (588). Morris & Korner recently demonstrated that physiological concentrations of glucose, stimulated proinsulin biosynthesis in isolated rat islets, in preference to other islet proteins (589) and have suggested that the glucose stimulation of proinsulin synthesis was not mediated by newly synthesised RNA (590).

Up to the time of, and during part of the work to be described here, investigation of the effect of ^{c}AMP on insulin and proinsulin biosynthesis had been limited to the work of Lazarus and coworkers, who, using human

insulinoma tissue *in vitro* had been able to demonstrate a db-^{c}AMP and theophylline enhanced release of newly synthesised proinsulin and insulin (591). An attempt was therefore made to investigate the effect of db-^{c}AMP, theophylline and NADPH on the high glucose induced *in vitro* biosynthesis of proinsulin and insulin in microdissected islets of normal and obese mice. The effect of NADPH was investigated, because this compound had been shown to stimulate the *in vitro* secretion of insulin *per se.* (235) and was also probably involved with the metabolism of glucose in islet tissue.

MATERIALS & METHODS.

Islets were microdissected from the pancreata of 20 - 25 week old normal and obese mice allowed free access to food and water. Islets were often pooled from several animals and only those with intact connective tissue capsules were used in experimental work.

Incubation.

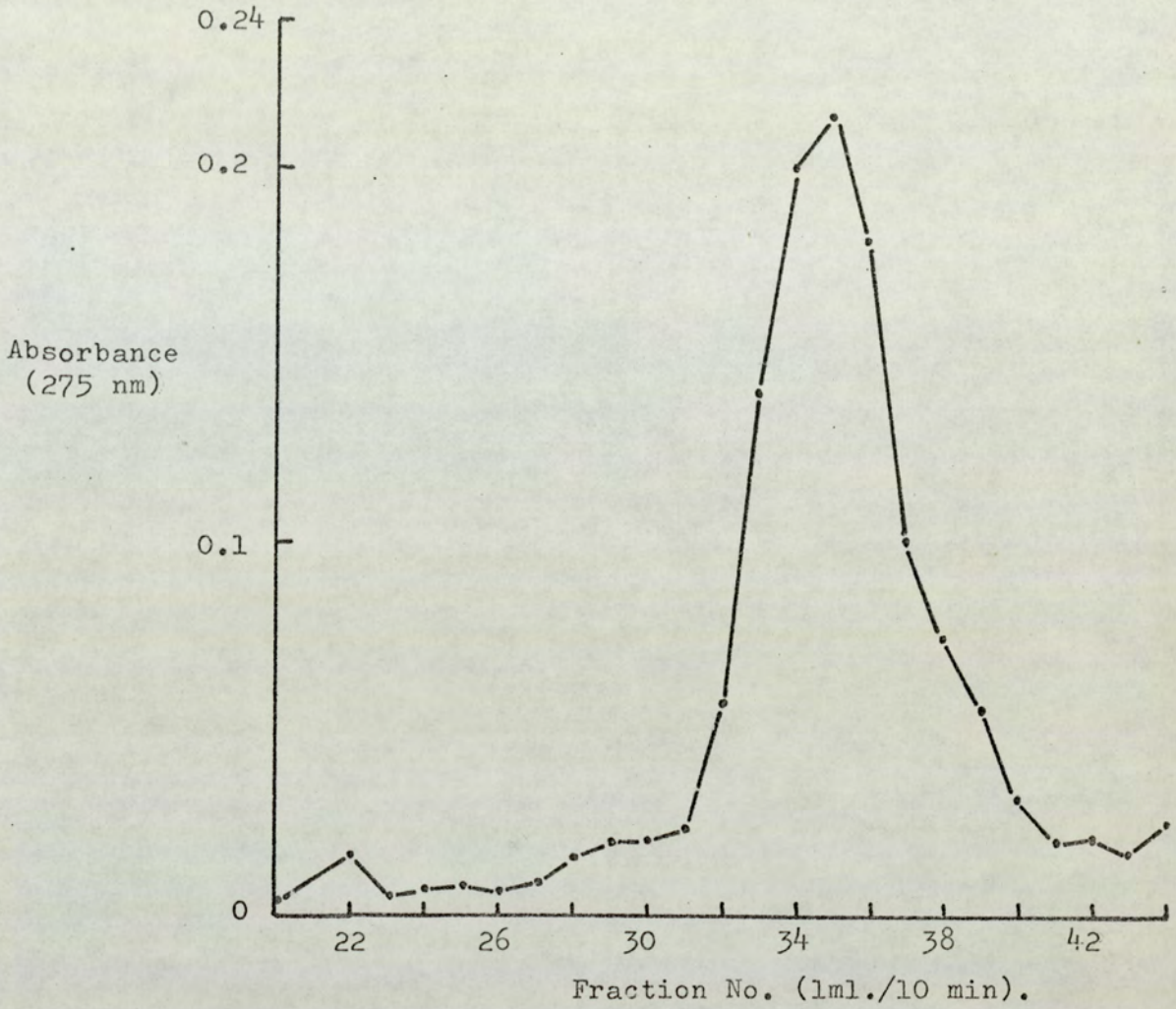
All glassware was treated with inert silicone compounds (Sigmacote or Siliclad). The procedures for the incubation of islets and the subsequent investigation of their capacity to incorporate $[^{3}H]$- leucine into proinsulin and insulin were similar to those described by Steiner and coworkers (579,582) and Lin & Haist (592). Groups of 20 intact islets were placed in small glass micro-homogeniser tubes (capacity - 2ml.). To each tube was added 0.5ml. of Gey & Gey buffer pH. 7.4 (110) containing 5μCi of dl-leucine 4,5$[^{3}H]$ (Specific activity, 19 Ci/mmole, final radioactive concentration 10mCi/litre, Radiochemical Centre, Amersham, Bucks.); bovine serum albumin V, 0.2 mg/ml; soya bean trypsin inhibitor, 20μg/ml. (Sigma type Is); glucose, 3g/litre; together with one of either db-^{c}AMP (5mmol/litre), theophylline (5mmol/litre) or NADPH (1mmol/litre, tetra-sod. salt, Boehringer) when appropriate. The microhomogeniser tubes were sealed with parafilm and incubated at 37°C for 3 h. with continual gassing ($95\%O_2/5\%CO_2$) via a long fine syringe needle. After incubation the tubes were cooled in ice and the contents

lightly centrifuged. The incubation medium was removed as completely as possible and retained in a capped 10ml. polythene centrifuge tube (M.S.E) at 4°C. The remaining islets were washed twice with Gey & Gey buffer containing 1 mg/ml. unlabelled dl-leucine and the supernatant discarded. A fresh charge (0.5ml.) of the original incubation medium (without labelled dl-leucine) was added to the islets and the latter completely homogenised over ice to prevent evaporation.

Extraction of insulin and proinsulin.

Insulin and proinsulin were extracted from homogenates and incubation media by the method of Davoren (593). To each was added 1.5ml. of acid/alcohol (750ml. of ethanol + 250ml. of distilled water and 15 ml. of ^{c}HCl) containing 2mg. of crystalline insulin (bovine, Sigma) and 0.5 mg. of unlabelled dl-leucine (Sigma) as carriers. The homogenates and incubation media were allowed to extract overnight at 4°C. The samples were then brought to pH. 8-8.5 (indicator paper) with a few drops of ammonium hydroxide (0.88 sp. grav.) and the white precipitate produced removed by light centrifugation at 0°C. Supernatants (≏ 1ml.) were transferred to silicone treated 10ml. polythene centrifuge tubes (M.S.E.) and the contents adjusted to pH. 5.5 (indicator paper) with 6N HCl. 0.5ml. of 2mol/litre ammonium acetate (pH. 5.3) was then added, followed by 2.5ml. of absolute alcohol and 5.5ml. of ether (anaesthetic grade). The tubes were capped and their contents allowed to react at -10°C overnight. The precipitate produced was collected by centrifugation at -5°C (10,000g, 30 minutes, M.S.E. superspeed 50). Alcohol and ether were allowed to evaporate off and the precipitate dissolved in 2ml. of 0.1N HCl. To this was slowly added 5ml. of 15% (mass/vol) sodium chloride solution, with stirring over 5 minutes. The solution was then centrifuged at +20°C for 1h. (12,00g, M.S.E. superspeed 50). The sodium chloride supernatant was discarded and tubes drained on filter paper. The precipitate was rewashed with ether, allowed to dry and then dissolved in 1ml. of

FIG. 25. Absorbance profile for bovine insulin.



1mol/litre acetic acid. This mixture was then transferred to the top of a 1 x 60cm. sephadex G.50 (fine) column with a syringe. The column itself was made of borosilicate glass treated with silicone, and a sample of 2mg. bovine serum albumin V + 2mg. bovine insulin had been passed through it prior to the addition of the test sample in order to reduce adsorbtion effects. The sephadex G-50 gel was prepared by heating with 1mol/litre acetic acid in a water bath for 1h. to remove air bubbles and then allowed to equilibrate at room temperature in 1mol/litre acetic acid prior to pouring. Samples were eluted from the column with acetic acid (1mol/litre) and 1ml. fractions collected (1 fraction/10 minutes) in sigmacote treated glass tubes (15ml), that had been washed out with a solution of 0.2mg/ml. bovine serum albumin in acetic acid (1mol/litre), in an attempt to obtain quantitative recovery of the labelled hormones. Fractions used for absorbance measurements were collected on an LKB-

ultrorac fraction collector in tubes that had not been previously treated with bovine albumin. The column had a void volume of 17-18 fractions, and after each use was flushed out for one day with acetic acid (1mol/litre). The absorbance of unlabelled insulin marker fractions was measured on a Beckman DB spectrophotometer at 275 nm. The elution position of bovine insulin but not its proinsulin contaminant was subsequently determined.

The radioactivity of fractions containing labelled insulin and proinsulin was measured by adding 10ml. of Bray's scintillation fluid (595) to the 1 ml. fraction in a 20ml. glass scintillation vial. The activity was counted on a packard scintillation counter, mean counting efficiency 10%. The radioactive yield was very low due to the lengthy extraction procedure (less than 1% of the theoretical amount of activity added). Blank vials contained 1ml. of acetic acid (1mol/litre) and 10ml. of scintillation fluid and their activity was substracted from test values after correction to 100% efficiency.

The effects of db-^{c}AMP, theophylline and NADPH were investigated on the glucose induced incorporation of labelled leucine into newly synthesised normal and obese islet insulin and proinsulin and the capacity of these islets to secrete newly synthesised hormone into the incubation medium in response to glucose during 180 minutes incubation.

It was rather unfortunate that towards the end of these preliminary studies a very comprehensive paper appeared by Tanese and colleages (594), covering most facets of the role of db-^{c}AMP in the biosynthesis and secretion of proinsulin and insulin from rat islets <u>in vitro.</u> As a consequence no further work was carried out on this particular aspect of study.

<u>Results:</u>

The low number of estimations carried out did not warrant statistical analysis. Blank values ranged from 550-750 d/min. and had a coefficient

of variation of 7%.

Verification of the absorbance profile for bovine insulin.

A 2mg/ml. bovine insulin marker in acetic acid (1mol/litre) was used to locate insulin on the elution profile. The absorbance of 1ml. fractions was estimated at 275 nm. The usual position of insulin on the elution profile is shown in Fig. 25. Fraction collecting tubes were not treated with bovine serum albumin V when their contents were to be used for absorbance measurements.

The sephadex G-50 gel filtration peaks for the proinsulin and insulin present in extracts of normal and obese islets and their respective incubation media after 180 minutes incubation in the presence of glucose alone and in the presence of glucose and db-^{c}AMP are shown in Figures 26 and 27 respectively. No formal electrophoretic or chromatographic verification of the insulin and proinsulin peaks was carried out, since their identity had been adequately established by a number of workers in the field (579,592,594). The peaks for insulin and proinsulin in the media samples were not easily definable and in most cases values of the activity associated with them were only educated estimates. However, well defined peaks could be identified for proinsulin and insulin from both normal and obese islet extracts.

For the interpretation of the limited number of results obtained it was assumed, although not guaranteed, that the efficiency of the hormone extraction procedure remained constant for all the incorporation experiments carried out. The effects of 180 minutes incubation; db-^{c}AMP; theophylline and NADPH on the percentile and absolute distribution of radioactivity between the sephadex G-50 peaks of insulin and proinsulin present in extracts of normal and obese islets and their incubation media have been summarised in Table 22.

After 180 minutes incubation, islet tissue rather than incubation media contained the greater proportion of the total incorporated radioactivity and this distribution was found to hold for both normal

FIG. 26. The effect of 180 minutes incubation on the glucose induced incorporation of labelled leucine into newly synthesised islet proinsulin and insulin, and the secretion of newly synthesised hormone into the incubation medium. (Glucose 3g/litre).

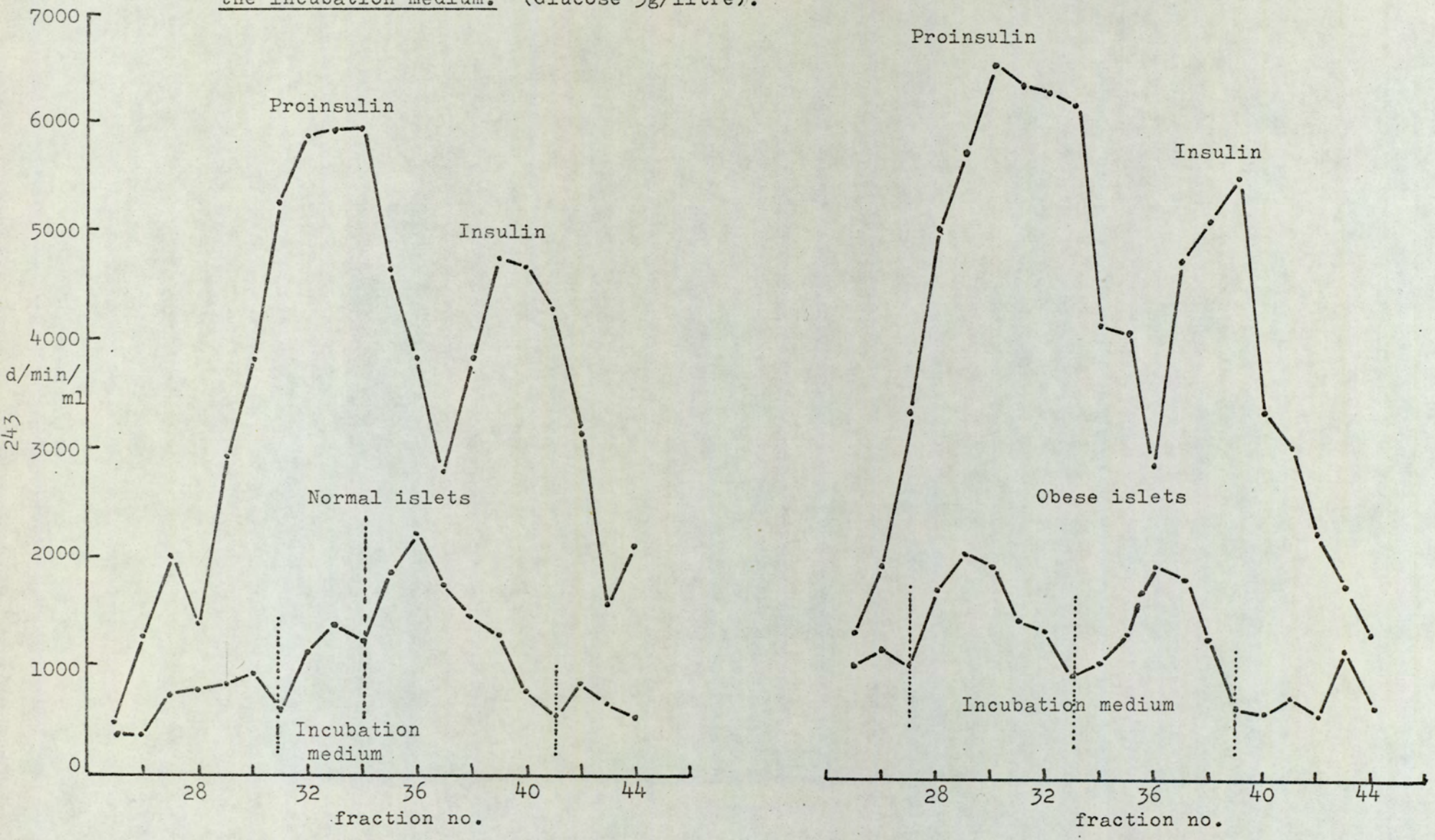


FIG. 27. The effect of 180 minutes incubation in the presence of db-^{c}AMP on the glucose induced incorporation of labelled leucine into newly synthesised islet proinsulin and insulin, and the secretion of newly synthesised hormone into the incubation medium. (Glucose 3g/litre).

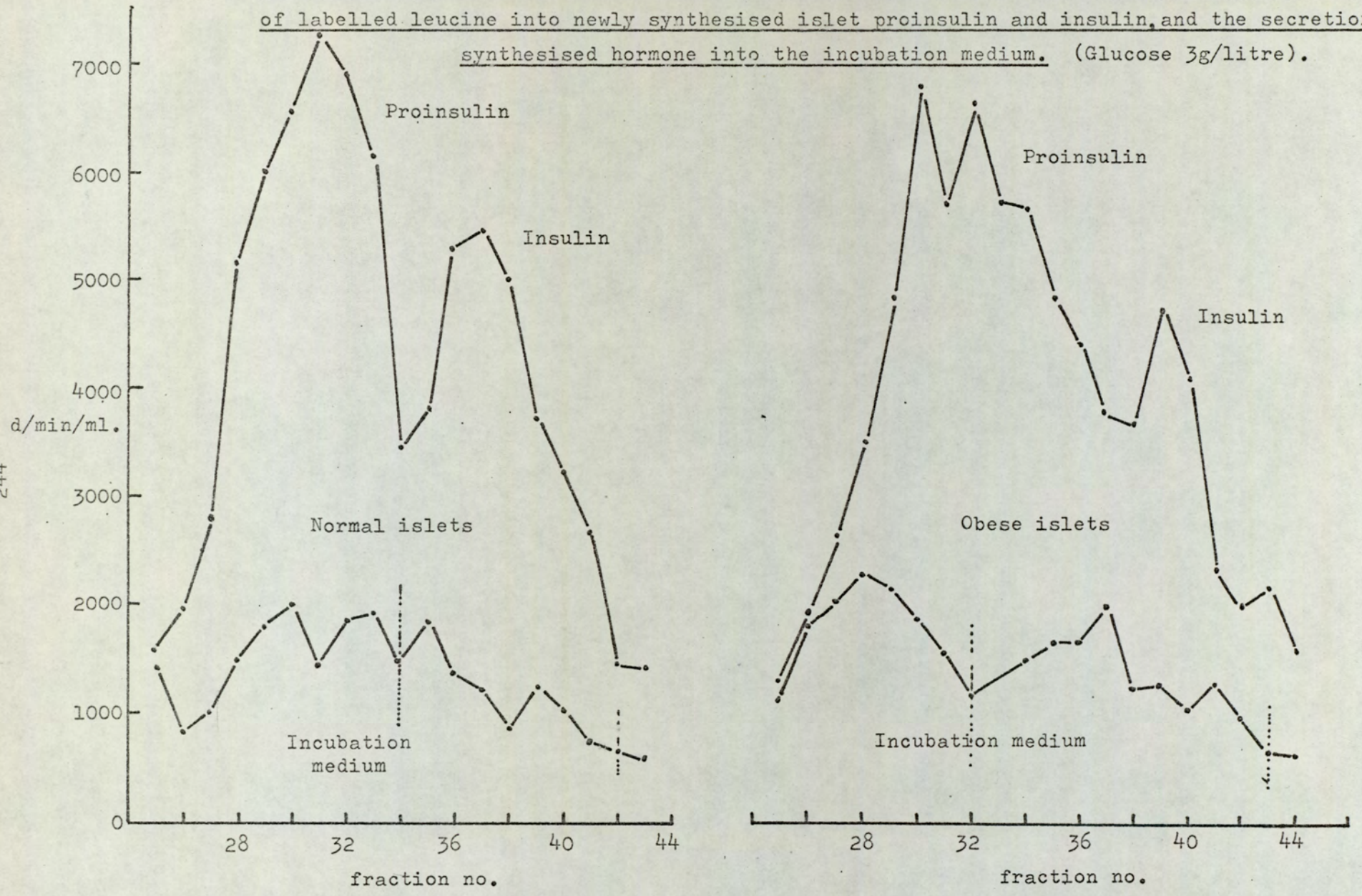


TABLE 22. The effect of 180 minutes incubation, db-^{c}AMP, theophylline and NADPH on the percentile and absolute distribution of radioactivity between newly synthesised proinsulin and insulin and thatsecreted from normal and obese islets in response to high glucose in vitro. (All incubations 180 minutes).

	Insulin (d/min).			Proinsulin (d/min).			Total incorporated radioactivity		
Normal islets	Medium *	Islets ‡	Total insulin synthesised †	Medium *	Islets ‡	Total proinsulin †	Medium ø	Islets ø	Total incorpn.
glucose 3g/litre	11137 (30%)	25006 (70%)	36143 (43%)	4341 (9%)	42332 (91%)	46673 (57%)	15478 (18%)	67338 (82%)	82816
+db-^{c}AMP (5mmol/litre)	9805 (22%)	34092 (78%)	43897 (40%)	15339 (24%)	48012 (76%)	63351 (60%)	25144 (23%)	82104 (77%)	107248
+theophylline (5mmol/litre)	7359 (28%)	18254 (72%)	25613 (35%)	15062 (31%)	32346 (69%)	47408 (65%)	22421 (30%)	50600 (70%)	73021
+NADPH (1mmol/litre)	5625 (11%)	41309 (89%)	46934 (49%)	13522 (27%)	34938 (73%)	48460 (51%)	19147 (20%)	76247 (80%)	95394
Obese islets									
glucose 3g/litre	9016 (23%)	30077 (77%)	39093 (37%)	10558 (16%)	54027 (84%)	64585 (63%)	19574 (18%)	84104 (82%)	103678
+db-^{c}AMP (5mmol/litre)	15560 (48%)	16819 (52%)	32379 (30%)	13958 (18%)	61359 (82%)	75317 (70%)	29518 (27%)	78178 (73%)	107696
+theophylline (5mmol/litre)	8477 (26%)	23769 (74%)	32246 (43%)	7423 (17%)	35139 (83%)	42562 (57%)	15900 (21%)	58908 (79%)	74808
+NADPH (1mmol/litre)	10379 (22%)	35150 (78%)	45529 (50%)	10196 (22%)	34525 (78%)	44721 (50%)	20575 (22%)	69675 (78%)	90250

* Secretion of newly synthesised hormone.
† Total hormone biosynthesis.
‡ % distribution of total hormone synthesised.
ø % distribution of total incorporated activity.

and obese islets in the presence of glucose alone, and in the presence of db-^{c}AMP, theophylline and NADPH. Obese islets compared to normal, showed the greater total glucose induced radioactive incorporation. Also, the total glucose induced incorporation into both normal and obese islets was marginally increased by db-^{c}AMP, but markedly reduced by theophylline. NADPH slightly increased the total glucose induced incorporation into normal islets while marginally decreasing the total incorporation into obese islets.

The greater percentage of the total incorporated activity was associated with glucose induced total proinsulin biosynthesis rather than total insulin biosynthesis and this distribution was found to hold for all normal islet treatments and all obese islet treatments except NADPH. The proportion of the total incorporated activity associated with glucose induced proinsulin biosynthesis was greater in obese islets than in normal. In both normal and obese islets, db-^{c}AMP increased the proportion of the total incorporated activity associated with glucose induced total proinsulin biosynthesis. However, theophylline only increased the proportion of the total incorporated activity associated with normal islet glucose induced total proinsulin biosynthesi In obese islets, theophylline reduced the proportion of the total incorporated activity associated with glucose induced total proinsulin biosynthesis. NADPH reduced the proportion of the total incorporated activity associated with the glucose induced total proinsulin biosynthesi in both normal and obese islets.

After 180 minutes incubation, obese islets secreted a greater proportion of their total newly synthesised proinsulin in response to glucose than did normal islets. This proportion secreted in response to glucose was increased markedly from normal islets, but only marginally from obese islets, by db-^{c}AMP, theophylline and NADPH.

The proportion of the total incorporated activity associated with glucose induced insulin biosynthesis was greater in normal islets than in obese. In both normal and obese islets db-^{c}AMP reduced the proportion of the total incorporated activity associated with glucose induced total insulin biosynthesis. However, theophylline only increased that proportion of the total incorporated activity associated with obese islet glucose induced total insulin biosynthesis. In normal islets, theophylline reduced that proportion of the total incorporated activity associated with total insulin biosynthesis. NADPH increased the proportion of the total incorporated activity associated with glucose induced total insulin biosynthesis in both normal and obese islets.

After 180 minutes incubation normal islets secreted a greater proportion of their total synthesised insulin in response to glucose than did obese islets. This proportion secreted in response to glucose by normal islets was marginally reduced by db-^{c}AMP and theophylline and markedly reduced by NADPH. The proportion secreted in response to glucose by obese islets was markedly increased by db-^{c}AMP, marginally increased by theophylline and little affected by NADPH.

Discussion:

In summary, db-^{c}AMP and theophylline acted on normal islets to increase both the proportion of the total incorporated activity associated with proinsulin synthesis and the secretion of newly synthesised proinsulin, while acting to reduce the proportion of the total incorporated activity associated with insulin synthesis and the secretion of newly synthesised insulin, compared to proportions observed in the presence of glucose alone. Db-^{c}AMP acted on obese islets to increase both the proportion of the total incorporated activity associated with proinsulin synthesis and the secretion of newly synthesised proinsulin, but while reducing the proportion of the total incorporated activity associated with insulin synthesis, db-^{c}AMP increased the proportion of newly synthesised insulin secreted. These observations

were consonant with a role for ^{c}AMP in the mechanism of insulin secretion and compared to normal, the higher levels of adenyl cyclase activity demonstrated for obese islets might account for their hypersecretory activity. However, in both normal and obese islets db-^{c}AMP would seem to be more involved with proinsulin synthesis and secretion than the synthesis and secretion of insulin. Theophylline acted on obese islets to reduce the proportion of the total incorporated activity associated with proinsulin synthesis, while increasing both the proportion of newly synthesised proinsulin secreted, and the proportions of the total incorporated activity associated with insulin synthesis and the secretion of newly synthesised insulin, compared to proportions observed in the presence of glucose alone. In both normal and obese islets NADPH acted to reduce both the proportion of the total incorporated activity associated with proinsulin synthesis and the secretion of newly synthesised insulin, but to increase the proportion of the newly synthesised proinsulin secreted and the proportion of the total incorporated activity associated with insulin synthesis compared to proportions observed in the presence of glucose alone. Obese islets seemed to be predisposed to the production and secretion of proinsulin rather than the conversion of proinsulin to insulin prior to secretion, while normal islets synthesised proinsulin but secreted a greater proportion of insulin in response to high glucose.

Db-^{c}AMP might be acting in both normal and obese islets to induce the preferential synthesis and secretion of proinsulin without conversion to insulin. Proinsulin might be secreted in preference to insulin in response to suddenly elevated and maintained blood glucose levels. The excessive synthesis and secretion of biologically less active proinsulin (596), in preference to insulin, might contribute to the observed hyperinsulinaemia and explain the prevailing hyperglycaemia in obese mice.

The very marginal proportional increase in normal islet proinsulin

synthesis and the proportional reduction in obese islet proinsulin synthesis in the presence of theophylline were consonant with the work of Halkerston and colleagues who have shown high concentrations of theophylline to cause an anomalous inhibition of protein synthesis in surviving rat adrenal tissue (597).

The proportional increase of both normal and obese islet glucose induced proinsulin biosynthesis by db-^{c}AMP might be interpreted as a translational effect. Indeed Permutt & Kipnis have suggested the acute stimulatory effect of glucose on insulin biosynthesis in rat islets to be translational rather than transcriptional (598).

Although db-^{c}AMP (272), theophylline (265) and NADPH (234,235) are established insulinsecretogogues, measurements of immunoreactable hormone have rarely been so specific as to determine whether the hormone secreted was insulin or proinsulin. The sephadex G-50 peaks of insulin and proinsulin from normal and obese islets might, in the future, be found to be composed of several types of insulin and proinsulin. Clark & Steiner have established the existence of two rat insulins and two rat proinsulins as well as several proinsulin intermediates and a free C-peptide (588). Tanese and coworkers have shown db-^{c}AMP to significantl increase the synthesis of all insulin moieties and to enhance their secretion (594). Insulin was found to be preferentially secreted to proinsulin from isolated rat islets.

In the work described here the fact that proinsulin was secreted into the incubation medium probably reflected the failure of the islet converting enzyme to keep pace with the increased proportion of glucose induced proinsulin synthesis seen in normal and obese islets in the presence of db-^{c}AMP. The preferential secretion of proinsulin over insulin might represent the preferential secretion of non-granular hormone over granule contents. Howell, Kostianovsky & Lacy have suggested that only insulin and double chain proinsulin intermediate forms were present in β-granules (599). Baird & Bauer have recently

shown ^{c}AMP to significantly stimulate [^{3}H]-leucine incorporation into proinsulin of isolated rat islets at normal physiological levels of glucose i.e. 70 - 100mg/100ml, but at 300mg/100ml. the glucose stimulatory effect of this agent was markedly diminished (600). Also at this glucose concentration NADPH was shown to inhibit theincorporation o labelled leucine into proinsulin. Consonant with this observation, NADPH reduced the proportion of the total incorporated activity associated with proinsulin biosynthesis in both normal and obese islets (Table 22). Baird & Bauer emphasised the rapid and specific regulatory role of glucose in proinsulin biosynthesis and suggested that ^{c}AMP might influence the latter by modulating glucose metabolism (600).

The synthesis and secretion of increased amounts of proinsulin, with high immunoreactivity and relatively low biological activity, might theoretically explain the marked hyperglycaemia and greatly elevated serum IRI levels seen in obese mice. However, Poffenbarger and colleagues using spontaneously diabetic mice characterised by early obesity, hyperinsulinaemia and progressive hyperglycaemia showed proinsulin to comprise only a small proportion of the total circulating insulin immunoreactivity (601,602). In consequence,the apparent biological ineffectiveness of the high circulating levels of immunoreactive insulin in the diabetic syndrome did not appear to be the result of a disproportionate secretion of biologically less potent proinsulin nor did there appear to be any detectable abnormality in proinsulin-insulin biosynthesis

Steiner & Clark have suggested that proinsulin served neither as a major storage nor secretory form of the hormone and that its primary function was to facilitate the efficient formation of the disulphide bonds of insulin (603). Insulin was the major storage form of the hormone and the conversion of proinsulin to insulin was believed to take place in the golgi apparatus or in the initial phase of granule maturation (604) by vesiculation from the golgi periphery (605) and the C-peptide and insulin produced by the conversion were secreted together into the blood stream.

^{c}AMP might be capable of influencing the conversion of proinsulin to insulin and might even be present in β-granules. Track and colleagues have proposed that t-RNA modulation might play a role in the regulation of the rate of proinsulin biosynthesis and that t-RNA modulation might be one of the regulatory mechanisms in insulin synthesis, in addition to a mechanism not involving the synthesis of RNA (606,607).

SECTION 3.

The effect of db-^{c}AMP on the glucose-6-phosphate dehydrogenase activity in normal and obese mouse islet tissue.

In an attempt to further investigate the possible role of ^{c}AMP (db-^{c}AMP) in the metabolism of glucose within isolated mouse islets, its effect was studied on the activity of a prominent member of the pentose-phosphate pathway, cytoplasmic glucose-6-phosphate dehydrogenase (G6PDH). Matschinsky, Kauffman & Ellerman have demonstrated the presence of an active pentose phosphate pathway in islet tissue (608). Consonant with the thesis that the effect of ^{c}AMP on insulin secretion might be mediated via alterations in the metabolism of glucose by the β-cell, glucose was required in the incubation medium for the effect of glucagon or theophylline on insulin section (41). Also Montague & Taylor have shown theophylline and glucagon to decrease G-6-PO_4 and increase 6-phosphogluconate levels in incubated rat islets in the presence of glucose (359). The increased concentration of 6-phosphogluconate was taken to indicate that there was an increased conversion of G-6-PO_4 into 6-phosphogluconate. The rise in the latter was suggested to reflect an increased islet G6PDH activity mediated directly or indirectly by ^{c}AMP. It was the aim of this study to investigate this possibility.

MATERIALS & METHODS

Histochemical control for G6PDH activity in islet tissue.

Enzyme activity was demonstrated using 8µm air dried cryostat sections. Glucose-6-phosphate (disod.salt) was used as the substrate with MTT (609).

Assay for glucose-6-phosphate dehydrogenase (EC. 1.1.1.49).

Islets microdissected from the excised pancreata of both 20-25 week old normal and obese mice were pooled and weighed wet, but with all adherent buffer removed. Islets were homogenised in phosphate buffer (Sørenson (610), pH. 7.5) mass/vol. ratio 1 : 9 and centrifuged for 30 minutes at 0°C and 20,000 rev/min. (M.S.E). This procedure provided sufficient supernatant for both G6PDH and total protein determinations. Protein was estimated by Lowry's procedure (218) using 1 cm light path microcuvettes in a Beckman DB Spectrophotometer at 750nm. A standard curve for protein estimation was prepared using fresh solutions of bovine serum albumin $\overline{V}$ in phosphate buffer, pH. 7.5 (Fig.28). Glucose-6-phosphate dehydrogenase was measured in 50µl samples of islet supernatant using a Biochimica test combination (UV-method, T.C.W. Cat. No. 15993. TGAC Boehringer) at 366 nm and 25°C (Unicam SP 800).

Calculation:

$$G\text{-}6\text{-}PO_4 + NADP \xrightarrow[Mg^{2+}]{G6PDH} \text{gluconate-6-}PO_4 + NADPH + H^+$$

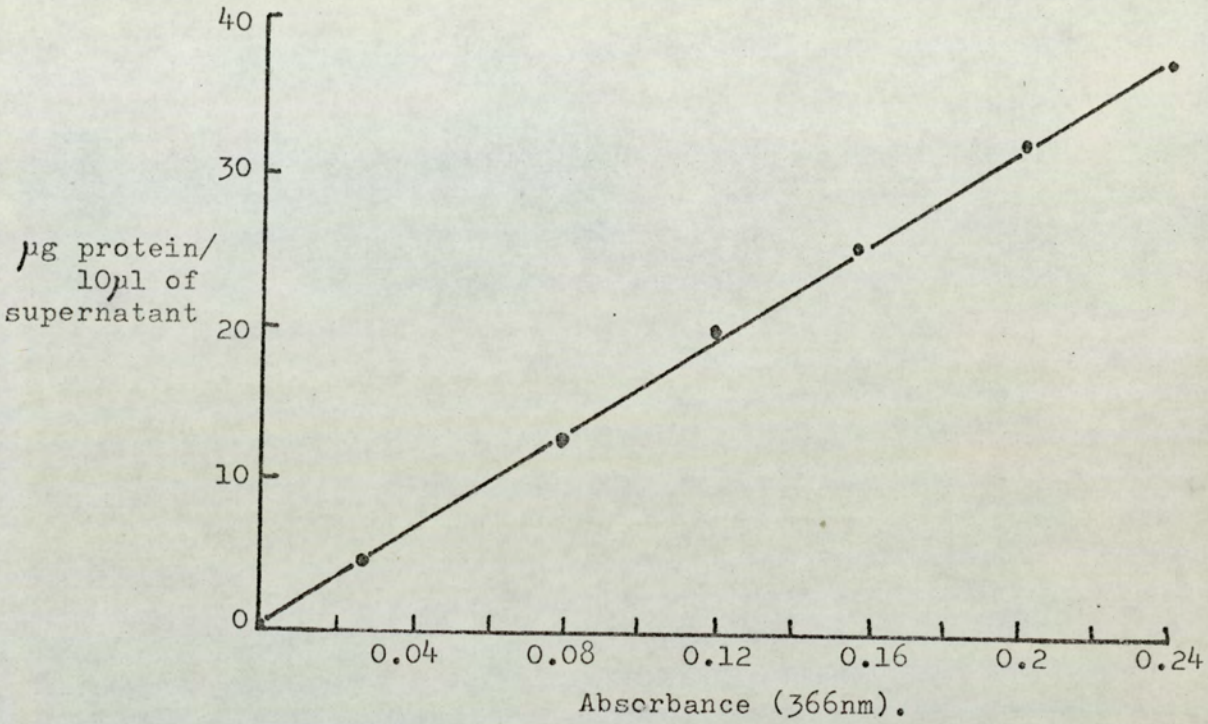
There was an increase in optical density with time. 10 µl and 50 µl supernatant samples were taken for protein and G6PDH determinations respectively. The protein content of the supernatant (µg/10µl) was obtained from the standard curve. This was converted to µg/50ul. The G6PDH absorbance/5 minutes was obtained from the SP 800 chart and the change in absorbance per minute Δ E/min evaluated.

Δ E 366nm/min x 955 x 20 = mU/ml serum equivalents.

Then $\frac{\text{mU/ml}}{20}$ = mU/50µl G6PDH activity.

and $\frac{\text{mU/50µl}}{\text{µg protein/50µl}} \times 1000 =$ mU G6PDH activity/mg islet protein 25°C, pH. 7.5

FIG. 28. Standard protein curve for G6PDH estimation.
(B.S. Albumin in phosphate buffer, pH. 7.5).



G6PDH units were expressed in terms of serum equivalents and islet protein as bovine serum albumin $\overline{V}$ equivalents. The unit of G6PDH activity was defined as the amount of enzyme which converted 1µmole of substrate per minute under highly optimal conditions. Measurements at 25°C related to 1ml. of serum.

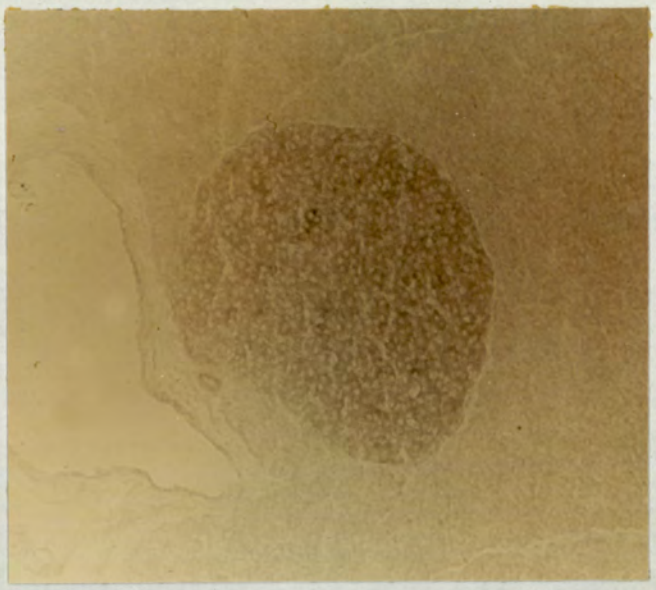
$$1U/ml = \frac{1\mu mole \text{ of substrate converted}}{1 \text{ min} \times 1 \text{ ml.}}$$

The G6PDH activity of fresh islet tissue and incubated islet tissue in the presence and absence of db-^{c}AMP (5mmol/litre) was estimated for both normal and obese mice.

Results:

G6PDH activity was demonstrated in islet tissue from both normal and obese mice. The histochemical reaction was more intense in islet tissue than exocrine (Plate 20). Similar results have been obtained by Kissane and colleagues (94) using rat pancreas and Petkov using guinea pig pancreas (611).

PLATE 20. Glucose-6-phosphate dehydrogenase activity in obese mouse islet and exocrine tissue (x40).



Microchemical estimations have shown G6PDH activity to be higher in fresh and incubated obese islets than in normal, Table 23.

TABLE 23. The effect of db-^{c}AMP on the glucose-6-phosphate dehydrogenase activity of normal and obese mouse islet tissue.
(Mean values ± S.E., islets pooled from several mice for each determination).

	N	Normal islets (mU/mg islet protein, 25°C, pH. 7.5).	CV	Obese islets (mU/mg islet protein, 25°C, pH. 7.5).	CV
Fresh islets	4	2.73 ± 0.09	7%	8.78 ± 0.84*	19%
Incubated 30 minutes + glucose ⌀	5	3.48 ± 0.24	16%	7.74 ± 0.20*	6%
Incubated 30 minutes + glucose ⌀ + db-^{c}AMP (5mmol/litre)	5	4.77 ± 0.41†	19%	9.05 ± 0.29*†	7%

* Denotes significant difference ($P<0.05$) between normal and obese islet values subjected to the same treatment.

† Denotes significant difference ($P<0.05$) between values obtained by incubating in the presence and absence of db-^{c}AMP for both normal and obese islets.

⌀ glucose concentration 100mg% normal islets, 300 mg% obese islets.

CV = Coefficient of variation.

The effect of 30 minutes incubation.

Islets were incubated for 30 minutes at 37°C in 2ml. of Gey & Gey buffer continuously oxygenated ($95\%O_2/5\%CO_2$). Normal islet incubations contained 100 mg/100ml. glucose and obese islet incubations 300mg/100ml. glucose.

30 minutes incubation produced a 27% increase and 11% decrease in the G6PDH activity of normal and obese islet tissue respectively. These values were used as untreated incubated controls for subsequent experiments with db-^{c}AMP.

The effect of 30 minutes incubation in the presence of db-^{c}AMP (5mmol/litre).

After 30 minutes incubation db-^{c}AMP significantly increased both normal and obese islet G6PDH activity by 37% and 16% respectively.

Discussion:

Compared to normal, the higher level of G6PDH activity in obese islets probably reflected their greater capacity for glucose metabolism via the pentose-phosphate pathway. The effect of db-^{c}AMP on normal islet G6PDH activity was some x2 greater than its effect on obese islet activity. However, in both cases db-^{c}AMP wouldproduce an increase in the rate of conversion of G-6-PO_4 to 6-phosphogluconate. One might suggest that db-^{c}AMP, by its action, increased the rate of glucose flux through the pentose phosphate pathway in both normal and obese islets.

The higher adenyl cyclase activity in obese islets compared to normal might have the effect, in these islets, of changing the bias of glucose metabolism from glycolysis and the TCA cycle to the pentose-phosphate pathway. The catabolism of glucose by this pathway would provide reducing equivalents in the form of NADPH for the resynthesis of ATP, and pentose phosphate for nucleotide and nucleic acid synthesis and possibly subsequent protein (insulin and proinsulin) synthesis. The increased flux of glucose through the pentose phosphate pathway might lead to the hypersecretory activity of obese islets and contribute to the prevailing

hyperinsulinaemia in obese mice. Montague & Taylor have suggested the pentose-phosphate pathway to be associated with glucose induced insulin secretion (359). The provision of NADPH by the pathway might be necessary for some phase of insulin secretion, either to provide energy for the process to occur or to stimulate the insulin secretion mechanism per se. (234,235). Work by Aleyassine measuring insulin secretion from incubated pieces of rat pancreas in the presence of glycolytic inhibitors and uncoupling agents of oxidative phosporylation (197) has shown the glucose stimulated secretion of insulin to be an energy dependent process. The energy requirement was supplied as ATP by the operation of oxidative phosphorylation in the mitochondria. ATP dependent secretory processes responsible for the release of granule bound substances have been described for catecholamine secretion from the adrenal medulla (612) and vasopressin and oxytocin release from the posterior pituitary (613).

The proportions of β-cells in normal mouse islets and obese mouse islets have been reported to be 80% (30) and 90% (63) respectively. Hence the enzyme activity studied in islet homogenate supernatants was assumed to be derived from β-cells, although the possible contribution from other cell types could not be excluded. Montague & Taylor using rat islets demonstrated a change in the activity of G6PDH when the extracellular glucose concentration exceeded 5mmole/litre (359) and this was thought to reflect an activation of the enzyme by glucose-6-phosphate or by a change in the NADPH / NADP ratio. Although the 300mg/100ml. glucose concentration used in the G6PDH incubation of obese islets might well have produced similar effects, they could have been physiological, since the blood glucose to which obese islets are normally exposed is of this order. Ashcroft & Randle suggested that the activities of mouse islet G6PDH and 6-phosphogluconate dehydrogenase indicated a high potential capability of mouse islets to oxidise G-6-PO_4 (614). The oxidation of glucose by the pentose phosphate pathway was thought to be subject to control in the intact islet and the NADPH / NADP+ ratio in the cytosol was suggested to be an important factor

in this control by influencing G6PDH activity. NADPH was found to be a potent inhibitor of mouse islet G6PDH activity and the role of the pentose-phosphate pathway was suggested to be the provision of NADPH for biosynthetic or release processes (614). However, other routes for NADPH generation have been demonstrated in islet tissue i.e. NADP-ICDH activity (615). Hence the islet NADPH / $NADP^+$ ratio might not be solely dependent on the activity of the enzymes of the pentose phosphate pathway.

SECTION 4.

The effect of db-^{c}AMP on the glycogen levels of normal and obese mouse islets.

The islet cells of normal and obese mice have been shown to contain appreciable quantities of glycogen (23,201). The glucose metabolite suggested to be involved in the mechanism of insulin secretion (98) could conceivably be derived from the degradation of stored glycogen in the β-cell (616). An adenyl cyclase/^{c}AMP phosphodiesterase system has been demonstrated in the islet tissue of normal and obese mice (285) and ^{c}AMP has been assigned a role in the mechanism of insulin secretion (276). The inhibitory effect of adrenaline on insulin secretion, on the other hand, has been associated with a significant increase in the amounts of β-cell glycogen (130). Malaisse and colleagues suggested that ^{c}AMP might activate glycogen phosphorylase in β-cells and provide an intracellular source of glucose from stored glycogen. Insulin secretion being associated in some way with the products of β-cell glycogen degradation (41). An investigation was therefore made into the possible effect of db-^{c}AMP on the islet glycogen levels of normal and obese hyperglycaemic mice.

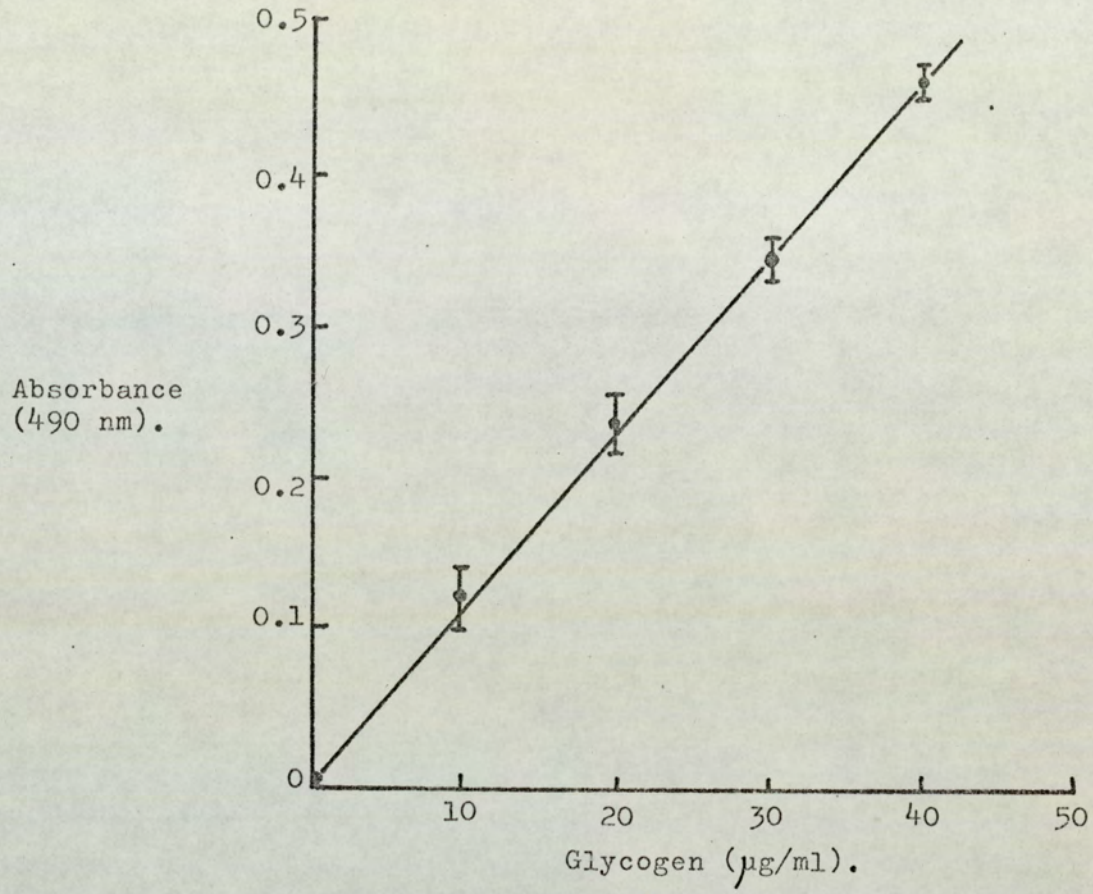
Materials & Methods.

Microdissected islets were pooled from many mice on perforated foil planchets. Buffer was removed from the planchet by placing on a filter

paper and the contained islets allowed to dry momentarily. Foil planchets and their contents were carefully immersed in iso-pentane chilled to its freezing point with liquid nitrogen. Once frozen the islet tissue was stored temporarily at -20°C with a dessicant. In this way a reasonable quantity of isolated islet tissue could be accumulated. Islet tissue was then freeze dried at -40°C, 0.001 mm Hg. for 18 h. (Pearse tissue dryer. Model 1, Edwards high vacuum Ltd., Sussex). Planchet and their contents were weighed on a ultramicrobalance at 22°C (Mettler UM 6, 0-10mg). The contents of several planchets were transferred to graduated glass centrifuge tubes (15ml.) and the glycogen content estimated according to the method of Lo, Russel & Taylor (620). To each centrifuge tube containing freeze dried islet tissue was added 0.5 ml. of KOH saturated with sodium sulphate, making sure that the tissue was completely immersed. The tubes were capped and placed in a boiling water bath until the tissue had disappeared. The tubes were then cooled in ice and 1 ml. of 95% ethanol added to each to precipitate glycogen from the alkaline digest. Capped tubes were allowed to stand in ice for 30 minutes prior to centrifugation at 3000 rev/min. for 30 minutes.

The supernatants were aspirated off and the faint glycogen precipitate dissolved in 1ml. of distilled water. To this was added 1ml. of 5% phenol solution followed rapidly by 5ml. of concentrated sulphuric acid. The yellow/brown contents were stirred with a glass rod and the tubes retained in a water bath at 25°C prior to reading on a Beckman DB spectrophotometer at 490 nm (621). Blank tubes contained 1 ml. of distilled water instead of prepared glycogen solution. A normal and obese islet glycogen extract was found to have an absorption maximum at 483nm. and a series of rabbit liver glycogen standard solutions, (Type iii, Sigma) was prepared in distilled water (5-50µg/ml). A typical standard curve is shown in Fig. 29.

FIG. 29. Standard curve for phenol-sulphuric acid-glycogen reaction.
(Rabbit liver glycogen, each point represents the mean of three determinations, ± S.E.).



Calculation:

Weight of islet tissue used, x mg./ml.

Absorbance of solution estimated and read off standard curve in terms of Y µg/ml glycogen.

$\frac{Y}{X}$ = g.of rabbit liver glycogen equivalents/Kg. freeze dried islet tissue.

Values were converted to glucosyl units by multiplying by 6.234 (mmole glucosyl units/Kg freeze dried islet tissue).

Results:

The glycogen contents of fresh islet tissue from normal and obese mice of 10-15, 20-25 and 40-45 weeks of age, together with the effects of increased exogenous glucose concentration and db-^{c}AMP on the glycogen contents of incubated islet tissue from 20-25 week old normal and obese mice, have been summarised in Table 24.

Obese islet glycogen levels were significantly higher than the corresponding normal islet values at 20-25 and 40-45 weeks of age, but

TABLE 24. The effect of animal age and incubation in the presence of glucose and db-^{c}AMP on the glycogen content of normal and obese mouse islets. (Mean values ± S.E., n = number of determinations, one determination carried out on islets pooled from several mice).

	N	Obese islets (g. of glycogen/Kg freeze dried islets).	CV	Normal islets (g. of glycogen/Kg freeze dried islets).	CV
Effect of Age					
10 - 15 weeks.	5	0.57 ± 0.04 (3.6)	17%	0.67 ± 0.03 (4.2)	12%
20 - 25 weeks.	7	0.88 ± 0.03 (5.5)	9%	0.61 ± 0.03 (3.8)*	13%
40 - 45 weeks.	5	1.08 ± 0.04 (6.7)†	9%	0.61 ± 0.04 (3.8)*	14%
Effect of 30 minutes incubation (Gey & Gey buffer, continuously oxygenated).					
20 - 25 week old mice.					
No glucose	5	0.34 ± 0.03 (2.1)	16%	0.21 ± 0.02 (1.3)*	22%
1 g/litre	5	0.49 ± 0.02 (3.1)	9%	0.39 ± 0.05 (2.4)	26%
3 g/litre	5	0.86 ± 0.05 (5.4)ø	12%	0.66 ± 0.07 (4.1)*ø	22%
Effect of 30 minutes incubation (Normal islets 1g/litre glucose, obese islets 3g/litre glucose).					
db-^{c}AMP (5mmol/litre).	5	0.48 ± 0.05 (3.0)≠	21%	0.25 ± 0.03 (1.6)*≠	23%

CV = Coefficients of Variation.

(-) Mean value in mmol. glucosyl units/Kg. freeze dried islet tissue.

* Denotes significant difference ($P<0.05$) between normal and obese islet values subjected to same treatment

† Denotes significant difference ($P<0.05$) between obese islet values at 40-45 and 10-15 weeks.

ø Denotes significant difference ($P<0.05$) between 30 minute incubated samples containing no glucose and 3g/litre glucose for both normal and obese mice.

≠ Denotes significant difference ($P<0.05$) between 30 minute incubated samples containing the appropriate amount of glucose alone and the appropriate amount of glucose +db-^{c}AMP.

not at 10-15 weeks. In addition, the islet glycogen levels in 20-25 week old obese mice were higher than the corresponding normal islet levels after 30 minutes incubation in the absence and presence of 3g/litre glucose and in the presence of the appropriate concentration of glucose and db-^{c}AMP. The glycogen content of obese islets increased steadily with age up to 40 weeks while the content of normal islets showed no significant change over this period.

Incubation in the absence of glucose seriously depleted the glycoge levels of both normal and obese islets. Only 30 minutes incubation in the presence of high glucose (3g/litre) proved sufficient to re-establish the usual fresh levels of normal and obese islet glycogen, Table 24.

In the presence of the appropriate concentration of exogenous glucose, db-^{c}AMP significantly reduced the glycogen levels of incubated normal and obese mouse islets by 35% and 44% respectively.

Discussion:

Glycogen has been demonstrated microchemically in the islet tissue of both normal and obese mice and probably represents a normal constituer of mammalian pancreatic β-cells. Although some glycogen was invariably lost during the microdissection procedure, the values obtained were of the same order as those demonstrated by Hellman & Idahl (23). The higher level of glycogen in obese islets probably reflected the adaptation of their β-cells to hyperglycaemia.

The observed changes in islet glycogen levels reflected the activities of both glycogen phosphorylase, that acts to degrade glycogen to G-1-PO_4, and glycogen synthetase, that acts to synthesise glycogen from this substrate via UDP-glucose. The process of glycogenesis was induced in normal and obese islet tissue in vitro by increasing the exogenous glucose concentration and this observation suggested the presence of an active glycogen synthetase complex.

Db-^{c}AMP, assumed to be imitating the effect of ^{c}AMP in vitro, reduce both normal and obese islet glycogen levels, presumably by activating

glycogen phosphorylase and suppressing the activity of the kinase responsible for the activation of glycogen synthetase. Similar observations have recently been made by Idahl & Hellman, who found db-^{c}AMP to actually prevent the accumulation of glycogen normally caused by high glucose concentration (286). These observations suggested the presence of an active, ^{c}AMP modulated glycogen phosphorylase complex in islet tissue capable by its activities of supplying glucose phosphates from the degradation of glycogen, either to provide energy for the process of insulin secretion via β-cell metabolism or to trigger off the secretion mechanism per se. However, it remains unclear as to whether the amounts of glycogen usually found in mammalian pancreatic β-cells would be adequate to provide sufficient glucose intermediates fast enough to initiate insulin secretion (617).

The absorption maximum of normal and obese islet glycogen was found to be 483nm. However, a value of 490nm was selected for absorbance measurements since Dubois and colleages had shown measurements at this wavelength to be free from ribose interference (621). In the glycogen estimation, the glycogen precipitate was broken down to glucose by concentrated sulphuric acid and the glucose condensed with two molecules of phenol to give a yellow-orange-brown colour. Some possibility existed that the alkaline (KOH) extraction used in the assay caused glycogen degradation. However, Lo, Russel & Taylor showed that caustic digestion did not cause any appreciable loss of glucose and the reagents used for glycogen isolation did not interfere with the colour reaction (620). Although Bell and coworkers also reported that alkaline extraction of glycogen from its parent tissue did not effect very serious degradation (622), Stetten and colleagues have demonstrated serious degradation of glycogens exposed to hot KOH (623).

Although glycogen could be estimated microchemically, ordinary histochemical staining methods have failed to demonstrate glycogen in

islet tissue (618,619). Kugler & Wilkinson demonstrated that there had to be a minimal quantity of glycogen present in the tissue before histochemical demonstration could be effected (624). Their work suggested that glycogen existed in tissues in two forms a protein bound form and a form readily soluble in tri-chloroacetic acid (625). Histochemical techniques for glycogen only detected the tri-chloroacetic acid fraction and this would account for the discrepancy between total tissue glycogen and histochemically demonstrable glycogen. Leloir & Goldemberg have demonstrated glycogen synthetase of liver to be bound to particulate glycogen (626) and this probably represented the protein bound form postulated by Kugler & Wilkinson (625). E/M studies have demonstrated glycogen in the β-cells of rabbits (627) and cats (628). Also, a recent review by Renold and colleagues has shown the ultrastructura accumulation of glycogen to be a prominent aspect of β-cell morphology in the presence of hyperglycaemia in sand rats, spiny mice and chinese hamsters. However little ultrastructural evidence of glycogen deposition was available for the diabetic mouse (dbdb) or the obese mouse (obob) (34).

Brolin & Berne using extremely sensitive assays have demonstrated glycogen synthetase and glycogen phosphorylase activity in islet tissue of NZO mice (629). Glycogenolysis has been suggested to be a way of contributing to the pool of hexosemonophosphates in islet tissue without the expenditure of ATP and probably offered more favourable conditions for energy formation than the uptake and phosphorylation of glucose. The extent to which this potential source of energy would be relevant would depend upon the size of the glycogen store and the enzymic capacity for its mobilisation. The low glycogen synthetase activity demonstrated in NZO islets by Brolin & Berne (629) indicated that the accumulation of glycogen would be a slow process taking place over a long period of time. But islet glycogen synthetase could be stimulated by the high levels of G-6-PO_4 demonstrated in islets during hyperglycaemia (201). Brolin & Berne have suggested that glycogen could be used as an energy store in

islet tissue for the reconstitution of ATP from ADP and have drawn attention to the possibility of a common trigger mechanism for the secretion of insulin in the form of a rapid production of ATP (630). For the utilisation of glycogen, phosphorylase was shown to be rate limiting and it was debatable whether a very fast flux would occur in islet cells. A role for ^{c}AMP in the stimulation of islet phosphorylase was likely, but whether the ^{c}AMP induced mobilisation of glycogen in islet tissue was sufficient to supply glucose intermediate(s) capable of triggering insulin secretion remained to be investigated.

CHAPTER 6.

The effect of age on aspects of normal and obese mouse islet morphology and metabolism.

Obesity has been defined as an increase in the percentage contribution of body fat to the total body weight (47). In the obese mouse, obesity could be identified between 3 and 4 weeks of age (75). This obesity increased with age, stabilising at 5-6 months. Elevated levels of circulating insulin were present by 1 month of age and reached a peak at about 6 months (51). Hyperglycaemia, a prominant feature of the fully developed ob/ob mouse, did not appear until after the onset of obesity and hyperinsulinaemia and reached its peak by about 12 weeks of age. Marked resistance to exogenous insulin was among the earliest observations on these animals (631), which could be overcome by food restriction (69) and alloxan therapy (632). Their life span could also be prolonged by limiting the intake of food (633). Over 90% of the increased weight in the obese mouse resulted from the accumulation of fat and the blood volume did not increase with obesity (634). Restriction of the caloric intake to or below that of lean

littermates did not normalise the content of fat, indicating that obese mice retained excessive quantities of fat even when their body weight was restricted to less than that of lean littermates. (56). The accumulation of fat implied that caloric intake exceeded expenditure and obese mice have been shown to eat more than their lean littermates (635). Not only was the food intake of obese animals increased, but so toowas the fraction of the ingested calories stored as fat (56).

The histological changes in the pancreas associated with obesity have been comprehensively reviewed (47). The β-cells of obese mice showed intense secretory and synthetic activity and their hyperplasia resulted in an 8-10 fold increase in islet volumes. The decreased frequency with which α-cells occurred suggested that the hyperplasia was restricted to β-cells and there was increased islet vascularity (636) The presence of hyperinsulinaemia, together with hyperglycaemia or even normal blood sugar suggested that the obese mouse was insensitive to the hypoglycaemic effects of insulin (631). At the height of the obese syndrome glucose tolerance was extremely poor, but this tended to improve with age and decreasing insulin levels (641). The hyperglycaemia and hyperinsulinaemia were maintained throughout life but were often observed to decrease in very old animals (51). Indeed, decreased serum insulin levels have been observed in old mice (61) and exhaustion of β-cells was assumed to be the cause. Westman observed no morphological signs indicating cellular necrosis, hyalinisation or fibrosis in islets of 5 month old animals or 20 month old animals (54). The proportion and appearance of cells other than β-cells (α_1 and α_2) were found to be about the same in the two groups of mice. Hellman observed a higher percentage contribution of islet tissue to the exocrine in adult obese mice (64) and this was subsequently confirmed by Westman for old obese animals (54). Compared to lean mice, obese mice were shown to have a lower α_1 cell frequency (81). It was demonstrated that the

proportion of α_1 cells was related to both obesity and age and old animals had lower values than young controls. It was suggested that α_1 cells might be influenced by the hyperglycaemic state of the experimental animals (637,638). Westman indicated that in the light of the normal β-cell appearance recorded for old obese mice, their decreased serum insulin level signified a lowered functional demand on the β-cells rather than a disturbed capacity for insulin secretion. (54).

Although many hypotheses have been advanced, to date no generally acceptable biochemical explanation has been proposed for any of the genetically transmitted forms of obesity. A strong case has been made for the primary genetic defect to be located in the pancreas of the obese mouse and the necessity of increased insulin secretion for weight gain has been demonstrated (639). The altered islet composition, which was remedied by alloxan (632) but not food restriction (640) suggested some defect in the pancreatic islet.

Insulin insensitivity by peripheral tissues has been reported for the obese mouse (632,642). However, this resistance could represent the effects of obesity rather than reflecting its cause, since both the response to insulin in vivo, as well as the response of adipose tissue to insulin in vitro was restored if the obesity was controlled or reversed by restricting foot intake (642). Alloxan has been shown to restore insulin sensitivity in vivo and in vitro without significant reduction in body or organ weight. Mahler & Szabo have suggested that the appearance of insulin sensitivity in obesity was not dependent on the body weight of the obese animal but might be associated with either a chronic over production of insulin per se. or a humoral substance emanating from the islet and secreted in parallel with insulin (632).

In the past β-cell hyperplasia in the obese mouse pancreas has been thought to represent a compensatory hypersecretion in response to

peripheral insensitivity through an undefined feedback mechanism. Mahler & Szabo have suggested that β-cell hypersecretion might be the cause rather than the result of peripheral insulin sensitivity and their work has shown insulin insensitivity in the obese mouse to depend upon hyperplastic hypersecretory β-cells (645). Recent work by Abraham & Beloff-Chain investigating the sensitivity and response to insulin of adipose tissue and diaphragm muscle of obese mice in vitro has indicated that a decreased sensitivity to insulin at the cellular level was not the primary defect in the obese hyperglycaemi syndrome and that the hypersecretion of insulin in response to the ingestion of food might more closely reflect the primary disorder (643,644).

The possible association between the aetiology of maturity onset obesity, hyperglycaemia and hyperinsulinaemia and some pancreatic islet defect in obese mice, prompted an investigation of certain morphological, physiological and metabolic islet tissue parameters with age. Because of time, studies were limited to normal and obese animals of 10-15, 20-25 and 40-45 weeks of age and included islet morphology, blood sugar and insulin levels; islet adenyl cyclase and ^{c}AMP-phosphodiesterase activity; islet Ca^{2+} and Mg^{2+} levels; islet total RNA and DNA levels and finally islet glycogen and ATP levels.

SECTION 1.

The functional morphology of normal and obese islet tissue with age.

Westman was unable to demonstrate any morphological signs of islet necrosis, hyalinisation or fibrosis in obese mice of either 5 or 24 months of age. The aldehyde fushsin positive secretion granules of obese islet β-cells were normal, suggesting a normal store of insulin. Also the proportion and appearance of cells other than β-cells seemed to be about the same in both groups of obese mice (51,54,66).

The observation of a less severe hyperinsulinaemia in old obese mice warranted an investigation of the changes in mouse islet morphology during the manifestation of the obese hyperglycaemic syndrome.

MATERIALS & METHODS.

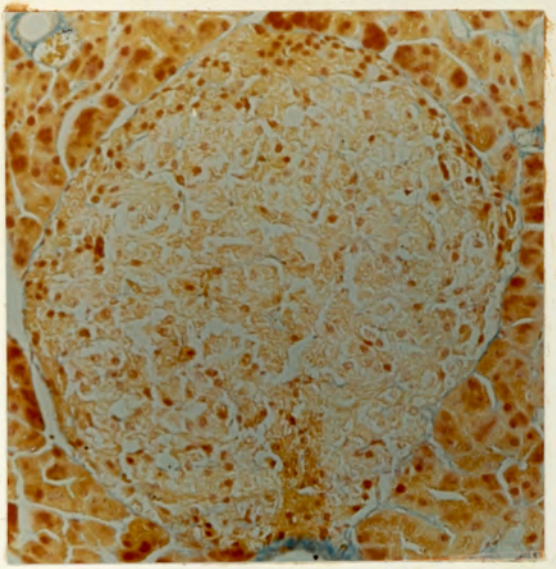
3-4μm paraffin sections of the pancreata of six normal and six obese mice, irrespective of sex, were processed for each age group. Representative sections for photography (Ektachrome B., Kodak, Zeiss photomicroscope fitted with grey filters) were chosen at random from each. General islet morphology was demonstrated using Heidenhain's "Azan" variant trichrome strain on formol fixed pancreas (646). β-cells were demonstrated using Gomori's aldehyde fuchsin with an orange - G counterstain instead of light green (647). α cells (α_2 and probably α_1) were demonstrated using a silver nitrate stain according to the method of Grimelius (648,649).

Results:

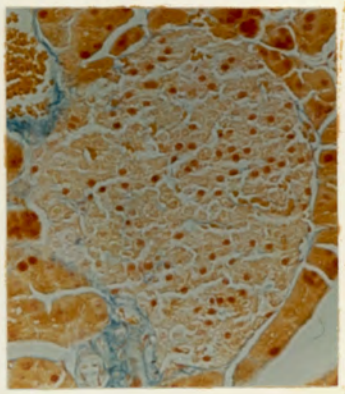
Gross islet morphology and the distribution of α and β-cells in islets of both normal and obese mice with age have been illustrated in Plates 21,22 and 23.

Normal islets showed no obvious alteration with age (Plate 21). Hyperplasia and increased vascularity were apparent in obese islets at 10-15 weeks. After 20-25 weeks of age fluid filled spaces appeared in obese islets accompanied by light fibrosis and marked thickening of the connective tissue capsules. Fibrous connective tissue became more obvious between clumps of islet cells. The spaces could have resulted from the dissolution of capillary walls, and their contents had the appearance of amyloid or fibrinoid material (650). The hypertrophy of obese islets after 20-25 weeks was the result of an increased penetration of fibrous connective tissue and the formation of fluid filled spaces, rather than the hyperplasia of islet β-cells. The actual β-cell volume of 40-45 week obese islets appeared to be reduced. Also, there was

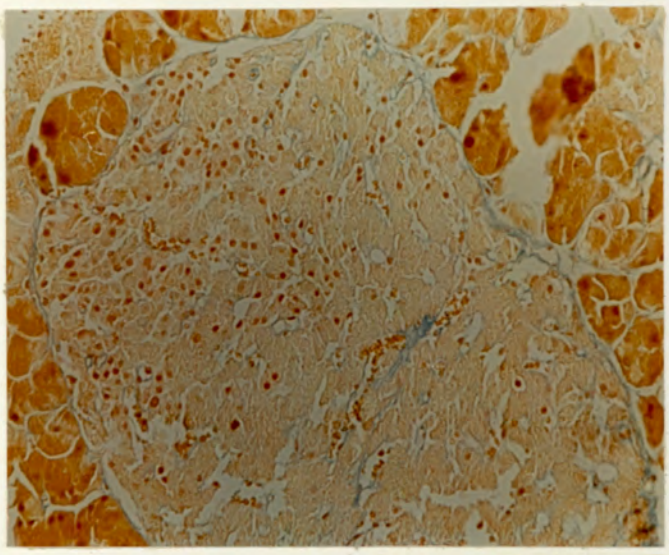
PLATE 21. MOUSE ISLET MORPHOLOGY AT 10, 20 AND 40 WEEKS OF AGE



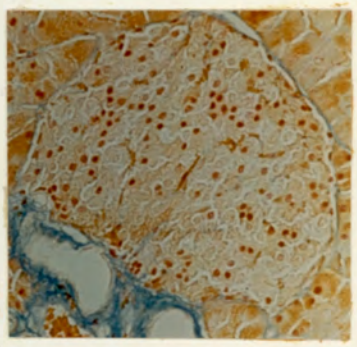
Obese 10 weeks. x50



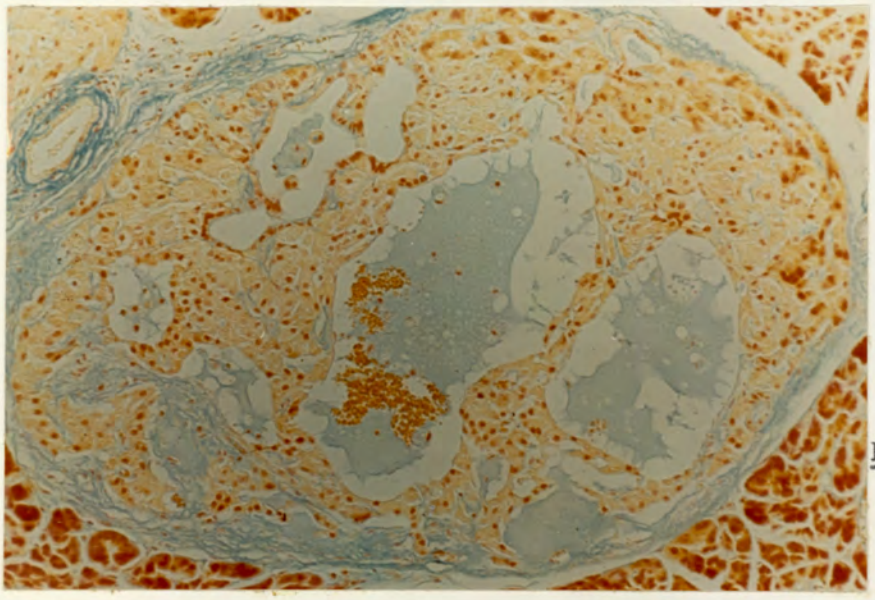
Normal 10 weeks. x50



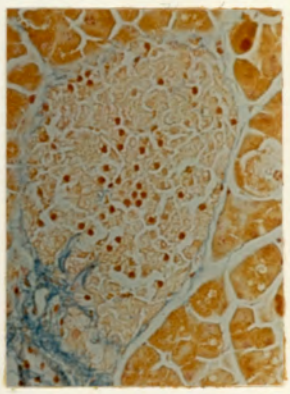
Obese 20 weeks. x50



Normal 20 weeks. x50

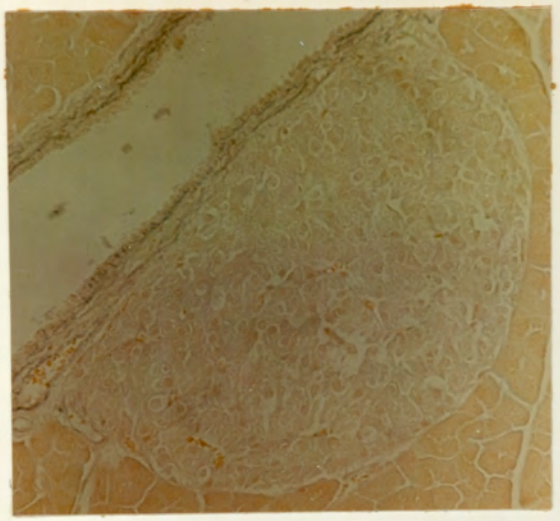


Obese 40 weeks. x40

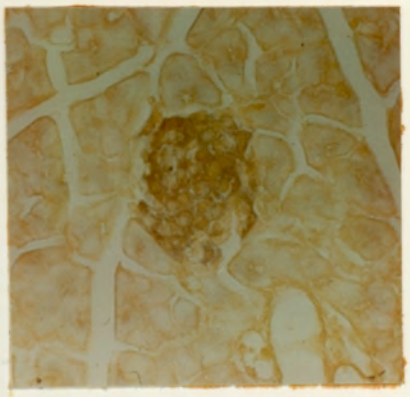


Normal 40 weeks. x50

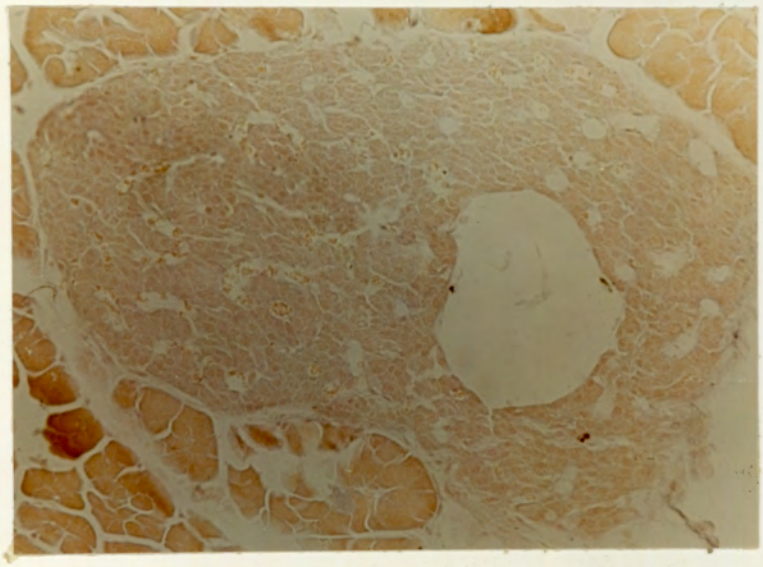
PLATE 22. DISTRIBUTION OF B-CELLS IN MOUSE ISLET TISSUE



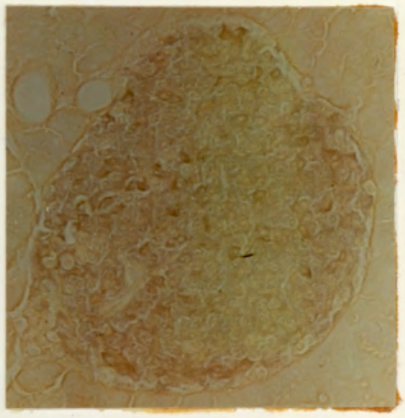
Obese 10 weeks. x50



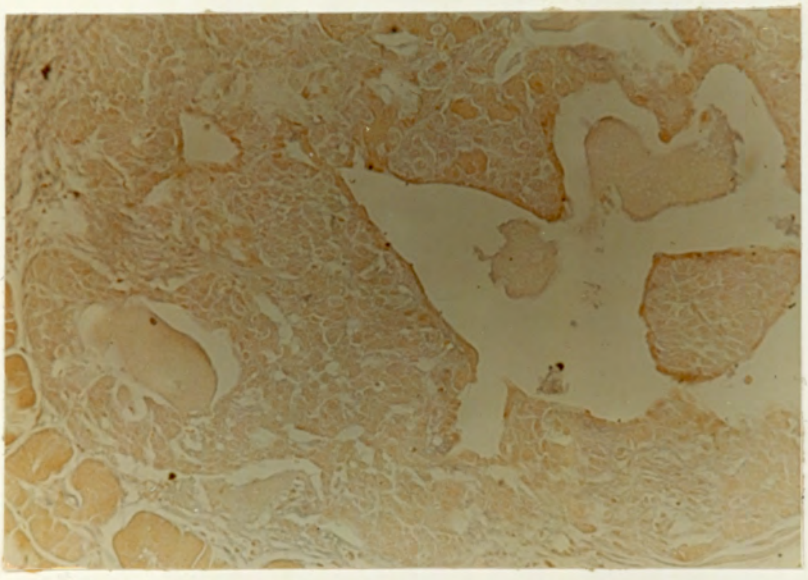
Normal 10 weeks. x80



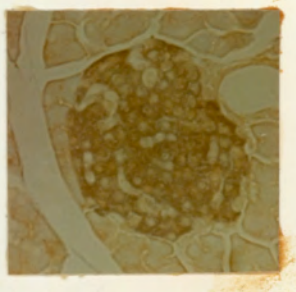
Obese 20 weeks. x50



Normal 20 weeks. x50

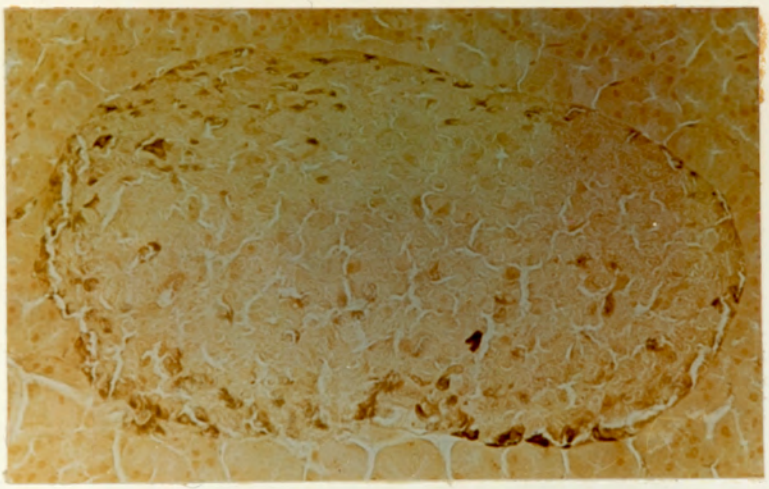


Obese 40 weeks. x50

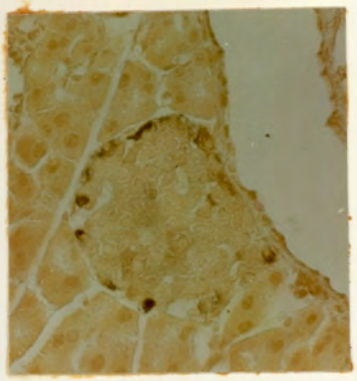


Normal 40 weeks. x63

PLATE 23. DISTRIBUTION OF ALPHA CELLS IN MOUSE ISLET TISSUE

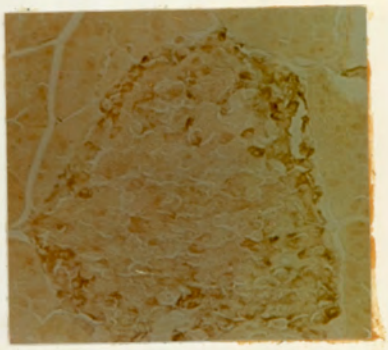


Obese 10 weeks. x50

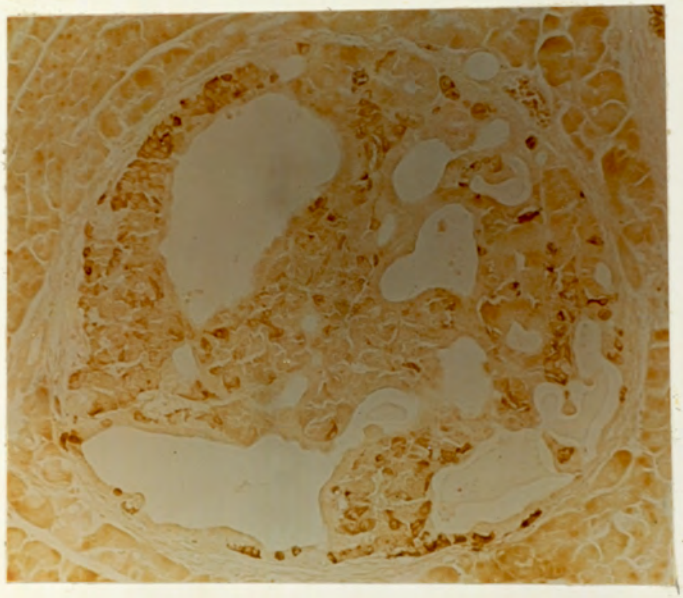


Normal 10 weeks. x80

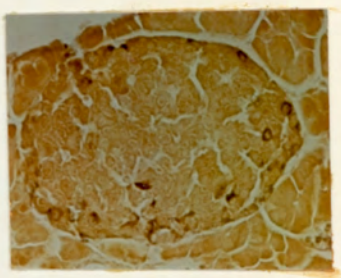
Obese 20 weeks. x63



Normal 20 weeks. x50



Obese 40 weeks. x40

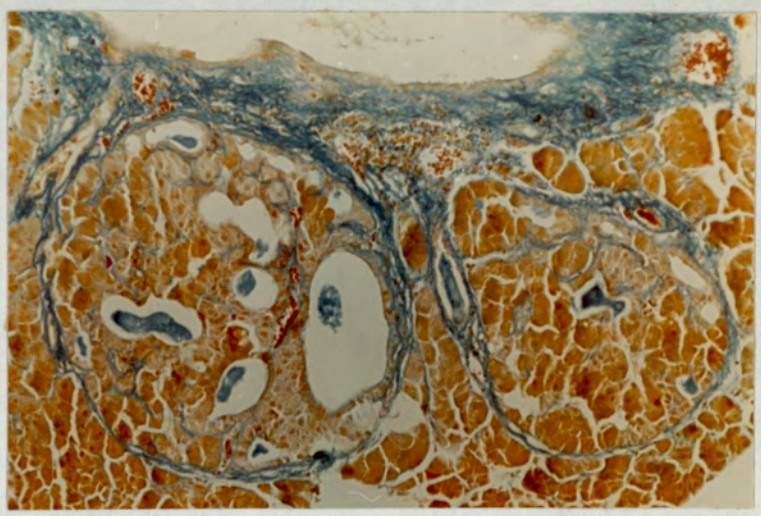


Normal 40 weeks. x50

evidence of exocrine type cells appearing around the periphery of islets, within theirconnective tissue capsules (Plate 24).

Aldehyde fuchsin staining of obese islet β-cells was not particularly intense, while normal islet β-cells at all three age groups gave strong positive purple reactions (Plate 22). Increasing age did not appear to change the β-cell volume of normal islets. β-cell hyperplasia was well demonstrated in islets from 20-25 week old obese mice. However, compared to the latter, islets from 40-45 week old obese mice appeared to contain a reduced β-cell volume. The majority of the 40-45 week old obese mouse islet being composed of fibrous tissue, exocrine type tissue and fluid spaces. However, the reduced β-cell volume did not necessarily imply a reduced secretory response, since the capacity of the remaining β-cells might have been increased.

PLATE 24. Exocrine type cells in the islets of a 40-45 week old obese mouse (Azan trichrome x40).



α-cells were demonstrated around the periphery of both normal and obese islets. They constituted only a very small part of the total islet volume in both cases. With age, in both normal and obese islets there appeared to be a redistribution of α-cells from the periphery to the central portions of islets. Although this could have been due to the penetration of fibrous connective tissue or the creation of fluid

spaces in old obese islets, the same explanation could not be used for the redistribution observed in islets of 40-45 week normal mice. Since no count was taken of α and β-cell numbers in this preliminary work, it was not possible to describe the apparent changes in islet cell distribution and volume in a quantitative fashion.

Discussion.

Morphological evidence has been presented to describe the degenerative changes occurring in obese islets with age. Despite the work of Westman (54), old obese islets were characterised by intra islet fibrosis; excessive thickening of the outer connective tissue capsule; the creation of fluid filled spaces from the disolution of capillaries and evidence of islet endocrine/exocrine cell transformation. It might be tentatively suggested that these degenerative changes were the cause of the less severe hyperinsulinaemia observed for old obese mice (54). The fibrosis and fluid filled spaces compensating for a reduced β-cell volume.

In obese islets compared to normal, at all three age groups, there was a higher percentage contribution by islet tissue to the pancreatic parenchyma. This observation confirmed similar work by Hellman and coworkers (64). Casual examination of obese islets showed no obvious change in α-cell numbers with increasing age.

The fluid filled spaces in obese islets made a more significant contribution to the total islet volume with age. The spaces contained material of semi-fluid consistency, probably hyalin/amyloid or fibrinoid in nature. Hyalinisation has been considered to be a most typical but not entirely specific pancreatic lesion in diabetes mellitus and its importance in the aetiology of the syndrome has been comprehensively reviewed (651). Hyaline, characteristic of obese maturity onset diabetic humans older than 35 years of age, has been suggested to be intracellular in origin, and composed of a combination of glycoprotein and acid muccopolysaccharide in tightly

packed fibrils. The hyaline was deposited in association with the supporting framework of the islets and occurred in association with, but was not responsible for the loss of β and often α-cells. Hyaline material was often seen in contact with islet capillaries and might have been derived from their contents.

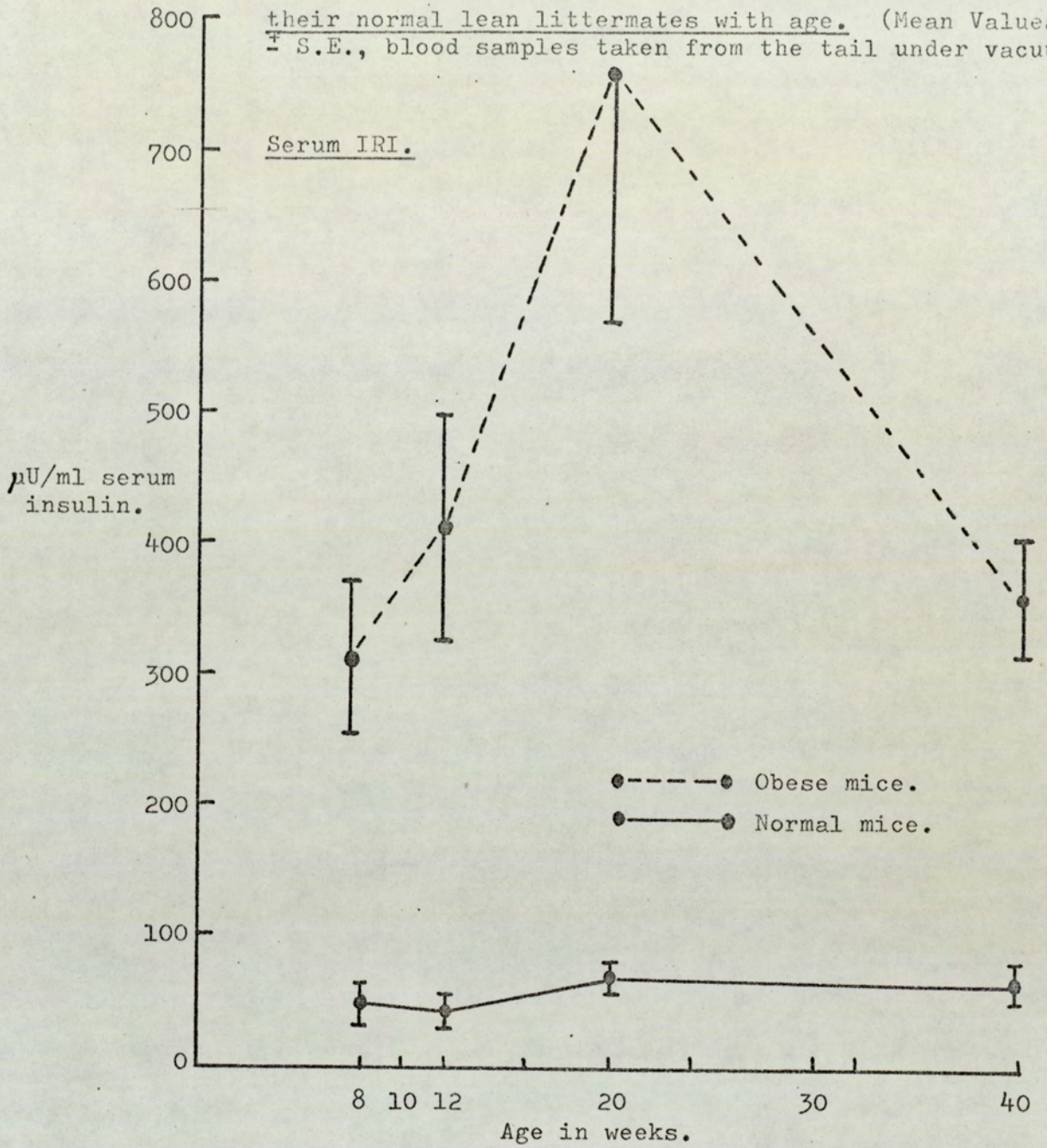
In sections of obese mouse pancreas at 40-45 weeks, the striking feature was the unequal distribution of islet morphological degeneration. Some islets were almost completely occluded by hyaline material and fibrous connective tissue while others were completely spared. Still others showed varying degrees of hyalinisation and fibrosis between the two extremes. This observation seemed to point to either striking inequalities of involvement or to the possible replacement of destroyed non-functional islets by new ones. When the process of hyalinisation became sufficiently marked as to destroy a number of β-cells or to separate them further than usual from the capillaries, diabetes might then be expected to occur. In the obese mouse a truly diabetic state was never observed since hyperinsulinaemia once established lasted throughout life. Hyalinisation could not have been the primary lesion of the obese hyperglycaemic syndrome in mice, but might have contributed to the less severe hyperinsulinaemia observed in old obese mice. Hyaline deposition might be secondary to fibrosis, and a result of fibroblastic activity or their dysfunction. The presence of increased mast cell numbers in islets of aged diabetic humans has been correlated with increased amyloid formation in pancreatic islets (652). Also hyaline or amyloid in islets could be a part of senile amyloid deposition similar to the amyloid deposition seen in other tissues with increasing old age (651).

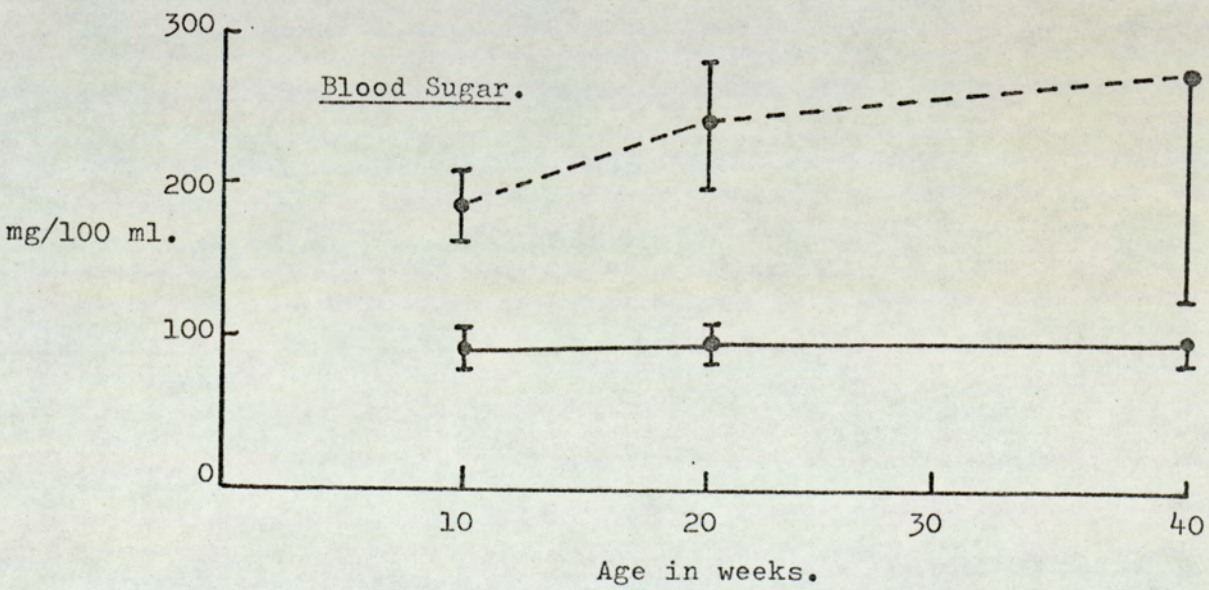
Lymphocytic infiltration was occasionally observed in the very large islets of 40 week old obese mice, generally accompanied by hyalinisation and fibrosis. The latter varied in degree and in its most extreme form resulted in the complete replacement of islet tissue.

α_2 and probably α_1 cells have been demonstrated in the islets of both normal and obese mice. Hellman indicated that compared with normal lean littermates, the islets of obese mice were characterised by a lower frequency of α_1 cells. (81). In addition, the proportion of gastrin producing α_1-cells was related to both obesity and age and lower numbers of α_1-cells were demonstrated in old obese animals than in 5 month old obese controls (54). Using sensitive silver techniques Hellman has shown glucagon producing α_2-cells to be practically absent from the islets of obese mice, and that about 5% of the endocrine pancreatic cells were α_1-cells, located mainly in the large islets (53). This observation was not consonant with islet glucagon as the normal physiological stimulator of the insulin secretion mechanism, although it did not rule out the possibility of a role for "entero-glucagon" in this context.

The presence of exocrine type cells within islets of old obese mice (Plate 24) suggested the possibility of endocrine cell redifferentiation. Whether dysfunction was a prerequisite for this transformation remained to be investigated. Shorr & Bloom have provided electron microscopic evidence for the presence of both zymogen and β-cell granules in the same cell in rat pancreas, the so called "Acino-insular cells" (653). Leduc & Jones have reported the presence of similar cells in an apparently normal pancreas from a C_3HF (Wellesby hybrid) mouse, which would normally show hyperglycaemia, glycosuria and hyperplastic islets (654). These ultrastructural observations of cells containing both zymogen and β-cell granules would suggest the likelihood of islet cell to exocrine cell transformation and vica-versa. Whether transformations of this kind would contribute to the manifestation of the obese hyperglycaemic syndrome in mice remains to be investigated. Presumably the presence of functional exocrine cells within islets would cause serious depletion of both stored and secreted insulin.

FIG. 30. Serum IRI and blood glucose of obese hyperglycaemic mice and their normal lean littermates with age. (Mean Values ± S.E., blood samples taken from the tail under vacuum).





Concomitant with the observed changes in islet morphology, obese mice were shown to have reduced serum IRI values in the presence of a maintained hyperglycaemia at 40-45 weeks of age (Fig. 30). A most severe hyperinsulinaemia was observed at 20-25 weeks of age in obese animals (150) and Malaisse and colleagues have obtained a similar serum IRI profile from Jackson obese mice (C57BL/6J) (655). The hyperinsulinaemia might have resulted in part from an adaptation to the increased amounts of peripheral tissue to be supplied with hormone. The reduced serum IRI in 40-45 week old obese mice suggested that either their β-cells were not able to make a sufficient and sustained increase in synthetic and secretory activity in response to hyperglycaemia or the functional demand for insulin at this age was reduced as a result of the somewhat lower body weight.

SECTION 2.

The effect of age on the adenyl cyclase and ^{c}AMP-phosphodiesterase activity of normal and obese mouse islets.

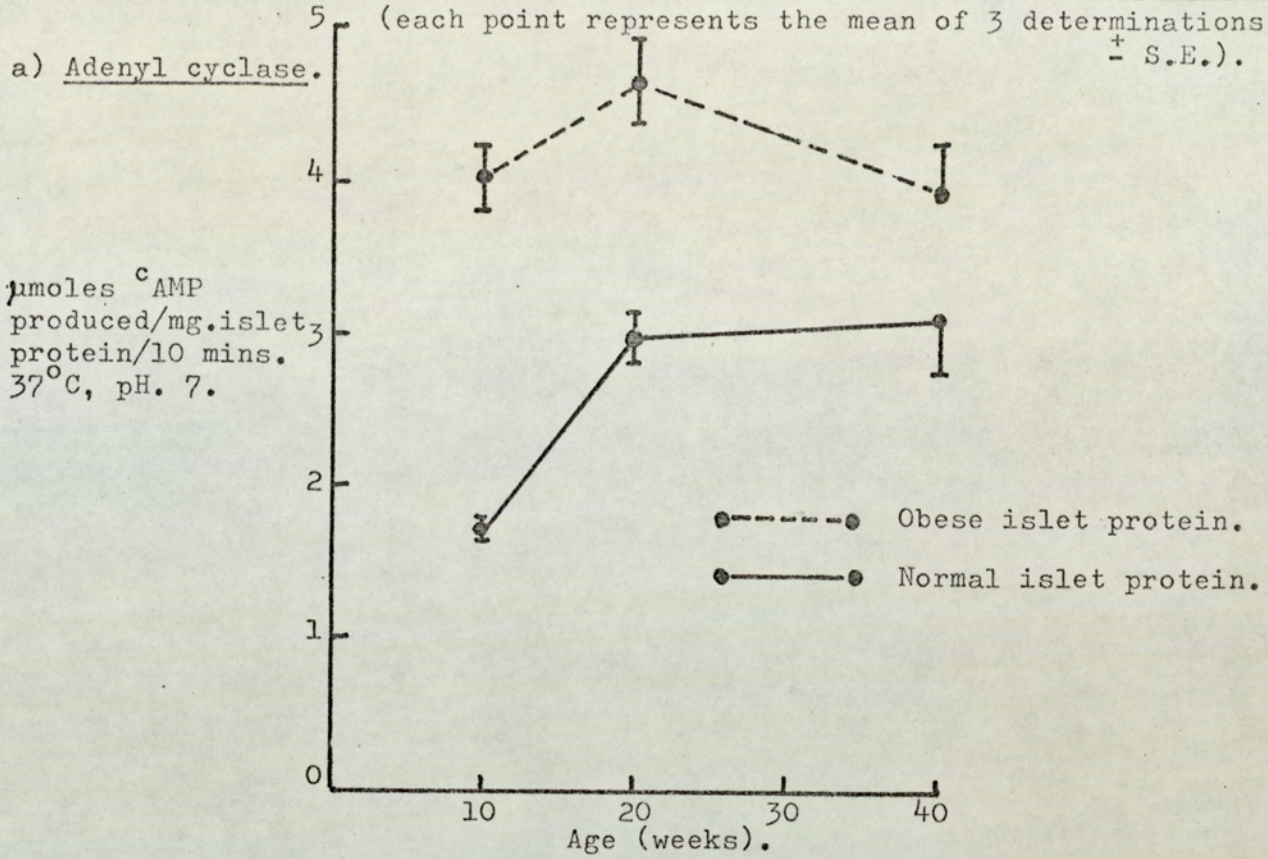
Hollinger has shown the adenyl cyclase activity in rat testis to be age dependent (656). Enzyme activity was progressively increased from 25 to 60 days of age, where upon it was decreased by the eightieth day. Forn and coworkers have shown NaF and noradrenaline stimulated adenyl cyclase activity to be much lower in epididymal fat pad cell homogenates of old rats than those of young rats (657). By contrast ^{c}AMP-phosphodiesterase activity was found to be higher in the epididmal fat cells of old rats and the overall effect was suggested to be a lowering of the ^{c}AMP levels in these cells with age.

At any one time the proposed insulinogenic effect of ^{c}AMP in islet tissue would be expected to depend upon the relative activities of islet adenyl cyclase and ^{c}AMP - phosphodiesterase. The contribution made by changes in the potential obese islet ^{c}AMP level with age to the manifestation of the obese hyperglycaemic syndrome in mice was investigate

FIG. 31. The effect of age on the adenyl cyclase and ^{c}AMP-phosphodiesterase activity in normal and obese mouse islet tissu

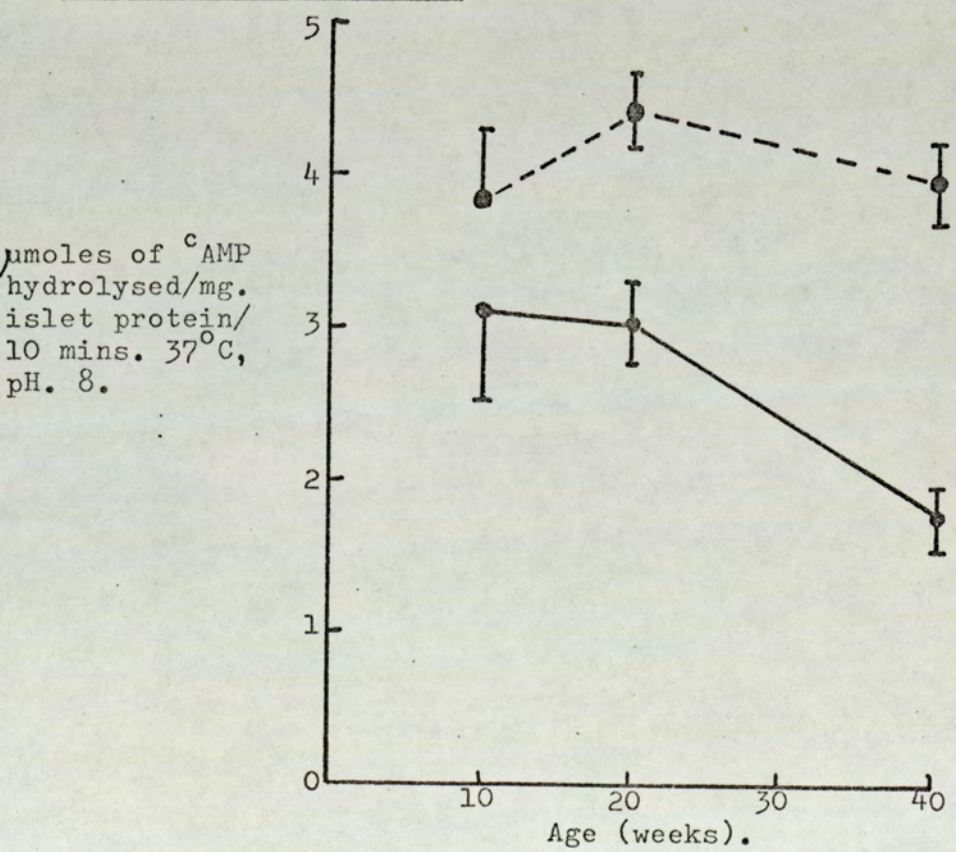
(each point represents the mean of 3 determinations ± S.E.).

a) Adenyl cyclase.



(islets pooled from many obese mice for each 10 week determination)

b) ^{c}AMP-phosphodiesterase.



MATERIALS & METHODS

The procedures for the radiometric assay of islet adenyl cyclase and ^{c}AMP-phosphodiesterase activity have been described previously, Chapter 3, Sections 2 and 3 respectively. The adenyl cyclase and ^{c}AMP-phosphodiesterase activity was similarly determined in the islet tissue of 10-15 and 40-45 week old normal and obese mice. However, the microdissection of islet tissue from 10-15 week old obese mice proved very difficult and islet tissue had to be pooled from 3 or even 4 animals for each determination.

Results and discussion

The values of adenyl cyclase and ^{c}AMP-phosphodiesterase activity in islet tissue from 10-15, 20-25 and 40-45 week old normal and obese mice have been summarised in Fig. 31, a & b. Obese islet adenyl cyclase activity was significantly higher than the corresponding normal islet value at both 10-15 and 20-25 weeks of age ($P < 0.05$), but not at 40-45 weeks. The maximum obese islet cyclase activity was observed at 20-25 weeks of age, while no significant difference was observed between normal islet cyclase activity at 20-25 and 40-45 weeks. Obese islet cyclase activity was reduced at 40-45 weeks to a value not significantly different from normal islet activity of the same age.

Obese islets ^{c}AMP-phosphodiesterase activity was significantly higher than the corresponding normal islet value at both 20-25 and 40-45 weeks of age, but not at 10-15 weeks. At 40-45 weeks normal islet ^{c}AMP-phosphodiesterase activity was significantly reduced.

The ratios of adenyl cyclase to ^{c}AMP-phosphodiesterase in islets of both normal and obese mice with age have been summarised in Table 25.

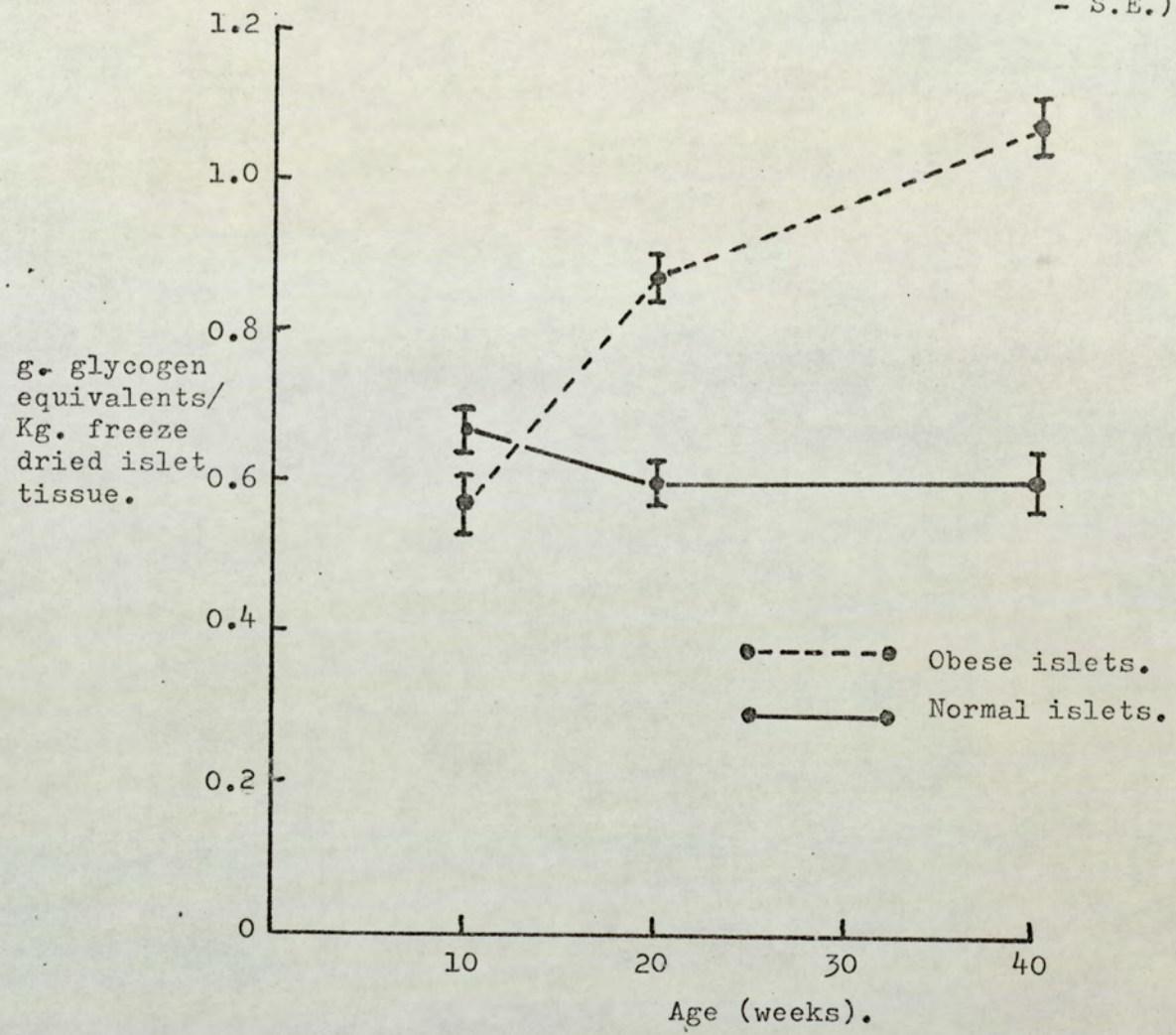
The low ratio in islet tissue from normal mice of 10-15 weeks of age suggested a greater capacity to degrade ^{c}AMP than to produce it. The presence of unbound ^{c}AMP in these islets would therefore be transitory although ^{c}AMP levels might be maintained by rapid complexing with protein kinase.

TABLE 25. The ratios of adenyl cyclase to ^{c}AMP-phosphodiesterase in islets of both normal and obese mice with age.

Age (Weeks)	Normal Islets	Obese Islets
10 - 15	0.55 : 1	1.04 : 1
20 - 25	0.97 : 1	1.04 : 1
40 - 45	1.7 : 1	0.99 : 1

The ratio of 1 : 1 observed for islets from normal mice of 20-25 weeks of age and obese mice of all three age groups, suggested that in these islets the capacity for ^{c}AMP degradation was no greater than the capacity for ^{c}AMP synthesis. In normal islets there was a gradual increase in ^{c}AMP'availability'. The function of which might have been to stimulate the replacement of enzyme systems that had become less efficient with age. The fact that the adenyl cyclase/^{c}AMP phosphodiesterase system was observed to be working at a higher level of activity in obese mouse islets of all three ages compared to normal, suggested the development of hyperinsulinaemia in the obese syndrome to be associated with a potentially increased islet ^{c}AMP level. However, that such a situation might be the sole cause of hyperinsulinaemia seemed doubtful, since 40-45 week old normal islets, with a adenyl cyclase/^{c}AMP-PDE ratio of 1.7 : 1, would also be expected to show hyperinsulinaemia as a result of potentially elevated ^{c}AMP levels. The somewhat reduced islet adenyl cyclase activity demonstrated for 40-45 week old obese mice was consonant with their less severe hyperinsulinaemia (Fig. 30). The potentially increased levels of ^{c}AMP in obese islets might have facilitated a greater involvement by phosphorylase in the degradation of obese islet glycogen. However, glycogen levels in obese islets were much higher than in normal at 20-25 and 40-45 weeks of age (Fig. 32), suggesting that the influence of hyperglycaemia on islet glycogenesis exceeded the capacity of available ^{c}AMP to bring about islet glycogenolysi Although islet glycogenesis might have been stimulated by the prevailing

FIG. 32. The glycogen content of normal and obese mouse islet tissue with age. (each point represents the mean of 5 determinations. ± S.E.).



hyperinsulinaemia via the stimulation of islet glycogen synthetase activity.

Consonant with the serum IRI and islet adenyl cyclase profiles of obese mice was the ATP profile for obese islets with age (Table 26). At all three ages the levels of ATP were significantly higher in obese islets than in normal and at 20-25 weeks the obese islet ATP level was significantly elevated above 10-15 and 40-45 week obese islet values. The reduced islet ATP levels of 40-45 week old obese mice were consonant with the previously described reduction in islet adenyl cyclase activity; the less severe hyperinsulinaemia and the observed morphological degeneration of islet tissue from these mice.

ATP could be a necessary prerequisite for the process of insulin secretion. Although Hellman and colleagues have demonstrated the sulphonylureas, HB419 and carbutamide to produce a marked reduction in β-cell ATP content possibly by uncoupling oxidative phosphorylation (131)

TABLE 26. ATP levels in the islets of normal and obese mice with age.

(Mean values ± S.E., n = number of determinations, islets pooled from several mice for each determination).

Age (weeks)	N	Normal Islets (mmoles ATP/Kg wet islet tissue)	CV	Obese Islets (mmoles ATP/Kg wet islet tissue)	CV
10 - 15	5	3.2 ± 0.3	19%	5.2 ± 0.3 *	15%
20 - 25	5	2.9 ± 0.4	27%	7.5 ± 0.4 *†	12%
40 - 45	5	2.7 ± 0.4	29%	4.8 ± 0.2 *	8%

* Denotes significant difference ($P < 0.05$) between normal and obese islet values in the same age group.

† Denotes significantly different ($P < 0.05$) from obese islet values at 10-15 and 40-45 weeks of age.

CV = Coefficient of Variation.

Islet homogenate ATP content was assayed using a Boehringer test kit, (Cat. No. 15979 TAAC).

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this result might also reflect a rapid turnover of ATP or its conversion to ^{c}AMP. Indeed recent work by Krzanowski and coworkers has shown no change in rat islet ATP or phosphocreatine content after tolbutamide when insulin output was maximal. This suggested that glucose metabolism was of no importance for tolbutamide action, or at least not for the initial response of the β-cells (658). The sulphonylurea depletion of islet ATP shown by Hellman & Idahl could hardly be explained on the basis of an energy consumption during the process of insulin secretion. Since adrenaline and diazoxide that inhibited insulin secretion, and db-^{c}AMP that stimulated insulin secretion, left the ATP content of β-cells unchanged (130).

R-Candela and colleagues have demonstrated an *in vitro* stimulation of insulin secretion from pieces of rat pancreas by ATP (13). Yet it has also been reported that the glucose stimulation of insulin secretion remained unimpaired under conditions in which the level of ATP would have presumably been reduced by the uncoupling action of dinitrophenol (659). Also Matschinsky & Ellerman have emphasised that the β-cell

response to hyperglycaemia could not be correlated with the intracellular content of ATP (201).

Attention has been paid to the possible significance of a fast glycolytic ATP formation for the rapid process of insulin secretion (630). While the substrate for glycolytic ATP formation was probably derived from the pentose phosphate pathway (133,660,661) the subsequent degradation of glucose in the β-cells of NZO mice was characterised by high enzyme activities in the ATP regenerating steps. A high ATP content of islets would seem to be essential for their capability of incorporating glucose into the islet metabolic pool, and an high ATP level might be regarded as a prerequisite condition for a stimulatory effect of glucose on insulin secretion. However, it would seem unlikely that the increased ATP level per se. would be the stimulus for insulin secretion. The dynamic equilibrium between ATP and ADP in the β-cell might play a regulatory role in the biosynthesis and secretion of insulin. The energy flux, caused by the interconversion between available amounts of ADP and ATP in islet cells, would depend on the substrate and the kinetics of the processes involved (663). The high content of energy rich adenosine phosphates in β-cells has confirmed the presence of sufficient substrate for adenyl cyclase activity and possibly ^{c}AMP-phosphodiesterase inhibition and a high metabolic capacity and readiness to react to stimulus. For the further evaluation of β-cell physiology, as linked to energy transfer, assessment of both ATP and ADP content will be required. The determination of the sum of ATP and ADP will be of interest as an indication of the potentiality to make energy available.

SECTION 3.

The calcium and magnesium concentration in islet tissue from normal and obese mice with age.

Calcium has been shown to be an essential requirement for insulin secretion (385,386). Rasmussen & Tenenhouse suggested that ^{c}AMP might

FIG. 33a. Normal and obese mouse islet Ca^{2+} content with age. (Mean Values ± S.E.).

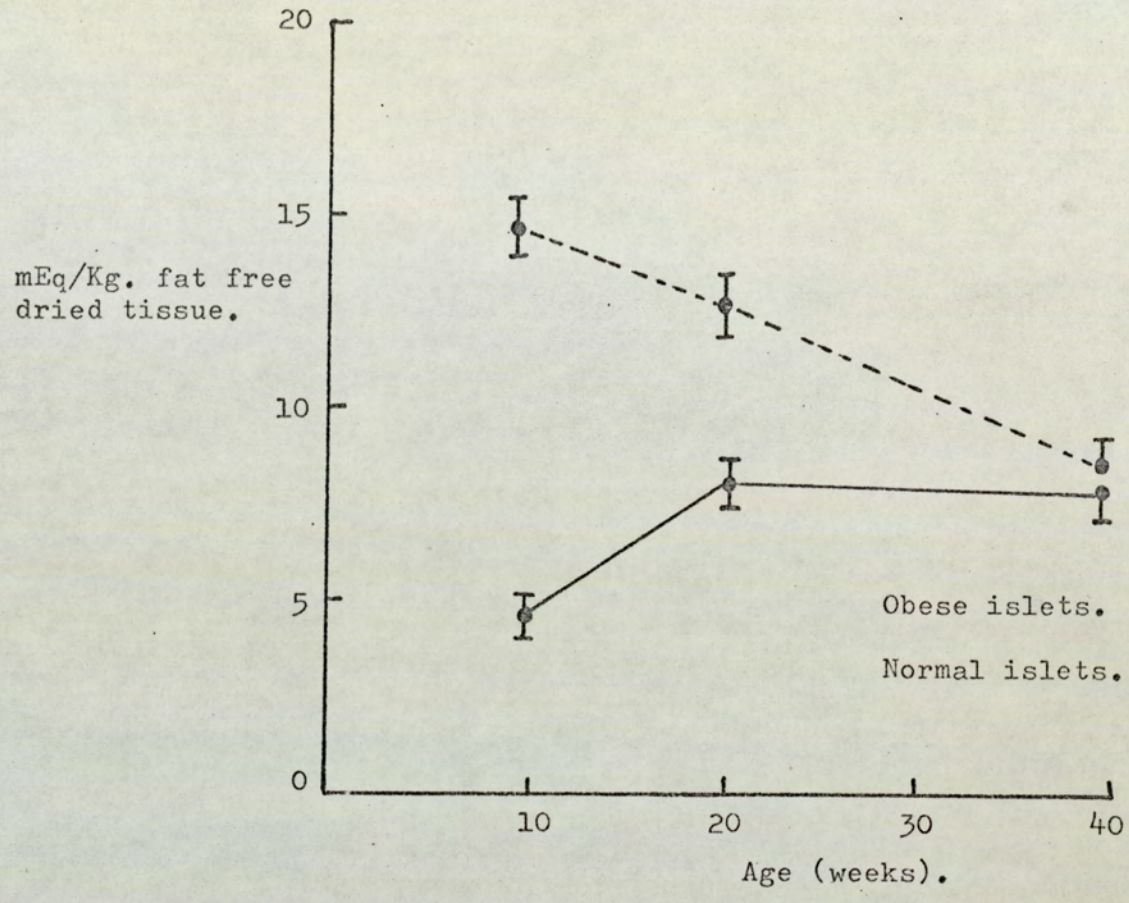
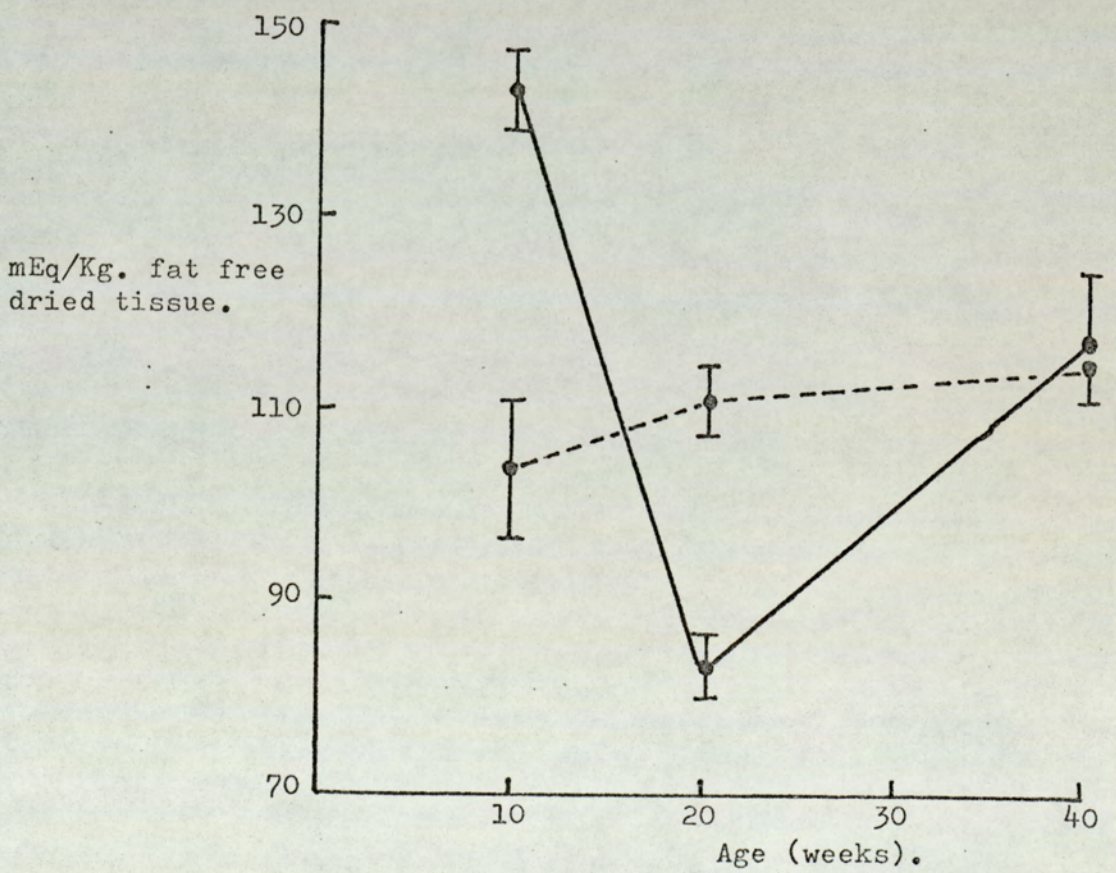


FIG. 33b. Normal and obese mouse islet Mg^{2+} content with age. (Mean Values ± S.E.).



facilitate the entry of Ca^{2+} into the cell by increasing the cell membrane permeability to this cation or be involved in the compartmentalisation of Ca^{2+} within cells (394). Ca^{2+} rather than ^{c}AMP might be the ultimate effector in the mechanism of insulin secretion. Since Ca^{2+} and Mg^{2+} may act competitively both for the active site of islet adenyl cyclase and in the stimulation of insulin secretion (664), the profile of islet Ca^{2+} and Mg^{2+} content was investigated for both normal and obese mice with age.

MATERIALS & METHODS.

The procedures for the estimation of Ca^{2+} and Mg^{2+} in islet tissue have been described previously (Chapter 4).

Results and Discussion.

The Ca^{2+} levels in islet tissue of both normal and obese mice with age are shown in Fig. 33a.

Obese islet Ca^{2+} levels were higher than the corresponding normal islet values at 10-15 and 20-25 weeks, but not at 40-45 weeks of age. Obese islet Ca^{2+} levels were highest at 10-15 weeks (Fig. 33a) and only marginally different from values obtained at 20-25 weeks. The 10-15 week period corresponded to the onset of hyperinsulinaemia in obese mice (51,54) and results suggested that an elevated islet Ca^{2+} content might have been a prerequisite for this to occur. Normal islet Ca^{2+} levels increased significantly from 10-15 weeks, stabilising at 20-25 and 40-45 weeks. At 40-45 weeks the Ca^{2+} level in obese islets had decreased to a value not significantly different from that of normal islets of the same age. This decrease correlated with the reduced β-cell volume observed for islets of 40-45 week old obese mice and was consonant with the latter's less severe hyperinsulinaemia. This observation also suggested that the majority of islet Ca^{2+} was localised in the β-cells.

The Mg^{2+} levels in islet tissue of both normal and obese mice are shown in Fig.33b. Obese islets showed no significant variation in Mg2+

content with age. This observation tended to dissociate Mg^{2+} from any causal role in obese islet hypersecretory activity. Indeed hypersecretory activity might only occur in the presence of a stable unchanging islet Mg^{2+} content. The profile of Mg^{2+} content with age for normal islets was irregular. Over the period of life considered, a maximum Mg^{2+} content was observed at 10-15 weeks, but 20-25 weeks saw an inexplicable reduction in Mg^{2+} content to a value significantly lower than in obese islets of the same age. No significant difference was observed between the Mg^{2+} content of normal and obese islets at 40-45 weeks of age. The absence of any reduction in Mg^{2+} content of obese islets at 40-45 weeks suggested the greater proportion of islet Mg^{2+} to be localised in cells other than β-cells, perhaps α-cells. On the other hand the Mg^{2+} content of invading connective tissue might have compensated for any loss incurred as a result of the reduced β-cell volume.

Mg^{2+} and Ca^{2+} might compete with each other in the β-cell for binding to β-granule membranes (664), only the binding by calcium facilitating the secretion of insulin. The higher level of Mg^{2+} compared to calcium in islet cells suggested that β-granule membrane sites would be more likely to be bound by Mg^{2+} than Ca^{2+} unless intra-β-cell compartmentalisation of the latter had taken place i.e. into the golgi apparatus. On the other hand the binding of Ca^{2+} in preference to Mg^{2+} would not favour β-granule membrane bound adenyl cyclase activation and might tend to rule out a role for ^{c}AMP within the β-granule.

SECTION 4.

The total RNA and total DNA content of normal and obese mouse islets with age.

It seemed reasonable to assume that in the fully differentiated β-cell the greater proportion of protein synthesis would involve the production of proinsulin and insulin. In the obese mouse at 20-25 weeks the hypersecretory behaviour of their islets and the prevailing

hyperinsulinaemia could be correlated with an increased total RNA content (Chapter 5, Section 1). With this in mind the total RNA and total DNA contents of normal and obese mouse islet tissue were estimated and correlated with the serum IRI profile of these mice with age.

MATERIALS & METHODS.

The methods for the determination of total RNA and total DNA have been described previously (Chapter 5, Section 1).

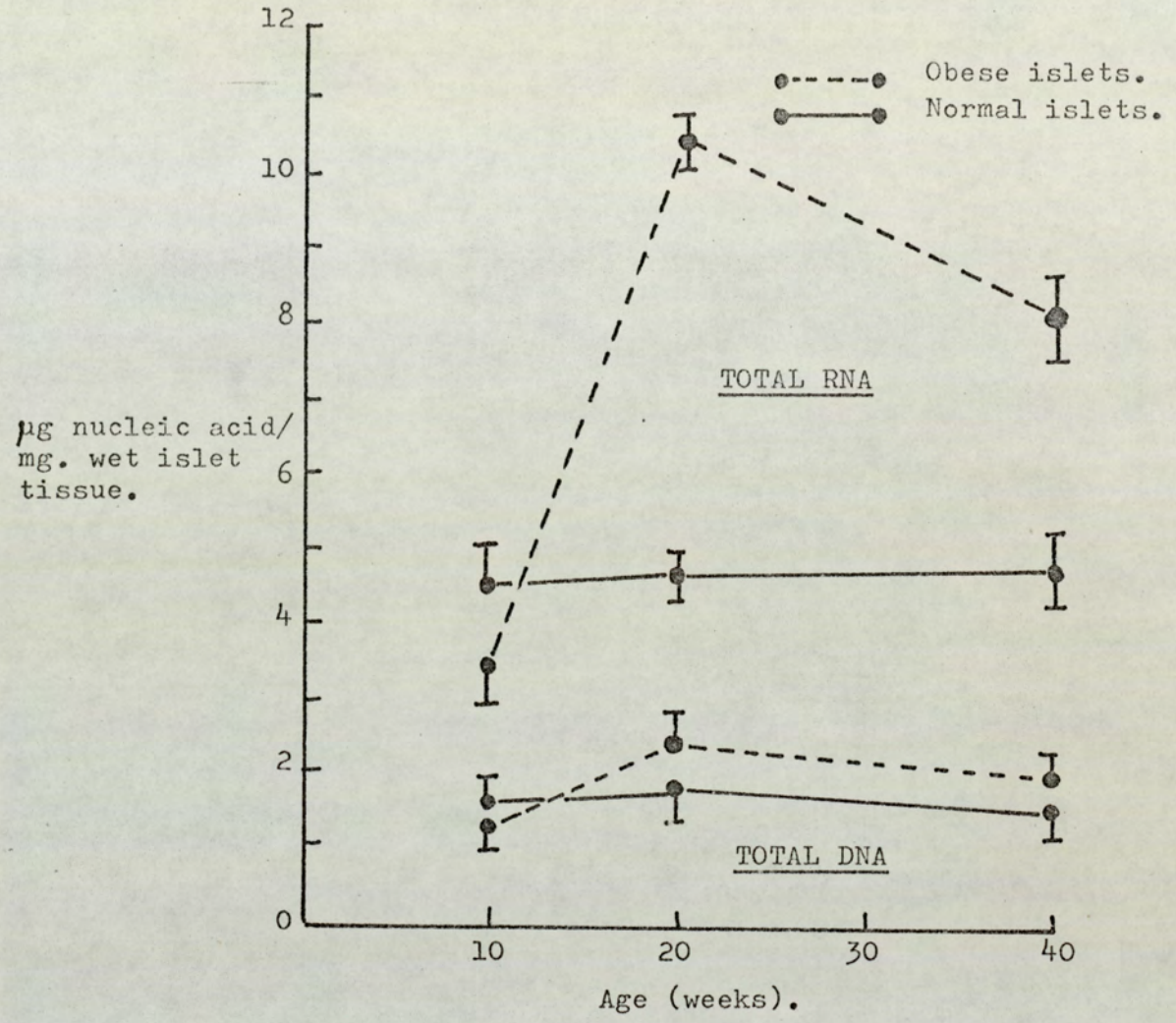
Results and Discussion.

The total RNA and total DNA levels in normal and obese islets with age are shown in Fig. 34. Normal islets showed no significant change in either total RNA or total DNA content with age. Similarly obese islet total DNA showed no significant variation with age. However, at 20-25 weeks, obese islet total RNA content was markedly increased and consonant with the characteristic hyperinsulinaemia of obese mice at this age. The reduced total RNA content of islets from 40-45 week old obese mice probably reflected their observed reduction in β-cell volume and might have contributed to the less severe hyperinsulinaemia observed for obese mice in this age group.

Summary of the changes in obese islet activity with age associated with the manifest hyperinsulinaemia of obese hyperglycaemic mice.

At the height of the syndrome (20-25 weeks), β-cell hyperplasia; serum IRI; the activity level of the islet adenyl cyclase/^{c}AMP-phosphodiesterase system and islet ATP, Ca^{2+} and total RNA levels were maximal. The maximum total RNA level probably reflected the increased insulin and proinsulin biosynthesis demanded by the hypersecretion of insulin in response to hyperglycaemia. Glycogen levels were submaximal, but increasing in response to the prevailing hyperglycaemia. In comparison, at 40-45 weeks; β-cell volume; serum IRI; the activity level of the islet adenyl cyclase/^{c}AMP-phosphodiesterase system and

FIG. 34. The total RNA and total DNA content of normal and obese mouse islet tissue with age. (each point represents the mean of 4 determinations ± S.E.).



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islet ATP, Ca^{2+} and total RNA contents were all reduced, but glycogen levels were maximal as a result of the maintained hyperglycaemia. It remains to be investigated whether increased islet levels of these potential secretory requirements either cause or are the result of the hypersecretion and subsequent hyperinsulinaemia maintained by a tissue resistance to insulin.

GENERAL DISCUSSION

It is not yet possible to give a complete mechanistic description of insulin secretion on a molecular level, but approaches to it have become possible. Similarly it is not yet possible to define the primary lesion responsible for the obese hyperglycaemic syndrome in mice.

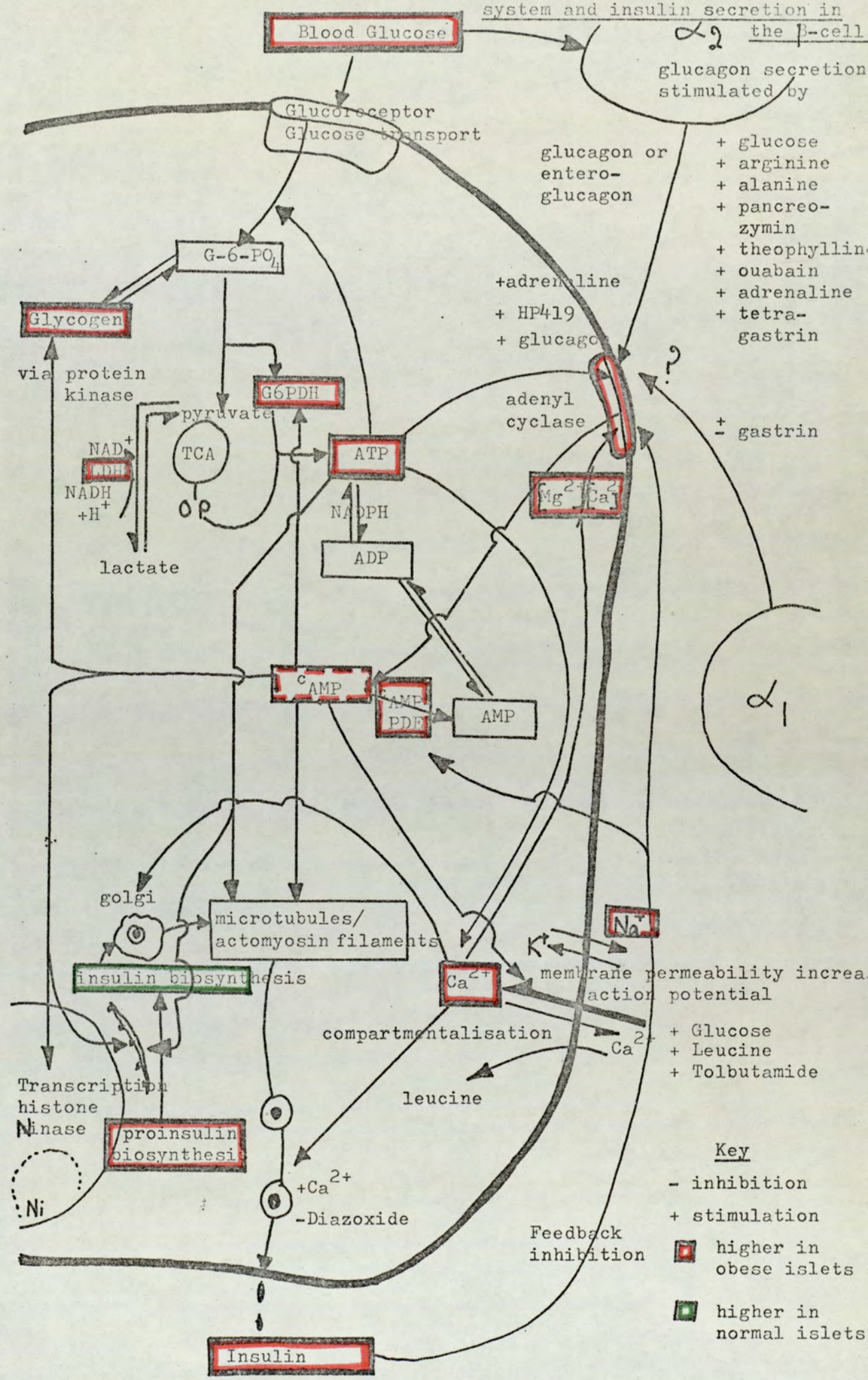
The work presented here has investigated certain aspects of islet physiology and metabolism in the light of an existing functional adenyl cyclase/^{c}AMP-phosphodiesterase system. That ^{c}AMP was probably involved in the secretion process was not only deduced from *in vitro* and *in vivo* animal experiments using glucagon, theophylline and adrenaline but also in clinical circumstances where in persons heavily pre-disposed to diabetes by genetic circumstances (genetically potential diabetes, prediabetes) glucose provoked insulin secretion, but sluggishly, phase I was delayed, and in such cases, theophylline corrected the slow response and temporarily re-established the normal rapid phase of secretion. In the process of insulin secretion β-granules might have been induced to discharge by localised shifts in calcium ions brought about by a rise in ^{c}AMP, and glucose or one of its metabolites might have been necessary for either the energetics or the production of a cofactor for these discharge reactions. It could be that an actomyosin like filament connected the granule to the cell membrane and the contraction of such a filament forced the granule into contact with the cell membrane, thus leading to the discharge of insulin by emiocytosis: Clearly an orderly release by such a mechanism would maintain the integrity of

appropriately placed β-cell membrane bound receptor(s). Whereas random emiocytosis could lead to the disruption of these receptors and the possible loss of response to insulinogenic stimuli. The involvement of Ca^{2+} and actomyosin filaments in insulin secretion and the analogy with muscle contraction might carry more weight than was at once thought, since the glucose initiated secretion of insulin has been shown to be preceeded by electrical phenomena resembling an action potential (544,546).

Although there was reasonable evidence to suggest the involvement of membrane receptors in insulin secretion there has long been evidence to suggest that glucose not only probably triggered insulin secretion but its metabolism was essential for the process to occur (4,665). Since secretion ultimately depended upon previous biosynthesis, glucose might be more importantly involved in biosynthesis than in the mechanism of secretion. On this basis any model of the mechanism of insulin secretion must allow for β-cell glucose metabolism and the transfer of energy from glucose metabolism to the biosynthetic and secretory mechanisms. Indeed the majority of the work of Lambert, Jeanrenaud & Renold on the foetal rat pancreas, recently reviewed, has clearly indicated that both ^{c}AMP and some energy source were required to elicit maximal insulin secretion in vitro (666). Glycogen stores in islets probably did not provide sufficient energy themselves to totally support the insulin secreting action of ^{c}AMP but they might contribute significantly to the provision of ATP via metabolism.

On thebasis of the experimental work described in this thesis, a tentative summary of the relationships that might be expected to exist in a β-cell between the adenyl cyclase system and glucose metabolism in order to facilitate the secretion of insulin has been illustrated in Fig. 35. The sequence basically resembled that first hypothesised by Cerasi & Luft (43,499) and indicated the possible dual

FIG. 35. The association between glucose metabolism, the adenyl cyclase system and insulin secretion in the β-cell



action of glucose as a metabolite and as an effector in relation to the secretion of insulin, but the association between the adenyl cyclase system and the metabolic activity of the β-cell has been elaborated. By this system the β-cell would use its own glucose metabolism as a parameter of glucose metabolism in the body as a whole and modulate the insulin stimulatory and biosynthetic pathways accordingly. Although the glucoreceptor and adenyl cyclase systems were illustrated as separate entities on the membrane, the metabolic and physiological consequences of their stimulation were closely linked. In biphasic insulin secretion, a stimulation via the adenyl cyclase system might initiate the rapid first phase and stimulation via the glucoreceptor the second or biosynthetic phase. The possible glucagon stimulation of β-cell adenyl cyclase has been emphasised and glucagon was suggested to modulate hyperglycaemia both by the stimulation of glycogenolysis in the liver and the stimulation of insulin secretion. The portions shown to be increased in obese mouse islet tissue compared to normal seemed to be those entities that would be required for hypersecretion to occur rather than being the primary cause of it. The rate of ^{c}AMP turnover or its binding to protein kinases might be more important than the level of free ^{c}AMP in β-cell metabolism and the mechanism of insulin secretion. ^{c}AMP might alter the transport of electrolytes such as K^{+}, Ca^{2+} and Na^{+} and perhaps of more importance, the nucleotide might control the intracellular distribution of Ca^{2+} in the β-cell. Such an alteration in distribution might result in changes in cell membrane permeability, in β-granule membrane permeability, or in the function as well as morphology of intracellular organelles; any or all of these changes could eventually be responsible for the secretion of insulin. Clearly the influence that ^{c}AMP has within the cell requires a great deal more investigation. It might be mentioned here in passing that db-^{c}AMP

has recently been shown to exert no direct action on β-cells *in vivo* in order to enhance or inhibit glucose stimulated insulin secretion (680). The physiological effects of the cyclic nucleotide might be traced eventually to its ability to activate or inactivate enzymes that catalyse key reactions of metabolism. It may be found that the sole function of ^{c}AMP in the β-cell is the activation of protein kinases. ^{c}AMP has been shown to stimulate the synthesis of various enzymes in foetal rat liver (667). In β-cells the role of ^{c}AMP might be to induce the synthesis of enzyme sequences at birth associated with the mechanism of insulin secretion. The post-natal presence of an adenyl cyclase system might be vestigial and play no part in the mechanism of insulin secretion or its prior biosynthesis in the adult animal. Also ^{c}AMP may not prove to be the only cyclic nucleotide capable of influencing β-cell metabolism and perhaps insulin secretion. Guanyl cyclase and the ^{c}GMP it produces could also be involved, although the levels of ^{c}AMP in tissues tend to exceed those of ^{c}GMP (668). Beavo and colleagues have shown ^{c}GMP to stimulate the activity of ^{c}AMP-PDE in rat liver extracts (669). This finding suggested the possibility that ^{c}GMP might be capable of controlling the level of ^{c}AMP and possibly the response to all target cells to hormones. For this reason studies of guanyl cyclase activity might reveal new insights into the regulation of ^{c}AMP mediated processes.

Although future research might show prostaglandins and possibly the pineal body to be involved in insulin secretion (670), there exists at present a reasonable amount of evidence to suggest a role for the hypothalamus in the stimulation of insulin secretion. It has long been known that destruction of the ventro-medial region of the hypothalamus in the rat results in hyperinsulinism (671) and enlargement of the

islets of Langerhans a few hours after making the lesion (672). Also thesedata, covering a wide range of hypothalamic function, were suggestive of a lesion in the hypothalamus of obese mice (47). Recent work by Idahl & Martin involving the exposure of isolated mouse islets to the exudate of pieces of mouse hypothalamus has demonstrated the existence of a ventro-lateral hypothalamic factor capable of stimulating insulin secretion (673). Its importance in the regulation of islet function and its possible involvement in the pathogenesis of diabetes remains to be investigated.

Obesity per se., whether regulatory or metabolic always tends to be accompanied by hyperinsulinaemia. The latter could be due to over production, but it could also be due to an increased sensitivity of the islets to the natural stimulus, glucose. Although the hypersecretion of insulin was essential for increased body weight, it did not necessarily reflect a primary genetic defect in the pancreas. A strong case could be made, however, for the primary genetic defect to be located in the pancreas of obese hyperglycaemic mice and the evidence for this has been extensively reviewed (47). Of importance in work on the obese mouse was the observation of an elevated level of adipose tissue glycerokinase activity (674). The lack of a lipostatic factor, capable of suppressing glycerokinase activity in obese mouse adipose tissue could provide an explanation for the increased concentration of glycerol phosphate and subsequently decreased glucose uptake, even in the presence of elevated plasma insulin levels. The resulting hyperglycaemia would cause the hyperplasia of islet β-cells, increased insulin production and elaboration, and constant β-cell degranulation. It seemed feasible that some component of normal islet tissue might indirectly regulate glucose catabolism probably through its action on lipogenesis in adipose tissue but the nature of this factor is as yet unknown.

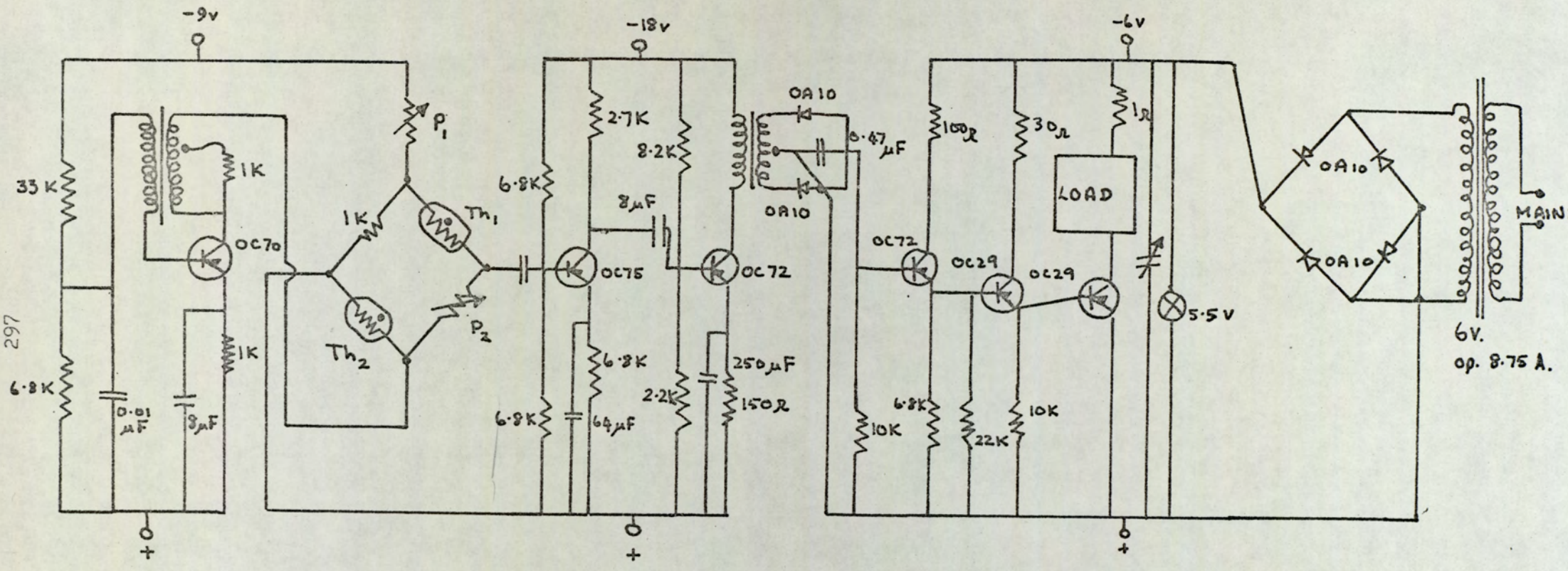
It also seemed possible that some gastrointestinal insulin secretogogue might potentiate the pancreatic response to oral glucose in obese mice. Overactivity of gastrointestinal insulinogenesis in response to eating could be the primary aberration of the obese mouse leading to hyperinsulinaemia, obesity, resistance to insulin and eventually hyperglycaemia.

Obesity is a significant promoting factor in diabetes mellitus and like the latter might have an inherited genetic component (675). The relationship between obesity and maturity onset diabetes has been known for a long time but remains poorly understood. Both syndromes are known to be associated with anomalies of insulin secretion and/or effectiveness. Obesity has been shown to be associated with fasting hyperinsulinism and hyperinsulinism in response to glucose or other stimuli (677). The presence of hyperinsulinism implied a relative ineffectiveness of the circulating IRI, and a relative ineffectiveness of exogenous insulin has been demonstrated in obesity and in maturity onset diabetes (678). In the obese syndrome the decreased responsiveness to insulin seemed to be most marked in striated muscle (56) and associated with a long lasting hyperinsulinaemia. The insulin resistance was most marked in adult or middle aged animals and imposed a permanent stress on the β-cells. The increased demand for insulin resulted in islet hyperplasia; hypertrophy and elevated plasma insulin levels following hypersecretion (679). The high prevalence of obesity in diabetes, and of diabetes in obesity, in both animals and man, as well as the number of shared metabolic characteristics in both disorders, suggested the possibility that some genes might be common to both conditions. Some might control insulin biosynthesis, profiles of insulin secretion, capacity for hyperplasia of β-cells, and also ease of deposition or mobilisation of lipids in adipose tissue, number of fat

cells and feeding habits.

It would seem that despite conceptual and methodological complexity the search for factors which decrease the sensitivity to insulin of tissues in general, or individual tissues in particular, might provide a more precise understanding of the aetiology of obesity and diabetes in animals and man.

APPENDIX. Circuit diagram for the microdissection apparatus.



Sub-stage electronics.

Power supply.

Operating instructions for the microdissection apparatus.

1) It is recommended that the solution to be maintained at low temperature be pre-chilled to avoid a long wait for the frigistor element to cool the solution from room temperature.

2) The concealed light is controlled by the mains switch. The lighting current is D.C. to avoid interference with the sub-stage electronics. If a subsidiary light is used i.e. dissecting lamp keep the mains cable and transformer away from the stage.

3) The brightness of the sub-stage light varies inversely with the current through the frigistor element.

4) The preset is not normally used in routine work. If it requires adjustment fill the stage-bath with warm water. Set the control knob to 100 and adjust the preset for minimum brightness of the substage light.

5) Make the connections between the stage platform and the power control unit. Ensure that the five pin microphone plug labelled in red is connected to the plug also labelled red.

6) The control knob is calibrated in degrees C approximately. 0 maintains a temperature of about 10°C while 100 very nearly freezes the stage bath. This calibration depends to a great extent on the ambient temperature. The bottom of the dissecting dish is about 2°C lower than the surface. The recommended value on the control knob is that reading that gives a temperature of 2 - 4°C in dissecting dish.

7) Ensure that water is flowing freely through the element jacket before switching on mains.

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Metabolic viability of freehand microdissected and collagenase-isolated islets of Langerhans. By T. Atkins and A. J. Matty. *Department of Biological Sciences, University of Aston in Birmingham, Costa Green, Birmingham*, 4

The discovery of the obese hyperglycaemic mouse strain with its numerous metabolic disturbances and hyperplastic islet β-cells has made possible the introduction of a freehand microdissection technique for fresh islets; also, the enzyme collagenase has been used to digest the collagenous framework of the pancreas and effectively separate whole islets from the surrounding exocrine tissue (Moskalewski, 1965). Although these methods are being applied to investigations of islet physiology it seemed of value to compare the metabolic rate, insulin content and rate of insulin secretion of islets obtained by freehand dissection and enzyme digestion before further investigation. Accordingly we have measured these parameters in obese hyperglycaemic mice, in their lean litter-mates and in normal albino mice.

Preparation of collagenase-isolated islets was carried out by a modification of the methods of Lacy & Kostianovsky (1967) and Howell & Taylor (1968) and that of freehand dissection by a technique similar to that described by Hellerström (1964). Insulin immunoassay was carried out by the double antibody technique and metabolic condition (viability) was determined by measuring oxygen consumption of the islets by micropolarography. All preincubations, test incubations and respiratory measurements were made in oxygenated bicarbonate buffer plus glucose. The concentration was 0·6 g./l. for low and 3·6 g./l. for high glucose.

The oxygen uptake of microdissected islets was about twice that of collagenase-isolated islets in both the absence of glucose and in the presence of low and high glucose (microdissected, low glucose $1{\cdot}78 \pm 0{\cdot}1$, high glucose $3{\cdot}17 \pm 0{\cdot}1$ Q_{O_2} μl./mg./hr.: collagenase, low glucose $1{\cdot}11 \pm 0{\cdot}02$, high glucose $1{\cdot}79 \pm 0{\cdot}02$ Q_{O_2} μl./mg./hr.) Also, the microdissected islets of obese hyperglycaemic mice had a much higher oxygen consumption than their lean litter-mates and normal albino mice. The normal tissue relationship of oxygen consumption to weight of tissue applied; there was no inverse correlation and there appeared to be no relationship between islet oxygen uptake and animal body weight with either preparation.

Increasing the glucose concentration of the incubation media stimulated insulin secretion in both preparations but in normal albino strain mice the increase of insulin secretion, in response to increased glucose, expressed as a percentage of the total content was found to be much greater in microdissected islets ($7{\cdot}4\,\% \pm 0{\cdot}5$ S.E.) than in collagenase-isolated islets ($1{\cdot}6\,\% \pm 0{\cdot}1$ S.E.). Also the initial total content of insulin of these two preparations, in response to increased glucose, differs in normal albino mice. In a sample of ten microdissected and ten collagenase-treated mice the initial total insulin content was about 142 m-u. insulin/mg. dry islet tissue for the former and about 120 m-u. dry weight for the latter. The difference was very significant ($P < 0{\cdot}01$).

Microdissected islets as well as having a greater insulin secretory rate than that of collagenase-isolated islets show a much greater response to increased concentration of glucose when incubated for 1 hr.

[P.T.O.

It appears that the microdissected islet is metabolically more active than those prepared by collagenase extraction and might be the preferred method of preparation for future investigations on islet physiology. Commercially available preparations of collagenase differ in their purity but all contain some protease and peptidase activity which might reduce islet insulin content and secretory rate.

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Adenyl cyclase and cyclic AMP-phosphodiesterase activity in the isolated islets of Langerhans of normal and obese mice. By T. Atkins and A. J. Matty. *Department of Biological Sciences, The University of Aston in Birmingham, Birmingham* 4, *England*

Adenyl cyclase and cyclic AMP phosphodiesterase activity have been measured radiometrically in microdissected islets of 25-week-old normal and obese mice using a modification of the method of Bär & Hechter (1969). Theophylline (10 mM) in the presence of 1 mM-cyclic AMP prevented the breakdown of cyclic AMP in the cyclase assay. The levels of labelled substrate used were in excess of the apparent K_m values. Cyclic AMP production and hydrolysis at 37 °C in the cyclase and phosphodiesterase assays respectively were found to be linear with time and protein concentration.

Islet cyclase and phosphodiesterase activity were found to be significantly higher in islets of obese mice than in normal mice; $4{\cdot}3990 \pm 0{\cdot}062$ and $3{\cdot}4812 \pm 0{\cdot}059$ μM-cyclic AMP produced/mg islet protein/10 min (pH 7·5) for cyclase and $4{\cdot}2228 \pm 0{\cdot}060$ and $3{\cdot}1979 \pm 0{\cdot}0264$ μM-cyclic AMP hydrolysed/mg islet protein/10 min (pH 8) for phosphodiesterase respectively. 10 mM-NaF increased cyclase activity in islets of obese mice by 16% while 10 mM-theophylline in the presence of 1 mM-cyclic AMP reduced phosphodiesterase activity by 90%.

Although the cyclase system seemed to be operating at a higher level in the islets of obese mice, the ratio of phosphodiesterase : cyclase in both normal and obese mice was the same (1:1). The balance of the system in islets remains to be clarified, especially in view of the many metabolites produced naturally as a result of ATP metabolism that are capable of inhibiting phosphodiesterase activity. Insulin itself may be capable of inhibiting cyclic AMP production in islets.

High glucose, propranolol in the presence of adrenaline, and phenylephrine in the presence of propranolol all reduced islet cyclase activity in both normal and obese mice. Adrenaline, phentolamine in the presence of adrenaline, isoprenaline in the presence of phentolamine and HB.419 all increased cyclase activity in normal and obese mice. The percentage stimulation by adrenaline was some three times higher in obese mice. Phentolamine in the presence of adrenaline, isoprenaline in the presence of phentolamine and HB.419 stimulated cyclic AMP production, which was some six, two, and six times greater, respectively, in normal islets than in those of obese mice. The stimulatory effect of phentolamine in the presence of adrenaline, and isoprenaline in the presence of phentolamine exceeded the effect of adrenaline alone in normal islets. These results show adrenergic stimulation of islet cyclase to be a β-adrenergic receptor function, and adrenergic inhibition an α-receptor function.

In diabetes, Cerasi & Luft (1970) suggested that the cyclic AMP-forming mechanism may be defective. The genetic defect may be at the level of adenyl cyclase or be the result of very high phosphodiesterase activity. Excessive phosphodiesterase activity has not been found in the islets of obese hyperglycaemic mice and, although pharmacologically unresponsive, the cyclase system in the obese mice appears normal.

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ADENYL CYCLASE AND PHOSPHODIESTERASE ACTIVITY IN THE ISOLATED ISLETS OF LANGERHANS OF OBESE MICE AND THEIR LEAN LITTER MATES: THE EFFECT OF GLUCOSE, ADRENALINE AND DRUGS ON ADENYL CYCLASE ACTIVITY

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SUMMARY

Radiometric estimations of adenyl cyclase and cyclic AMP (cAMP) phosphodiesterase showed significantly higher enzyme activities in the islet tissue of obese–hyperglycaemic mice than in that of their normal litter mates, but the ratios of the two enzymes in both types of islets were found to be the same, i.e. 1:1.

Adenyl cyclase and phosphodiesterase activity increased linearly with incubation time and protein concentration. Sodium fluoride (10 mmol/l) increased cyclase activity in the islets of obese mice by 33·4 %; theophylline (10 mmol/l) in the presence of cAMP (1 mmol/l) reduced phosphodiesterase activity by almost 100 %.

Glucose (2 g/l) reduced, while adrenaline (10^{-5} mol/l) increased cyclase activity in islets from both normal and obese animals. An evaluation of the effects of α- and β-adrenergic receptor stimulating and blocking agents on islets of both normal and obese mice showed the stimulation of adenyl cyclase to be a β-adrenergic receptor function and the inhibition an α-adrenergic receptor function. The possible role of the adenyl cyclase system in diabetes mellitus and insulin secretion is discussed.

INTRODUCTION

Cyclic adenosine-3′,5′-monophosphate (cAMP) has been shown to be a key regulating factor in a number of metabolic processes (Sutherland & Robison, 1966; Robison, Butcher & Sutherland, 1967) and its presence has been established in a variety of mammalian tissues (Sutherland & Rall, 1960). As an intracellular second messenger, cAMP not only mediates the actions of a number of hormones but is also implicated in the secretion of certain peptide hormones (Robison, Butcher & Sutherland, 1968). To date evidence for a role of cAMP in regulating the release of insulin has come from work showing the stimulation of insulin release by exogenous

cAMP (Sussman & Vaughan, 1967; Sussman, Vaughan & Stjernholm, 1969; Malaisse, Malaisse-Lagae & Mayhew, 1967). Corticotrophin, which stimulates adenyl cyclase in the adrenal cortex and in adipose tissue (Grahame-Smith, Butcher, Ney & Sutherland, 1967; Butcher, Baird & Sutherland, 1968), has also been shown to stimulate insulin release (Sussman & Vaughan, 1967; Malaisse *et al.* 1967).

Tanese, Lazarus, Devrim & Recant (1970), using rat islets, have shown an increase in both the release of newly synthesized pro-insulin and insulin, and the biosynthesis of these two hormones by the dibutyryl derivative of cAMP. Available evidence suggests that α-adrenergic stimulation inhibits insulin release, while β-adrenergic stimulation increases insulin release from the pancreatic islet (Porte, 1967*a*, *b*, 1969). Turtle & Kipnis (1967) have shown that α-adrenergic receptor stimulation decreases the tissue concentration of cAMP while β-adrenergic receptor stimulation increases the concentration of cAMP in isolated rat islets. Cerasi & Luft (1970) have suggested that in diabetes, the inherited factor is a reduced ability of the β-cells to produce cAMP, due either to defective adenyl cyclase or to excessive phosphodiesterase activity. However, both the adenyl cyclase and cAMP phosphodiesterase systems in islets may be normal and a basic cause of the diabetic syndrome may be the amyloid barriers described by Lacy (1967). The purpose of the present work was to demonstrate the presence of an adenyl cyclase–cAMP phosphodiesterase system in the islets of obese mice and their normal lean litter mates and to investigate the effect of glucose, adrenaline and some pharmacologically active agents on adenyl cyclase activity. A preliminary account of this work has been given (Atkins & Matty, 1970). The work described below has been performed using whole islet homogenates and until a method is available for separating α and β cells in both normal and obese mouse islets then enzyme activity can only be expressed in terms of the whole islet.

MATERIALS AND METHODS

Male normal and obese mice (Institute of Animal Genetics, Edinburgh), 20–25 weeks old, were starved overnight (water was available *ad libitum*) before removal of the islets of Langerhans by freehand microdissection in bicarbonate buffer, pH 7·2 (Hellerström, 1964). Mean blood sugar values for these mice were 85 mg/100 ml for normal animals and 298 mg/100 ml for obese animals (unpublished results).

Isolated islets were weighed and homogenized at 2 °C in 1 mM-potassium bicarbonate (tissue : volume ratio, 1 : 9) and their protein content determined by the method of Lowry, Rosebrough, Lewis Farr & Randall (1951). Islet protein concentration was adjusted to 2 μg/10 μl with 1 mM-potassium bicarbonate before use in assays.

Adenyl cyclase assay

Adenyl cyclase and cAMP phosphodiesterase activity were measured radiometrically by the method of Bär & Hechter (1969) with some modifications described below. Assays were performed on fresh islet homogenates within 1 h of isolation. The final concentration of reaction components in 50 μl were: 40 mM-tris–HCl, pH 7·0, at 37 °C; 5 mM-$MgCl_2$; 0·1 % crystalline bovine albumin V; 10 mM-theophylline; 1 mM-cAMP; pyruvate kinase II, 0·05 mg/ml (Sigma); phosphoenolpyruvate trisodium salt (8 mmol/l) (Sigma); 1·9 mM-[^{3}H] ATP (final specific activity 7·9 mCi/

mmol) (Radiochemical Centre, Amersham, England). To the reaction mixture was added 10 μl of islet protein. Blank assays contained 10 μl of 1 mM-potassium bicarbonate instead of protein, together with any test substance used. Incubations were carried out in Parafilm-sealed tubes for 10 min at 37 °C. The reaction was stopped by immersion in a boiling water bath for 2 min. A carrier containing ATP, ADP, AMP, cAMP, adenosine, inosine and IMP (2–5 mmol/l) was added to the incubation mixture and after centrifugation the contents of the supernatant were separated by two-dimensional ascending thin layer chromatography using precoated cellulose F thin layer sheets (20 × 20 MERCK): first dimension, isopropanol:ammonia:water (7:1:2); second dimension, isobutyric acid:ammonia:water (66:1:33). IMP co-chromatographed with ATP in these two systems. Spots were identified in ultra violet light and their activity counted on a Packard Liquid Scintillation Counter. The average radioactive yield was about 66 %.

Phosphodiesterase assay

Cyclic AMP phosphodiesterase activity was estimated using 1·74 mM-[^{3}H]cAMP (final specific activity, 8 mCi/mmol), 40 mM-tris–HCl, pH 8·0, 5 mM-$MgCl_2$ and 0·1 % bovine albumin V, in a total volume of 50 μl. Incubations were carried out for 10 min at 37 °C. After centrifugation a carrier containing AMP, cAMP, IMP, adenosine and inosine (2–5 mmol/l) was added. The contents of the supernatant were separated by a single descending chromatography on Whatman G.F. 81 glass fibre paper in the solvent system *n*-butanol:isopropanol:ammonia (7:2:1). In this system IMP cochromatographed with AMP. Spots were identified and counted as for adenyl cyclase. The percentage radioactive yield was about 81 %. Although the radioactive yield was fairly high, a recent report by Therriault & Winters (1970) has indicated a more comprehensive separation of the incubation products by running in a second dimension with *n*-butanol:isopropanol:water (52:25:25).

Results were expressed in terms of μmol cAMP hydrolysed (phosphodiesterase) or produced (adenyl cyclase)/mg islet protein/10 min.

The effect of incubation time, pH and protein concentration on adenyl cyclase and cAMP phosphodiesterase activity was investigated before making a comparison of the activity of the two enzymes in normal and obese mice and an investigation of the effects of certain agents on adenyl cyclase activity. These agents were added to the tris buffer of the incubation medium at the concentrations shown in Table 2.

RESULTS

Determination of optimal conditions for islet cyclase and cAMP phosphodiesterase assays

Incubation time

Non-specific ATP breakdown in the islet homogenate was measured in the adenyl cyclase assay in order to determine the optimal incubation time and effectiveness of the ATP regenerating system (Fig. 1). Cyclase incubations were carried out for 0–20 min and at 5-min intervals the amounts of ADP, AMP, cAMP, IMP, adenosine and inosine produced were measured. In both obese mice and their normal lean litter mates, cAMP production increased almost linearly with time, while the increases in

adenosine, inosine and AMP were more gradual. High ADP levels resulting from high initial ATPase activity were reduced by the ATP regenerating system. Because of these results, 10 min was chosen as the optimal incubation time for subsequent adenyl cyclase assays.

The optimum incubation time (10 min) for phosphodiesterase was determined in a similar way, by measuring the breakdown of cAMP (Fig. 2). Total cAMP phosphodiesterase activity was taken as the sum of the activity of AMP, IMP, adenosine and inosine, and the hydrolysis of cAMP was found to increase with time over the range used (Fig. 3).

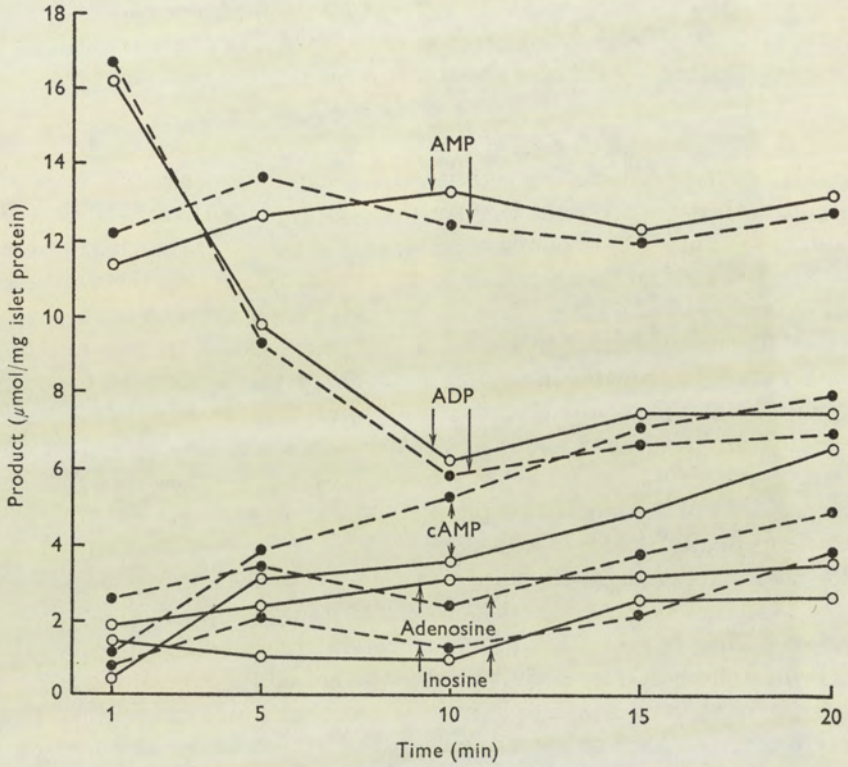

Fig. 1. Time-course of hydrolysis of ATP by adenyl cyclase at 37 °C and pH 7·0. ●– – –●, Obese mouse islet protein; ○——○, normal mouse islet protein.

Variation of pH

Adenyl cyclase and cAMP phosphodiesterase assays using islet protein of only obese mice were carried out with tris–HCl buffers giving pH values of 6, 7, 8, 9 at 37 °C (Fig. 4). Incubations were carried out for 10 min and a blank without protein was used for each pH. Adenyl cyclase had a pH optimum of 7·0 and cAMP phosphodiesterase one of 8·0.

Islet protein concentration

At the optimal incubation time and pH both enzymes were found to be dependent on islet protein concentration (Fig. 5*a*, *b*). The tissue:volume ratio used (1:9) in homogenization gave an adequate working volume from which initial protein concentrations could be prepared. Over the range of protein concentrations used adenyl cyclase activity increased with concentration. However, at any one concentration the activity of the protein from obese animals was greater than that of their lean litter mates. Phosphodiesterase activity showed a similar increase with islet protein from obese mice.

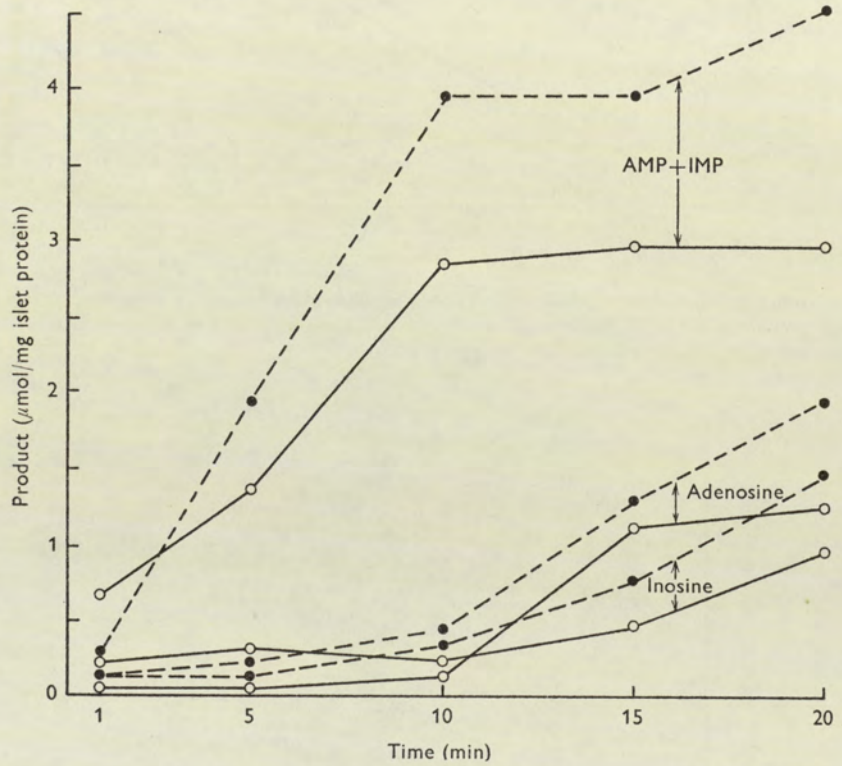

Fig. 2. Time-course of hydrolysis of cAMP by cAMP phosphodiesterase at 37 °C and pH 8·0. ●---●, Obese mouse islet protein; ○——○ normal mouse islet protein.

Comparison of islet adenyl cyclase and cAMP phosphodiesterase activity from normal and obese mice

Using the enzyme optima previously determined, a comparison was made of cyclase and phosphodiesterase activity in the islets of normal and obese mice. The effect of sodium fluoride (NaF) on adenyl cyclase activity, and the effect of theophylline in the presence of cAMP on cAMP phosphodiesterase activity (Table 1) were also determined using protein from only obese animals.

Both adenyl cyclase and phosphodiesterase activity were found to be significantly

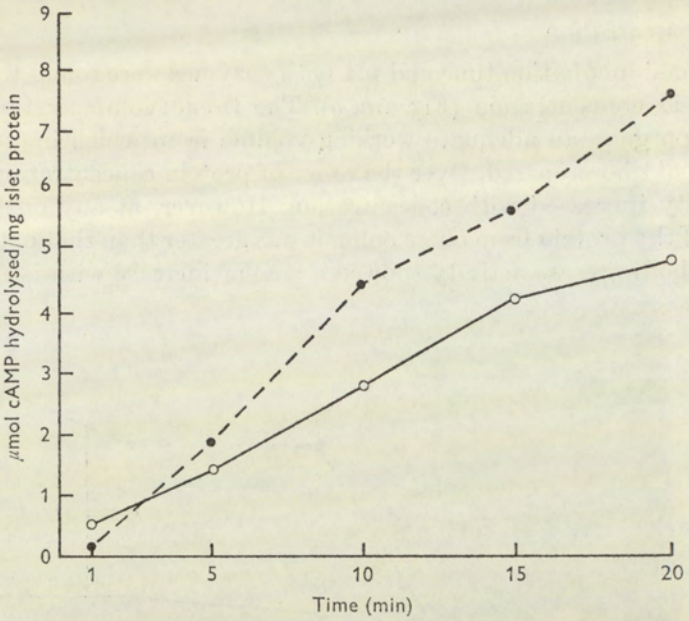

Fig. 3. Time course of cAMP hydrolysis (cAMP phosphodiesterase activity expressed as the sum of the activities of AMP, IMP, adenosine and inosine at 37 °C and pH 8·0). ●---●, Obese mouse islet protein; ○——○, normal mouse islet protein.

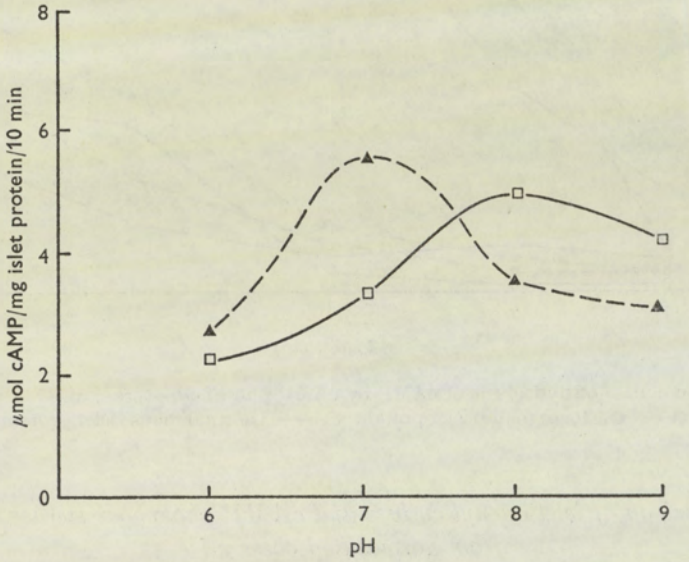

Fig. 4. The effect of pH on adenyl cyclase and cAMP phosphodiesterase activity in islet tissue from obese mice at 37 °C. Each point represents the mean of three determinations. ▲---▲, adenyl cyclase activity (cAMP production); □——□, cAMP phosphodiesterase activity (cAMP hydrolysis).

higher in islets from obese than in islets from normal mice. NaF (10 mmol/l) increased cyclase in the former by 33·4% while 10 mM-theophylline in the presence of 1 mM-cAMP reduced phosphodiesterase activity by almost 100%. Although adenyl cyclase and phosphodiesterase activities were higher in islets from obese mice, the ratio of cyclase to phosphodiesterase in islets from both normal and obese animals was found to be about the same, i.e. 1:1.

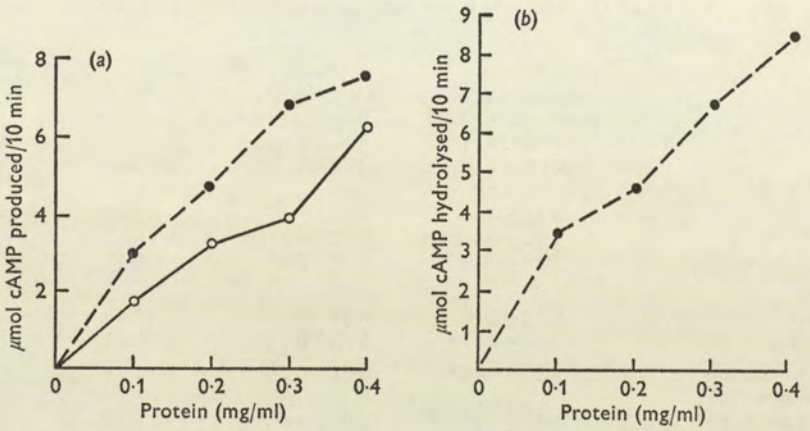

Fig. 5. (*a*) The effect of protein concentration on adenyl cyclase activity at pH 7 and 37 °C. (*b*) The effect of protein concentration on cAMP phosphodiesterase activity at pH 8 and 37 °C. Each point is the mean of two determinations. Blank assays contained no protein. ●---●, Obese mouse islet protein; ○——○, normal mouse islet protein.

Table 1. *Comparison of adenyl cyclase and phosphodiesterase activity in islet tissue from normal and obese mice, and the effect of sodium fluoride on adenyl cyclase, and theophylline+cAMP on phosphodiesterase of islets from obese mice* (*means* ± S.E.M.; *one determination per mouse from homogenized islets*)

Islet homogenate	No. of mice	Adenyl cyclase activity (μmol cAMP produced/ mg islet protein/ 10 min; pH 7, 37° C)	No. of mice	cAMP phosphodiesterase activity (μmol cAMP hydrolysed/ mg islet protein/ 10 min; pH 8, 37° C)	Ratio of phospho-diesterase: adenyl cyclase	% deviation from usual values
Normal	7	3·07 ± 0·18	6	2·99 ± 0·25	1:1·03	—
Obese	7	4·78 ± 0·26***	6	4·37 ± 0·22**	1:1·10	—
Obese + 10 mM-NaF	3	5·87 + 0·22†	—	—	—	+33·4%
Obese + 20 mM-NaF	3	4·71 ± 0·47	—	—	—	—
Obese + 10 mM theophylline + 1 mM-cAMP	—	—	6	0·27 ± 0·13†††	—	−93·0%

Significance of difference between values from normal and obese animals: $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.
Significance of difference between values from treated-obese and naturally obese animals: † $P < 0{\cdot}02$, ††† $P < 0{\cdot}001$.

The effects of glucose and drugs on adenyl cyclase activity

Glucose (1–4 *g/l*) (*Table* 2)

Glucose reduced adenyl cyclase activity at all three concentrations used. The greatest percentage reduction in both normal and obese mice occurred at 200 mg/100 ml.

Adrenaline (10^{-7}–10^{-5} *mol/l*) (*Table* 2)

Adrenaline increased adenyl cyclase activity in islets from both normal and obese animals. The greatest percentage stimulation occurred at 10^{-5} mol/l in both cases.

Table 2. *Effect of metabolic and pharmacological agents on islet adenyl cyclase activity in normal and obese mice* (*means* ± S.E.M.; *one determination per mouse from homogenized islets*)

	No. of mice	Normal (μmol cAMP produced/mg islet protein/10 min; pH 7, 37 °C)	Obese (μmol cAMP produced/mg islet protein/10 min; pH 7, 37 °C)	% deviation from usual values	
				Normal	Obese
Glucose:					
1 g/l	3	2·81 ± 0·40	4·26 ± 0·40	−8·37	−11·22
2 g/l	3	1·97 ± 0·08**	3·42 ± 0·01**	−35·82	−28·78
4 g/l	3	2·79 ± 0·49	4·22 ± 0·46	−9·11	−12·17
Adrenaline:					
10^{-7} mol/l	3	3·94 ± 0·08*	5·82 ± 0·43	+28·40	+21·29
10^{-5} mol/l	3	4·07 ± 0·27*	5·88 ± 0·10*	+32·43	+22·48
10^{-4} M-Phentolamine + 10^{-5} M-adrenaline	3	3·91 ± 0·13*	5·12 ± 0·25	+27·24	+6·57
10^{-2} M-Phentolamine + 10^{-5} M-adrenaline	3	5·61 ± 0·07***	6·07 ± 0·42*	+82·51	+26·51
10^{-4} M-Propranolol + 10^{-5} M-adrenaline	3	2·75 ± 0·43	4·51 ± 0·27	−10·59	−6·00
10^{-2} M-Propranolol + 10^{-5} M-adrenaline	3	2·06 ± 0·01**	3·59 ± 0·31*	−32·85	−15·25
10^{-6} M-Isoprenaline + 10^{-2} M-phentolamine	3	3·65 ± 0·49	4·88 ± 0·03	+18·70	+1·59
10^{-4} M-Isoprenaline + 10^{-2} M-phentolamine	3	4·56 ± 0·11***	6·13 ± 0·43*	+48·38	+29·78
10^{-6} M-Phenylephrine + 10^{-2} M-propranolol	3	2·88 ± 0·22	3·94 ± 0·18	−6·14	−17·87
10^{-4} M-Phenylephrine + 10^{-2} M-propranolol	3	2·05 ± 0·21*	3·46 ± 0·34*	−33·23	−27·83

Significance of difference compared with the normal values in Table 1: * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

Phentolamine (10^{-4} *or* 10^{-2} *mol/l*) *in the presence of adrenaline* (10^{-5} *mol/l*)

Phentolamine (an α-adrenergic receptor blocker) in the presence of adrenaline increased cyclase activity in islets of both normal and obese mice. The greatest percentage stimulation was shown at 10^{-2} M-phentolamine and was about three times greater in the islets from normal animals. The stimulatory effect of phentolamine in the presence of adrenaline exceeded the effect of adrenaline alone in both types of islets.

Propranolol (10^{-4} *or* 10^{-2} *mol/l*) *in the presence of adrenaline* (10^{-5} *mol/l*)

Propranolol in the presence of adrenaline reduced islet cyclase activity in both types of islets. The greater reduction occurred at the higher concentration.

Isoprenaline (10^{-6} *or* 10^{-4} *mol/l*) *in the presence of phentolamine* (10^{-2} *mol/l*)

Isoprenaline in the presence of phentolamine increased adenyl cyclase activity in islets from both normal and obese mice. The greater percentage stimulation occurred at a concentration of 10^{-4} M-isoprenaline. The stimulation was twice as great in islets from normal animals and exceeded the effect of adrenaline alone in both types of islets.

Phenylephrine (10^{-4} *or* 10^{-6} *mol/l*) *in the presence of propranolol* (10^{-2} *mol/l*)

This combination reduced adenyl cyclase activity in both types of islets. The greater percentage reduction occurred at 10^{-4} mol/l in islets of both normal and obese animals.

The results suggest that stimulation of the α-adrenergic receptor results in a reduction of adenyl cyclase activity, while β-receptor stimulation causes an increase.

DISCUSSION

These results demonstrate the presence of adenyl cyclase and cAMP phosphodiesterase activity in the isolated islets of both normal and obese hyperglycaemic mice operating at a pH similar to that found in other tissues (Bär & Hechter, 1969; Dorrington & Baggett, 1969). Under the conditions used, enzyme activity would appear to be proportional to both incubation time and protein concentration. Adenyl cyclase and phosphodiesterase activity was found to be higher in islets from obese mice but the ratio of cyclase to cAMP phosphodiesterase in both obese hyperglycaemic mice and their normal lean litter mates was about the same, i.e. 1:1.

The metabolic balance of this enzyme system in the islet cells remains to be clarified, especially since many metabolites produced naturally as a result of ATP metabolism can inhibit phosphodiesterase activity.

Dousa & Rychlik (1970) have found that ADP, ATP and pyrophosphate inhibit phosphodiesterase and contribute to the accumulation of cAMP. They believe that fluoride, in addition to stimulating adenyl cyclase directly, without affecting the apparent K_m (Bär & Hechter, 1969), maintains the level of substrate for adenyl cyclase by blocking the breakdown of both ATP and ADP. In our experiments, 10 mM-NaF brought about a 33·4% increase in adenyl cyclase activity of islets from obese mice. Sodium fluoride is a very acceptable alternative to the enzymatic ATP regenerating system used in the cyclase assay.

The investigation of the effects of other agents on islet adenyl cyclase activity produced some perplexing results. Glucose, the physiological stimulus for insulin biosynthesis and release, reduced adenyl cyclase activity. Montague & Cook (1970) have also shown that increasing levels of glucose have no effect on the intracellular levels of cAMP in incubated rat islets. Clearly then there is no effect by glucose on insulin secretion mediated by adenyl cyclase in the β cell. However, the role of adenyl cyclase, and the cAMP it produces, may be to influence or even regulate the way in which glucose is metabolized in the β cell.

Adrenaline, as in other tissues, increased adenyl cyclase activity (Marinetti, Ray & Tomasi, 1969; Triner, Vulliemoz, Verosky & Nahas, 1970). It may also increase the

capacity of the islet cells to use cAMP and this might account for the low cAMP concentration found by Turtle & Kipnis (1967) in rat islets after adrenaline treatment. An evaluation of the effects of α- and β-adrenergic blocking agents and drug combinations designed specifically to stimulate discrete α- and β-adrenergic receptors showed the increase of cyclase activity to be a β-receptor function and its reduction an α-receptor function.

Normal islet cyclase showed the greater response to adrenergic receptor stimulation. The unresponsiveness of cyclase in islets from obese mice may be a character of the obese hyperglycaemic syndrome.

Many observations have led to the hypothesis that an adenyl cyclase system in the β cell may be involved in insulin secretion. Various agents (glucagon, theophylline and adrenergic stimulants) that are thought to act through or stimulate adenyl cyclase directly in liver and fat, have been found to influence islet cAMP and to stimulate insulin secretion *in vivo* and *in vitro* (Karam, Grasso, Wegienka, Grodsky & Forsham, 1966; Porte, 1967*a*; Ketterer, Eisentraut & Unger, 1967; Turtle, Littleton & Kipnis, 1967; Malaisse *et al.* 1967). Theophylline increases the cAMP level in pancreatic islets by reducing phosphodiesterase which degrades cAMP to inactive adenosine 5′-monophosphate. In the present study, 10 mM-theophylline in the presence of 1 mM-cAMP produced almost 100% inhibition of cAMP phosphodiesterase activity.

Many quantitative investigations have shown that when the glucose concentration is altered *in vitro* there is a parallel change in insulin release from pancreatic islets. Glucose does not require the addition of a hormone or cAMP in order to stimulate insulin release, and current investigations centre on the hypothesis that some direct or indirect product of glucose metabolism provides the actual stimulus. The role of cAMP in β cell metabolism has yet to be established. It is conceivable that cAMP acts either by increasing the rate at which glucose is converted into an active metabolite or else by promoting the action of the active metabolite without actually being required for the process to occur. Glucose, however, may act on the A_2 cells of the islet producing glucagon which in turn stimulates insulin release via an adenyl cyclase system in the β cell. Glucagon has been shown by Lambert, Jeanrenaud & Renold (1967) to increase insulin secretion in the absence of added glucose and this has suggested that cAMP generated in the β cells in response to glucagon may play an essential role in the insulin releasing mechanism. Our own unpublished results indicate that glucagon stimulates islet adenyl cyclase activity.

Malaisse *et al.* (1967) have suggested that cAMP could activate glycogen phosphorylase in β cells and so provide an intracellular source of glucose from stored glycogen. The presence of glycogen in both normal and obese islets has been demonstrated by Hellman & Idahl (1969) and this implies that insulin release may be associated in some way with glycogen breakdown.

Whether a single adenyl cyclase molecule is endowed with many types of receptors or whether there is a whole group of hormone–effector adenyl cyclase systems remains to be clarified. Tomasi, Koretz, Ray, Dunnick & Marinetti (1970), using plasma membrane from rat liver, have shown that hormone receptor proteins are separate from adenyl cyclase. Separate adenyl cyclase systems have been demonstrated for glucagon and adrenaline in rat liver (Bitensky, Russell & Robertson, 1968; Becker &

Bitensky, 1969; Marinetti *et al.* 1969) and for thyroid hormones and noradrenaline in cat myocardium (Levey & Epstein, 1969).

As previously mentioned, Cerasi & Luft (1970) have indicated that in genetically potential diabetes the cAMP-forming mechanism may be defective. This state then may be the result of reduced adenyl cyclase activity or the result of excessive phosphodiesterase activity. No very high phosphodiesterase activity has been found in the obese hyperglycaemic mouse and although pharmacologically less responsive, the obese cyclase system at 20–25 weeks appears normal. However, even if the cyclase and phosphodiesterase systems are normal, amyloid deposits shown by Lacy (1967) in maturity-onset diabetic islet tissue may inhibit insulin secretion or the activation of islet adenyl cyclase.

In this study, adenyl cyclase and phosphodiesterase activity has been measured in the presence of high concentrations of insulin. However, insulin has been shown to reduce adenyl cyclase activity in plasma membranes of rat liver (Ray, Tomasi & Marinetti, 1970). It must be appreciated though that the action of exogenous insulin may be quite different from the role of endogeneous insulin in the β cell. Also, it may be that the level of adenyl cyclase activity in whole islets was found to be so high because the capacity of glucagon to stimulate adenyl cyclase in the homogenate exceeds that of insulin in the homogenate to reduce it.

Islets of Langerhans of mice have been shown to be highly complex and heterogenous structures containing some eight morphologically different cell types (de Hoyos–Guevara, 1969). Even though Gepts, Christophe & Mayer (1960) have shown that islets of obese mice contain 90 % β cells, cytochemical verification or the microchemical assay of islet cell types is required to locate the distribution of cAMP and adenyl cyclase in islet tissue. In future work the role of adenyl cyclase and cAMP in islet metabolism and the part these two substances play in the process of insulin secretion will be investigated.

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