GLUCOSE STIMULUS INSULIN SECRETION COUPLING IN THE PANCREATIC ISLETS OF LEAN AND OBESE MICE

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The mechanism by which the B-cell recognises glucose as a stimulus for the release of insulin has been studied using pancreatic islets isolated by collagenase digestion from lean and obese hyperglycaemic mice.

2-desoxy-D-altromethylose (digitoxose) specifically and competitively inhibited glucose stimulated insulin secretion from lean and obese mouse islets without affecting the rates of glucose oxidation or oxygen consumption. Obese mouse islets were marginally more resistant to the inhibitory effect of digitoxose than lean mouse islets. Digitoxose thus provided a means for the dissociation of metabolic and secretory roles of glucose.

A novel perifusion system involving vibrating reed electrometer and ionization chamber was designed for the simultaneous measurement of the rates of glucose oxidation and insulin release by lean and obese mouse islets. Perifused lean mouse islets responded rapidly to increasing glucose concentrations in terms of insulin release and rates of glucose oxidation however, insulin release was sluggish from obese mouse islets whilst glucose oxidation was unimpaired. This reduced secretory response to glucose in obese mouse islets suggests a glucoreceptor abnormality. Digitoxose was found to have no effect on glucose oxidation but simultaneously inhibited insulin release. Mannoheptulose on the other hand inhibited both glucose oxidation and insulin release. These observations confirm that glucose metabolism per se is not a necessary prerequisite for the initiation of release but is essential for sustaining the secretory process.

The interaction between Ca^{2+}-calmodulin and cAMP in the process of insulin release was studied using lean mouse islets. When cAMP levels were raised by forskolin or theophylline, the calmodulin inhibitors, trifluoperazine and MB17108 were found to have no significant effect on glucose stimulated insulin release. This suggested that high intracellular cAMP levels were capable of stimulating insulin release independently of any involvement by Ca^{2+}-calmodulin.

Key Words: Islets of Langerhans, insulin release, glucose metabolism, digitoxose, glucoreceptor.

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CHAPTER 1

GLUCOSE STIMULUS INSULIN SECRETION COUPLING IN THE PANCREATIC ISLETS OF LANGERHANS

The secretory response of the B-cell to an increased extracellular glucose concentration is mediated by a coordinated set of intracellular events. This sequence of intricate events may be viewed as the process of stimulus-secretion coupling. When the extracellular glucose concentration is increased, the B-cell undergoes a number of changes: depolarization of the membrane, which creates electrical activity, ionic fluxes Na^+, K^+, Mg^{2+}, phospholipid turnover, the production of cAMP, increased intracellular Ca^{2+}, the phosphorylation and dephosphorylation of proteins, contractile activity of the cell web, fusion of the insulin granules with the plasma membrane and insulin release. This complex set of events constitutes glucose stimulus insulin secretion coupling, its regulation and integration is discussed in more detail below.

The Structure and the Function of B-cell

The B-cells of pancreatic islets of Langerhans were first described by Lane in 1907 (1). Originally the B-cell was characterized by its content of cytoplasmic granules which were soluble in alcohol and preserved in tissues fixed in chrome sublimate bestowing the cell with specific staining characteristics (2). Today

however, the B-cell is defined not only by its histological or cytochemical staining characteristics but also by the ability to express a complicated set of genes which provide the B-cell with a unique mechanism for the synthesis and release of insulin in exact concert with peripheral demand. Recently the B-cell has been shown to express a certain set of antigens on the plasma membrane which are specific for the B-cell and which may initiate autoimmune reaction (3).

The pancreatic islets of Langerhans are scattered throughout the acinar pancreas and constitute only about 1% of the total pancreatic parenchyma (4). Islets vary considerably in their form and size, the diameter of normal mouse islets ranges from 50 to 300 microns. The average dry weight of a medium sized mouse islet with a diameter of 150 microns is 0.67µg and there are approximately 2200 cells per islet. Rat islets are somewhat larger than the mouse islets and are estimated to contain some 5700 cells each (5,6). The proportion of B-cells in islets varies from species to species, in normal and genetically obese mouse (ob/ob) islets the B-cells comprise approximately 80 and 90% respectively of the total cell population (4). The rest of the cells are glucagon producing A cells, somatostatin secreting D cells and PP cells which contain pancreatic polypeptide 97). In other species, eg. rabbit and gunea pig B-cells may account for only 60-70% of the entire cell population (8).

The islets are richly supplied with blood vessels. The vascularization consists of anastomising capillaries that make the islet well suited for the rapid delivery of hormonal secretion into the bloodstream and also facilitates rapid sensing and uptake of nutrients

from the blood into islets. There are two basement membranes separating the B-cells from adjacent capillaries and the secreted insulin must traverse both before entering the bloodsteam. Hyalination and thickening of the two basement membranes has been reported in diabetic patients (9), thus creating a transport barrier for insulin secretion into blood. Whether these membranes play a regulatory role in the normal release process remains speculative.

Figure 1 shows a possible scheme for signal input to the B-cell. The synthesis, storage and the release of insulin is regulated by a complex set of incoming signals which influence and coordinate the B-cells to release insulin in precise relation to the needs of the body. Within this system several different types of signals can be envisaged. These include nutrient factors and hormones arriving via the blood, together with neural signals autonomic in nature (adrenergic and cholinergic) transmitted via afferent axons (10). In addition paracrine influences from the neighbouring A, D and PP cells seem to play a modulating role (10). The physical association between B-cells themselves may also influence the control of insulin release by electrical coupling via intracellular gap junctions (11).

Features of Glucose Stimulated Insulin Release

The relationship between extracellular glucose concentration and the rate of insulin release from mouse islets is sigmoidal (12), Figure 2. Glucose concentrations below 3 mmol/l do not influence the

Figure 1. A possible scheme for the signal input into the B-cell (from Ref 10)

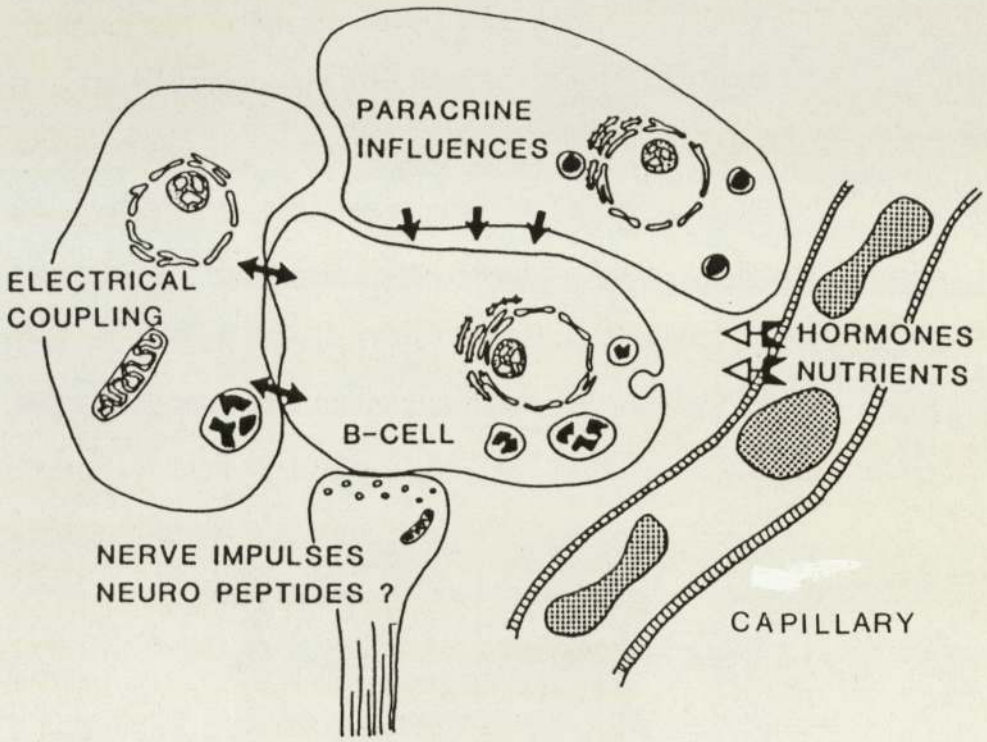
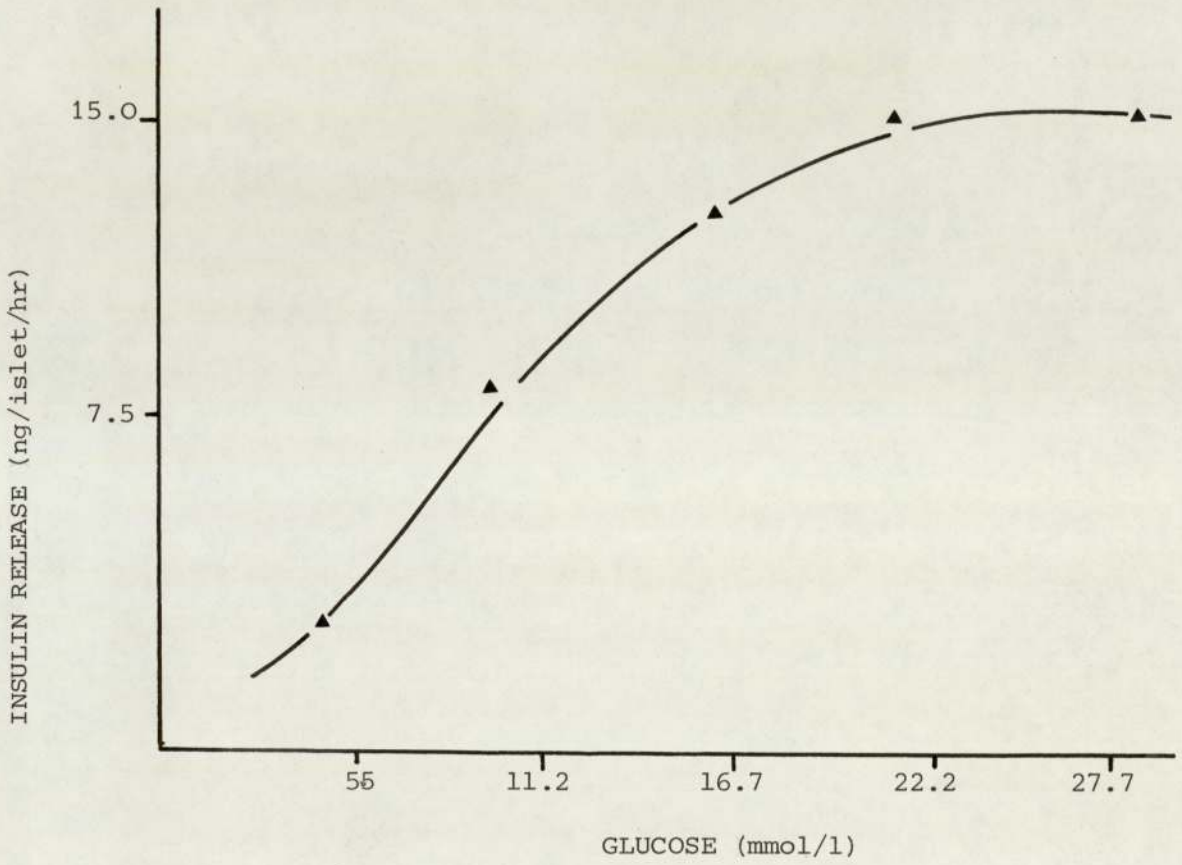


Figure 2. The sigmodal relationship between the extracellular glucose concentration and insulin release from isolated mouse islets (from Ref 90)



insulin secretory rate, the threshold concentration is around 4 mmol/l and the largest increase in insulin secretion occurs between 4 to 17 mmol/l, indicating a very sensitive response to small incremental changes in glucose concentration within the physiological range. At still higher concentrations greater than 16.7 mmol/l the curve tends to reach a plateau. Half maximum rates of insulin secretion are obtained in response to 6 and 8 mmol/l of glucose for the rat and mouse islets respectively (12).

D-glucose is the major physiological stimulus for insulin release and sugars like fructose, N-acetyglucosamine and sorbitol are potentiators or secondary stimuli since they are ineffective alone but increase the insulin secretory response in the presence of glucose.

The response of the B-cell to a square wave increase in glucose concentration is characteristically biphasic and characterized by a short spike of insulin release (within five minutes), followed by a nadir and a slowly rising second phase. Wollheim and Sharp (56) have made certain observations about the possibile mechanisms underlying the biphasic response. Firstly such a response may be the result of two compartments of insulin-containing granules, one of which is more readily released than the other. Secondly it may be that two distinct classes of B-cells exist, distinguished by their differential sensitivity to glucose. In addition the initial burst of insulin may cause a negative feedback, thus generating a nadir between the two phases of insulin release. The latter postulate is very unlikely since

the monophasic release in response to certain other secretagogues may be as pronounced as that evoked by glucose. An example is the monophasic second phase insulin release evoked by 3-isobutylmethyxanthine (IBMX) in the presence of low glucose (13). Another postulate is that since glucose stimulates the release of both insulin and somatostatin from the islets, a partial inhibition of insulin release by somatostatin might occur. Because insulin and somatostatin release can be dissociated this alternative also seems unlikely. An attractive explanation of the biphasic phenomenon is the close correlation between the glucose induced bursts in B-cell membrane potential (spike potential) and the biphasic profile of insulin release. It has therefore been suggested that the activation and the inactivation of the ionic permeability of the B-cell plasma membrane, particularly to Ca^{2+} could explain the biphasic pattern of insulin release. The striking parallelism between glucose stimulated insulin release and the stimulation of $^{45}Ca^{2+}$ efflux from islets indicates a biphasic change in the concentration of ionized Ca^{2+} in some critical cytosolic pool. Therefore the relationship between elevated cytosolic Ca^{2+} and the stimulation of insulin secretion is of central importance in the understanding of the mechanism of insulin secretion.

The Relationship Between Ca^{2+} and Phospholipid Turnover

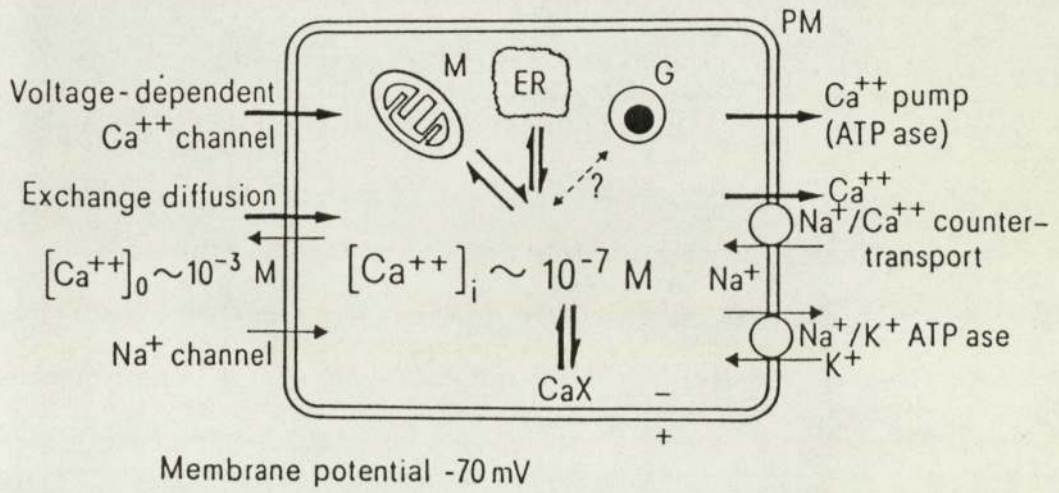
The presence of extracellular Ca^{2+} for the stimulation of insulin release is essential and well established (14,15). Milner and Hales

(16) demonstrated a bell shaped activation curve with a maximum of Ca^{2+} concentration of 2.5 mmol/l for glucose induced insulin release. Both Ba^{2+} and Sr^{2+} can substitute for extracellular Ca^{2+} and elicit normal glucose induced insulin release, whereas 8-12 mmol/l Mg^{2+} has an antagonizing effect on the normal stimulatory effect of Ca^{2+} (17-19). Figure 3 illustrates a current model for Ca^{2+} handling by the B-cell (18).

In a normal situation Ca^{2+} enters the B-cell by diffusion along an inwardly directed electrochemical gradient. The maintenance of this ionic gradient depends on the ability of Ca^{2+} extruding mechanisms at the level of the plasma membrane and Ca^{2+} sequestration by intracellular organelles. At the level of plasma membrane two processes are thought to be involved, a calcium pump and sodium pump. The Ca^{2+} pump derives its energy from ATP and Na^{+}/Ca^{2+} countertransport and is driven by an inward Na^{+} gradient. In turn this gradient is maintained by the activity of a Na^{+} pump (Na^{+}/K^{+} ATPase). The presence of both of these pumps has been demonstrated in rat islets (20,21).

More than 99% of the total cytosolic Ca^{2+} is bound to cytoplasmic proteins in the mitochondria, endoplasmic reticulum and secretory granules (1 ß). The stimulation of insulin release raises intracellular cytosolic Ca^{2+} ($[Ca^{2+}]i$) by several different means. An increased permeability of the B-cell plasma membrane to Ca^{2+} by the voltage-dependent or receptor activated Ca^{2+} channels is associated with the passive entry of Ca^{2+} into the B-cell along its

Figure 3: Ca^{2+} handling by the B-cell. The abbreviations are: M, mitochondria; ER, endoplasmic reticulum; G, insulin-containing secretory granules; PM, plasma membrane; CaX, calcium buffering by cytoplasmic components including calmodulin and small molecules. In addition, calcium is also bound to membrane phospholipids (from 18)



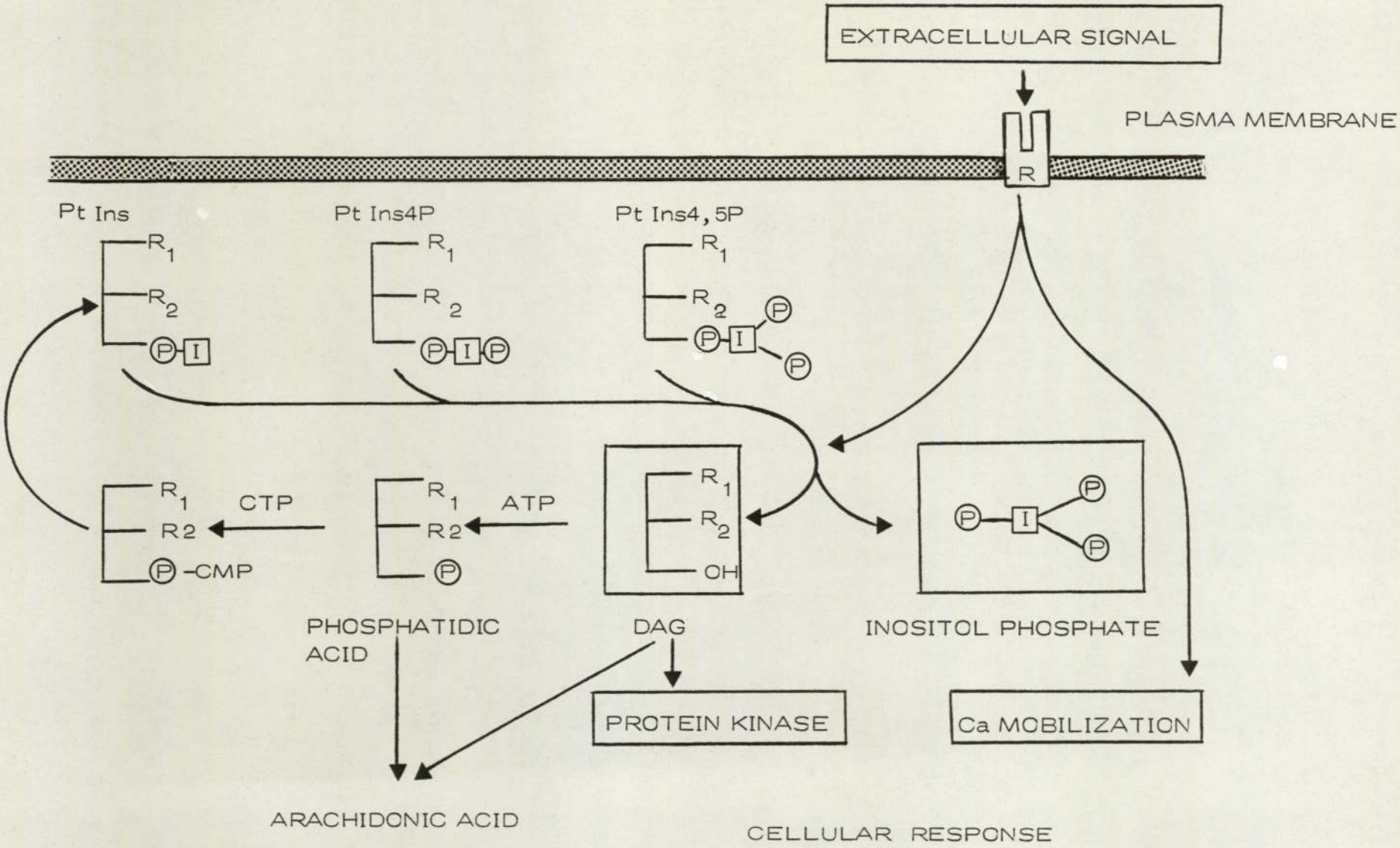
electrochemical gradient. The inhibition of Ca^{2+} efflux from the B-cell across the plasma membrane, the mobilization of Ca^{2+} from intracellular stores and the inhibition of Ca^{2+} sequestration by these stores will all lead to raised B-cell $[Ca^{2+}]i$.

Although the exact effect of glucose on Ca^{2+} handling by B-cell is unknown, it is thought that glucose enhances Ca^{2+} influx (22), and inhibits Ca^{2+} efflux (23) thereby increasing $[Ca^{2+}]i$ levels. Studies using ionophores have also demonstrated an increased B-cell $[Ca^{2+}]i$ as a critical factor for glucose stimulated insulin release. The liposoluble ionophone A23187, which complexes divalent cations and facilitates Ca^{2+} influx into cells by acting as a carrier in the plasma membrane has been shown to stimulate insulin release at non-stimulatory Ca^{2+} concentrations (24,25). using the Quin-2 technique for measuring $[Ca^{2+}]i$ it has been shown that glucose mobilizes both intra- and extracellular Ca^{2+} to raise B-cell cytosolic Ca^{2+} levels (26). However, the exact mechanisms of regulation of Ca^{2+} handling by the various organelles is not clearly understood. Subcellular fractionation studies have been hampered by the extremely small quantity of pancreatic islet tissue available from rodents. Until recently the link between signal reception and the organelles involved in the regulation of intracellular Ca^{2+} was unknown. However, the new finding that some neutrotransmitters and hormones induce a rapid and Ca^{2+}-dependent breakdown of the polyphosphoinositide leading to the accumulation of inositol 1,4,5 triphosphate (IP3) has provided a possible hint as to the mechanism

involved (Figure 4). It has been shown that the addition of IP3 to RIN m5F cells (a radition induced insulinoma cell line) released Ca^{2+} from a non- mitochondrial pool (27). The converging evidence from several studies strongly suggests that IP3 is the second messenger for Ca^{2+} mobilizing agonists (28). The identification of the target organelle for IP3 has been achieved using rat insulinoma (26,29). When IP3 was added to microsomal fraction of the insulinoma a rapid release of Ca^{2+} occurred suggesting that IP3 mobilizes Ca^{2+} exclusively from endoplasmic reticulum.

Enhanced ^{32}P-labelling of phospholipids was first demonstrated by Fex and Lernmark (30). These workers found that islet fractions containing phosphatidylinositol and phosphatidylserine were predominantly labelled when islets were exposed to high glucose concentration. Freinkel and colleagues reported that glucose stimulated ^{32}P-labelling of phosphatidylinositol, phosphatidic acid and phosphatidlethanolamine in rat islets (31). In such studies it was assumed that the measurement of enhanced labelling of these phospholipids with ^{32}P reflected the turnover of the polar head group, or more specifically their resynthesis following breakdown (Figure 4). An alternative approach has been adopted by Clements and Rhoten (32). These workers pre-labelled islet phospholipids with ^{3}H-inositol and observed the subsequent fall in labelled lipids upon exposure to glucose. This effect was accompanied by an increased production of watersoluble derivatives of ^{3}H-inositol and suggested that glucose provoked a breakdown of inositol phospholipids in islets.

Figure 4. Inositol phosphate turnover and signal transduction. Abbrev: Pt ins, phosphatidyl inositol; DAG, diacylglycerol; R, receptor. (Ref 34)



Several insulin secretagogues including glucose, mannose, leucine, α-ketoisocaproate, acetylcholine and carbamylcholine have been shown to enhance the turnover of inositol phospholipids (33). It has already been mentioned above that one of the consequences of enhanced phospholipid turnover is the mobilization of intracellular Ca^{2+} by IP3. However, there is yet another physiologically important breakdown product of phospholipids, namely diacylglycerol (DAG). It has been suggested that the formation of this compound upon cell activation may play a key role in a number of subsequent cellular reactions (33). For example increased concentrations of DAG in a cell membrane could have profound effects on membrane fluidity and fusion and it is interesting to note that glucose increased membrane fluidity in islets (23). Another responsive target for DAG has recently been identified as a Ca^{2+}-phospholipid dependent protein kinase (c-kinase) (34-36). The activation of this enzyme by DAG suggests yet another mechanism by which inositol breakdown may regulate B-cell function. Recently Lord and Ashcroft (35) have isolated and characterised the physical and kinetic properties of the enzyme from rat pancreatic islets and cloned hamster B-cells (HIT-T15 cells). The enzyme was activated by unsaturated DAG as well as by the tumour promoting agent 12-O-Tetrodeconyl-phorbol-13-acetate (TPA). This substance is also a potent insulinotrophic agent, suggesting a possible connection between the activation of c-kinase and and insulin release. These workers also found several peptides whose phosphorylation was

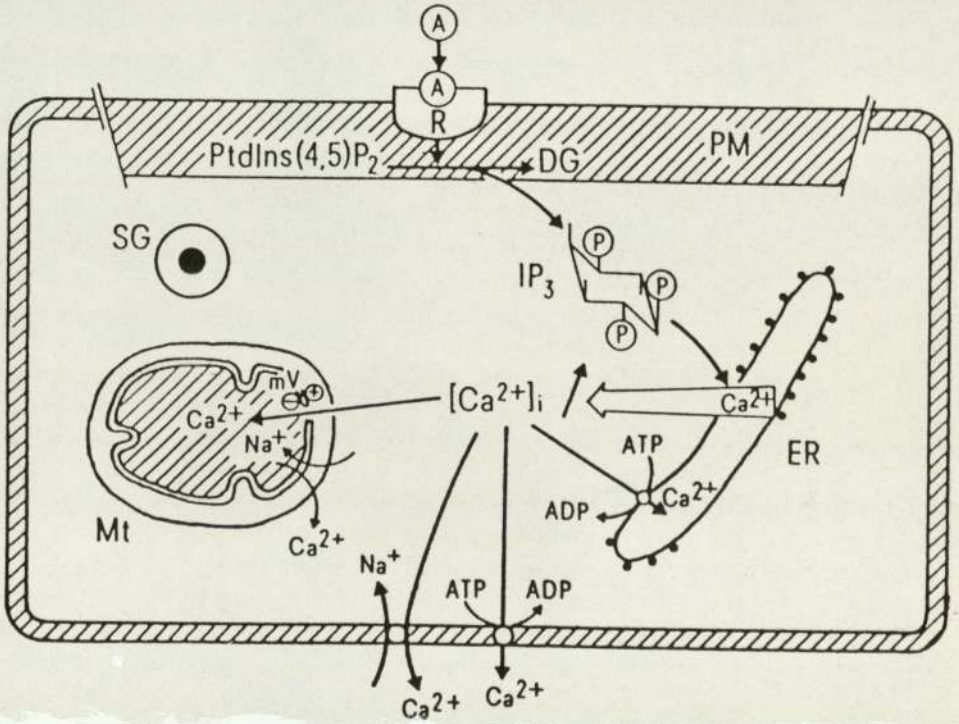
enhanced by c-kinase. The Mr's of these peptides were found to be 38, 35, 20, 15 and 11 KDalton.

Figure 5 summarizes the possible role of IP3 in the regulation of Ca^{2+} homeostasis and subsequent insulin release. Agonist-receptor interaction activates phospholipase C and results in the breakdown of phosphatidylinositol, 4,5-bisphosphate to diacylglycerol and IP3. The latter acts on the endoplasmic reticulum to release Ca^{2+}. The elevated $[Ca^{2+}]i$ triggers insulin release as well as activating mitochondrial Ca^{2+}-sensitive enzymes (pyruvate dehydrogenase) thus stimulating oxidative metabolism to provide energy for sustaining the insulin secretory process.

Ca^{2+} Responsive Target Systems

So far mechanisms by which glucose might increase cytosolic Ca^{2+} have been discussed but it is also pertinent to consider Ca^{2+}-responsive target systems in the B-cell. At the present moment there is little information about the actual components of the insulin secretory machinery of B-cells. But consideration of other secretory systems suggests that the possible components likely to be the direct mediators of Ca^{2+} action are calmodulin and microfilamentous-microtubule system (34,35,36).

Figure 5. Inositol 1,4,5 triphosphate and the regulation of cytosolic Ca^{2+} homeostatis in the B-cell. The abbreviations are: A, agonist, eg. carbamylcholine; R, membrane receptor; Ptd Ins (4,5)P_2 phosphatidylinositol, 4,5-bisphosphate; DG, diacylglycerol; PM, plasma membrane; IP_3 inositol 1,4,5-trisphosphate; ER, endoplasmic recticulum, Mt, mitochondria; SG, secretory granules (from Ref 18)

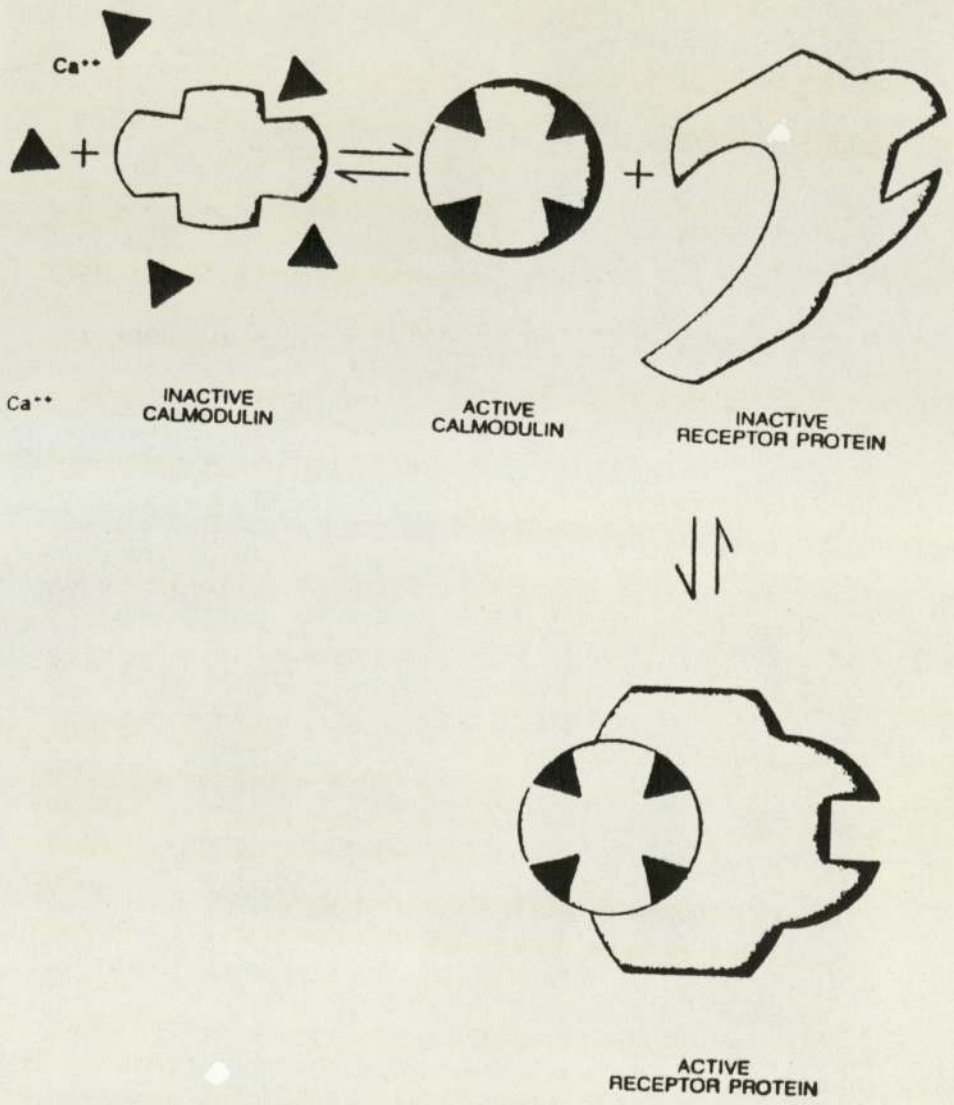


The Role of Calmodulin in Insulin Secretion

The way in which Ca^{2+} might regulate the process of insulin release has become clearer in the last 4-5 years with the finding that calmodulin (a calcium-dependent regulatory protein) has a pivotal role in the secretory process.

In 1979 Sugden, Christie and Ashcroft (39) and another group of workers Valverde, Sener and Malaisse (40) independently reported the presence of calmodulin in rat pancreatic islets. These studies indicate that calmodulin acts on several islet enzymes. There is considerable evidence to suggest that calmodulin might influence cyclic nucleotide synthesis and degradation in a variety of cells including islet cells (39-45). The important features of calmodulin are its ability to bind Ca^{2+} with a dissociation constant (Kd) in the range of 10^{-6} to 10^{-5} mol/l and the significant conformational changes which occur as a result of Ca^{2+}-binding. The conformational changes induced by Ca^{2+}-binding allow the Ca^{2+}-calmodulin complex to bind to the enzymes and activate them. Figure 6 shows the possible mechanism by which calmodulin is activated by Ca^{2+} and resulting Ca-calmodulin complex interacts with inactive receptor protein thus activating it. The physiological significance of the Kd values become apparent when examination is made of the changes in free intracellular Ca^{2+} concentration during insulin release. In the resting state the cytosolic free Ca^{2+} concentration is of the order of 10^{-8} to 10^{-7} mol/l; upon stimulation this rises to 10^{-5} mol/l.

Figure 6. Mechanism by which calmodulin mediates the biological action of calcium ions. Neither calcium alone nor calmodulin alone is active. The binding of four calcium ions to calmodulin activates it. The activated calmodulin then interacts with other proteins (Ref 92)



Harrison, Ashcroft, Christie and Lord (36) have suggested that Ca^{2+} may act as a second messenger via calmodulin by activating specific protein kinases. Using exogenous substrates (such as histones) it has been possible to identify two Ca^{2+}-calmodulin dependent protein kinases from islet tissue - namely myosin light chain kinase (MLCK) (46-48) and phosphorylase kinase (36). MLCK from rat islets (47) and rat insulinoma (48) has been partially purified by ion exchange chromatography and calmodulin affinity chromatography. Micromolar concentrations of Ca^{2+} have been found to stimulate the phosphorylation of MLCK in the presence of calmodulin. Trifluoperazine, a calmodulin inhibitor has been shown to prevent phosphorylation. In smooth muscle and other non muscle cells phosphorylation of myosin is a prerequisite to actomyosin ATPase activation and the subsequent filament contraction. In the B-cell the phosphorylation of MLCK is thought to facilitate the interaction of actin and myosin and resultant ATPase activation provides the motile force for the movement of insulin secretory granules to the periphery of the cell (49).

Harrison and Ashcroft (50) have shown that islets contain a Ca^{2+}-calmodulin dependent protein kinase whose major endogenous substrate is a polypeptide with a mw of 53 Kdaltons (P53). Maximal phosphorylation of P53 was shown to occur in the presence of 2 µm Ca^{2+} and 0.7 µm calmodulin. Incorporation of the label into P53 was inhibited by trifluoperazine but not by cyclic AMP dependent protein kinase inhibitor.

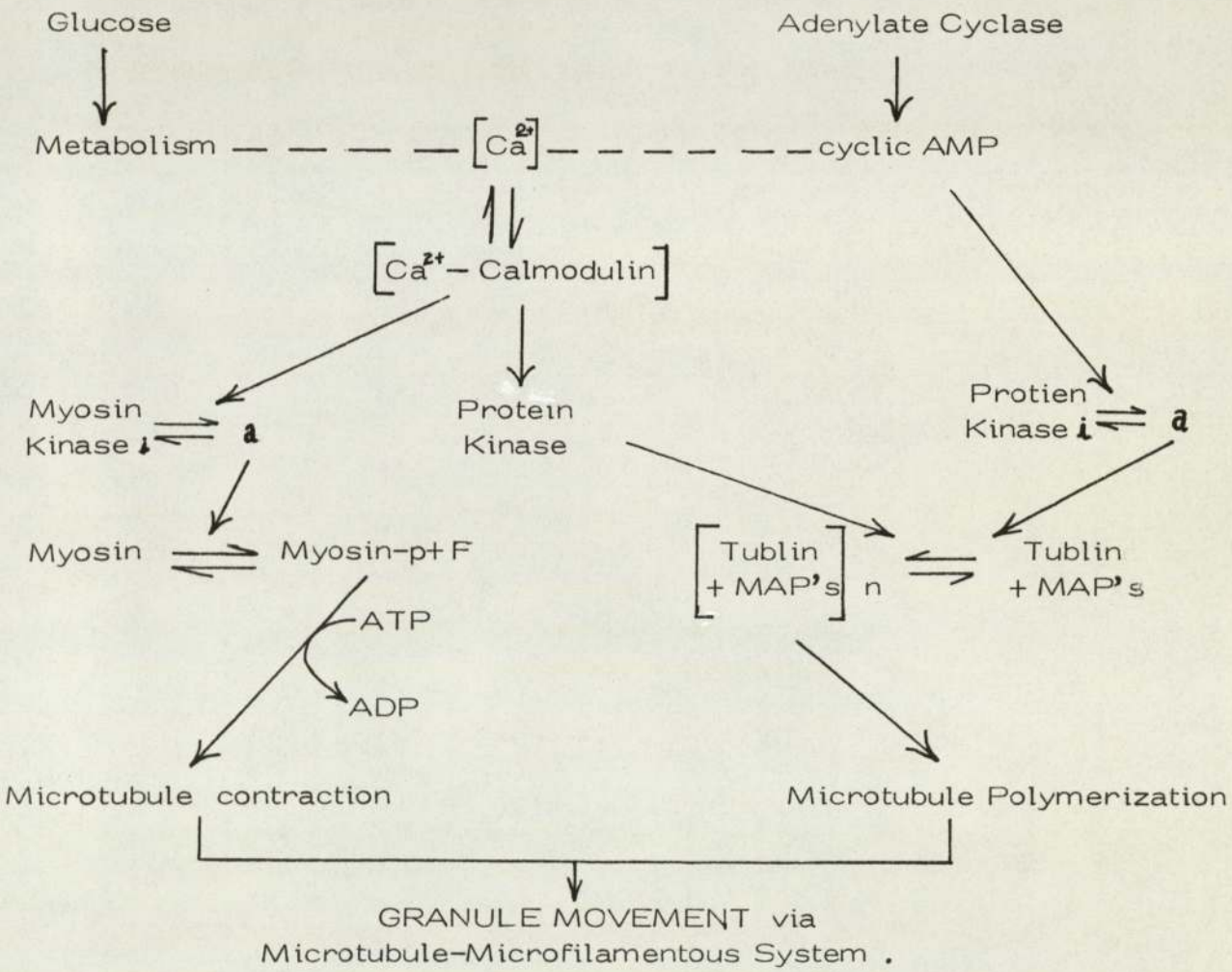
Although the exact location and the nature of the endogenous substrate for the kinases is unresolved it has been suggested that the substrate may be subunits of tubulin. The microtubular-microfilament system has been implicated in the movement of secretory granules in B-cells (51-53) and the Ca^{2+}-calmodulin complex has been shown to both prevent the assembly and cause the depolymerization of microtubules *invitro* (54). Hence the motile force required for the movement of insulin secretory granules could be provided by the microfilaments (55). The contraction of microfilaments would be mediated through the activation of myosin Mg^{2+}-ATPase by actin. This process is dependent on the phosphorylation of the light-chains of myosin, catalyzed by MLCK, an enzyme activated by Ca^{2+}-calmodulin complex.

It appears therefore that both the intracellular pathways and the motile force required for the transport of granules to the plasma membrane might be controlled by Ca^{2+}-calmodulin (see Figure 7).

The Recognition of Glucose by the B-Cell as a Signal for Insulin Secretion

A most controversial and unresolved issue in the field of islet research is the question as to how the B-cell recognizes glucose as a stimulus for insulin release. Two hypotheses have been proposed to explain the molecular mechanism by which the pancreatic B-cell recognizes glucose as the physiological signal. According to the first hypothesis, the substrate site hypothesis the signal for release is

Figure 7. Diagram showing the potential link between cyclic AMP cytosolic Ca^{2+} and the microbutule-microfilaments effector system for granule movement. Abbrev. i; inactive and a; active forms of kinases. (Ref 38)



generated by the metabolism of glucose in the B-cell in the form of a metabolite or a cofactor (59). In more general terms stimulus recognition has been suggested to be a function of redox potential, the intracellular pH and availability of NAD^+/NADH (37,58). The alternative glucoreceptor hypothesis postulates that glucose combines with a specific receptor protein located on the B-cell membrane (57).

The Substrate Site Hypothesis for Glucose Recognition

Coore and Randle (61) using pieces of rabbit pancreas proposed that "the phosphorylation of glucose and a pathway of glucose-6-phosphate metabolism may provide the signal for insulin release induced by glucose". Since its conception this hypothesis has given a considerable impetus to research in the area of islet metabolism and in particular to the process of glucose-stimulus insulin-secretion coupling.

The major experimental evidence for this idea has been extensively reviewed (37,38,57-60). The following observations appear to corroborate this hypothesis: The relative insulinotrophic activity of several hexose sugars correlates with their capacity to be metabolized in the islets. Thus in general non-metabolizable sugars do not stimulate insulin release, whilst readily metabolizable sugars glucose, mannose, N-acetylglucosamine, glyceraldehyde and fructose stimulate insulin release (58). The metabolism of these sugars has

been monitored in various ways including $^{14}CO_2$ evolution from ^{14}C-labelled sugars as an index of oxidation; tritated H_2O production from ^{3}H-labelled sugars as an estimate of glycolysis; the ability to maintain ATP levels as a measure of fuel function and lactate output. Rates of glucose utilization, oxidation and lactate formation have been reported to be closely related to rates of insulin release in response to varying glucose concentrations. However, limited correlation between the metabolic and insulin releasing efficiency of glucose has also been reported (173). Another observation which appears to support the substrate site hypothesis is the finding that it is possible to stimulate insulin release in the absence of extracellular glucose by merely provoking glycogenolysis from endogenous B-cell glycogen stores. Glycogenolysis was achieved by raising B-cell intracellular cAMP levels with theophylline (63). However, this study has not yet been confirmed. The effect of metabolic inhibitors specially mannoheptulose has also porovided supportive evidence for substrate site hypothesis. Mannoheptulose has been shown to inhibit the oxidation of glucose and mannose and the insulin release elicited by these sugars. However, other effects of mannoheptulose such as its influence on carrier mediated transport of glucose across the B-cell plasma membrane cannot be ruled out 95). A similar equivocal study has been reported involving the effect of phloretin (a competitive inhibitor of glucose transport) on glucose induced insulin release (64). The authors suggested that one

of the actions of phloretin in the B-cells might be to interact with a component of the B-cell glucose-sensor mechanism distinct from glucose metabolism.

These pieces of evidence constitute the major part of the case for the substrate site hypothesis and the glucose sensing step or the glucoreceptor is identified with the rate limiting step for glucose metabolism. It has been suggested that this rate limiting step might be phosphorylation of glucose by hexokinase (Km, 7.0 mmol/l). The rate of glucose transport is much faster (Km, 50 mmol/l) than its rate of utilization and phosphorylation. Therefore the phosphorylation of glucose is the rate limiting step for glucose metabolism and may constitute the glucose-sensor mechanism (37).

Recently emphasis has shifted away from searching for specific metabolites that may be triggers for insulin release to more general metabolic parameters (such as NADPH/NADP ratio) which might transmit the secretory signal to the insulin releasing machinery of the B-cell.

Based on somewhat inconclusive studies Ashcroft (37) speculated that the elevated extracellular glucose concentration lead via metabolism to increased phosphoenol pyruvate (PEP) and reduced nucleotide (NADPH) concentrations. The latter increases the permeability of the B-cell membrane to K^+, resulting in depolarization and subsequent Ca^{2+} influx. The resultant increase in cytocolic Ca^{2+} triggers exocytosis and the process is maintained by the restraining effects of PEP on mitochondrial Ca^{2+} uptake (Figure 8).

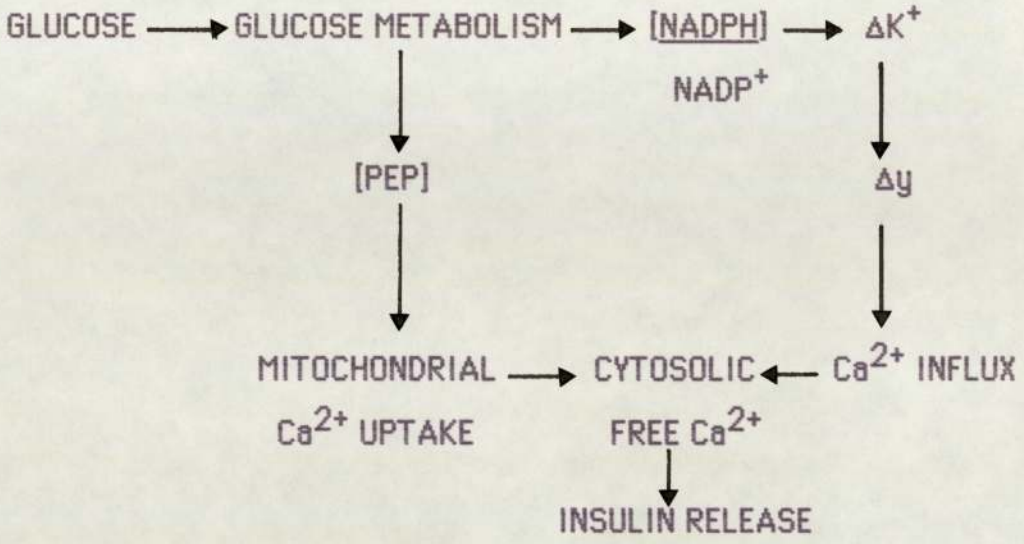


FIGURE 8
Metabolic model for glucose-stimulus insulin-secretion coupling in the B-cell (from Reference 37).

The Regulator Site Hypothesis for Glucose Recognition

There is a great deal of literature to support the existence of glucoreceptor protein in the B-cell membrane (65-78). The glucoreceptor has been defined as "the molecular species in the B-cell that, by virtue of its ability to bind glucose, confers on the B-cell, sensitivity to changes in extracellular glucose concentrations" (60). It is thought that the binding of glucose to its receptor leads to a conformational change which activates the secretory machinery via second messengers such as Ca^{2+}, IP3 and cyclic AMP. Some of the most convincing experimental evidence in support of the glucoreceptor hypothesis has come from elegant

studies on the anomeric specificity of hexose stimulated insulin release. Niki, Niki and Miwa (65) showed that the α-anomer of D-mannose is a more potent insulin secretagogue than the β-anomer. Yet the glycolytic enzyme Mannose-6-phosphate isomerase exhibits preferential affinity for the β-anomer, ie. b-D-mannose-6-phosphate undergoes glycolysis at a higher rate than a-D-mannose. In the case of glucose the α-anomer is a more potent stimulant of insulin release and this coincides with its higher rate of metabolism than the β-anomer. This is observed for both glucose and mannose-induced insulin release despite the opposite anomeric specificities of the isomerases responsible for the further metabolism of glucose-6-phosphate and mannose-6-phosphate. These observations suggest that the B-cell directly recognizes the hexose molecule as a signal for insulin release, most probably at receptor sites postulated on the cell membrane.

Davis and Lazarus (67) assembled an *invitro* cell free secreting system consisting of plasma membranes prepared from Cod Brockman bodies and b-granules from mouse islets, Cod Brockman bodies were chosen because of the difficulty of obtaining sufficient islet cell membrane from mouse islets. Glucose metabolism was not demonstratable in the system but for insulin release to proceed the latter did show an absolute requirement for a physiological concentration of Ca^{2+}. ATP in the presence of Ca^{2+} stimulated insulin release, and D-glucose, like ATP was able to increase insulin release above that found with Ca^{2+} alone. D-glucose in the presence

of Ca^{2+} and ATP produced a large increase in the amount of insulin liberated and similar effects were observed with D-mannose, D-fructose and D-glyceraldehyde. On the other hand L-glucose, D-galactose and 5^1-thio-D-glucose were not effective secretagogues. Among various phosphorylated intermediates of glucose tested, only phosphoenolpyruvate and glucose-6-phosphate were capable of releasing insulin. Glucose is as effective a secretagogue in the presence of non-phosphorylating analogues of ATP as it is in the presence of ATP. The effect of ATP on glucose initiated insulin release is thus considered to be allosteric. Glucose-6-phosphate produces insulin release only in the presence of ATP but not in the presence of non-phosphorylating analogues of ATP, suggesting that ATP is necessary as a phosphoryl donor.

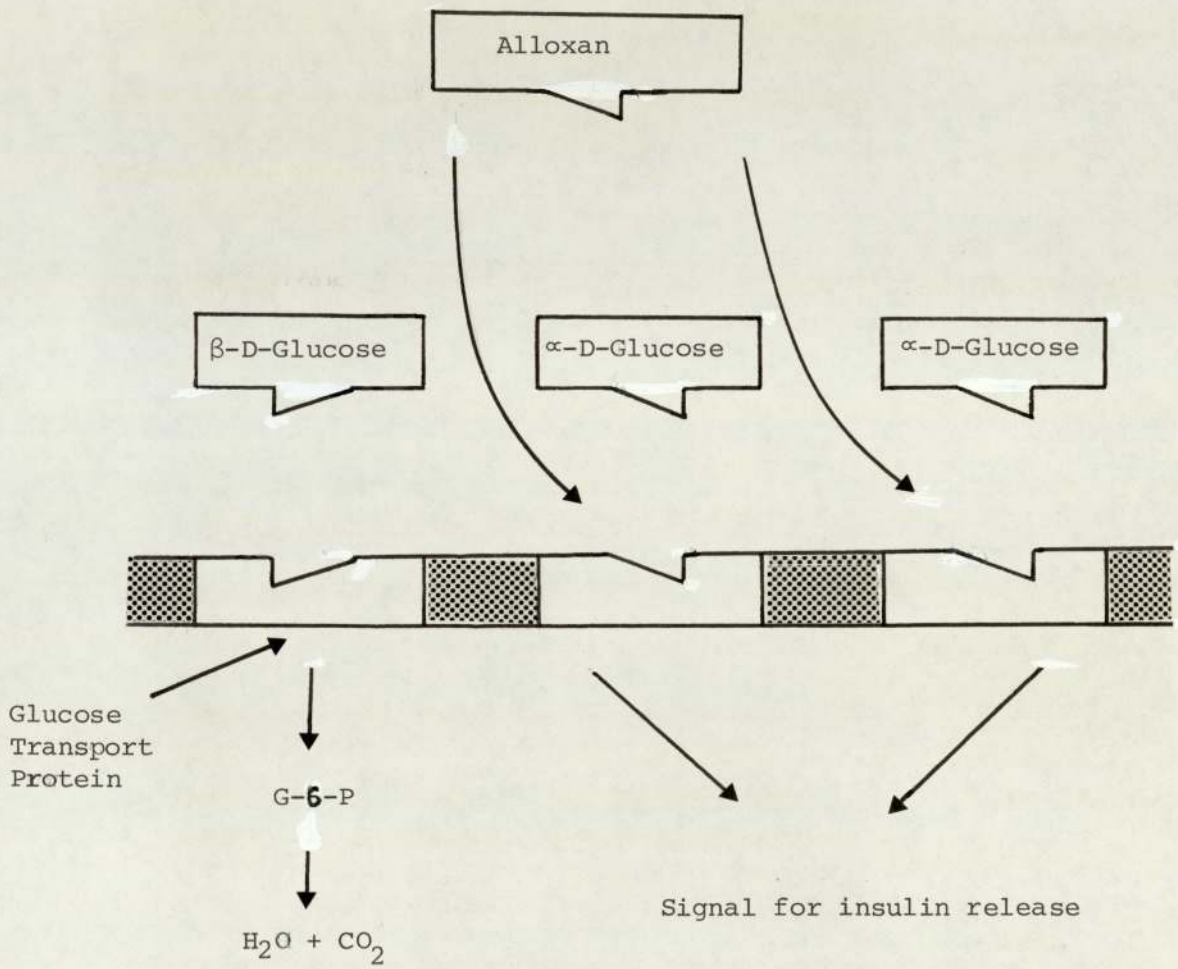
To explain their findings Davis and Lazarus postulated a "cascade theory" of insulin release. According to which glucose initiates insulin release, first by interaction with a glucose receptor on the plasma membrane and simultaneously enters the cell to undergo metabolism. Although simple and attractive theory, caution should be exercised when extrapolating observations obtained from cell free systems with the *invitro* situation. Furthermore, these studies with isolated b-granules and plasma membranes were the product of activity at both sides of the membrane, hence the system may have reflected a pharmacological rather than physiological situation.

Another method of approach adopted to try to understand how the B-cell recognizes glucose as a signal for insulin release has been to use alloxan as a probe (for the identification of the glucoreceptor). Alloxan is a cytotoxic, diabetogenic agent that destroys pancreatic B-cells (68). Its diabetogenic effects are known to be ameliorated by the concomitant presence of glucose, 3-O-methylglucose and mannose (79-82). It is thought that in the presence of these sugars the receptors would be occupied and therefore protected from the harmful effects of alloxan. Niki and Niki (57) demonstrated that α-D-glucose provides more protection against alloxan than the β-anomer suggesting that the receptor has preferential affinity for α-anomer. The possible interaction of the receptor with D-glucose anomers and alloxan are illustrated in Figure 9.

Other studies have suggested that the action of alloxan is directed against thiol groups located in the receptor (85). However, these studies do not rule out the possibility that thiol groups associated with the glucose transport system may also be involved (83,84). The finding that neither the prior exposure to nor the concomitant presence of alloxan altered the rate of glucose transport into rat islets (85) suggests that the site of action of alloxan is not the glucose transport system (86) but rather the glucoreceptor site on the B-cell membrane.

Weaver and colleagues (70) have demonstrated the structure activity relationship between various agonists and antagonists of insulin release. They have employed a receptor mapping approach in which the structure of D-glucose, alloxan and ninhydrin were

Figure 9. The protection of the glucoreceptor by glucose (from Ref 57)



analyzed by a computer for their molecular similarity. The three compounds were found to share the following molecular properties: An oxygen at C^1; an hydroxyl at C^2 either equatorial or axial; an equatorial oxygen at C^3 and electron density at position 5, Figure 10. Further structural analysis revealed that alloxan and ninhydrin would require little, if any, additional volume than that required for the recognition of active hexoses, D-glucose and D mannose.

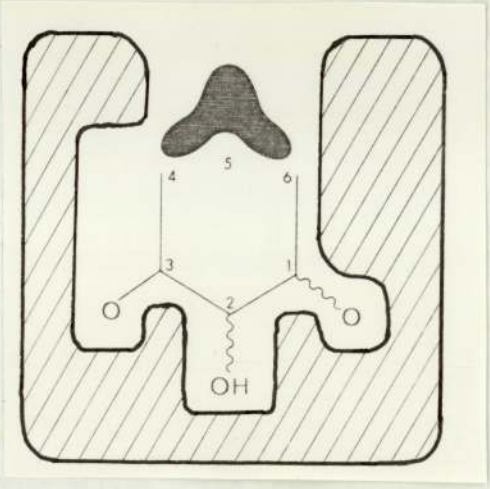


Figure 10

The Pharmacophore for the Glucose Recognition at the Glucoreceptor (from Reference 70).

Non insulinotrophic hexoses (D-galactose, D-allose, Lyxose and L-glucose) were found to vary in their structure from the active

hexoses. The authors concluded that glucose, alloxan and ninhydrin interact at a common receptor to initiate the first phase of insulin release.

If there is a glucoreceptor on the B-cell then it should be possible given sufficient tissue to solubilize, separate and characterize it from the plasma membrane. Several attempts have been reported (74-76). Price (74) found that glucose and mannose formed complexes with solubilized cell membranes of dog pancreatic islets. Sugars that are not insulin secretagogue such as D-galactose and 3-O-methylglucose did not form complexes. Price speculated that the complex might be a putative glucoreceptor. In another study (75) the binding of ^{14}C-labelled glucose to purified fish islet membranes has been investigated. The membranes were found to bind ^{14}C-glucose but not ^{14}-C-L-glucose binding. Similar results have been observed by Kobayashi, Yoshida and Tsumura (76) using rat islet cell membranes. They found that D-[^{14}C]-glucose binding to the plasma membrane was dose dependently inhibited by D-glucose but not by either L-glucose, D-galactose or 3-O-methylglucose. In addition both phloridizin and alloxan were found to inhibit D-[^{14}C]-glucose binding.

A major disadvantage of these binding studies is their inability to differentiate between the glucose transport and the glucoreceptor protein. However it may be that both these moieties are intimately linked. Any approach that may be used to dissociate glucose transport system from glucose binding to its receptor would require the blockade of glucose transport system with phloridizin followed by photo affinity labelling of glucoreceptor. A certain

degree of success has been reported using this approach for the identification and characterization of taste receptors (87-89). Several studies have confirmed the presence of glucoreceptors for sweet taste on the tongue. The interaction between proteins extracted from rat and bovine tongue and various sugars shows a good correlation with behavioural and electrophysiological studies. Furthermore there is experimental evidence for the existence of glucoreceptors in liver and hypothalamus both of which appear to be involved in the regulation of food intake (90). The impaired activity of glucoreceptors associated with satiety centre appears to result in enhanced food intake leading to excessive obesity especially in laboratory animals (88). On the basis of such studies on glucoreceptors Niki and Niki (57) have proposed the hypothesis, that diabetes mellitus (in particular Type II) may well be understood as a generalized disorder of membranal glucoreceptors.

Another approach used to demonstrate the existence of glucoreceptors on the B-cell membrane has been to prevent glucose entry into the B-cell and see whether glucose still initiates insulin secretion. Newton (77) used this kind of approach when he incubated lean and obese hyperglycaemic mouse islets with a solution of glucose saturated lentil lectin LcH-B. The glucose bound to lectin was unable to penetrate the B-cell membrane. The lectin per se did not stimulate insulin release, however glucose saturated lectin was shown to elicit insulin release from lean mouse islets equivalent in magnitude to that induced by 30 mmol/l glucose alone. No significant stimulatory response was observed from obese mouse

islets. This latter observation suggested that the constant hyperglycaemia in obese mice might have desensitized the membranal glucoreceptors leading to an insensitivity of the B-cell to elevated glucose.

The Genetically Obese Diabetic Mouse (Genotype ob/ob)

The recessively inherited obese-hyperglycemic syndrome in mice was first described in 1950 (93). The manifest syndrome is a result of a single autosomal recessive gene (symbol ob/ob) on chromosome 6, linkage group X1 (Plate 1). Some of the conspicuous features of the syndrome are an abnormal deposition of fat, hyperglycaemia, hyperinsulaemia insulin resistance and greatly enlarged functionally hyperactive islets of Langerhans (94). The pronounced obesity and metabolic disturbances in the obese hyperglycaemic mouse are representative of certain types of human obesity and maturity onset diabetes (95) and therefore it provdes a useful animal model to study the human condition.

The ob mutation was transmitted to the C57BL/6J and C57BL/KsJ strains at the Jackson Laboratory, Bar Harbour, Main (96). The metabolic abnormalities of the obese mice are determined not only by the ob gene but also arise as a consequence of its interaction with the background genome (97,90). Accordingly, the characteristic features of the syndrome may vary slightly depending on the genetic background in which the mutation is maintained. The

PLATE 1. The hyperglycaemic mouse and its lean littermate (20 weeks of age)

strain used throughout these studies has been maintained at Aston University for the past 15 years as inbred stock on the C57BL/65 background. The characteristic features of the Aston obese mouse have previously been described (99). One of the earliest signs of the syndrome is a reduction in core temperature and oxygen consumption, demonstratable as early at 10-14 days. This property is used to distinguish the obese from lean mice at this early stage when there is no other visual difference (100). The impaired thermogenesis and reduced energy expenditure may partially account for the increase in fat cell size visible after 14-20 days, but the cause of obesity seems to be the high rate of lipogenesis in the liver and the adipose tissue (101,102). Obesity is only visibly detectable after 25-28 days (101). At day 17-21 there is a slight hyperinsulinaemia accompanied by hypoglycaemia (94). Thereafter plasma insulin levels continue to rise rapidly with the gradual appearance of insulin resistance and hyperglycaemia. The obese mouse gains weight rapidly from day 35 and hyperinsulinaemia, hyperglycaemia and hyperphagia increase during this dynamic phase of the syndrome, with the progressive development of glucose intolerance, fasting hyperglycaemia and insulin resistance. With age (<25 weeks) weight gain slows down and even stops (99). Flatt and Bailey have noted that at 40 weeks of age plasma insulin concentrations are lowered and insulin resistance is reduced (103). Additionally these workers found that longevity is reduced in obese mice that do not shed weight and animals rarely live longer than 40 weeks. The cause of insulin resistance in the obese mouse is not

clear, however the factors responsible for its development may be of primary importance since it is an early feature of the syndrome. Moreover, tissue resistance to the action of insulin underlies the manifest symptoms of non insulin-dependent diabetes in man and it is therefore possible that the primary abnormality or abnormalities in question are associated with the link between obesity and diabetes. In the obese mouse the insulin resistance is thought to be the result of hyperinsulinaemia causing a reduction in the concentration of insulin receptors at the target tissue membrane (104-106). However, this down regulation of insulin receptors by hyperinsulinaemia is unlikely to wholly account for the reduced insulin sensitivity as only a limited number of receptors (10-30%) need to be occupied to produce a maximal response to insulin (106). Furthermore, it has been demonstrated that insulin insensitivity and obesity persist even after the reduction of circulating insulin levels (107,108). The defect(s) could lie in more physiologically significant sites of regulation of insulin action distal to receptor binding, known as postreceptor events (such as glucose transport, glycogen synthesis). Indeed, it has been shown recently that the development of insulin resistance in the obese mouse is due primarily to a postreceptor defect(s) in the action of insulin (106).

Morphological studies of the islets of Langerhans from the obese mouse show a marked hypertrophy and hyperplasia (109-111). The islet volume in the adult mouse may be ten times higher than that of their lean littermates (112) and the B-cells may comprise >90% of the total islet volume (111). There is a certain degree of

degranulation of B-cells (112) and increased nuclear and nucleolar size (113). Furthermore the B-cells contains about three times more glycogen than do B-cells from their lean littermates (114). In the Aston obese mouse there is increased vascularization, metaplasia of the exocrine ductal cells, scattered areas of fibrosis and the appearance of hyaline-fibrinoid-filled lacunae (114-117).

The number of a_2-cells in obese mouse islets is increased (113) and provides morphological support for the idea that hypersecretion of glucagon might be one of the factors responsible for hyperglycaemia in the obese mouse (116,117). It has been suggested that the hyperinsulinaemia is also secondary to an increased glucagon secretion which continuously stimulates the B-cell and thus contributes to the hyperinsulinaemia (116). This observation is supported by a study in which high glucose concentrations were shown to paradoxically stimulate glucagon secretion from the perfused pancreas of obese mice (121). This observation is corroborated by the observation of high liver glycogen phosphorylating activity (118), as increased rate of glycogenolysis (119) and an enhanced glycogon turnover in livers of obese mice (120). However, the precise role played by glucogon in the aetiology and pathogenesis of the obese hyperglycaemic syndrome remains to be elucidated.

The present work was initiated to extend ourunderstanding of the way the physiological stimulus glucose initiates the release of insulin from the B-cell. Digitoxose has been chosen for these studies because it is reported to be a non-metabolizable epimer of

glucose and its effect(s) on glucose induced insulin release should provide new insight into the mode of action of glucose in the process of insulin release. The effects of digitoxose on islet metabolism in particular, the rate of glucose oxidation and oxygen consumption were also studied in order to discover if the metabolic role of glucose can be dissociated from its role as a signal for insulin release. A comparative study of insulin release and glucose metabolism from isolated pancreatic islets of obese hyperglycaemic mice and their lean littermates should provide information on the integrity of the insulin secretory process and islet metabolism in an animal model of non-insulin dependent diabetes. Ca^{2+}-calmodulin and cAMP have been suggested to play an important role in the mechanism of glucose induced insulin secretion however, the relative importance and the interaction of these two systems remains speculative. In order to establish the role of these two systems in insulin secretion the adenylate cyclase stimulator forskolin and calmodulin inhibits trifluoperazine and MB17108 have been used.

CHAPTER 2

GENERAL MATERIALS AND METHODS

1 Animals

Homozygous obese (ob/ob) and homozygous lean (+/+) mice from the colony maintained at Aston University in Birmingham were used throughout these studies. The origin and the characteristics of the obesity syndrome have been described by Flatt and Bailey (103).

Animals were housed in air-conditioned rooms at 22± 2^{0}C with a regular lighting schedule of 9.5h light (0800-1730h) and 14.5h dark. Tap water and a standard 3/8" pellet diet (mouse breeding diet, Heygate and Sons Ltd, Northampton) were supplied *ad libitum*.

The obese animals (ob/ob) used in these studies were taken from litters of known heterozygous (ob/+) monogamous pairs which provide the mendelian ratio of 1 obese (ob/ob): 2 lean heterozygous (ob/+): 1 lean homozygous (+/+). Lean homozygous mice (+/+) were used from the litters of established homozygous (+/+) parents. Care was taken to use only the homozygous animals as a gene-dose effect of the ob mutation has been documented (122,123). All animals were fasted overnight prior to the experiments.

2 Chemicals

Reagents of analytical grade and double distilled water were used throughout. The chemicals and their sources were as follows:

N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), D-glucose, mannoheptulose, thiomersalate from Sigma (London) Chemicals, Poole, UK; Collagenase (type IV) from Flow Laboratories, Irvine, Scotland; Mouse Insulin Standard from Novo Research Institute, Bagsvaerd, Denmark; Insulin binding reagent from Wellcome Reagents Ltd, UK; D-(U-^{14}C) glucose (250 mci/mmol) from Amersham International, Amersham, UK; Insulin free bovine serum albumin (fraction V) from Miles Laboratories Ltd, Slough, UK; Digitoxose from Aldrich Chemicals Co Ltd, Gillingham, UK; Sodium dithionite, Arginine and Leucine from British Drug Houses; Glibenclamide from Hoechst Pharmaceuticals, London, UK.

3 The Isolation of Pancreatic Islets of Langerhans

The large amount of information presently available about the physiology, morphology and biochemistry of pancreatic islets has mainly been derived from *invitro* studies which have clear advantages over *invivo* studies. In the *invitro* system the pancreatic islets are kept in an artificially controlled environment and can be subjected to different conditions, metabolites and active substances and the subsequent hormonal response analyzed.

Techniques for the perfusion of the isolated rat pancreas were first introduced by Anderson and Lang in 1947 (124). Since then the technique has been extensively used to study the effect of insulinotrophic agents on the islets in terms of the kinetic

aspects of insulin, glucagon and somatostatin release (125). In the early sixties Coore and Randle used pieces of rabbit pancreas to study the regulation of insulin secretion (61). The technique was later refined by employing a perifusion system in which finely cut pieces of pancreas were contained in a small chamber and perifused with the medium containing test substances, the perifusate was collected and assayed for insulin or glucagon (126). This perifusion technique eliminated the complicated surgery required for the perfusion of the whole pancreas but had two drawbacks. Firstly the secretory dynamics were poor because some fragments contained no islets. There was also the danger of protease release from the damaged acinar tissue and it proved difficult to quantitate the rate of insulin release.

The islets of Langerhans are dispersed throughout the pancreas with a total volume of only 1% of the total gland. A more appropriate approach to *invitro* study of regulation of insulin release would be to use isolated islets free of acinar tissue. Hellerstrom in 1964 was the first to use a freehand microdissection technique for the isolation of mouse islets (127). Islets isolated by this technique were used for *invitro* biochemical studies concerning enzymatic activity, oxygen consumption and secretory activity (128). A little later Moskalewski pioneered a technique in which pancreatic acinar tissue of guinea pig was digested with the enzyme collagenase (129). Lacy and Kostainovsky improved the technique by carrying out the prior injection of buffer solution into the pancreatic duct

to disrupt the acinar tissue and to increase the surface area of subsequent digestion with collagenase. The islets were subsequently either sedimented in Hanks buffer or separated by centrifugation on a discontinuous sucrose gradient (130).

4 The Collagenase Digestion Technique for the Isolation of Islets

The technique used for the isolation of pancreatic islets in the present studies is a modification of the method described by lacy and Kostainovsky (130). Mice were culled by cervical disslocation, laid ventral side up and the abdominal cavity opened with a midline incision to display the vicera. The pancreas was located near the spleen and distended by injecting Krebs-Hepes buffer (pH 7.4) using an 18 gauge hypodermic needle. The pancreas was then carefully excised from the surrounding vicera using butterfly scissors. Usually the pancreata from three mice were used for each digestion. The pancreata were trimmed of fat and connective tissue, washed and cut into very small pieces with round shaped scissors. Any floating fat was removed with pasteur pipette and the pieces of pancreas transferred to a conical flask (25 ml) containing 8 ml of buffer and 40 mg of Worthington collagenase. The contents were then vigorously shaken in a shaking waterbath (120 cycles/min) at 37^{0}C for 20-30 minutes. After digestion the mixture was diluted with 15 ml of fresh ice-cold buffer to stop further enzymatic digestion. The contents were then transferred to a larger test tube and the digest allowed to sediment. The

supernatant was discarded and the sediment resuspended in 10-15 ml of Krebs-Hepes buffer. In order to remove the collagenase as much as possible the separation procedure was repeated once more and the final sediment resuspended in 10 ml of buffer and transferred to a glass petri dish. At this stage DNAase (200 µg/digest) was added to prevent the clumping of digested debris. Islets free of acinar tissue were picked out using a drawn out pasteur pipette under a disecting microscope (magnification x 10). Approximately 200 islets could be harvested in a small black watchglass containing ice cold oxygenated buffer until required.

5 Static Incubation of Islets of Langerhans

After isolation the islets were preincubated in KRB-Hepes buffer supplemented with 5.5 mmol/l glucose plus bovine serum albumin (1 mg/ml) at 37^{0}C to establish a steady metabolic state of secretion. At the end of a 30 minute preincubation, the buffer was removed and batches of 5-8 islets were transferred to small glass vials (volume 5 ml) containing 1 ml of oxygenated test buffer. The test vials were gently agitated (60-70 cycles/min) in a shaking waterbath constantly aerated (95% O_2: 5% Co_2) using a gassing hood. At the end of the incubation period a 100 µl aliquot of the test buffer was removed from each vial and diluted with 500 µl of buffer B and stored at -20^{0}C for the subsequent assay of insulin. The dilution was necessary to bring the insulin content of the samples within the range of the insulin standard curve.

6 Insulin Release from Perifused Islet of Langerhans

The perifusion system used in the present study was a modification of the method described by Lacy, Walker and Fink (133). Swinnex filter holders (13 mm diameter, Millipore Ltd, Wembly, Middlesex) were used as tissue chamber. Usually 10 islets were placed in the centre of a millipore filter (pore size 8µ) which had been uniformly punctured with a 15 mm gauge hypodermic needle. The filter was supported on a stainless steel grid sandwiched between two teflon gaskets and the two component parts of the tissue chamber. The assembled unit provided a filter area of 0.8 cm^2 and a total chamber volume of 300 µl (Plate 2).

The tissue chamber was fitted into the perifusion system as shown in Figure 11. Krebs-Hepes buffer supplemented with the appropriate test substance was drawn into the islet chamber via polyethylene tubing (ID 0.58 mm, pp 50; Portex Ltd, Hythe, Kent) at a constant flow rate of 0.5 ml/min by means of a peristaltic pump (LKB 2132, Microperpex Ltd; LKB Ltd, Sweden). The islet chamber and the buffer reservoir were maintained at 37^{0}C in a water bath. The perifusate was collected in 0.5 ml aliquots at 1 minute intervals on a programmable fraction collector (LKB 2211 Superac: LKB Ltd, Sweden) and subsequently assayed for insulin content. Total dead space of the perifusion system was 1 ml and a lagtime of 2 minutes between closing the valves and the test buffer

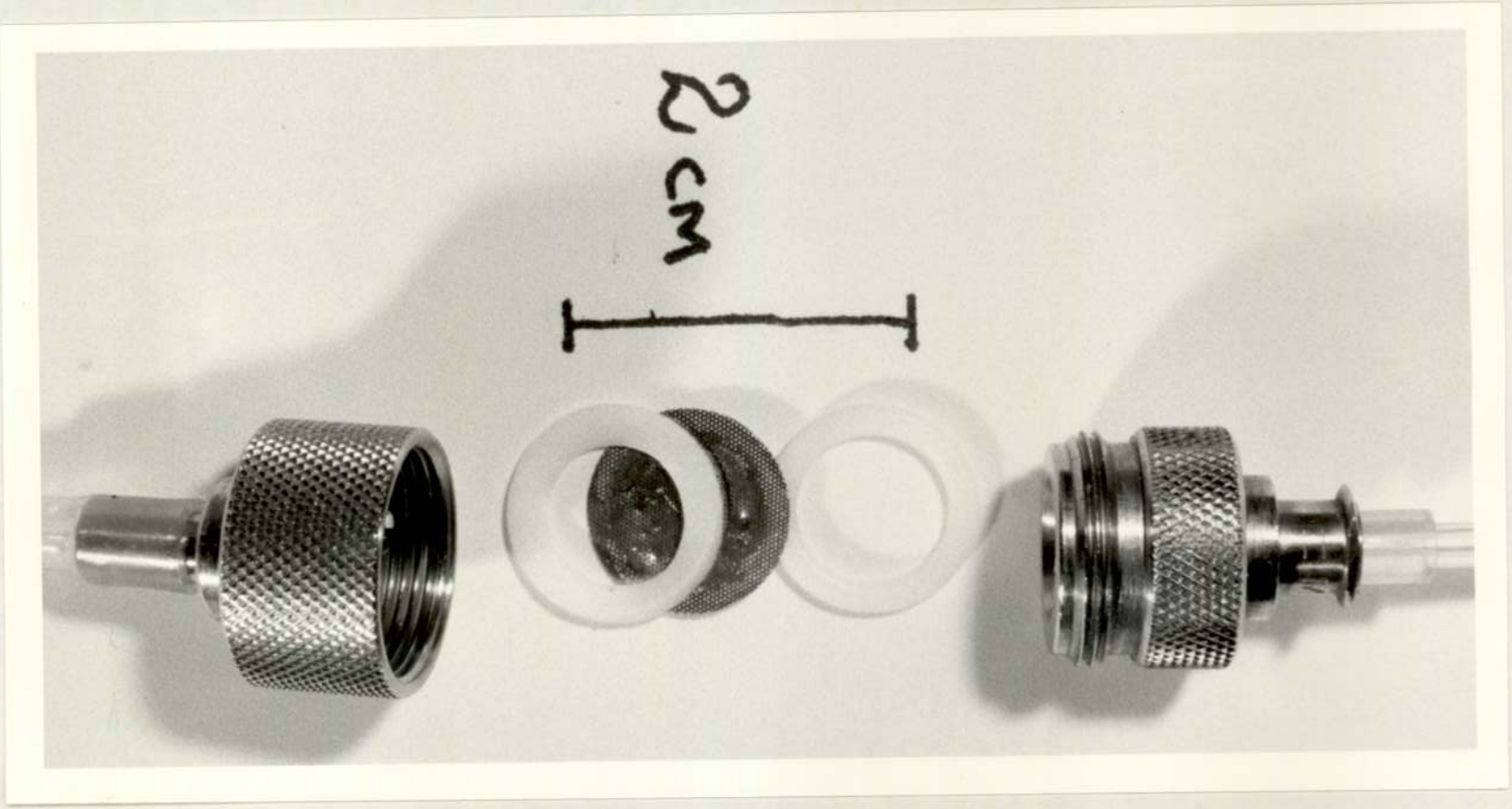
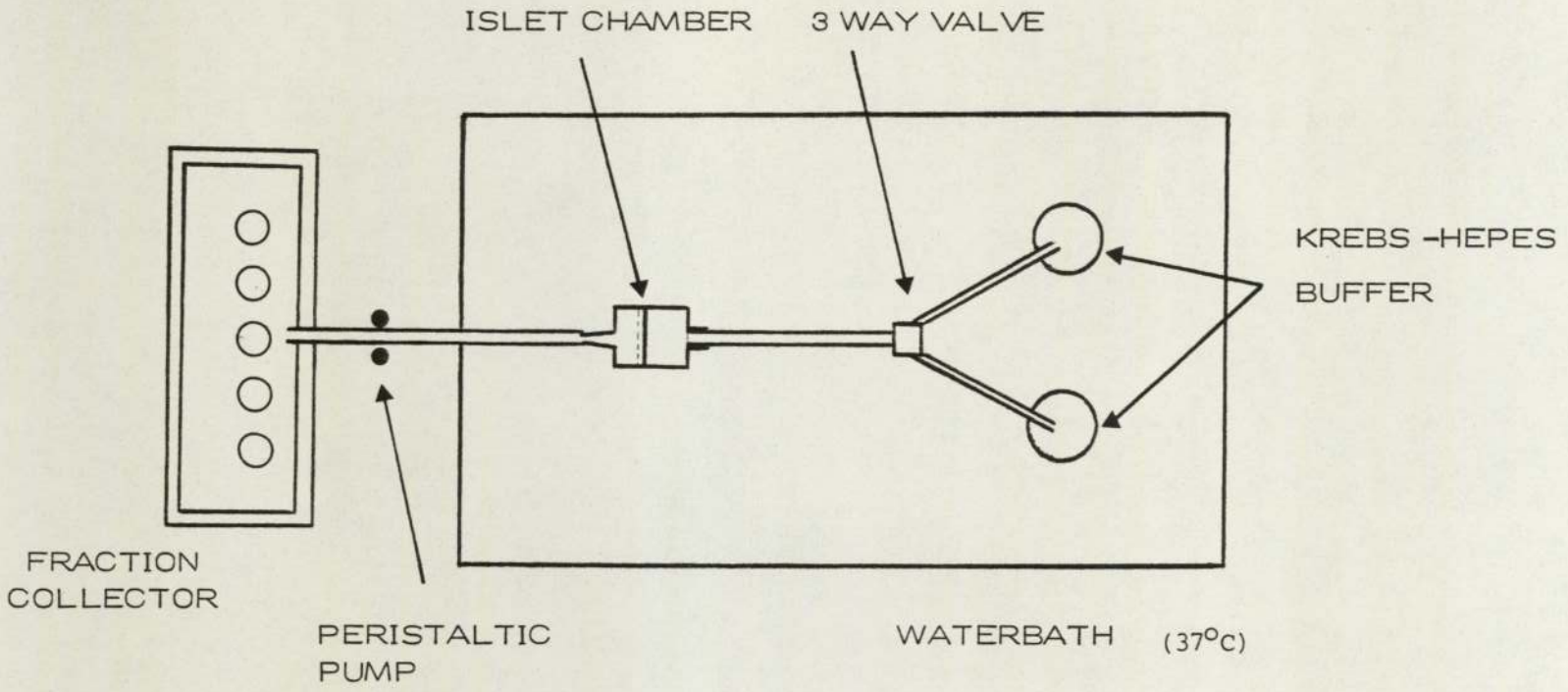


PLATE 2. Exploded view of the islet chamber used in perifusion studies.

(a) teflon gaskets

(b) stainless steel grid

Figure 11. Top view of the perifusion system



reaching the islets. This was allowed for by closing the valve 2 minutes before the test perifusion was due to begin.

The islets were preperifused for 45 minutes with KRB-Hepes buffer supplemented with 5.5 mmol/l glucose and BSA (1.0 mg/ml) in order to establish steady state secretory dynamics. During the prepreifusion period samples were taken after every five minutes. At the end of pre-perifusion period the 3-way vlave was switched to allow the perifusion of islets with the buffer containing various test substances.

7 The Determination of Islet Dry Weight

In order to make direct comparisons between the rates of insulin secretion from lean and obese mouse islets the dry weight was determined. Since it was found difficult to quantitatively recover all the islets from perifusion chambers and incubation vials, 20 islets were kept aside from each digest, then washed (to remove albumin and other buffer salts) dried and their weight determined as follows: 20 islets were transferred with a pasteur pipette to a small boat made from aluminium foil. The boats were made by moulding foil around the tip of a 4 mm diameter glass rod and trimming down with scissors to an approximate size of 3 mm in depth. The bottom of each boat was perforated with a fine gauge needle to allow the buffer to drain through into adsorbent filter paper placed underneath thus leaving the islets trapped inside the boat. The boats containing the islets were dried at

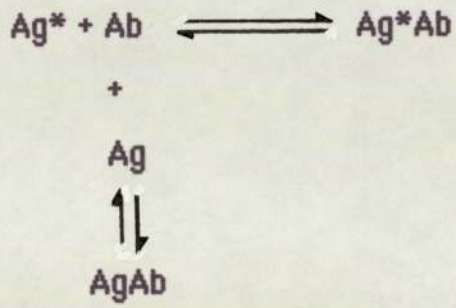
80^{0}C for 24 hours in an oven and subsequently weighed on a Mettler UM6 microbalance (Gallenkamp, London) with a sensitivity of 0.1 µg. After weighing, the boat was unfolded and the dried islets scraped off with the aid of a hypodermic needle, under a dissecting microscope. The cleaned foil was reweighed and the islet dry weight calculated by difference.

8 The Radioimmunoassay of Insulin

Radioimmunoassay (RIA) procedures for the measurement of hormones and other substances of biological interest, constitute one of the most important and rapidly expanding analytical techniques in the biochemical field. RIA has become the method of choice for determining minute quantities of such compounds as hormones, drugs and enzymes in plasma and other body fluids. RIA is a very sensitive technique, enabling detection down to picogram/ml of a substance. This very high sensitivity makes it possible to assay biological substances for which no other analytical method is available. Furthermore, like all other immunological techniques RIA has a very high specificity (134,135).

Most radioimmunoassays depend on the ability of unlabelled antigen (Ag) to inhibit the binding of labelled antigens (Ag*) when a limited amount of a specific antibody (Ab) is present. This reaction is one of simple competition in which Ag reduces the

amount of free Ab, thus reducing the availability of Ab to Ag*. In performing the assay Ag* and Ab are incubated in the presence and



absence of samples containing Ag. After reaching the equilibrium of the above equation, free Ag* and the antibody bound Ag* are separated and the radioactivity of one or the other is determined. The antigen concentration in the unknown is measured by the diminuition of Ag* binding produced by Ag in the unknown sample, to that of a standard curve obtained by adding graded, known amounts of Ag to the assay system.

The important elements of a radioimmunoassay are the specificity of antibody, the species specific labelled hormone and the separation of bound and free labelled hormone. In the case of insulin, most investigators have successfully used guinea pig anti-porcine insulin systems. This arrangement being derived from earlier studies showing that guinea pig antisera reacted strongly with human insulin (136) and that antisera raised in rabbits was not very sensitive to the human hormone (137). As with other antisera, those of insulin are characterised in terms of their titre, affinity and specificity (138). The optimal titre is

found by noting the dilution of antiserum that allows approximately 50% binding of labelled hormone in the absence of unlabelled hormone (a bound to free ratio of 1:1). In general, this concentration will give an optimal ratio of sensitivity and precision in a radioimmunoassay (139). The presence of tyrosine residues in the insulin molecule has facilitated its iodination by substituting radioisotopes of iodine, either ^{125}I or ^{131}I, for the hydrogen atoms of the tyrosine ring. Although iodine monochloride (140) and peroxidase (141) methods have been used to iodinate insulin, the chloramine T method originally described for growth hormone (142) is still the most popular. Freychet, Roth and Neville (143) modified this technique improving the immunochemical characteristics of the labelled insulin and enhancing its stability in storage. The isotopes of iodine have a high specific activity and it has been suggested that one iodine atom per insulin molecule is the optimal concentration of label that will give a high specific activity whilst maintaining optimal immunoreactivity (144). The iodine isotope ^{125}I is preferred to ^{131}I because it has longer half life (60 days) than the latter (8 days) and the counting efficiency of ^{125}I is greater than that of ^{131}I (135). Furthermore, compounds containing ^{125}I tend to be more stable than those with ^{131}I (135). The final step of radioimmunoassay involve the measurement of bound or free labelled antigen. This requires the physical separation of the bound and the free antigen. In the case of insulin, early methods of

separation were by paper electrophoresis and chromoelectrophoresis (136,146). A variety of other more versatile separation methods have since been developed. These include the adsorption of free insulin to solid phase materials such as uncoated (146) or dextran coated charcoal (147), talcum powder or other silicates (146); salting out techniques (148); adsorption or complexing of antibody to soild phase material such as glass, styrene or sephadex (149). Perhaps the most widely used method for insulin assay, is the double antibody technique of Hales and Randle (132). The method depends on the ability of anti-immunoglobulin antibodies raised in rabbits to bind to soluble antigen-antibody complexes and cause precipitation of the entire complex, thus separating bound labelled antigen that is present from the unbound labelled antigen. Hales and Randle (132) used anti-γ-globulin, raised in rabbits to soluble guniea pig antibodies, which precipitates the insulin antibody complex. As the insulin antibody complex precipitated with anti-γ-globulin serum was still capable of reacting with insulin, Hales and Randle (132) used a pre-precipitated insulin antibody.

Insulin Radioimmunoassay Protocol

The insulin radioimmunoassay method used in this study was a modification of the method first described by Hales and Randle (132). Separation of the insulin-antibody complex from the unbound label was achieved by centrifugation and careful decanting of the supernatant which contained the free label.

(i) The Components of Insulin Radioimmunoassay

All the reagents were prepared in a double distilled water, as variations in the quality of ordinary distilled water may give rise to considerable variations in the amount of insulin bound to the antibody and the reagents were stored at either -20^0C or 4^0C.

(a) Diluent Phosphate Buffer (Buffer B)

A phosphate buffer (40 mmol/l) was used for the dilution of insulin standards, radioiodinated insulin and where necessary the dilution of samples. The composition of the buffer was sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$); 6.2 g/l; insulin free bovine serum albumin, 5 g/l; sodium chloride 9 g/l and sodium ethyl mercurithiosalicylate (thiomersalate - an antibacterial agent) 0.25 g/l. The buffer was adjusted to pH 7.4 with sodium hydroxide (2 mol/l).

(b) Insulin standards

Mouse insulin standard (Novo Research Institute, Bagsvaerd, Denmark) was supplied in vials containing 0.1 mg lypholized mouse insulin. A stock solution of which was prepared in buffer B and diluted to yield a concentration of 10 ng/ml (250 µV/ml). 1 ml aliquots of this stock solution were dispensed into stoppered tubes and stored at -20^0C. Immediately prior to assay, one tube was thawed and dilutions made to give a range of working standards of 10, 5, 2.5, 1.0 and 0.5 ng/ml (or 250, 125, 62.5, 25 and 12.5 µV/ml).

(c) Insulin binding reagent

The insulin binding reagent (Wellcome Reagents Ltd, Beckenham, Kent) was obtained in a lypholyzed form as an immunprecipitate of guniea pig anti-insulin serum and rabbit anti-guniea pig globulin serum. The binding reagent was reconstituted with 8 ml of double distilled water.

(d) Labelled Insulin

Labelled insulin was prepared in our laboratory by the chloramine-T iodination method (143) to an average specific activity of 247 µci/µg and diluted to 50 µci/µg by the addition of an appropriate amount of unlabelled insulin. It is important not to add a significantly greater amount of labelled insulin as the resulting saturation of insulin binding sites severely reduces the assay sensitivity.

(ii) Procedure

Small polystyrene tubes (LP3) 9.5 x 64 mm (Luckham Ltd, Burgeshill, Sussex) were used for the insulin assay. All the reagents were added in 50 µl aliquots using a variable automatic pipette (Anachem Ltd, Luton, Beds). Each standard was assayed in triplicate and each test sample in duplicate. The assay tubes consisted of: zero, blank, total counts, standards and test sample tubes, their contents are shown in Table 1.

Table 1. A protocol table for insulin RIA. Each + indicates 50 µl of the reagents

Reagents TUBES	Standard Insulin	Test Sample	Insulin Binding Reagent	Buffer B	Labelled Insulin
ZERO	-	-	+	-	+
STANDARDS	+	-	+	-	+
SAMPLES	-	+	+	-	+
BLANKS	-	-	-	++	+
TOTALS	-	-	-	-	+

The reactants were vortex mixed (Whirlmixer, Fisons Scientific Apparatus Ltd, Loughborough, Leicester) then incubated at 4^0C for 5 to 6 hours. This incubation period and temperature increases the equilibrium constant for the insulin-antibody reaction (150), and allows the antibody to react with the unlabelled insulin prior to the addition of the labelled insulin. This procedure has been reported to improve the sensitivity of the reaction (151). 50 µl of labelled insulin were then added to all tubes including the "zero", "blank" and "total tubes". The reactants were again vortex mixed and incubated at 4^0C for a further 16-18 hours. Following the second incubation period, the precipitate was washed by adding 0.5 ml of buffer B to all the tubes except the "totals". The insulin-antibody complex was then sedimented by centrifugation

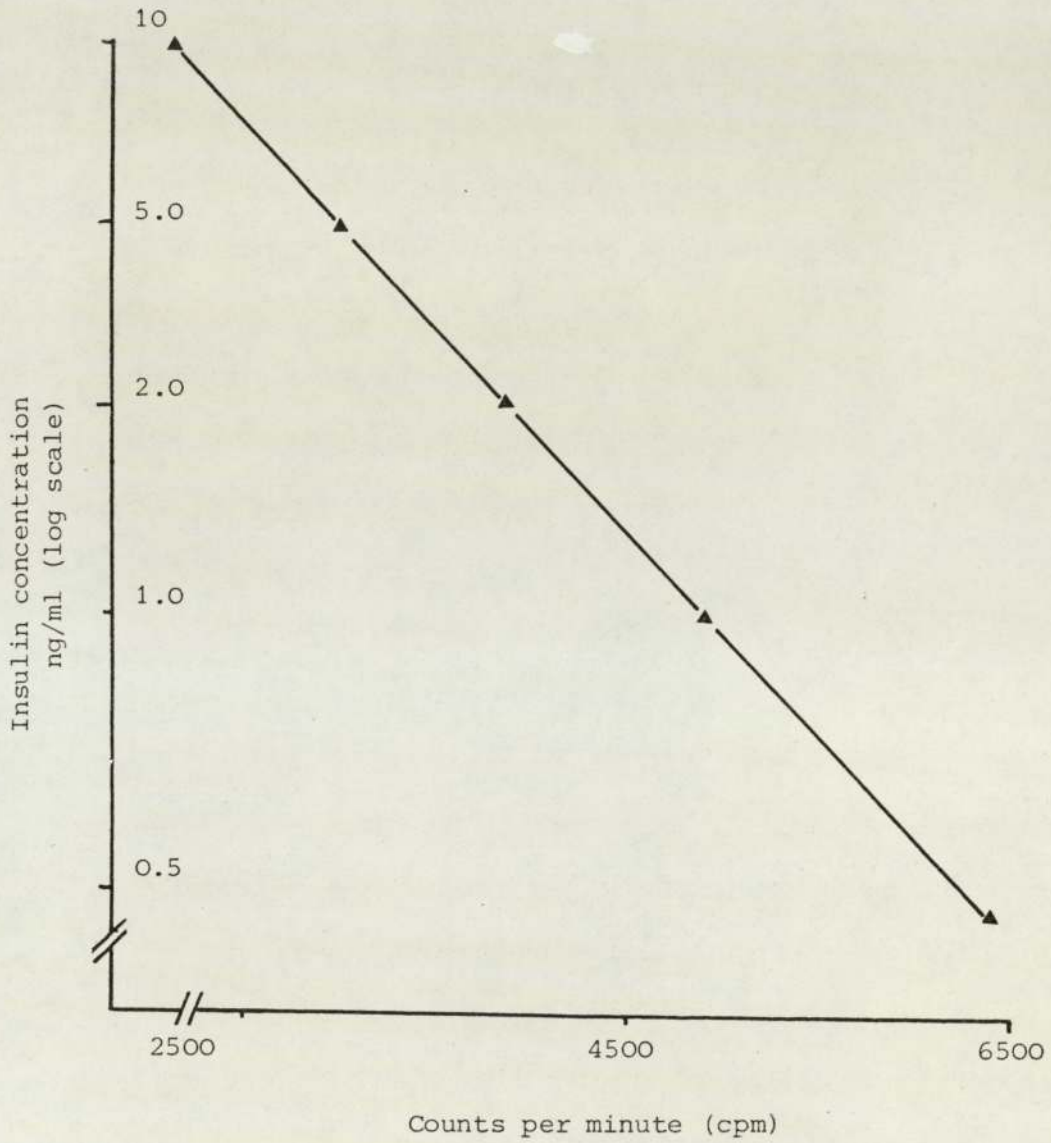
at 1800 g (2900 RPM) for 30 minutes in a MSE bench centrifuge (MSE Ltd London). The supernatant, containing both labelled and unlabelled free insulin was decanted and the remaining few drops aspirated with a pasteur pipette attached to a water vacuum pump. The tubes were inverted and allowed to dry at room temperature for 4 to 6 hours. The resulting radioactivity was determined on an ICN gamma set 500 Gamma Spectrometer (ICN Tracer Lab, Walton-on-Thames, Surrey). Each tube was counted for one minute. The counting time was sufficient to give 10,000 CPM for the "totals" and 1,000 CPM for the tube containing the highest standard insulin concentration.

Computation of Results

A computer program was written (see Appendix) to calculate the concentration of insulin in each unknown sample. The background was subtracted from all tubes and the program constructed a standard curve for insulin by plotting counts per minute (CPM) for each insulin standard against the logarithm to base 10 of the respective insulin concentration (Figure 12). The program then calculated the regression coefficient which it subsequently used to work out the amount of insulin for a given CPM.

In our laboratory the minimum sensitivity of the assay was between 0.05 - 0.12 ng/ml with coefficient of variation of 7 to 10% for intra and inter assay variation respectively.

Figure 12. A typical standard curve for insulin produced by the insulin double-antibody radioimmunoassay.



CHAPTER 3

THE EFFECTS OF DIGITOXOSE ON INSULIN RELEASE, GLUCOSE OXIDATION AND OXYGEN CONSUMPTION FROM PANCREATIC ISLETS OF LEAN AND GENETICALLY OBESE DIABETIC MICE

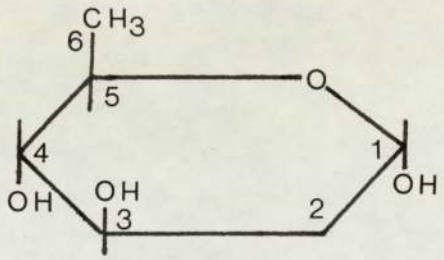
Introduction

Subtle models have been offered to reconcile both the regulator and substrate site theories for insulin release (57,67,152). Substantial support in favour of the regulator site hypothesis would be provided by a sugar that has the capacity to inhibit glucose stimulated insulin release without affecting glucose metabolism (64). 2-desoxy-D-altromethylose (digitoxose), the sugar moiety of the cardiac glycoside digitoxin has been shown to partially inhibit glucose-stimulated insulin release from rat islets without inhibiting glucose oxidation (153,154). Therefore digitoxose may provide a means of dissociating the signal and fuel functions of glucose.

Structure and Origin of Digitoxose

Digitoxin and digoxin are two of the most commonly used cardiac glycosides for the treatment of congestive heart failure, atrial fibrillation, atrial flutter and paroxysmal tachycardia (155). Each glycoside represents the combination of an aglycone with three molecules of digitoxose (155). The aglycones have chemical structure similar to bile acid, sterols and sex and adrenocortical

hormones and are responsible for the pharmacological activity of the glycosides, whilst the sugars attached to the aglycone modify the water solubility, cell permeability and the potency of the resulting glycosides (155). The aglycone can be released from the cardiac glycosides by hydrolysis. The structural formula of digitoxose is shown below (154).



2-Desoxy-D-altromethylose (DIGITOXOSE)

In order to elucidate the mechanism by which digitoxose induces the inhibition of insulin secretion the following studies were undertaken: firstly the effect of digitoxose on glucose stimulated insulin release, secondly its effect on the rate of islet oxygen consumption and glucose oxidation and finally the specificity of digitoxose inhibition of glucose-stimulated insulin release.

Materials and Methods

1 Isolation and incubation of pancreatic islets

Islets were prepared by the collagenase method as described previously (page 41) from overnight fasted obese mice and their lean littermates. All the incubations were carried out in Krebs-Hepes buffer containing bovine serum albumin (1 mg/ml) and glucose (5.6 mmol/l). For the measurements of insulin release batches of 5-8 islets were incubated in 1 ml of the buffer supplemented with the appropriate glucose concentrations (5.6, 11.2, 16.7, 22.2 or 27.2 mmol/l) in the absence or presence of digitoxose.

For the dose-response experiments islets were similarly incubated in Krebs-Hepes buffer containing digitoxose (5.6, 11.2, 16.7, 22.2 or 27.2 mmol/l) and glucose concentration of 16.7 mmol/l. In the experiments on specificity of digitoxose inhibition of insulin release the incubation medium contained digitoxose (22.2 mmol/l) and either one of the three test substances (L-Leucine (36.5 mmol/l), L-Arginine (19 mmol/l) and glibenclamide (8.1 mmol/l)).

2 Measurement of the rate of oxygen consumption by isolated islets

The Clarke type oxygen electrode used in these studies was designed for following the uptake or the production of oxygen by

cell suspensions, subcellular particles or enzyme systems. The principle of operation has been described by Lessler and Brierly (157). Oxygen diffuses through a thin teflon membrane and is reduced at the platinum electrode immediately in contact with the membrane:

$$O_2 + 2_e + 2H^+ \longrightarrow H_2O_2$$

The other half cell consisting of Ag-AgCl electrode is also incorporated in the base of the incubation vessel and further reduces the hydrogen peroxide to water:

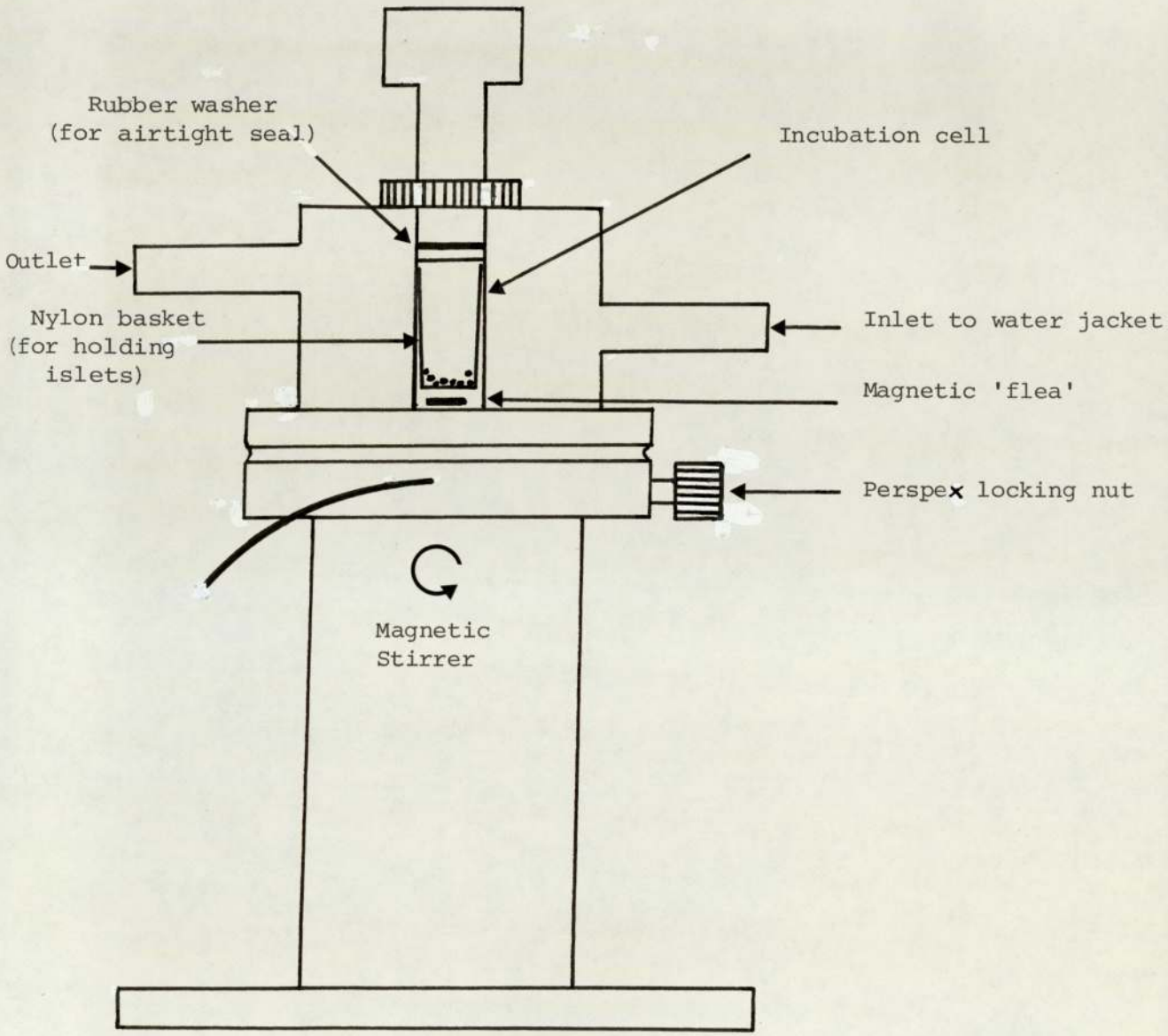
$$H_2O_2 + 2_e + 2H \longrightarrow 2H_2O$$

Figure 13 is a line diagram of the oxygen electrode used in these studies (Rank Brothers, Cambridge, England).

Assembly and Calibration of the Oxygen Electrode

The base of the incubation chamber was detached by unscrewing the perspex locking nut. The platinum and silver electrodes were cleaned with cotton wool and household silver polish (Silvo) and rinsed several times with distilled water. The two electrodes were then moistened with saturated KCl and

Figure 13. The oxygen electrode assembly



covered with lens tissue followed by a square piece of teflon membrane (1 sq cm). Finally the membrane was locked into place by closing the locking nut and the whole assembly was then connected to a potentiometric recorder (Servoscribe RE 551).

The outerjacket of the incubation chamber was connected to the circulator unit of a waterbath heater in order to maintain the electrode temperature at 37^0C. A polarizing voltage of 0.6 V was then applied across electrodes. To calibrate the electrode, the incubation chamber was first filled with double distilled water at 37^0C and a few crystals of sodium dithionite to remove all the dissolved oxygen. The recorder was then zeroed. The chamber was emptied and thoroughly washed with distilled water at 37^0C. Next the incubation chamber was refilled with a known volume of air-saturated distilled water (ie. water which had been stirred at 37^0C for an hour) and the sensitivity of the recorder adjusted to obtain a maximum deflection. The concentration of dissolved oxygen in distilled water at 37^0C was found from tables (157). The electrode was now calibrated and ready for use.

Measurement of Oxygen Uptake by Isolated Pancreatic Islets

A small plastic basket was made to fit inside the incubation cell. The basket was made from blue anachem pipette tips by removing the tapered end and fastening a nylon mesh (pore size 40 microns) to the cut end, the islets were supported on the nylon mesh. After electrode calibration the basket was inserted inside

the cell and the latter filled with 2 mls of Krebs-Hepes buffer, pH 7.4 supplemented with the appropriate test substance. The electrode was allowed to stabilize for 20 minutes followed by a 30 minute blank experiment to determine the extent of electrode drift. This was necessary since the oxygen electrode was found to consume oxygen at a constant rate (approximately 200 ngO_2/h). At the end of the blank experiment the medium was removed and the cell refilled with 2 mls of fresh medium at 37^{0}C. About 100-150 islets were then transferred to the incubation basket and the oxygen uptake of the islets monitored for 30 minutes. At the end of this period a 50 µl aliquot of the medium was removed and stored at -20^{0}C for the subsequent assay of insulin. The nylon basket was removed and the contained islets washed with Krebs-Hepes buffer prior to being reinserted into the incubation cell with fresh buffer supplemented with either glucose (16.7 mmol/l) alone or with digitoxose (22.2 mmol/l) for a further 1 hour incubation. A 50 µl aliquot of incubation buffer was removed for the measurement of cumulative insulin release and the new rate of oxygen consumption determined.

At the end of the incubation the basket was removed and the islets collected in a black watchglass. The islets were then transferred to a boat as described previously and their dry weight determined.

Technical Considerations and Calculation of Results

Since the O_2 electrode is a "percentage response" instrument, the values obtained during the course of an experiment can be used for the direct calculation of the initial amount of O_2 in the medium and the amount of O_2 utilized by the tissue. This procedure involves calibrating the recorder to 100 divisions with a known volume of air-saturated water (as described above).

Many biochemists prefer to express the rate of oxygen consumption in gravimetric (µg atoms) rather than volumetric (µl) form. The former approach was used in the present study. This was done by calibration of the electrode with air-saturated water and using the conversion factors of Lessler and Brierly (157). For example, when the incubation cell contained 2 ml of air-saturated distilled water at 37^0C a full scale deflection of 20 cm was obtained. On the basis that 1 ml of air-saturated water contains 6.9 µg O_2/ml (157) the following equation is obtained:

$$\frac{2 \text{ ml} \times 6.9 \text{ µg } O_2/\text{ml}}{20 \text{ cm deflection}} = 0.69 \text{ µg } O_2/\text{ cm deflection}$$

So 10 cm reduction in recorder response in 30 minutes caused by metabolizing islets will represent both the rate of oxygen uptake by the islets and amount consumed by the electrode ie. 6.9 μg O_2 consumed per 30 minutes.

The electrode had a 90% response time of 5 seconds and its sensitivity was sufficient to allow the measurement of oxygen uptake from 80-100 mouse islets. The use of an air/5% CO_2 mixture was dictated by the greater stability of the medium prepared at ambient PO_2 and the greater relative changes produced by islet respiration. Lowering the medium PO_2 to this extent did not alter the oxidation of D-[U-^{14}C] labelled glucose or the insulinotrophic action of glucose (158).

3 Measurement of glucose oxidation from pancreatic islets

The term glucose oxidation as used here refers to the conversion of glucose carbon into carbon dioxide and is calculated from the incorporation of ^{14}C from D-[U-^{14}C] glucose into $^{14}CO_2$ and the specific activity (μci/μg) of glucose in the medium. The method used in these studies was a modification of the method previously described by Ashcroft, Hedeskov and Randle (159). Batches of 20-30 isolated islets were incubated inside small plastic reaction tubes (5 x 35 mm, Luckhams Ltd, Burgess Hill, UK) containing 100 μl of Krebs-Hepes buffer, pH 7.4, supplemented

with either glucose (16.7 mmol/l) alone or with digitoxose (22.2 mmol/l) and D-[U-^{14}C] glucose (4 µci/ml). The reaction tubes were placed inside glass scintillation vials. The scintillation vials were tightly sealed with self-seal rubber caps (Suba Seal, Gallenkamp, London) and incubated for 75 minutes at 37^{0}C in a shaking water bath (100 cycles/min). After the incubation the vials were removed and chilled. 1 ml of NaOH (1 mol/l) was injected into the outer compartment and the islet metabolism stopped by a further injection of 200 µl of HCl (0.1 mol/l) into the islet containing reaction tube. The scintillation vials were left at room temperature overnight for the absorption of $^{14}CO_2$. The following day the vials were opened and the inner islet containing tubes removed. 9 ml of toluene based Scintillant (NE 260: Nuclear Enterprises Ltd, Edinburgh, Scotland) was added to each scintillation vial and the radioactivity measured on a liquid scintillation counter (Tricarb 2660, Packhard Instruments Ltd, Amersham, UK) with counting efficiency of >95%. The efficiency was calculated by the method of external standard channels ratio (ESR) from a curve relating efficiency to ESR. The curve was generated by counting a series of differently quenched samples containing a known amount of D-[U-^{14}C] glucose. The resulting quench curve was stored in the counter's "memory". For each sample CPM were converted to dPM via the following equation (160):

$$Y = B/E$$

where Y = DPM of the radionuclide, B = total CPM and E = efficiency.

Three blank incubations without islets were carried through the whole procedure in each experiment and blank values were subtracted from experimental values. Glucose oxidation rates were calculated as p mole/l of D-[U-^{14}C] glucose oxidised per µg dry weight of islets per hour from the following formula:

$$\text{D-[U-}^{14}\text{C] glucose oxidised (p mol/l)} = \frac{^{14}CO_2 \text{ formed (dPM)}}{\text{Specific activity of D-[U-}^{14}\text{C] glucose (dPM/P mol/l)}}$$

RESULTS

1 The Effect of Digitoxose on Glucose Stimulated Insulin Release

Figure 14 shows the effect of increasing concentrations of digitoxose on glucose stimulated insulin release from lean and obese mouse islets. Digitoxose at concentration of 5.6 mmol/l had no significant inhibitory effect whilst 11.2, 16.7 and 22.2 mmol/l of the antagonist produced a significant inhibition of insulin release. The maximum inhibitory effect of digitoxose was obtained at concentrations greater than 16.7 mmol/l with lean mouse islets and greater than 22.2 mmol/l with obese mouse islet producing 53% and 46% inhibition of glucose stimulated insulin release respectively. A double reciprocal plot of the same data gave values for the inhibitor constant (Ki) of 16 and 20 mmol/l digitoxose and values for maximum percentage inhibition of 83% and 77% for lean and obese mouse islets respectively.

In order to find the type of inhibition produced by digitoxose, the effects of 11.2, 16.7 and 22.2 mmol/l digitoxose were investigated on a series of glucose dose-response curves (Figure 15). The dose-response curves for glucose stimulated insulin release from lean mouse islets were sigmoidal. 5.6 mmol/l glucose slightly stimulated insulin release above basal (release in the absence of glucose) and appeared to be the threshold concentration for stimulation. The largest increase in the rate of insulin release occurred between 5.6 and 16.7 mmol/l, suggesting

Figure 14. Dose response for digitoxose inhibition of glucose stimulated insulin release from islets of lean (▲—▲) and ob/ob (■——■) mice. Each point represents the mean of six determinations ± SEM. a $P < 0.05$ compared with lean at each digitoxose concentration.

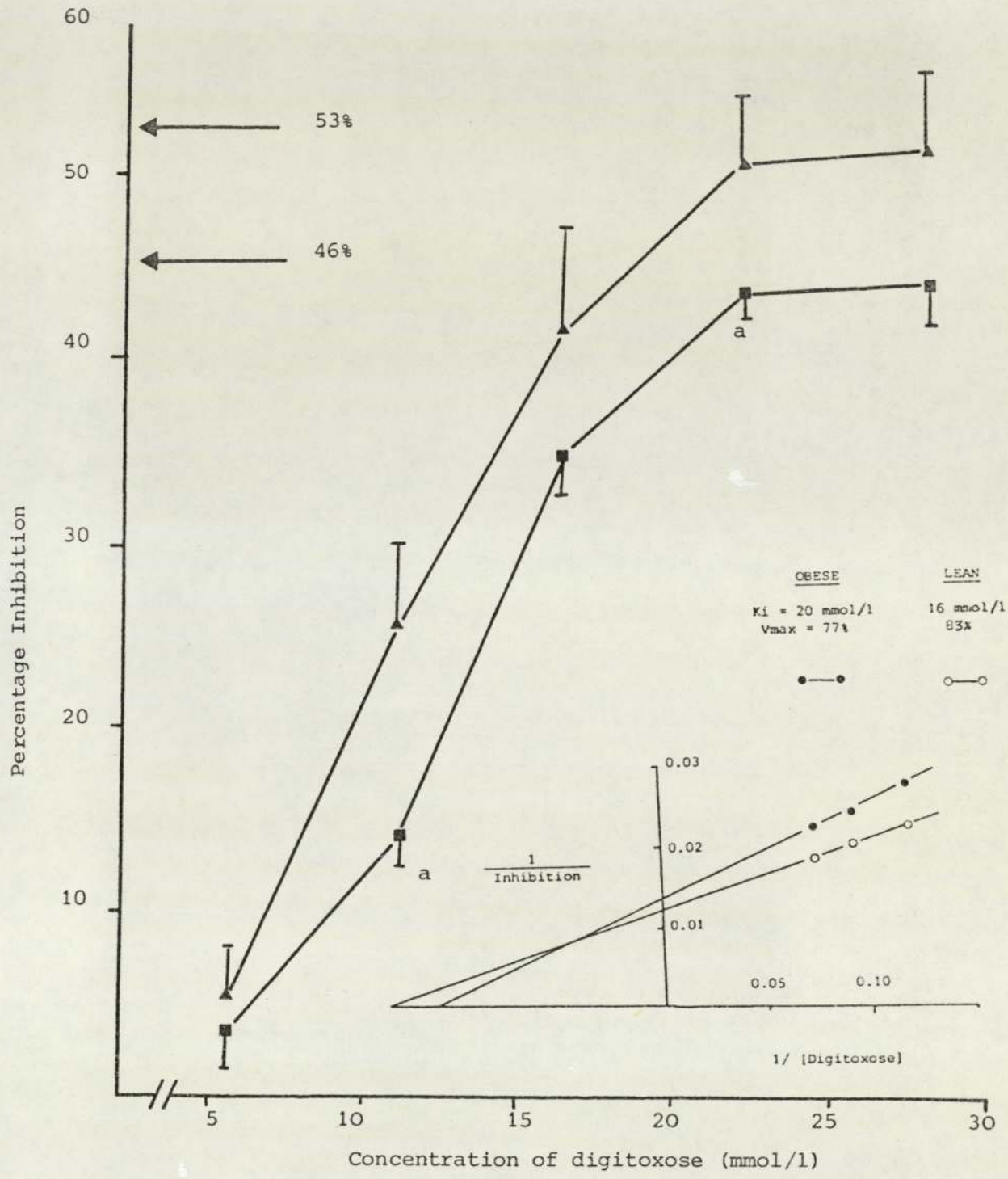
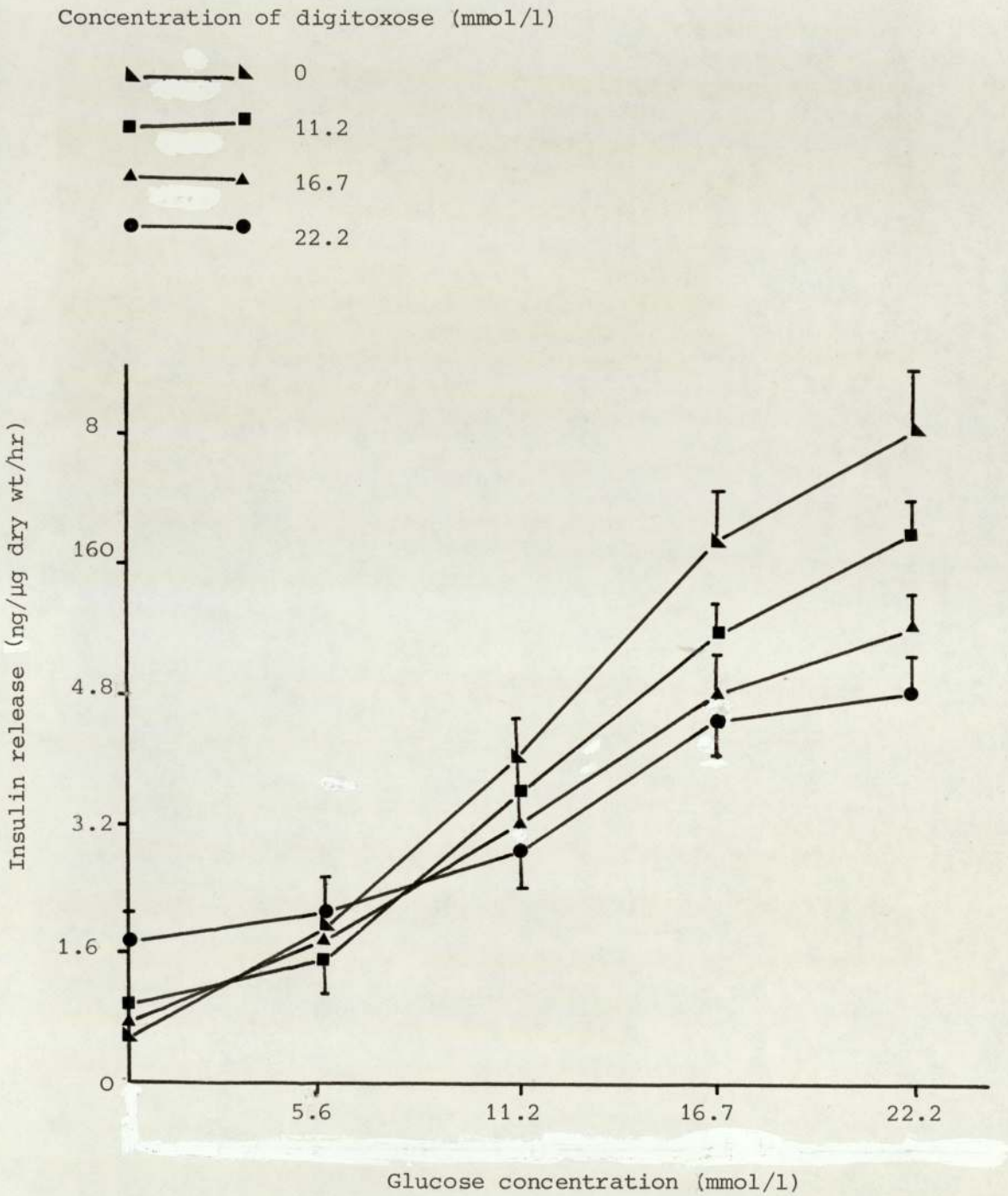


Figure 15. The effect of different concentrations of digitoxose on the glucose-dose response curve for insulin release from lean mouse islets. Mean values ± SEM of 4 separate experiments.



that the B-cells were acutely sensitive to small changes in glucose concentration within this range. At still higher concentrations of glucose the curve tended to reach a plateau. In the presence of increasing concentrations of digitoxose (11.2, 16.7 and 22.2 mmol/l) the curves were progressively shifted towards the right. This is a characteristic feature of competitive inhibition, whereas the Vmax (in terms of the rate of insulin release) is only slightly reduced but the Km (glucose concentration required to produce half Vmax) rises moderately with increasing concentrations of digitoxose. A Line-Weaver Burke plot of these data gave Km values of 10, 13.2, 15.7 and 19.0 mmol/l for glucose in the presence of digitoxose concentrations of 0, 11.2, 16.7 and 22.2 mmol/l respectively.

2 The Effect of Digitoxose on the Rate of Oxygen Uptake by lean and Obese Mouse Islets

The rate of oxygen consumption by lean mouse islets was significantly greater than that of obese mouse islets in the presence of 5.6 mmol/l but not in the presence of 16.7 mmol/l glucose, Table 2. On the other hand, the cumulative insulin release by lean mouse islets was significantly greater than that for obese mouse islets in the presence of 16.7 mmol/l glucose but not in the presence of 5.6 mmol/l glucose, Table 3. Increasing the glucose concentration from 5.6 to 16.7 mmol/l significantly increased the rate of oxygen consumption, Table 2 and cumulative insulin

release, Table 3, by both lean and obese mouse islets. Digitoxose (22.2 mmol/l) had no significant effect on the rate of glucose (16.7 mmol/l) stimulated oxygen consumption by either lean or obese mouse islets, Table 1, but significantly reduced the cumulative insulin release in response to glucose (16.7 mmol/l) from both lean and obese mouse islets by 41% and 38% respectively.

Table 2. The Effect of Digitoxose (22.2 mmol/l) on the Rate of Glucose Stimulated Oxygen Consumption by Lean and ob/ob Mouse Islets

		Oxygen Consumption ($ngO_2/\mu g$ dry weight of islets/h)		
Glucose (mmol/l)	n	lean mouse islets	n	ob/ob mouse islets
5.6	6	11.2 ± 1.0^a	6	8.2 ± 0.6
16.7	4	14.9 ± 0.5^b	5	12.8 ± 0.8^b
16.7 + Digitoxose (22.2 mmol/l)	4	15.7 ± 1.1	5	13.2 ± 1.2

Results are expressed as means ± SEM

a: $p < 0.05$ compared with corresponding value for ob/ob mouse islets.

b: $p < 0.05$ compared with values in the presence of 5.6 mmol/l glucose.

Table 3. The Effect of Digitoxose (22.2 mmol/l) on Cumulative Insulin Release from Islets of Lean and ob/ob Mice

	Cumulative Insulin Release (μU/μg dry weight of islets/h)			
Glucose (mmol/l)	n	lean mouse islets	n	ob/ob mouse islets
5.6	7	2.2 ± 0.3	7	2.1 ± 0.25
16.7	4	4.9 ± 0.5	5	3.75 ± 0.4
16.7 + Digitoxose (22.2 mmol/l)	4	2.8 ± 0.3	5	2.3 ± 0.2

Results are expressed as mean ± SEM

a: $p < 0.05$ compared with the corresponding value for ob/ob mouse islets.

b: $p < 0.05$ compared with values in presence of 16.7 mmol/l glucose alone.

3 The Effect of Digitoxose on the Rate of Glucose Oxidation by Lean and Obese Mouse Islets

In preliminary studies the effect of increasing glucose concentrations (5.6, 11.2, 16.7 and 22.2 mmol/l) on the rate of glucose oxidation by lean and obese mouse islets was investigated and the results summarized in Figure 16. The rate of glucose oxidation was found to depend markedly on the extracellular glucose concentration. The most striking feature of these dose-response curves is their sigmoidal shape relating extracellular glucose concentration and the rate of glucose oxidation. Increasing the glucose concentration from 5.6 up to 16.7 mmol/l led to a marked increase in the rate of glucose oxidation. At concentrations above 16.7 mmol/l the rate of glucose oxidation tended to a plateau. A double reciprocal plot of these data revealed that the concentration of glucose required for the half maximum rate of glucose oxidation was 9.0 and 12.0 mmol/l for the lean and the obese mouse islets respectively and the values for the Vmax were 32.3 and 31.2 pmole/µg dry weight/h for the lean and obese mouse islets respectively. The lean mouse islets showed a slightly higher rate of glucose oxidation than obese but this difference was not statistically significant.

Table 4 shows the effect of digitoxose (22.2 mmol/l) on the rate of glucose oxidation. Digitoxose (22.2 mmol/l) had no significant effect on the rate of glucose oxidation by islets from either lean or obese mice.

Figure 16. Effect of increasing glucose concentrations on the rate of glucose oxidation by lean and obese mouse islets. Mean values ± SEM of 4 separate experiments.

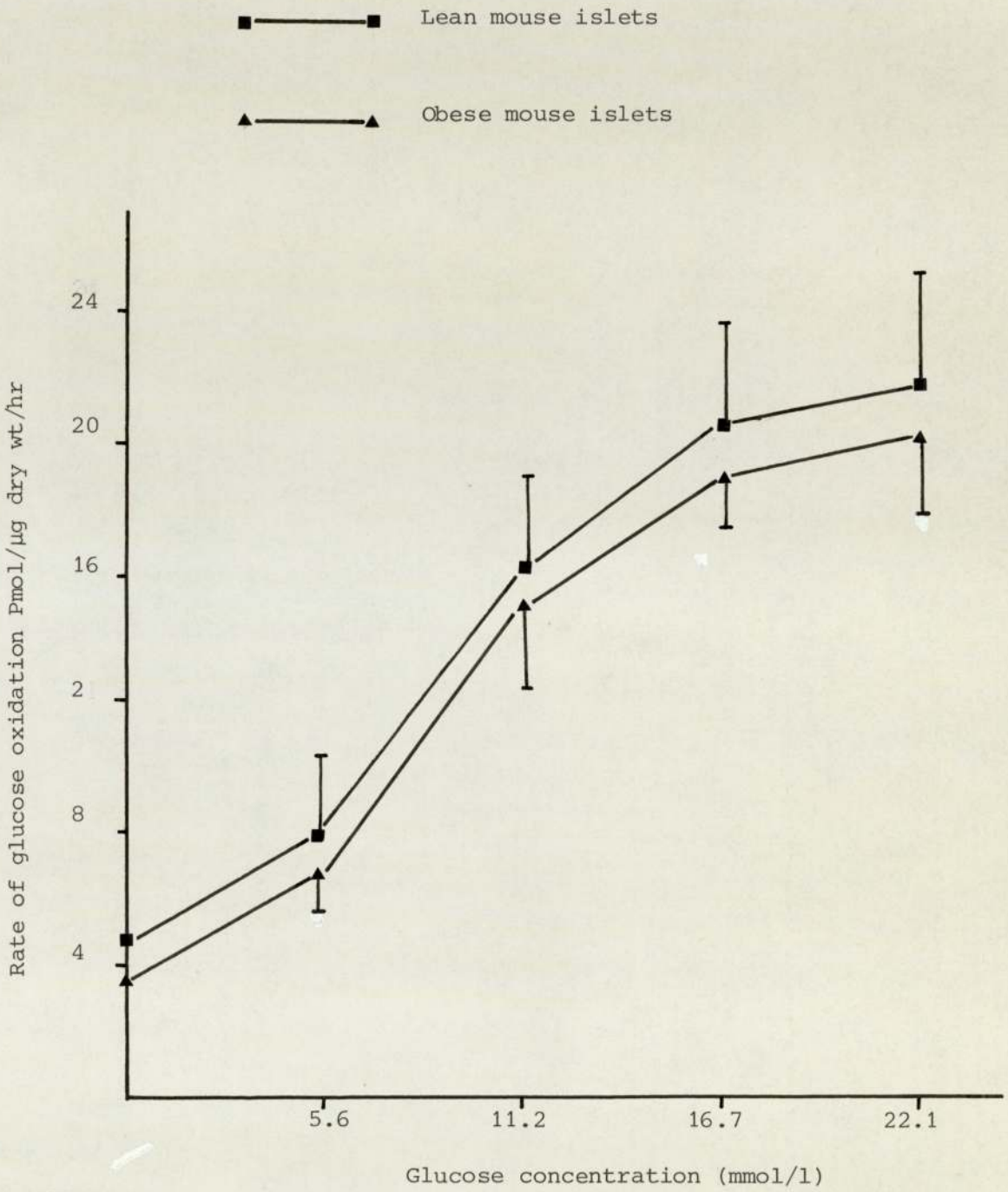


Table 4. The Effect of Digitoxose (22.2 mmol/l) on the Rate of D-[U-^{14}C] Glucose Oxidation by Lean and ob/ob Mouse Islets

		D-[U-^{14}C] Glucose Oxidation (pmol/l/µg dry weight of islets/h)	
Glucose (mmol/l)	n	lean mouse islets	ob/ob mouse islets
16.7	5	14.0 ± 1.5	12.3 ± 2.7[a]
16.7 + Digitoxose (22.2 mmol/l)	5	12.9 ± 0.7	11.9 ± 2.4[a]

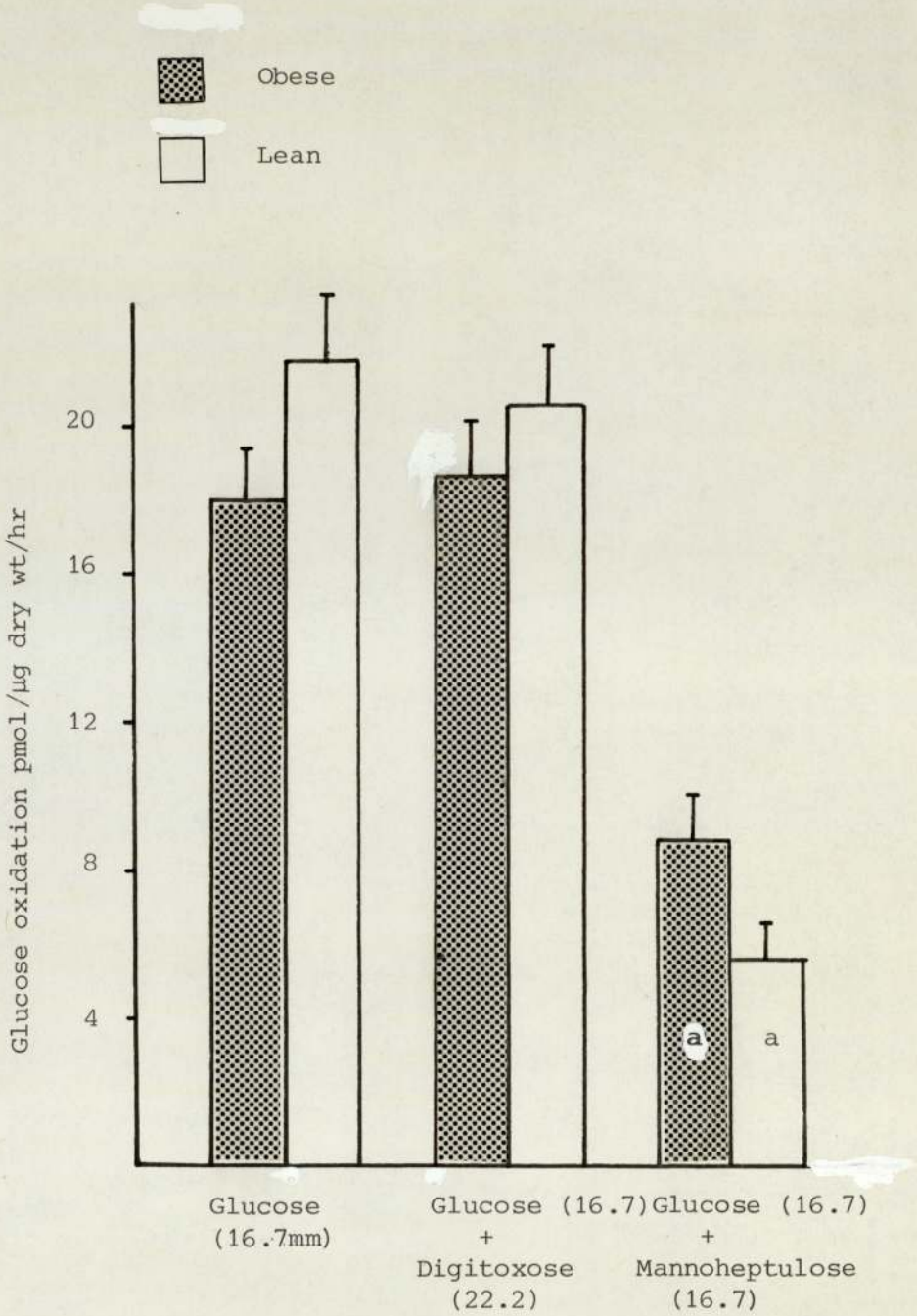
Results are expressed as mean + SEM

a: NS compared with lean mouse islets

Figure 17 shows the results of another series of experiments performed to compare the effects of digitoxose (22.2 mmol/l) and mannoheptulose (16.7 mmol/l) on rate of glucose oxidation by lean and obese mouse islets. Whilst digitoxose was without any effect, mannoheptulose significantly inhibited the rate of glucose oxidation by both lean and obese mouse islets. The percentage inhibition was 75 and 52% in the lean and obese mouse islets respectively.

The term glucose oxidation as used in the present work refers to the conversion of glucose carbon into carbon dioxide, calculated from the incorporation of ^{14}C derived from D-[U-^{14}C] glucose into carbon dioxide using the specific activity of glucose in the medium. However, the true rate of glucose oxidation may exceed this estimate because of the presence of pools of unlabelled metabolites of glucose in the islets at the beginning of the incubation and the possibility of some delay in temperature equilibration of the incubation medium.

Figure 17. Effect of digitoxose and mannoheptulose on the rate of glucose oxidation by lean and obese mouse islets. Mean values ± SEM of 5 separate experiments. a $P < 0.001$ compared to values in the absence of mannoheptulose.



4 The Specificity of Digitoxose Induced Inhibition of Insulin Release From Lean Mouse Islets

It is possible that digitoxose may be interfering with the insulin secretory process distal to the glucose recognition stage. In order to investigate this possibility lean mouse islets were incubated in the presence of a number of well documented insulin secretagogues, L-leucine, L-arginine and glibenclamide. Table 5 shows that digitoxose specifically inhibited glucose (16.7 mmol/l) stimulated insulin release but was without effect on the insulin secretory response elicited by either L-leucine, L-arginine or glibenclamide. Providing further evidence for the hypothesis that the digitoxose interacts specifically with a membranal glucoreceptor.

Table 5. Specificity of Digitoxose (22.2 mmol/l) Inhibition of Insulin Release from Lean Mouse Islets

	Insulin Release (µU insulin/µg dry weight of islets/h)			
Secretagogue	n	-Digitoxose	n	+ Digitoxose
None	5	22 ± 2.5	4	30 ± 3.9
D-glucose (16.7 mmol/l)	4	123 ± 6.1	7	73 ± 3.2[a]
L-leucine (36.5 mmol/l)	10	104 ± 7.6	11	96 ± 5.3
L-arginine (19 mmol/l)	8	102 ± 9.4	10	100 ± 6.5
Glibenclamide (8.1 µmol/l)	8	83 ± 2.7	7	79 ± 4.8

Results are expressed as mean ± SEM

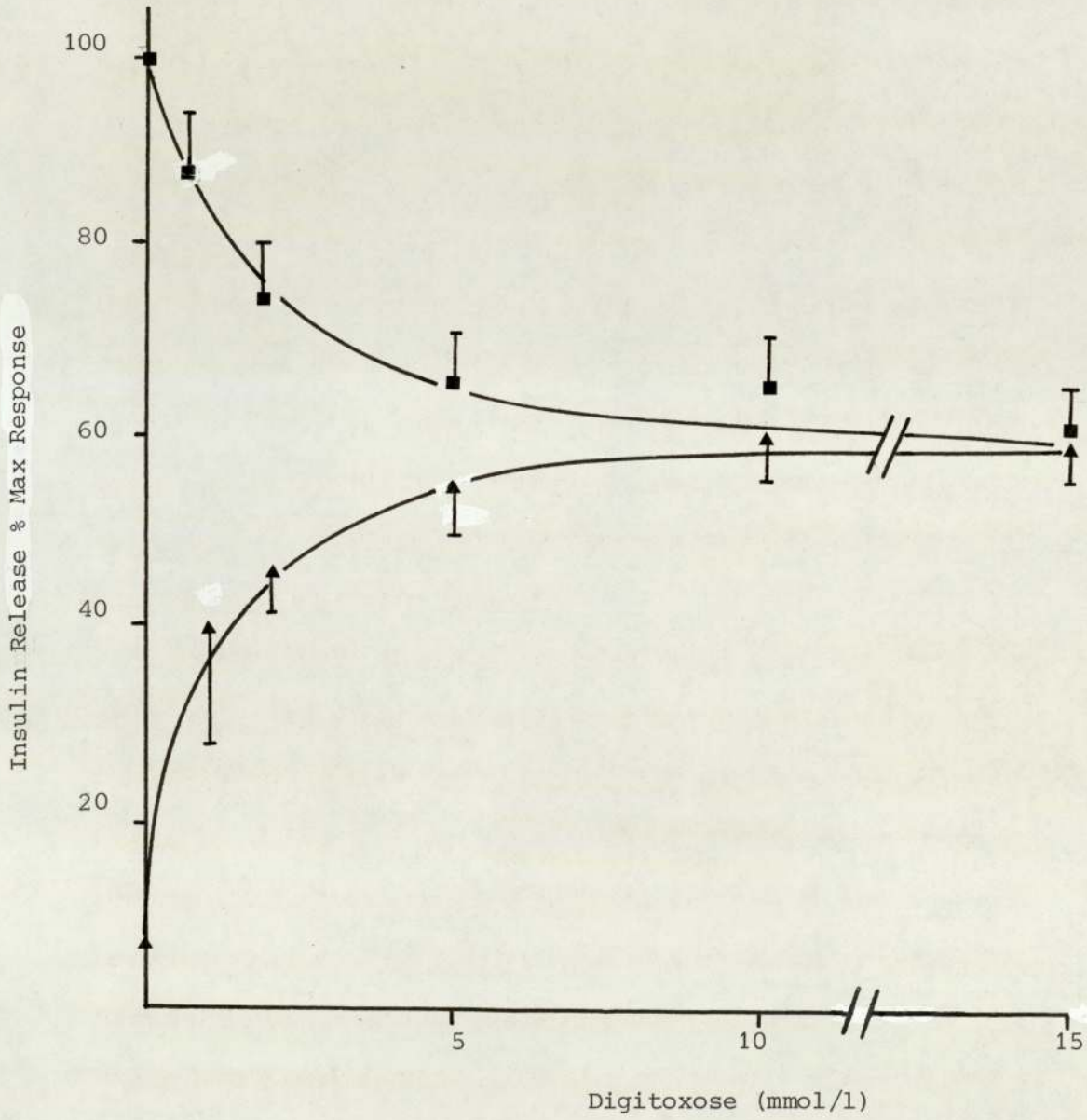
a: $p < 0.05$ compared with value in the absence of digitoxose.

DISCUSSION

The results indicate that digitoxose specifically and competitively inhibits glucose stimulated insulin release from islets of both lean and obese mice and these data are consistent with the previous observations made on the isolated rat islets (153,154). In Figure 14 it can be seen that 22.2 mmol/l digitoxose exhibits a weak stimulatory effect on insulin release suggesting that at high concentrations digitoxose behaves as a partial agonist (ie. an agonist to which the maximal response is less than that produced by the active agonist acting on the same set of receptors, 161). When a partial agonist produces its maximal response all the receptors are assumed to be occupied, but the efficacy (ie. the capacity of a drug to initiate a response once it occupies the receptor sites) is such that the stimulus produced is not sufficient to provide the maximal response potentially obtainable in the system, digitoxose at high concentrations appears to exhibit this feature of a partial agonist.

Recently Dr N R Lazarus (personnel communication) has observed a similar but much marked effect of digitoxose on insulin release from isolated rabbit islets. His data are summarized in Figure 18. 10 mmol/l digitoxose produced a stimulation of insulin release from rabbit islets equivalent to 60% of the maximum response obtained with glucose (16.7 mmol/l) alone. Whereas 22.2 mmol/l digitoxose produced a stimulation of insulin release from mouse islets equivalent to 25% of the maximum response obtained with glucose (16.7 mmol/l) alone (Figure 15). Digitoxose exhibited

Figure 18. The effect of digitoxose on insulin release from isolated rabbit islets. In the absence of glucose (▲—▲) and in the presence of glucose (■—■). From Lazarus personal communication.



a weaker partial agonist activity in mouse islets than with rabbit islets. This is not surprising, since it is well known in pharmacology that the same drug tested either on different tissues in the same species or the same tissue in different species can behave as an active agonist, partial agonist or an inhibitor (161-163). The explanation of this phenomenon appears to be that there is a change in receptor number either from tissue to tissue or from species to species (164). This theory may explain the difference in the partial agonistic behaviour of digitoxose with rabbit and mouse islets. The percentage inhibition of glucose stimulated insulin release by digitoxose from statitically incubated obese mouse islets was less (46%) than that for lean mouse islets (53%) suggesting obese mouse islets to be marginally more resistant to the inhibitory effect of digitoxose than lean mouse islets.

The oxygen uptake values obtained in the present study were somewhat higher than those reported in previous studies (see Table 6). A possible explanation for these differences could be the presence of bicarbonate ions in the incubation medium, since the ion has been shown to increase the rate of oxygen uptake in mouse pancreatic islets by 80% (165). Pancreatic islets contain a HCO_3-sensitive ATPase, and the ion has also been shown to be essential for glucose stimulated insulin release (167). A further explanation for these high oxygen uptake values obtained in the present study may be the continuous stirring of the incubation medium. It has been shown that thorough stirring of incubation

medium increases the rate of oxygen uptake values by 40% compared with non-stirred medium (168). The rapid stirring probably prevents the accumulation of inhibitory secretion products in the vicinity of islets. Futhermore, species differences should also be taken into consideration when comparing the data in Table 5. This comparison however, above may not be totally valid since in the present study the rate of oxygen uptake was measured with an apparatus based on a principle wholly different from that of cartesian divers.

Table 6. A comparison of the rate of oxygen uptake obtained by other workers with values obtained in this study. References are shown in paranthesis. All values are expressed as ngO_2/µg dry islet weight/hr.

Glucose (mmol/l)	Obese mouse islets (169)	Albino mouse islets (165)	Albino mouse islets (168)*	Present Study	
				Lean	Obese
Endogenous	-	1.7	-	-	-
Basal	-	-	3.9	-	-
2.5	-	2.4	-	-	-
5.6	-		4.5	11.2	8.2
16.7	6.2	4.3		14.9	12.8
20.0	-	-	8.9	-	-

*Bicarbonate free incubation medium

The increase in extracellular glucose concentration from 5.6 to 16.7 (mmol/l) resulted in a significant increase in the rate of oxygen uptake by 33% and 36% for lean and obese mouse islets respectively. The fact that oxygen uptake is intimately linked with oxidative phosphorylation has been confirmed by the demonstration that 2,4-dinitrophenol stimulated the rate of O_2 consumption whilst antimycin A and rotenone inhibit islet respiratory activity (158). Digitoxose (22.2 mmol/l) was found to have no effect on oxygen uptake but significantly inhibited glucose-induced insulin release. The fact that digitoxose had no effect on the oxidative metabolism of the islets but significantly inhibited the insulin secretory process suggest a dual role for glucose (namely, the provision of energy for the secretory process and as a stimulus for insulin release).

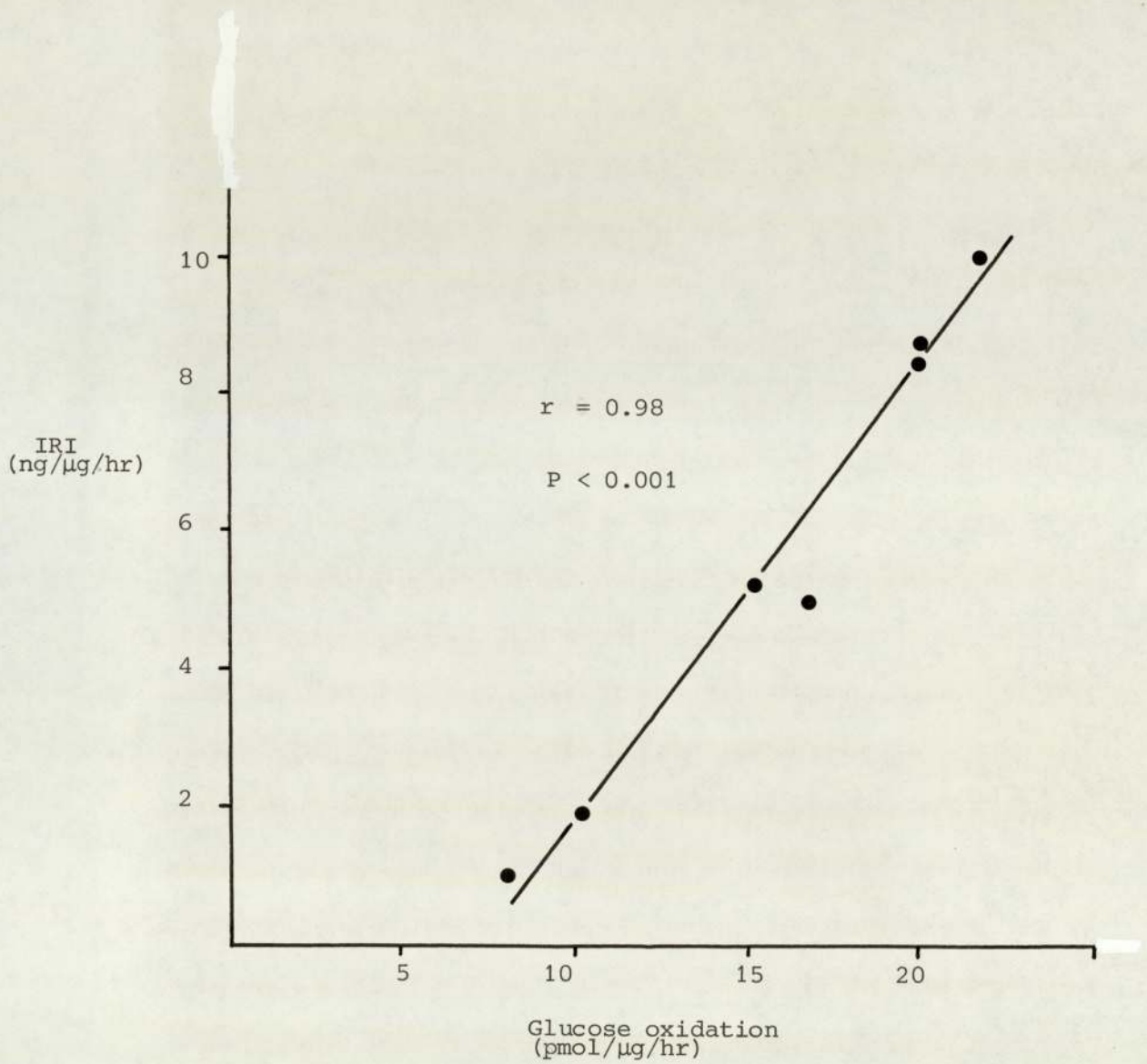
Digitoxose was found to have no effect on glucose oxidation by both lean and obese mouse islets Table 4. However, mannoheptulose significantly inhibited glucose oxidation (Figure 16). It has been reported that mannoheptulose competitively inhibits glucose phosphorylation in mouse (170) and rat (171) islets. However, it has also been emphasized that caution should be used when interpreting the effects of mannoheptulose because, although it inhibits glucose oxidation and lowers glucose-6-phosphate concentrations markedly in islets, it does not decrease lactate output (165). Moreover, the kinetics of mannoheptulose inhibition of hexokinase do not completely account for the profound inhibition of glucose oxidation by this sugar

(172). Mannoheptulose has also been shown to inhibit the oxidation of pyruvate (165) and this observation might explain why glucose oxidation to CO_2 is completely blocked by mannohpelulose, when glucose phosphorylation is only inhibited by 20% and lactate output not reduced at all. Mannoheptulose may also influence the carrier-mediated transport of glucose across the B-cell (173).

Since digitoxose has been shown to have no effect on the rate of glucose oxidation it is speculated that digitoxose has no effect on any of the processes intimately involved in glucose oxidation. This observation supports the previous finding that digitoxose has no effect on the rate of oxygen consumption by islets and supports the proposal that digitoxose dissociates the fuel function of glucose from its signal function in the mechanism of insulin release.

A statistically significant positive correlation exists between the rate of glucose oxidation and insulin release, Figure 19, ($r = 0.98$, $p < 0.001$). A similar positive correlation has been reported by Ashcroft, Hedeskov and Randle (159) but this positive correlation between metabolic and secretory parameters does not absolutely prove a cause and effect relationship. In fact such close correlation between metabolic and secretory processes is well documented for other tissues, for example the acid secretion from parietal cells is an highly energy dependent process which is initiated by the interaction of secretagogues such as acetylcholine, gastrin, secretion and cholecystokinin with specific

Figure 19. The relationship between the rates of glucose oxidation and insulin release from lean mouse islets (data from Figure 15 and 16)



receptors (174, 175). In the B-cell glucose carries out both fuel and signal function by interacting with a glucoreceptor to initiate the secretory process and serving as a substrate for the intermediary metabolism to provide energy for the secretory process.

In conclusion, the present data suggest that digitoxose, which is not metabolized to any extent by isolated islets (154), inhibits glucose stimulated insulin release from mouse islets without affecting the oxidative glucose metabolism. Therefore digitoxose is able to dissociate glucose stimulated insulin release from metabolism of glucose. This property is not exclusive to digitoxose, since the α-adrenergic agonist clonidine will also produce a dose related inhibition of glucose stimulated insulin release without affecting glucose oxidation (176). In addition, glucose releases 30 fold more insulin from islets than from single B-cells and the dependency of glucose induced insulin release upon the functional co-ordination between islet cells is not mediated through changes in glucose metabolism alone, suggesting that the latter are not sufficient for the appropriate regulation of insulin release by glucose (177). The fact that B-cell rich obese mouse islets appear to be more resistant to the inhibitory effect of digitoxose than lean, suggests that the process of glucose recognition may be altered in obese mouse islets, indeed the latter have been shown to be less sensitive than lean to lower concentrations (1-9 mmol/l) of a gradient of α-D-glucose (178).

CHAPTER 4

SIMULTANEOUS MEASUREMENT OF GLUCOSE OXIDATION AND INSULIN RELEASE FROM LEAN AND OBESE MOUSE ISLETS

Introduction

Data presented so far suggest that digitoxose is capable of dissociating secretion signal from the fuel function of glucose in the process of insulin secretion. These observations support the concept of glucoreceptor on the membrane of B-cell. However, more unequivocal evidence would be provided by a study in which the rates of glucose oxidation and insulin release were measured simultaneously and continuously. A comparison of the effect(s) of digitoxose on these two parameters might help to clarify any potential link between glucose metabolism and insulin release.

To date there have been no studies in which the rates of glucose oxidation and insulin release have been measured simultaneously and continuously. For this purpose a method has been developed based on that originally described for the simultaneous measurement of acid secretion and substrate oxidation by isolated dog gastric mucosa (179,180). The gastric mucosa was contained inside a tissue chamber and perifused with ^{14}C-labelled substrates, the perifusate was pumped into a gassing chamber using a peristaltic pump. Here a constant stream of gas (95% O_2 and 5% CO_2) removed and swept the $^{14}CO_2$ (produced by the metabolizing gastric mucosa) into the ionization chamber. The perifusate was pumped out from the gassing chamber at a rate

equal to its inflow and collected in tubes for determination of acid secretion. The $^{14}CO_2$ swept into the ionization chamber induced a tiny current which was detected by the vibrating reed electrometer. Since the instrument was calibrated the exact amount of substrate oxidation was calculated.

In the present study the effects of digitoxose and mannoheptulose on the rates of glucose oxidation and insulin release from pancreatic islets have been investigated.

MATERIALS AND METHODS

1 Animals

Homozygous obese (ob/ob) and homozygous lean (+/+) mice from the Aston colony were used for this study. The conditions under which the animals were maintained, their characteristics and the origins of the colony have previously been described (103).

Chemicals

Mannoheptulose was purchased from Sigma Co Ltd, Poole, Dorset; D-[U-^{14}C] glucose (specific gravity 270 mci/mmol) from Amersham, Bucks, England; $Ba^{14}CO_3$ from NEN, Boston, Mass, USA. All other chemicals were of analytical grade and purchased from British Drug House, Atherstone, England.

2 The Apparatus for Simultaneous Measurement of the Rates of Glucose Oxidation and Insulin Release

The apparatus designed for the simultaneous measurement of the rate of glucose oxidation and insulin release is shown in Figure 20 and consisted of a Vibrating Reed Electrometer Cary 401 Type (VRE), an ionization chamber of volume 275 ml (Varian Associates Ltd, Surrey), a gassing chamber (plate 4) and an incubation vessel. The vibrating reed electrometer was used without critical damping and a voltage of 90V applied across the terminals of the ionization chamber with 10 9V Ever Ready batteries (PP3/6F22) connected in series. A high resistance leak method using a 10^{12} ohm resistor was employed to measure the tiny ionization current generated by the $^{14}CO_2$ produced from D-[U-^{14}C] glucose by the metabolizing tissue. As the radiolabelled CO_2 passes through the ionization chamber it ionizes the gas inside the chamber, the application of 90V voltage across the electrodes of the chamber causes this tiny current to flow towards the cathode of the chamber. The electrode detects and amplifies this minute current. The VRE is a very sensitive instrument capable of detecting electron current as small as 10^{-17} Amps (approximately 100 electrons) (181). The output from the electrometer was connected to a potentiometric chart recorder (Serro Sorbic RE 540, Smith Industries Ltd, London). The rate of carrier gas flowing through the system was determined with the aid of a floating ball type gas flowmeter (Platon Flowbits Ltd, Basingstoke).

Figure 20. Diagram of the apparatus for the simultaneous measurement of the rate of glucose oxidation and insulin secretion from islets of Langerhans

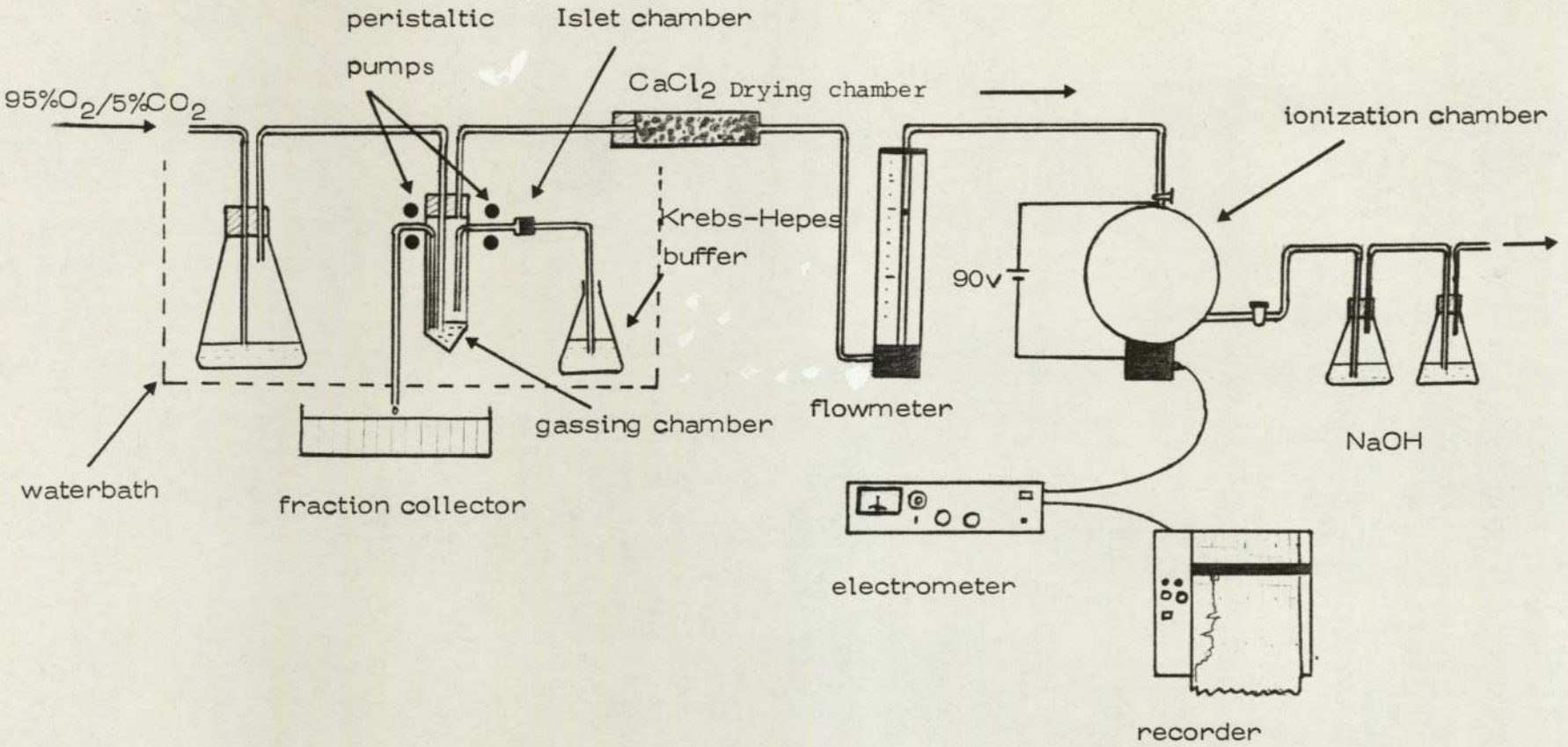


PLATE 3. Apparatus for the simultaneous measurement of glucose oxidation and insulin release from isolated islets.

(a) gassing
(b) islet
(c) drying
(d) ionization chambers
(e) electrometer.

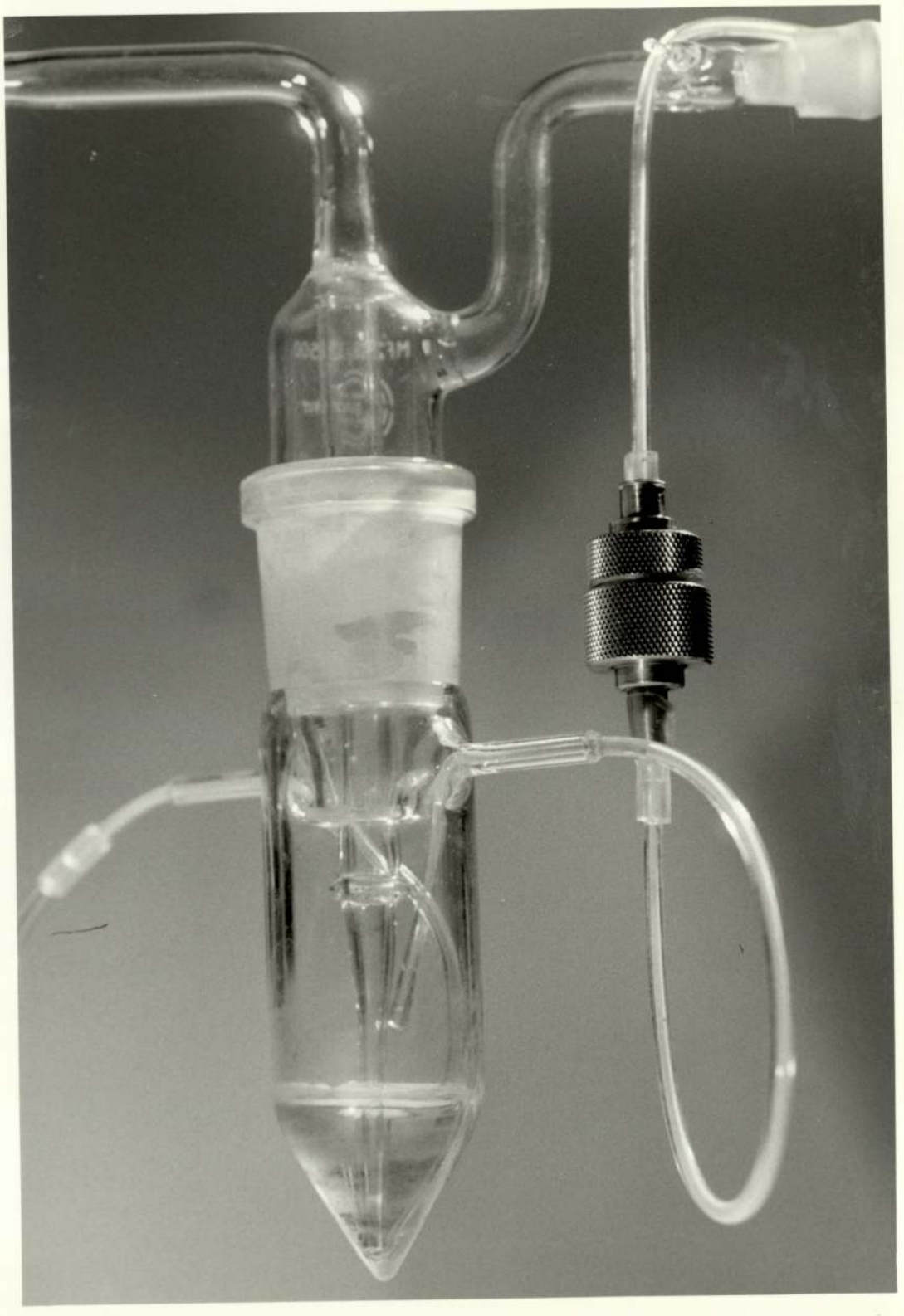


PLATE 4. The gassing chamber.

(a) Islet chamber

(b) Inlet

(c) Outlet

The islets were incubated in a Swinnex Filter Holder as described previously in Chapter 2. The islet chamber was fitted into the system as shown in Figure 20 and Plate 3. Krebs-Hepes buffer was drawn through the islet chamber with the aid of a peristaltic pump at a constant flow rate of 0.4 ml/min. The perifusate passed into the gassing chamber where it was thoroughly aerated and any $^{14}CO_2$ thus liberated was carried through the drying chamber into the ionization chamber. The perifusate was purged from the gassing chamber with a second peristaltic pump maintained at the same flow rate and collected in tubes for insulin assay. Using this set up the rates of perifusate inflow and outflow were kept constant throughout the experiment. The gassing chamber, islet incubation chamber and Krebs-Hepes buffer were maintained at 37^0C in a water bath.

Principle of Operation and Setting up of the Vibrating Reed Electrometer

The Cary 401 Vibrating Reed Electrometer is a versatile instrument capable of measuring either electrostatic charge, current, potential or resistance (Figure 21). It detects charge as small as 5×10^{-6} Coulomb, current as low as 10^{-17} ampere, potentials down to 2×10^{-5} volts and resistance in excess of 10^{12} ohms.

The Cary 401 VRE measures electrons (charge) as they collect on the charge collecting capacitor Cc (Figure 22). This input signal is converted to alternating current by the vibrating reed

Figure 21. Cary 401 Vibrating Reed Electrometer

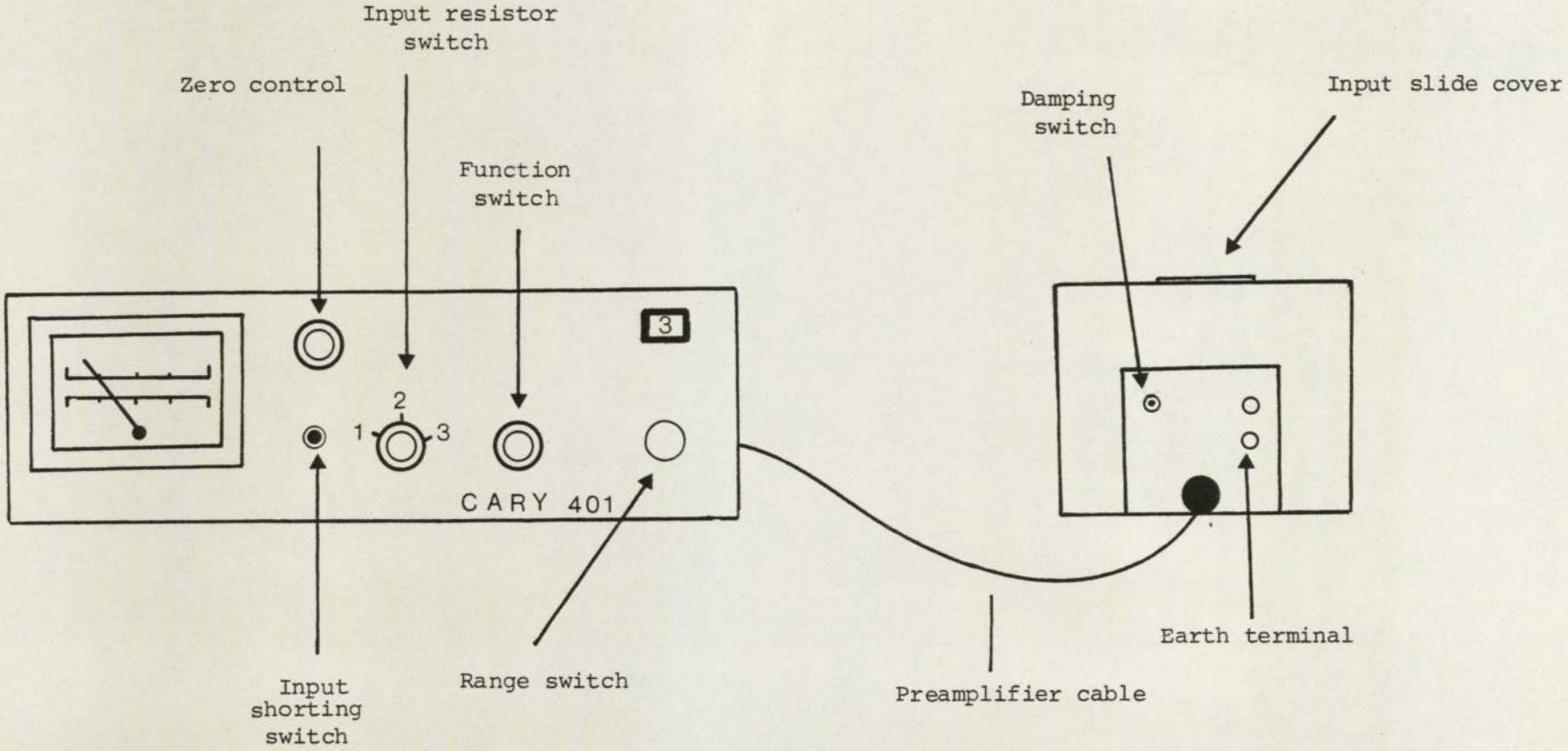
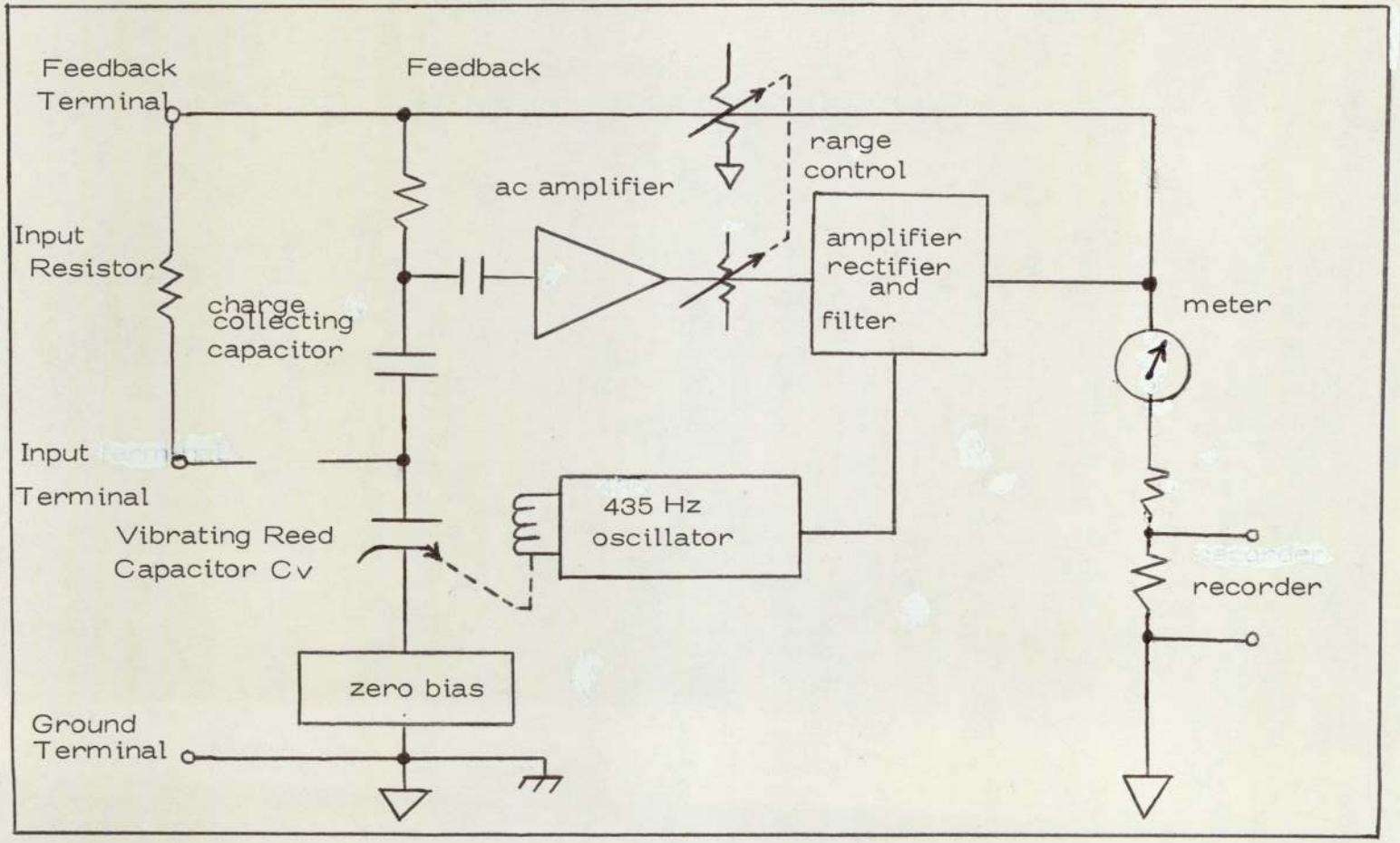


Figure 22. Electrical Circuit for Cary 401 Electrometer



capacitor Cv. The AC signal produced is proportional to the accumulated charge and is amplified by an AC amplifier, synchronously rectified, filtered and used to drive the indicating meter or an on line recorder.

Operating Procedure to Measure an Ionization Current

The Cary 401 VRE can be used for the measurement of radioactivity using ionization chamber. It involves the measurement of a very small ionization current generated by $^{14}CO_2$ inside the ionization chamber. Figure 21 shows a diagram of the electrometer and its controls. The operational procedure is briefly outlined below:

The amplifier was connected to the preamplifier as shown and the FUNCTION switch was rotated to the CURRENT position. The INPUT SHORTED switch was closed and DAMPING switch turned off. The RANGE switch is rotated to position marked 3V and power switched on, the pilot should light, then RANGE switch is rotated in steps from 3V to 1mV position and ZERO control switch is rotated simultaneously to maintain the panel meter reading at zero. The INPUT RESISTOR switch is positioned at 3 which is 10^{12} ohms. Finally the INPUT SHORTED switch is opened. The electrometer is now ready to measure the ionization current generated by $^{14}CO_2$ (liberated by the tissue or from $Ba^{14}CO_3$) passing through the ionization chamber.

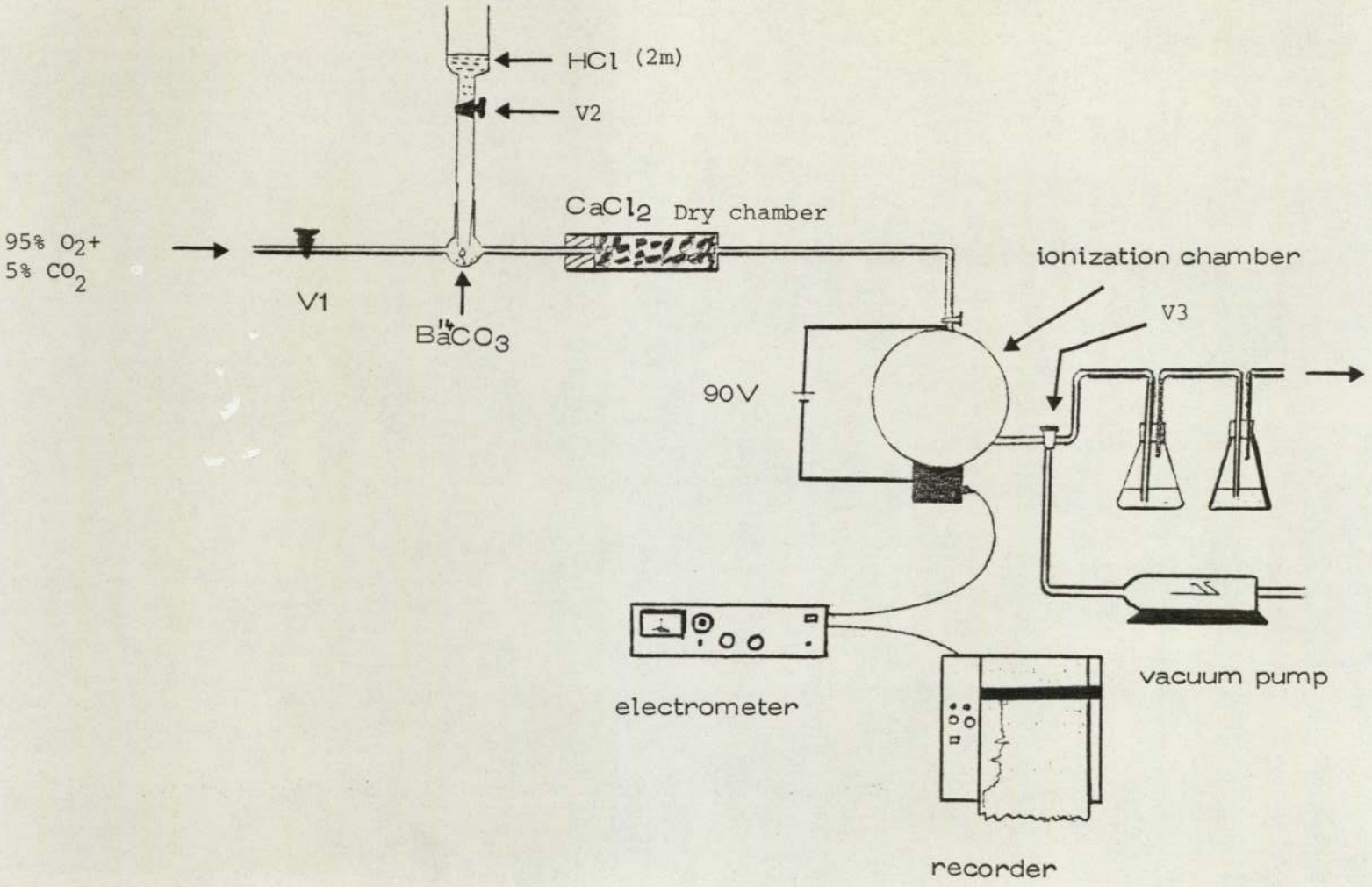
3 The Calibration of the Ionization Chamber

The ionization chamber was calibrated with $^{14}CO_2$ generated from a known amount of $Ba^{14}CO_3$ (specific activity 0.27 μCi/mg). The apparatus used for calibration is shown schematically in Figure 23 and pictorally in Plate 5.

Table 7 outlines the sequence of events in calibration procedure. Initially valve V1 was closed in order to evacuate the system followed by closure of valve V3 and the vacuum pump turned off. A few drops of 2M HCl were reacted with 1 μCi of $Ba^{14}CO_3$ by momentarily opening valve V2. The evolution of $^{14}CO_2$ from $Ba^{14}CO_3$ was completed within 30 seconds then valve V1 was briefly opened to allow approximately 300 ml of carrier gas to flow into the reaction chamber and carry the $^{14}CO_2$ into the ionization chamber. A 90V voltage was then applied across the terminals of ionization chamber and current generated by the radiolabelled CO_2 was detected by the electrometer and recorded on potentiometric chart recorder.

At the end of calibration valves V1 and V3 were opened to allow carrier gas to enter and purge the $^{14}CO_2$ into the scrubbers containing 2M NaoH. The calibration was carried out five times and the mean of the five determinations used to calculate the calibration factor, which is defined as Unit radioactivity (μci) per unit volume (ml) per volt.

Figure 23. Apparatus for the calibration of the ionization chamber.



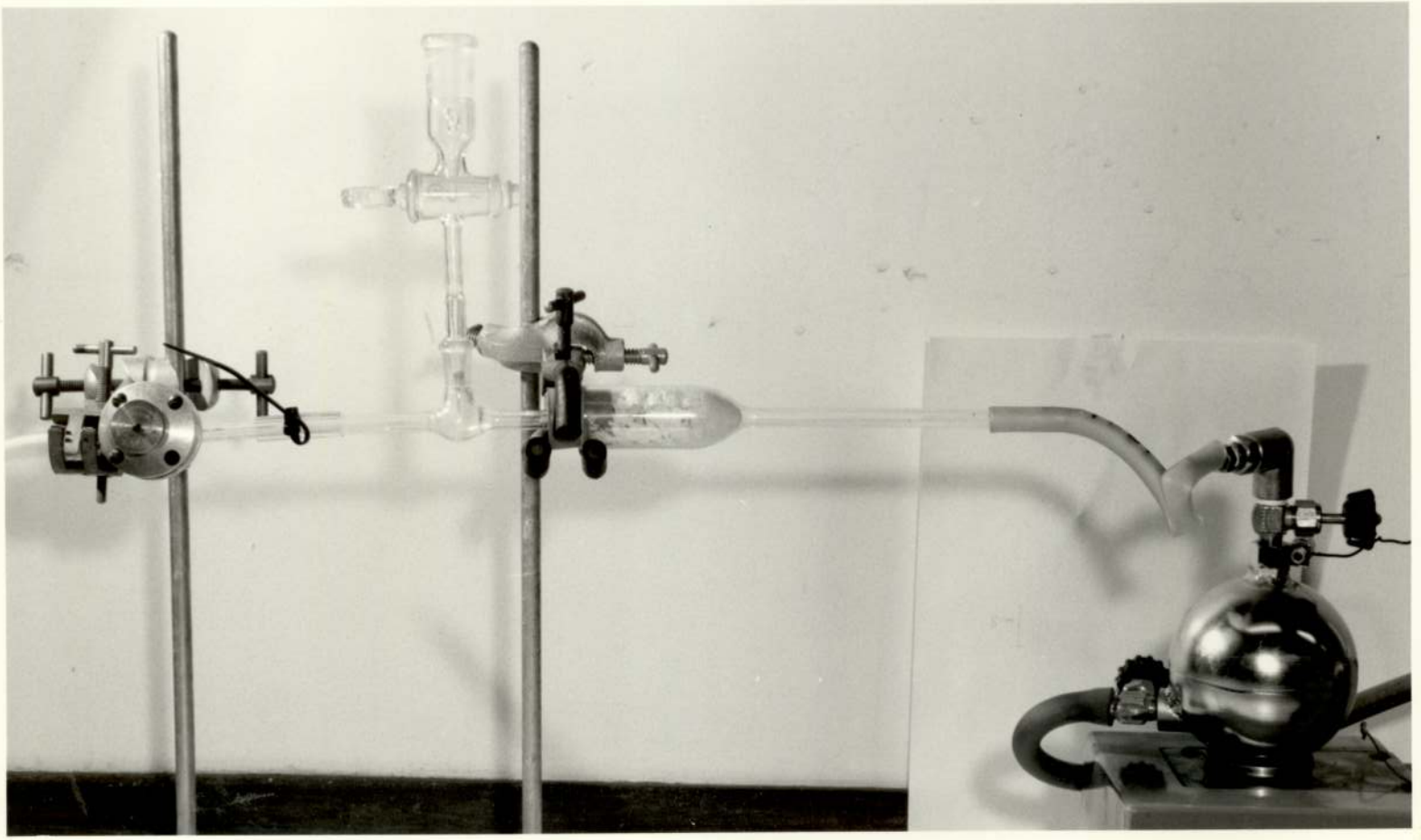


PLATE 5. Apparatus for the calibration of ionization chamber.

(a) Valve VI

(b) Reaction chamber containing $Ba^{14}CO_3$

(c) Tap funnel containing HCl (2m)

(d) Drying chamber (e) Tap V2 (f) Valve V3

The $^{14}CO_2$ evolved from 1 µci of $Ba^{14}CO_3$ produced a mean response of 2.55 V (range 2.56 - 2.74V), the total volume of the system was 285 ml (ionization chamber 275 ml and remainder of system 10 ml). Since all the variables were known the calibration factor was calculated in terms of µci/ml/mV. The calibration factor was found to be 1.37×10^{-6} µci/ml/mV.

Table 7. Summary of calibration procedure: X denotes valve closed, O denotes valve open.

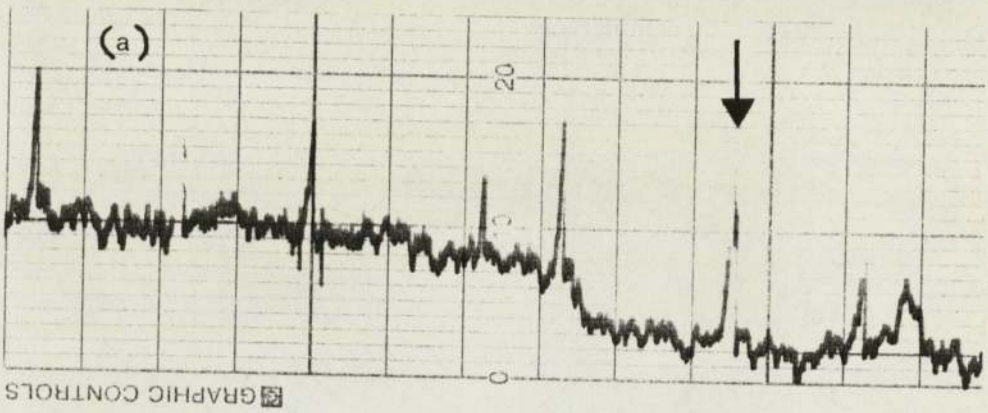
Stages	Valve V1	Valve V2	Valve V3
1 System opened	O	X	O
2 System closed for vacuum	X	X	O
3 System evacuated	X	X	X
4 $Ba^{14}CO_3$ reacted with acid	X	O	X
5 Gas allowed to flow momentarily	O	X	X
6 Followed by voltage applied to ionization chamber	X	X	X

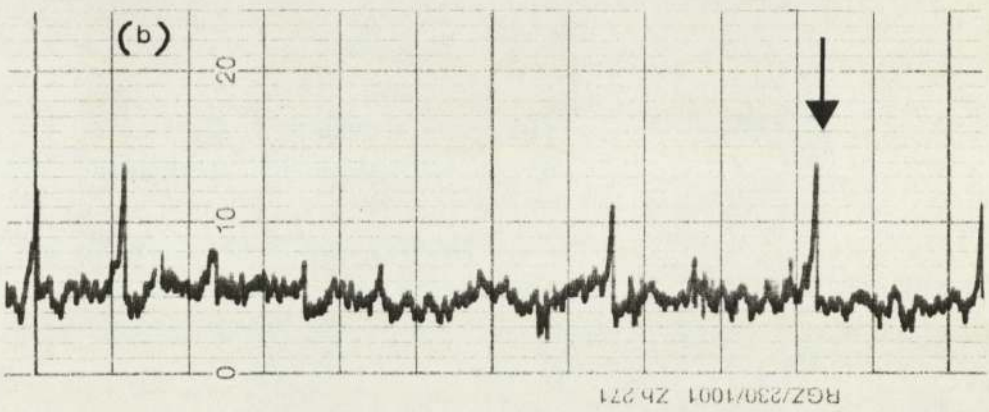
4 Purification of D-[U-^{14}C] Glucose

In preliminary experiments the D-[U-^{14}C] glucose from Amersham was found to be contaminated with small amounts of labelled volatile compounds. These compounds needed to be removed to avoid the generation of falsely high values for the rates of glucose oxidation. Purification was carried out using a rotary evaporator. The sealed vial containing the D-[U-^{14}C] glucose was opened under sterile conditions, transferred via pipette to a sterile 3 ml pearshaped flask and the latter connected to a rotary evaporator. A slight vacuum was created in the flask using a vacuum pump and the flask rotated slowly in a water bath at 55^{0}C. The contents of the flask evaporated completely within 10 minutes leaving behind purified and dried D-[U-^{14}C] glucose. The latter was then reconstituted to the original volume with sterile double distilled water. The purified labelled glucose was then aliquoted into small tubes (50 µci/tube) in amounts suitable for a single experiment. The tubes were stored at -20^{0}C until required.

To confirm that all volatile impurities had been removed, 2 mls of Krebs-Hepes buffer pH 7.4 containing 5 µci of labelled glucose was aerated inside the gassing chamber and the response from the ionization chamber monitored for 30 minutes. The response obtained was not significantly different from control experiments using 2 ml of Krebs-Hepes buffer containing no labelled glucose (Figure 24).

Figure 24. To show the presence of non-volatile compounds in ^{14}C-glucose (a) before purification (b) after purification. Arrows show the addition of labelled glucose.





5 The Isolation and Storage of Pancreatic Islets for Glucose Oxidation and Studies on Insulin Release

Islets were isolated from overnight fasted obese and lean mice as described previously (Chapter 2). Since a large number of islets (approximately 700-1000) were required for a single experiment it was found necessary to isolate the islets on one day and use them on the next. Islets were isolated under sterile conditions and kept in sterile plastic petri dishes (size 60 x 15 mm, Sterilin, Fison Scientific and Medical Equipments, Loughborough, Leicester) containing 5 ml of culture medium (Medium 199, Wellcome Diagnostics, Beckenham) supplemented with 11.2 mmol/l glucose, penicillin 100 IU/ml and streptomycin 100 µg/ml. The petri dish containing islets was retained in a tissue culture gassing jar with an atmosphere of 95% O_2 and 5% CO_2 overnight at 37°C. On the day of experiment the islets were examined under stereomicroscope prior to use to ensure sterility. Islets that were damaged or contaminated usually burst and disappeared overnight.

Preliminary Studies

In order to confirm that the system was capable of measuring small changes in the rate of glucose oxidation a series of preliminary studies was carried out using obese mouse epididymal fat pads and pieces of mouse exocrine pancreas. The modified system for the measurement of the rate of glucose oxidation is

shown in Figure 25. In this system the tissue was actually incubated in the gassing chamber.

In order to establish a background or a baseline rate of glucose oxidation a known wet weight of the fat pads was preincubated for 30 minutes in the gassing chamber containing 2 ml of Krebs-Hepes buffer pH 7.4 supplemented with glucose (5.6 mmol/l) and no labelled glucose. A carrier gas flow rate of 80 ml/min was used throughout these experiments. After the preincubation the Krebs-Hepes buffer was aspirated and fresh prewarmed buffer containing 2 µci D-[U-^{14}C] glucose was added and the rate of glucose oxidation was measured in the absence or the presence of test substances. The rate of glucose oxidation was expressed in terms of nmole of glucose oxidized per hour per gram of tissue.

The Effect of Insulin on the Rate of Glucose Oxidation by Isolated Mouse Epididymal Fat Pads

300 mg of epididymal fat pads were cut into small pieces and washed in Krebs-Hepes buffer pH 7.4. The pieces of fat were then transferred to the gassing chamber using pasteur pipettes and preincubated for 30 minutes as described above. At the end of preincubation period fresh buffer containing 2 µci of labelled glucose was added. After approximately 50 minutes 30ng/ml of bovine insulin was added and the rate of glucose oxidation monitored for a further 60 minutes. The rate of glucose oxidation was calculated as follows: a smooth continuous curve of best fit as judged by eye was drawn through the trace in Figure 26. After

Figure 25. Apparatus for the measurement of the rate of glucose oxidation by pieces of mouse pancreas and epididymal fat pad.

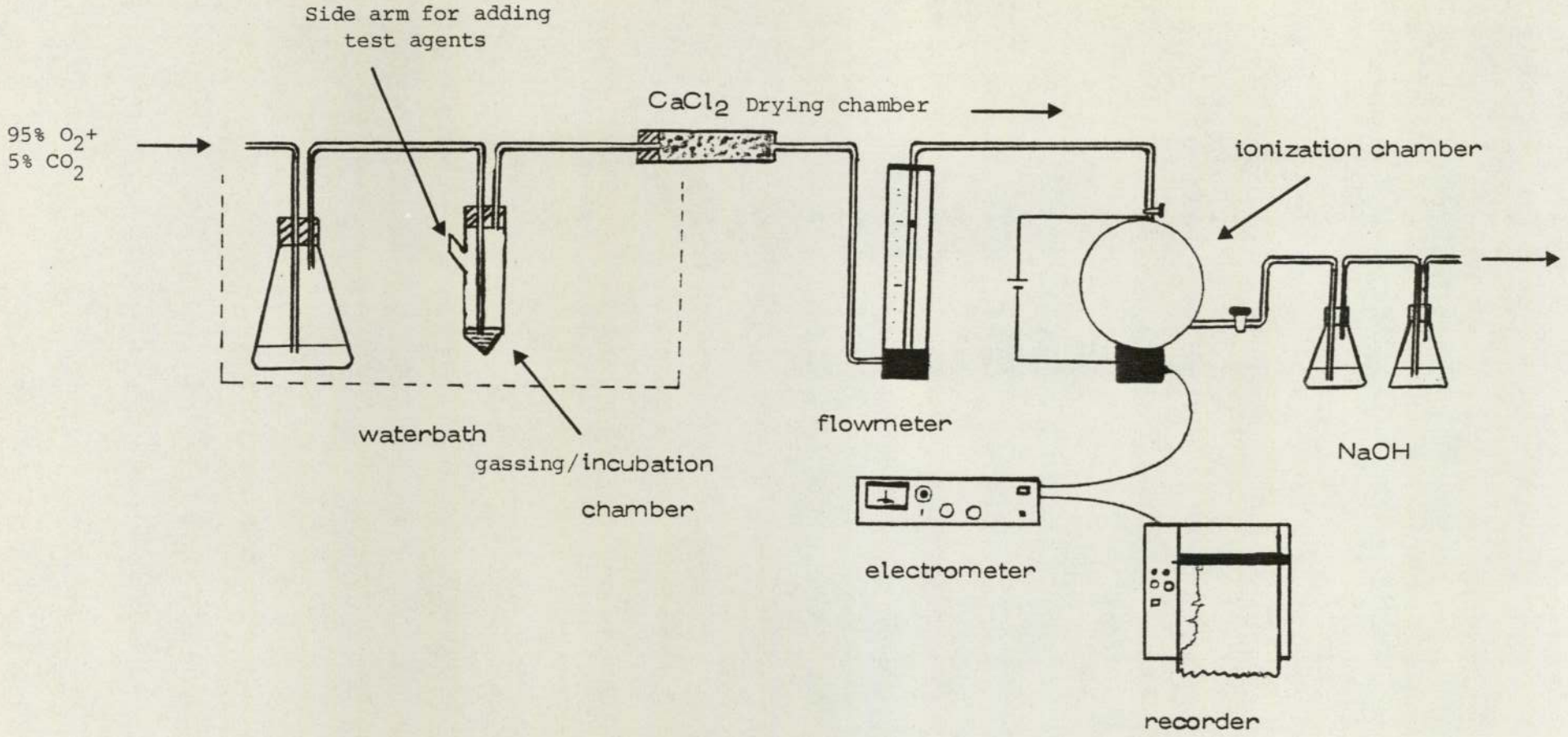
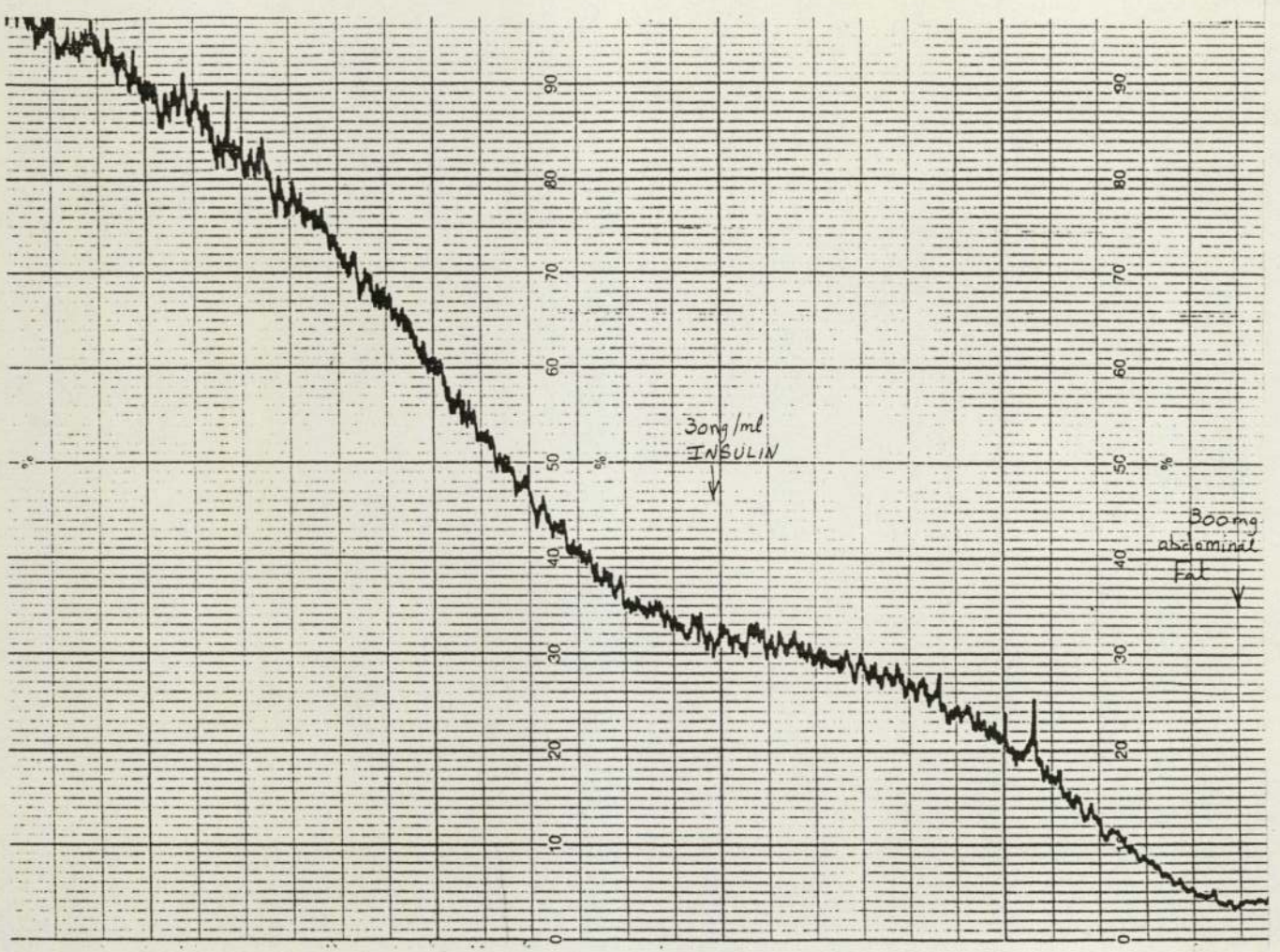


Figure 26. Original recorder trace showing the effect of insulin on the rate of glucose oxidation by pieces of epididymal fat pad.



the subtraction of the background reading, the rate of glucose oxidation was calculated for each point at 2 minute intervals along the entire curve using the following equation (180):

$$\text{Rate of Glucose Oxidation } (\mu\text{Mole/h/g}) = \frac{\text{GFR} \times \text{SA} \times \text{CF} \times \text{ER}}{\text{wt. of tissue}}$$

where:

GFR is the gas flow rate in ml/hr

SA is the specific activity in µci/µmol

CF is the calibration factor 1.37×10^{-6}, µci/ml/mV

ER is the electrometer response in mV.

All the points were then replotted as shown in Figure 27.

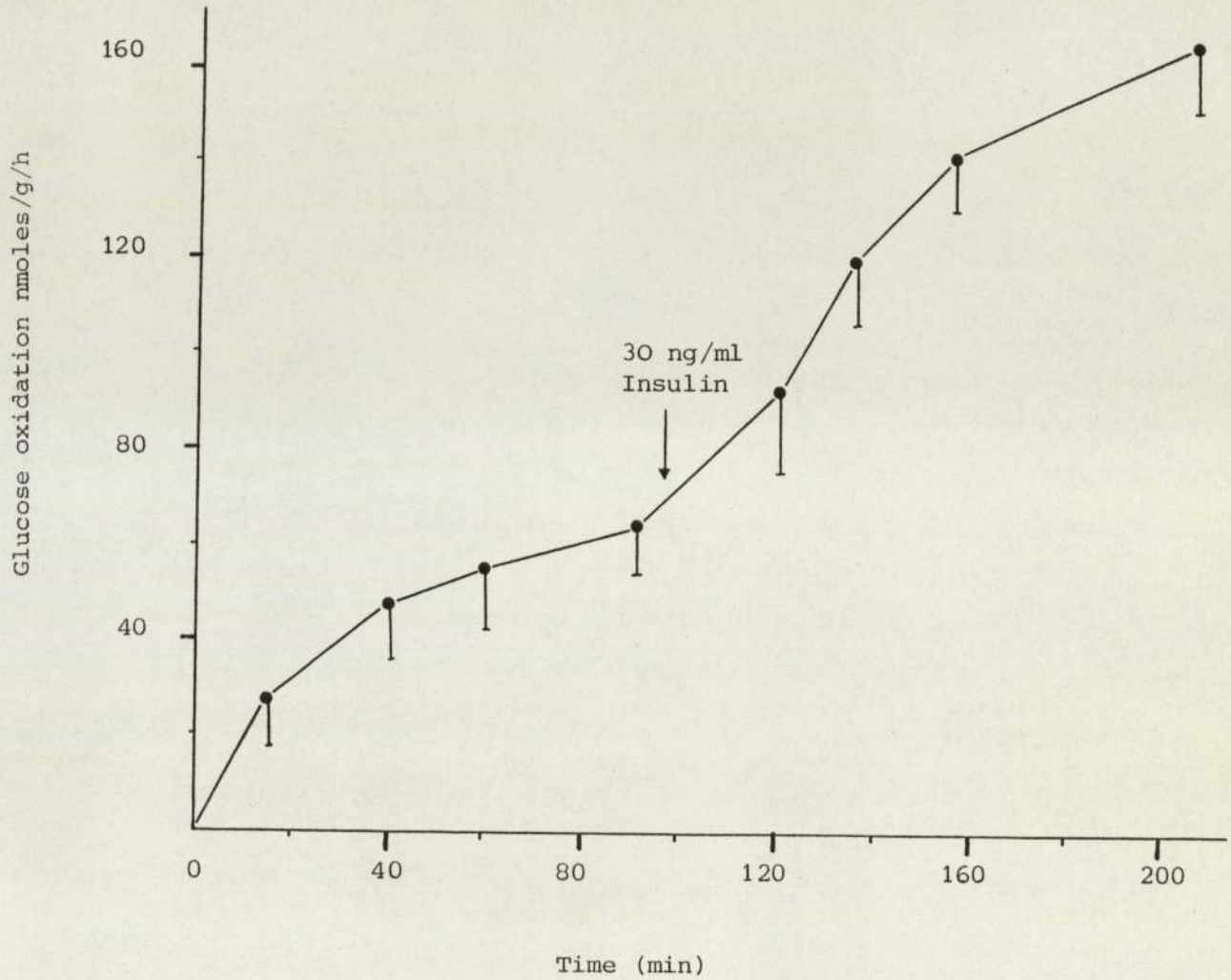
Glucose Oxidation by Pieces of Obese Mouse Exocrine Pancreas

Similar experiments as described above were performed with pieces of obese mouse exocrine pancreas.

The effect of iodoacetate on the rate of glucose oxidation by mouse pancreas.

30 mg of pancreas was cut into small pieces and incubated as described above. At 45 minutes iodoacetate (final concentration 1 mmo/l) was added to the incubation vessel.

Figure 27. The rate of glucose oxidation by obese mouse epididymal fat pad (mean values ± SEM of 4 determinations).



RESULTS

Some examples of curves for the rate of glucose oxidation in obese mouse epididymal fat pad and pieces of mouse pancreas are shown in Figures 26 to 29. For the obese mouse fat pad the rate of glucose oxidation reached a peak at about 1 hour whereas for the pieces of pancreas the rate of glucose oxidation reached a plateau within 40 minutes. Furthermore the rate of glucose oxidation during the steady state (plateau) was significantly higher in the pieces of pancreas than the epididymal fat pad, the respective values were 117 and 60 nmoles/hr/gram of tissue.

In order to determine whether the system would measure small changes in the rate of glucose oxidation brought about by the addition of insulin, 30ng/ml of insulin was added to the incubation medium. The results are shown in Figures 26 and 27. Bovine insulin produced a 3 fold stimulation of the rate of glucose oxidation. On the other hand iodoacetate (1 mmol/l) a metabolic inhibitor reduced the rate of glucose oxidation to basal levels within 30 minutes in pieces of mouse pancreas, Figure 28. Figure 29 shows a similar rate of glucose oxidation by a larger piece of pancreas than that used in Figure 28, the rate of glucose oxidation increased proportionally to the weight of tissue. The removal of the tissue from the incubation chamber produced a rapid decline in the rate of glucose oxidation.

Figure 28. The effect of iodoacetate on the rate of glucose oxidation by pieces of mouse exocrine pancreas (30 mg)

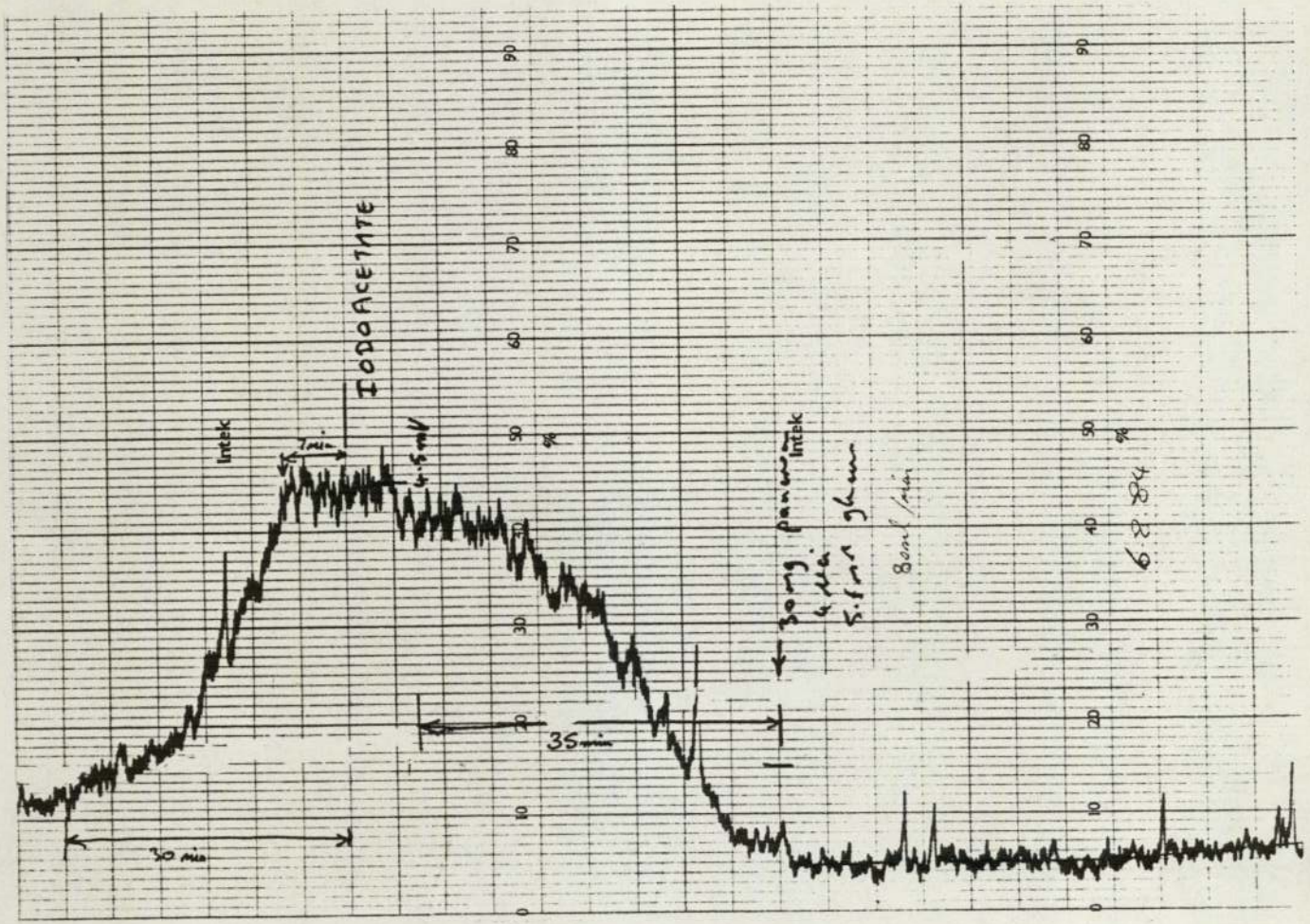
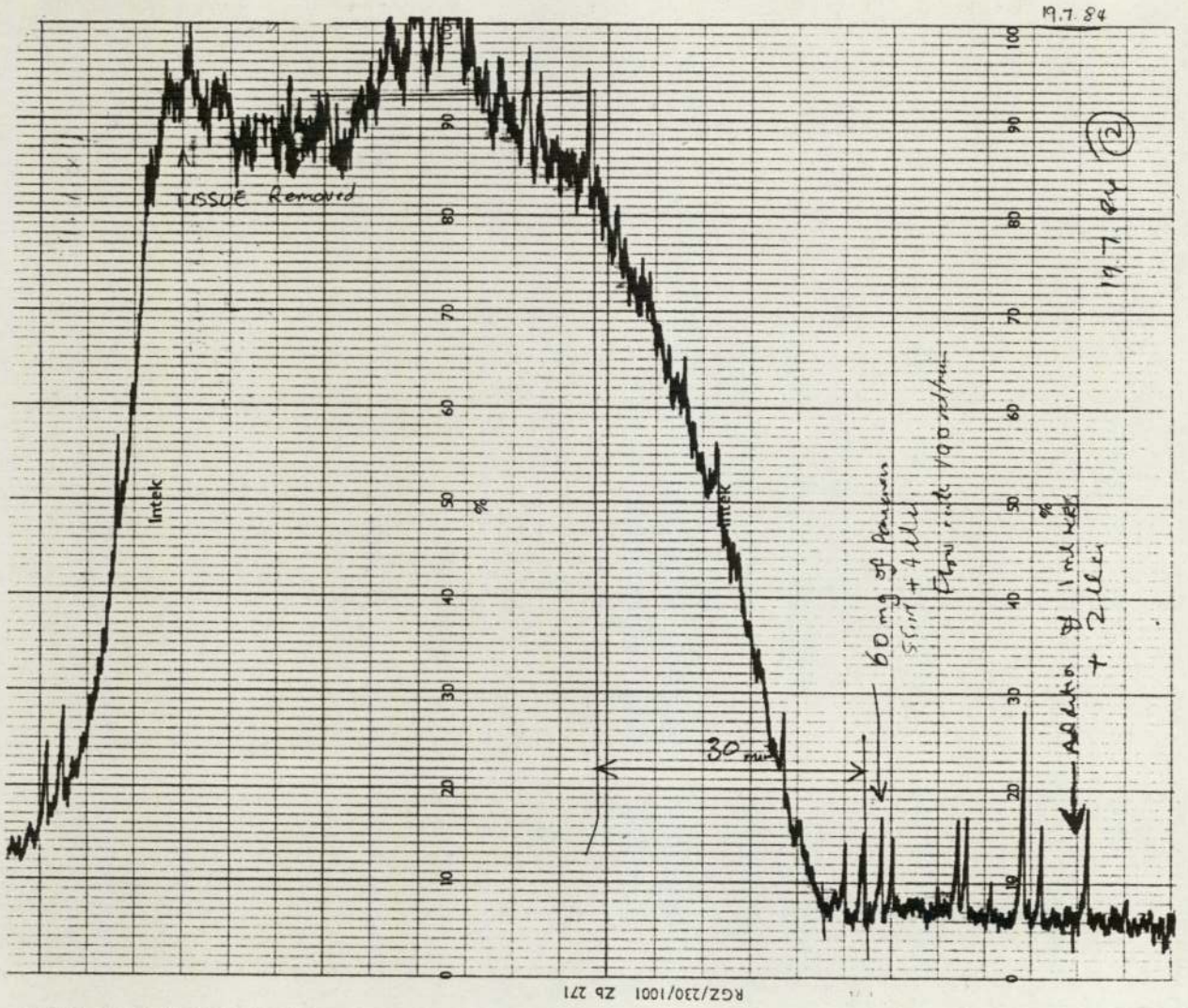


Figure 29. The rate of glucose oxidation by pieces of mouse exocrine pancreas (60 mg)



DISCUSSION

These results indicate that this technique is a convenient method for the measurement of the rate of glucose oxidation by small amounts of tissue. The major advantage of the technique is that it monitors the rate of glucose oxidation continuously and allows the investigator to add an appropriate stimulator or an inhibitor of glucose metabolism at will during the experiment. In addition each piece or pieces of tissue act as their own control. Furthermore, the tissue and the incubation medium are accessible at all time for biopsy or sampling or for the study of some other tissue function.

Simultaneous Measurement of the Rate of Glucose Oxidation and Insulin Release from Lean and Obese Mouse Islets

Introduction

Preliminary studies with pieces of epididymal fat pad and exocrine pancreas confirmed the possibility of using the generation of ionization current (by $^{14}CO_2$ produced from metabolizing tissue) as a means of measuring the rate of glucose oxidation and its modification for the simultaneous measurement of insulin release. This section describes experiments in which pancreatic islets from lean and obese mice were used in the system to study the effects of increasing concentrations of glucose, iodoacetate, digitoxose and mannoheptulose on the rate of glucose oxidation and the simultaneous release of insulin.

Materials and Methods

The description and the operational procedure of the apparatus designed for the simultaneous measurement of glucose oxidation and insulin release and isolation and storage of pancreatic islets has been given in the previous section. The islets (approximately 700-1000) were isolated on one day and stored overnight and used the next day. The islets were examined to ensure sterility and then loaded into the Swinnex filter holders with the aid of a pasteur pipette. The islet chamber was then fitted into the system as shown in Figure 20. The islets were preperifused for 45 minutes in Krebes-Hepes buffer containing glucose 5.6 mmol/l before beginning the measurement of glucose oxidation and insulin release. This preperifusion period served to establish a baseline for both background radioactivity and insulin release.

RESULTS

The effect of increasing glucose concentrations on the rates of glucose oxidation and insulin release from lean mouse islets is shown in Figure 30. In the presence of 5.6 mmol/l glucose both the rate of glucose oxidation and insulin release reached a plateau within 20 minutes. The addition of 11.2 mmol/l glucose produced a rapid increase in insulin release (approximately 275% of that at 5.6 mmol/l of glucose) which stabilized after a further ten minute period, a similar trend was observed with the glucose oxidation curve. Increasing the glucose concentration to 16.7 mmol/l produced a sharp rise in both the rate of glucose oxidation and insulin release. The percentage increase in the rate of glucose oxidation and insulin release above basal (5.6 mmol/l glucose) with increasing glucose concentrations has been summarized in Figure 31.

Iodoacetate (1 mmol/l) produced a prompt 50% decrease in the rate of glucose oxidation within 10 minutes whilst the reduction in the rate of insulin release was not profound and amounted to a reduction of 12% over 10 minutes.

The effect of increasing the glucose concentration from 5.6 to 16.7 mmol/l and the effects of digitoxose (22.2 mmol/l) and mannoheplulose (16.7 mmol/l) on the rates of glucose oxidation and insulin release by lean mouse islets has been summarized in Figure 32. Increasing the glucose concentration from 5.6 to 16.7 mmol/l produced a rapid increase in insulin release within six minutes, the response reaching the maximum within 20 minutes.

Figure 30. The effect of increasing glucose concentration on the rate of glucose oxidation (■—■) and insulin release (▲—▲) from lean mouse islets. Mean values ± SEM of 4 determinations.

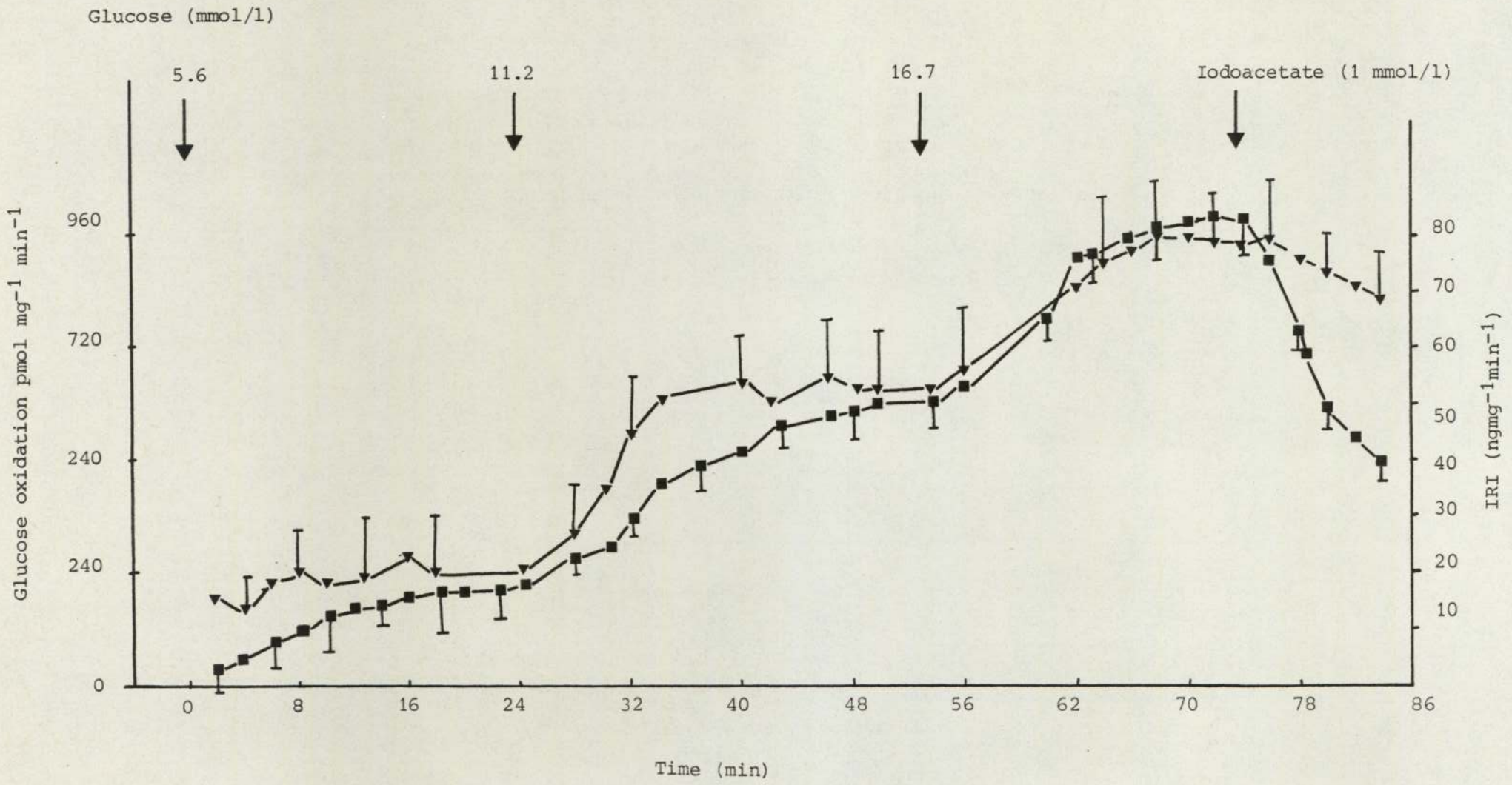


Figure 31. The effect of increasing glucose concentration on rates of glucose oxidation and insulin release from lean mouse islets. Mean values ± SEM of 4 determinations. a, $P < 0.01$ compared with 5.6 mmol/glucose
b, $P < 0.01$ compared with 11.2 mmol/glucose
data from Figure 30.

NB Basal = 5.6 mmol/l glucose

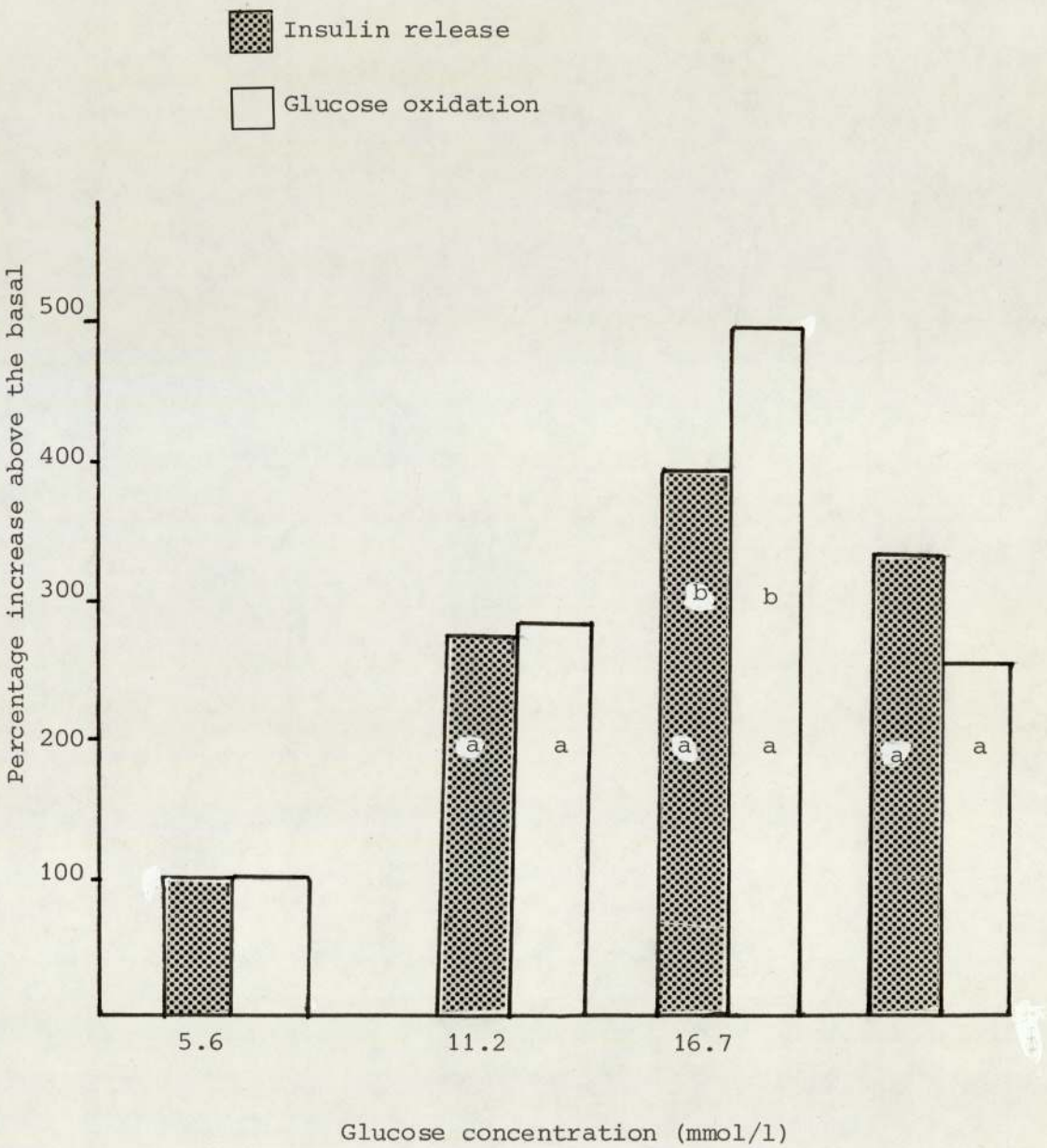
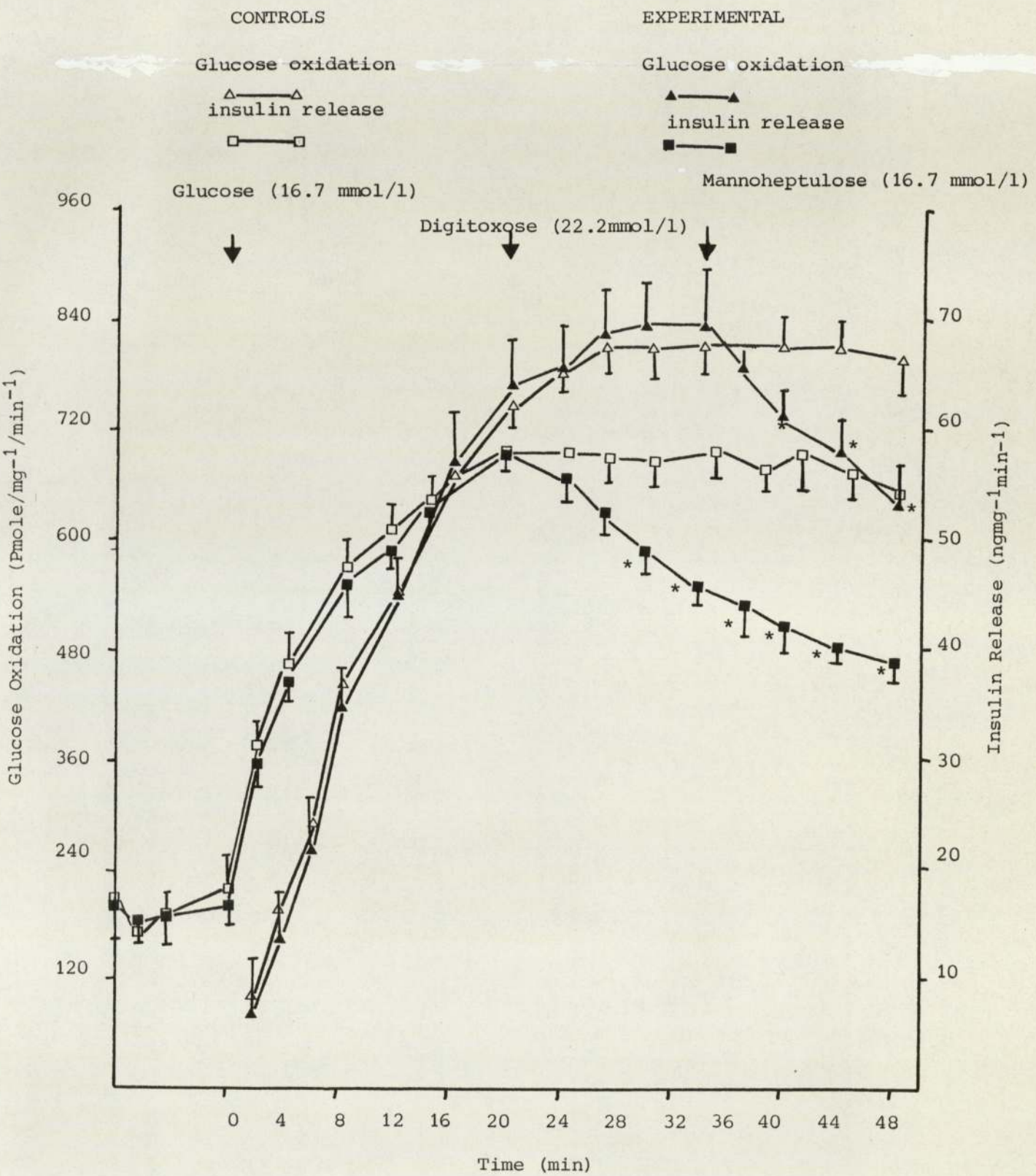


Figure 32. The effect of glucose, digitoxose and mannoheptulose on glucose oxidation and insulin relea from lean mouse islets. (Mean values ± SEM of 4 determinations)
* P < 0.05 compared to controls.



In addition there was a concomittant rapid increase in the rate of glucose oxidation in response to the increased glucose concentration. The addition of digitoxose after 20 minutes produced a prompt 20% reduction in the rate of insulin release but did not significantly effect the rate of glucose oxidation. Mannoheptulose after 34 minutes significantly inhibited both the rate of glucose oxidation and insulin release from lean mouse islets.

The effect of increasing the glucose concentration 5.6 to 16.7 mmol/l, digitoxose and mannoheptulose on the rate of glucose oxidation and insulin release from the obese mouse islets is shown in Figure 33. Again digitoxose had no effect on the rate of glucose oxidation but significantly reduced the rate of insulin release, mannoheptulose influenced both the rate of glucose oxidation and insulin release. Figure 34 is a comparison of rates of insulin release from lean and obese mouse islets. The lean mouse islets reached a plateau within 16 minutes and the obese mouse islets required 34 minutes to reach maximum secretory rate clearly showing a sluggish insulin response. The comparison of rates of glucose oxidation in lean and obese mouse islets is shown in Figure 35. The two curves in Figure 35 are nearly parallel although the rate of glucose oxidation in obese mouse islets is lower than in lean islets, but is not statistically significant.

Figure 33. The effect of glucose, digitoxose and mannoheptulose on glucose oxidation and insulin release in obese mouse islets (n = 4) (Mean values ± SEM of 4)

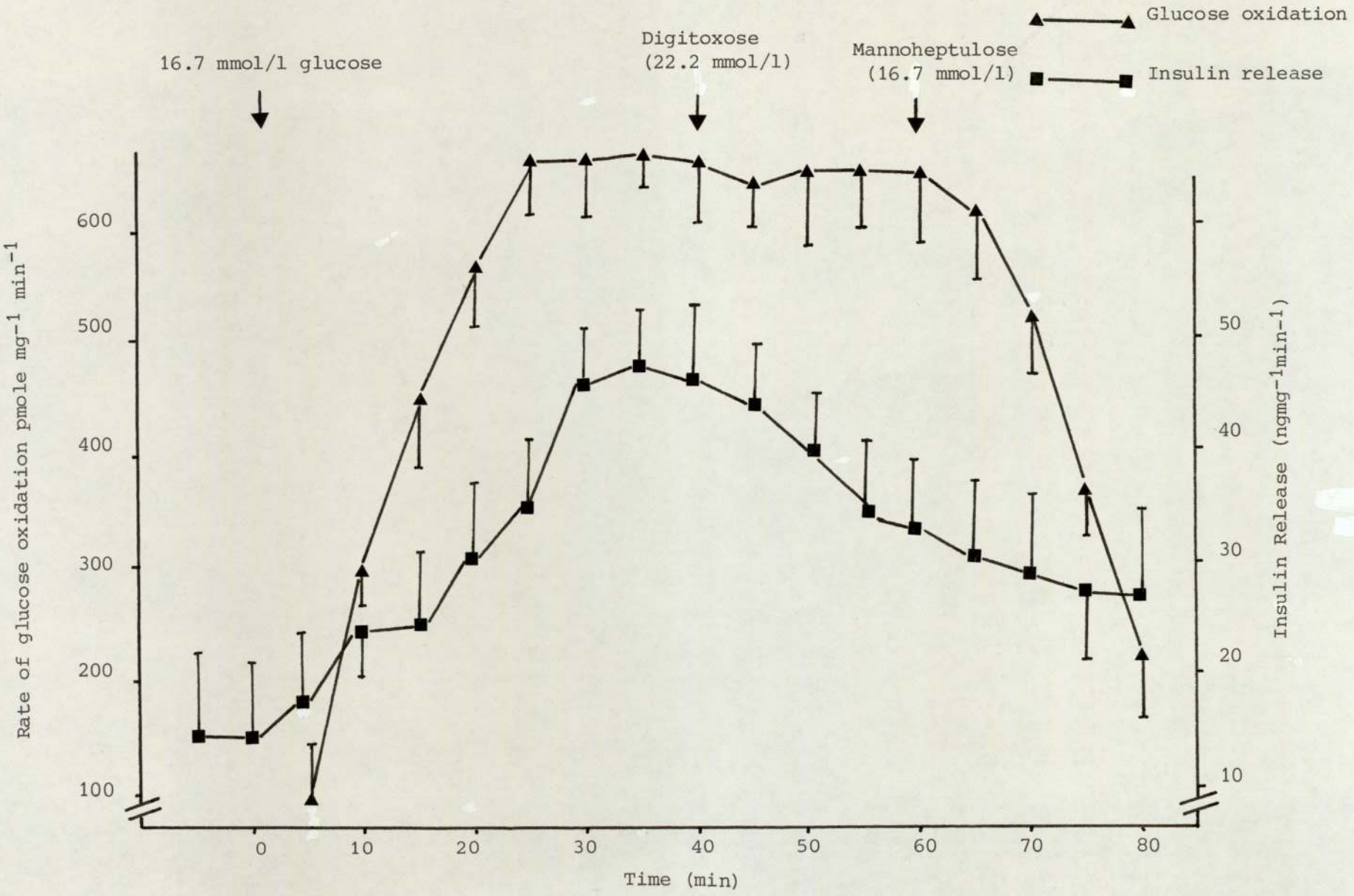


Figure 34. Rates of insulin release from lean and obese mouse islets.
* P < 0.01 compared with lean mouse islets

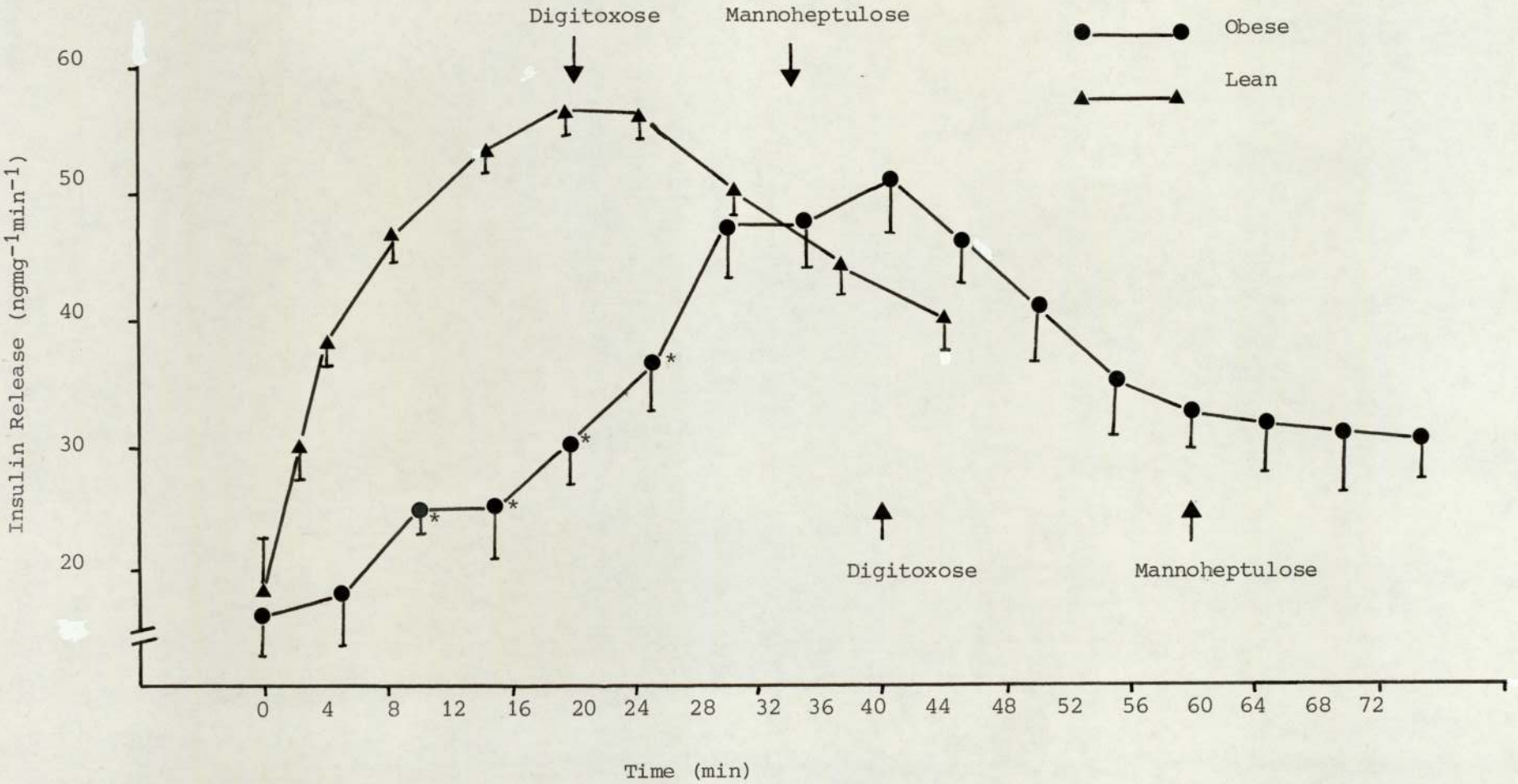
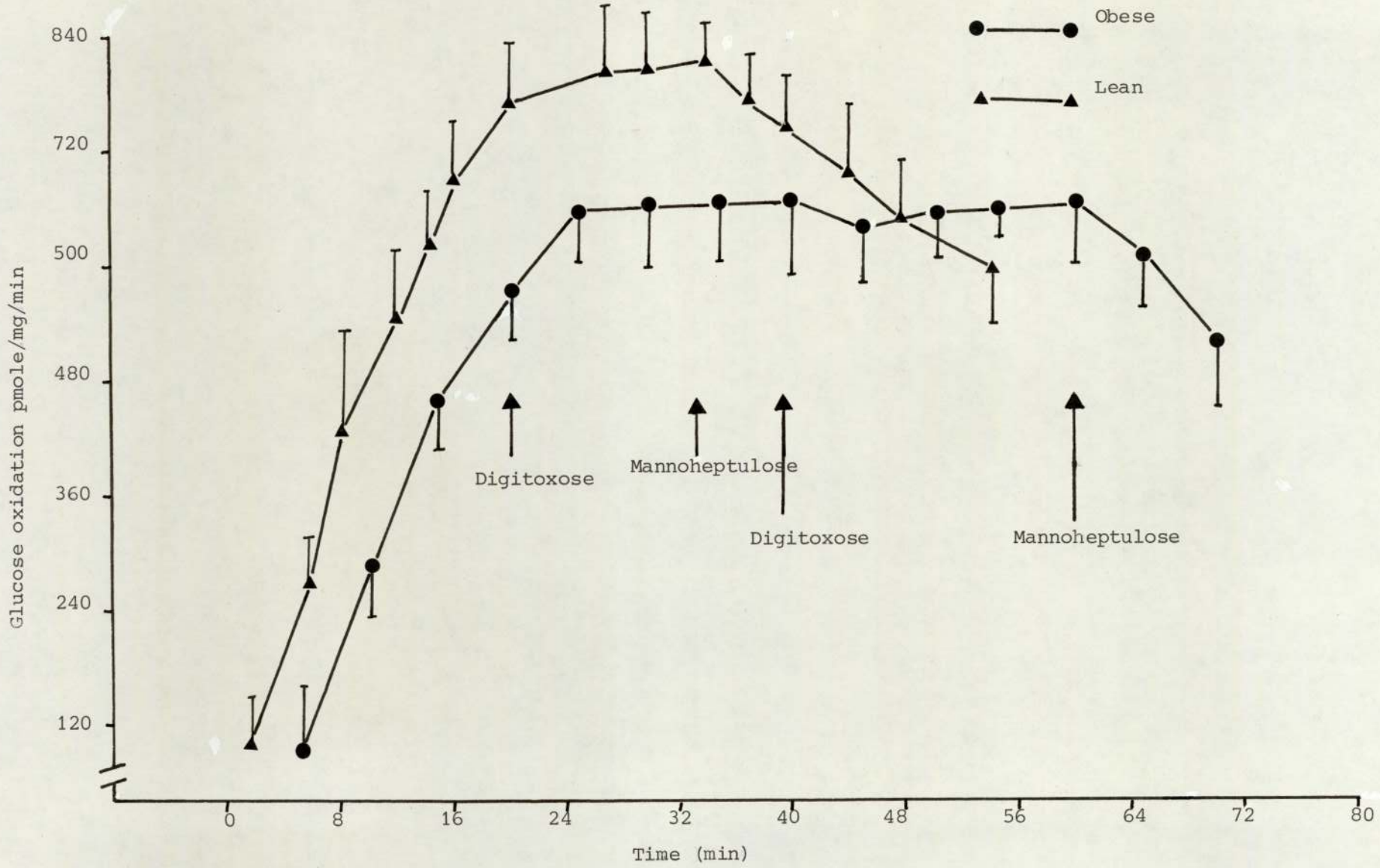


Figure 35. Rates of glucose oxidation from lean and obese mouse islets



DISCUSSION

In the past the very difficulty of dissociating the fuel function of glucose from its insulin releasing activity has provided a measure of support for the substrate site hypothesis. It is clear that support for the regulator site hypothesis (glucoreceptor) would be greatly strengthened if a sugar could be found which specifically inhibited glucose-induced insulin release but had no effect on the rate of islet glucose oxidation (64).

The present study clearly demonstrates that digitoxose can dissociate the fuel function of glucose from the latter's role as a signal for insulin release. Digitoxose was found to significantly inhibit insulin release yet the glucose oxidation was unaffected in both lean and obese mouse islets. These studies confirm the limited data provided by Hermida and Gomez-Acebo (153) and Lazarus *et al*(154).

The current work is the first to describe the simultaneous and continuous monitoring of the rate of glucose oxidation and insulin release from lean and obese mouse islets. It has previously been shown (page 78) that digitoxose specifically inhibits glucose induced insulin release and has no significant effect on amino acid or glibenclamide induced insulin release. It is speculated that digitoxose binds to a membranal glucoreceptor site and in competition with glucose prevents the activation of the glucoreceptor thus inhibiting the glucose induced insulin release, glucose oxidation however is unimpaired. This implies that glucose oxidation per se is not a necessary pre-requisite for insulin release, however, insulin release is a highly energy

dependent process (like all other secretory processes) and therefore uses glucose as an energy source.

The dependency of insulin secretion on the provision of energy by glucose oxidation is confirmed by the action of mannoheptulose on glucose oxidation and insulin release. By inhibiting glucose phosphorylation (182) mannoheptulose blocks glucose oxidation thereby depriving the insulin secretory process of energy consequently the latter process is inhibited too.

The fact that glucose may exert metabolic and signal function in the mechanism of insulin release is by no means unique. For example the amino acids L-glycine, L-asparate and L-glutamate are metabolic substrates and are also neurotransmitters in the central nervous system. The presence of receptors in the brain for these amino acids as well as those for adenosine and ATP is well established (183-186). Other tissues having well defined glucoreceptors exist in the hypothalamus (90,184), the liver (185,186) and on the tongue (187). Hiji, Kobayashi and Sato (187) demonstrated the presence of a sweet-sensitive protein in the gustatory cells of rat tongue and suggested that the interaction between sugars and this protein is the initial process in the receptor mechanism for sugars.

The Effect of Glucose on the Rate of Glucose Oxidation and Insulin Release from a Clonal Hamster B-cell Line (HIT T15)

Introduction

Our understanding of insulin release and B-cell metabolism is derived essentially from *invitro* studies using isolated islets of Langerhans and in few instances from islet cell monolayer cultures. Such studies have been limited by the difficulty of preparing significantly large quantities of islets and the cellular hetrogeniety of islets. Islets contain A, B and other cells. Studies on the mechanism of insulin release would be greatly facilitated by the availability of glucose sensitive permanent B-cell lines possessing functional characteristics of differentiated B-cells (188). Attempts to establish stable insulin-producing cell lines from primary islet monolayer cultures (189-191), insulinomas (192) and hybrids of islet cells and continuous cell lines have met with a limited success. Simian virus 40 (SV40) transformation of rat islet B-cell has yielded a continuous cell line that generates a 30,000 dalton protein antigenically related to insulin (193). A continuous glucose sensitive B-cell line has also been established from SV40 transfromed syrian hamster islet B-cell, that produces substantial quantity of insulin and proinsulin *invitro* This B-cell line is referred to as the HIT-T15 cell line (a 6-thioguanine resistant subclone isolated from HIT 5B5 cells at passage 27). The HIT-T15 cell line was established by the treatment of primary cultures of syrian hamster islet B-cells with ethyl

methanesulphate and subsequent transformation with SV40 virus. The insulin content of the HIT cells was found to be 0.3 to 2.5 µg insulin/mg of protein, which is approximately 2.5-20 orders of magnitude less than normal hamster islets (194) and glucose-stimulated HIT-cell cultures released 100ng of insulin per 10^6 cells per hour. This was observed to be 5 orders of magnitude less than that released by similarly treated normal hamster islet cell monolayer cultures. Insulin release from HIT-T15 B-cell was stimulated 2-fold by 10 mmol/l glucose compared with nearly 7 fold from isolated hamster islets, suggesting that alterations in secretory process might have occurred during the transformation procedure. However, in spite of the weak stimulation of insulin release by glucose the HIT cells do retain most, if not all, the characteristic differential functions of normal B-cells. Furthermore they can potentially provide unlimited amount of material for the study of insulin secretion. Since the present system for the simultaneous measurement of the rate of glucose oxidation and insulin release required large numbers of islets the HIT cell line is ideally suited for use in the system provided the cells can be restrained without damage in a perifusion system.

Another advantage of the isolated B-cell line is that it removes the element of heterogeniety of cell types seen with intact isolated islets and dispersed islet cells. Hence insulin release can be studied without influence from local interfering hormone-pancreatic effects, ie. glucagon, somatostatin and polypeptide P.

Materials and Methods

HIT-T15 Culture Maintenance Schedule

HIT-T15 cells were supplied generously by Dr R Santerne of El-Lilly Research (USA). Stock cultures of HIT-T15 cells were routinely maintained in Ham F12 medium (Flow Lab, Rickmansworth, Herts, England) supplemented with 15% horse serum, 2.5% foetal calf serum (Gibco Ltd, Paisley, Scotland), 0.1 µmol/l selenous acid (BDH Chemicals Ltd, Poole, England), 10 µg/ml glutathione (Sigma, Poole, Dorset, UK), 100 µg/ml streptomycin and 100 iU/ml penicillin (Flow Lab Ltd) using 250 ml costar tissue culture flasks. Culture flasks were seeded with 4×10^{-6} cells in 20 ml of F12 medium and maintained at $37^{0}C$ in humidified 5% CO_2/air mixture. Stock cultures were fed three times a week and passaged once a month.

Prior to experimental work stock cultures were trypsinized with 0.5 ml trypsin/EDTA solution for 5-7 minutes, the resultant suspension was then centrifuged at 250g in the medium described above. After centrifugation the supernatant was discarded and the pellet resuspended in 5 ml of fresh medium. The cell concentration was confirmed with a Neubaur haemocytometer and $2-4 \times 10^{6}$ cells were transferred with sterile siliconize glass pasteur pieptte into sterile swinnex filter units containing filters with pore size of 0.22 microns. The reassembled swinnex was then attached to the system as shown in Figure 20.

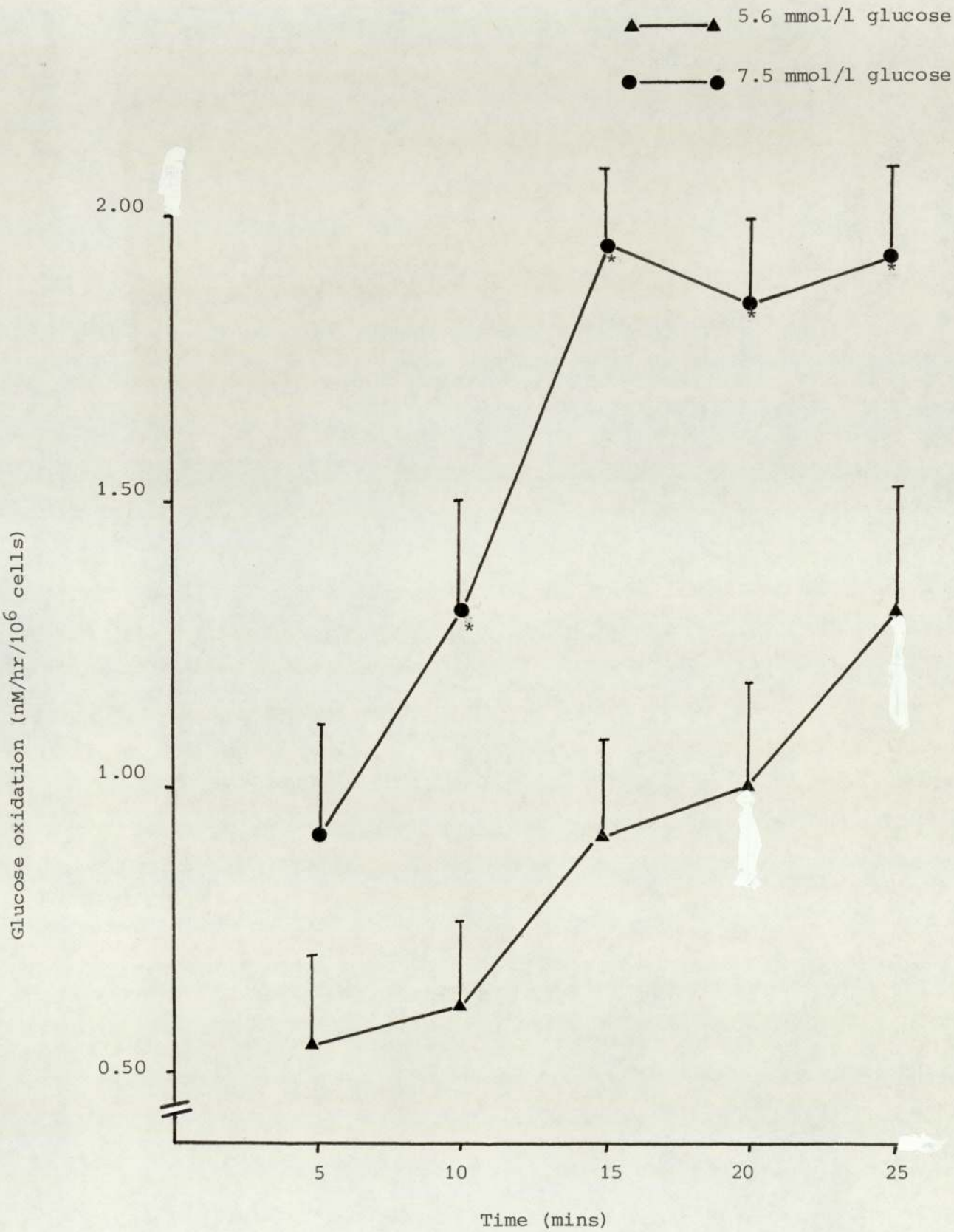
The cells were preperifused for 45 minutes with 5.6 mmol/l glucose to obtain steady state conditions prior to being perifused with D-[U-^{14}C] glucose. The rate of glucose oxidation was monitored as previously described (page 96) and perifusate collected for determination of insulin release.

RESULTS AND DISCUSSION

The rates of glucose oxidation by HIT-T15 cells in response to 5.6 and 7.5 mmol/l glucose are summarised in Figure 36. The rate of glucose oxidation increased with time in the presence of both concentrations of glucose. In the presence of 5.6 mmol/l glucose the rate of glucose oxidation established a plateau after 25 minutes which in the presence of 7.5 mmol/l glucose the response was significantly greater and more rapid, the plateau was established after 15 minutes.

Insulin release from perifused HIT-T15 cells was very low and could not be very reliably detected by the insulin radioimmunoassay, this is why it is not shown in Figure 36. The concentration of insulin in the perifusate was below 0.1 ng/minute/10^6 cells, which was outside the sensitivity range of insulin standard curve. Static batch type experiments confirmed that the release of insulin from HIT-T15 cells in response to 7.5 mmol/l glucose was very low (13 $\pm$ 1.6 ng/hour/10^6 cells). HIT-T15 cells appear to lose their sensitivity to glucose with passage. The passage number used in the current work was number 73 and it has since been found that the earlier passages are significantly more sensitive to glucose. For example in our laboratory HIT-T15 cells passage number 48 released approximately 120 ng/hour/10^6 in response to 7.5 mmol/l glucose whereas passage number 73 produced only 13 $\pm$ 1.6 ng/hour/10^6 cells (Lambert D personal communication).

Figure 36. Glucose oxidation in HIT-T15 cells at glucose concentrations of 5.6 and 7.4 mmol/l. n = 3, values are mean ± SEM
* $P < 0.05$ compared with 5.6 mmol/l glucose.



CHAPTER 5

THE ROLE OF cAMP AND Ca^{2+}-CALMODULIN IN THE MECHANISM OF INSULIN RELEASE FROM LEAN MOUSE ISLETS

Introduction

The adenylate cyclase -cAMP system is believed to play an important role in the mechanism of insulin release (196). The involvement of cAMP is clearly demonstrated by the many reports indicating that hormones which activate the adenylate cyclase system in a variety of tissues will also stimulate insulin release or potentiate glucose-induced insulin release (197-201). The finding that cAMP enhanced the B-cell insulin response to glucose came from studies involving the stimulation of insulin release by glucagon. In these studies the insulin response to glucagon was shown to be greater during hyperglycaemia than during normoglycaemia (196-198). On the basis of the observed stimulating effect of glucagon on the adenylate cyclase system in liver, it was suggested that cAMP was involved in the mediation of the stimulating effect of glucagon on insulin release. Since then many reports have confirmed the effects of glucagon on insulin release and the similarities of action between those agents which raise intracellular cAMP levels, such as glucagon (200); B-adrenergic agents (202); phosphodiesterase inhibitors (papaverine and methylxanthines, 203, 204) and cholera toxin and the effects of exogenous cAMP or dibutryl cAMP on insulin release (205). It is clear from these diverse reports that when the

intracellular cAMP concentration of the B-cell is raised, by whatever means, insulin release is enhanced. To understand the role of adenylate cyclase-cAMP system in insulin release it is pertinent to consider the components of the system, adenylate cyclase enzyme, its product cAMP, the activity of cyclic nucleotide phosphodiesterases which breakdown cAMP and finally the possible mechanisms of action of cAMP on the process of insulin release.

Adenylate cyclase which catalyzes the formation of cAMP from ATP is a cell membrane bound enzyme and is stimulated by hormones interacting at the external surface of the plasma membrane with specific receptor molecules (205). Thus, one control of cAMP concentration is at the level of circulating hormones. The B-cells have specific receptors for hormones such as glucagon, ACTH, B-adrenergic agents and vasoactive intestinal peptide (VIP) (196) all of which are thought to act via cAMP. A second means of regulating intracellular cAMP concentrations in islet cells is via the activity of cAMP dependent phosphodiesterases which catalyze the breakdown of cAMP to 5^1 AMP (201). Several phosphodiesterases have been characterised in the B-cell, based on their Km values and by their location. Thus, soluble and particulate, high and low Km phosphodiesterases have been described. Some 70% of the soluble phosphodiesterase has been shown to be present in the post microsomal (105,000g, 60 min) supernatant (206). The remaining 30% has been shown to be present in the pellet of low speed centrifugation and very little in mitochondria or microsomes. It has been suggested that the low

Km phosphodiesterase activity is largely in control of cAMP breakdown in the B-cell because estimations of the intracellular cAMP concentrations have been shown to be micromolar (196).

The major receptors for cAMP in mammalian cells are the protein kinases, which catalyze the phosphorylation of proteins using the -phosphate of ATP. The cAMP dependent protein kinases (cAMP-Prk) consist of regulatory subunits and catalytic subunits. The binding of cAMP to the regulatory subunits results in the dissociation of the subunit complex and the activation of dissociated catalytic unit. In this way, the concentration of cAMP within the cell is able to determine within limits the activity of protein kinase. A further control point has been suggested to be the so called "inhibitory protein" (207). This protein is thought to act by combining with a component of regulatory protein binding site on the catalytic unit. Thus after cAMP activation and dissociation of the subunits the dissociated catalytic subunit might well be influenced by the inhibitory protein. Protein kinase activity in islets was first documented by Montague and Howell in 1972 (208). Since then its presence has been confirmed by several workers (209). The properties of cAMP-PrK in isolated rat islets have been studied in some detail (209). Using DEAE-cellulose chromatography it was possible to demonstrate two isoenzymes of cAMP-PrK in rat islets, corresponding to the Type I and Type II described in other tissues. Christie and Ashcroft defined the substrates for cAMP-PrK as being particulate peptides of mw 23, 30 and 32 K dalton. These authors concluded that the potentiation

of insulin secretion that occurs when islet cAMP is elevated is accompanied by the phosphorylation of these particulate peptides suggesting that protein phosphorylation might play an important role in the mechanism of insulin release. However, it still remains to be established what role these phosphorylated proteins might play in the mechanism of insulin release. It has been suggested that a cAMP-induced phosphorylation of proteins could lead to an increased cytosolic Ca^{2+} concentration and this may augment the increase in cytosolic Ca^{2+} caused by glucose (196). Such a simple model assumes that whi le cAMP can raise cytosolic Ca^{2+}, it cannot raise the level sufficiently in the absence of glucose to cause sustained release - hence its dependence on glucose. Experimental evidence suggests that cAMP can inhibit $^{45}Ca^{2+}$ uptake by granule, mitochondrial and microsomal fractions of islets (210). The mitochondria have been thought to exert a major controlling role over the translocation of Ca^{2+} by virtue of their ability to take up Ca^{2+} rapidly and in large quantities (211).

The possible roles cAMP and glucose play in the mechanism of insulin release have been summarized by Sharp (196). Glucose inhibits the efflux of Ca^{2+} across the B-cell membrane thereby increasing cytosolic Ca^{2+} concentrations and stimulating insulin release. cAMP has been shown to inhibit the uptake of cytosolic $^{45}Ca^{2+}$ into intracellular organelles such as mitochondria which effectively raise the cytosolic Ca^{2+} concentrations and stimulates the release of insulin only when the rate of Ca^{2+} efflux across the

cell membrane is reduced by glucose. Such a mechanism would adequately explain the glucose dependency of the action of cAMP on insulin release.

The effects of glucose on the concentration of cAMP in isolated islets has also been studied. Charles, Franks and Grodsty (212) found that glucose at 16.7 mmol/l caused a 2.5 fold increase in the concentration of cAMP in perifused islets. This increase was detectable within two minutes of adding glucose (16.7 mmol/l) and was still present 20 minutes after the addition of glucose. Theophylline (10 mmol/l) increased the cAMP concentration four fold but did not increase the rate of insulin release to the same extent. Since theophylline caused a greater increase in the concentration of cAMP but a smaller increase in insulin release than 16.7 mmol/l glucose, Charles *et al* (212) concluded that cAMP had only a minor role to play inthe direct stimulation of insulin release. Since glucose (16.7 mmol/l) and theophylline (10 mmol/l) combined increased the rate of insulin release and islet cAMP levels to a greater extent than in the presence of glucose alone a prominant role has been ascribed to cAMP modulation of glucose-induced insulin release. Grill and Cerasi (213) have also reported an increase in islets cAMP content upon stimulation with glucose. This observation is supported by the fact that alpha anomer of D-glucose has been shown to stimulate both islet cAMP levels and the release of insulin (214, 215) to a greater extent than the B-anomer of glucose.

The Integration of cAMP and Ca^{2+}-Calmodulin in the Mechanism of Insulin Release

There is now considerable evidence to suggest calmodulin exerts effects on both the synthesis and degradation of cyclic nucleotides in a variety of cell systems (216). Calmodulin is a member of the so called "troponin-C-superfamily" of calcium binding proteins which have a fundamental role in muscle contraction and relaxation and probably in intestinal calcium transport. It seems possible that the effects of Ca^{2+}-calmodulin on glucose-induced insulin release are linked in some way to its interaction with adenylate cyclase system. Indeed a calmodulin activated adenylate cyclase has been found in pancreatic islets (217). However, reduced concentrations of cAMP have not been found in isolated islets under conditions of impaired insulin secretion brought about by trifluoperazine (218, 219). Trifluoperazine an antipsychotic drug strongly binds to calmodulin in a dose and time dependent manner. Similarly, glucagon stimulated insulin release which is associated with elevated islet cAMP levels, was not inhibited by trifluoperazine (220, 221). In addition, trifluoperazine had no effect on insulin release provoked by phosphodiesterase inhibitors, 3-isobutylmethyl-xanthine and theophylline which was accompanied by a marked elevation in the islet cAMP concentration (219). These observations could be taken to indicate that cAMP might be able to stimulate insulin release without involving calmodulin-mediated events. Alternatively it

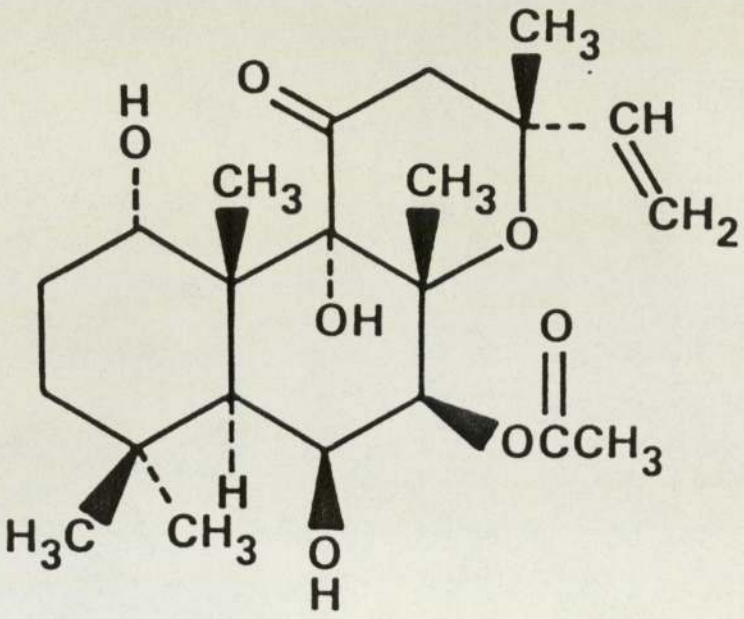
may be that cAMP requires Ca^{2+} ions but not calmodulin for its stimulatory role in insulin release.

The aim of the present study was to determine whether agents capable of increasing islet cAMP levels stimulated insulin release independently of calmodulin. Islet cAMP levels were increased with either theophylline or forskolin, the latter is a specific stimulator of islet adenylate cyclase. Concommitantly islet Ca^{2+}-calmodulin was inhibited with trifluoperazine and a new calmodulin inhibitor MB17108 obtained from May and Baker Ltd (Dagenham, Essex).

Forskolin (7β-acetoxy-8, 13 α-epoxy, 1α, 6β, 9αtrihydroxylabd-14-ene-11-one) is a diterpene of the labdane family and is the commercial preparation extracted from the root of the plant Coleus Forskohlii (222). The structural formula of forskolin is shown in Figure 37. Foskolin has been shown to be a specific and a direct stimulator of adenylate cyclase in a number of different cell types, 3 to 20 fold increase in enzyme activity has been reported in cell free preparations (223). It has also been shown to produce a 6-400 fold increase in the levels of cAMP in intact cells (222). The activation is rapid and reversible with Km of 10-40 μmol/l. The increased accumulation of cAMP produced by forskolin can be further augmented by adding cAMP phosphodiesterase inhibitor (222).

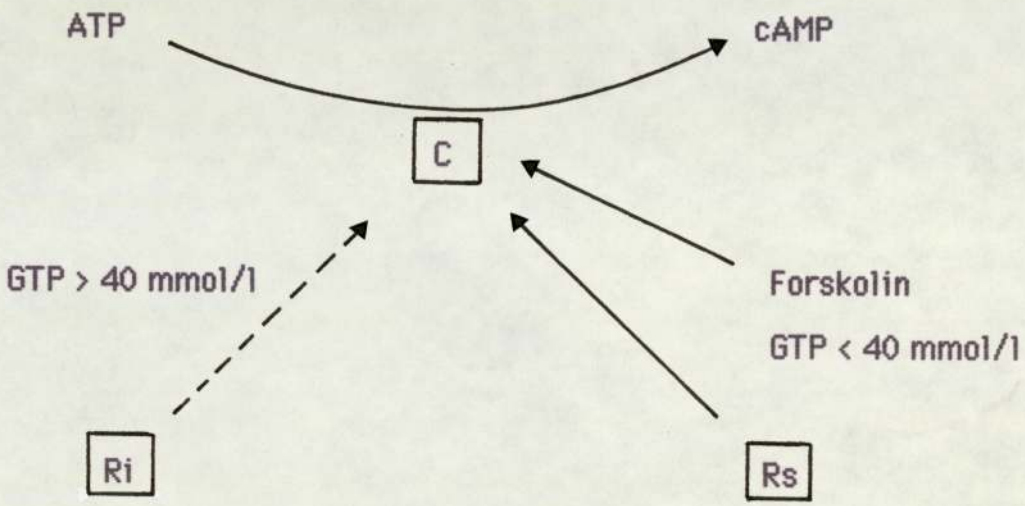
Hormone-responsive adenylate cyclase consists of three functionally distinct subunits; the catalytic subunit which converts MgATP to cAMP; and two guanine nucleotide regulatory

Figure 37. Strucutre of Forskolin



subunits (stimulatory and inhibitory), which bind guanine nucleotides and are the required mediators of hormone receptor input to the catalytic subunit; and hormone receptors which bind hormones specifically, Figure 38.

Figure 38. The three subunits of adenylate cyclase; C, catalytic; Ri, inhibitory regulatory; Rs, stimulatory regulatory subunit (from Reference 222).



Activation of cAMP generation is not blocked by a variety of receptor blockers and therefore does not occur through the intermediacy of any of the major classes of receptors. Similarly it has been shown that forskolin does not interact with the regulatory subunits to activate adenylate cyclase, a catalytic subunit of rat brain adenylate cyclase which has been fractionated to remove the guanine regulatory subunits is still potently activated by forskolin (222). Therefore Forskolin appears to exert its effect by a direct action on catalytic subunits.

MATERIALS AND METHODS

Chemicals

Trifluoperazine was purchased from Smith Kline and French Lab Ltd, Welwyn Garden City, Hert; Forskolin from Calbiochem, Behring, Hoeschst Corp, La Jolla, California; MB17108 compound was a kind gift from Dr T Brown (May and Baker). Theophylline was brought from Sigma Co Ltd, Poole Dorset.

Preparation of Stock Solutions of Forskolin, Theophylline and the Calmodulin Inhibitors MB17108 and Trifluoperazine

Since these compounds were not readily soluble in Krebs-Hepes buffer, they were dissolved first in a small quantity of dimethylsulphoxide (DMSO) and then diluted to the appropriate concentration with Krebs-Hepes buffer. An appropriate aliquot of this stock solution was then added to the incubation vials to obtain the desired concentrations. Stock solution of forskolin was prepared by adding 1 ml DMSO to 10 mg foskolin and then adding 9 ml Krebs-Hepes buffer. This stock solution was then aliquoted into small tubes and stored at $-20^{0}C$ until required. In order to obtain the desired final concentration of 10 µmol/l of forskolin in the incubation vials 4.1 µl of the stock solution were added to 1 ml of incubation medium. Other agents were similarly prepared and the concentrations worked out using the formula:

$$m_1 v_1 = m_2 v_2$$

where:

m_1 is the desired final molarity (mol/l)

m_2 is the molarity of stock solution (mol/l)

v_1 is the volume of stock solution required (l)

v_2 is the total incubation volume (l)

The final concentration of DMSO in the incubation vials never exceeded 0.4% and this amount was always added to control incubations. It has been shown that low DMSO concentrations of this order have no effect on insulin release from isolated pancreatic islets (224).

Insulin Release from Statically Incubated Islets of Langerhans

Islets from overnight fasted lean mice were isolated and preincubated as previously described (page 41). Following 30 minute preincubation batches of 5-8 islets were transferred to Idram vials containing 1 ml of Krebs-Hepes buffer pH 7.4 supplemented with glucose (16.7 mmol/l), forskolin (10 µmol/l) or theophylline (2 mmol/l), trifluoperazine (15 µmol/l) or MB17108 (10 µmol/l) and incubated for 60 minutes at 37°C in shaking waterbath (100 cycles/min). After the incubation 50 µl aliquots of the incubation medium were removed and frozen for the subsequent assay of insulin. The results were expressed as µU/µg dried islet weight per hour.

Perifusion of Isolated Islets of Lean Mice

Islets were perifused as previously described (page 43). 10 isolated lean mouse islets were pipetted into the tissue perifusion chamber and preperifused for 45 minutes with Krebs-Hepes buffer pH 7.4 supplemented with 5.6 mmol/l glucose. This was followed by 40 minutes of test perifusion with either 5.6 mmol/l glucose (control) or 16.7 mmol/l glucose alone or 16.7 mmol/l glucose plus theophylline (2 mmol/l) or with calmodulin inhibitor as detailed in Figure 39. Aliquots of perifusate were taken for insulin assay at every 5 minute interval.

After perifusion islets were removed from the tissue chambers and their weights determined after oven drying as described earlier (page 46). The results were expressed as ng/µg insulin islet weight/min.

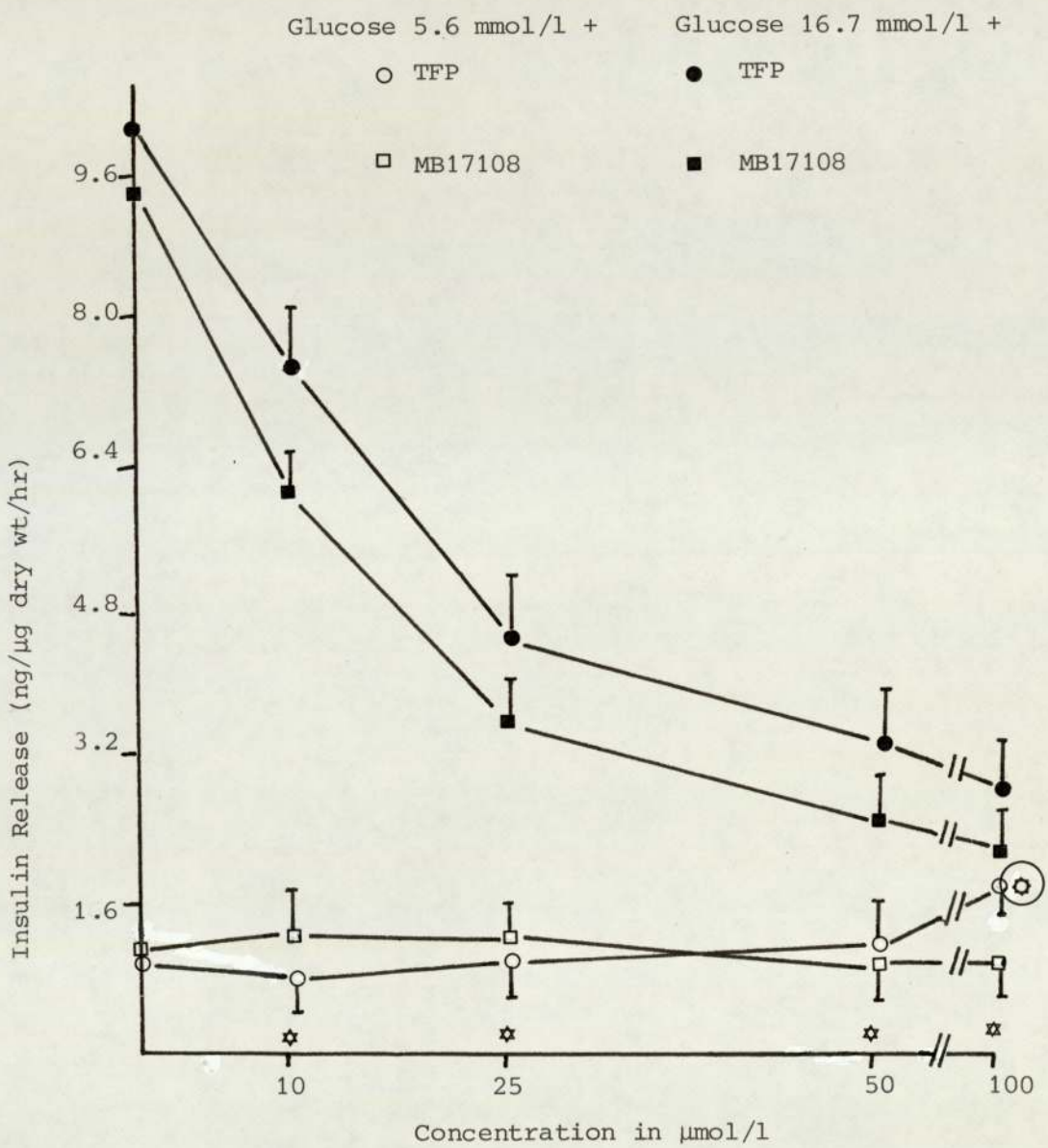
RESULTS

The effects of trifluoperazine and MB17108 on insulin release from lean mouse islets statistically incubated are summarized in Figure 39. In the presence of 5.6 mmol/l glucose, basal insulin release was not affected significantly by 10, 25 and 50 µmol/l trifluoperazine, but was increased from 0.9 ± 0.2 to 1.8 ± 0.6 ng/µg dry weight per hour ($p < 0.05$) by 100 µmol/l trifluoperazine. In the presence of 16.7 mmol/l glucose, trifluoperazine produced a dose-dependent inhibition of insulin release. The lowest dose tested was 10 µmol/l which produced a 35% inhibition of the maximum insulin release. The concentration required to bring about a 50% inhibition of insulin release (Ki) was found by Line-Weaver and Burke plot and found to be 15 µmol/l.

The inhibition of insulin release produced by MB17108 was similar to that shown by TFP (Figure 39). However unlike TFP, high concentrations of the drug (100 µmol/l) had no effect on basal insulin release. The Ki was 10 µmol/l somewhat lower than that of TFP.

Figure 40 summarizes the effects of TFP on the dynamics of glucose stimulated insulin release. The addition of 15 or 30 µmol/l TFP during the second phase of release produced a progressive dose dependent fall in the rate of insulin release. 15 and 30 µmol/l TFP produced a 50 and 65% reduction in insulin release respectively.

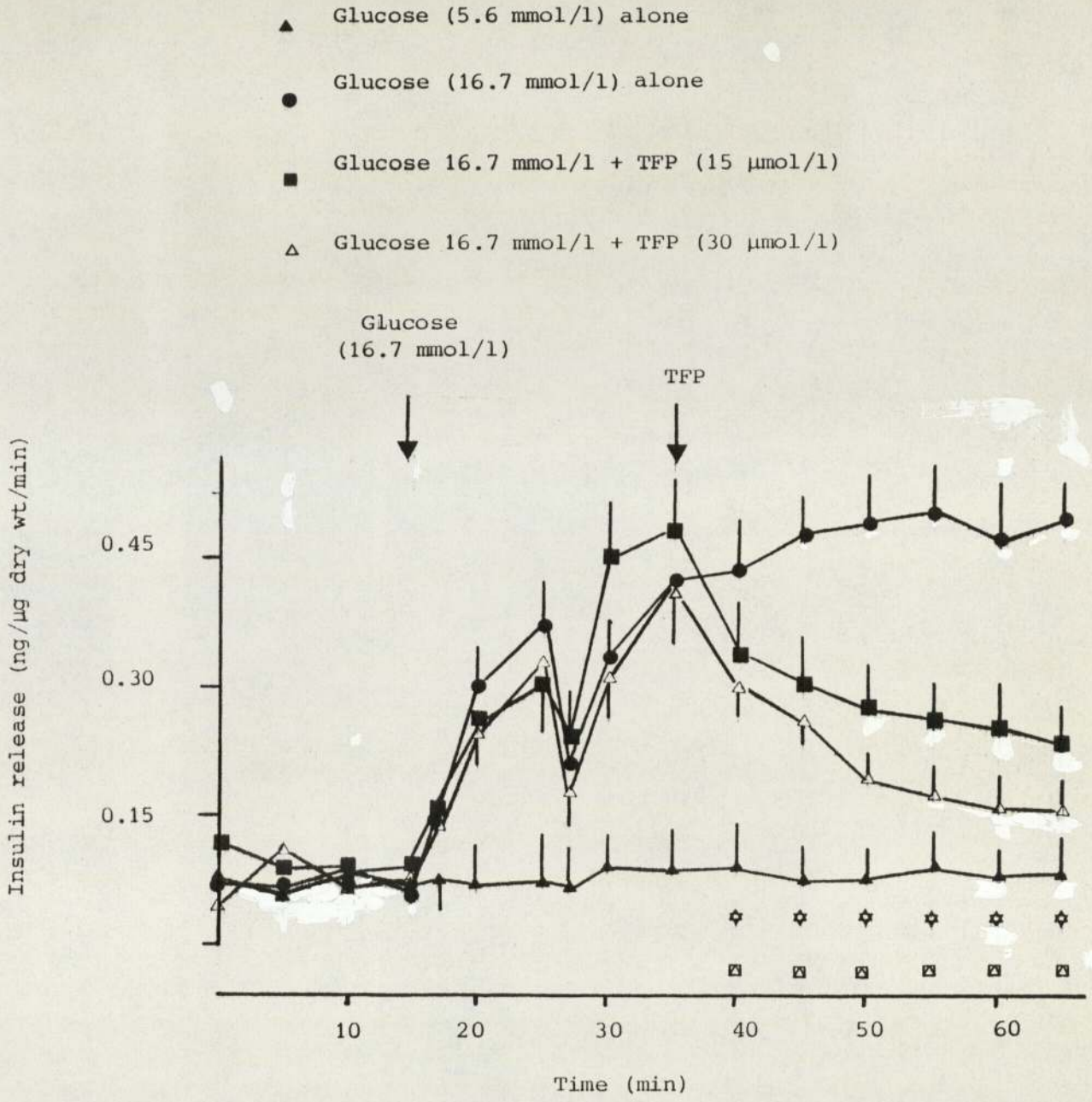
Figure 39. The effects of increasing concentrations of trifluoperazine and MB17108 on insulin release from lean mouse islets. Mean values ± SEM of 7 separate experiments.



✡ Indicates significant difference from glucose at 5.6 mmol/l

⊛ Indicates significant difference from zero TFP.

Figure 40. The effect of trifluoperazine on the dynamics of glucose stimulated insulin release. Profiles represent mean values ± SEM of 4 separate experiments.



The effects of glucose and theophylline on insulin release are summarised in Figure 41. Glucose (16.7 μmol/l) produced a typical biphasic insulin response whilst glucose in the presence of theophylline did not produce a clear biphasic response. However the latter insulin response was increased significantly ($p < 0.001$) compared to the profile obtained in the presence of glucose alone. The effects of 5, 10 and 20 μmol/l forskolin on the basal and glucose stimulated insulin release are summarized in Figure 42. Forskolin had no significant effect on basal insulin release but significantly potentiated glucose stimulated insulin release. A maximal insulin response was obtained with 5 μmol/l forskolin and there appeared to be no dose-dependent effect.

Both TFP (15 μmol/l) and MB17108 (10 μmol/l) inhibited glucose stimulated insulin release from isolated lean mouse islets by 49 and 55% respectively. Forskolin potentiated glucose-stimulated insulin release both in the presence and absence of calmodulin inhibitors, Figure 43.

The combined effect of forskolin and theophylline on glucose stimulated insulin release from lean mouse islets in the presence of TFP and MB17108 is summarized in Table 8. Theophylline (2 mmol/l) plus forskolin (10 μmol/l) significantly elevated glucose induced insulin release ($p < 0.001$ compared with glucose (16.7 mmol/l) alone). On the other hand the calmodulin inhibitors, TFP and MB17108 both significantly reduced glucose induced insulin release ($p < 0.005$ compared to 16.7 mmol/l glucose), however in the presence of forskolin and theophylline neither TFP or MB17108 significantly affected glucose stimulated insulin release.

Figure 41. The effect of glucose and theophylline on insulin release from lean mouse islets. Profiles represent mean values ± SEM of 4 determinations. Arrow shows the addition of glucose 16.7 mmol/l alone or plus theophylline.

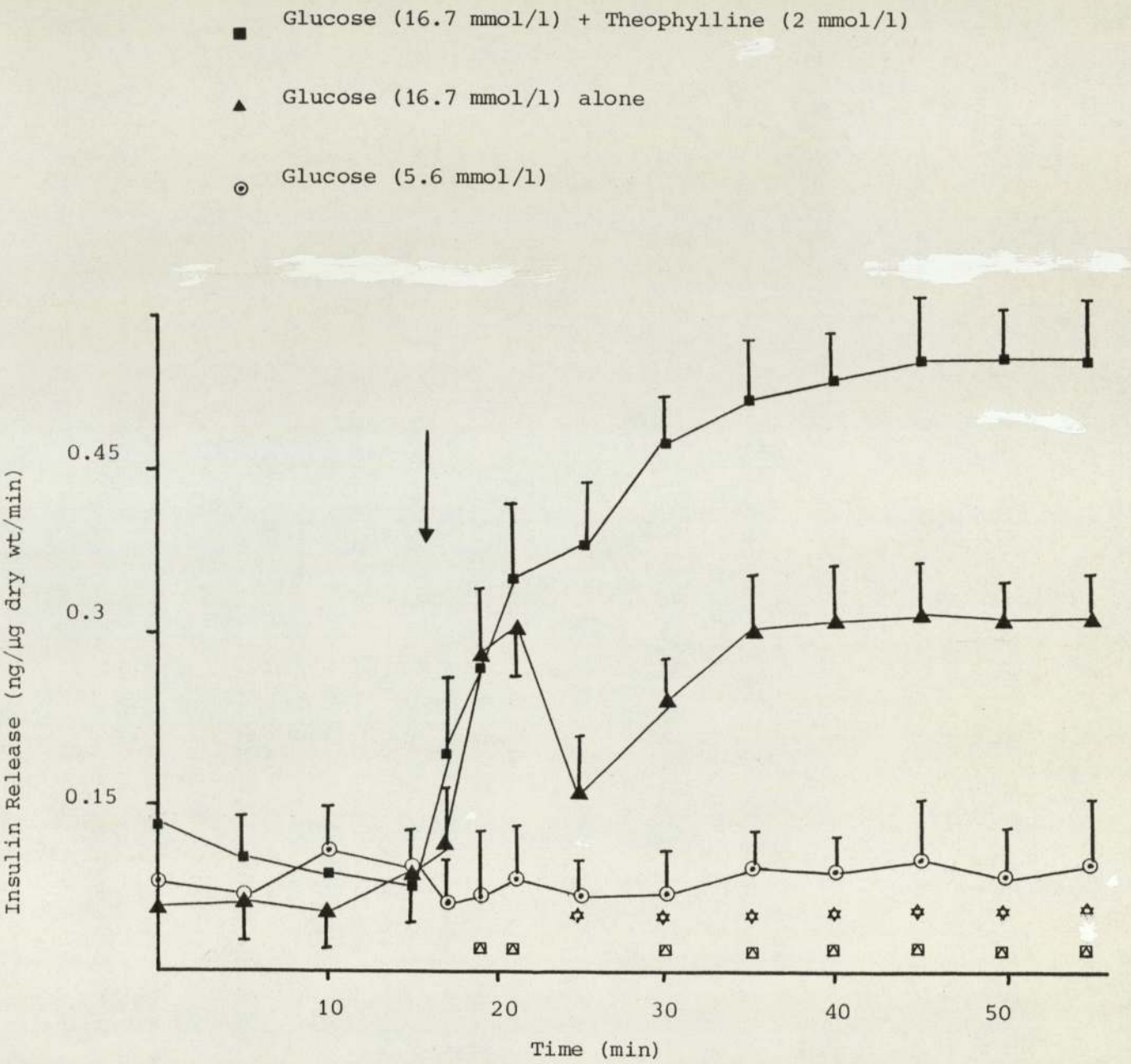


Figure 42. The effect of forskolin on glucose stimulated insulin release from lean mouse islets. Mean values ± SEM of 7 separate experiments.

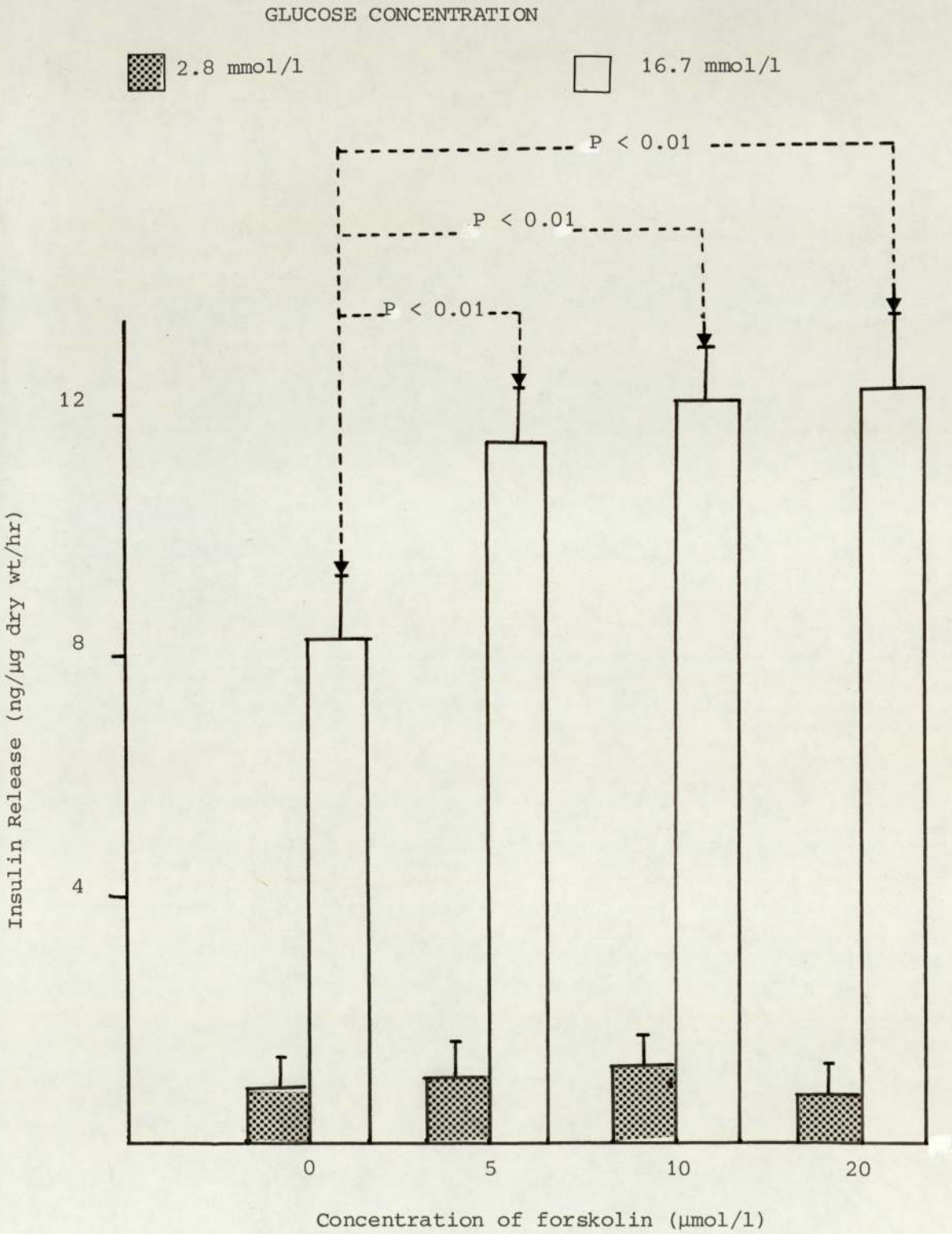


Figure 43. The effect of calmodulin inhibitors and forskolin on glucose induced insulin release from lean mouse islets. Mean values ± SEM of 8 determinations.

a and b $P < 0.01$ compared with glucose alone.

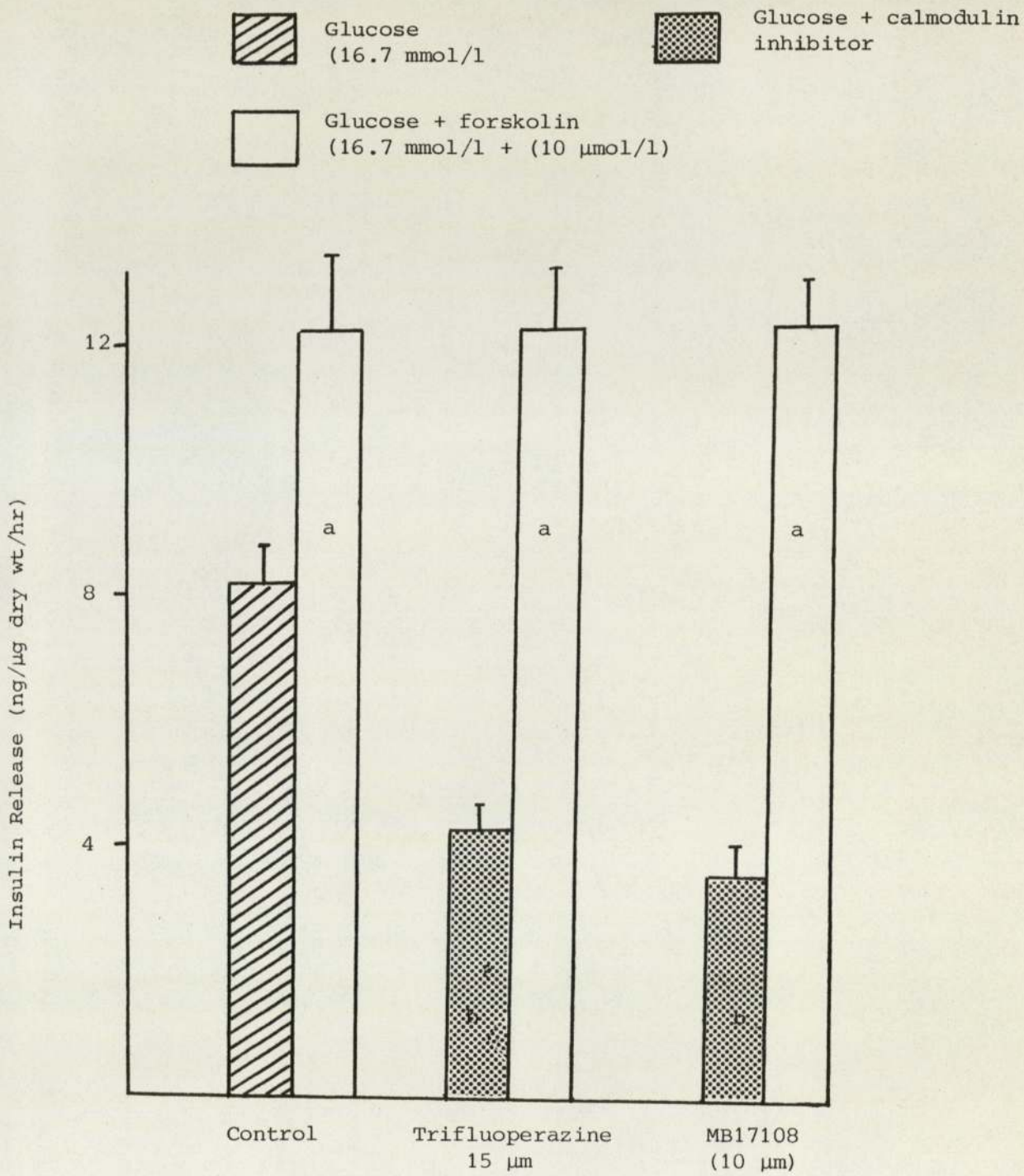


TABLE 8 COMBINED EFFECT OF FORSKOLIN AND THEOPHYLLINE ON GLUCOSE STIMULATED INSULIN RELEASE IN THE PRESENCE OF CALMODULIN INHIBITORS
(N = 9, FOR EACH TREATMENT. VALUES ± SEM).

CALMODULIN INHIBITORS	INSULIN RELEASE UU/UG/H	
	GLUCOSE 16.7 MMOL/L	GLUCOSE (16.7 MMOL/L + THEOPHYLLINE (10 MMOL/L) + FORSKOLIN (10 UMOL/L)
NONE	223 ± 25	357 ± 29 *
TRIFLUOPERAZINE (15 UMOL/L)	143 ± 11	321 ± 31 *
MB17108 (10 UMOL/L)	114 ± 19	347 ± 37 *

* $P < 0.001$ COMPARED WITH GLUCOSE ALONE

DISCUSSION

The present study demonstrated that the calmodulin inhibitors, TFP and MB17108 dose-dependently inhibit glucose stimulated insulin release and forskolin and theophylline both individually and in combination potentiated glucose induced insulin release. The calmodulin inhibitors had no effect on insulin release in the presence of either forskolin or theophylline or the two in combination.

From Figure 42 it is clear that forskolin is capable of potentiating glucose induced insulin release, but cannot initiate insulin release per se in the absence of glucose. This is consistent with the view that cAMP is not an initiator of insulin release (196). It has previously been demonstrated that forskolin causes a concentration dependent increase in cellular cAMP concentrations (225). Henquin and Miessner (226) showed that forskolin produced a rapid dose-dependent increase in mouse islet cAMP levels, which was not influenced by the prevailing glucose concentration and did not require extracellular Ca^{2+}. That forskolin will activate adenylate cyclase independently of extracellular Ca^{2+} has recently been confirmed by Garcia-Morales, Sener, Dufrane, Valverde and Malaisse (227). Using the Quin-2 technique for the measurement of intracellular Ca^{2+}, it has been reported that the elevation of the intracellular cAMP levels by forskolin, dibutryl cAMP and theophylline did not influence intracellular Ca^{2+} in spite of doubling the rate of insulin release (228). This finding, however, contradicts the previously held view that cAMP raises

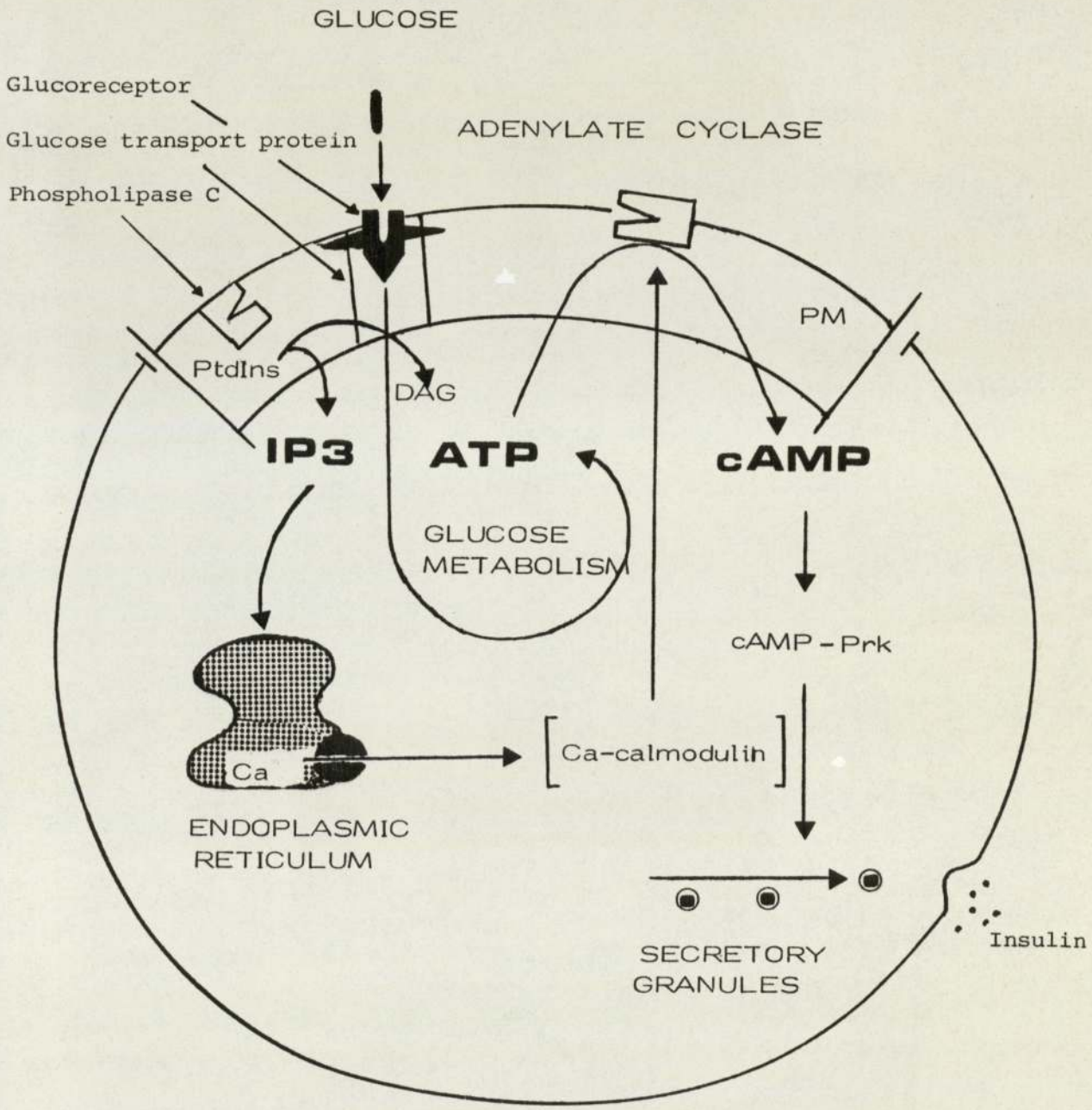
intracellular Ca^{2+} levels (201). Since cAMP has been shown to have no effect on the rate of Ca^{2+} uptake (227) and intracellular Ca^{2+} concentrations, it has been suggested that cAMP may potentiate insulin release by sensitizing the secretory mechanism of the B-cell to Ca^{2+} without actually affecting cytosolic Ca^{2+} levels (228).

Trifluoperazine, a phenothiazine derivative is a calmodulin inhibitor. The specificity of the binding of trifluoperazine to calmodulin has been studied by Levin and Weiss (229). They found that trifluoperazine bound to calmodulins prepared from several species and tissues. From a variety of purified proteins with molecular weights ranging from 125 K dalton to 240 K dalton only calmodulin displayed a significant Ca^{2+}-dependent binding with trifluoperazine. These observations clearly confirm the specificity of trifluoperazine binding to calmodulin. However, TFP is a hydrophobic compound and the possible interaction of TFP with a hydrophobic environment such as the plasma membrane needs to be considered in intact cell system. For example, in voltage clamp studies using frog sciatic nerve, prochloroperazine (a penothiazine similar to trifluoperazine) decreased Na^{+} conductance in a manner similar to that seen with local anaesthetics (230). Although, it has been difficult to eliminate the possibility of calmodulin independent actions of trifluoperazine of insulin release, the following findings rule out such a possibility: firstly trifluoperazine has been shown to have no effect on islet glucose utilization and

insulin biosynthesis (209). Secondly, the compound N-(6-aminohexyl)-5-chloronapthalene-1-sulphonamide (W7), a specific calmodulin inhibitor and chemically related to phenothiazines, also inhibits glucose-induced insulin release (231). Thirdly, the present study has not relied on the potential activities of a single drug but has used a new specific calmodulin inhibitor, MB17108. The latter compound has been shown to selectively inhibit the calmodulin-dependent but not the Ca^{2+}-phospholipid dependent phosphorylation of proteins from rat anterior pituitary (Dr T J Brown, May and Baker Ltd, Personal Communication).

This study has demonstrated that glucose-stimulated insulin release is not blocked by the calmodulin inhibitors trifluoperazine and MB17108 in the presence of either forskolin or theophylline agents known to facilitate elevated levels of intracellular cAMP. There are two possible explanations of this observation: firstly it may be that the elevated intracellular cAMP levels produced by forskolin and/or theophylline antagonize the activities of the calmodulin inhibitors on insulin release or secondly it may be possible that under conditions of elevated cAMP levels the secretory process is activated independently of calmodulin. However, this does not rule out the possibility that under normal physiological conditions calmodulin may still be able to influence the cAMP generation system by activating adenylate cyclase. Figure 44 is a speculative model for the interaction of cAMP and Ca^{2+}-calmodulin. The requirement of stimulatory concentrations

Figure 44. Possible model for the interaction between cAMP and Ca-Calmodulin in insulin release. Abbrev: Ptd Ins, phosphatydyle inositol: PM, plasma membrane DAG, diacylglycerol; IP_3, inositol triphosphate



of glucose by forskolin and theophylline for the initiation of insulin may be explained on the basis that cAMP generation in B-cell depends upon a high ATP/ADP ratio which in turn is dependent on the metabolism of glucose. So glucose needs to serve two functions in the B-cell, firstly an initiator of insulin release via the glucoreceptor and secondly a generator of ATP via metabolism. ATP would also serve as the substrate for adenylate cyclase to produce cAMP which in turn would activate cAMP-dependent protein kinases and thus lead to the stimulation of insulin release. Glucose might also act via IP3 to raise intracellular Ca^{2+} levels which would activate calmodulin, this in turn would further stimulate adenylate cyclase to raise cAMP levels. In the presence of calmodulin inhibitors this process would be inhibited and cause inhibition of insulin release, however when cAMP levels are elevated by forskolin or theophylline this inhibition is compensated.

CHAPTER 6

GENERAL DISCUSSION

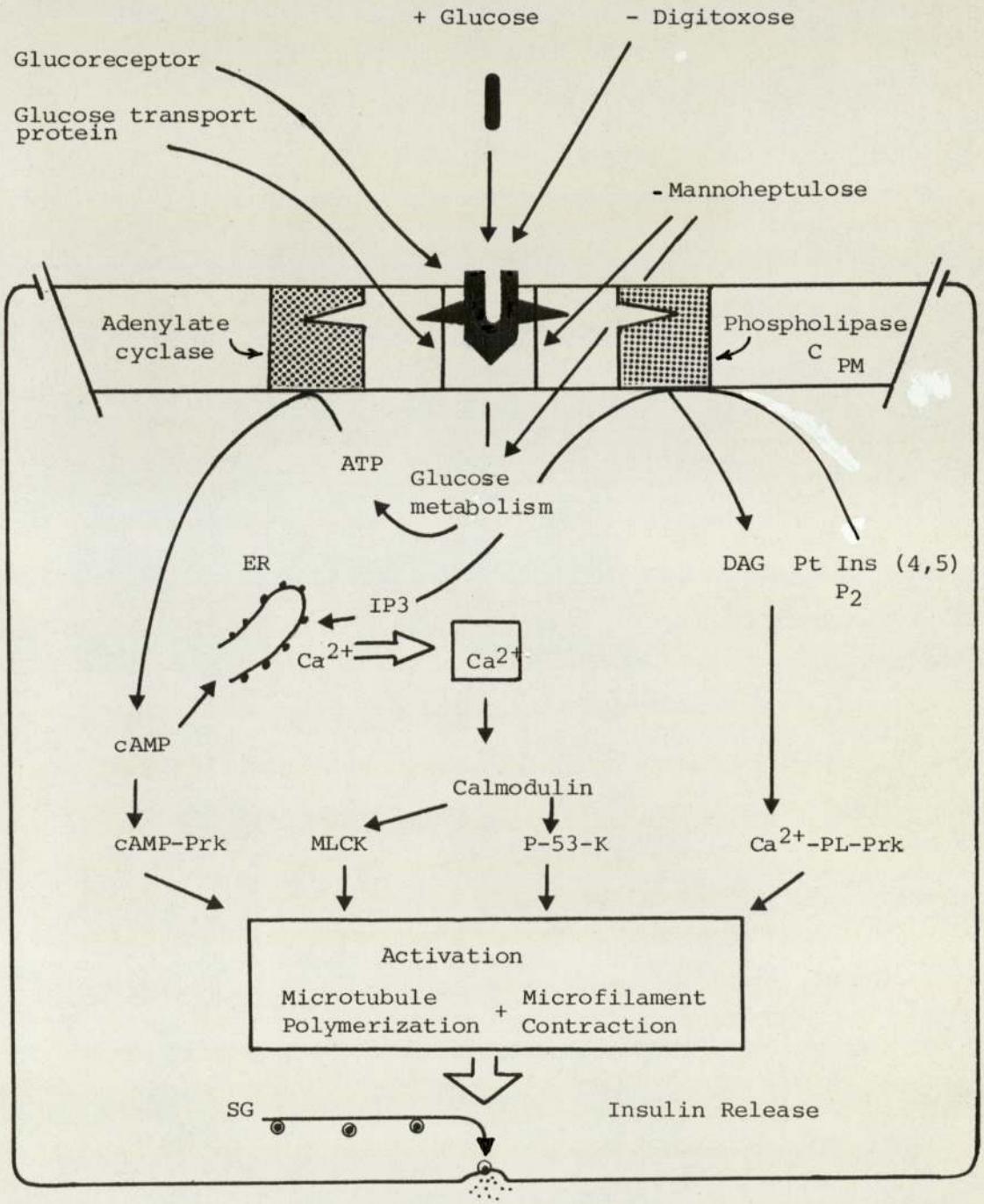
Over the last twenty years two contrasting theories have been advocated concerning the mechanism by which the B-cell recognizes glucose as the physiological stimulus for insulin secretion. According to the first theory "the regulator site hypothesis", the glucose molecule serves as the signal for insulin release by interacting with the putative stereospecific glucoreceptor located at the level of B-cell membrane (65-78). According to the second theory "the substrate site hypothesis, the metabolism of glucose or cofactors associated with glucose metabolism rather than glucose itself, triggers insulin release from B-cells (57-60). Subtle models have also been offered to reconcile both theories (64,67). However, any hypothesis put forward to explain the mechanism of glucose stimulated insulin release is received with a conflicting and highly critical response. The present work with digitoxose provides some measure of support for the existence of a putative membranal glucoreceptor on the B-cell. Digitoxose specifically and competitively inhibited glucose stimulated insulin release from islets of both lean and obese mice without affecting the rate of glucose oxidation or the rate of glucose stimulated oxygen consumption. Therefore, digitoxose provides a means of dissociating glucose stimulated insulin release by isolated islets from their metabolism of glucose suggesting that glucose metabolism *per se* is not a

necessary pre-requisite for the initiation of insulin release but is required to fuel the insulin secretory process.

Studies involving the simultaneous measurement of the rates of insulin release and glucose oxidation from lean and obese mouse islets provide additional support for glucoreceptor hypothesis. The method is unique in that the relationship between insulin release and glucose oxidation can be studied simultaneously. In this system digitoxose significantly inhibited insulin release from both lean and obese mouse islets but had no effect on the rate of glucose oxidation. Based on these observations a speculative model for glucose stimulus insulin secretion coupling involving a membranal glucoreceptor is suggested and illustrated in Figure 45. The model assumes an interaction between glucose and digitoxose and glucose and mannoheptulose at the level of the B-cell membrane with the putative glucoreceptor. It is speculated that digitoxose interacts with the glucoreceptor and partially inhibits the glucoreceptor thereby depressing glucose induced insulin release but leaving the oxidation of glucose within the B-cell unimpaired. The concommitant entry and subsequent metabolism of glucose in the B-cell provides the energy to sustain the secretory process. The dependency of insulin release on supply of energy from glucose oxidation was confirmed by the experiment with action of mannoheptulose. Mannoheptulose is believed to inhibit glucose phosphorylation in islets (182) and by its action inhibits subsequent glucose oxidation thereby depriving the insulin

Figure 45. A speculative model to show the modulation of glucose induced insulin release by digitoxose and mannoheptulose as well as the possible links between membranal glucoreceptor and intracellular processes involved in the release of insulin.

- = inhibition
+ = stimulation



Abbreviations are: Ptd Ins (4,5)P_2, phosphatidyl inositol, 4,5 bisphosphate; PM, plasma membrane; ER, endoplasmic reticulum SG, secretory granules.

secretory process of metabolic energy and consequently inhibiting insulin release.

The possibility that glucose may exert both a signal and metabolic function in the process of insulin release is by no means unique. It is not an uncommon occurrence in biological processes to find one and the same molecule acting as a substrate on one hand and a regulator on the other. This phenomenon is well illustrated by the amino acids L-glycine, L-asparate and L-glutamate which are substrates for metabolic pathways as well as being neurotransmitters in the central nervous system (183). Hence the amino acids have two distinctive effects on brain cells, metabolic effects and regulatory effects. As a metabolic substrate the amino acid molecule participates directly in a substrate reaction and is chemically transformed. When executing a regulatory effect, the amino acid molecule acts as a regulatory signal, an allosteric effector, and its effect does not depend upon its chemical transformation. Like these amino acid glucose might have a dual role in the B-cell.

The mechanism by which specific information is transferred from extracellular space via the B-cell glucoreceptor to the B-cell interior where it triggers the process of insulin secretion is as controversial as the glucoreceptor concept per se. Cyclic AMP has been suggested to be one of the intracellular links between the putative glucoreceptor and the secretory machinery. Within the last three years several other possible second messengers have

been proposed including inositol trisphosphate (26), diacyglycerol (28) and Ca^{2+} (38). Grill and Cerasi (214) showed that glucose directly increases the intracellular concentration of cAMP in rat islets and these workers found that α-D-glucose produced a greater accumulation of islet cAMP than either B-D-glucose or mutarotated glucose. In the present study, the marked potentiation of glucose induced insulin release from lean mouse islets by the specific adenylate cyclase stimulator forskolin suggests an important role for cAMP in the transmission of extracellular information to cell interior. The observation that forskolin shows its greatest effect only in the presence of stimulatory glucose concentrations suggests that the glucoreceptor must first be activated for insulin release to proceed. The inability of calmodulin inhibitors to block insulin release potentiated by either forskolin or theophylline suggests an important role for cAMP in the mechanism of insulin release which is independent of Ca^{2+}-calmodulin (see Figure 44).

A speculative model describing the mechanism by which a putative membrane glucoreceptor might be linked to intra B-cell processes involved in the release of insulin is shown in Figure 45. The concept proposed in this model implies that glucose participates in two independent processes. Firstly it is transported into and is used as a metabolic substrate in the B-cell as in other cells of the body. Secondly, glucose acts on the glucoreceptor which in turn activates phospholipase C and adenylate cyclase. The activation of phospholipase C results in

the breakdown of B-cell membrane phosphatidyl inositol, 4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3). The activation of adenylate cyclase by glucoreceptor results in an increased intracellular cAMP concentration. The elevated concentrations of B-cell cAMP and DAG then activate cAMP and Ca^{2+}-phospholipid dependent protein kinases respectively. IP3 then releases Ca^{2+} from B-cell endoplasmic reticulum and the raised intracellular Ca^{2+} levels activate Ca^{2+}-calmodulin dependent protein kinases, in particular myosin light chain kinase resulting in the phosphorylation and subsequent stimulation of the components of secretory machinery such as microtubules and microfilaments leading to movement of B-granules to the plasma membrane and the release of insulin.

In the present work studies involving the effects of digitoxose on insulin release from obese mouse islets revealed the latter to be more resistant to the inhibitory effect of digitoxose than lean mouse islets suggesting that the activity of the glucoreceptor may be altered in obese mouse islets. Simultaneous measurements of the rates of glucose oxidation and insulin release from obese mouse islets showed a sluggish insulin release but a normal rate of glucose oxidation in response to stimulatory glucose concentration perhaps suggesting a reduced sensitivity in these animals. Indeed obese mouse islets have been shown to be less sensitive than lean to a linear gradient of α-D-glucose(178).

These observations on the normal and altered physiology of insulin release in experimental animal models might be fruitfully

applied to understanding the aetiology of non-insulin dependent diabetes (NIDD). In NIDD glucose fails to induce a normal insulin release, the response is both delayed and reduced (232, 233). The observation that theophylline restored the impaired glucose induced insulin release of prediabetic patients lead Cerasi and Luft (234) to propose that the impaired insulin release in diabetes is caused by a selective impairment of the glucoreceptor for the activation of adenylate cyclase. Hence, the cellular abnormality in diabetes might lie in the inability of the B-cell to transmit extracellular stimulus to the B-cell interior where this information brings about the activation of the insulin secretory machinery (235).

Niki and Niki (67) have postulated that NIDD is due to a generalized disorder of glucoreceptor activity in the neurone-paraneurone system (this refers to secretory and sensory cells which share common morphological physiological and cytochemical features with neurones). For example, the α-anomer of D-glucose is more potent than B-anomer as an inhibitor of spontaneous or arginine-stimulated glucagon release from isolated perfused rat pancreas (236, 237) and in NIDD patients the ability of glucose to suppress the secretion of glucagon is impaired (238). Similarly the α-anomers of D-glucose and D-mannose are reported to be sweeter than their B-anomers (239) and it is generally assumed that these sugars act on the membranal sweet-receptors located on gustatory cells (240). In NIDD patients the sensitivity

of the taste receptor system for glucose appears to be low compared to that in normal subjects (241). Dastoli (242) has been able to isolate proteins sensitive to glucose in bovine tongue and he concluded that glucose interacts with a receptor protein on the tongue and a neural response is elicited which is then transmitted to the taste centres in the brain. A similar situation might occur in the B-cell when glucose interacts with the putative membrane glucoreceptor, the glucose stimulus is transmitted to the insulin secretory machinery to evoke insulin release. The present study provides evidence to support the existence of membranal glucoreceptors on the B-cells of pancreatic islets and extends our understanding of the way the B-cell recognizes glucose as a physiological secretagogue for the release of insulin and the possible perturbation in this system which may contribute to the aetiology of diabetes mellitus.

Information on the mechanism of glucose stimulus insulin secretion coupling is far from complete, however, it is hoped that the present study offers, at least, a framework for the basis of future investigation. Since our understanding of stimulus-secretion coupling will improve our understanding of the aetiology of non-insulin dependent diabetes and contribute to the prognosis and treatment of the syndrome.

APPENDIX

1 BASIC INCUBATION MEDIUM

The incubation medium used throughout these studies was Krebs-Hepes bicarbonate having the following composition:

(the molarity in mmol/l is shown in parenthesis). NaH_2PO_4 (0.2), $NaHCO_3$ (20), Na_2HPO_4 (1.8), KCL (4.5), NaH_2PO_4 (0.2), $MgSO_4$ (1.2), $CaCl_2$ (2.56). After gassing the buffer for 20-30 minutes with 95% O_2, 5% CO_2, HEPES (16 mmol/l) was added and pH adjusted to 7.4 ± 0.5 with 2M NaoH or 2M HCL.

2 STATISTICAL ANALYSIS

(i) Students t-test (unpaired)

In order to determine whether two sample means taken from normal populations were different from each other the Students t-test was used. For example when comparing glucose stimulated insulin release from islets of lean and obese mice. The calculation of t was based on the formula below (232, 233) and the calculated value of t was compared with the table of t values for a given number of degrees of freedom $(n_1 + n_2 - 2)$.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad \text{where } \bar{X}_1 - \bar{X}_2 = \frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}$$

$\bar{X}_1, \bar{X}_2$ = means; S_1, S_2 = standard deviations

n_1, n_2 = number of observations

(ii) Students Paired t-test

In certain studies these data could be paired for example when comparing glucose-stimulated insulin release from lean mouse islets exposed to 5.5 and then 16.7 mmol/l glucose. Pairing was justified, in this instance, as the observations were made on the islets from the same animal in each experiment. In a paired t-test the calculation of t was based on the formula below and the calculated value of t was compared with a table of t values for a given number of degrees of freedom (n -1).

$$t = \frac{\Sigma D}{\sqrt{\frac{(n \Sigma D^2 - (\Sigma D)^2)}{n - 1}}}$$

ΣD, the sum of difference values

ΣD^2, the sum of the squares of the difference values

In both paired and unpaired student t-tests the two groups are treated as samples from two separate populations and efforts were made to establish whether these populations had different means. The value of 't' is a measure of the difference in the sample means. Probability levels of $P < 0.05$ were taken as statistically significant.

A4.3 Linear Regression Analysis

To estimate the degree of relationship between two variables, a relationship in which one variable is dependent on the magnitude of the other, is termed regression and can be analysed by calculating the correction coefficient r, using the formula below and comparing this with a table of r values with n-2 degrees of freedom.

$$r = \frac{\Sigma XY}{\sqrt{\Sigma X^2 \Sigma Y^2}}$$

Σx, the deviation of each x value from the mean of X.

Σy, the deviation of each y value from the mean of Y.

3. A computer program in BASIC for the analysis of RIA data using either human or mouse standards.

```
  5 REM              INSULIN ASSAY
 10 REM
 15 REM
 20 REM
 25 REM  DATA statements : "Species", "units", no. of standards, standard concns.
 30 DATA "Mouse", "ng/ml", 5, .5, 1, 2.5, 5, 10
 35 DATA "Human", "uU/ml", 5, 12.5, 25, 50, 100, 200
 40 INPUT "Do you want printout ", PR$
 45 IF ASCII(CVT$$(PR$,32)) = 89 THEN 55
 50 LET PR$ = "N" : GOTO 140
 55 LET PR$ = "Y"
 60 PRINT : INPUT "File name for printout ", FL$ : IF FL$ = "INS" THEN 60
 65 ON ERROR GOTO 85
 70 GENERATE FL$
 75 ON ERROR GOTO 0
 80 ASSIGN #11 = FL$ : OPEN #11 : GOTO 115
 85 IF ERROR >< 2150 THEN DO : ON ERROR GOTO 0 : GOTO 70 : DOEND
 90 PRINT:PRINT"Area already exists - do you want to delete existing data in it"
 95 INPUT "( WARNING - do not delete a program area with this name ) ", ANS
100 IF ASCII(CVT$$(ANS,32)) >< 89 THEN 60
105 ELIMINATE FL$
110 GOTO 70
115 PRINT : PRINT "Experiment title etc. for printout"
120 INPUT TI$ : PRINT #11 TI$
125 REM ************************************************
130 REM Input standard cpm's & compute best line
135 REM ************************************************
140 INPUT "STANDARDS : Mouse or Human or Both ( M or H or B ) ", MHB$
145 LET MHB$ = CVT$$(MHB$,32)
155 IF MHB$ = "H" THEN 185
160 IF MHB$ >< "B" THEN 140
165 GOSUB 235
170 LET INM = IN
175 LET SLM = SL
180 IF MHB$ = "M" THEN 440
185 RESTORE
190 READ R$, R$, RX
195 FOR JX = 1 TO RX
200 READ R
205 NEXT JX
210 GOSUB 235
215 LET INH = IN
220 LET SLH = SL
225 GOTO 440
230 REM ******************** SUB
235 READ Sp$, Un$
240 PRINT : PRINT Sp$
245 PRINT "Standards","  cpm       (if value not available enter '0')"
250 PRINT Un$
255 IF PR$ = "N" THEN 275
260 PRINT #11 : PRINT #11 Sp$
265 PRINT #11 "Standards","  cpm"
270 PRINT #11 Un$
275 LET X,Q,Y,S,W,NX = 0
280 READ StNX
285 FOR JX = 1 TO StNX
290 READ StCo ! Standard concentration
295 PRINT StCo," ";
300 INPUT StCpm
305 IF StCpm >< 0 THEN 320
310 IF PR$ = "N" THEN 365
315 PRINT #11 USING "####.#                -", StCo : GOTO 365
320 IF PR$ = "N" THEN 330
325 PRINT #11 USING "####.#     ##############", StCo,StCpm
330 LET StCo = LOG10(StCo)
335 LET X = X+StCpm
340 LET Q = Q+StCpm^2
345 LET Y = Y+StCo
350 LET S = S+StCo^2
355 LET W = W+StCo*StCpm
360 LET NX = NX+1
365 NEXT JX
370 LET SL = (W-X*Y/NX)/(Q-X^2/NX)
375 LET IN = (Y-SL*X)/NX
380 LET CoCo =(W-X*Y/NX)/SQR((Q-X^2/NX)*(S-Y^2/NX))
385 PRINT : PRINT "Computed best line : Intercept (cpm = 0)      = "; IN
390 PRINT TAB(22),"Slope",TAB(46),"= "; SL
395 PRINT TAB(22),"Correlation coefficient = "; CoCo
400 IF PR$ = "N" THEN 420
```

(continued)

```
405 PRINT #11 : PRINT #11 "Computed best line : Intercept (cpm = 0)      = "; IN
410 PRINT #11, TAB(22), "Slope", TAB(46), "= "; SL
415 PRINT #11, TAB(22), "Correlation coefficient = "; CoCo
420 RETURN
425 REM ************************************************
430 REM                  Print headings
435 REM ************************************************
440 PRINT : PRINT "Input sample name , or 'END' to stop program"
445 PRINT TAB(21), "or 'DIL' to change dilution factor"
450 IF PR$ = "N" THEN 460
455 PRINT TAB(21), "or 'SUBH' to insert subheading for printout"
460 PRINT "followed by cpm"
465 PRINT : PRINT "'*' indicates result outside linear region of curve"
470 PRINT "    and will be incorrectly calculated by this program"
475 REM Limits of linearity set in 640, 645, 690, 695
480 IF MHB$ = "B" THEN 545
485 PRINT : PRINT TAB(38), "Insulin concn. "
490 PRINT TAB(38), "ref. "; Sp$; " standard"
495 IF PR$ = "N" THEN 510
500 PRINT #11 : PRINT #11 TAB(39), "Insulin concentration"
505 PRINT #11 TAB(27), "Dilution     ref. "; Sp$; " standard"
510 IF MHB$ = "H" THEN 530
515 PRINT "Sample", "  cpm", "   ng/ml", "  uU/ml"
520 IF PR$ = "N" THEN 600
525 PRINT #11 "Sample", "  cpm     factor       ng/ml          uU/ml" : GOTO 580
530 PRINT "Sample", "  cpm", "   uU/ml"
535 IF PR$ = "N" THEN 600
540 PRINT #11 "Sample", "  cpm     factor", TAB(45), "uU/ml" : GOTO 580
545 PRINT : PRINT TAB(38), "Insulin concentration    Insulin concn. "
550 PRINT TAB(38), "ref. Mouse standard      ref. Human st. "
555 PRINT "Sample", "cpm", " ng/ml", "uU/ml", " uU/ml"
560 IF PR$ = "N" THEN 600
565 PRINT #11:PRINT #11 TAB(38), "Insulin concentration    Insulin concentration"
570 PRINT #11 TAB(26), "Dilution     ref. Mouse standard     ref. Human standard"
575 PRINT #11 "Sample", "cpm     factor       ng/ml          uU/ml", TAB(69), "uU/ml"
580 PRINT #11
585 REM ************************************************
590 REM          Calculate sample concentrations
595 REM ************************************************
600 PRINT : INPUT "Dilution factor ", DF : PRINT
605 LET MX$, HX$ = "*"
610 INPUT Sam$
615 IF PR$ = "N" THEN 630
620 IF CVT$$(Sam$, 32) >< "SUBH" THEN 630
625 INPUT TI$ : PRINT #11 : PRINT #11 "  "; TI$ : PRINT : GOTO 610
630 IF CVT$$(Sam$, 32) = "END" THEN 780
635 IF CVT$$(Sam$, 32) = "DIL" THEN 600
640 PRINT " ", " "; : INPUT Cpm : PRINT Sam$, Cpm, " ";
645 IF MHB$ = "H" THEN 700
650 LET CoM = INT(EXP((INM+SLM*Cpm)*LOG(10))*10+.5)/10
655 IF CoM < .5 THEN 670
660 IF CoM > 12.8 THEN 670
665 LET MX$ = " "
670 IF MHB$ = "B" THEN 700
675 PRINT USING "!####.#       !#####.#", MX$, DF*CoM, MX$, 25*DF*CoM
680 IF PR$ = "N" THEN 695
685 LET FMM$ = "\                \#####     #####      !#####.#      !######.#"
690 PRINT #11 USING FMM$, Sam$, Cpm, DF, MX$, DF*CoM, MX$, 25*DF*CoM
695 IF MHB$ = "M" THEN 605
700 LET CoH = INT(EXP((INH+SLH*Cpm)*LOG(10))+.5)
705 IF CoH < 12.5 THEN 720
710 IF CoH > 320 THEN 720
715 LET HX$ = " "
720 IF MHB$ = "B" THEN 745
725 PRINT USING "!######", HX$, DF*CoH
730 IF PR$ = "N" THEN 605
735 LET FMH$ = "\                \#####     #####          !########"
740 PRINT #11 USING FMH$, Sam$, Cpm, DF, HX$, DF*CoH : GOTO 605
745 LET FMBT$ = "!####.#       !#####.#          !#####"
750 PRINT USING FMBT$, MX$, DF*CoM, MX$, 25*DF*CoM, HX$, DF*CoH
755 IF PR$ = "N" THEN 605
760 LET FMB1$ = "\               \#####     #####      "
765 LET FMB$ = FMB1$+"!#####.#      !######.#           !########"
770 PRINT #11 USING FMB$, Sam$, Cpm, DF, MX$, DF*CoM, MX$, 25*DF*CoM, HX$, DF*CoH
775 GOTO 605
780 IF PR$ = "N" THEN STOP
785 PRINT : PRINT "File for printout : "; FL$
```

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THE EFFECT OF DIGITOXOSE ON INSULIN RELEASE, GLUCOSE OXIDATION AND OXYGEN CONSUMPTION BY ISLETS OF LEAN AND GENETICALLY OBESE DIABETIC (OB/OB) MICE

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Digitoxose specifically and competitively inhibited glucose stimulated insulin release from islets of both lean and obese mice without affecting either the rate of glucose oxidation or the rate of glucose stimulated oxygen consumption. Obese mouse islets were marginally more resistant to the inhibitory effect of digitoxose than lean mouse islets.

Digitoxose provides a means for dissociating glucose stimulated insulin release by isolated islets from their metabolism of glucose confirming that glucose metabolism per se is not a necessary prerequisite for the initiation of insulin release but is required to fuel the insulin secretory process.

Over the last 20 years two contrasting theories have been advocated concerning the mechanism by which the B-cell recognises glucose as the physiological stimulus for the secretion of insulin. According to the first theory, 'the regulator site hypothesis', the glucose molecule itself serves as the signal for insulin release by interacting with a putative stereo-specific glucoreceptor located at the level of the B-cell membrane (1,2,3). According to the second theory, 'the substrate site hypothesis', the metabolism of glucose or cofactors associated with glucose metabolism rather than glucose itself, triggers insulin release from B-cells (4,5). Subtle models have also been offered to reconcile both theories (6,7,8). Substantial support in favour of the regulator site hypothesis would be offered by a sugar that would have the capacity to inhibit glucose stimulated insulin release without affecting glucose metabolism (9). D-(+)-digitoxose (2-desoxy-D-altromethylose), the sugar moiety of the cardiac glycosides digitoxin and

Abbreviations: ob/ob, genetically obese diabetic mice, genotype ob/ob. (QO_2), rate of oxygen consumption.

digoxin has been shown to inhibit glucose stimulated insulin release from rat islets without inhibiting glucose metabolism (10). The present study examines the dose response and specificity of digitoxose induced inhibition of insulin release and the effect of digitoxose on the rate of D-(U-^{14}C)-glucose oxidation and oxygen consumption by islets from lean and genetically obese diabetic (ob/ob) mice to provide information on the activity of the putative regulator site mechanism for glucose stimulated insulin release in an experimental model of non insulin requiring diabetes mellitus.

MATERIALS AND METHODS

Animals:

Overnight fasted male genetically obese diabetic (ob/ob) mice and homozygous lean (+/+) mice from the Aston colony were used, aged between 14 - 16 weeks. The origin and characteristics of Aston ob/ob mice have been described elsewhere (11,12). All mice were housed in an air conditioned room at 22 ± 2°C and maintained on a standard pellet diet (Mouse breeding diet, Heygate & Sons, Northampton, U.K.) and tap water ad libitum.

Chemicals:

Reagents of analytical grade and double distilled water were used throughout. The chemicals and their sources were as follows: N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), α-D-glucose from Sigma (London) Chemicals, Poole, U.K.: collagenase (K4C5577) from Flow Laboratories Ltd, Irvine, Scotland: mouse insulin standard from NOVO Research Institute, Bagsvaerd, Denmark: insulin binding reagent (Lot K896210) from Wellcome Reagents Ltd, U.K.: D-(U-^{14}C) glucose (250 mCi/mmol) from Amersham International, Amersham, U.K.: insulin free bovine serum albumin (fraction V, batch 318) from Miles Laboratories Ltd, Slough, U.K.: 2-desoxy-D-altromethlyose (Digitoxose) from Aldrich Chemical Co Ltd, Gillingham, U.K.: sodium dithionite from British Drug Houses and other reagents from Fisons Scientific Apparatus, Loughborough, U.K.

Experimental procedures:

The specificity and dose response for digitoxose inhibition of glucose stimulated insulin release was initially evaluated and the rates of D-(U-^{14}C)-glucose oxidation, glucose stimulated oxygen consumption (Qo_2) and cumulative insulin release by isolated islets from lean and ob/ob mice determined in the absence and presence of an optimal inhibitory concentration of digitoxose.

Preparation of isolated islets:

Islets were separated from the pooled pancreas of two mice by collagenase digestion (13) in oxygenated Krebs-Hepes buffer, pH 7.4 (14) containing glucose (5.6 mmol/l) and bovine serum albumin (0.1 g/l) and harvested with the aid of a drawn out pasteur pipette. A yield of 100-150 clean islets was obtained within two hours and maintained in oxygenated buffer at 2°C prior to use.

Dose response for digitoxose inhibition of glucose stimulated insulin release:

Batches of 5-8 isolated islets from either lean or obese mice were preincubated for 30 min at 37°C in oxygenated Krebs-Hepes buffer, pH 7.4 containing glucose (5.6 mmol/l) and bovine serum albumin (0.1 g/l). The preincubation buffer was subsequently replaced with fresh (oxygenated buffer at 37°C containing glucose (16.7 mmol/l) alone or glucose (16.7 mmol/l) supplemented with one of a range of digitoxose concentrations (5 6, 11.2, 16.7, 22.2, 27.2 mmol/l) and islet incubation continued for a further 1 hr in a shaking water bath (100 cycles/min). After 1 hour 50 ul aliquots were removed for the determination of insulin (15). Incubated islets were dried at 70°C overnight on aluminium foil and their dry weight calculated by difference. The rate of insulin release was expressed as uU of insulin released per ug. dry weight of islets per hour. Values for the percentage inhibition of glucose stimulated insulin release from islets of lean and ob/ob mice produced by the various concentrations of digitoxose were calculated and these data used to construct dose response curves. Lineweaver and Burk analysis of these data was used to provide values for the inhibitor constant (Ki) and the maximum percentage inhibition (16).

The specificity of digitoxose induced inhibition of insulin release from lean mouse islets:

Batches of 5-8 islets were initially preincubated for 30 min at 37°C in oxygenated Krebs-Hepes buffer, pH 7.4 containing glucose (5.6 mmol/l) and bovine serum albumin (0.1 g/l) and subsequently test incubated for 1 hour in fresh buffer supplemented with one or other of the following secretagogues, D-glucose (16.7 mmol/l), L-leucine (30.5 mmol/l), L-arginine (19 mmol/l) and glibenclamide (8.1 umol/l) in the absence and presence of digitoxose (22.2 mmol/l). After incubation 50 ul aliquots were removed for the determination of insulin and values expressed as uU of insulin released per ug dry weight of islets per hour.

Glucose oxidation:

Batches of 25 islets were incubated in small plastic reaction tubes (5x35mm, Luckhams Ltd, Burgess Hill, UK.) (17) containing 100 ul Krebs-Hepes buffer, pH 7.4, supplemented with either glucose (16.7mmol/l) alone or with digitoxose (22.2 mmol/l) and D-(U-^{14}C) glucose (4 uCi/ml). The reaction tubes were placed inside glass scintillation vials and the atmosphere gassed with 95% O_2 : 5% Co_2 for 10 min. The scintillation vials were sealed and incubated for 75 min at 37°C in a shaking water bath (100 cycles/min.). After incubation the vials were removed and chilled. 1ml of NaOH (1 mol/l) was pipetted into the outer compartment and islet metabolism stopped by the injection of 200 ul of HCl (0.1 mol/l) into the reaction tube. The vials were then tightly sealed and left at room temperature overnight for the absorption of $^{14}Co_2$. Radioactivity trapped by the NaOH was subsequently measured on a liquid scintillation counter (Tricarb 2660, Packhard Instruments Ltd., Caversham, U.K., counting efficiency > 95%). Three blank incubations without islets were carried through the whole procedure in each experiment and blank values were subtracted from experimental values. Glucose oxidation rates were calculated as pmol/l of D-(U-^{14}C)-glucose oxidised per ug. dry weight of islets per hour from the following formula:

$$\text{D-(U-}^{14}\text{C)-glucose oxidised (pmol/l)} = \frac{^{14}CO_2 \text{ formed (dpm)}}{\text{Specific radioactivity of D-(U-}^{14}\text{C)-glucose (dpm/pmol/l)}}$$

Oxygen consumption and cumulative insulin release.

Islet oxygen consumption (QO_2) was measured using an electrode assembly (Rank Brothers, Cambridge, U.K.) specifically designed for monitoring the consumption of dissolved oxygen by cell suspensions (18). The electrode was calibrated by filling the incubation cell with distilled water at 37°C, Figure 1. A baseline oxygen depleted setting was first obtained by adding a few crystals of sodium dithionite. The incubation cell was then purged and refilled with air-saturated water at 37°. The sensitivity of the electrode response was adjusted to obtain maximum deflection and the electrode calibrated daily. The dissolved oxygen content of air saturated water was determined by the Winkler method (19). Since the oxygen electrode is a percentage response instrument, values of electrode current obtained during the course of experimental work could be used for the direct calculation of the initial amount of dissolved oxygen in the incubation medium and subsequently the rate of oxygen consumption (QO_2) by isolated islets. The oxygen electrode used in this study had a 90% response time of 5 seconds and consumed only negligible amounts of oxygen (< 200 ng O_2/h). After electrode calibration the incubation cell was filled with Krebs-Hepes buffer, pH 7.4 and the drift in electrode current monitored for 30 min. The content of the incubation cell was replaced with fresh buffer containing 100-150 isolated islets from either lean or ob/ob mice and the steady state basal rate of oxygen consumption recorded for a further 30 min. At the end of this time a 50 ul aliquot of incubation buffer was removed for insulin determination (15). The nylon basket was removed and the contained islets washed with Krebs-Hepes buffer prior to being reinserted into the incubation cell with fresh buffer supplemented with either gluocse (16.7 mmol/l) alone or with digitoxose (22.2 mmol/L) for a further 1 hour incubation. A 50 ul aliquot of incubation buffer was removed for the measurement of cumulative insulin release and the new rate of oxygen consumption determined. The dry weight of incubated islets was determined as described previously and the rate of oxygen consumption expressed as ng O_2 consumed per ug dry weight of islets per hour. The most commonly encountered difficulty with the oxygen electrode was its tendency to age. This was aggravated by the presence of divalent cations and albumin in the incubation medium and blank experiments without islets were carried out routinely to monitor any non-specific upward drift in the basal rate of oxygen consumption.

Statistical analysis:

Results were expressed as the mean ± SEM. Student's t-test was used for statistical comparison and differences were considered to be significant if $P < 0.05$.

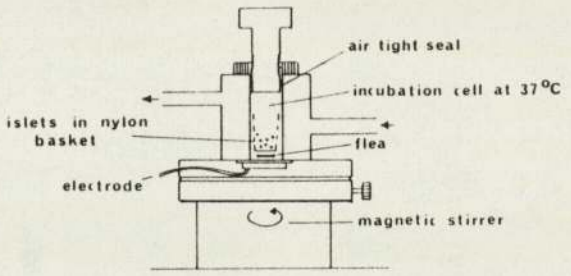

Figure 1. Electrode assembly for the measurement of oxygen consumption and cumulative insulin release.

RESULTS

Figure 2 shows the effect of increasing concentrations of digitoxose on glucose stimulated insulin release from lean and ob/ob mouse islets. The maximum inhibitory effect of digitoxose was obtained at concentrations greater than 16.7 mmol/l with lean mouse islets and greater than 22.2 mmol/l with ob/ob mouse islets producing 53% and 46% inhibition of glucose stimulated insulin release respectively. A double reciprocal plot of the same data gave values for the inhibitor constant (Ki) of 16 and 20 mmol/l digitoxose and values for maximum percentage inhibition of 83% and 77% for lean and ob/ob mouse islets respectively. In a separate series of experiments the dose response curve for glucose stimulated insulin release from lean mouse islets was shifted towards the right by increasing concentrations of digitoxose, a characteristic feature of competitive inhibition. Lineweaver and Burk plots of these data gave Km values of 10, 13.2, 15.7 and 19.0 mmol/l for glucose in the presence of digitoxose concentrations of 0, 11.2, 16.7 and 22.2 mmol/l respectively. In all subsequent studies digitoxose was used at a

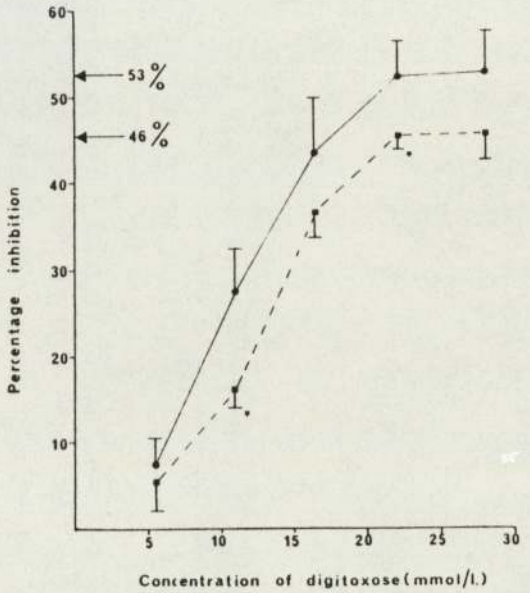

Figure 2. Dose response for digitoxose inhibition of glucose stimulated insulin release from islets of lean ●——● and ob/ob ■– –■ mice. Each point represents the mean of six determinations ± SEM. * $P < 0.05$ compared with lean at each digitoxose concentration.

Table 1. Specificity of digitoxose (22.2 mmol/l) inhibition of insulin release from lean mouse islets

Secretagogue	Insulin release (uU insulin/ug dry weight of islets/h)			
	n	- Digitoxose	n	+ Digitoxose
None	5	22 ± 2.5	4	30 ± 3.9
D-glucose (16.7 mmol/l)	4	123 ± 6.1	7	73 ± 3.2 [a]
L-leucine (36.5 mmol/l)	10	104 ± 7.6	11	96 ± 5.3
L-Arginine (19 mmol/l)	8	102 ± 9.4	10	100 ± 6.5
glibenclamide (8.1 umol/l)	8	83 ± 2.7	7	79 ± 4.8

Results are expressed as mean ± SEM.

a, $P < 0.05$ compared with value in the absence of digitoxose.

concentration of 22.2 mmol/l. Indeed digitoxose at this concentration specifically inhibited glucose (16.7 mmol/l) stimulated insulin release from lean mouse islets but was without significant effect on the insulin secretory response elicted by either L-leucine, L-arginine or glibenclamide, Table 1.

The rate of glucose oxidation by ob/ob mouse islets was significantly greater than that of lean mouse islets, Table 2 and digitoxose (22.2 mmol/l) had no significant effect on the rate of glucose oxidation by islets from either lean or ob/ob mice.

The rate of oxygen consumption (QO_2) by lean mouse islets was significantly greater than that of ob/ob mouse islets in the presence of 5.6 mmol/l glucose but not in the presence of 16.7 mmol/l glucose, Table 3. On

Table 2. The effect of digitoxose (22.2 mmol/l) on the rate of D-(U-^{14}C)-glucose oxidation by lean and ob/ob mouse islets

Glucose (mmol/l)	n	D-(U-^{14}C)-glucose oxidation (pmol/l/ug dry weight of islets/h)	
		lean mouse islets	ob/ob mouse islets
16.7	5	21.1 ± 1.6	36.8 ± 3.1 [a]
16.7 + Digitoxose (22.2 mmol/l)	5	18.2 ± 0.8	34.6 ± 2.6 [a]

Results are expressed as mean ± SEM.

a, $p < 0.05$ compared with lean mouse islets.

Table 3. The effect of digitoxose (22.2 mmol/l) on the rate of glucose stimulated oxygen consumption (QO_2) by lean and ob/ob mouse islets

Glucose (mmol/l)	Oxygen consumption (QO_2) (ngO_2/ug dry weight of islets/h)			
	n	lean mouse islets	n	ob/ob mouse islets
5.6	6	11.2 ± 1.0 [a]	6	8.2 ± 0.6
16.7	4	14.9 ± 0.5 [b]	5	12.8 ± 0.8 [b]
16.7 + Digitoxose (22.2 mmol/l)	4	15.7 ± 1.1	5	13.2 ± 1.2

Results are expressed as means ± SEM.

a, $p < 0.05$ compared with corresponding value for ob/ob mouse islets.

b, $p < 0.05$ compared with values in the presence of 5.6 mmol/l glucose.

the other hand the cumulative insulin release by lean mouse islets was significantly greater than that for ob/ob mouse islets in the presence of 16 7 mmol/l glucose but not in the presence of 5.6 mmol/l glucose, Table 4. Increasing the glucose concentration from 5 6 to 16.7 mmol/l significantly increased the rate of oxygen consumption, Table 3 and the cumulative insulin release, Table 4 by both lean and ob/ob mouse islets. Digitoxose (22.2 mmol/l) had no significant effect on the rate of glucose (16.7 mmol/l) stimulated oxygen consumption by either lean or ob/ob mouse islets, Table 3, but significantly reduced the cumulative insulin release in response to glucose (16.7 mmol/l) from both lean and ob/ob mouse islets by 41% and 38% respectively, Table 4.

Table 4. The effect of digitoxose (22.2 mmol/l) on cumulative insulin release from islets of lean and ob/ob mice

Glucose (mmol/l)	Cumulative insulin release (uU/ug dry weight of islets/h)			
	n	lean mouse islets	n	ob/ob mouse islets
5.6	7	55 ± 6 [b]	7	52 ± 6 [b]
16.7	4	123 ± 12 [a]	5	92 ± 7
16.7 + Digitoxose (22.2 mmol/l)	4	73 ± 4 [b]	5	57 ± 5 [b]

Results are expressed as mean ± SEM.

a, $p < 0.05$ compared with the corresponding value for ob/ob mouse islets.

b, $p < 0.05$ compared with values in presence of 16.7 mmol/l glucose alone.

DISCUSSION

The results indicate that digitoxose specifically and competitively inhibits glucose stimulated insulin release from islets of both lean and ob/ob mice (Ki values of 16 and 20 mmol/l and maximum inhibition of 83% and 77% respectively) and these data are consistent with previous observations made on isolated rat islets (10,20). However, digitoxose (22.2 mmol/l) had no effect on either the rate of glucose oxidation or the rate of glucose stimulated oxygen consumption by islets from either lean or ob/ob mice. The percentage inhibition of glucose stimulated insulin release by digitoxose (22.2 mmol/l) from statistically incubated ob/ob mouse islets (46%) was less than that for lean mouse islets (53%) suggesting ob/ob mouse islets to be marginally more resistant to the inhibitory effect of digitoxose than lean mouse islets.

Islet viability was confirmed by the significantly increased rates of oxygen consumption and cumulatative insulin release observed for both lean and ob/ob mouse islets in response to an increase in the glucose concentration from 5.6 to 16.7 mmol/l. The rates of glucose oxidation shown by ob/ob mouse islets were always significantly greater than those of lean mouse islets both in the presence and absence of digitoxose. On the other hand the rates of glucose stimulated oxygen consumption and cumulative insulin release were generally higher for lean mouse islets, although this depended upon the concentration of glucose present.

The rates of oxygen consumption observed for mouse islets in the present study were somewhat higher than values obtained by previous workers (21,22) and may be accounted for by the expression of data in terms of the dry weight of islet tissue, the use of bicarbonate in the incubation medium (23) and by thorough stirring of the medium to prevent electrode stagnation and the build up of inhibitory secretory products in the vicinity of the islets (22).

The present data suggest that digitoxose, which is not metabolised to any extent by isolated islets (20), inhibits glucose stimulated insulin release from mouse islets without affecting the rate of glucose oxidation. Therefore digitoxose is able to dissociate glucose stimulated insulin release by

isolated islets from their metabolism of glucose. This property is not exclusive to digitoxose, since the the α-adrenergic agonist clonidine will also produce a dose related inhibition of glucose stimulated insulin release without affecting glucose oxidation (24). In addition, glucose releases 30 fold more insulin from islets than from single B-cells and the dependency of glucose induced insulin release upon the functional co-ordination between islet cells is not mediated through changes in glucose metabolism alone suggesting that the latter are not sufficient for the appropriate regulation of insulin release by glucose (25). The fact that B-cell rich ob/ob mouse islets (26) appear to be more resistant to the inhibitory effect of digitoxose than lean suggests that the process of glucose recognition may be altered in ob/ob mouse islets and indeed the latter have been shown to be less sensitive than lean to the lower concentrations (1-9 mmol/l) of a gradient of α-D-glucose (27).

The studies described here support the concept of a regulator site mechanism for glucose stimulated insulin release and confirm that glucose metabolism per se is not a necessary prerequisite for the initiation of insulin release but is required to provide energy for the insulin secretory process.

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ABSTRACT 1

Diabetic Medicine 1(2):125A

THE EFFECT OF 2, 6 DIDESOXY - D - ALLOSE (DIGITOXOSE) ON OXYGEN UPTAKE AND INSULIN RELEASE FROM ISLETS OF LEAN AND OBESE MICE.

Simultaneous measurements of oxygen uptake (QO_2) and insulin release have been made on isolated islets from lean and obese mice. Islets were incubated in Kreb's-Hepes buffer pH 7.4 supplemented with either 5.5 or 16.7 mM glucose in the presence and absence of 16.7 mM 2,6 didesoxy-D-Allose for 60 minutes. The QO_2 was recorded continuously using a Clark type oxygen electrode and 200 ul aliquots of medium were removed for insulin assay. Obese mouse islets gave QO_2 ± SEM values of 8.2 ± 0.7 and 12.8 ± 0.8 (ngO_2/ug-dry wgt./hr.) in the presence of 5.5 and 16.7 mM glucose respectively. The equivalent values for lean mouse islets were 14.4 ± 0.9 and 23.2 ± 1.6 respectively. Digitoxose had no effect on glucose stimulated QO_2 but significantly reduced glucose (16.7 mM) stimulated insulin release from both lean and obese mouse islets by 37%. The ability of digitoxose to dissociate the oxidative metabolism of glucose from its effect on insulin release would suggest that oxidative glucose metabolism is not an essential stage in glucose stimulus insulin secretion coupling.

ABSTRACT 2

Diabetic Medicine 1(3);125A

The effect of calmodulin inhibitors on glucose induced and forskolin potentiated insulin release from mouse pancreatic islets.

Ca^{2+} calmodulin and cyclic AMP are believed to be involved in the mechanism of glucose induced insulin release. The aim of the present study was to determine whether the effect of elevated cyclic AMP on insulin release is calmodulin dependent. Mouse islets were isolated by collagenase digestion and incubated at 37°C in oxygenated Krebs buffer supplemented with 16.7 mmol/ℓ glucose. Calmodulin inhibition was effected with either trifluoperazine (TFP) or MB 17108 (a new phenoxy propylamine derivative). Both agents inhibited glucose induced insulin release dose dependently, with inhibitor constants (K_I) of 25 umol/ℓ and 15 umol/ℓ respectively. Glucose (16.7 mmol/ℓ) stimulated insulin release was significantly increased by forskolin (10 umol/ℓ) from 208 ± 22, uU $islet^{-1}$ hr^{-1}, ($P<0.01$). When either calmodulin inhibitor was added, glucose induced insulin release was reduced by ≃ 50%. However, in the presence of either forskolin or theophylline (2 mmol/ℓ) or the two in combination, the calmodulin inhibitors had no effect on insulin release. These data suggest that agents capable of raising cyclic AMP levels in islets stimulate glucose induced insulin release independently of Ca^{2+} calmodulin.

ABSTRACT 3

Diabetic Medicine 1(4);

Simultaneous measurement of glucose oxidation and insulin release from lean and obese mouse islets; the effects of digitoxose and mannoheptulose.

In order to establish whether glucose oxidation is a necessary prerequisite for glucose induced insulin release, the rates of glucose oxidation and insulin release by mouse islets were monitored continuously and simultaneously in the presence of digitoxose (22.2 mmol/l) and mannoheptulose (16.7 mmol/l) using a novel perifusion system. Perifusate insulin was measured by radioimmunossay. Digitoxose had no effect on the rate of glucose oxidation by either lean or obese mouse islets but inhibited glucose induced insulin release from both by 40%. Mannoheptulose significantly inhibited both glucose oxidation and insulin release from lean and obese mouse islets ($P<0.05$). The rates of glucose oxidation and glucose induced insulin release were always greater for lean mouse islets than for obese. The differential effects of digitoxose and mannoheptulose on glucose oxidation and insulin release support a regulator site mechanism for insulin release. A sluggish insulin response to glucose by obese mouse islets compared to lean with no change in the rate of glucose oxidation suggests that the glucoreceptor may be altered in these animals.

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