THE GASTROINTESTINAL STIMULUS TO INSULIN SECRETION IN LEAN AND OBESE HYPERGLYCAEMIC MICE

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CONTENTS

Summary

A shortened, simplified modification of the insulin radioimmunoassay method of Hales & Randle has been established.

Intraperitoneal Glucose tolerance tests (ipGTT) confirmed hyperglycaemia, hyperinsulinaemia and impaired glucose tolerance in obese hyperglycaemic mice. Secretin and pancreozymin, but not gastrin elevated plasma insulin levels and reduced blood glucose levels during ipGTT.

Secretin, pancreozymin and glucagon stimulated the secretion of insulin from both lean and obese mouse islets in static incubation and during perifusion. Pancreozymin and glucagon stimulated insulin secretion was accompanied by a parallel rise and fall in islet cyclic AMP content and efflux. Glucose and secretin did not significantly influence islet cyclic AMP content.

It is suggested that gastrointestinal hormones might stimulate the secretion of insulin from a small labile pool via cyclic AMP, providing an early warning signal to the β-cell and a fine tuning of glucose-induced insulin secretion.

Although obese mouse islets showed an exaggerated insulin response to glucose and gastrointestinal hormones, lean and obese mouse islet cyclic AMP levels were not significantly different.

The incorporation of lean and obese mouse everted jejunal sacs into the islet perifusion system indicated that factors released from the jejunum in response to glucose and amino acid might be capable of stimulating the release of insulin.

Obese mouse duodenal-jejunal extracts contained higher insulinotrophic activity and higher levels of bioassayable secretin and pancreozymin than extracts from lean mice.

Histological and electronmicroscopical studies on lean and obese mouse intestine confirmed the presence of enteroendocrine cells.

The possible role of the entero-insular axis in the development of hyperinsulinism and the pathogenesis of the obese-hyperglycaemic syndrome in mice has been discussed.

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Statistical Analysis

All statistical analyses were carried out on an Olivetti Programma 101 Bench Computer. Mean values ± standard error of the mean were computed for groups of data and the significance of the difference between these groups evaluated by students t-test. Probability levels of $p<0.05$ were taken as statistically significant. In perifusion studies, statistical comparisons were made at all points along insulin secretion, cyclic AMP content and efflux profiles. Lines of best fit were computed by the method of exponential curve fit.

CHAPTER I

GENERAL INTRODUCTION

The gastrointestinal tract is the largest and probably the most complex of the endocrine organs. Indeed, the discovery in 1902 by Bayliss and Starling (1) of Secretin, the first hormone to be characterised, laid the very foundations of endocrinology. It was thought that Secretin, released from the gut mucosa in response to certain stimuli, exerted a humoral control over the exocrine portion of the pancreas. It was also apparent that the gastrointestinal tract was in some way involved in the maintenance of carbohydrate homeostasis. In 1896, Beidl (2) demonstrated that glycosuria was more easily produced by an intravenous glucose load than by glucose administered orally. It was observed that certain disorders of carbohydrate metabolism, such as diabetes mellitus, often presented abnormalities of the duodenal mucosa (3). Macallum (4) suggested that an excessive release of insulin from the endocrine pancreas as a result of stimulation by enteric factors might lead to islet β cell exhaustion and subsequently to diabetes mellitus. Pancreatic islet hyperplasia has been observed in some peptic ulcer cases (5), possibly as a result of hyperacidity and hypersecretinaemia. In addition, certain forms of duodenal ulcer have been associated with an increased secretion of insulin (6).

The effects of gastrointestinal surgery on glucose homeostasis are less clearly defined. Increased levels of circulating insulin have been recorded after jejunoileostomy (7) and gastrectomy (8, 9), although conflicting reports (10,11) suggest that glucose tolerance is impaired in such cases, perhaps as a result of the loss of intestinal insulinotropic factors. These observations, although contradictory, do suggest

collectively that gastrointestinal surgery might, in some way, influence endocrine pancreatic function.

In 1906, Moore (12) prompted by the discovery of secretin, and in particular its postulated action on the exocrine pancreas, proposed that the duodenum elaborated a hormone or series of hormones that stimulated the secretion of the endocrine pancreas. This postulate was based upon the finding that the urine of subjects with diabetes mellitus became glucose-free when these patients were administered extracts of duodenal mucosa. Subsequently, Heller (13) in 1929 demonstrated that duodenal extracts injected intravenously into rabbits reduced the hyperglycaemia produced by an intravenous glucose challenge. Laughton and Macallum (14) were also able to demonstrate the hypoglycaemic activity of an extract of enteric origin. These authors suggested that neither secretin nor insulin were responsible for this hypoglycaemic activity. Perhaps the most numerous and significant contributions concerning the relationship between the gastrointestinal tract and insulin secretion were made by La Barre and his colleagues (15). They showed that the intravenous injection of secretin into dogs was followed by a distinct lowering of blood glucose levels. Upon further purification, the secretin preparation used by these workers was separated into two fractions. One fraction was active upon the exocrine part of the pancreas, the other having marked hypoglycaemic properties. La Barre proposed the terms 'excretin' and 'incretin' for these fractions respectively.

After the work of La Barre, the concept of an enteroinsular axis became discredited, largely as a result of criticisms made by Loew and colleagues (16). Interest was revived after thirty years by the discovery that an oral glucose load resulted in a lower absolute blood

glucose level than the same dose of glucose administered intravenously (17,18,19,20). It was subsequently suggested that this observed difference between oral and intravenous glucose tolerance might be due to the limitation of glucose absorption at the level of the small intestine (21). Scow and Cornfield (19) contended that an increased hepatic uptake of glucose following the oral route of administration might account for the lower blood glucose levels. However, it later became apparent that hepatic glycogenolysis could not have accounted for such an increase in glucose uptake into the liver. In 1950, Somersalo (22) showed that an oral administration of glucose, but not an intravenous injection, markedly increased the rate of disposal of a subsequent glucose load. This observation was also confirmed by Dupré (23), and suggested that an alimentary factor was probably involved in the enhanced rate of glucose disposal following an oral route of administration.

With the advent of the radioimmunoassay for insulin (24) it became evident that an oral or intestinal glucose load produced significantly higher immunoreactive insulin levels than intravenously administered glucose, despite similar initial blood glucose concentrations (25,26). These findings suggested that an alimentary mechanism, activated by the intestinal absorption of glucose, might be enhancing the normal glycaemic stimulus to the secretion of insulin. Perley and Kipnis (27) estimated that β-cytotropic gastrointestinal hormones could account for approximately fifty percent of the insulin released after an oral glucose load, and since they were able to demonstrate similar results in portacaval shunt patients, they concluded that the mechanism was predominantly alimentary and not hepatic.

A similar, though not necessarily identical mechanism is thought to exist for the augmentation of insulin secretion in response to the ingestion of proteins, amino acids (28,29,30,31) and possibly also fats (32,33). An intestinal enhancement of pancreatic glucagon secretion must also be implicated by these observations, especially since amino-acids have been shown to be powerful stimulants of glucagon release in vivo (34).

The work of Marks and Samols (32) provides convincing evidence in favour of some form of intestinal modulation of islet function. They noticed that galactose, an epimer of glucose and a sugar which shares a common intestinal transport mechanism with glucose (32), produced a stimulation of insulin secretion, whereas intravenously administered galactose did not. Fructose, on the other hand, which produces higher blood glucose levels than galactose, but does not share the same transport mechanism, was without effect on insulin secretion after oral or intravenous administration. Thus, the hypothesis of an enteroinsular axis is a very attractive one. Nevertheless, it must be appreciated that different species probably exhibit differing gastrointestinal physiology, and whilst an alimentary augmentation of the insulin response to ingested glucose is readily shown in man, it has proved less easy to demonstrate in other species, especially the dog (35).

Many investigations have been conducted into the insulin-releasing activities of various gut mucosal extracts. Dupré (36) showed that a porcine intestine mucosal extract improved intravenous glucose tolerance in man to an extent comparable to that produced by an oral glucose challenge. The same extract also produced a significant enhancement of insulin secretion in response to intravenous glucose. Similar extracts of intestinal mucosa were subsequently shown to

possess insulinotropic activity both in vivo (36) and in vitro (37,38,39). Buchanan, Vance and Williams (40), however, were unable to demonstrate an insulinotropic effect of crude porcine duodenal-jejunal extract upon isolated rat islets.

Many elegant in vitro experiments have been performed using a section of small intestine in series with a perfused pancreas preparation. These studies have shown (41,42,43) that the small intestine, in response to glucose, releases a substance, or several substances, which enhance the secretion of insulin from the pancreatic islet.

Several gastrointestinal hormones were believed to be acting as insulin secretagogues. Secretin had already been implicated as a component of the enteroinsular axis (13), and subsequently, the insulinotropic activities of other intestinal hormones were examined. The large number of reports which have emerged on this topic have been reviewed extensively (44). The majority of the data indicate that secretin (45-55) and pancreozymin (47,48,49,51,55,56) are capable of stimulating the release of insulin in vivo in both man and animals. Other investigators (57) have been unable to demonstrate an enhanced release of insulin after secretin or pancreozymin injection, although these negative findings may be due to the extremely rapid and short-lived insulin response to these hormones.

The possible role of gastrin in stimulating insulin secretion is less conclusive. Many different species of this hormone have been investigated, including the biologically active C-terminal tetrapeptide. Many authors have reported elevated levels of circulating insulin following gastrin administration (51,55,58,59,60). Other workers, however, have suggested that gastrin administration has little significant effect (61), and in some cases might even produce an

inhibition of glucose-stimulated insulin secretion (62). The results obtained from work with gastrin were largely dose-dependent, and it was suggested that its effects might be largely pharmacological, or possibly due to the mobilisation by gastrin of other insulinotropic factors (58).

Many techniques have been adopted to investigate the effects of gastrointestinal hormones on the secretion of insulin in vitro. These include incubated pancreas pieces (38, 63, 64, 65) perfused whole rat pancreas (66), incubated isolated islets of Langerhans (40,64, 67,68,69,70) and perifused islets (68). Results obtained in vitro appear to be largely a function of the various experimental conditions employed. Many studies have failed to demonstrate a gastrointestinal hormone stimulation of insulin secretion from islets isolated from the pancreatic exocrine tissue (40,68,71). However, in the presence of exocrine tissue, an insulinotropic effect was observed (71). In addition, the stimulatory effects of secretin and pancreozymin have been shown to be impaired in human (73,74) and in experimentally-induced exocrine insufficiency (75,76). These observations substantiate the view of Pfeiffer and colleagues (62) that the gastrointestinal modulation of insulin secretion might be dependent upon the presence of pancreatic acinar tissue. Since glucose-induced insulin secretion was unaffected in the above circumstances, it was further suggested that the β-cell 'glucoreceptor' was independent of exocrine function. On the other hand, the action of gastrointestinal hormones on the hypothetical "enteroreceptor" of the β cell was thought to involve the exocrine tissue, possibly via channels of some kind between the two types of cell (77,78). This concept was not supported, however, by numerous reports of a direct action of secretin and pancreozymin on

isolated islets of the rat (64,67) and of the mouse (69). It has also been reported that the sensitivity of the islet to secretin and pancreozymin was retained in chronic pancreatitis (75), even in cases which exhibited an impaired islet response to glucose (79,80). Thus, it is still uncertain whether the exocrine pancreas plays any role in the intestinal modulation of insulin secretion.

In vitro investigations have done little to clarify the role gastrin might play in the enteroinsular axis. Again, the effects of the hormone appear to be largely dose-related, there being reports of stimulatory (81,82,83), inhibitory (84,85) and of null effects (40,64) upon the secretion of insulin. The observation that gastrin could inhibit glucose-stimulated insulin release in vitro from mouse pancreatic slices (84) and from mouse islets (85) led these workers to speculate that the argyrophilic gastrin-like cells (α_1 - cells) previously observed in rat pancreatic islets (86) may be involved in modulating the secretion of insulin from the adjacent β-cells (87).

In 1965, Samols, Marri and Marks (88) demonstrated that glucagon, produced by the pancreatic α_2 cells, was capable of stimulating insulin secretion in man in the absence of hyperglycaemia. This finding has subsequently been confirmed both in vivo (61,89-92) and in vitro (40,68,82,93-97).

Samols and colleagues also showed that an oral glucose load caused an elevation of serum immunoreactive glucagon levels, and suggested that an enteric species of the hormone ('enteroglucagon') might be involved in the augmentation of insulin release in response to oral glucose (88,98). This work recalled the earlier observations of Sutherland and DeDuve (99) and of Kenny and Say (100), demonstrating that the upper intestinal tract of man and certain other species

contained a substance biologically similar to pancreatic glucagon.

A rise in circulating plasma glucagon levels has been recorded after the alimentary but not intravenous administration of galactose (101). This implied that the glucagon so-released might be causing the accompanying stimulation of insulin secretion. Furthermore, intrajejunal glucose infusion has been shown to produced elevated plasma glucagon levels in a totally depancreatised patient (102) and in depancreatised dogs (103,104). In an attempt to distinguish between intestinal and pancreatic species of glucagon, Unger and colleagues (104) showed that intrajejunal glucose produced a rise in glucagon-like immunoreactivity (GLI) in plasma taken from the mesenteric vein, whereas that in the pancreatoduodenal vein was simultaneously depressed. Hence, the GLI produced in response to an oral glucose load appeared to be of enteric origin.

In 1961, Unger and co-workers (105) first observed glucagon-like immunoreactivity in canine intestinal extracts. Since then, extensive investigations have been carried out in an attempt to characterise 'enteroglucagon' and distinguish it from the pancreatic hormone. Glucagon-like immunoreactivity has been found throughout the gastro-intestinal tissues of all species studied including man (103, 106, 107) and in especially high concentrations in the gastric antrum, pylorus and duodenum (108). Pancreatic and enteric glucagon have been shown to differ immunologically, although the cross-reactivity between the two appears to increase as the gut descends (103, 107). The problem of cross-reactivity between the two glucagon species was largely remedied by the discovery in 1968 of an antiserum highly specific to pancreatic glucagon and designated G58 (109). Gut glucagon-like immunoreactivity

has been separated into two components (110). Fraction I, with a calculated molecular weight of about 7,000 appears to have no glycogenolytic activity. Fraction II has a molecular weight similar to that of pancreatic glucagon; about 3,500, and appears to possess similar biological activity. The relationship between the two fractions remains unclear, although it has been suggested that the larger molecular weight fraction might be a precursor of the smaller (111). However, Valverde and colleagues (112) have shown that glucose absorption through the small intestine appears to release approximately equal amounts of each fraction.

The status of pancreatic and enteric glucagon as physiological modulators of insulin secretion remains ill-defined. Samols, Tyler, Megyesi and Marks (107) suggested that pancreatic glucagon has an insulinotropic role, and indeed the close proximity of the α_2 and β cells in the pancreatic islet would be consonant with this hypothesis. However, the vast majority of available literature suggests that the release of pancreatic glucagon, unlike enteroglucagon, is suppressed by an elevation of circulating glucose levels (104, 113, 114, 115) and stimulated by hypoglycaemia (115, 116, 117, 118). The picture has been clarified somewhat by the use of antibodies which can differentiate between glucagons of pancreatic and intestinal origin (119). Thus, it has been proposed that oral glucose acts as a signal for the release of enteroglucagon, and at the same time, pancreatic glucagon release is inhibited, confirming the earlier observations of Unger et al (104). It is therefore questionable whether pancreatic glucagon is involved in augmenting the insulin response to the ingestion of glucose. It seems possible, however, that pancreatic glucagon might participate in the insulin response to amino acid and protein ingestion (120),

especially since the protein component of a meal is thought to stimulate the release of pancreozymin, which is a known alphacytotropin (121).

The physiological role of alimentary glucagon in stimulating insulin secretion directly is also subject to considerable doubt. Early experiments with crude acid-ethanol extracts containing gut GLI suggested that enteroglucagon might be the active component of the enteroinsular axis (104). Buchanan et al (40), however, reported that acid-ethanol extracts containing IRG were devoid of insulin-releasing activity. Moody and colleagues (122) also reported insulin-releasing activity associated with gut GLI, although there was a lack of correlation between the amount of GLI and its insulin-releasing capacity. Marco, Faloona and Unger (123) found that after an oral glucose load, the elevation of circulating insulin levels occurred prior to the rise in glucagon concentration. Furthermore, galactose, which has been shown to produce an increase in GLI, was seen to have little or no effect upon insulin release (123). Both these observations tend to dismiss enteroglucagon as a physiological insulin secretagogue. Further purification and identification of the components of entero-glucagon will be necessary before the real biological significance of the substance can be clarified.

The lack of conclusive evidence of a physiological role for gastro-intestinal hormones in the modulation of insulin release has prompted several recent studies involving other enteric factors. Several investigations have shown that whereas crude gastrointestinal hormone preparations could stimulate insulin release, purer forms were often inactive (124-127). These observations have resulted in the discovery and isolation of several closely related intestinal

polypeptides including insulin-releasing polypeptide (IRP) (128), Gastric Inhibitory Polypeptide (GIP) (129) and Vasoactive Intestinal Polypeptide (VIP) (130). It is believed that these substances might be present in intestinal extracts which were shown to be free of the known gastro-intestinal hormones, yet still possessed insulinotropic activity (131). Subsequent purification and examination of these polypeptides have shown IRP (128, 132, 133, 134) and GIP (134, 135) in particular to have insulin-releasing activity, and might thus be components of the enteroinsular axis. There is some evidence to suggest that serotonin, a biogenic amine present in gastrointestinal tissues, might also be capable of modulating the release of insulin. However, in this respect there have been reports of both stimulatory (136-139) and inhibitory (138, 139) effects of serotonin on insulin release, and it remains to be confirmed whether serotonin plays a physiological role in regulating insulin secretion.

Rehfeld (139) has suggested that certain criteria are observed for investigating the insulin-releasing activity of a particular gastro-intestinal hormone. The use of pure hormone preparations in physiological doses was advocated, complemented by reliable determinations of the hormone concentrations in the blood. A large amount of work has also been carried out in order to examine the effects of endogenous intestinal hormones upon the release of insulin. The original observation of Loew et al (16) that the endogenous release of secretin stimulated by an acidification of the duodenal mucosa was unaccompanied by changes in blood sugar concentration was subsequently confirmed by other authors (140, 141). Circulating insulin levels were also unaffected by a stimulation of endogenous secretin (141). Conversely, data from other sources (142, 143) report a stimulatory effect of

endogenous secretin upon insulin release. The literature describing the effects of endogenous pancreozymin on insulin secretion suffers from similar contradictions. The stimulation of pancreozymin release from the intestinal mucosa by the intraduodenal instillation of peanut oil was shown to increase the concentration of circulating immunoreactive glucagon in both man and dogs, with a concomitant rise in plasma insulin levels (144). Raptis and co-workers (31) found that intraduodenal amino acid application stimulated pancreatic enzyme secretion and increased duodenal bilirubin content, whereas the volume of pancreatic juice and bicarbonate content rose only slightly. These workers concluded that the release of pancreozymin might account for the enhanced secretion of insulin following oral, as compared to intravenous, administration of amino acids.

The stimulation of endogenous gastrin secretion by the oral administration of glycine failed to influence circulating serum insulin levels (145), although the work of Rehfeld (59) suggested that during protein ingestion, gastrin might stimulate the secretion of insulin significantly.

An essential criterion for an enteroinsular factor is that its release must be effected by the ingestion of an appropriate food substance. However, investigations in this area have been limited owing to the chemical similarity of the gastrointestinal hormones, and the subsequent problems encountered in their radioimmunoassay.

From the available data, it appears that the ingestion of glucose may cause either a rise (142, 146, 147), a fall (148), or finally have null effect (149, 150) upon serum secretin levels. The early study of Wang and Grossman (151) which showed that intraduodenal glucose failed to stimulate the secretion of pancreatic juice or amylase in dogs has been recently confirmed in man (152). These findings cast

some doubt on the ability of glucose to induce secretin release.

It has been reported that circulating levels of pancreozymin are elevated following the ingestion of a protein and fatty meal (153, 154), and in addition a large oral glucose load (200g) (155). The ingestion of food substances, particularly glucose (59) and amino acids (156) also appeared to be associated with elevated serum gastrin levels (157).

Of the more recently discovered gastrointestinal polypeptides, the level of Gastric Inhibitory Polypeptide has been shown to rise after oral glucose (158) and following a standard meal (159). Recent work by Felber, Zermatten and Dick (160) has demonstrated that the β-cytotropic activity of rat duodenal mucosal extract is markedly increased by the prior intragastric administration to the same rat of glucose or arginine. These authors did not, however, specify which gastrointestinal hormone, or hormones, were associated with the β-cytotropic activity.

Further clarification of the gastrointestinal stimulus to insulin secretion must await systematic studies, including investigations of the combined actions of several gastrointestinal hormones. The current literature suggests that the gastrointestinal tract plays a physiologically important role in modulating endocrine pancreatic function, possibly involving a complex interaction of humoral and neural factors. It is thought that several branches of the enteroinsular axis exist, providing an integrated control of insulin secretion in response to the ingestion of dietary carbohydrates, amino acids and fats. It has been suggested (161) that such a system would provide an 'early warning' and 'fine tuning' of the endocrine pancreas and assist in the maintenance of nutrient homeostasis under widely varying conditions of dietary intake.

The Obese-Hyperglycaemic Syndrome in Mice

The obese-hyperglycaemic mouse was first observed in the Jackson Colony, Bar Harbour, and was initially described by Ingalls (162). The syndrome is inherited as an autosomal recessive gene with complete penetrance (163). The homozygous animal (gene symbol ob/ob) is characterised by excessive fat deposition, hyperphagia, hyperglycaemic, low metabolic rate, hyperinsulinanaemia, insulin resistance and sterility (163-168). The obese mouse shows a marked accumulation of adipose tissue at about 5 weeks of age (169), increasing further in size until it reaches approximately twenty times the body fat of the lean littermate. It has been shown that the adipose tissue of obese mice has the unique ability of being able to phosphorylate glycerol directly (170) and this might be the primary anomaly leading to excessive fat accumulation, assisted by hyperphagia. In fact, a time sequence study by Chlouverakis, Dade and Batt (171) showed that adiposity preceeded hyperinsulinaemia and hyperglycaemia in the development of the obese-hyperglycaemic syndrome in mice. However, other workers (165) have interpreted the abnormal α-glycerokinase activity as an adaptation to the dietary and hormonal conditions established in early life. Obesity is most probably the end product of hyperphagia, high alimentary efficiency and relative immobility (172), although Chlouverakis (173) has shown that mice maintained on a restricted diet could still become obese, indicating that hyperphagia was not the only factor responsible for the obesity. The elevation in hyperglycaemia and glucosuria develops in obese mice at approximately eight weeks, reaching levels of 300mg/100ml and above (174). The hyperglycaemia could be reduced to almost normal levels by an overnight fast (174) and in addition became less pronounced in older animals (175).

Plate 1. The obese-hyperglycaemic mouse and its lean littermate (25 weeks of age).

The simultaneous presence of hyperglycaemia and hyperinsulinaemia (176) indicated that the obese mouse developed a peripheral insulin insensitivity (177, 178). Mayer, Bates and Dickie (178) demonstrated that obese-hyperglycaemic mice developed an increased tolerance to exogenous insulin with increasing age. Subsequently, it was postulated that some form of this resistance to insulin might reside in the muscle tissue of the obese mouse (179, 180), whilst a reduced insensitivity shown by the adipose tissue might be responsible for increased body weight (180). It seems possible that a peripheral tissue resistance to insulin might be the primary abberation in the obese-hyperglycaemic syndrome (180), leading to hyperinsulinism and eventual islet cell hyperplasia (175).

It has also been suggested that a structurally defective insulin molecule might be a possible cause of the tissue resistance in the obese mouse (181). However, Stauffacher and colleagues (180) have shown pancreatic and circulating plasma insulin from both obese and age-matched normal mice to be biologically and immunologically comparable. More recent work by Kahn, Neville and Roth (182) has shown obese mouse liver plasma membrances to be capable of binding only 20-25% as much insulin as those of the lean littermate. This reduction in the number of insulin binding sites correlated well with the degree of insulin resistance, although it still remains to be established whether this phenomenon is a primary causative factor or secondary to hyperinsulinism and obesity. From the available literature, the latter possibility appears to be the most likely. Islets of Langerhans from young obese mice show an increased rate of insulin biosynthesis prior to the insulin responsiveness of the animals tissues becoming impaired (183). This suggested that islet hyperplasia might precede rather than follow insulin

resistance. Furthermore, Mahler and Szabo (184, 185) have shown pancreatic islet cell hyperplasia to be a prerequisite for insulin insensitivity, since the latter disappeared if islet cell hyperplasia was suppressed by alloxan treatment. The observation that insulin resistance is reduced by loss of body weight after fasting (186, 187) has lead to the suggestion that a primary abnormality of islet β cell function, leading to an elevated insulin secretory rate, might be the initial aberration in the obese-hyperglycaemic syndrome. This could then conceivably lead to peripheral insulin insensitivity, (187, 188). Sims and Danforth (189) have suggested that a rise in the concentration of circulating insulin might lead directly to a reduction in the number of insulin receptors and a lowering of tissue insulin sensitivity.

In a series of experiments performed by Strautz (190, 191), it was shown that an unidentified factor present in normal but not obese mouse islets, was capable of normalising glucose and lipid metabolism, at the same time reducing insulin levels and insulin resistance and stabilising weight gain in obese animals. Gates and co-workers (192) repeated these studies using New Zealand Obese (N.Z.O.) mice, and obtained similar results. Furthermore, islets pretreated with streptozotocin (a β-cytotoxic agent) produced a significant weight reduction in the NZO mice, suggesting that the unknown factor might lay not specifically in the β-cell, but elsewhere in the islet (193).

Hellman (194) has demonstrated the presence of a third type of endocrine cell in mouse islets of Langerhans, designated the α_1 cell. An extract of pigeon α_1 cells was found to inhibit the *in vitro* release of insulin (87) and it was postulated that these cells might secrete a gastrin-like substance which exerted a local inhibitory effect on the adjacent β-cells (85, 87). Since Hellman (194) had shown the islets

of obese mice to be deficient in argyrophilic α_1 cells, Strautz (191) suggested that these cells might be the source of the unknown pancreatic factor missing from the obese mouse islet. Westman (195) has suggested that the proportion of α_1 cells in pancreatic islets of obese mice might be related in some way to the level of blood glucose.

Much attention has been focussed on glucagon as a factor which might contribute towards the aetiology of the obese-hyperglycaemic syndrome. Clarke, Wrenshall and Mayer (196) proposed that the development of obesity in the hereditary obese mouse could be caused by the excessive secretion of a "hyperglycaemic-glycogenolytic" factor (possibly glucagon) from the pancreatic islets of these animals. This factor might also contribute towards the hyperinsulinaemia present in these animals, by stimulating the release of insulin from the β cells (197). At variance with this hypothesis, however, several investigations have revealed that the obese mouse pancreatic islet is deficient in glucagon-secreting α_2 cells (198, 199, 200). In a recent study, Laube, Fussganger and Pfeiffer (201) reported that a high glucose concentration produced a paradoxical rise in glucagon secretion from the isolated, perfused pancreas of the obese mouse. This observation appears consonant with earlier reports of increased liver phosphorylase activity (202) and an increased turnover of hepatic glycogen (203) in these animals. The precise role of glucagon in the development of the obese hyperglycaemic syndrome remains to be established.

The islets of Langerhans of the obese mouse are eight to ten times the size of those in the lean littermate (204). It is also well-established that the pancreatic β-cells, which in the obese mouse islet comprise over 90% of the total islet volume, show a marked hypertrophy and hyperplasia (166, 205, 206). Histologically, the obese mouse β cells

display a pronounced, though not complete, degranulation (207) and increased nuclear and nucleolar size (208); in total, a picture of intense synthetic and secretory activity. It is still not certain whether this elevated functional activity is a primary lesion of the β cell, or an adaptation to increased metabolic demand. Petersson and Hellman (209) demonstrated that longterm caloric restriction normalised the size and hyperplasia of the β-cells in obese mice. However, Mahler (206) suggested that the obese mouse β-cell was caused initially to oversecrete insulin far beyond peripheral demand, possibly under the influence of external factors. The obese mouse exhibits elevated basal insulin levels (210) and is in addition hyper-responsive to a whole range of biological insulin secretagogues (210-213). Beloff-Chain, Newman and Mansford (213) demonstrated that this abnormal response to stimuli was retained in animals maintained on restricted diet, suggesting that a primary defect might reside in the obese mouse pancreatic islet. This hypothesis was consonant with that of Genuth (187) who noted that hyperinsulinaemia was the first abnormality manifest in the development of the obese-hyperglycaemic syndrome. The observation of Stauffacher (180) that short term fasting dramatically lowered the plasma insulin levels in obese mice even though blood glucose levels fell only slightly, pointed to the possible implication of gastro-intestinal factors in promoting hyperinsulinism (188). Furthermore, whilst oral glucose produced an elevated plasma insulin concentration, a comparable dose of glucose administered intravenously was without effect (188). These observations indicated that oral feeding was integral to the development of the obese hyperglycaemic syndrome. Certain morphological (214) and functional (215) aspects of the gastro-intestinal tract in the obese mouse support this hypothesis. Compared

with the lean littermate, the obese mouse gastrointestinal tract exhibits endocrine cell hyperplasia (216, 217) and an increased rate of glucose transport (215). Hence, increased food intake during the postweaning period leading to exaggerated gastrointestinal insulinogenesis might be one of the contributory factors in the initial development of hyperinsulinism, obesity, insulin resistance and eventual hyperglycaemia (188).

It is attractive to postulate that the hyperphagia and hyperinsulinaemia are both due to some sort of hypothalamic disturbance (187). Chlouverakis and colleagues (215) demonstrated that ventro-medial hypothalamic lesions (induced by Goldthioglucose) in normal mice caused insulin and glucose levels to rise, and this was accompanied by an accumulation of body fat. Similar treatment of obese mice produced no effect. The resistance of the hypothalamic region of obese mice to the action of goldthioglucose has been interpreted as being the result of an insulin resistance by the hypothalamic satiety centres, producing a raised satiety threshold and hyperphagia (216). However, hypothalamic obesity produced by chemical destruction of the satiety centre has not led to such extreme degrees of hyperinsulinism (217) and insulin resistance (218) in other strains of mice. Studies in rats have shown that ventromedial hypothalamic lesions enhance the responsiveness of the pancreatic β-cells to stimuli independent of changes in food intake, suggesting that there might exist a direct effect of the hypothalamus on the islet (219). Idahl and Martin (220) have demonstrated a hypothalamic factor, present in both fed and fasted animals, capable of stimulating insulin secretion in vitro. Experiments involving parabiosis of obese with both diabetic and normal mice have shown that obese mice in parabiotic union with diabetic mice became

hypoglycaemic and died. Obese mice united with normal littermates, however, survived, becoming fatter less quickly and eating less (221). It was inferred that the normal mouse provided a satiety factor which regulated food consumption in the obese animal. In addition to hypothalamic hyperphagia, there remains also the possibility that hyperinsulinism might induce hyperphagia (179). Mayer (222) suggested that the development of hypothalamic, or regulatory obesity in which the regulation of food intake was deranged, differed from that of metabolic obesity, as seen in the obese mouse, since in the latter case, hyperphagia was secondary to a primary metabolic defect.

It is known that hyperactivity of the adrenal cortex in mice can result in obesity and hyperplasia of the islet β-cells (223). Indeed, it has been shown that the adrenals of American obese mice are approximately twice as large as those of their lean littermates (224) and contain elevated levels of corticosteroids (225). Solomon and Mayer (226) have demonstrated that adrenalectomy results in improved glucose tolerance, lowered body weight and lowered insulin resistance in obese mice, suggesting that the adrenal glands could be involved in the expression of the ob/ob genome. However, other workers (224, 225) have suggested that adrenal function is unlikely to be of primary aetiological significance in the development of the obese-hyperglycaemic syndrome.

The observation that the obese mouse is hypothyroidic (227) has suggested a role for the thyroid gland in the aetiology of obesity. There is as yet, however, little evidence to substantiate this hypothesis.

The pathogenesis of the obese-hyperglycaemic syndrome in mice shows similarities, both morphological and metabolic, to the development of obesity and maturity-onset diabetes in humans (207) although one

must appreciate the complex interactions of genetic and environmental factors in the human condition. In human obesity, there often exists a hyper-responsiveness of the pancreatic islet to insulinotropic stimuli, including glucose (228-232), glucagon (231-233), secretin (234) and amino acids (235, 236). Because of this, it is generally accepted that obesity, like pregnancy, represents a diabetogenic stress (237) leading eventually, possibly by exhaustion of the islets, to a relative insulin deficiency (238). Whether this condition results from an excessive stimulation of the endocrine pancreas or from its hyper-responsiveness remains uncertain.

This thesis attempts to examine more closely the hypothesis that an increased activity of the enteroinsular axis might contribute to the development of hyperinsulinism, and subsequently to the aetiology and pathogenesis of the obese-hyperglycaemic syndrome in mice.

THE RADIOIMMUNOASSAY OF INSULIN

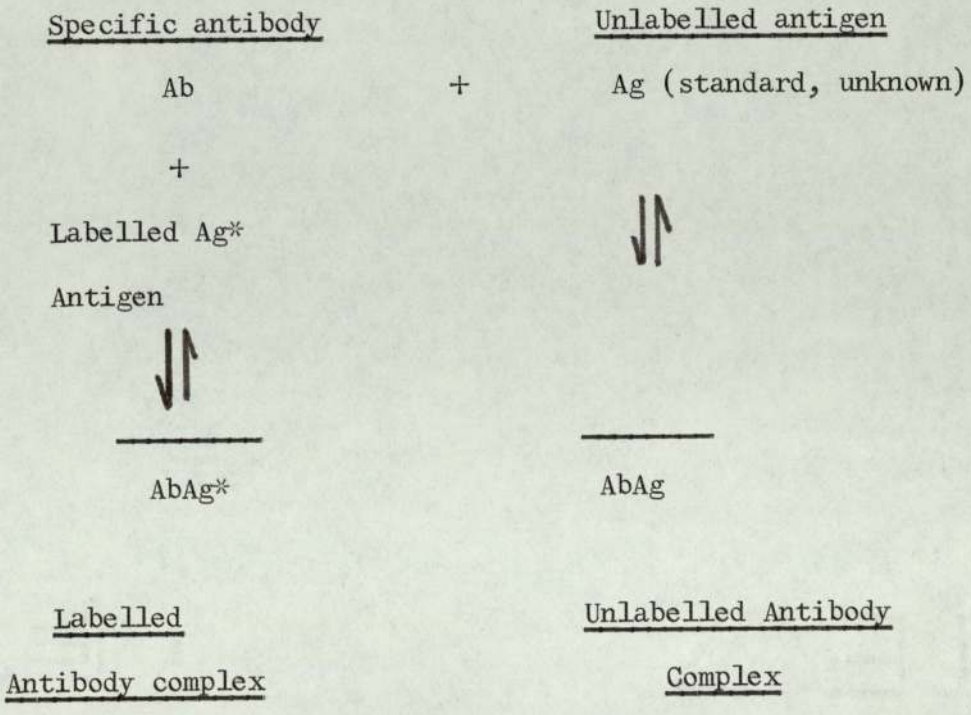
The earliest attempts to estimate the concentration of insulin in blood and other body fluids were based on the biological activity of the hormone (239). *In vivo* bioassay systems exploited the ability of the hormone to produce hypoglycaemic convulsions in mice and in rabbits (240). These methods did not, however, have the sensitivity necessary to detect the minute quantities of insulin present in blood. *In vitro* bioassays were subsequently developed and utilised the measurement of glucose uptake or glycogen deposition by isolated rat diaphragm (241) and the oxidation of ^{14}C-labelled glucose by rat epidydimal fat pads (242). Both methods proved to have poor reproducibility and sensitivity, and were expensive.

The measurement of hormones in blood and other body fluids is now carried out almost exclusively by radioimmunoassay, which eliminates many of the disadvantages of other assay systems. The radioimmunoassay for insulin was first developed by Yalow and Berson (24) in 1960. The principle was that of isotope dilution and involved the competition between labelled and unlabelled hormone for the binding sites on an antibody specific to insulin (see Fig.1). The radioactivity associated with the antibody was shown to be inversely proportional to the concentration of insulin in the unknown sample or standard. By reference to a standard curve, the insulin concentration in the unknown sample could then be estimated.

There are three critical requirements for a radioimmunoassay. These are:

1. A specific antibody to the species of insulin to be measured.
2. A labelled, species-specific antigen.
3. A technique for separating antibody-bound and free labelled insulin.

Fig. 1 The competitive antigen-antibody reaction upon which radioimmunoassay is based.



Insulin antibodies

Insulin antibodies are members of the group of serum proteins referred to as gamma-globulins, or immunoglobulins (243). They are commonly produced by the immunization of a number of animals (selected for high sensitivity to the antigen) with insulin mixed with Freund's adjuvant (244).

Antibodies produced by guinea pigs in response to immunization with insulin are believed to be the most efficacious in reacting with foreign antigen (245) and are therefore commonly employed in some types of insulin radioimmunoassay.

Important criteria for an antibody are high specificity and affinity for the antigen (246). The antibody solution is diluted to give a final concentration that allows approximately 50% binding of labelled hormone in the absence of unlabelled hormone (246).

Labelled insulin

The insulin used for radio-labelling should be the most highly purified source available (MRC research standard). Since the insulin molecule contains tyrosine residues, it can conveniently be labelled with radioisotopes of iodine either ^{125}I or ^{131}I by substituting for the hydrogen atoms of the tyrosine ring. These isotopes have the advantage of a high specific activity, and the method preserves maximum antigenicity of the hormone. It has been suggested that the optimum concentration of label that will give a high specific activity whilst maintaining optimal immunoreactivity is an average of one iodine atom per insulin molecule (247). Both ^{125}I and ^{131}I have distinct advantages for their use as tracers. Since ^{125}I has a longer half-life (60 days) than ^{131}I (8 days) and generally a higher counting efficiency, ^{125}I is the isotope most commonly used in commercial radioimmunoassays.

Radiolabelling of the hormone is usually carried out by the Chloramine-T method of Greenwood, Hunter and Glover (248). The labelled hormone is finally purified by column chromatography to remove degraded insulin and unreacted isotope material.

Separation of bound and free labelled insulin.

The separation of the free labelled insulin from the antibody-bound labelled insulin is necessary to enable the measurement of the radioactivity bound to the antibody. Many methods of separation have been investigated in an effort to achieve accuracy, reproducibility and simplicity. Yalow and Berson (24) employed a chromatoelectrophoretic technique based on the differential migration of the free and bound labelled insulin. Subsequent separation methods have employed Sephadex G75 gel filtration (249), adsorption of the free labelled insulin onto dextran-coated charcoal (250, 251), proteolysis of the free labelled insulin with insulinase (252) and precipitation of the insulin-antibody complex with sodium sulphite (253). Perhaps the most widely used method for the separation of free and antibody-bound labelled insulin is the double antibody technique of Hales and Randle (254). This technique uses an anti-γ -globulin raised in rabbits to guinea pig anti-insulin serum, which precipitates the insulin-antibody complex (255), a process called immuno-precipitation. Hales and Randle (254) used a pre-precipitated insulin antibody, since it was found that the insulin antibody precipitated with anti-γ-globulin retained its ability to react with insulin.

The time required for the establishment of equilibrium of the insulin-antibody reaction (i.e. the incubation period) varied between a few hours and several days (246). Some radioimmunoassay procedures have been found to be more effective if the incubation is not carried to the point of

equilibrium (246). It is assumed that the reaction between the hormone and specific antibody should ideally obey the law of mass action (256). On this basis, the binding sites on the antibody would be occupied by either labelled or unlabelled insulin depending on their respective molar concentrations. However, it must be appreciated that insulin molcules of different species may have different binding affinities, which might alter the final position of equilibrium. On the same basis, it is essential that the unknown sample insulin and standard insulin have similar affinity for the antibody (246, 257). In practical terms, this usually means that in order to obtain an absolute measurement of the hormone concentration, the insulin species used for the preparation of standards must be identical to that present in the sample to be measured. The specificity of the assay is not, however, dependent upon the immunological identity of the labelled and unlabelled insulins (246, 257). Other conditions necessary for a reliable radioimmunoassay system include optimization of pH, temperature and ionic environment.

Radioimmunoassay systems for the measurement of insulin provide an estimate of insulin immunoreactivity which is distinct from its biological activity (258). It is assumed that non-biologically active material (e.g. insulin fragments) possess cross-immunoractivity and provide an overestimate of the absolute concentration of insulin present in samples.

CHAPTER II

GENERAL MATERIALS AND METHODS

Animals

Obese hyper-glycaemic mice (genotype ob/ob) and their lean littermates (l.l.m.), and when necessary Tyler's original strain (white T.O.) were used in experimental work. All animals were bred and accommodated in the animal house of the University of Aston, maintained at 25 ± 2°C with a regular lighting schedule. Water and a standard pellet diet (41B, Pilsbury's Ltd., Birmingham) were supplied ad libitum. Animals were used at 20-25 weeks of age, at the height of the obese-hyperglycaemic syndrome and killed by rapid cervical dislocation. Overnight fasting was not adopted, since starvation has been shown to reduce glucose-stimulated insulin release (259, 260), both in vivo and in vitro, possibly owing to changes in a glucose-inducible enzyme system in the pancreatic β-cell (261).

Microdissection of Islets of Langerhans

Several methods have been adopted for the study of insulin secretion in vitro, each possessing advantages and disadvantages. These techniques may be generally divided into four groups; namely incubation of pancreas pieces (262, 263), perfused whole pancreas (264-266), cultured foetal pancreas (267, 268) and statically incubated and perifused isolated islets of Langerhans, (269-271). The latter two preparations were chosen for all subsequent experimental work, because of their reproducibility and ease of use.

The isolation of intact islet tissue can be effected using two basic techniques. Hellerström (269) employed a method of freehand microdissection for the removal of islets. This technique was particularly suitable for animals, such as the mouse, in which the islets are

relatively large and easily visible within the pancreatic parenchyma. Keen, Sells and Jarrett (270) employed pancreatic duct ligation to facilitate the subsequent microdissection of islets from the atrophied acinar tissue.

As an alternative to microdissection, Lacy and Kostianovsky (271) developed a technique which involved the digestion of surrounding pancreatic exocrine tissue with collagenase, and the harvesting of the remaining islet tissue by sedimentation. This method had the advantage of producing a large number of islets in a short period of time. However, it was necessary to carefully control the time of exposure of the tissue to the enzyme in order to avoid erosion of the islet capsule and its contents. Furthermore, there is evidence to suggest that islets obtained by collagenase digestion exhibit an impaired secretion of insulin (272, 273, 274.). The isolation of islets by freehand microdissection was considered to be most suitable for the present studies.

Microdissection Technique

The microdissection procedure was carried out in a square black plastic dish containing bicarbonate buffer (277) supplemented with 3mM glucose. The temperature of the dissection medium was maintained at 2-4°C by means of a water-cooled themoelectric element (Frigistor), which ensured maximal viability of the tissue during microdissection. The dissecting area was viewed through a steromicroscope at 8X magnification and illumination of the area provided by a variable-intensity slide lamp. The dissecting tools consisted of fine watchmakers' forceps (no.5), fine scissors, a 20-gauge hypodermic needle (mounted on a glass rod) and a fine pasteur pipette.

The excised pancreas was washed in cold, oxygenated dissecting buffer, all fat removed, and transferred to the dissecting dish. The

tissue was then cut into manageable portions (approximately 4mm square) using fine butterfly scissors. The islets were distinguished against the black background as spherical or ovoid bodies dispersed in the more translucent acinar tissue. The obese mouse islets were large, pink, highly vascularised and particularly suitable for microdissection because of their size and looseness of attachment to the acinar tissue. Using fine watchmakers' forceps to hold the tissue, the islets could be teased free of the surrounding exocrine tissue with the cutting edge of the hypodermic needle, taking care not to damage the delicate islet capsule. With some practice, it became possible to isolate 40-50 islets in an hour's microdissection. Using a finely-drawn out pasteur pipette the isolated islets were then transferred to a black solid watchglass containing cold oxygenated buffer, for temporary storage prior to subsequent experimental work.

Advantages of Microdissected Islets

Freehand microdissection provides a simple, rapid and reproducible method for the isolation of pancreatic islets virtually free from exocrine tissue. The removal of exocrine pancreatic tissue was of particular importance in the present studies, since there is evidence that the action of gastrointestinal hormones on the islet might be mediated via the exocrine tissue (68, 71, 72). The absence of exocrine tissue also eliminates the problem of interference by the insulin-lytic substance believed to be released from pancreatic acinar tissue of some rodents (272). It has also been suggested that insulin secretory measurements can be more accurately quantified using islets completely isolated from exocrine tissue, especially since the endocrine portion of the gland represents only a few per-cent of the total pancreatic volume (269). The technique of microdissection is limited, however, by the amount of

tissue obtainable, since this is essentially governed by the rate of isolation of the islets and the condition of the exocrine tissue. There is also a restriction in the choice of animals, as microdissection is feasible only when the islets can be visibly distinguished within the pancreas.

Attention has been drawn to the fact that the isolation of rat islets of Langerhans may be facilitated by the prior administration of pilocarpine to the animal (279). Pilocarpine is an imidazole alkaloid which stimulates the secretion of pancreatic juice, causing a rapid, marked depletion of pancreatic acinar tissue within one hour. Following pilocarpine administration to the animal, the islets of Langerhans are more clearly distinguishable and may be easily isolated from the exocrine pancreatic tissue. However, a study of the effects of pilocarpine on insulin secretion in normal and obese- hyperglycaemic mice revealed that the drug could influence insulin secretory activity both in vivo and in vitro (see Appendix 1). Consequently, in the present studies, pilocarpine pre-treatment was not employed to facilitate the microdissection of islets of Langerhans.

Insulin Radioimmunoassay

The insulin radioimmunoassay system adopted in the present studies was based on the double antibody technique of Hales and Randle(254). The modification of Quabbe (277) was employed, which involved sedimenting the insulin-antibody complex by centrifugation and decanting off all the supervatant containing the unbound labelled insulin.

Reagents

All solutions were prepared with double-distilled, deionized water ('Elgastat', Elga Products Ltd.) since the inconsistency of ordinary distilled water has been shown to affect the binding of insulin to the antibody (254).

Buffer Systems used in Radioimmunoassay

Buffer A1

A 40 mM phosphate buffer containing 6.2 g $NaH_2PO_4 . 2H_2O$; 5.1 g insulin free bovine serum albumin (Armour Pharmaceuticals Limited, Eastbourne, Sussex) and 0.25g sodium ethyl mercurithiosalicylate (antibacterial agent) per litre. The buffer was adjusted to pH 7.4 with 2N NaOH. Buffer A1 was subsequently used to dilute the labelled insulin.

Buffer B1

An isotonic phosphate buffer was used to dissolve and dilute the insulin standards, and to dilute the assay samples where necessary. The following components: 5.77g. $Na_2HPO_4 . 2H_2O$, 1.05g. $NaH_2PO_4 . H_2O$, 60.0g. Human Albumin (Sigma Chemical Co.Ltd., London), 0.24g. sodium ethylmercurithiosalicylate and 6.0g NaCl were dissolved in double distilled-deionized water, adjusted to pH 7.4 and made up to 1 litre.

Buffer C1

A high protein buffer consisting of equal volumes of Buffer A1 and Horse Serum (No.5, Welcome Reagents Ltd., Beckenham, Kent), was used conventionally for washing the antibody precipitate. The buffer was filtered through Kieselguhr if cloudy and used as a clear solution.

Insulin Antibody (insulin binding reagent)

The insulin antibody was supplied lyophilised with the second, precipitating antibody by the Radiochemical Centre, Amersham, Bucks. The insulin binding reagent was reconstituted by the addition of 8 ml. double distilled-deionized water and careful mixing. Each assay tube contained 100 µl of this solution.

Labelled Insulin

^{125}I-labelled bovine insulin (potency = 24.3 μU/ng) was supplied by the Radiochemical Centre, Amersham, Bucks. The labelled hormone was prepared by a modification of the method of McFarlane (278) and supplied in vials containing 100 ng of labelled insulin dissolved in 5 ml buffer A1 at a minimum specific activity of 1μCi/ml. A working solution was prepared by diluting this stock solution 1 : 8 with buffer A1. Each assay tube contained 100 μl of this working solution (i.e. 250 pg labelled insulin).

Insulin Standards

Mouse Insulin standards (Novo Research Institute, NovoHallë, DK2880 - Dagsvaerd, Denmark) were used throughout. The mouse insulin (potency = 22.9 μU/ng) was supplied in vials containing 0.1 mg lyophilised mouse insulin standard with 1 mg. human albumin. A stock solution was prepared by dissolving the contents of one vial in one litre buffer B1. From this stock solution, a range of standards (10-250 μU/ml) was prepared by further dilution with buffer B1. These standards were stored deep frozen in 1 ml aliquots and thawed immediately prior to use.

Procedure

All reagents were added in aliquots of 100 μl using an automatic pipette (Eppendorf Gerätebau Netheler + Hinz GmbH. 2000 Hamberg 63 (Hummelsbüttel) B.P.630324. The nature of the assay allowed all the reactions and counting to be performed in 9.5 x 63.5 mm polystyrene immunoassay tubes (Hopkin and Williams, Romford).

Tubes were set up as follows:

1. Blank or Control tubes

Contained labelled insulin and buffer B1 but no insulin binding

reagent. These tubes were used to evaluate the efficiency of the washing procedure.

2. Zero tubes

Contained labelled insulin, insulin-binding reagent and buffer B1 but no unlabelled insulin.

3. Insulin standards

Standards were set up containing 10, 20, 50, 100, 150 and 250 μU/ml mouse insulin.

4. Test samples

These were pre-diluted if necessary with buffer B1 prior to assay in order to fit onto the appropriate part of the standard curve. All tubes were assayed in triplicate or in duplicate. 100 μl Insulin Binding Reagent was added to all tubes except the washing blank and the reactants vortex mixed (Whirlimixer, Fisons Scientific Apparatus Limited, Loughborough). The tubes were then incubated at 4°C for 12 hours. It had previously been established that the equilibrium constant for the hormone-antibody reaction was increased when the incubation temperature was lowered to 4°C (256). There was also evidence to suggest that the sensitivity of the assay could be significantly improved if the antibody was allowed to react with the unlabelled insulin prior to the addition of the labelled insulin (254, 279).

Following the initial incubation, 100 μl of ^{125}I-labelled insulin (approx. 0.0125 μCi) was added to each tube in turn. The contents were again vortex-mixed and incubated at 4°C for 24 hours. Three tubes were prepared containing 100 μl labelled insulin only to provide a measure of the total activity added to each tube (the total counts). After the second incubation, the insulin-antibody precipitate was sedimented by centrifugation at 1500 r.p.m. and 4°C for one hour in an M.S.E. 4L refrigerated centrifuge (Measuring and Scientific Equipment Ltd.,London).

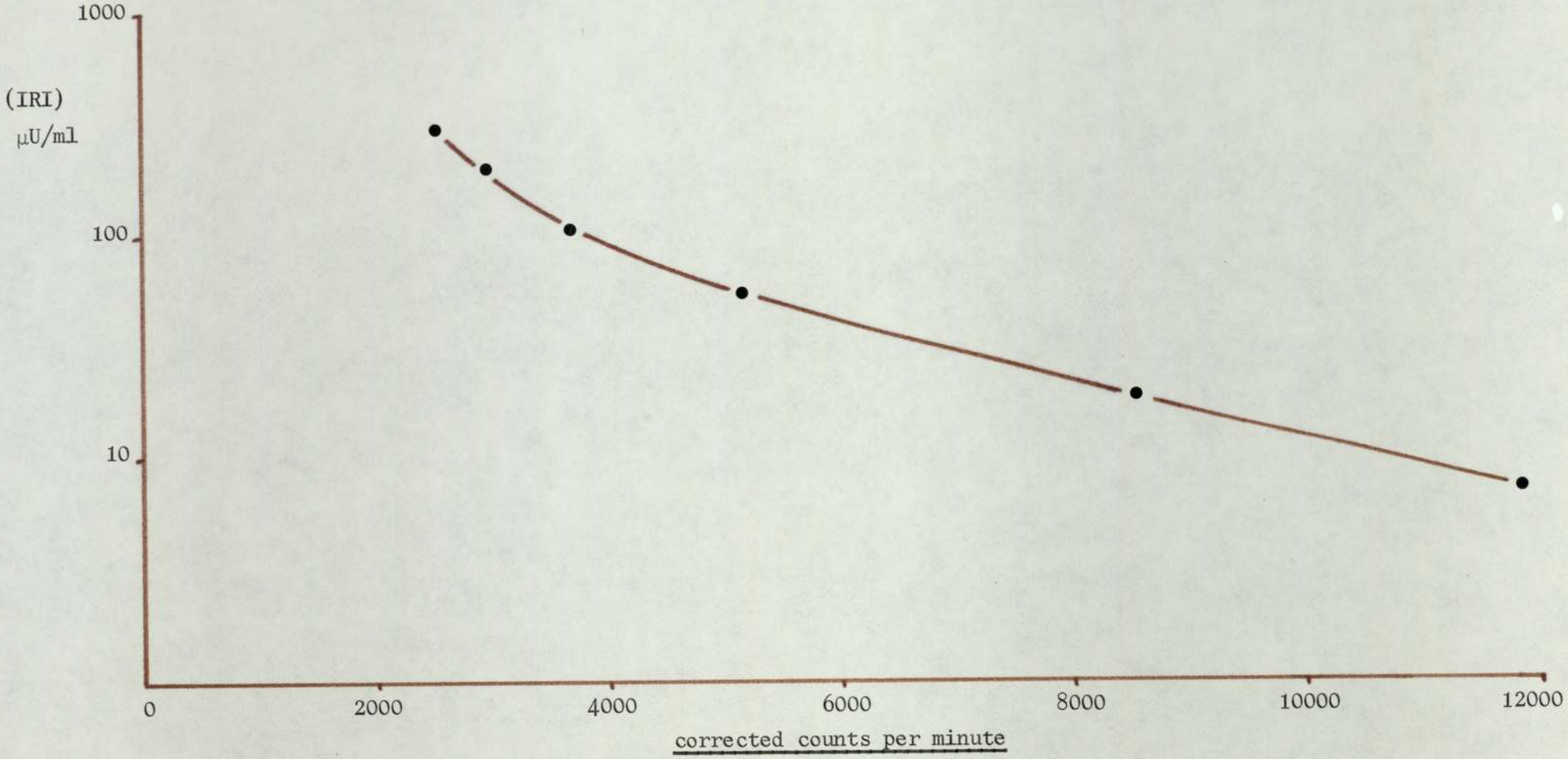
The supernatant, consisting of unbound labelled and unlabelled insulin, was then carefully decanted off, the last drop being aspirated with a pasteur pipette connected to a water vacuum pump. The insulin-antibody precipitate was then washed with 1 ml buffer C1 at 4°C, vortex mixed and re-centrifuged as previously. The washing procedure was repeated twice and the final precipitate allowed to dry in air for 12 hours at room temperature (22°C).

The radioactivity of the precipitate was counted for a period of 10 minutes using an I.C.N. gammaset 500 gamma spectrophotometer (I.C.N. (Tracerlab), Walton-on-Thames). The counts were corrected for radioactive decay and the replicates averaged using an Olivetti Programma 101 desk computor (British Olivetti Ltd., London).

The Standard Curve

The value in counts/minute obtained for each of the insulin standards was plotted against the logarithm of the respective insulin concentration using 3-cycle semi-logarithmic paper (see Fig.2). By referance to this standard curve, the insulin concentration in the unknown samples could be estimated. A satisfactory assay was indicated by good agreement between replicates, washing blank tubes contributing less than 5% of the total counts and the zero tubes containing approximately 40% of the total counts.

Fig.2 TYPICAL STANDARD CURVE FOR MOUSE INSULIN



Modification of the double antibody radioimmunoassay method for the measurement of insulin

Using the double antibody technique of Hales and Randle (254), the separation of free and antibody-bound labelled insulin was accomplished by a lengthy (3 day), tedious separation and washing procedure. Thus, the simultaneous assay of a large number of samples proved to be a very time-consuming process. Furthermore, the method did not readily permit triplicate insulin assays upon samples of limited volume (e.g. plasma samples obtained from lean and obese mice by tail tip amputation).

In an attempt to overcome these restrictions, the following modifications of the existing insulin radioimmunoassay were investigated:

1. The use of a smaller reaction volume.
2. The use of shortened incubation periods.
3. A much simplified separation and washing procedure.

The reagents used and their preparation were identical to those described previously for radioimmunoassay (see Chapter II, Page 31). Tubes were set up for blanks, zeros and mouse insulin standards of 10; 20; 50; 100; 250 and 500 μU/ml). 50 μl sample volume was used throughout. All tubes were assayed six times by both the Hales and Randle technique and by the modified method.

50 μl of Insulin Binding reagent was added to all tubes except the washing blank. The tubes were then vortex mixed and incubated for 45 minutes at 4°C. 50μl of labelled insulin was then added to each tube. The reactants were mixed thoroughly and incubated for $2\frac{1}{2}$ hours at 4°C. At the end of the second incubation period, 1 ml. of buffer A1 was added to each tube. The contents were then vortex mixed and the insulin-antibody complex separated by a 15 min. centrifugation at 2500 r.p.m. and 4°C. The supernatant was then carefully decanted off and

the last drop aspirated with a pasteur pipette attached to a water vacuum pump. The tubes were allowed to drain inverted for 30 minutes on filter paper. The final precipitate was allowed to dry in air for 12 hours at room temperature (22°C) and the radioactivity of the precipitate measured as described previously (page 34).

Results

Table 1 shows a comparison of the original and modified methods for insulin radioimmunoassay using the same range of mouse insulin standards. The respective standard curves are shown in Fig.3. The efficiency of the washing procedure, indicated by the counts for the washing blanks (radioactivity in the absence of antibody) appeared to be higher using the original Hales and Randle method. The counts per minute for the insulin standards were significantly higher for the modified method (except in the case of the zero) than for the original Hales and Randle method. In general, the standard errors of the mean of six determinations for each standard were slightly greater using the modified procedure than when using the original method.

The standard curves obtained for both original and modified methods (Fig.3) were of comparable shape and slope, although a steeper curve was obtained at high insulin concentrations (250-500 μU/ml) using the modified method. Subsequent work has shown no significant difference between values obtained by the two methods for a series of test insulin samples of unknown concentration.

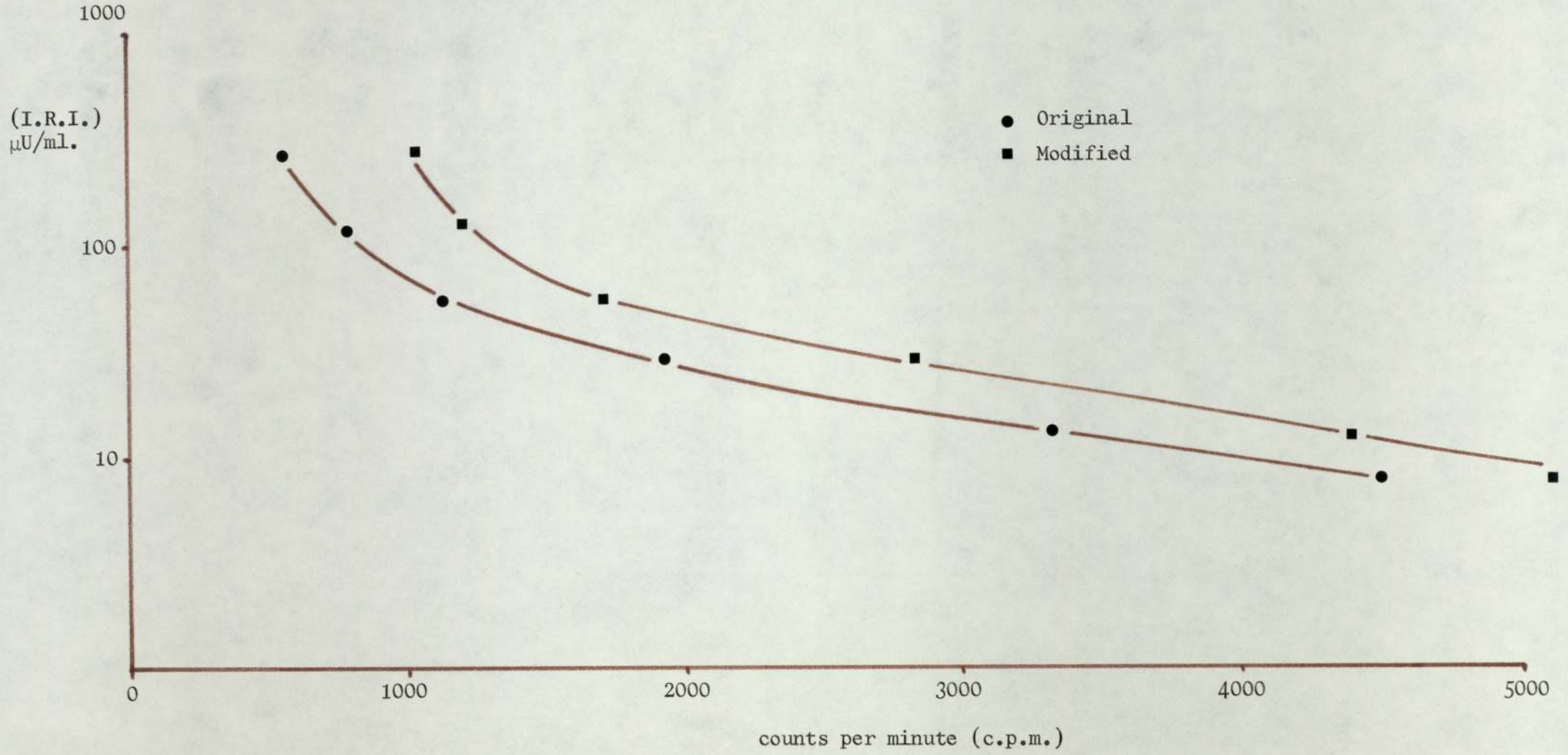
Discussion

The modification described above has proved to be an accurate and rapid method for the radioimmunoassay of insulin in large numbers of samples despite a slight loss in washing efficiency and a small but statistically insignificant increase in the S.E.M. value about the mean

TABLE 1 Radioimmunoassay of Mouse Insulin standards using the original Hales and Randle and the modified methods.

		C. P. M.		
	Tube	Mean	±	S.E.M. (n = 6)
ORIGINAL METHOD (50µl aliquots)	Total Counts	15904		
	Washing Blank	602	±	122
	Zero	5828	±	54
	10µU/ml	4558	±	36
	20 "	3199	±	27
	50 "	1839	±	28
	100 "	1240	±	29
	250 "	770	±	25
	500 "	537	±	7
MODIFIED METHOD (50µl/aliquots)	Total Counts	16202		
	Washing Blank	896	±	131
	Zero	5737	±	103
	10µU/ml	4997	±	82
	20 "	4303	±	56
	50 "	2713	±	61
	100 "	1638	±	38
	250 "	1057	±	39
	500 "	1007	±	22

Fig.3 STANDARD CURVES FOR MOUSE INSULIN USING THE ORIGINAL AND MODIFIED RADIOIMMUNOASSAY METHODS



of several replicates.

1. The effect of reducing the reaction volume

The reduction in reaction volume seems unlikely to be a major source of error. A reduction in the sample volume does, however, confer the advantage that twice the number of samples can be assayed for approximately the same price, and allows replicate assays on small serum samples.

2. The effect of shortening the incubation periods

The binding curve for the reaction

$$Ab + Ag \rightleftharpoons AbAg$$

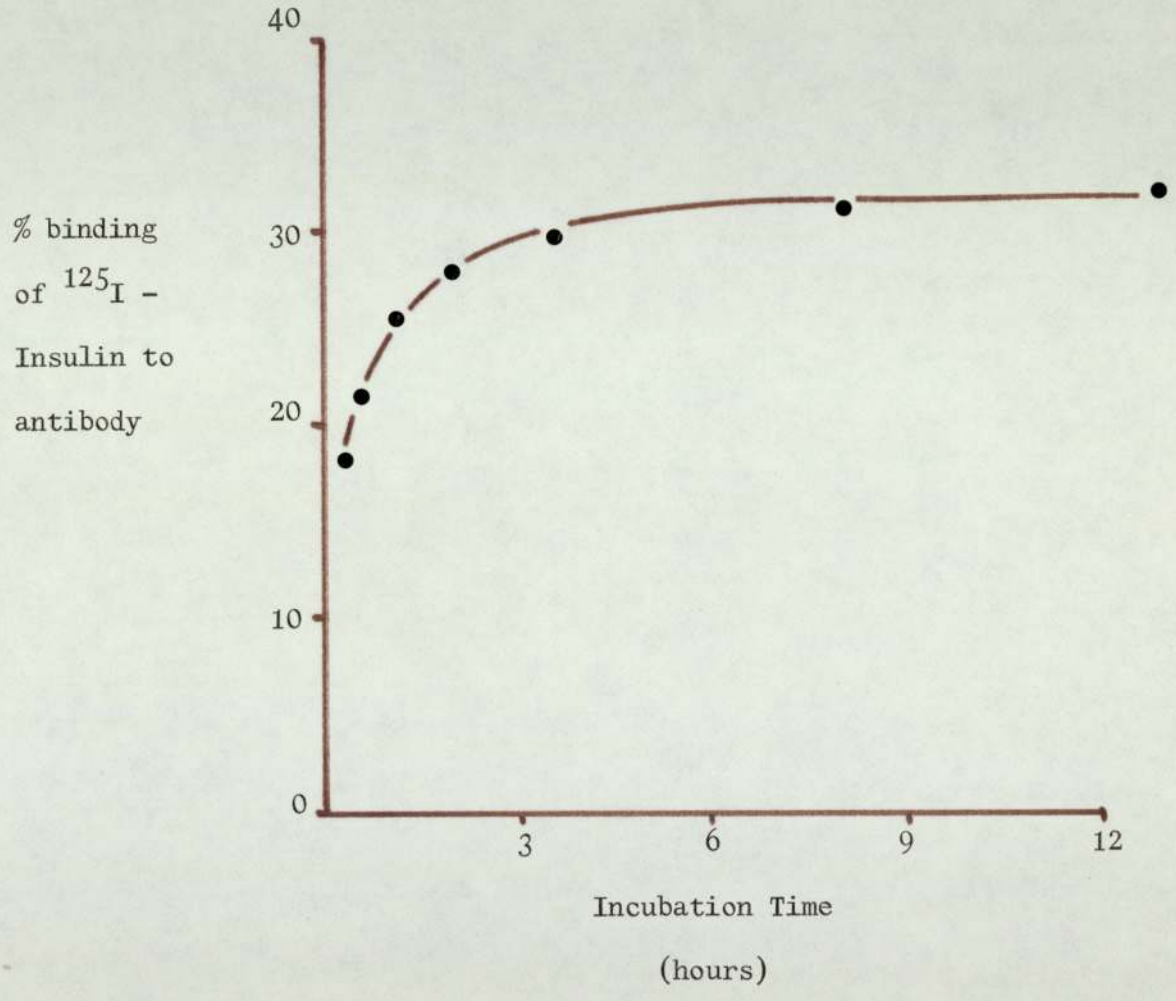
Insulin-Antibody Complex

is shown in Fig.4. After $2\frac{1}{2}$ hours, a 29% binding of insulin to the antibody was achieved, so that a reduction in the time of the second incubation period from 24 to $2\frac{1}{2}$ hours would appear to have little effect upon the position of the equilibrium. As in the original Hales and Randle assay, sensitivity was improved by allowing the antibody to react with the unlabelled insulin prior to the addition of labelled hormone.

3. The effect of simplifying the washing procedure

This was the most important factor because of its contribution to the elevated levels of radioactivity associated with the insulin-antibody precipitate in the modified technique. Because the number of precipitate washes had been reduced from three to one in the modified method, the amount of unbound radioactivity remaining in tubes was inevitably higher. This was reflected by the higher values for the washing blank in the modified method. As a consequence, all residual supernatant was removed by allowing the tubes to drain onto filter paper for 30 minutes prior to drying and counting.

Fig.4 Percentage of ^{125}I - Insulin bound to antibody with time using the double antibody technique.



In the shortened assay, all samples and standards were treated identically, and it was assumed that any errors resulting from residual unbound activity would be small and constant.

The standard curves obtained with the original and shortened assays showed greatest difference at high insulin concentrations (Fig.3). Consequently, when the modified technique was employed for *in vitro* experimental work, samples were diluted so that all insulin values obtained were contained within the range 10-100 μU/ml insulin.

To summarise, the shortened insulin radioimmunoassay method offered the advantages of a shorter and simpler procedure, greater economy and the estimation of insulin in 50 μl samples. The modified assay showed no serious loss of accuracy or reproducibility and has formed the basis of the new Insulin Radioimmunoassay Kit (Code I.M.78) recently introduced by the Radiochemical Centre, Amersham, Bucks.

CHAPTER III

The effect of Secretin, Pancreozymin, Gastrin and Glucagon on the secretion of Insulin from islets of obese-hyperglycaemic mice and their lean littermates.

There is substantial evidence to suggest that the gastrointestinal tract exercises a hormonal modulation of insulin secretion in response to the ingestion of carbohydrates, proteins and possibly fats (62, 125, 161). Most attention has been focussed on the enteric hormones secretin, pancreozymin and gastrin as possible components of the enteroinsular axis, although more recently, several other polypeptides, insulin releasing polypeptide (IRP), gastric inhibitory polypeptide (GIP) and vasoactive intestinal polypeptide (VIP) have been examined in this capacity (132, 134, 136). However, it remains to be established whether any of these factors is of physiological importance in modulating the release of insulin.

It has been proposed that the secretion of insulinotropic gastrointestinal factors in response to excessive food intake might be instrumental in causing hyperinsulinism, which is thought to be a primary factor in the development of the obese-hyperglycaemic syndrome in mice (188, 206). *In vitro* studies have shown the endocrine pancreas of the obese mouse to be hyperresponsive to glucose and amino acids (213). The present investigation describes the effects of the hormones secretin, pancreozymin, pancreatic glucagon and gastrin pentapeptide upon the basal and glucose-induced insulin secretory activity of islets of Langerhans isolated from obese-hyperglycaemic mice and their lean littermates. In addition, *in vivo* investigations were performed to evaluate the influence of gastrointestinal hormones on intraperitoneal glucose tolerance.

Section A The effect of secretin, pancreozymin and gastrin pentapeptide on intraperitoneal glucose tolerance in obese hyperglycaemic mice and their lean littermates.

The most common parameter used to evaluate endocrine pancreatic function is the glucose tolerance test (G.T.T.). Several variations of this test has been devised, i.e. oral, intravenous and intraperitoneal, differing mainly in the route of glucose administration and the site of blood sampling.

Materials and Methods

For practical convenience, a simplified intra-peritoneal glucose tolerance test (i.p. G.T.T.) has been employed as the basis for these experiments. Plasma insulin profiles were also monitored during the tolerance tests (177). All glucose tolerance tests were performed at 14.00 hours to eliminate the effects of circadian variation in plasma insulin and glucose levels (177a). Animals were deprived of food for 6 hours before each experiment. Mice were then weighed, taking care to minimise stress during this and subsequent handling. Glucose was administered by intraperitoneal injection (2g/Kg body weight in a 40% w/v aqueous solution of sterile double distilled water at 37°C).

Purified secretin and pancreozymin-cholecystokinin (Prof. J.E. Jorpes, G.I.H. Research Institute, Karolinska, Sweden) and gastrin pentapeptide (Calbiochem. Ltd., London) were dissolved in physiological saline (0.9% w/v) and administered intraperitoneally immediately prior to glucose loading. The hormone doses were related to animal weight. Thus, lean mice received either 0.75 clinical units of secretin, 0.75 clinical units of pancreozymin-cholecystokinin or 10 μg gastrin pentapeptide, whilst obese animals received either 2.0 clinical units of

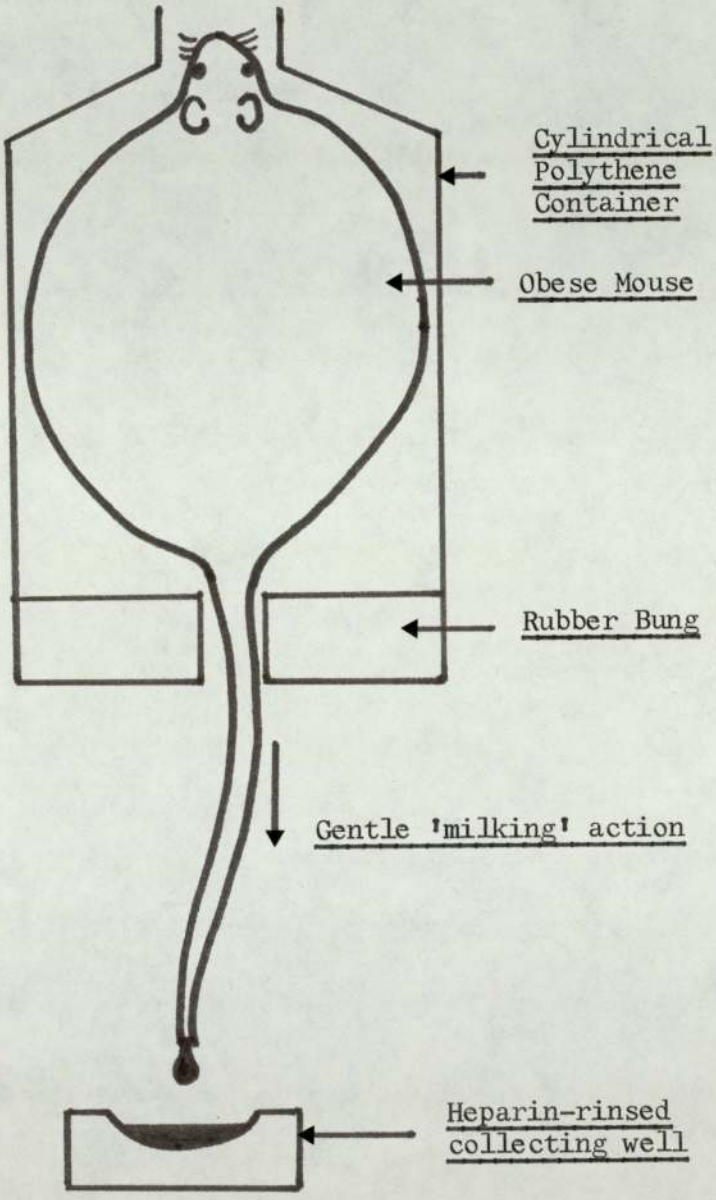
secretin, 2.0 clinical units of pancreozymin cholecystokinin or 30 μg gastrin pentapeptide. Control animals received saline only. Blood samples (0.2 ml) were withdrawn 10 and 25 minutes after injection using the tail-tip amputation method (280). The mouse was introduced into a cylindrical polythene container and held loosely with the tail protruding by means of a rubber bung (Fig.5). The animals were familiarised with the containers prior to the experimental work. The extreme tip of the tail (1-2mm) was removed with a sharp scalpel blade, and the blood obtained drop-wise by a gentle, downward 'milking' action. It was usually possible to obtain 0.2 ml of whole blood within one minute. The blood samples were collected in a Microtest II watchglass (Becton, Dickinson - U.K. Ltd., Middlesex) which had been pre-washed with a solution of heparin-saline (80 U/ml). Each 0.2 ml blood sample was then treated as follows: 50 μl of the whole blood sample was diluted 1:10 with a preservative-anticoagulant solution (Heparin, 60 U/ml + sodium fluoride, 0.1% w/v) and assayed for glucose content.

The remainder of the blood was rapidly centrifuged in a Beckman 152 microfuge using small polythene heparin-washed centrifuge tubes (Beckman) and duplicate aliquots of 20 μl quick frozen for the subsequent radio-immunoassay of insulin.

Determination of blood glucose concentration

In this study, blood glucose was determined by the ferricyanide reduction method of Hoffman (281) adapted for use on a Technicon autoanalyser (282). The principle is based on the partial reduction of yellow ferricyanide to the colourless ferrocyanide. The reaction is followed colorimetrically at 420 nm. Since all reducing substances in the blood might be detected by this technique, estimations of

Fig.5 Diagrammatic representation of blood sampling by tail-tip amputation



'blood glucose' concentration will also include other reducing sugars and possibly urea and creatinine, although these substances are only present in the blood in relatively small amounts.

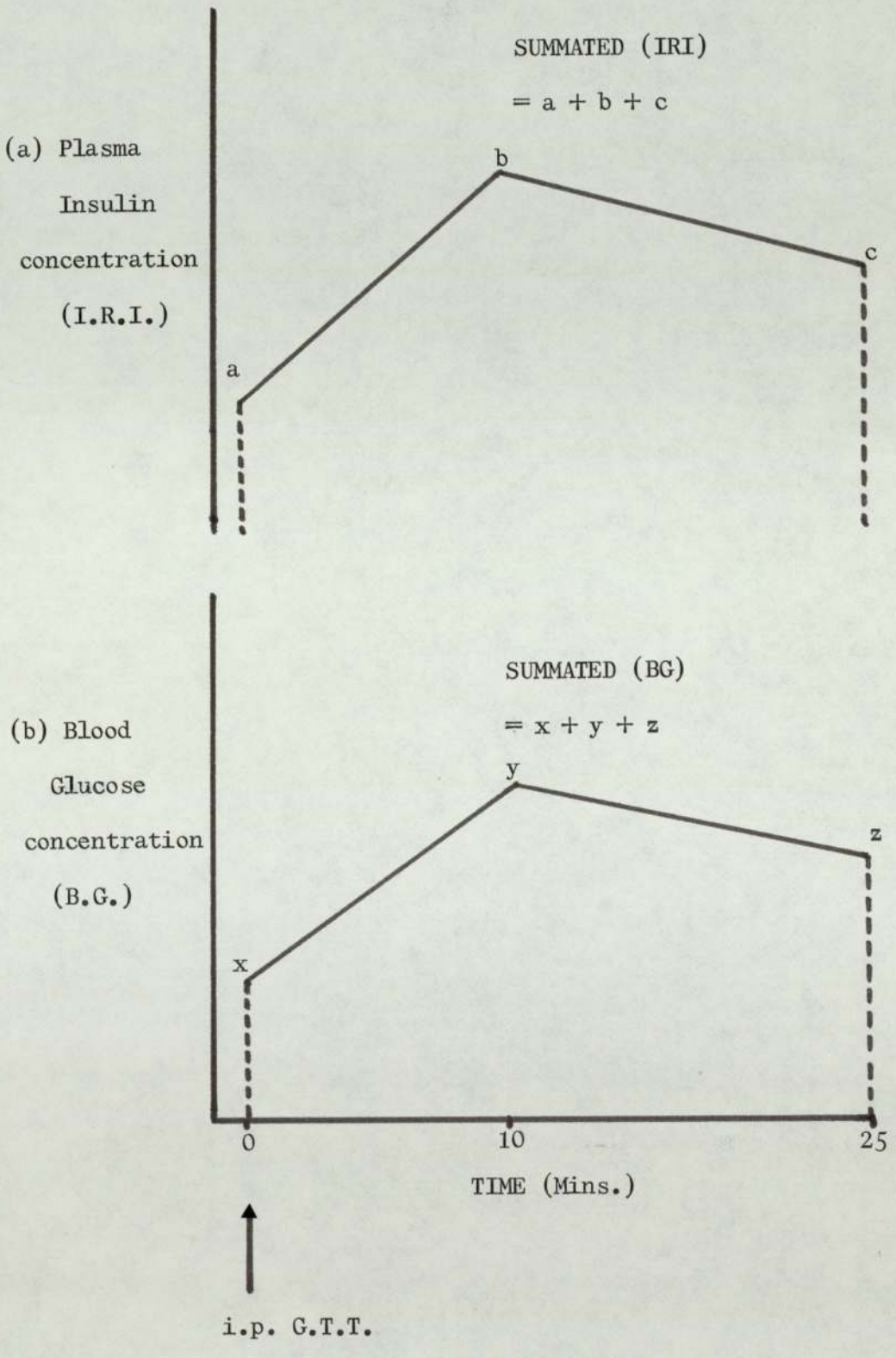
Blood glucose concentrations were calculated using a standard curve and expressed in mM.

The determination of plasma insulin concentrations

Plasma insulin concentrations were measured using the shortened modification of the double-antibody technique previously described (Chapter II, p 35). Because of the limited volume of mouse plasma available for insulin assay, the modified radioimmunoassay method was particularly suitable for the duplicate estimation of small samples. Plasma insulin concentrations were expressed in μU/ml.

In order to evaluate the results of the intra-peritoneal glucose tolerance tests performed, the comparison was made of the total plasma insulin and blood glucose after 0, 10 and 25 minutes for each experimental group. Results were represented as the total area beneath the insulin or glucose curve as shown in Fig.6, i.e. as the summated IRI or summated BG.

Fig.6 Typical Plasma Insulin and blood glucose profiles following i.p. G.T.T.



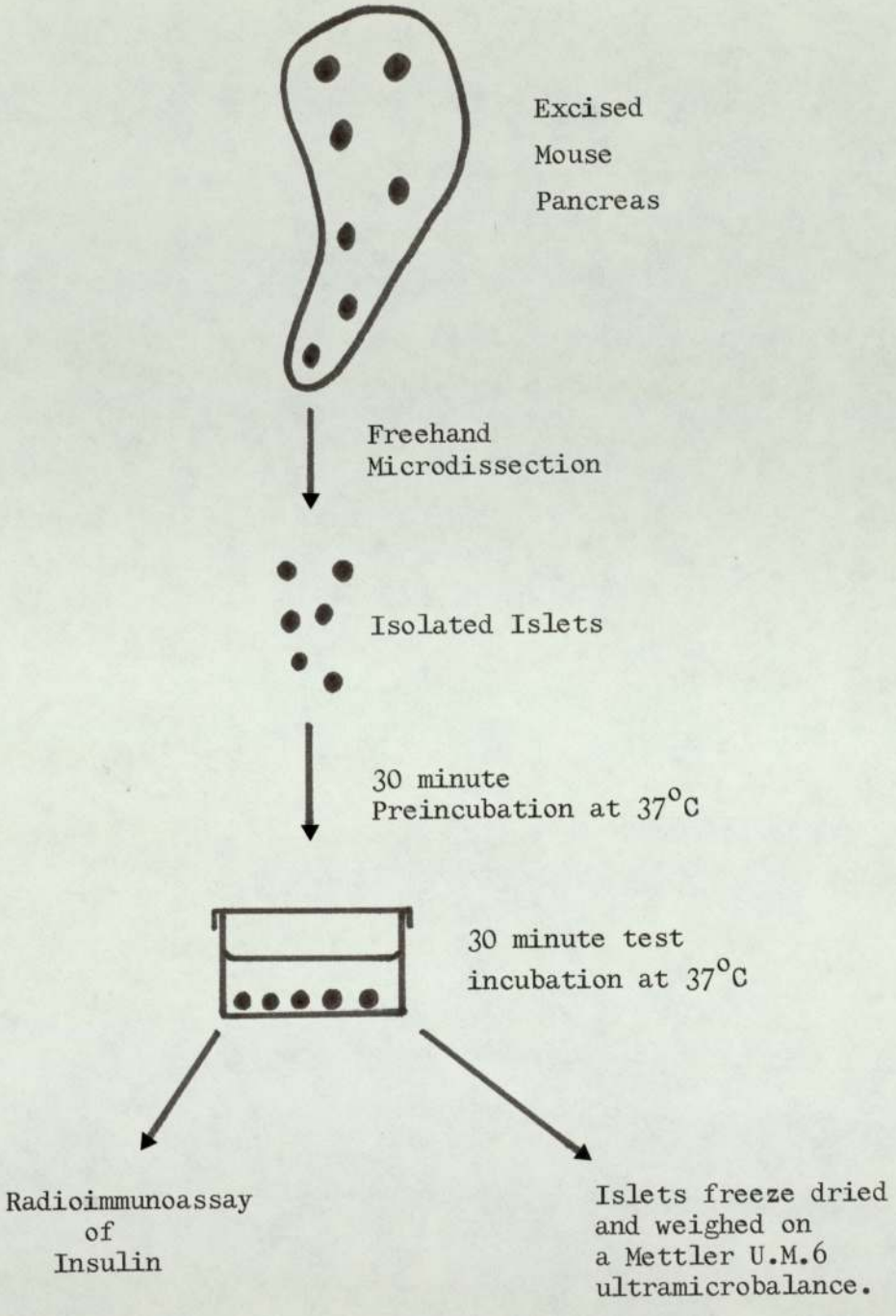
Section B The effect of secretin, pancreozymin, gastrin pentapeptide and pancreatic glucagon on the release of insulin from isolated islets of obese-hyperglycaemic mice and their lean littermates.

The effects of gastrointestinal hormones upon the release of insulin *in vitro* were investigated using isolated mouse islets of Langerhans in a static incubation system. The procedure adopted for these *in vitro* studies is shown in a flow diagram (Fig.7). Pancreatic islets were obtained by microdissection (Chapter II, page 27) and incubated in Gey and Gey bicarbonate buffer (277). The incubation medium was supplemented, as appropriate, with secretin (G.I.H. Research Institute, Karolinska; 0.1 and 0.2 clin. units/ml), pancreozymin-cholecystokinin (G.I.H. ; 0.75 and 1.5 Ivy Dog U/ml), gastrin pentapeptide (Calbiochem. Ltd., London ; 0.5 and 2.0 μg/ml) and pancreatic glucagon (Sigma Chemical Co.Ltd., London). The effects of each hormone was examined on basal and glucose (9mM and 18mM) induced insulin secretion.

The medium was aerated with a mixture of oxygen (95%) and CO_2 (5%). All incubation vials were pre-rinsed with 1% (w/v) bovine serum albumin (fraction V, insulin-free; Armour Pharmaceuticals Ltd., Eastbourne, Sussex) in order to prevent the loss of insulin by glass adsorption.

Twenty microdissected islets were pre-incubated for 30 minutes at 37°C in 10 ml of oxygenated Gey and Gey buffer supplemented with 3mM glucose. Using a finely drawn-out pasteur pipette, groups of five islets were then transferred to one dram glass vials (Johnson & Jordensen Ltd., London) each containing 2 ml of oxygenated buffer at 37°C. 30 minute test incubations were used throughout with continual

Fig.7 Protocol for in vitro studies



oxygenation. The islets were gently agitated during incubation since there is evidence to suggest that this increases the release of insulin by dispersing prereleased insulin already in the medium and eliminating localised concentration gradients (283). At the end of each test incubation period, triplicate 100 µl samples were removed from the incubation medium, placed in polystyrene immunoassay tubes and rapidly frozen at -20°C for the subsequent radioimmunoassay of insulin. Islets were then transferred to small perforated aluminium foil planchets using a fine wire loop, and all adhering buffer removed with filterpaper. The islets were then freeze-dried overnight at -70°C and 0.001 mm Hg ("Speedivac" Pearse Tissue Drier, Edwards High Vacuum Ltd., Crawley, Sussex). The weight of the freeze-dried islet tissue was estimated by difference using an ultramicrobalance (Mettler U.M.6, range 0.1 µg-10mg). Insulin secretion was expressed in terms of µU insulin/µg freeze dried islet tissue/30 minutes.

Results

A. The effects of gastrointestinal hormones on intraperitoneal glucose tolerance in obese-hyperglycaemic mice and their lean littermates.

The results of intraperitoneal glucose tolerance tests on lean and obese mice are shown in Figure 8. The influence of secretin, pancreozymin and gastrin pentapeptide upon the summated plasma insulin and blood glucose responses of lean and obese mice to an intraperitoneal glucose tolerance test are shown in Figures 9 and 10. In the case of both lean and obese mice, a 2g/Kg glucose load administered intraperitoneally significantly ($p<0.05$) elevated both plasma insulin and blood glucose concentrations. Secretin (0.75 clinical units) and pancreozymin (0.75 Ivy Dog units) significantly ($p<0.05$) increased the summated plasma insulin response to a simultaneous intraperitoneal glucose challenge, and also reduced the summated blood glucose concentration in leam mice. In obese mice, secretin (2.0 clinical units) significantly ($p<0.05$) enhanced the insulin response to a simultaneous i.p. G.T.T., whilst pancreozymin (2.0 Ivy Dog Units) lowered the summated blood glucose concentration. Gastrin pentapeptide had no significant effect upon either the summated insulin or glucose response, in either lean or obese mice.

Fig.8 Intraperitoneal glucose tolerance tests (i.p. G.T.T.) in lean and obese hyperglycaemic mice

Each point represents the mean of n = 6 determinations. 6 mice were used per profile.

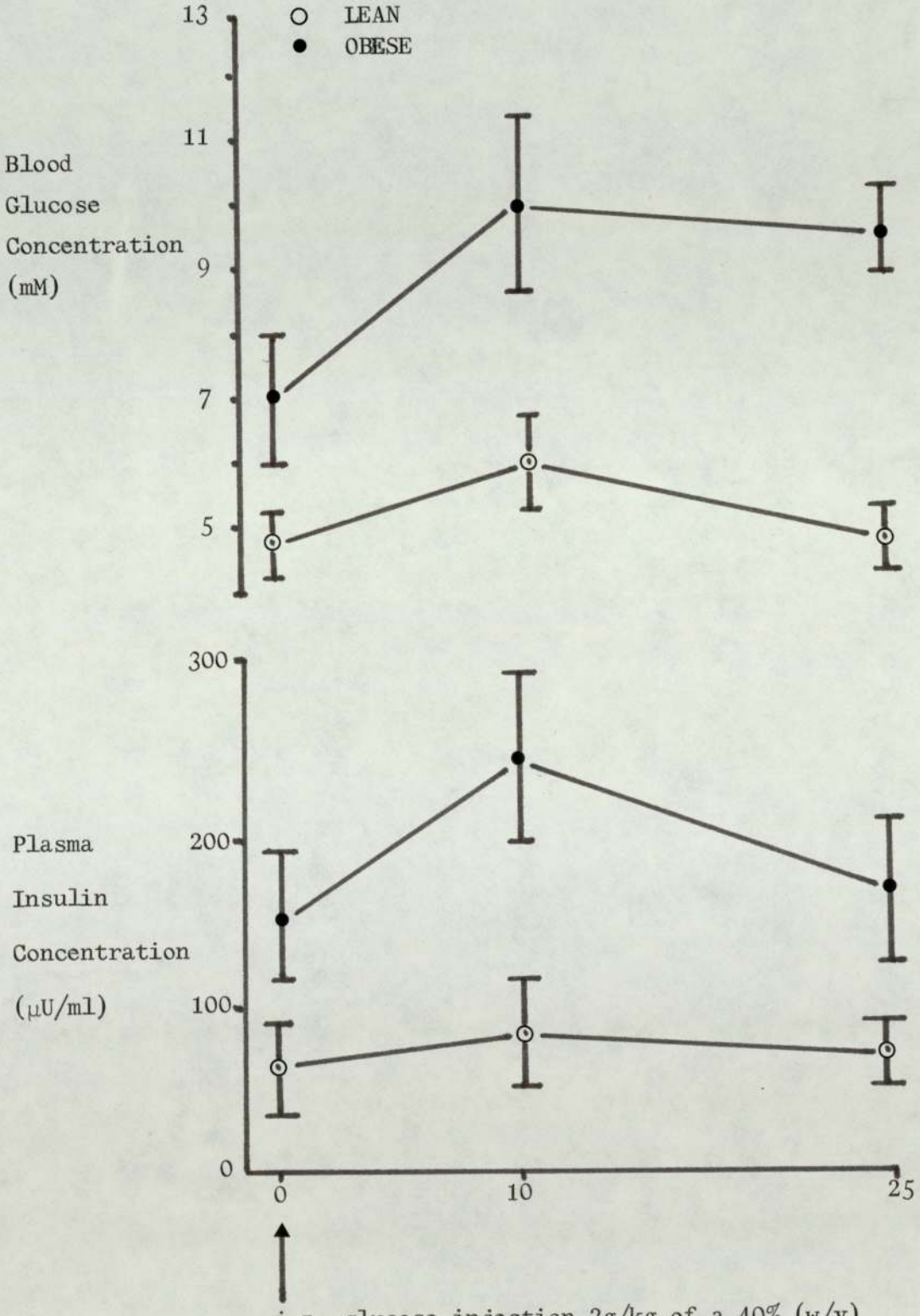
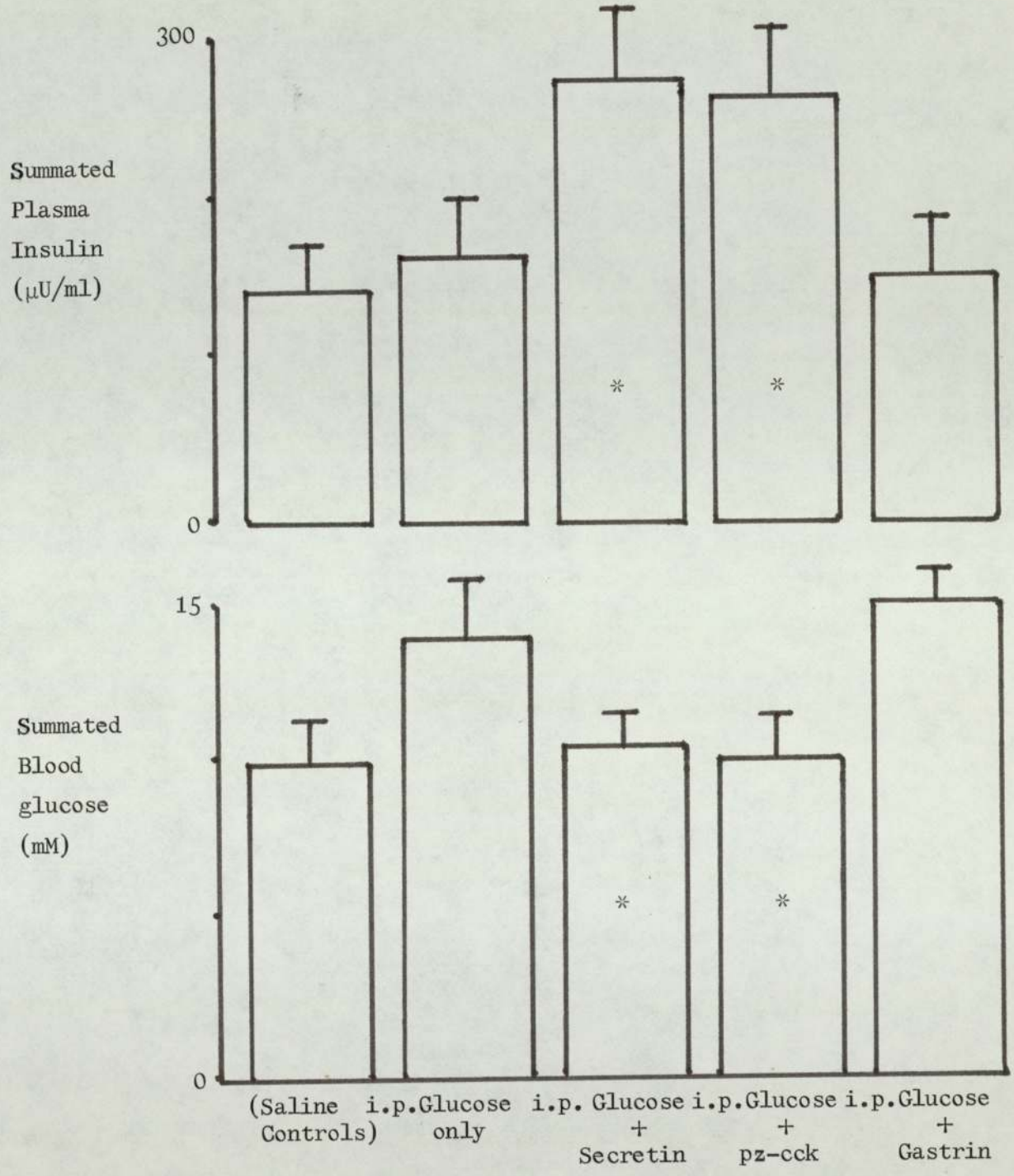


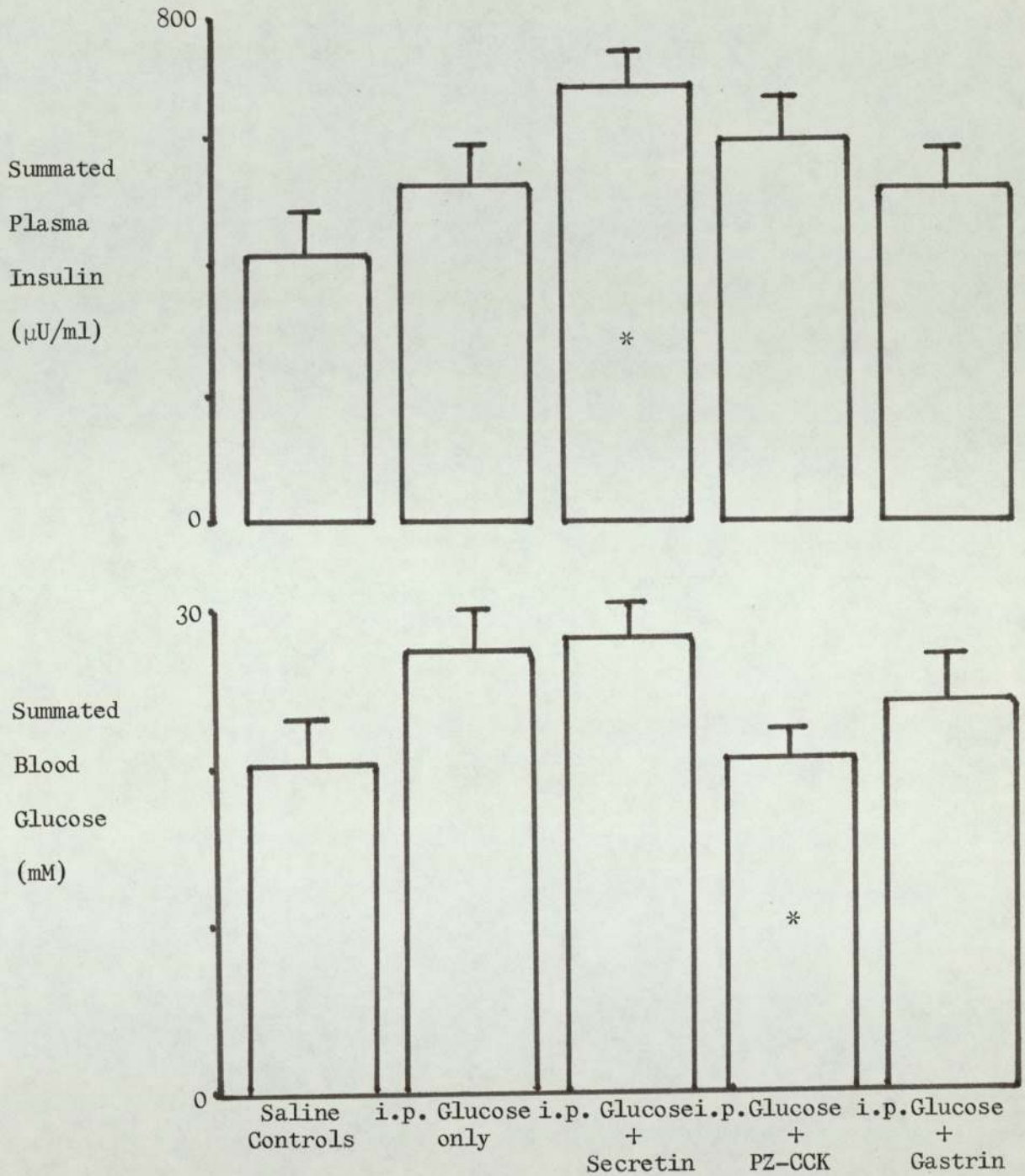
Fig.9 The effect of secretin, pancreozymin and gastrin on the summated plasma insulin and blood glucose levels following i.p. G.T.T. performed on lean mice (n = 6).



* Denotes significant difference ($p<0.05$)

Compared to glucose only treated animals.

Fig.10 The effect of secretin, pancreozymin and gastrin on summated plasma insulin and blood glucose levels following i.p. G.T.T. performed on obese mice (n = 6).



* Denotes significant difference ($p<0.05$) compared to glucose only - treated animals.

B. The effect of gastrointestinal hormones on the release of insulin from isolated islets of obese-hyperglycaemic mice and their lean littermates.

The results of the action of secretin, pancreozymin, gastrin pentapeptide and pancreatic glucagon on basal and glucose-induced insulin secretion from islets of lean and obese-hyperglycaemic mice are given in tables 2 to 5 and summarised in Figure 11. Glucose, at concentrations of 9 mM and 18 mM stimulated the secretion of insulin from the islets of both lean and obese mice compared to control values obtained in the absence of glucose.

The presence of secretin (0.1 and 0.2 clinical U/ml) or pancreozymin (0.75 and 1.5 Ivy Dog U/ml) in the incubation medium enhanced both basal and glucose-induced insulin release from islets of both lean and obese mice. A more powerful insulinotropic effect was observed with the higher hormone concentration in each case.

The results obtained with gastrin pentapeptide were less conclusive (Table 4). At a concentration (0.5 μg/ml), the hormone produced no significant effect on either basal or glucose-induced insulin secretion from lean mouse islets. However, at the same concentration, gastrin pentapeptide significantly inhibited both basal and glucose-induced insulin release from islets of obese mice. The higher concentration of gastrin pentapeptide (2.0 μg/ml) significantly ($p<0.05$) stimulated basal, but had no effect on glucose induced insulin release from islets of lean mice. On the other hand, in the case of obese mouse islets, the higher concentration of gastrin pentapeptide had no significant effect on basal, but significantly depressed the rate of insulin secretion in the presence of 9 and 18 mM glucose.

Pancreatic glucagon (0.5 μg/ml) stimulated the rate of basal insulin secretion from islets of lean mice but was without effect on lean islet glucose induced release. The same concentration of glucagon enhanced 9 mM glucose induced insulin release from obese mouse islets but was without effect on obese islet basal release and 18 mM glucose induced release, Table 5.

Glucagon at the higher concentration (5.0 μg/ml) stimulated basal and glucose-induced insulin secretion from both lean and obese mouse islets.

The amount of insulin secreted from obese mouse islets was always significantly greater than that secreted from lean mouse islets in both the presence and absence of glucose and gastrointestinal hormones.

The percentage stimulation of insulin secretion in the absence and presence of glucose and the various gastrointestinal hormones was no greater for obese mouse islets than for lean mouse islets.

TABLE 2 The effect of secretin on basal and glucose-induced insulin secretion from mouse islets in static incubation.

μU Insulin/μg freeze dried islets/30 min.

		GLUCOSE CONCENTRATION (mM)		
	n	0	9	18
Lean				
Control	(8)	12.2 ± 1.4 (Basal release)	20.7 ± 2.3	27.9 ± 4.5
0.1 clin.U/ml	(5)	27.6 ± 2.9*	38.4 ± 2.3*	41.4 ± 2.4*
0.2 clin.U/ml	(5)	46.1 ± 5.3*	56.3 ± 6.7*	83.7 ± 4.3*
Obese				
Control	(8)	20.8 ± 1.9	30.7 ± 5.1	49.7 ± 3.3
0.1 Clin.U/ml.	(5)	47.8 ± 3.5*	58.3 ± 5.7*	78.1 ± 4.1*
0.2 Clin.U/ml.	(5)	57.1 ± 11.8*	87.7 ± 4.7*	112.2 ± 8.9*

n = number of mice.

* denotes significant difference ($p<0.05$) from control valves.

′ denotes significant difference ($p<0.05$) between lean and obese mouse islet secretory activity.

TABLE 3 The effect of pancreozymin on basal and glucose-induced insulin secretion from mouse islets in static incubation.

μU Insulin/μg freeze dried islets/30 min.

	n	GLUCOSE CONCENTRATION (mM) 0	9	18
Lean				
Control	(8)	12.1 ± 1.4′	20.7 ± 2.3′	27.9 ± 4.5′
0.75 Ivy Dog U/ml	(6)	19.7 ± 3.7*′	26.0 ± 7.9′	35.3 ± 3.6′
1.5 Ivy Dog U/ml	(6)	40.4 ± 3.5*′	53.2 ± 3.7′	66.1 ± 8.4*
Obese				
Control	(8)	20.8 ± 1.9′	30.7 ± 5.1′	49.6 ± 3.3′
0.75 Ivy Dog U/ml	(6)	42.5 ± 5.6*′	46.9 ± 2.2*′	56.9 ± 3.2′
1.5 Ivy Dog U/ml	(6)	61.0 ± 6.2*′	72.3 ± 8.7*′	82.4 ± 8.2*

n = number of mice

* denotes significant difference ($p<0.05$) from control values.

′ denotes significant difference ($p<0.05$) between lean and obese mouse islet secretory activity.

TABLE 4 The effect of gastrin pentapeptide on basal and glucose-induced insulin secretion from mouse islets in static incubation.

μU Insulin/μg freeze dried islets/30 min.

		GLUCOSE CONCENTRATION (mM)		
	n	0	9	18
Lean				
Control	(8)	12.2 ± 1.4 (basal release)	20.7 ± 2.3 /	27.9 ± 4.5 /
0.5μg/ml	(6)	15.3 ± 4.9	18.7 ± 3.9	28.2 ± 2.6
2.0μg/ml	(6)	21.7 ± 3.6*	21.9 ± 4.1	29.6 ± 3.4
Obese				
Control	(8)	20.8 ± 1.9 /	30.7 ± 5.1 /	49.6 ± 3.3 /
0.5μg/ml	(6)	14.1 ± 1.8*	20.1 ± 3.2*	29.8 ± 2.4*
2.0μg/ml	(6)	20.0 ± 3.9	18.9 ± 1.7*	30.7 ± 3.6*

n = number of mice.

* denotes significant difference ($P<0.005$) from control values.

/ denotes significant difference ($P<0.05$) between lean and obese mouse islet secretory activity.

TABLE 5 The effect of pancreatic glucagon on basal and glucose-induced insulin secretion from mouse islets in static incubation.

μU Insulin/μg freeze dried islets/30 min.

		GLUCOSE CONCENTRATION (mM)		
	n	0	9	18
Lean				
Control	(8)	12.4 ± 1.4′	20.7 ± 2.3′	27.9 ± 4.5′
0.5μg/ml	(6)	19.8 ± 1.6*	28.2 ± 3.5′	33.5 ± 8.0′
5.0μg/ml	(6)	29.8 ± 4.2*′	45.0 ± 3.3*′	54.2 ± 6.1*′
Obese				
Control	(8)	20.8 ± 1.9′	30.7 ± 5.1′	49.6 ± 3.3′
0.5μg/ml	(6)	26.6 ± 4.1	45.2 ± 3.6*′	72.0 ± 8.1′
5.0μg/ml	(6)	58.6 ± 5.8*′	75.5 ± 8.1*′	99.1 ± 7.8*′

n = number of mice.

* denotes significant difference ($p<0.05$) from control values.

′ denotes significant difference ($p<0.005$) between lean and obese mouse islet secretory activity.

Fig.11 Summary of the effects of gastrointestinal hormones on basal and glucose-induced insulin secretion.

Insulin Secretion	No Treatment	SECRETIN 0.1 U/ml	SECRETIN 0.2U/ml	PZ-CCK 0.75 U/ml	PZ-CCK 1.5 U/ml	GASTRIN 0.5 µg/ml	GASTRIN 2.0 µg/ml	GLUCAGON 0.5 µg/ml	GLUCAGON 5.0 µg/ml
1. LEAN									
BASAL		↑	↑	↑	↑	—	↑	↑	↑
9mM Glucose Induced	↑	↑	↑	—	↑	—	—	—	↑
18mM Glucose Induced	↑	↑	↑	↑	↑	—	—	—	↑
2. OBESE									
BASAL		↑	↑	↑	↑	↓	—	—	↑
9mM Glucose Induced	↑	↑	↑	↑	↑	↓	↓	↑	↑
18 mM Glucose Induced	↑	↑	↑	—	↑	↓	↓	—	↑

Discussion

The mean fasting insulin level observed for obese mice (140μU/ml) represented a threefold increase over the lean mouse control value. This observation is consistent with the work of Malaisse et al (217), but is lower than values reported previously by several other investigators (163, 179, 188). The resting mean blood glucose value of 7mM for obese mice was also lower than had previously been reported in the literature (163, 174, 186, 190). These discrepancies may have resulted from using 6 hr - fasted animals in the present study.

Obese-hyperglycaemic mice showed an impaired tolerance to an intraperitoneally administered glucose load. This was presumably a consequence of the peripheral insulin resistance (particularly of muscle) which is characteristic of these animals (179, 180, 187).

Some criticism may be levelled at the validity of the intraperitoneal glucose tolerance test as an indicator of pancreatic endocrine function and glucose tolerance. Certainly, the method lacks some sensitivity and accuracy, largely because the test substances are not introduced directly into the blood circulation of the animal. In addition, intraperitoneal injection might lead to some proteolytic degradation of the polypeptide hormones, although this was assumed to be partially eliminated by using supra-physiological concentrations.

Nevertheless, it was felt that the method had some value in a preliminary examination of the potential actions of gastrointestinal hormones upon insulin secretion in vivo. Both secretin and pancreozymin elevated the summated plasma insulin responses of lean and obese mice, and reduced the summated blood glucose response in lean mice. In obese mice, only pancreozymin was capable of lowering the summated blood glucose response following an i.p. G.T.T. These results are

consonant with the literature that suggests gastrointestinal hormones to be capable of augmenting insulin secretion in both man (284) and in anaesthetised dogs (55).

Islets isolated from lean and obese hyperglycaemic mice and used in a static incubation system were responsive to glucose at concentrations of 9 and 18 mM (Fig.11). However, the insulin stimulatory effect of glucose on both lean and obese mouse islets was less pronounced than those reported by previous investigators (87, 285). Malaisse and Malaisse-Lagae (286) have suggested that the poor response of the mouse islet to glucose might be a consequence of the high basal release of insulin, possibly a result of biochemical or nutritional differences in this animal compared to other rodents. The results obtained in the present studies for both lean and obese mouse islets appear to support this hypothesis.

The results of the present in vitro work suggest that both secretin and pancreozymin, in the absence or presence of glucose, stimulate the secretion of insulin by a direct action upon the pancreatic islet. These findings have recently been substantiated by Danielsson et al (69) also using isolated mouse islets. Direct insulinotropic activity of secretin, pancreozymin and gastrin has been demonstrated with isolated rat islets (64), although several conflicting reports have suggested that gastrointestinal hormones exert no stimulatory effect upon isolated rat islets of Langerhans. It has been postulated (68, 71) that the action of enteric hormones on the pancreatic islet might require the presence of intact, functional exocrine tissue. The present data do not conform with this theory since the islets used were denuded almost completely of pancreatic acinar tissue.

Secretin and pancreozymin were shown to be capable of stimulating insulin secretion from islets of lean and obese mice in the absence of glucose, but their effects were potentiated by increasing the glucose concentration,(Fig. 11).

Gastrin pentapeptide had no significant effect upon either summated plasma insulin responses or summated blood glucose responses following an i.p. G.T.T. There have been reports suggesting both that gastrin is without effect (145) and also capable of elevating (59, 60) circulating plasma insulin levels. Rehfeld (287) has suggested that gastrin might only influence the secretion of insulin at gastrin concentrations above the measured physiological range. Hayes and colleagues (288) have shown that fluctuations in serum gastrin levels bear little relationship to the concentration of circulating insulin. This might suggest that gastrin has little, if any, physiological importance in modulating the release of insulin.

Results from the present *in vitro* studies indicate that gastrin pentapeptide might be capable of inhibiting basal and glucose- induced insulin secretion from obese mouse islets but not from lean mouse islets. These data are at variance with those of Jung *et al* (81) who reported on insulinotropic effect of gastrin pentapeptide on isolated lean mouse islets. However, Lernmark *et al* (85) have reported an inhibitory effect of gastrin on glucose induced insulin secretion from obese mouse islets. It has been proposed that gastrin, possibly secreted from the islet α_1 (or D) cell might have a local inhibitory effect on the secretion of insulin from adjacent β-cells (87). Hellman (194) has shown that the islets of obese-hyperglycaemic mice contain reduced numbers of silver-staining (argyrophil) cells, which presumably include gastrin-secreting α_1 cells, compared to islets of lean mice. It would seem feasible that

the hypersecretory activity of the obese mouse pancreatic islet might be a consequence of the reduced activity of some β-cell restraining factor. Indeed, elegant studies by Strautz (190, 191) using isolated lean mouse islets implanted into obese mice have suggested that a pancreatic factor present in lean, but not in obese mouse islets, was capable of normalising weight gains and reducing hyperglycaemia in obese mice. The physiological significance of gastrin as a local regulator of insulin secretory activity remains to be established.

It has recently been demonstrated that a group of cells in the pancreatic islets produce somatostatin (289) (Growth Hormone Release Inhibiting Hormone), a peptide that inhibits the secretion of insulin induced by glucose (290), secretin, pancreozymin (291) and glucagon (292). Thus, it is possible that somatostatin may be of physiological importance in the local regulation of insulin release (291) and may be secreted from a second type of D or α_1 cell.

Pancreatic glucagon at a concentration of 5 μg/ml stimulated the secretion of insulin from both lean and obese mouse islets both in the presence and absence of glucose. Samols et al (95) originally demonstrated that an injection of glucagon in man was followed by a rise in circulating insulin levels, and these workers consequently proposed that glucagon, of pancreatic and possibly enteric origin, might be a physiological insulin secretagogue in response to glucose ingestion (98, 107). More recently, glucagon has been shown to be capable of enhancing insulin secretion both in vivo (90, 91, 92) and in vitro (40, 93, 94, 95). It seems very likely that pancreatic glucagon is in some way implicated in the regulation of insulin secretion, and future studies using separated α_2 and β-cells will clarify this relationship.

Mayer and colleagues (197) have suggested that excessive glucagon

secretion might be an important factor in the development of hyperinsulinaemia in obese-hyperglycaemic mice. Laube and colleagues (201) have demonstrated that the α_2 cells in the obese mouse islet are hypertrophied, and that they respond to glucose with a paradoxical release of glucagon. It therefore appears likely that hyperglucagonaemia might be an important aetiological factor in the deranged carbohydrate metabolism of obese-hyperglycaemic mice.

The concentrations of gastrointestinal hormones used in the present investigations were supraphysiological, although not necessarily pharmacological. It remains to be established what levels of gastrointestinal hormones the pancreatic islet is exposed to, since these levels are likely to be much greater than the concentrations of the hormones measured in peripheral serum or plasma.

Islets of obese mice incubated *in vitro* always showed a higher basal rate of insulin secretion than lean mouse islets, and were hyperresponsive to glucose, secretin, pancreozymin and pancreatic glucagon. An exaggerated insulin response to various stimuli, including glucose, amino acids and possibly glucagon, has been demonstrated both in obese-hyperglycaemic mice (210, 213) and in human obesity (293 - 302) and it has been suggested that this abnormal response might be a primary factor in the aetiology of obesity (163, 206, 213, 303).

The present data substantiate previous hypotheses (187, 188, 206) that an elevated gastro-intestinal stimulation of insulin secretion in response to hyperphagia and an enhanced sensitivity of obese mouse islets to gastrointestinal hormones might be responsible for the hyperinsulinism and islet cell hyperplasia seen in the obese-hyperglycaemic mouse.

CHAPTER IV

Section A The effects of gastrointestinal hormones on the insulin secretion dynamics of isolated lean and obese-hyperglycaemic mouse islets of Langerhans.

Introduction

The *in vitro* secretion of insulin in response to glucose stimulation has been shown to occur in two distinct phases (304). Each of the two phases of the insulin secretory profile is thought to represent separate metabolic and secretory events (304) and it has thus become desirable to study dynamic aspects of insulin release in order to gain insight into the mechanism of stimulation of the various insulin secretagogues. It has been proposed that the secretion of insulin occurs from two separate compartments or pools within the islet β-cell (305, 306). Thus, the secretion of stored and *de novo* synthesised insulin is thought to represent the first and second phases of insulin release respectively (305, 307). From studies of insulin secretory dynamics following glucose stimulation, Cerasi, Fick and Rodemo (308) have derived a mathematical model for glucose-induced insulin release. These workers suggested that when the pancreatic islet is exposed to hyperglycaemia, the β-cell responds with an instantaneous release of insulin. This is followed by a short refractory period and a gradual potentiation of insulin release to a level determined by the glucose concentration. Other investigators have proposed that the islet might operate as a morphological and functional syncytium, secreting insulin in 'pulses' (309) and the phasic nature of insulin secretion might be related to ultrastructural factors within the islet (310).

Many techniques have been devised for the study of insulin secretory dynamics *in vitro*. These techniques differ fundamentally in the tissue preparation used, and can be divided into four general types:

1. Perfusion of whole pancreas

In 1947, Anderson and Long (311) described a technique for investigating the phasic nature of insulin secretion using an isolated rat pancreas perfused with heparinised rat blood containing various concentrations of glucose. The perfusate was supplied at a constant rate via the aorta, and collected from the hepatic portal vein. The technique has subsequently been modified and adopted for use with pancreata isolated from the rat (312, 313, 314), mouse (315) and dog (82). Other workers have described the perfusion of the rat pancreas *in situ*, either alone, or in conjunction with a section of duodenum (316, 317).

The advantages of pancreas perfusion are several-fold. Since the supply of metabolic substrates is optimal and the accumulation of hormones and waste products minimised, the perfused pancreas has a greater total insulin secretory capacity than static incubation systems (314). Furthermore, the perfused organ is not too far removed from the normal physiological situation.

2. Perifusion of pancreas fragments (Superfusion)

This technique employs the passage of a buffered medium through a small chamber containing finely chopped-up pieces of pancreas (213, 318, 319, 320). The technique eliminates the complicated surgery involved in whole pancreas perfusion, but presents less well defined insulin secretory dynamics (319) - because not all pieces of pancreas contain islets. In addition, with this preparation there is the danger of secreted insulin being degraded by enzymes released from the cut edges of the acinar tissue.

3. Perifusion of Isolated Islets of Langerhans

Hudson and Beck (320) originally described a system for perifusing rat islets of Langerhans isolated by collagenase digestion. Five islets were incubated in a watertight perifusion chamber immersed in a water bath at 37°C, and buffer containing albumin and appropriate test substances passed through from a gravity feed reservoir. Subsequent modifications of this technique have differed in the number of islets employed, the nature of the perifusion chamber, the flow rate and perifusion pressure, and the method used to pass the perifusate through the chamber. Lacy, Walker and Fink (321) used a system consisting of isolated rat islets perifused in a small polythene chamber. The perifusion medium was pumped through at a constant rate by means of a peristaltic pump. Idahl (322, 323) devised a microperifusion apparatus with a quick freeze facility that allowed concomitant recordings of intermediary metabolites and insulin release. Perifusion systems have also been used to investigate the dynamics of insulin secretion from isolated islets of rabbits (310) and reptiles (324).

The perifusion of isolated islets of Langerhans has several advantages over other techniques for studying insulin secretory dynamics. Perifusion systems allow more accurate control of insulin secretory dynamics, a high degree of versatility (the ability to rapidly alter the environment of the islets) and the ability to correlate changes in metabolite levels with changes in insulin secretion. Furthermore, since the pancreatic exocrine tissue is removed, the problem of the enzymatic degradation of insulin is eliminated and the exchange of substrates, oxygen and hormone by the tissue accelerated. Perifusion of isolated islets also reduces haemodynamic problems which are a characteristic of perfused pancreas preparations.

4. Islet "Dipping" Apparatus

Bessman, Beigelman and Thomas (325) devised a technique by which individual islets secured by suction to a micropipette were dipped successively into small vessels containing buffer. Aliquots from each vessel were then assayed for insulin to reveal the periodicity of insulin release. However, the mechanical stimulus caused by the constant dipping of the islet was thought to cause an artifactual rythmic 'spike' secretion of insulin.

In the present study, a perifused islet system was employed to investigate the effect of gastrointestinal hormones and extracts of mouse intestine on glucose induced insulin secretory dynamics of isolated islets of Langerhans of lean and obese-hyperglycaemic mice.

Materials and Methods

The perifusion of isolated mouse islets

The perifusion system used in the present studies is illustrated in Figure 12 and Plate 2 . The technique adopted followed the original method of Lacy, Walker and Fink (321). Groups of 15 islets microdissected from lean or obese-hyperglycaemic mice were placed in Millipore filter chambers (type 01200; Millipore, Wembley, Middlesex) containing a cellulose ester Millipore filter, pore size 5 μ punctured uniformly with the end of a needle. Stainless steel filter chambers were used in preference to polythene because of their greater conductive capacity. The perifusion chambers were clamped beneath the surface of a water bath maintained at $37\pm0.5^{\circ}C$ by a temperature control unit. Reservoirs containing perifusion medium were maintained at $37^{\circ}C$ in the water bath. Perifusion medium was drawn through the tissue chambers via polythene tubing (pp50, Portex Ltd) by means of a multi-channel, variable flow rate peristaltic pump (Watson & Marlow, Falmouth, Cornwall). In initial

Figure 12 ISLET PERIFUSION SYSTEM FOR STUDIES OF INSULIN SECRETORY DYNAMICS

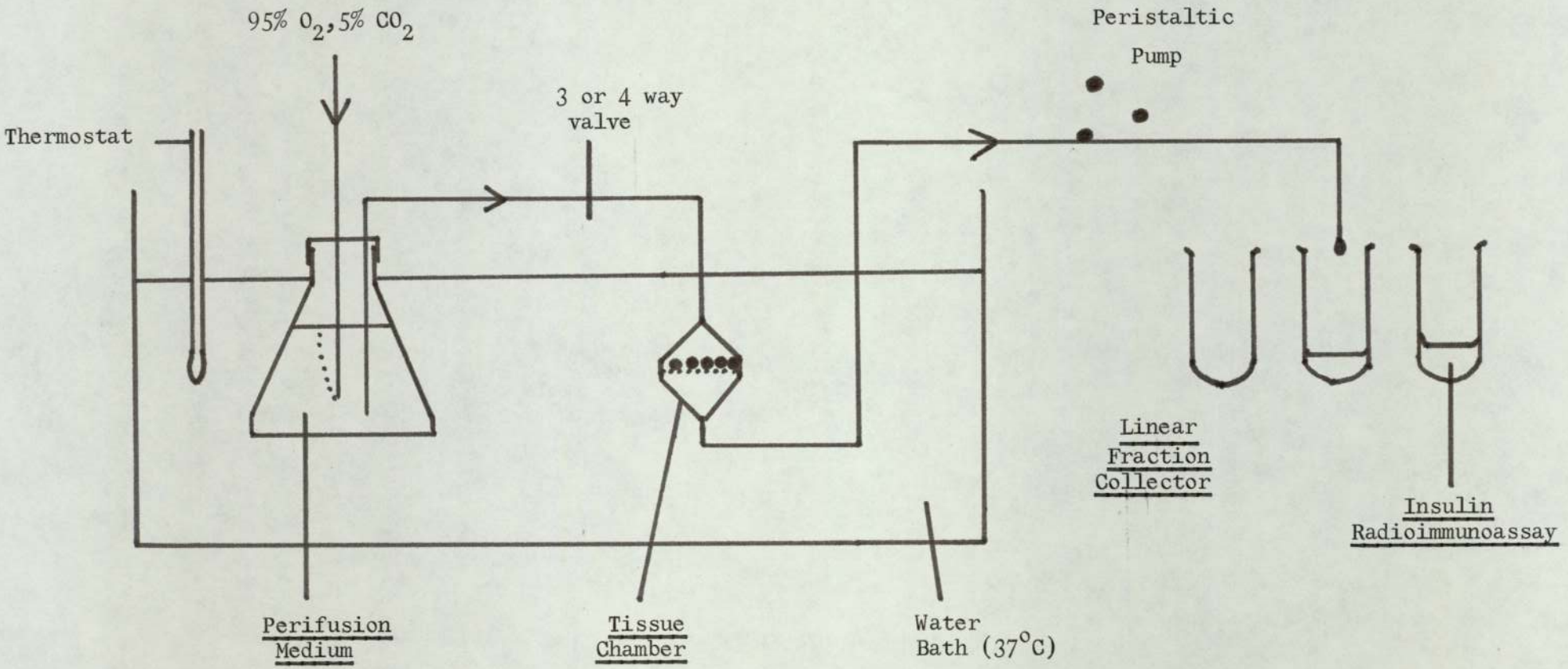
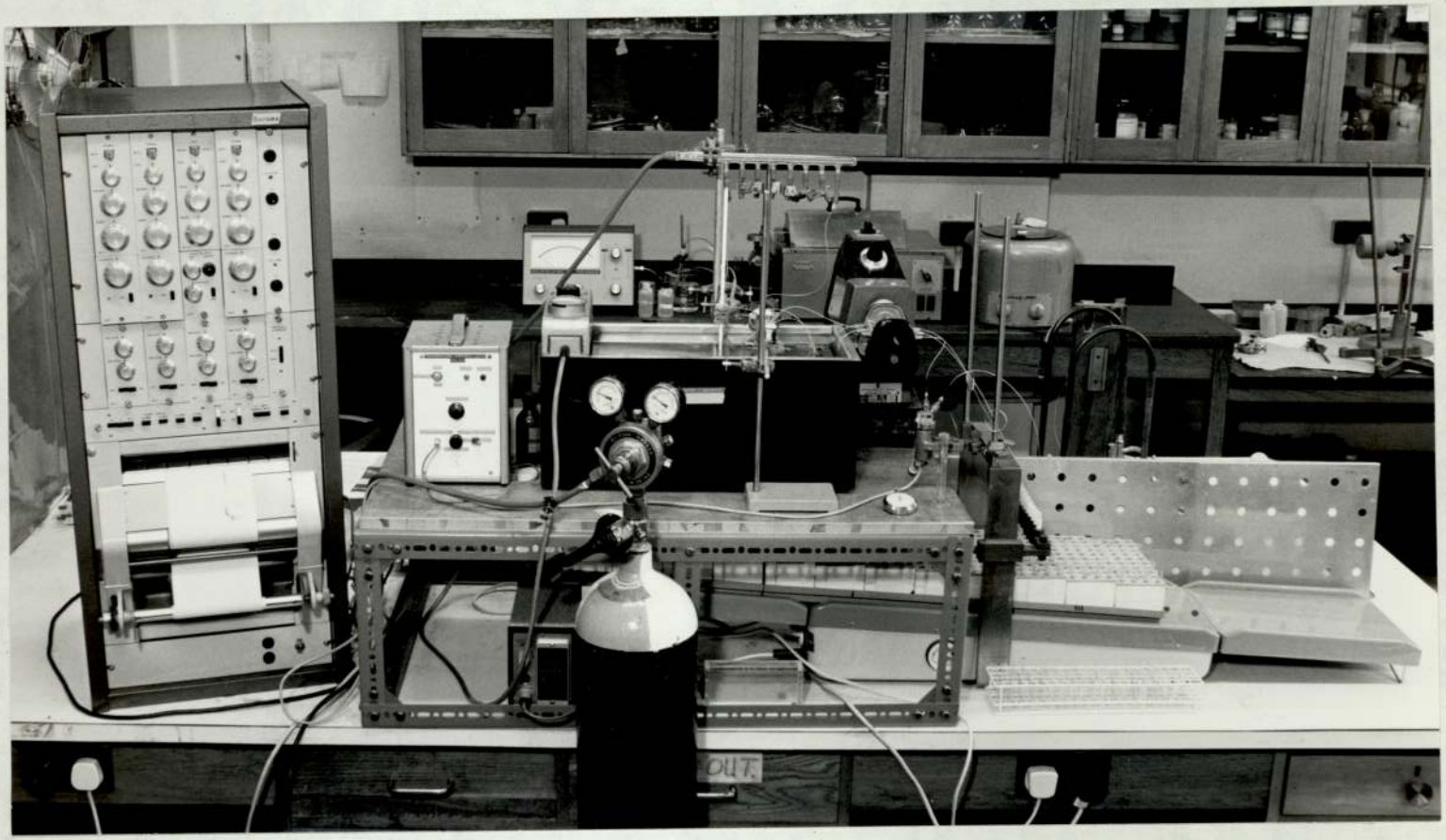


Plate 2. System used in the present studies for the perifusion of isolated lean and obese mouse islets of Langerhans.



experiments, a separate channel was connected to a pressure transducer (Bell and Howell Ltd., Basingstoke) in order to monitor the perifusion pressure in the system. A flow rate of 0.5 ml/min was used at a perifusion pressure of 12 mm Hg. These parameters were recorded on a Devices multichannel input recorder. Perifusate was collected at 1-minute intervals on an automatic linear fraction collector (10 line capacity; Serva-Technik, Malsch bei Heidelberg) and duplicate samples of 100µl taken from each 0.5 ml aliquot of perifusate and rapidly deep-frozen for the subsequent radioimmunoassay of insulin. The islets were pre-perifused for 40 minutes with 3mM glucose Gey and Gey bicarbonate buffer prior to perifusion with test solutions, in order to establish steady state insulin secretory dynamics. In early experiments, test perifusate was introduced into the system by stopping the pump for approximately 2 seconds and manually transferring the portex capillary tube to an alternative reservoir. This was initially found to be the simplest and most satisfactory method since it eliminated hydrostatic effects and air gaps. In later experiments, a 3-way valve (LV-3 lab. valve, Pharmacia Fine Chemicals, Uppsala, Sweden) was incorporated into the system. In all experiments, two channels were run simultaneously; one was employed to investigate the effects of a test substance whilst the second channel acted as a control. At the end of each experiment, the islets were removed from the perifusion chamber using fine watchmakers' forceps and a wire loop under a stereo-microscope, (Olympus, Sterozoom x7-x35). The islets were placed in small perforated aluminium foil planchets, freeze-dried and weighed as described in Chapter III.

Reagents

The perifusion medium consisted of Gey and Gey (277) bicarbonate buffer pH 7.4 supplemented with 1% (w/v) insulin free bovine serum albumin fraction V, glucose (9, 18 or 25 mM) and gastrointestinal hormones. The concentrations of gastrointestinal hormones used in the dynamic studies were as follows:-

Secretin and Pancreozymin - Cholecystokinin, both highly purified (G.I.H. Research Unit, Karolinska Institute, Stockholm, Sweden) 0.2 clinical units/ml and 1.5 Ivy Dog units/ml respectively and crude Secretin and Pancreozymin (Sigma Chemical Co.Ltd., London) 0.2 clinical units/ml and 1.5 Ivy Dog units/ml. respectively, together with gastrin pentapeptide (Calbiochem. Ltd., London) 2.0 μg/ml, synthetic human gastrin I (I.C.I.) 1.0 μg/ml and pancreatic glucagon (Sigma Chemical Co.Ltd., London) 5.0 μg/ml. Preparations of crude, mouse intestinal mucosal extracts were also investigated.

Preparation of a crude duodenal - jejunal extract('T.O.T.')

Crude extracts of duodenal-jejunal mucosa were prepared by the method of Moody et al (131).This extract was designated "T.O.T." by these authors and this nomenclature has been retained for the present studies. The whole duodenum and jejunum was rapidly excised from either lean or obese-hyperglycaemic mice, stripped of all pancreatic tissue, fat and blood vessels, and flushed with ice-cold (2-4°C) distilled water. The washed tissue was sliced into 75% ethyl alcohol containing 1.5% H_3PO_4 (5 ml acid-alcohol solution/g wet weight of tissue), and extracted for two hours with constant stirring at room temperature (21°C). The residue was removed by centrifugation (2,500 g) and extracted again with 60% ethyl alcohol containing 0.5% H_3PO_4 at room temperature for one hour. The combined extracts were filtered under vacuum through Kieselguhr

and the ethanol evaporated in vacuo. The concentrate was then filtered again through Kieselguhr and solid trichloracetic acid added to give a final concentration of 10% (w/v) TCA. The precipitate was removed by centrifugation (2,500 g) and extracted for one hour at 4°C with 50% and subsequently with 75% ice cold acetone. The acetone extracts were combined and further acetone added to give a final concentration of 97% (v/v) acetone. 4N HCl was then added and the flocculent precipitate isolated by centrifugation (3,000 g). The precipitate was washed with acetone, dried in a vacuum dessicator and stored in sealed ampoules. This material was designated 'T.O.T.'

The preparation of duodenal-jejunal extracts containing crude immunoreactive glucagon (I.R.G.)

Preparations of crude intestinal extract designated "I.R.G." was prepared by a modification of the method employed by Kenny (326). These extracts have been shown to contain glucagon-like immunoreactivity by Buchanan and colleagues (40). Duodenum and jejunum excised from either lean or obese-hyperglycaemic mice was dissected free of pancreatic tissue, fat and blood vessels and washed with ice cold saline (0.9% w/v). The tissue was homogenised in 5 ml acid-ethanol (750 ml. absolute alcohol + 250 ml. distilled water + 15 ml. conc. HCl) per g wet weight of tissue using a Waring Blender. The intestinal material was homogenised thoroughly and allowed to stand overnight at 4°C. The residue was centrifuged off (2,500 g) and re-extracted with 4 ml. acid-alcohol/g original wet weight of tissue for one hour. The sediment was again removed by centrifugation (2,500 g) and extracted for a further hour with 3 ml. acid-alcohol/g. original wet weight of tissue. The residue was finally separated by centrifugation (2,500g) and discarded, whilst the three supernatants were pooled and retained. The pH of the combined supernatant was adjusted to 7.5 with NH_4OH and allowed to stand for two hours

at room temperature. Any precipitate produced was then removed by centrifugation (2,500 g) and discarded. The protein fraction containing glucagon-like immunoreactivity was subsequently precipitated by the addition of 68 ml absolute ethanol and 112 ml ether to 40 ml aliquots of the extract and allowing to stand overnight at 4°C. The residue was then centrifuged off (3,000 g), dried in a vacuum dessicator, weighed and taken up in Gey and Gey bicarbonate buffer pH 7.4 (1 ml/g intestinal tissue). The product was then dialysed against the same buffer in Visking 18/32" tubing for 48 hours at 4°C. The dialysed material was stored at -20°C prior to use. The extracts so prepared were used in studies of insulin secretory dynamics at a final concentration of 100 μg extract/ml buffer.

Results

The results of studies on insulin secretion dynamics are illustrated in figures 13 to 36. The results are expressed as the concentration of insulin in the perifusate following perifusion with glucose or test hormone per mg freeze dried islet tissue (μU IRI/ml/mg freeze dried islet tissue). Statistical comparisons of test insulin concentration with control (i.e. 3mM glucose) levels were made at specific points along the insulin secretory profiles and probability levels of less than 0.05 taken as significant. Control insulin secretion profiles in the presence of 3mM glucose for both lean and obese mouse islets are shown in figure 13. Raising the glucose concentration in the perifusate from 3mM to 9 and 18 mM produced a significant ($p<0.05$) first phase release of insulin from obese mouse islets compared to control profiles and this was followed by a more gradual second phase release (figures 14a and b). Islets isolated from lean mice, however, showed no significant response to 18 mM glucose compared to controls (figure 14b).

A higher concentration of glucose (25mM) produced a significant, sustained simulation of insulin secretion compared to control levels from both lean and obese mouse islets (figure 15). A biphasic pattern of insulin secretion was not always observed. In most cases, however, a small peak occurred within one minute of raising the glucose concentration.

Crude preparations of secretin (0.2 clin.u/ml) and pancreozymin (1.5 Ivy Dog u/ml) in the presence of a non-stimulatory concentration of glucose (3mM) produced an immediate, significant stimulation of insulin secretion from islets of both lean and obese mice (figures 16 and 19). Stimulation by the hormone preparations generally lasted some 20 minutes and was potentiated and maintained by increasing the glucose concentration further to 9mM and 18mM (figures 17, 18, 20 and 21).

Purified secretin and pancreozymin (0.2 clin.u/ml and 1.5 Ivy Dog u/ml respectively) also significantly stimulated insulin secretion in the presence of 3mM glucose (figures 22 and 24). Both purified hormone preparations were significantly less effective insulin secretagogues than the crude hormone preparations. The presence of 18mM glucose potentiated and maintained the insulin stimulatory effects of purified secretin and pancreozymin (figures 23 and 25) on both lean and obese mouse islets compared to controls (figure 14b).

Pancreatic glucagon (5.0 μg/ml) in the presence of 3mM glucose produced a significant, sustained rise in insulin secretion from both lean and obese mouse islets (figure 26). This response was significantly enhanced in the presence of 9 and 18mM glucose (figures 27 and 28).

The insulin secretory responses of obese mouse islets to glucose, crude and purified secretin and pancreozymin, and pancreatic glucagon were consistently higher than those of lean mouse islets. Also, the

responses of both lean and obese mouse islets to gastrointestinal hormones were enhanced by the presence of 18mM glucose compared to 3mM glucose.

Gastrin pentapeptide (2.0μg/ml) in the presence of 3mM glucose produced a slight, though insignificant early stimulation of insulin secretion from islets of obese mice, and a gradual, insignificant rise in insulin release from lean mouse islets (figure 29). Gastrin pentapeptide, at the same concentration, significantly potentiated 18mM glucose-induced insulin secretion from both lean and obese mouse islets (figure 30).

Synthetic Human Gastrin I (1.0 μg/ml) produced a weak, slightly elevated but statistically insignificant effect on the secretion of insulin from lean and obese mouse islets (figure 31). In the presence of 18mM glucose, however, Synthetic Human Gastrin I initiated a significant potentiation of the early phase of insulin secretion from both lean and obese mouse islets (figure 32).

The effects of intestinal extracts on Insulin Secretion Dynamics

Crude extracts of mouse duodenum-jejunum designated ('T.O.T.') in the presence of 3mM glucose produced a significant stimulatory effect on the secretion of insulin from islets of both lean and obese mice (figures 33 and 34).

Intestinal extracts of lean and obese mouse duodenum-jejunum containing glucagon like immunoreactivity (I.R.G.) significantly enhanced the secretion of insulin from lean and obese mouse islets in the presence of 3mM glucose (figures 35 and 36). Islets isolated from obese mice were found to be significantly more sensitive to these extracts than lean mouse islets. Statistical comparisons of the insulin responses to the intestinal extracts revealed that obese mouse 'T.O.T.' and 'I.R.G.'

Figure 13 The effect of 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

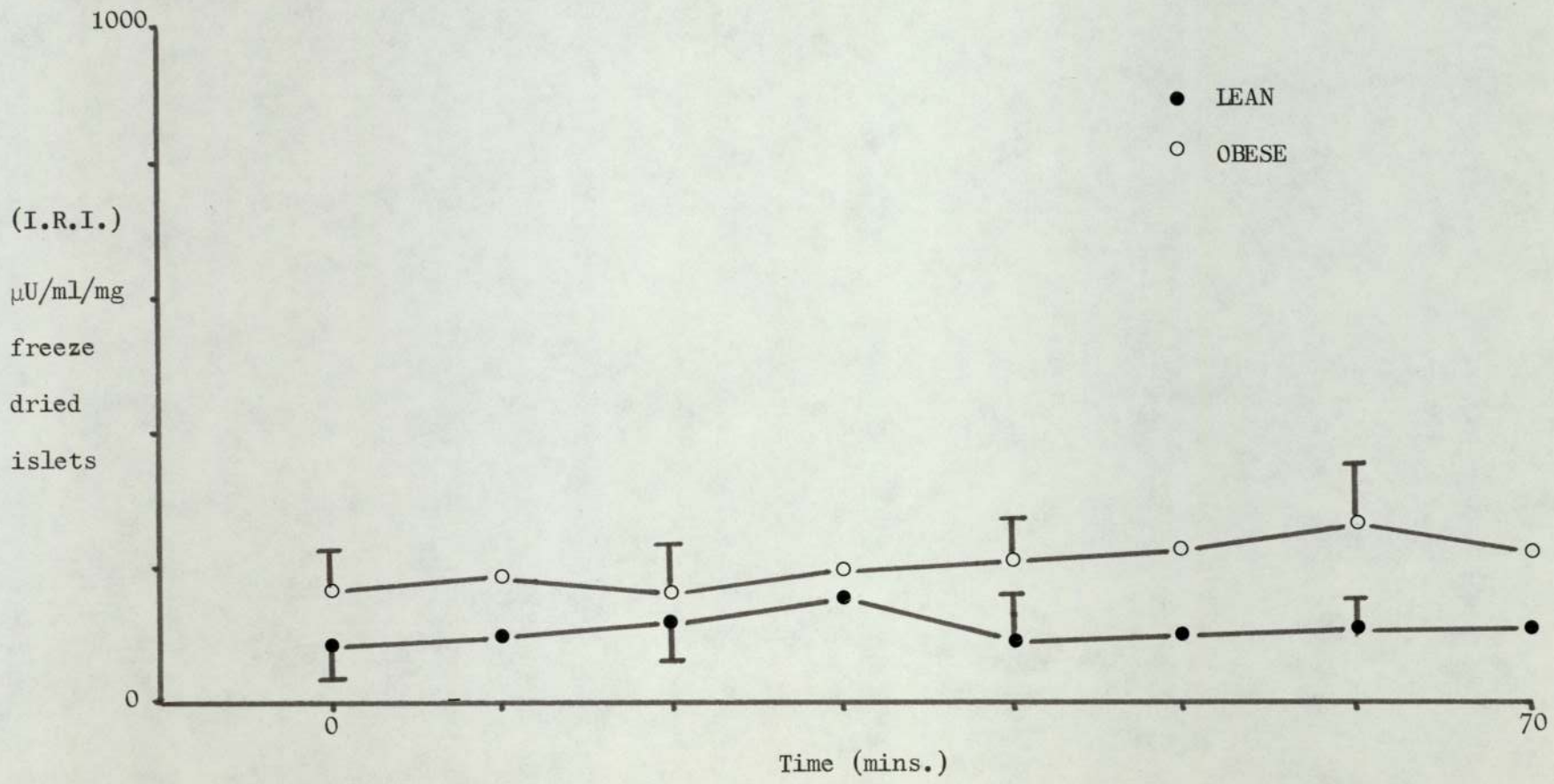


Figure 14(a) The effect of 9mM glucose on insulin secretion dynamics from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 4 separate profiles ± s.e.m., one profile per mouse)

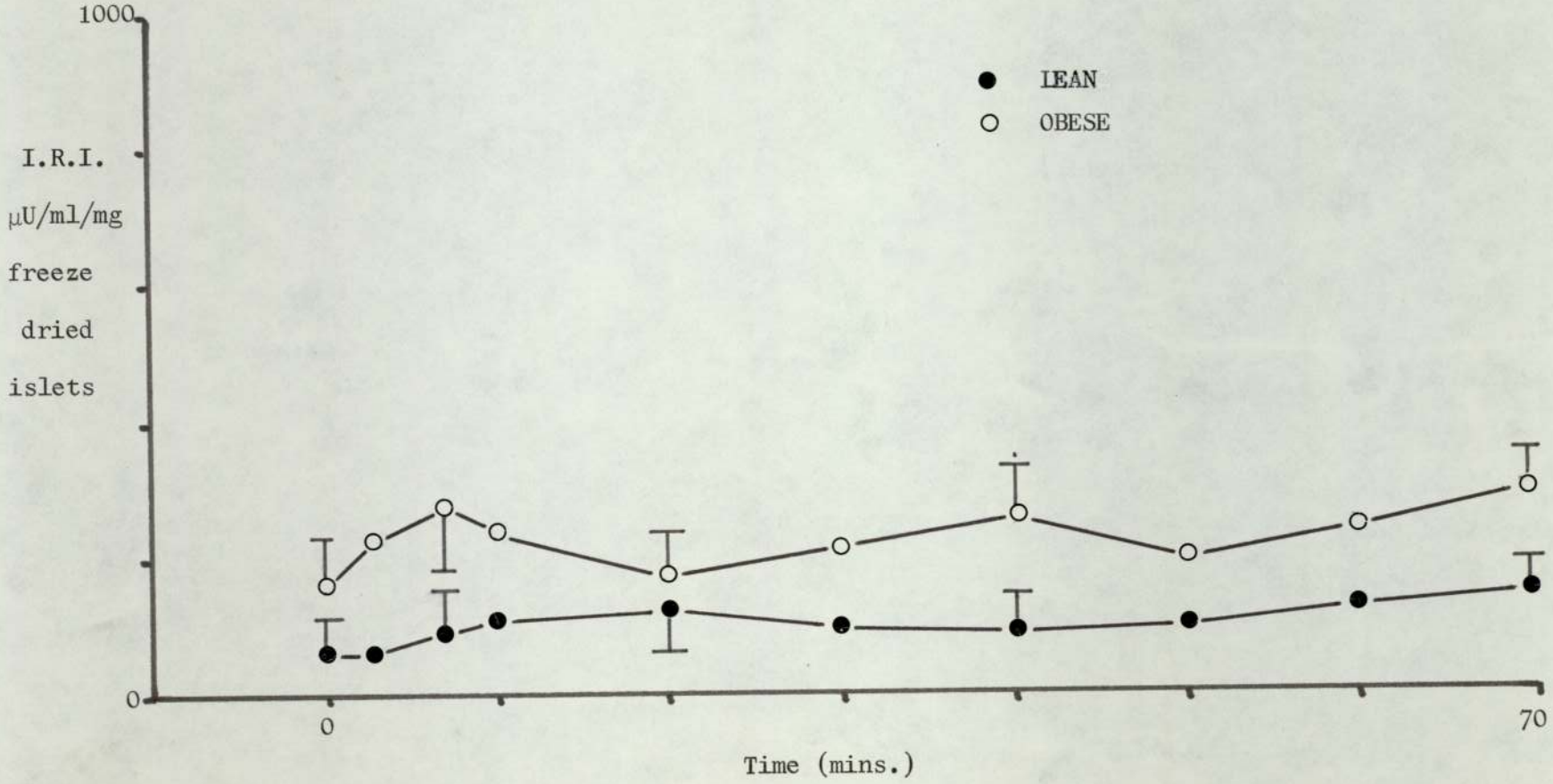


Figure 14(b) The effect of 18mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

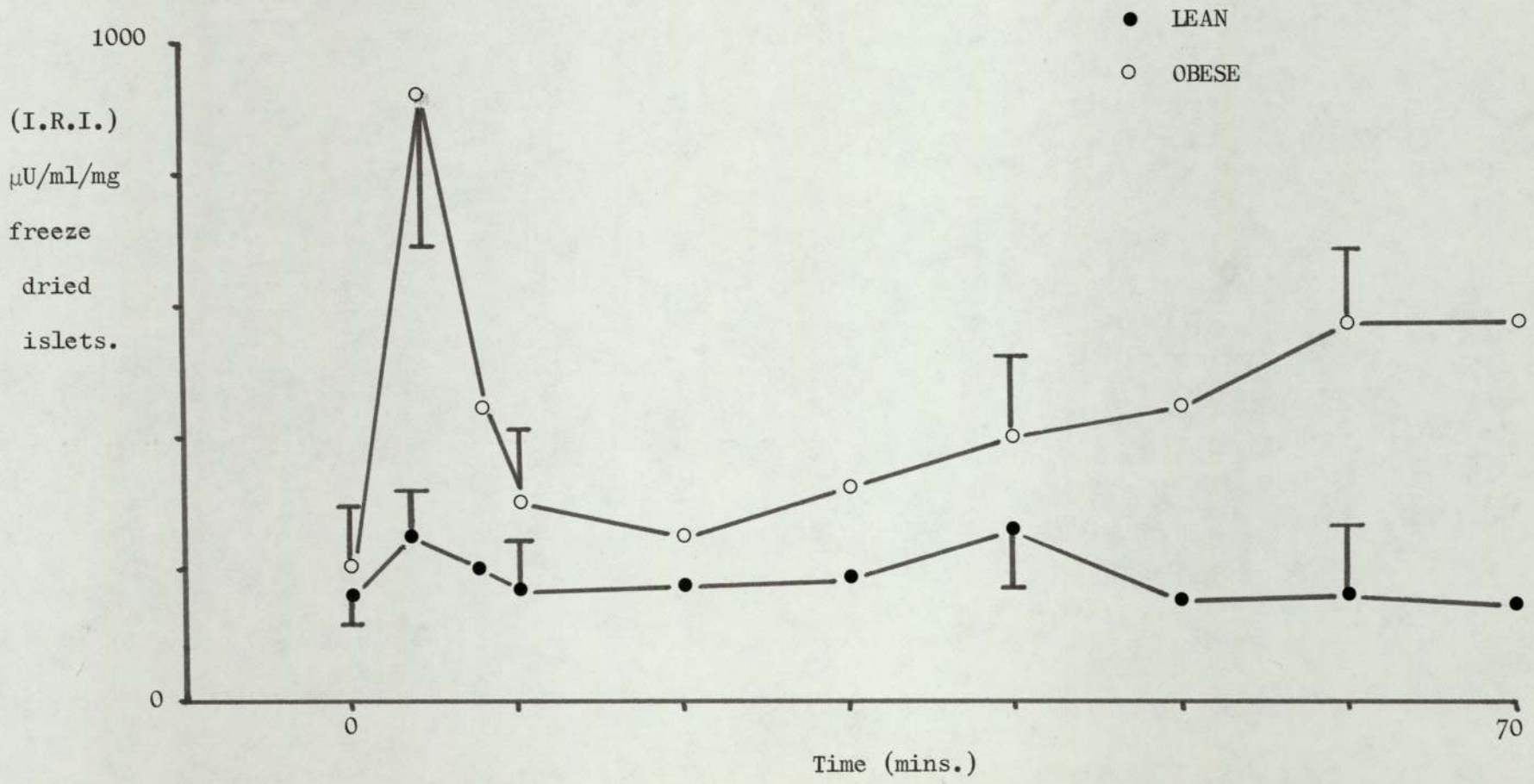


Figure 15 The effect of 25 mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

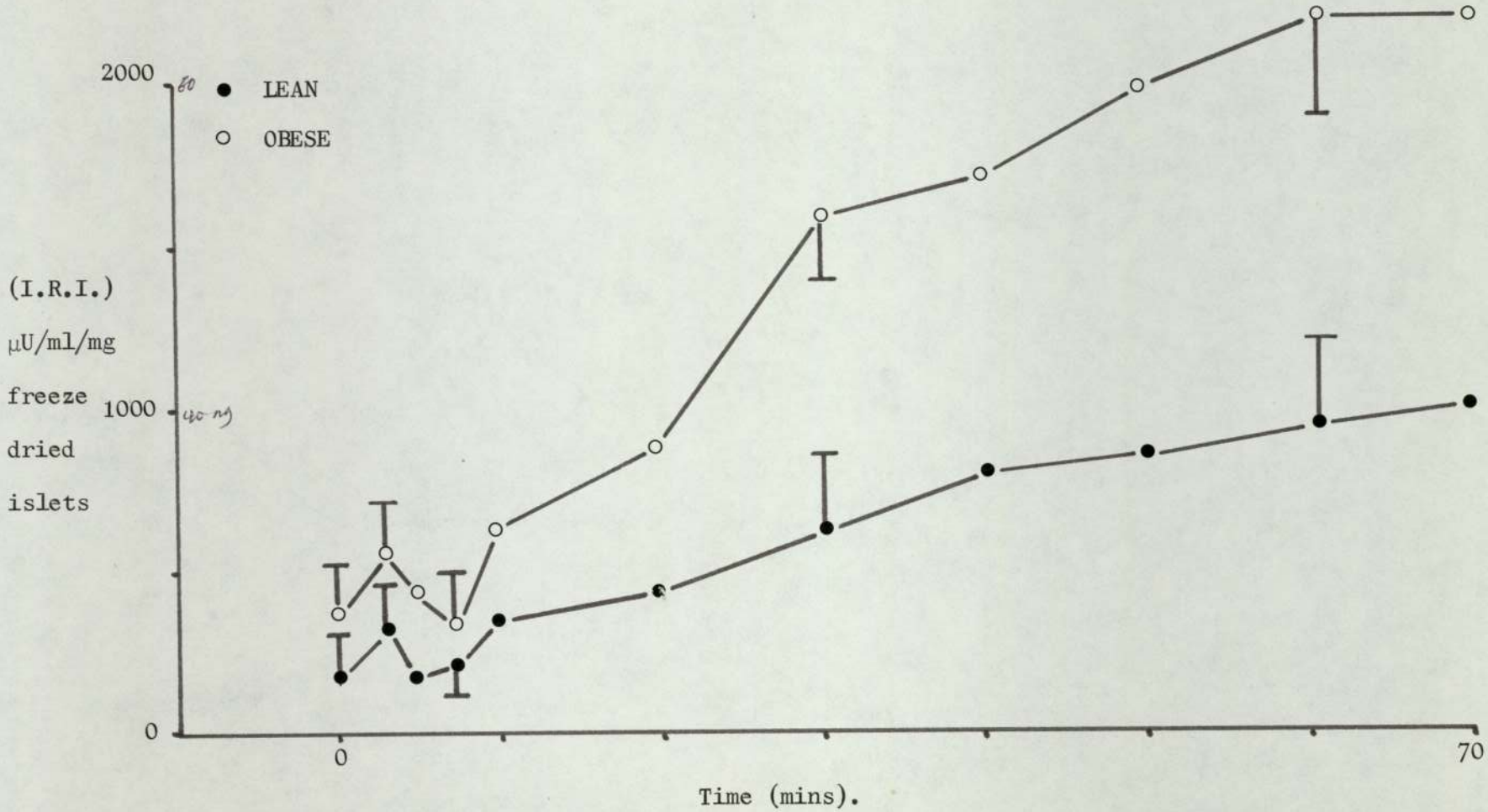


Figure 16 The effect of crude secretin (1.5 clin.U/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

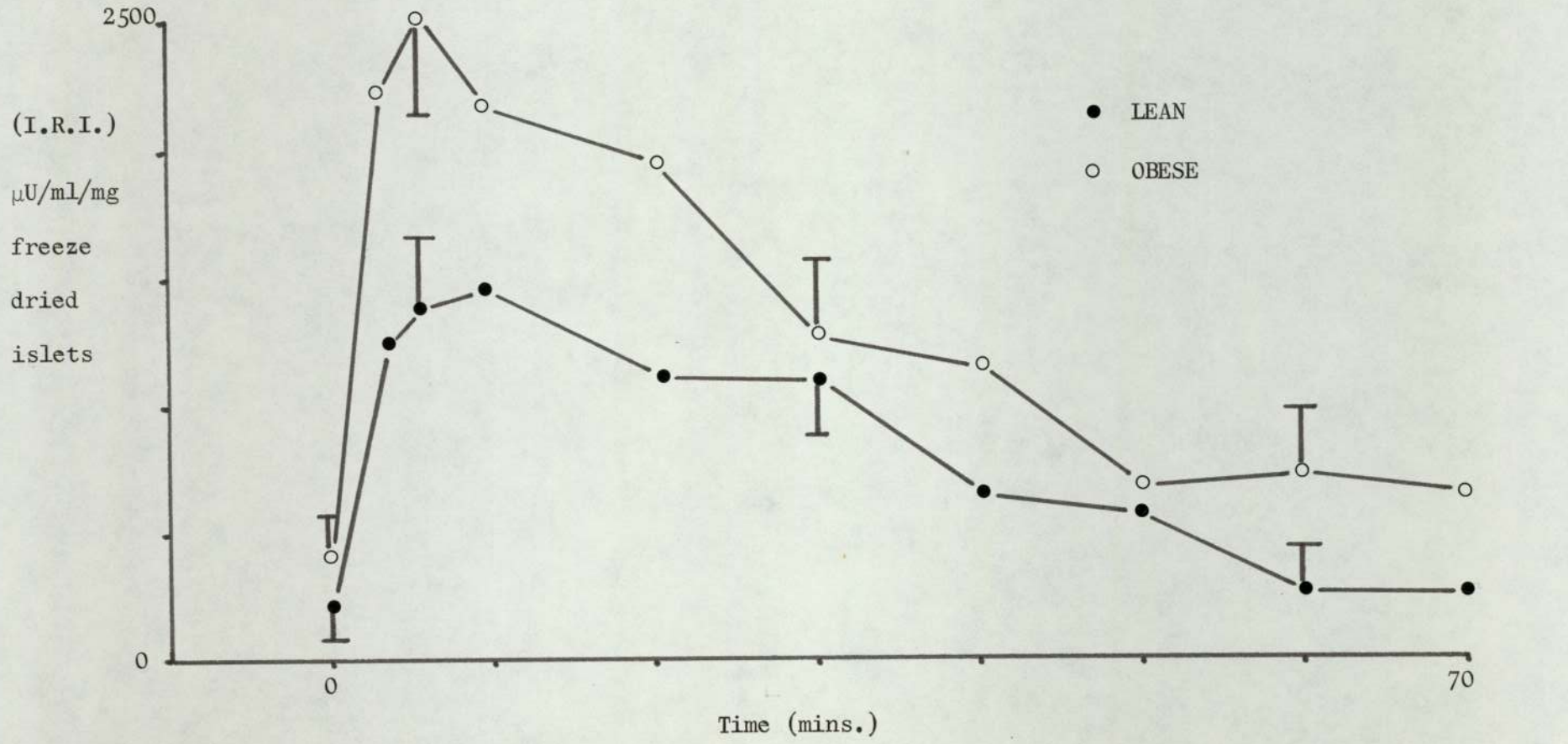


Figure 17 The effect of Crude Secretin (1.5 U/ml) + 9mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

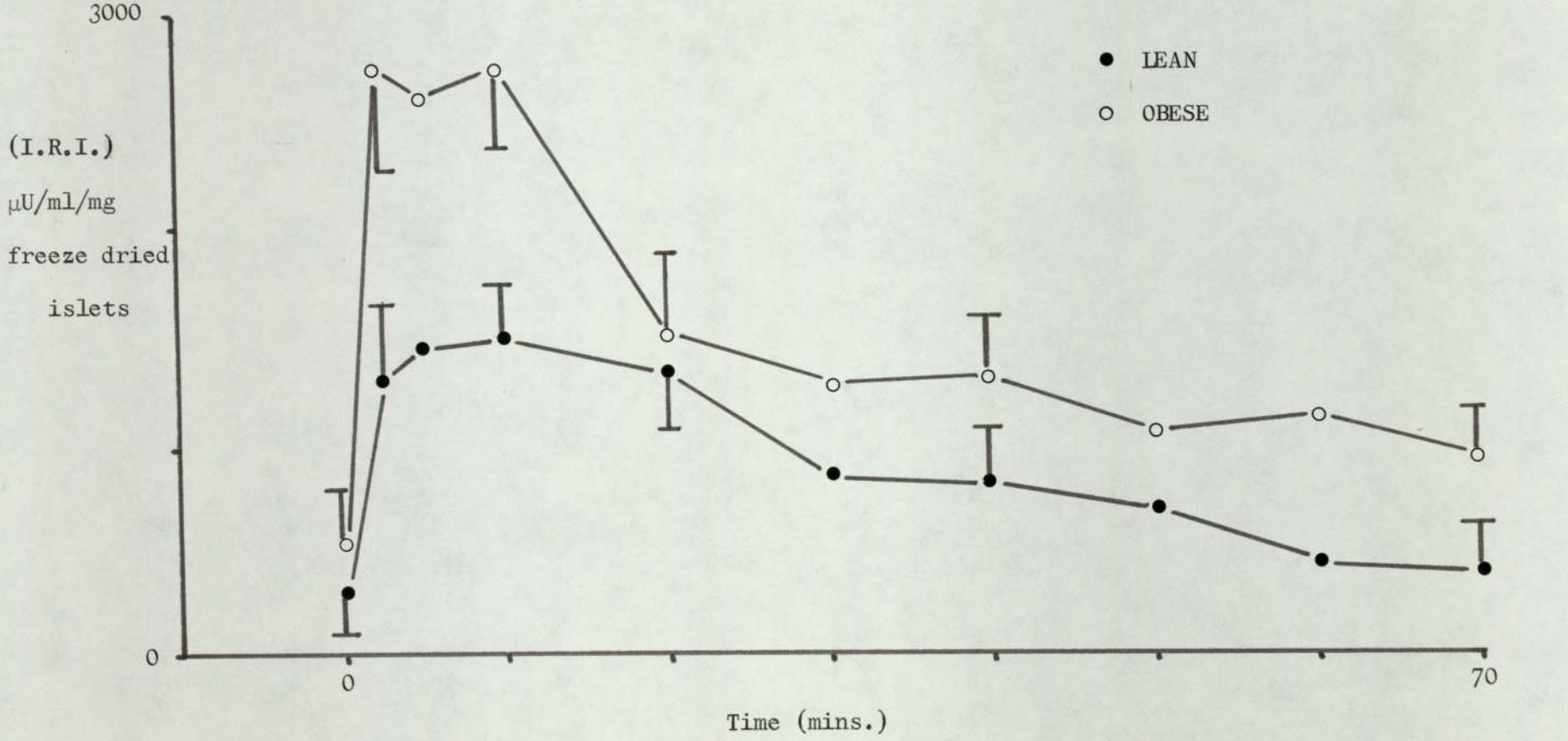


Figure 18 The effect of Crude Secretin (1.5 clin.U/ml) + 18 mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse.)

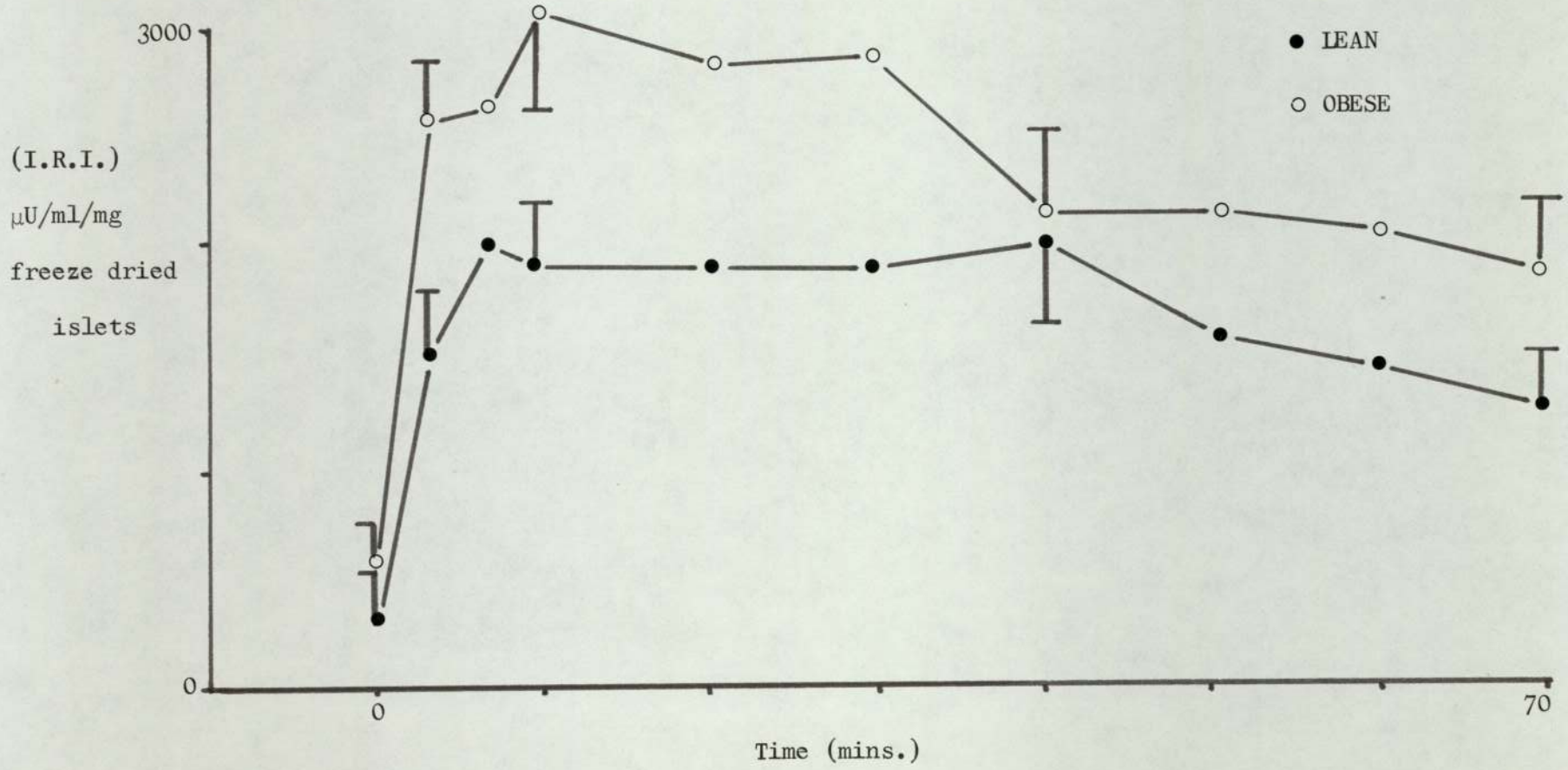


Figure 19 The effect of Crude Pancreozymin (1.5 Ivy Dog U/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

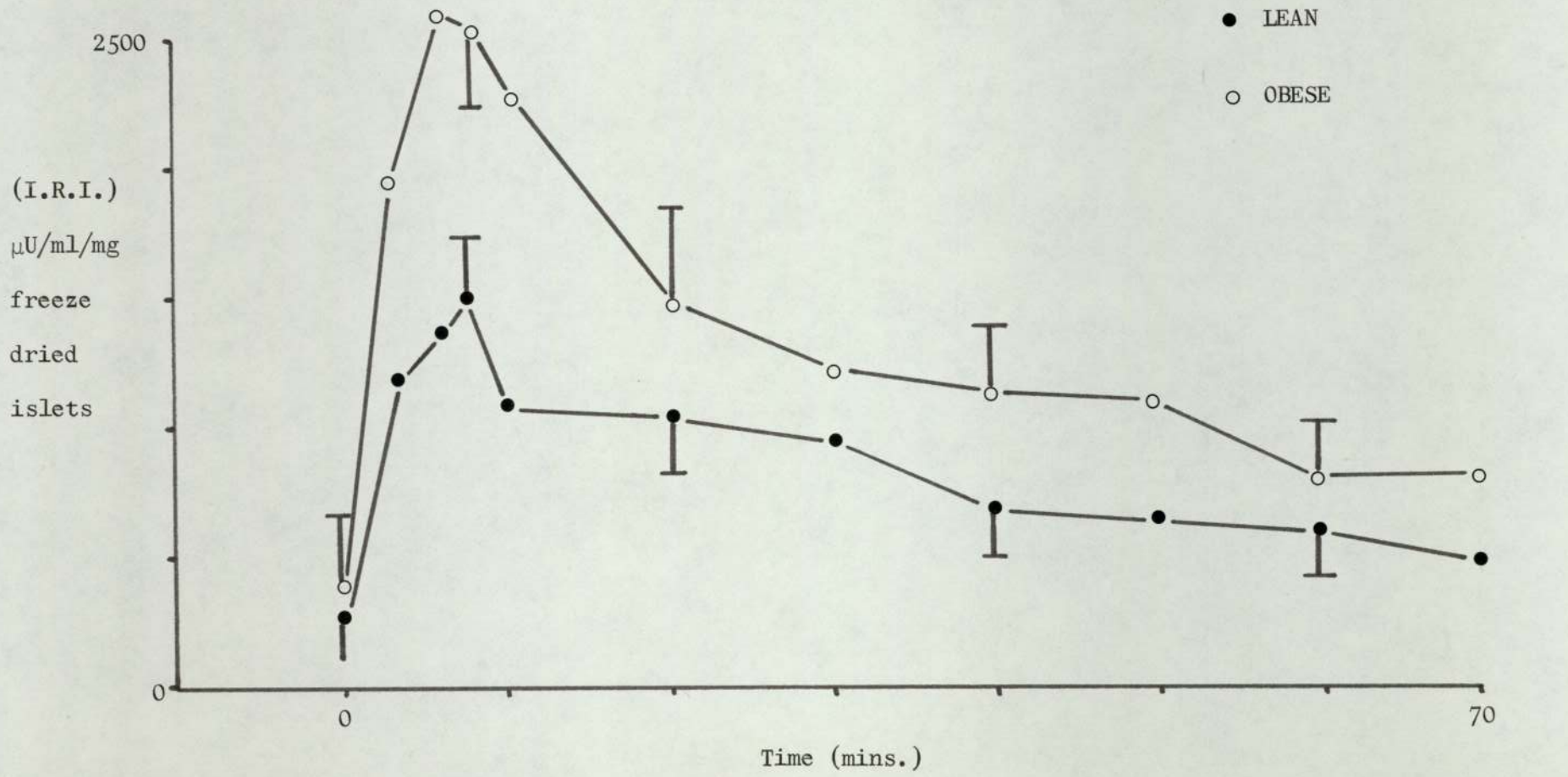


Figure 20 The effect of Crude Pancreozymin (1.5 Ivy Dog U/ml) + 9mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

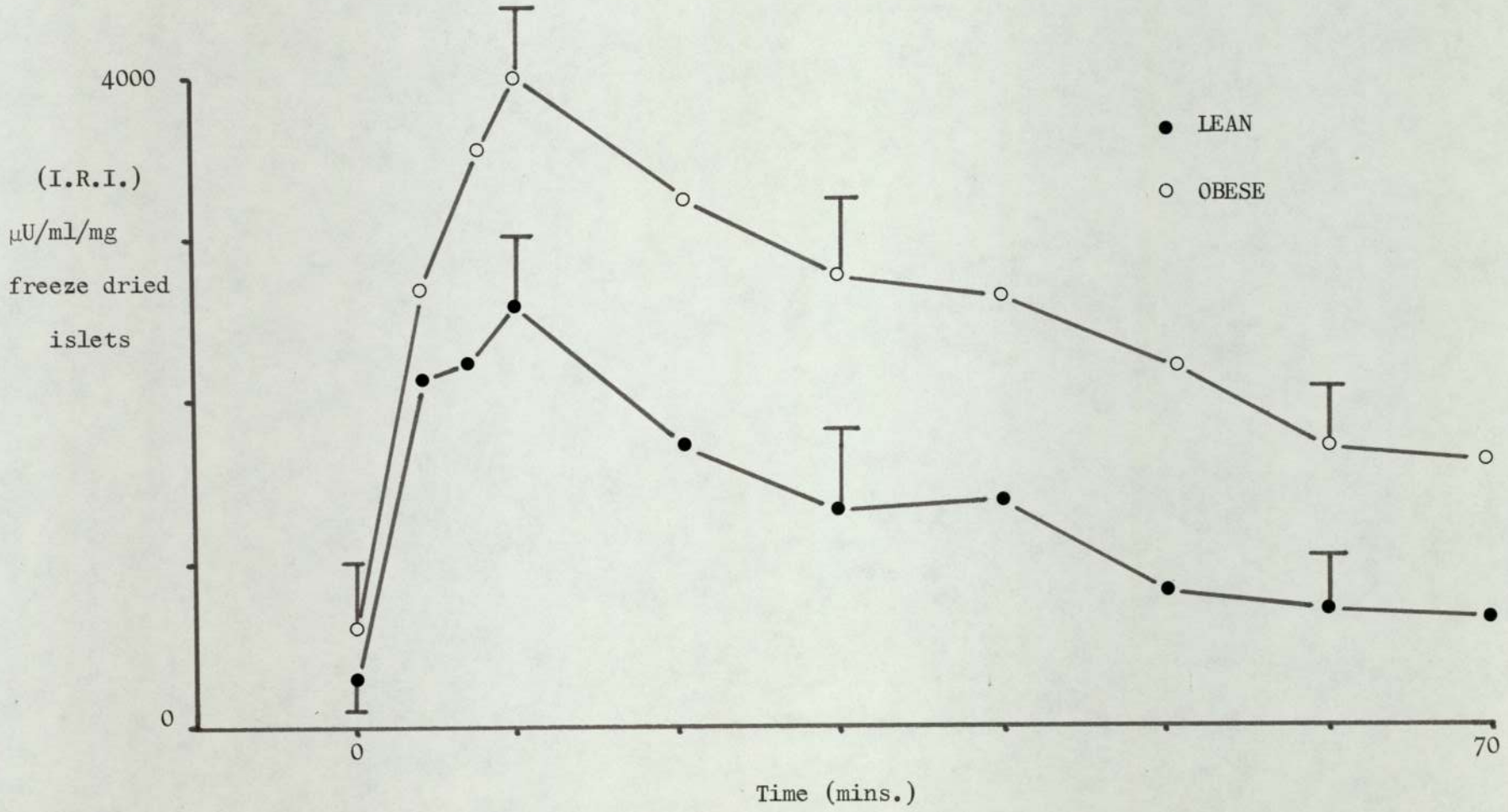


Figure 21 The effect of Crude Pancreozymin (1.5 Ivy Dog U/ml) + 18mM Glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

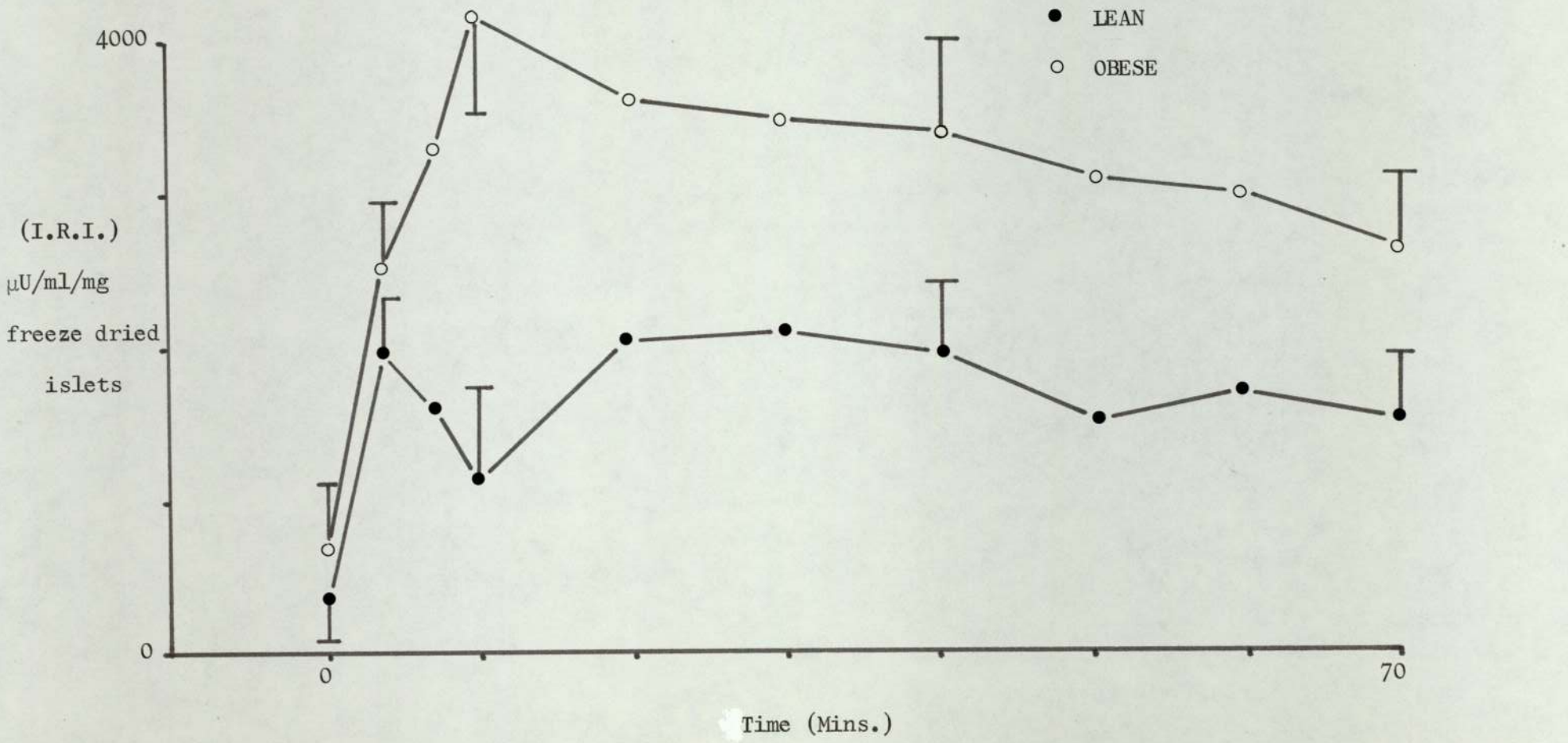


Figure 22 The effect of Purified Secretin (0.2 clin.U/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

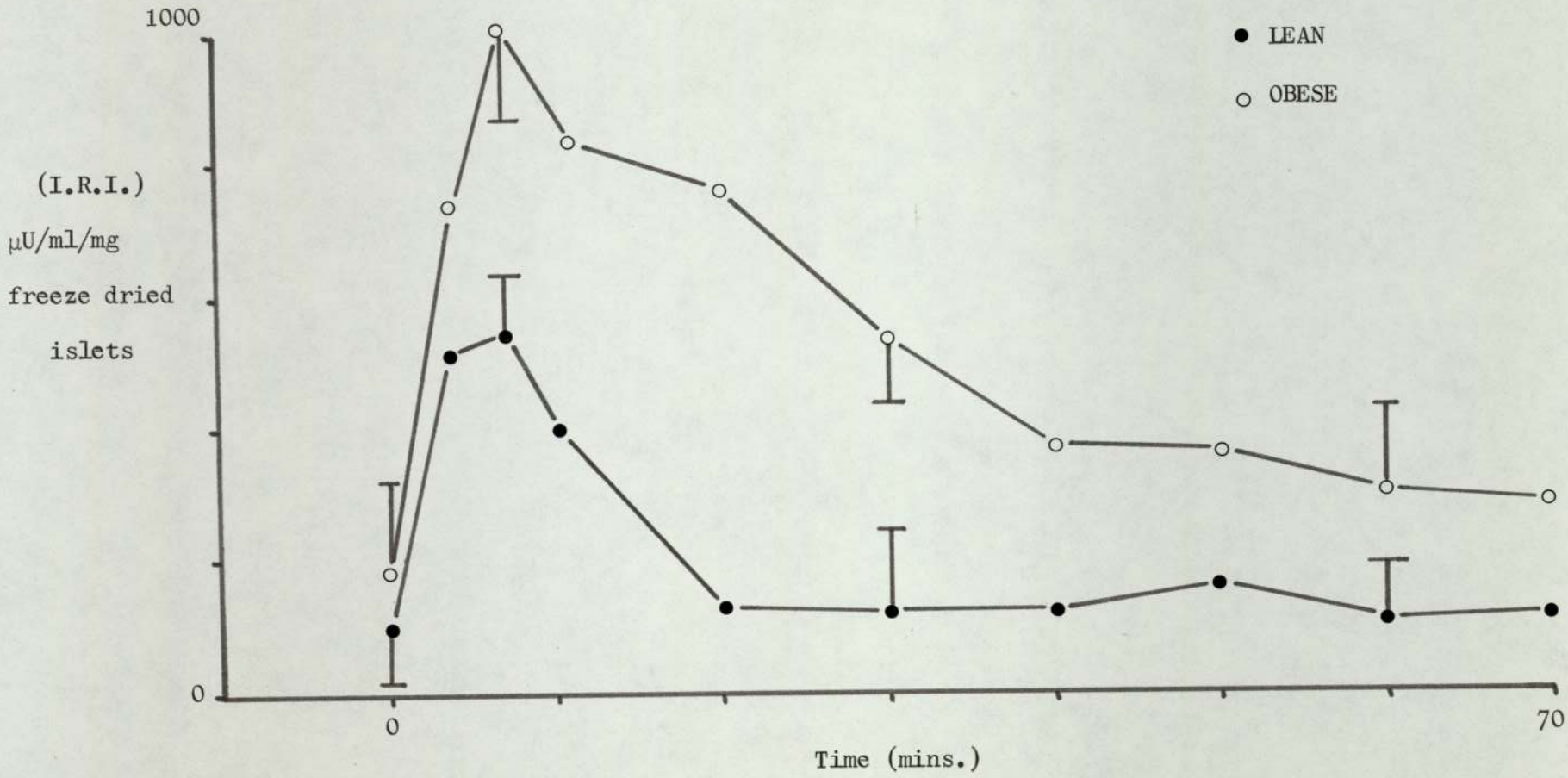


Figure 23 The effects of Purified Secretin (0.2 clin. U./ml) + 18mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

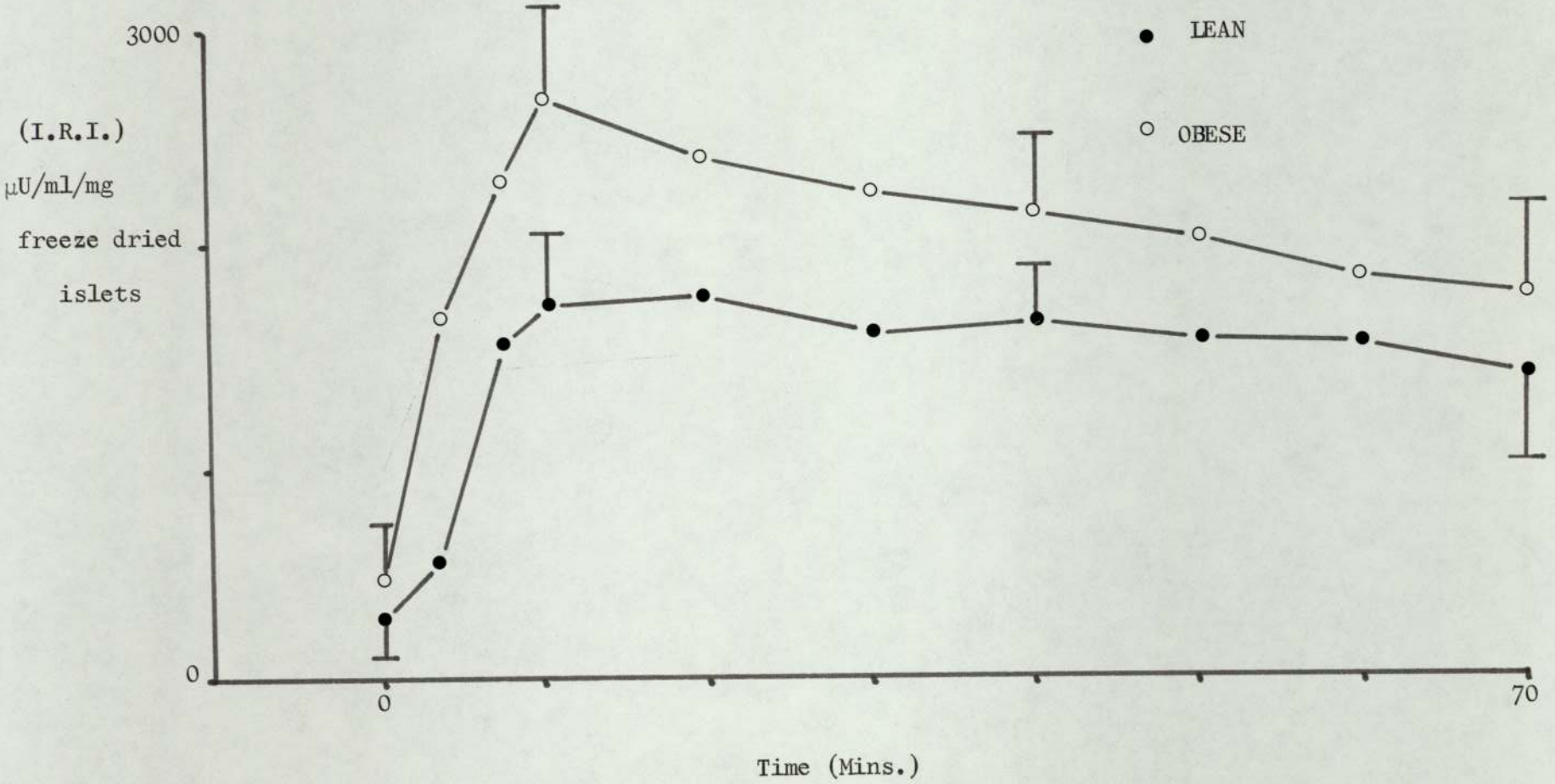


Figure 24 The effect of Purified Pancreozymin (1.5 Ivy Dog U/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

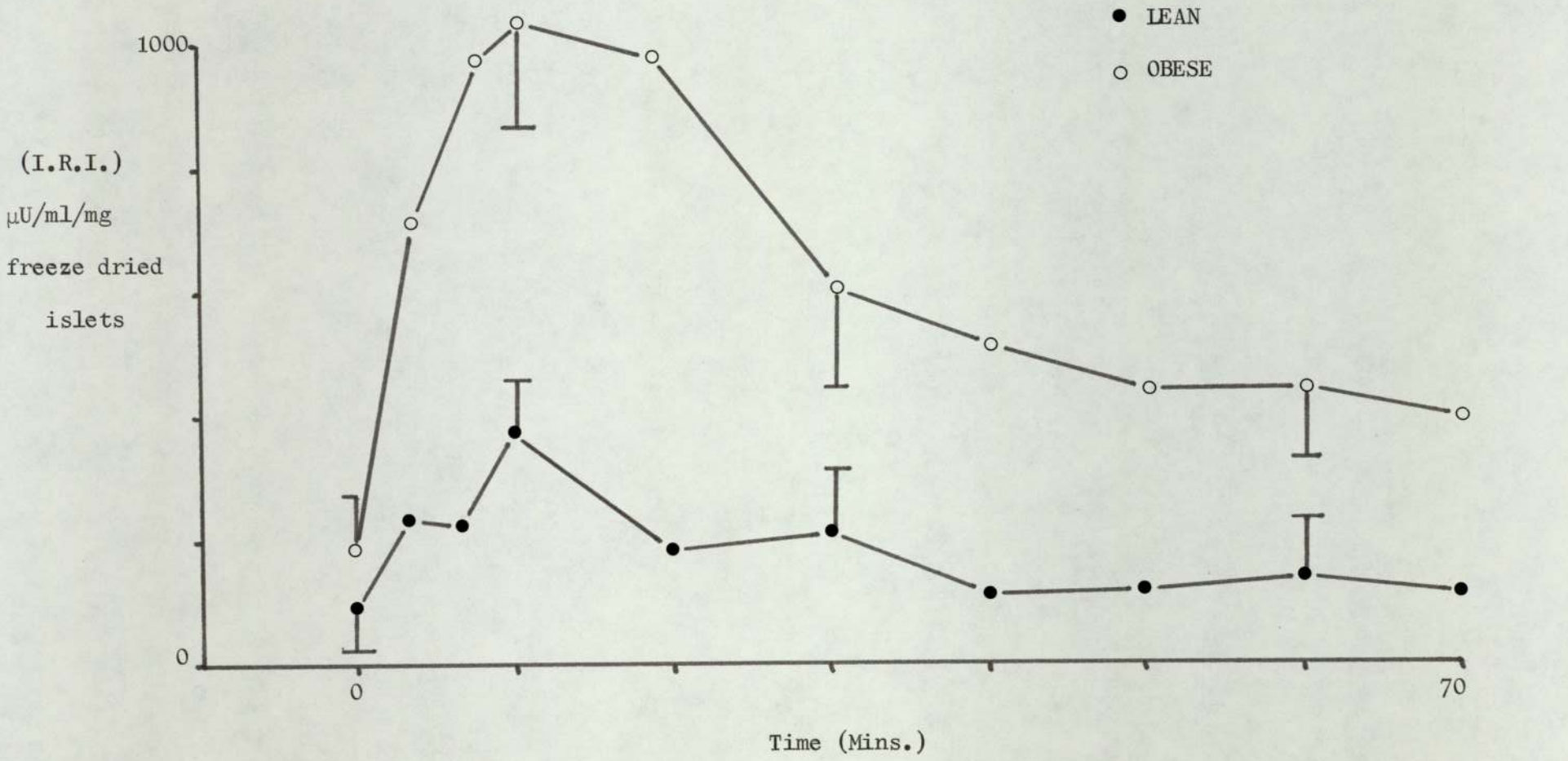


Figure 25 The effect of Purified Pancreozymin (1.5 Ivy Dog U/ml) + 18mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

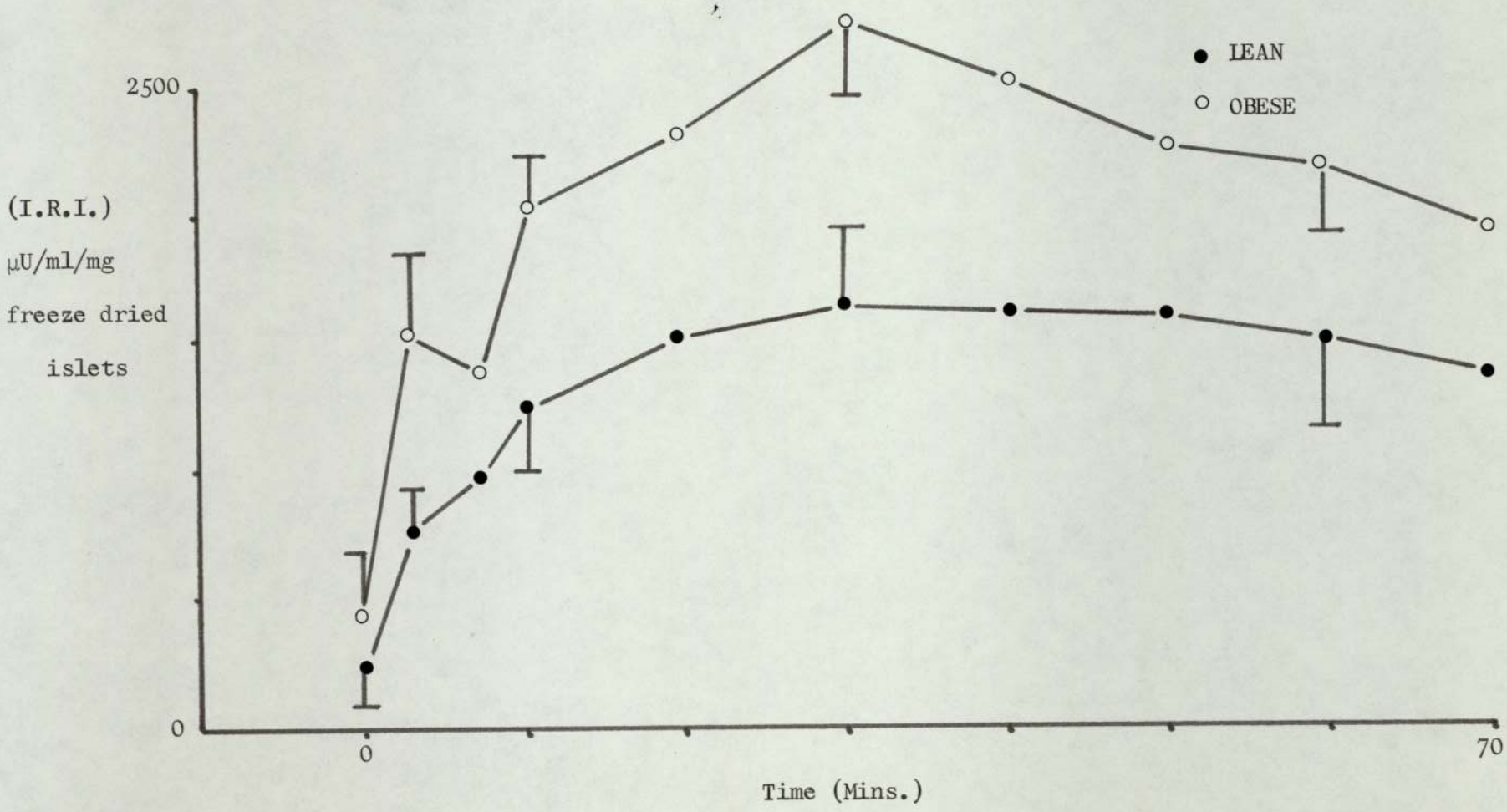


Figure 26 The effect of Pancreatic Glucagon (5.0 μg/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

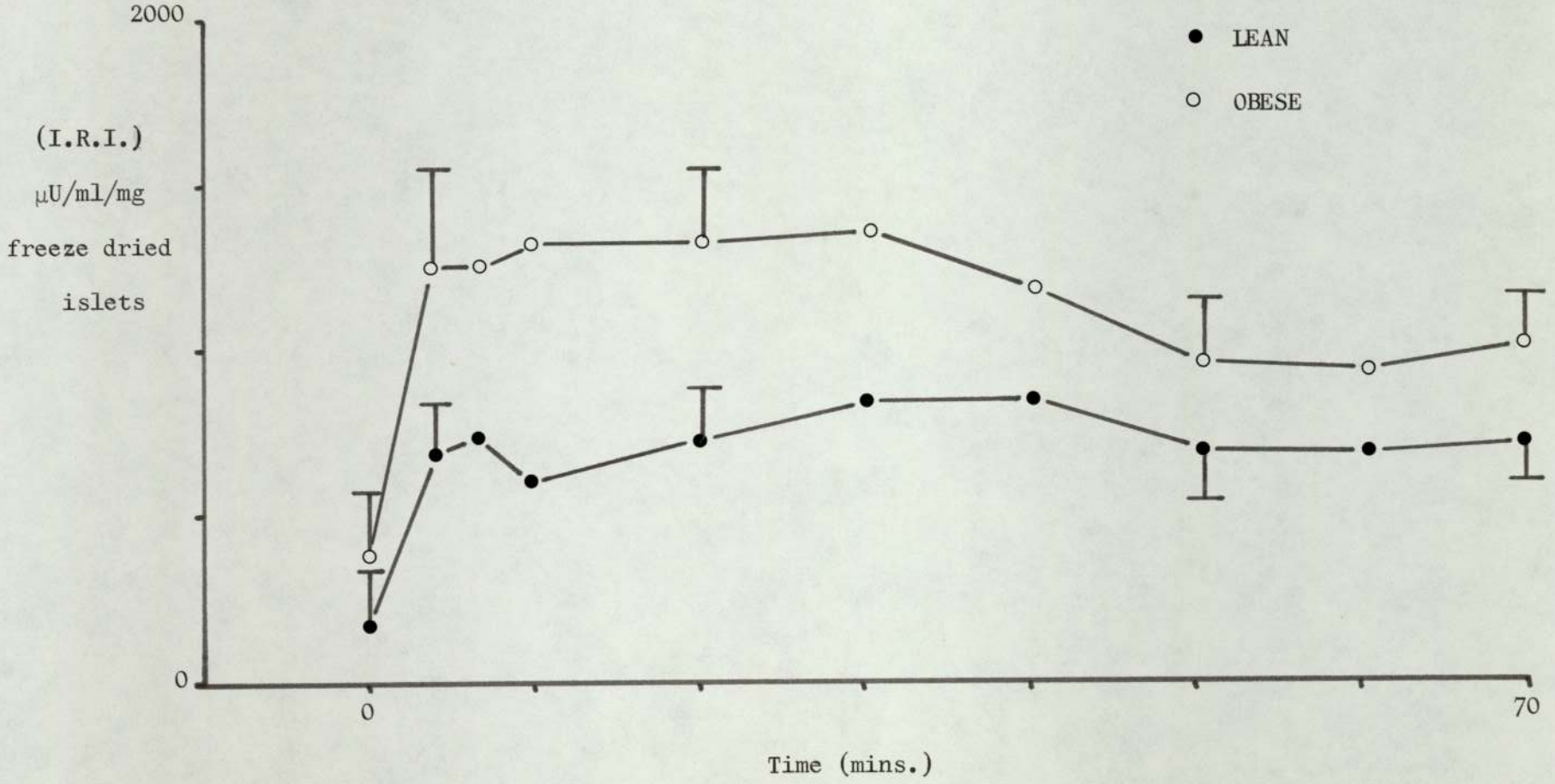


Figure 27 The effects of Pancreatic Glucagon (5.0 μg/ml) + 9mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

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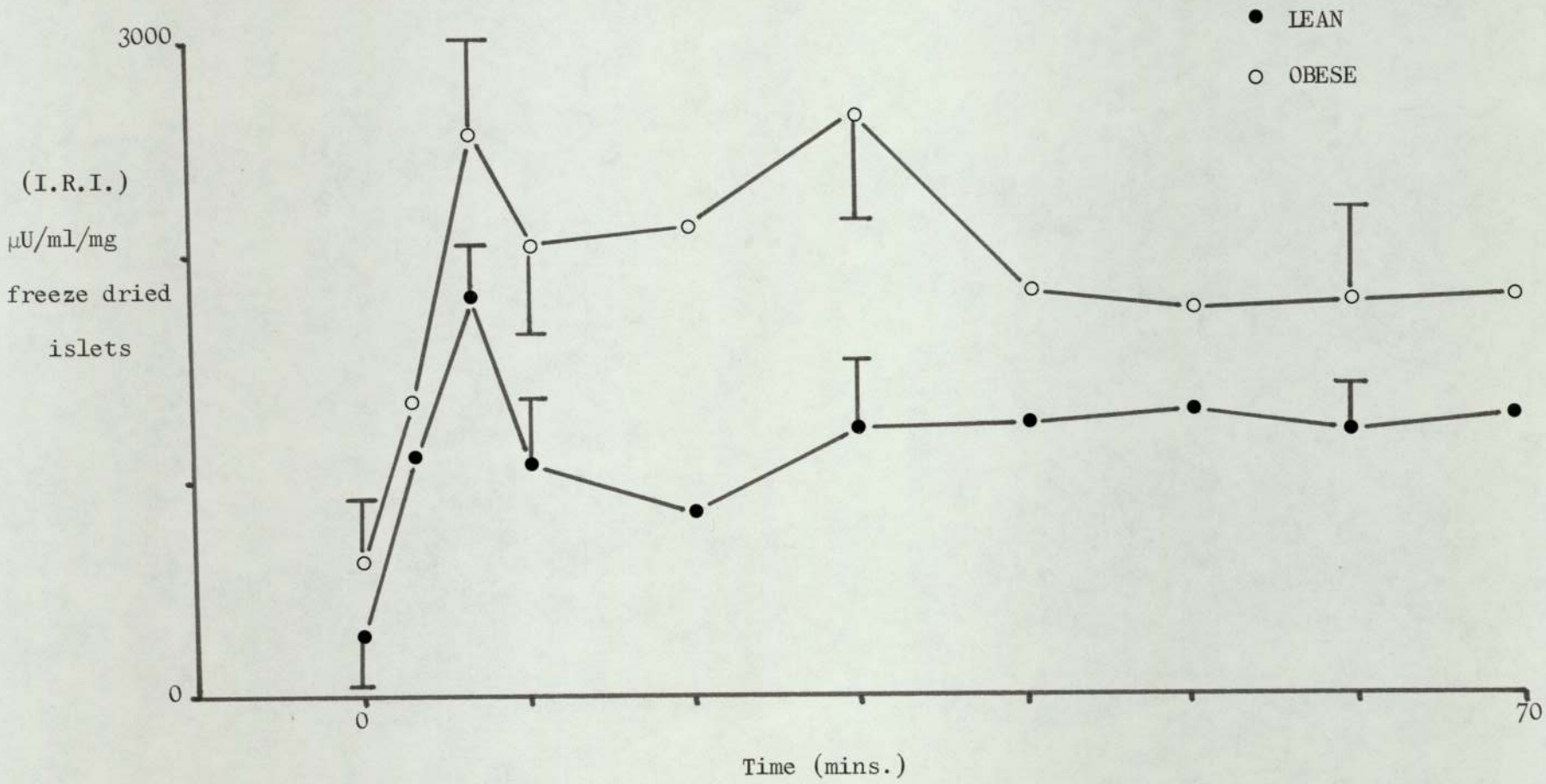


Figure 28 The effect of pancreatic glucagon (5.0 μg/ml) + 18mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

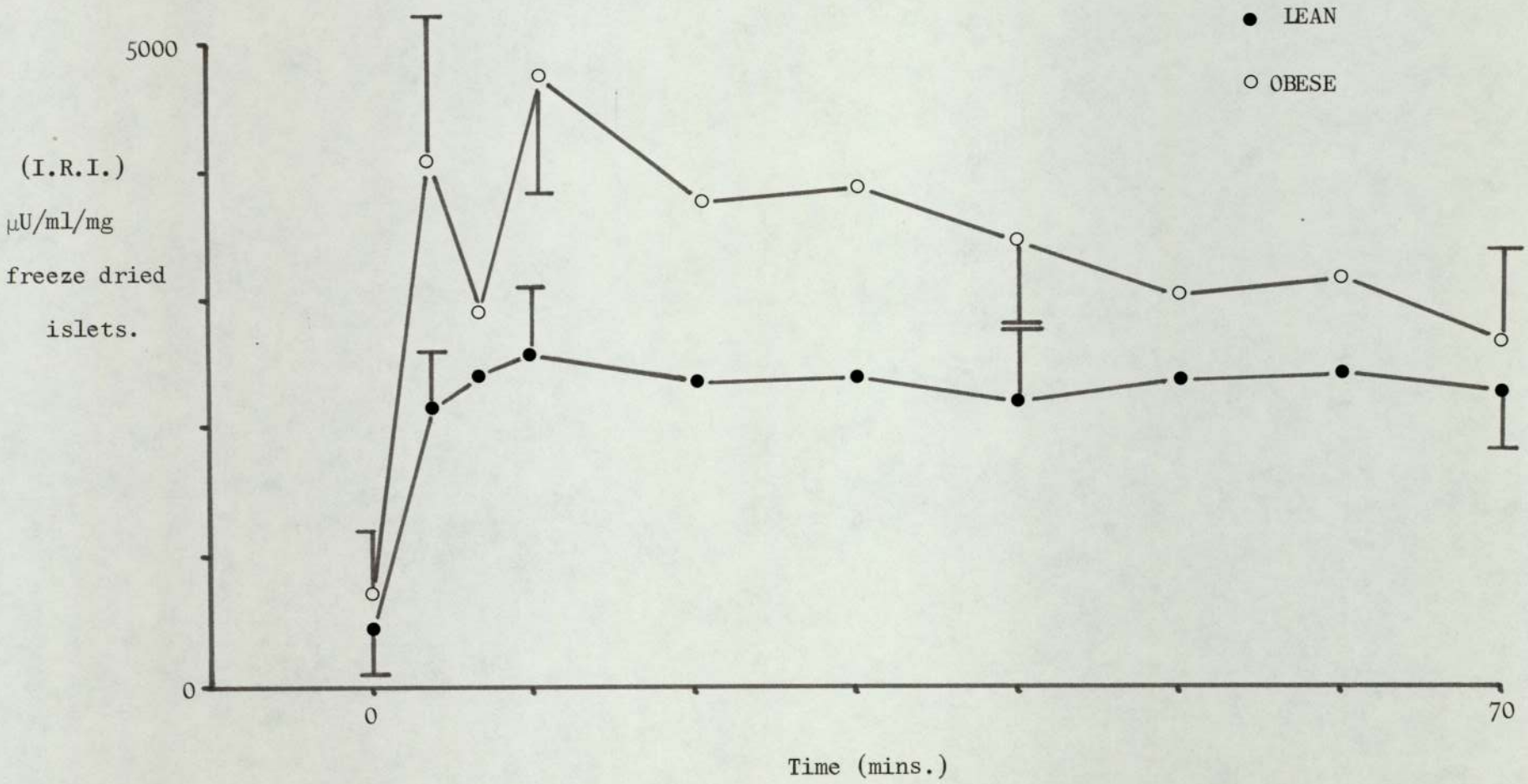


Figure 29 The effect of Gastrin Pentapeptide (2.0 μg/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

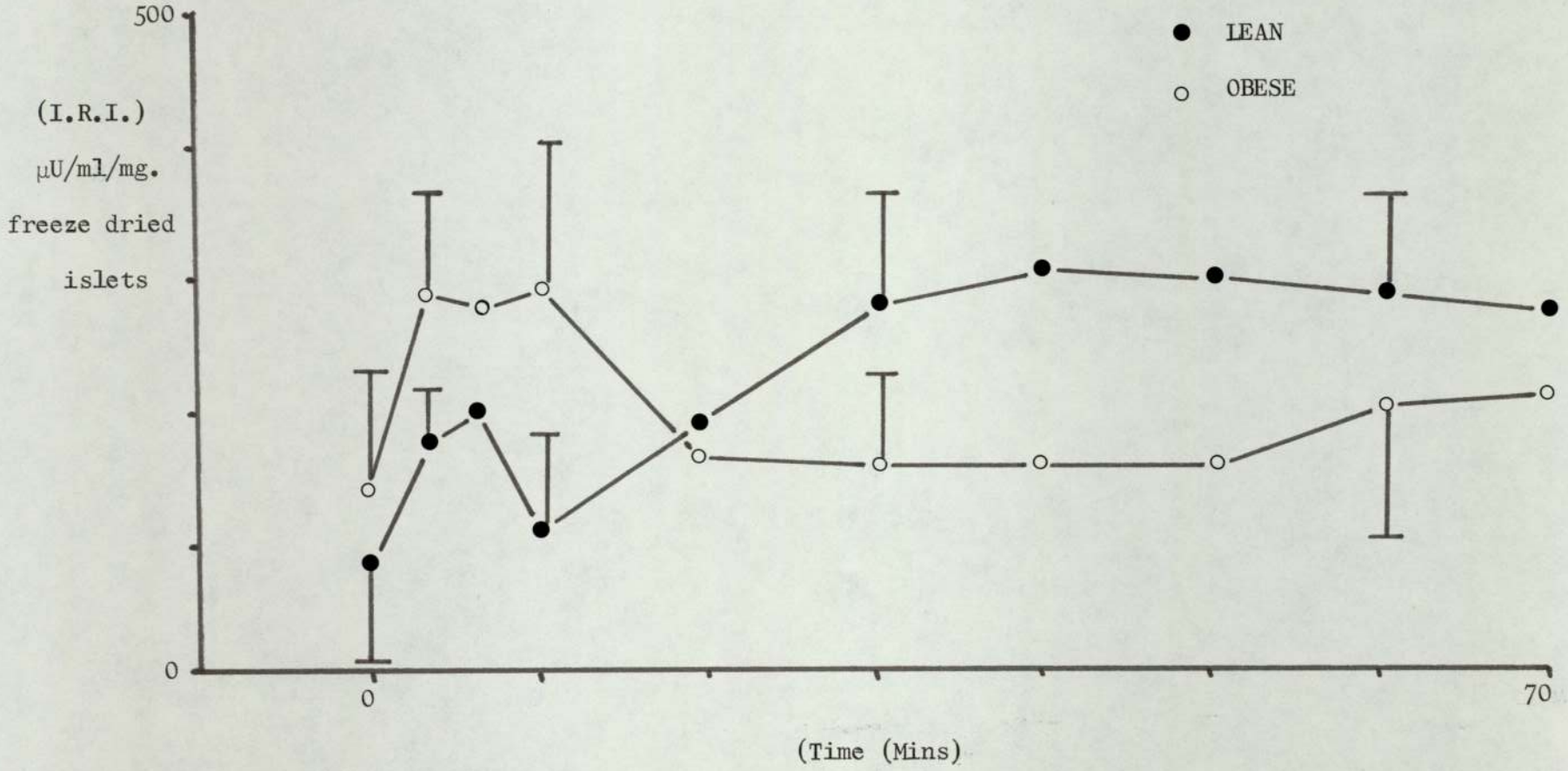


Figure 30 The effect of Gastrin Pentapeptide (2.0 μg/ml) + 18mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

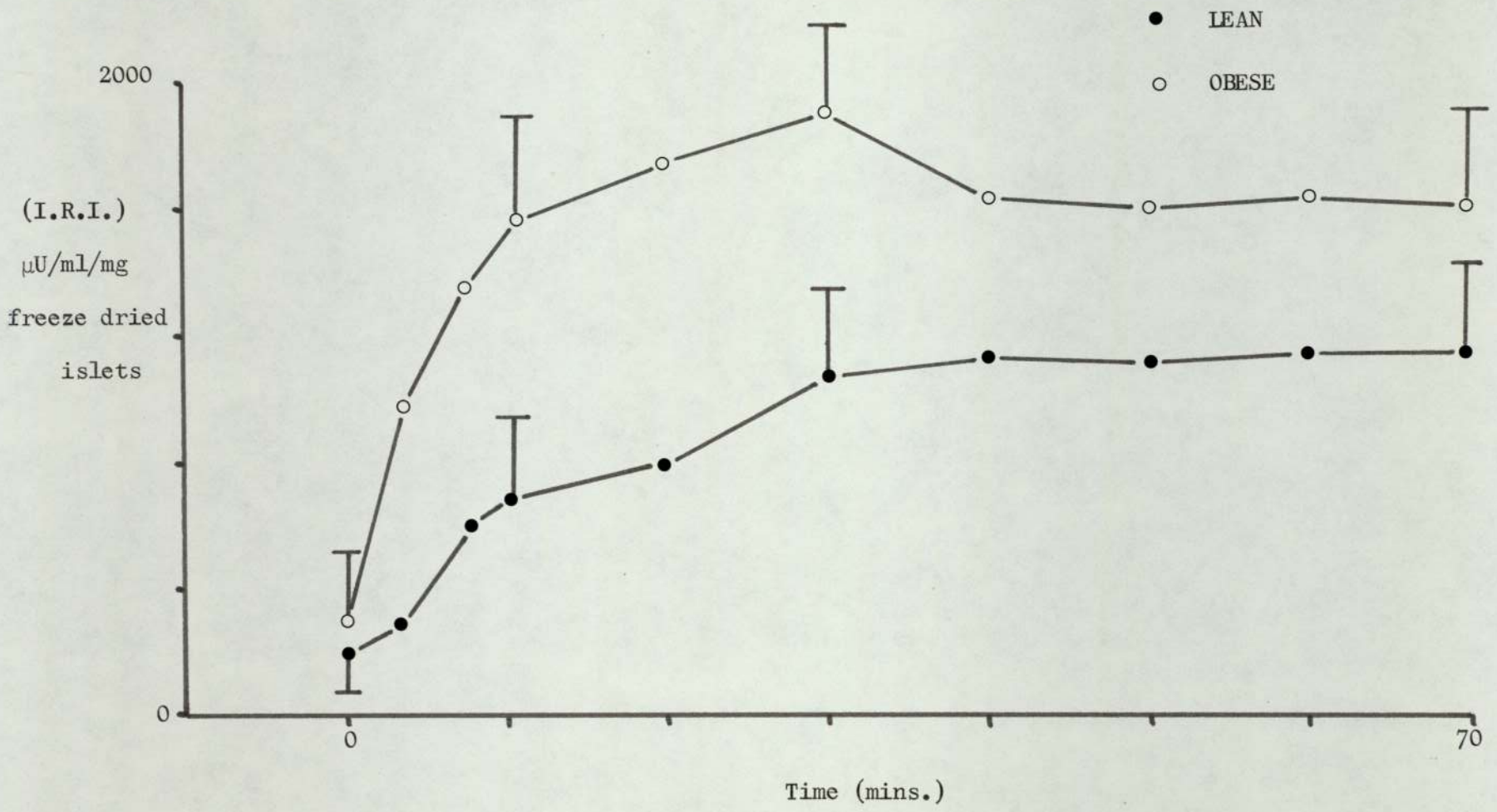


Figure 31 The effect of Synthetic Human Gastrin I (1.0μg/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

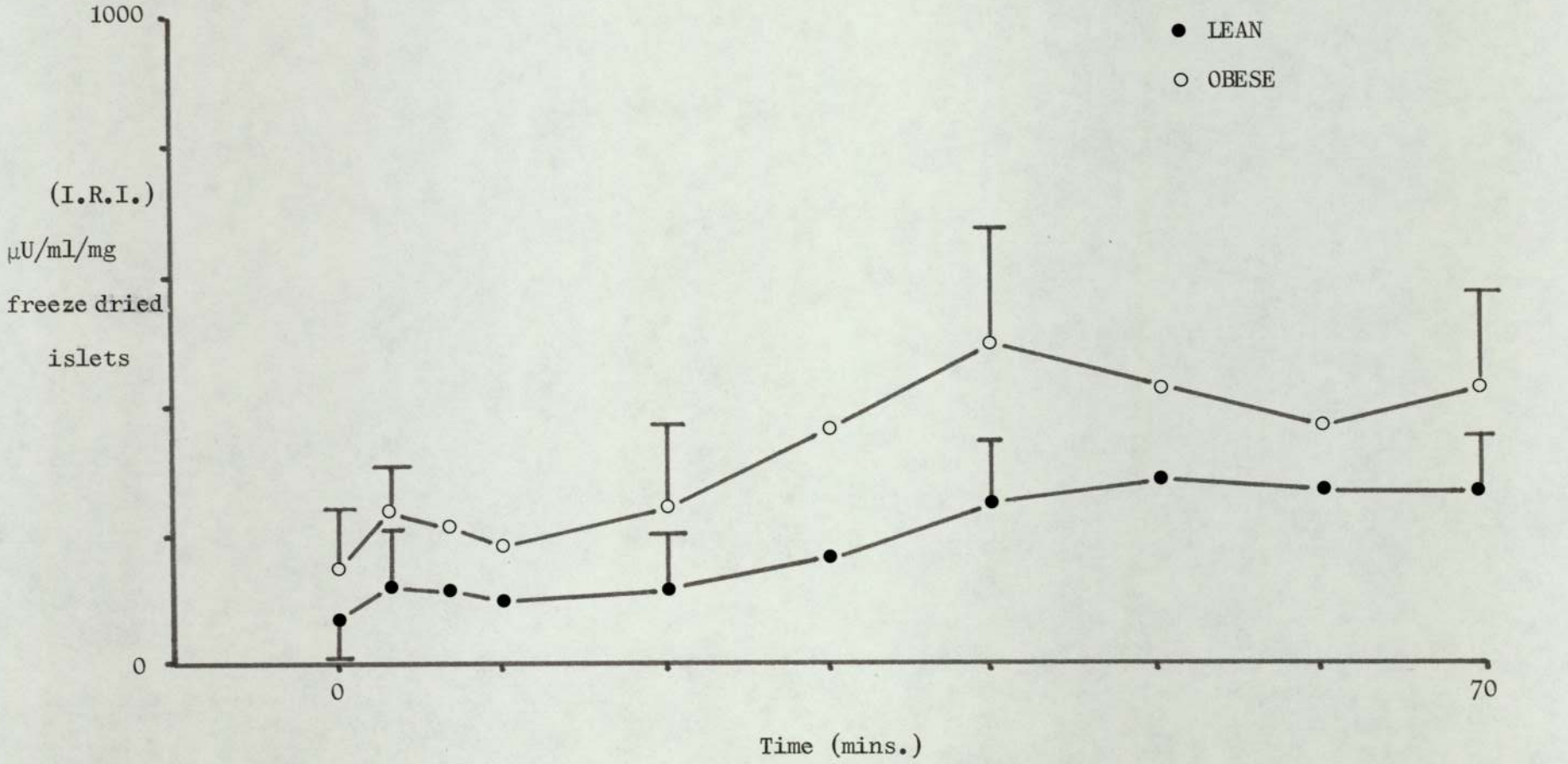


Figure 32 The effect of Synthetic Human Gastrin I (1.0 μg/ml) + 18 mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice

(Each profile illustrates the mean of 5 separate profiles ± s.e.m. One profile per mouse)

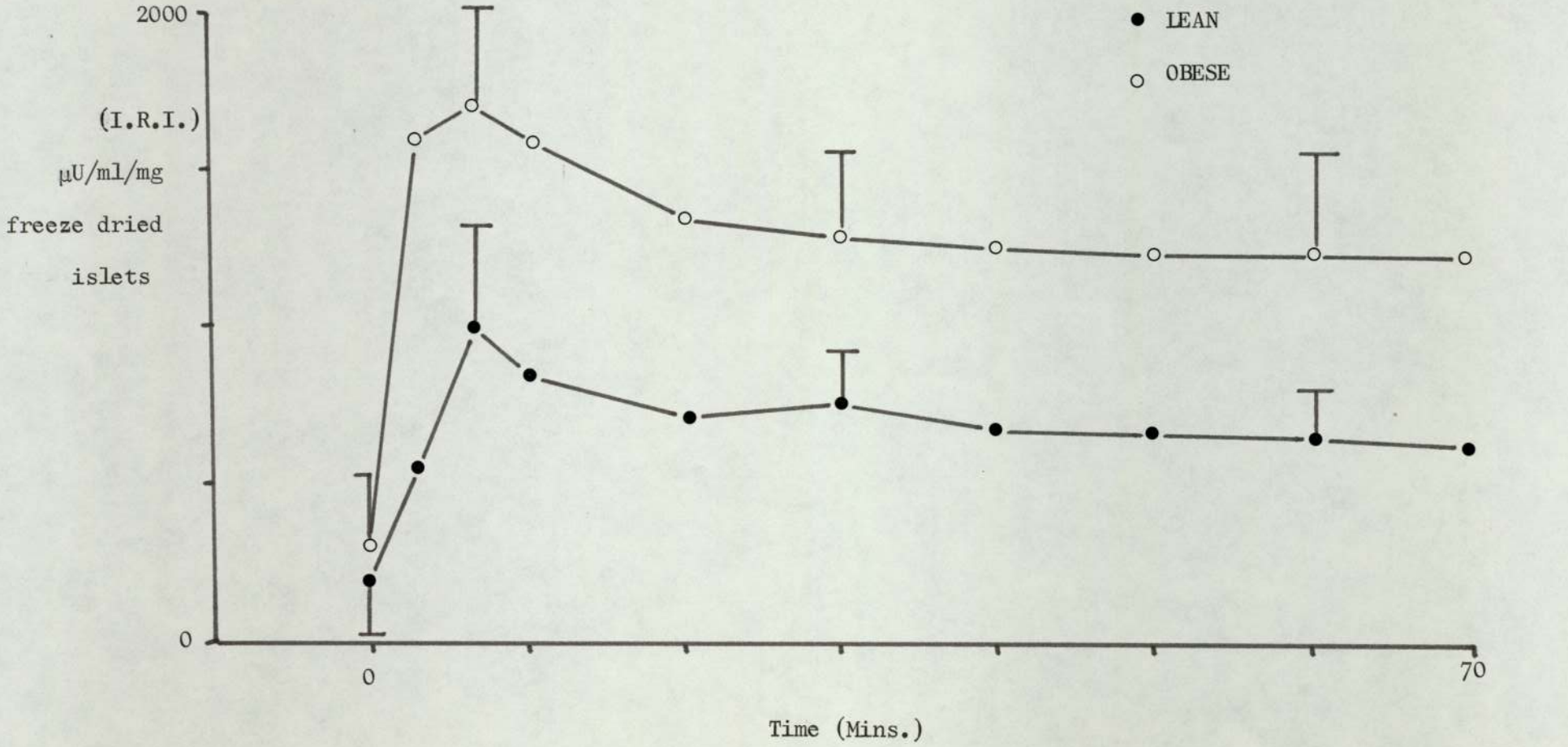


Figure 33 The effect of lean mouse intestinal extract ('T.O.T.' ; 100μg/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 4 separate profiles ± s.e.m., one profile per mouse)

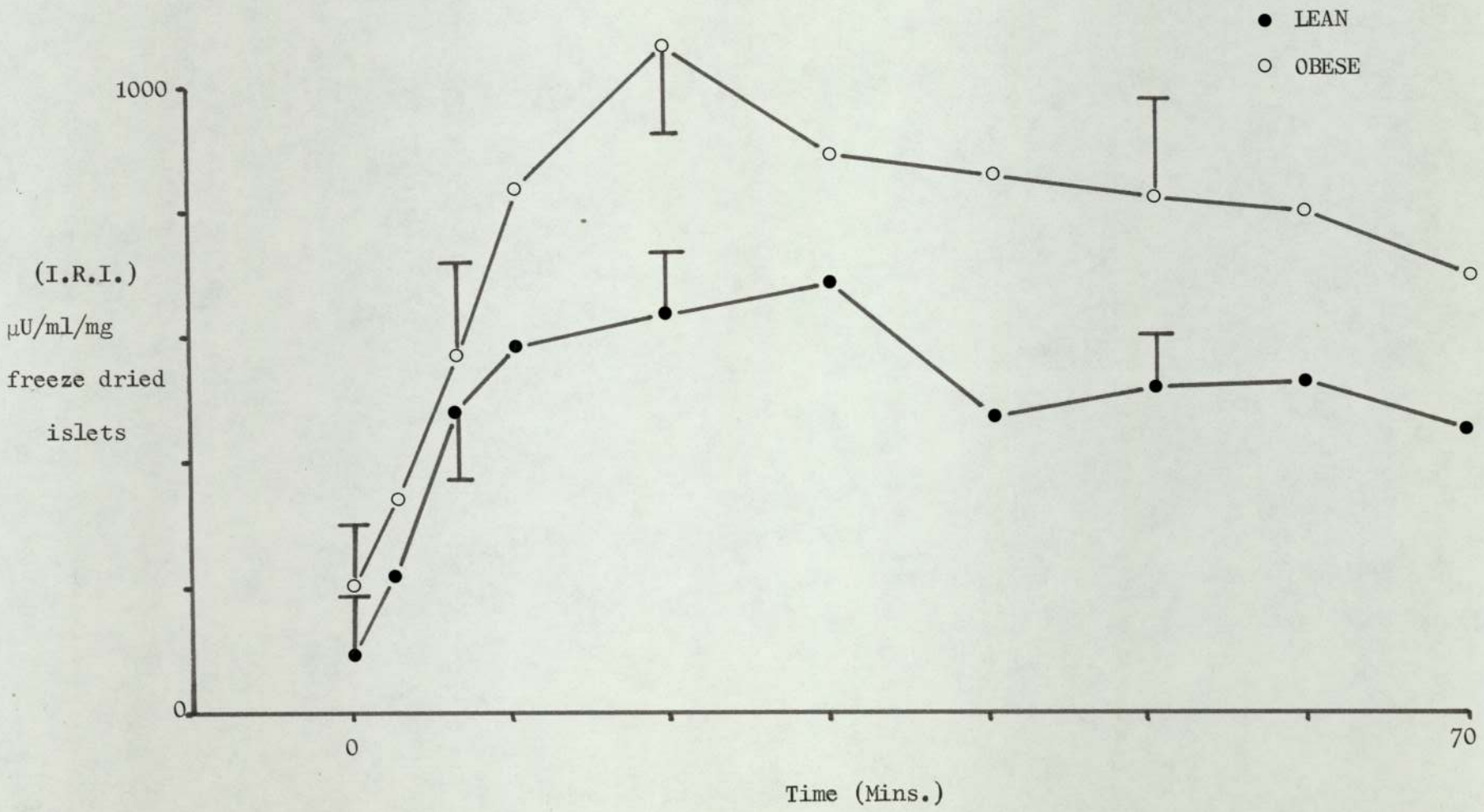


Figure 34 The effect of obese mouse intestinal extract ('T.O.T.'; 100μg/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

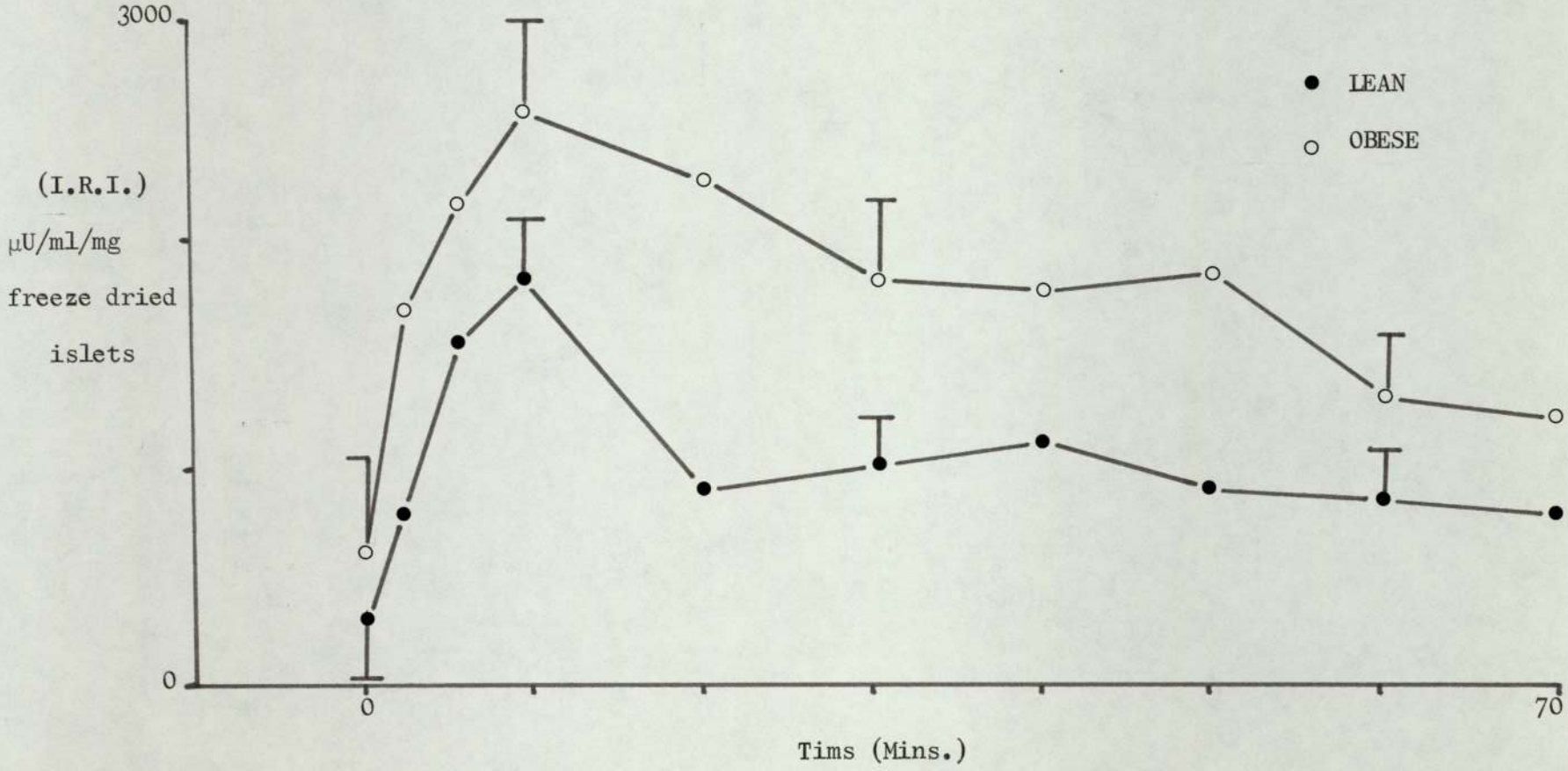


Figure 35 The effect of lean mouse intestinal extract ('I.R.G.' ; 100 μg/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m., one profile per mouse)

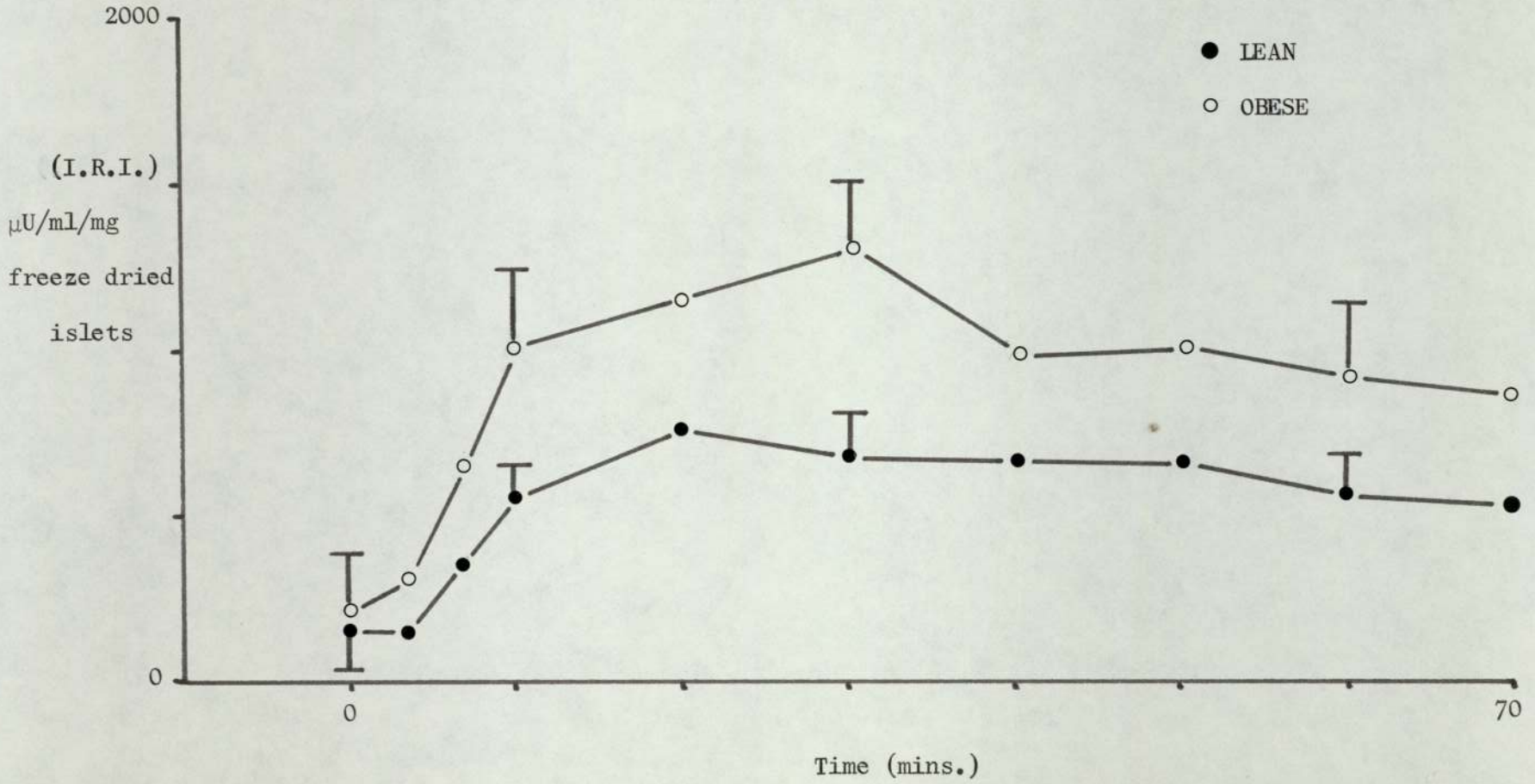
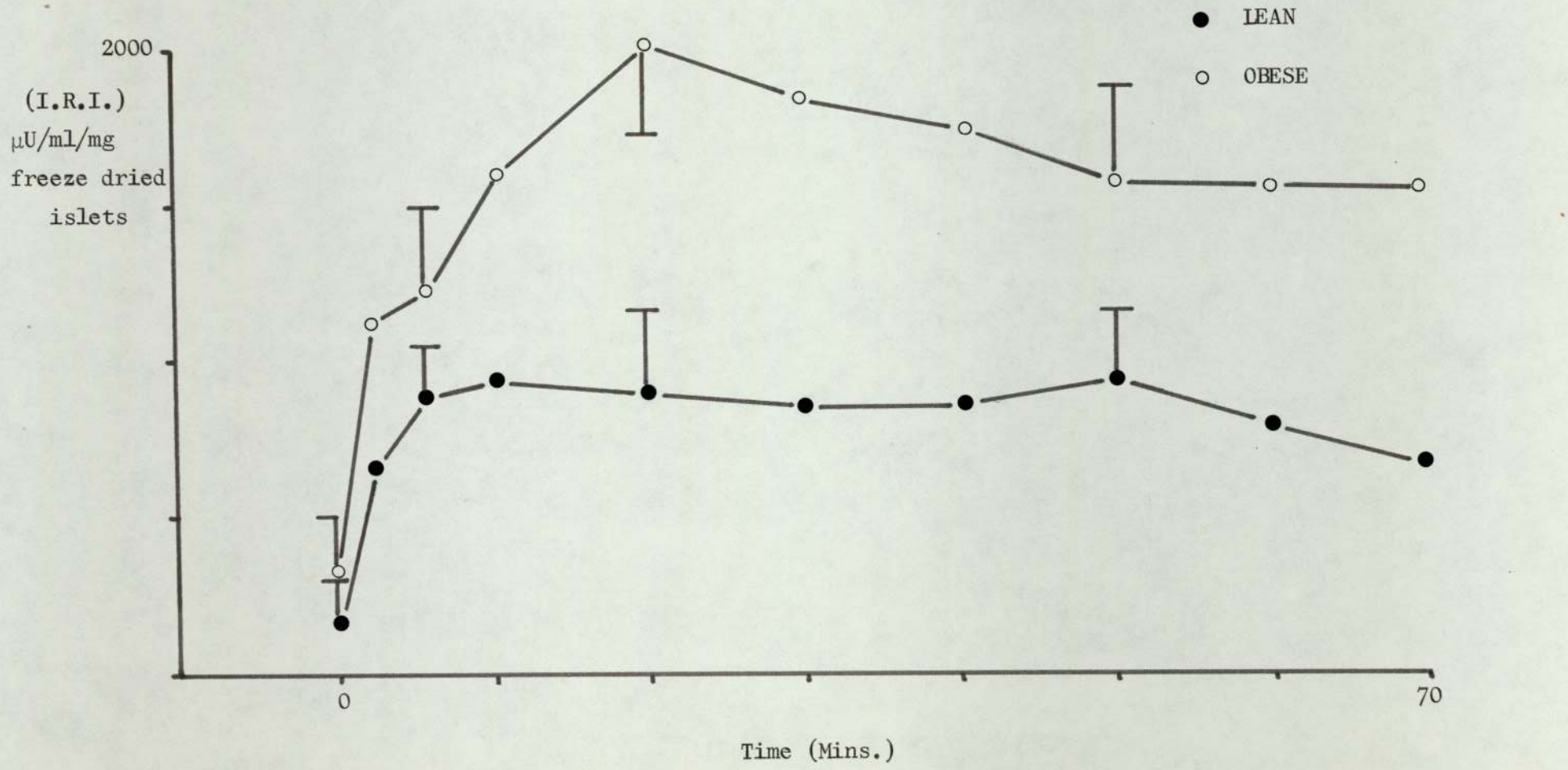


Figure 36 The effect of obese mouse intestinal extract ('I.R.G.' ; 100μg/ml) + 3mM glucose on insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile illustrates the mean of 5 separate profiles ± s.e.m. One profile per mouse)



extracts possessed a significantly higher ($p<0.05$) insulinotropic activity than those prepared from lean mouse intestine.

Discussion

The perifusion system used in the present studies was found to be suitable for monitoring the insulin secretory profiles of isolated mouse islets for at least 60 minutes. High concentrations of glucose (25mM) stimulated the rate of insulin secretion from both lean and obese mouse islets, although only obese mouse islets responded to 18mM glucose. The relatively low sensitivity of mouse islets to glucose has previously been observed by Beloff-Chain *et al* (213). Idahl (323) has proposed that the absence of metabolic fuels in the perifusion medium might blunt the insulin response of perifused lean mouse islets to glucose. Idahl found that a complete tissue culture medium, whilst having no significant effect *per se* on insulin secretion, restored the stimulatory effect of glucose.

Investigation of insulin secretion dynamics in response to glucose, using both perfused and perifused rat pancreatic tissue, has revealed that glucose-induced insulin secretion occurs as a biphasic process (305, 314, 321). To explain this phenomenon, it has been suggested that insulin might exist in two or perhaps more functional pools within the β-cell (305, 306). The first rapid, short-lived phase of insulin secretion was thought to involve the release of hormone from a small, labile pool of pre-formed insulin. The second phase could represent the mobilisation and release of insulin from a larger storage pool, or possibly the *de novo* synthesis of insulin. It has been suggested that the short refractory period between the two phases might be a physical phenomenon resulting from the time lag that occurs before the initiation

of the second phase of insulin secretion.

In the present studies, the biphasic insulin release pattern following glucose stimulation was not consistently observed with perifused lean or obese mouse islets. Hellman and colleagues (327) have reported a monophasic insulin release profile by perifused mouse islets in response to glucose. Davis and Lazarus (328) have suggested that the β-cell glucoreceptor (a hypothetical receptor in the β-cell membrane by which glucose is thought to initiate the first phase of insulin secretion; 328, 329) might be less evident in mice, and this might contribute towards the low sensitivity of mouse islets to glucose and also to the occasional absence of the first phase of insulin secretion.

In order to observe quantitatively minute changes in islet secretory activity, it was found desirable to minimise the volume of the perifusion chamber in order to reduce mixing of secreted insulin. In the present experiments, batches of 15 islets were incubated in Millipore filter chambers of internal volume 0.95 ml. This number of islets did not interfere with the flow rate.

Secretin and pancreozymin both produced an immediate, significant insulin secretory response in the presence of high and low concentrations of glucose. These observations were consonant with the work of Iverson (82) and Pfeiffer et al (78) who demonstrated the insulinotropic activities of secretin and pancreozymin using perfused pancreas of dog (82) and rat (78). It was of interest to note that purified secretin and pancreozymin in the presence of 3mM glucose were significantly less effective insulin secretagogues than the crude hormone preparations. Similar results have been obtained by Rabinovitch and Dupre (124) and by Hedner (330). A polypeptide impurity has recently been isolated from crude pancreozymin preparations, and designated G.I.P. (Gastric Inhibitory

Polypeptide). G.I.P. has been shown to be capable of stimulating insulin secretion both *in vitro* (134) and *in vivo* (135), and might therefore contribute towards the insulinotropic effect of a crude pancreozymin preparation.

In the presence of 3mM glucose, secretin and pancreozymin elicited only a transient stimulation of insulin secretion. An early, uniphasic response to secretin has been previously demonstrated *in vivo* (331) and it has been implied that this gastrointestinal hormone might stimulate the release of insulin from a small, labile pool (331, 332). Pancreatic glucagon was also found to initiate an immediate significant stimulation of insulin secretion. This supports the work of previous investigators using both perfused and perifused rat pancreas preparations (318, 333).

The present data show glucagon-stimulated insulin secretion to be significantly more prolonged than that produced by secretin or pancreozymin. Bennett and Grodsky (334) have demonstrated that glucagon appeared to exert its major influence on the second phase of insulin secretion. This effect was not abolished by the presence of puromycin, suggesting that glucagon might stimulate the release of pre-formed insulin, and not necessarily the *de novo* synthesis of insulin (96). Glucagon has been shown to elevate intracellular levels of adenosine 3', 5' cyclic monophosphate (cyclic AMP) by activating membrane bound adenylate cyclase in many tissues including pancreatic islets (328, 335, 336, 337, 338) and there is evidence that the action of glucagon on insulin secretion might be mediated via this cyclic nucleotide.

The insulin response to gastrin pentapeptide (the biologically active C-terminal portion of gastrin) and synthetic human gastrin I in the presence of 3 and 18 mM glucose were not easy to explain.

Iverson (82) showed that synthetic human gastrin I was capable of producing a large, instantaneous secretion of insulin from the perfused canine pancreas, but Hinz and colleagues (68) were unable to demonstrate a stimulatory effect of gastrin pentapeptide on insulin release from perifused rat islets. It is possible that the insulinotropic effects of gastrin are only of pharmacological importance.

Both gastrin and pancreozymin have been shown to be capable of stimulating the release of pancreatic glucagon from the perfused canine pancreas (82) raising the possibility that gastrin and pancreozymin might influence insulin secretion via their effect on glucagon release.

Intestinal extracts ('T.O.T.') from lean and obese mice elicited insulin secretory responses from mouse islets that were very similar to the profiles observed for gastrointestinal hormone preparations. These data indicate that the duodenal-jejunal extracts of lean and obese mice contain one or more factors capable of stimulating the secretion of insulin *in vitro*. Investigations by Moody *et al* (39) using an extract of porcine intestine revealed that the fractions most active in promoting insulin release were devoid of secretin and contained very little pancreozymin-like activity. It therefore seemed unlikely that these two hormones were responsible for the insulinotropic activity of the extracts used in the present study. Moody fractionated his porcine intestinal extract and suggested that the insulin-releasing activity might be associated with enteric glucagon-like immunoreactivity (G.L.I.).

In the present studies, crude intestinal 'I.R.G.' preparations were shown to be capable of stimulating insulin release from lean and obese mouse islets. The insulin secretory response to I.R.G. was found to resemble that produced by pancreatic glucagon. The immunological similarities between pancreatic and enteric glucagon-like immunoreactivity might suggest that they have other biological properties in

common, in particular, the ability to enhance the release of insulin. Buchanan, Vance and Williams (40) have demonstrated a stimulation of insulin secretion *in vitro* in response to a crude G.L.I. - containing extract of rat intestinal mucosa. It must be emphasised, however, that the mucosal extracts of Buchanan, and the extracts used in the present study were probably contaminated with gastrointestinal hormones other than enteroglucagon, which could have contributed towards the insulin secretory response observed.

The 'T.O.T.' and 'I.R.G.' extracts derived from the duodenal-jejunal mucosa of obese mice possessed a greater insulinotropic activity (per unit weight of extract) than those prepared from lean mice. It has recently been demonstrated that the gastrointestinal mucosa of the obese-hyperglycaemic mouse exhibits a hyperplasia of most of the endocrine cell types (339). Whether this phenomenon is a primary feature of the obese-hyperglycaemic syndrome in mice, or whether it is secondary to hyperphagia remains to be established, but it seems possible that hyperactive intestinal endocrine cells might aggravate the hypersensitivity of the obese mouse islet, leading to an excessive secretion of insulin.

Section B The perfusion of everted intestinal sacs in series with perifused islets of lean and obese-hyperglycaemic mice.

Introduction

Several gastrointestinal hormones, including secretin, pancreozymin, gastrin, Gastric Inhibitory Polypeptide and Insulin-releasing polypeptide have been shown to possess insulinotropic activity (161, 134). However, whilst the development of sensitive radioimmunoassay systems for these hormones (150) has permitted measurements of their circulating levels, it has not been possible to establish with any certainty which, if any, of the known gastrointestinal hormones are released in sufficient quantities after food ingestion to act as physiological potentiators of insulin secretion. There have been very few reports in the literature describing the effect of food substances on the synthesis and release of gastrointestinal hormones. However, it has been demonstrated that the presence of glucose in the duodenal lumen increases the potential insulinotropic activity of subsequently prepared intestinal mucosal extracts (340). Penhos and colleagues (341) using a rat pancreas - small intestine preparation, showed that the passage of glucose through the intestine initiated the release of a humoral substance from the mucosa which produced a marked elevation of insulin secretion when perfused *in situ* through the vascular system of the pancreas. It remains to be established, however, whether the substances responsible for this insulinotropic activity are identical to any of the known gastrointestinal hormones. Young and colleagues (155) demonstrated that the intestinal absorption of glucose was followed by an increase in the serum secretin concentration, although this has been disputed by subsequent investigators (148, 149).

In the present study, an attempt was made to establish whether the release of an insulinotropic factor from the intestinal mucosa could be demonstrated *in vitro*, using a perfused segment of *everted* mouse jejunum in series with isolated mouse islets of Langerhans incubated in perifusion chambers. The effect of varying the glucose and amino acid concentration of the external, or mucosal, fluid bathing the portion of intestine (i.e. mimicking the ingestion of carbohydrates and protein) was also investigated. The insulin secreted from the islets in response to any intestinal mucosal factors was measured by radioimmunoassay.

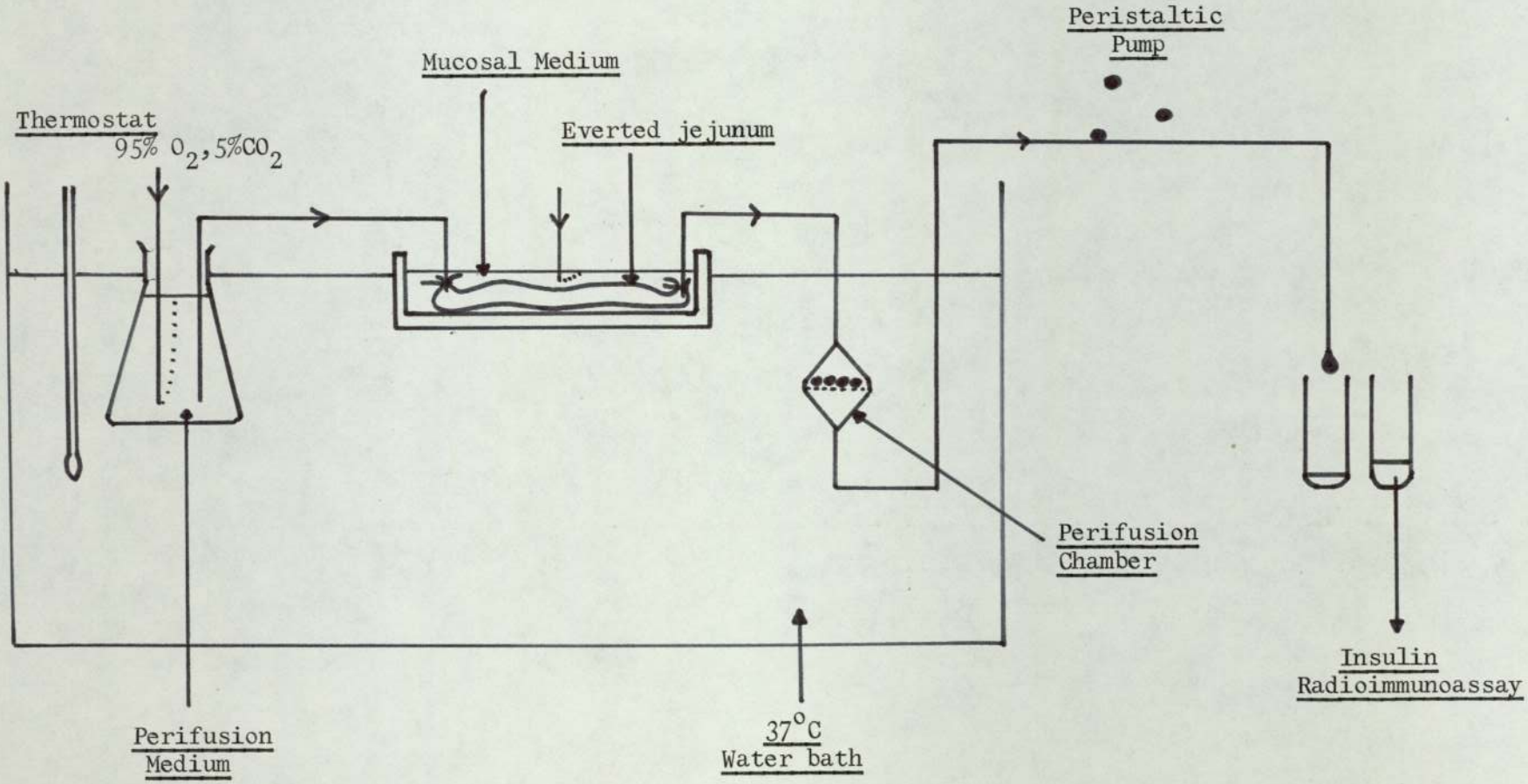
The everted, perfused jejunum preparation

The everted gut sac preparation has been used extensively for the study of intestinal physiology *in vitro*. Classically, the preparation has been used for investigations of intestinal absorption (342). The everted intestinal sac has the advantage that the absorption of substances in the normal, physiological direction (i.e. mucosal to serosal) will result in the accumulation of these substances within the sac. Furthermore, any release of gastrointestinal hormones in response to glucose and amino acids will also be into the 'lumen' or central cavity of the everted gut sac. Thus, using an everted jejunal sac perfused in series with isolated mouse islets, the insulinotropic activity of gastrointestinal factors released from the mucosa in response to glucose and amino acids can be investigated.

Materials and Methods

The perfused, everted jejunal sac - perifused islet preparation is shown diagrammatically in Figure 37. In this investigation, groups of 5 islets were perifused and the flow rate lowered to 0.2 ml/minute. It was assumed that this minor alteration in the dynamics of the system

Figure 37 The perfusion of everted mouse jejunum with perifused mouse islets in series.



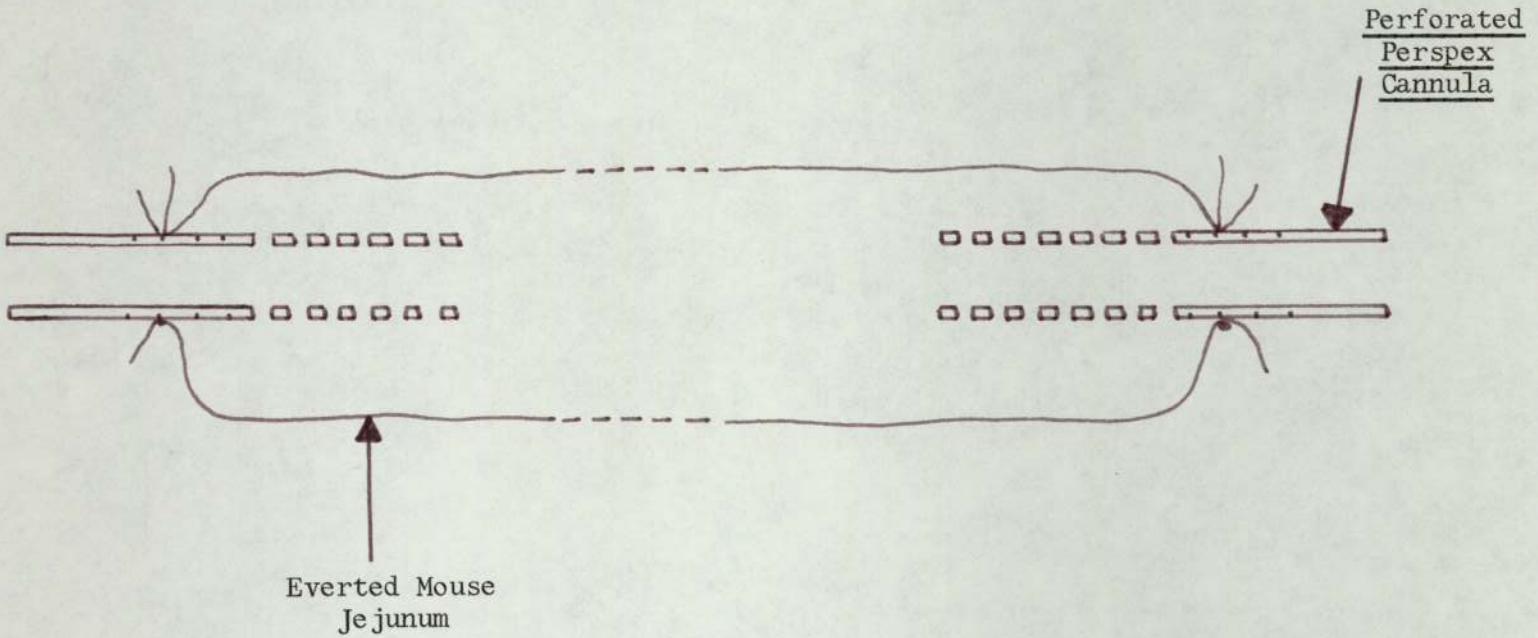
might magnify the effects of any insulinotropic substances released in low concentrations from the intestinal sac preparation. A preliminary examination of the modified system in the absence of an intestinal sac preparation but in the presence of mouse islet tissue, revealed similar insulin secretion kinetics to those observed for the original perifusion system used in Chapter IVa, page 56.

The islets were pre-perifused for 40 minutes with low glucose (3mM) Gey and Gey buffer pH 7.4 before the installation of the everted intestinal sac preparation into the circuit. The everted jejunal sacs of both lean and obese hyperglycaemic mice were prepared during this pre-perifusion period.

Preparation of jejunal sacs

Obese-hyperglycaemic mice and their lean littermates were allowed food and water ad libitum. Animals were killed by cervical dislocation, and the small intestine carefully removed, dissected free of all fat, pancreatic tissue and blood vessels and placed in a dish containing 0.9% (w/v) NaCl at room temperature (22°C). The intestine was then flushed gently with saline to remove all debris and carefully everted using a 20cm x 1.5 mm stainless steel rod. The everted intestine was then blotted on filter paper and the duodenum (first 3 cm) removed. The next 8 cm of jejunum was utilised for the perfused intestinal sac. Each end of the jejunal segment was cannulated with a 2cm perforated perspex tube specially designed to keep the everted jejunal sac patent, Figure 38. The preparation was then carefully immersed in a perspex bath containing oxygenated (95% O_2; 5% CO_2) Gey and Gey bicarbonate buffer (pH 7.4) supplemented with 3mM glucose at 37°C. The jejunal sac was pre-perfused at a flow rate of 0.2 ml/min for 10 mins with oxygenated

Figure 38 Everted Cannulated Mouse Jejunum Preparation



Gey and Gey buffer (pH 7.4) containing 3mM glucose, and the perfused effluent discarded. After this ten minute equilibration period, the jejunal sac was transferred to another perspex reservoir containing oxygenated Gey & Gey bicarbonate buffer (pH 7.4) supplemented with the following components:-

18 mM Glucose

7 mM Phenylalanine

5.5 mM Methionine

12.8 mM Valine.

In control experiments, the jejunum was transferred to a reservoir containing medium supplemented with 3mM glucose only.

The efferent cannula of the perfused jejunal sac was then connected in series to the perifusion chamber containing the pre-perifused islets. This step of the procedure was designated time zero. Perifusate was collected at one minute intervals. The volume of each perifused aliquot was measured to check the flow rate, and duplicate 50μl samples taken and quick frozen for the subsequent radioimmunoassay of insulin. During each experiment, several 50 ml samples of perifusate effluent were collected for the estimation of glucose content using the autoanalyser described in Chapter III. Six groups of experiments were performed as described in the protocol table 6. The results are expressed as the concentration of insulin in the perifusate following the incorporation of the test everted jejunal sac preparation into the system, (i.e. I.R.I./ml/mg freeze dried islet tissue) and compared to insertion of control sac preparations (exposed to 3mM glucose only) into the system.

TABLE 6 The perfusion of everted jejunal sacs in series with perifused mouse islets of Langerhans.

	Protocol				
Experiment	Perifusate	Mouse Jejunum	Mucosal Solution	Islets	Resultant effects on Insulin Secretion
1. (Fig.41)	3mM glucose	Lean	3mM glucose	Lean	No effect
2. (Fig.42)	3mM glucose	Obese	3mM glucose	Obese	No effect
3. (Fig.43)	3mM glucose	Lean	18mM glucose + Amino Acids	Lean	No effect
4. (Fig.44)	3mM glucose	Obese	18mM glucose + Amino Acids	Obese	Significant elevation p(0.05)
5. (Fig.45)	3mM glucose	Lean	18mM glucose + Amino Acids	Obese	Significant elevation p(0.05)
6. (Fig.46)	3mM glucose	Obese	18mM glucose + Amino Acids	Lean	No effect

Results

Figures 39 and 40 illustrate the control insulin secretory profiles of lean and obese mouse islets perifused with 3mM glucose with no jejunal sac preparation in series. The mean profiles for the 6 experimental groups, represented in the protocol Table 6, are shown in figures 41 to 46. The perfusate from in series everted jejunal sacs of lean mice maintained in a 3mM glucose medium had no significant effect upon the insulin secretory dynamics of lean mouse islets when compared to the mean control profile obtained in the absence of a jejunal sac (Fig.41). Statistical significance ($p<0.05$) was tested at 10 minute intervals along test and control profiles. The perfusion of everted obese mouse jejunum in a 3mM glucose mucosal solution produced a slight though insignificant increase in insulin output from perifused obese mouse islets compared to the mean control profile obtained in the absence of a jejunal sac (figure 42). Perfusate from everted lean mouse jejunum incubated in medium containing glucose (18mM); phenyl alanine (7mM); methionine (5.5mM) and valine (12.8mM) also had no significant influence on the release of insulin from perifused lean mouse islets (figure 43). However, a preparation of everted perfused obese mouse jejunum incubated in a 18mM-glucose and amino acid supplemented medium produced a significant ($p<0.05$) stimulation of insulin secretion from perifused obese mouse islets after approximately 15 minutes (figure 44). The secretion of insulin from perifused obese mouse islets was slightly, though significantly ($p<0.05$) enhanced 10 minutes after the in series attachment of lean mouse jejunal sacs incubated in a 18mM glucose-amino acid supplemented mucosal medium (figure 45). The perfusate from an obese mouse jejunal sac incubated in a 18mM glucose-amino acid supplemented mucosal medium had no significant effect on the secretion of insulin from perifused lean mouse islets (figure 46).

Figure 39 The secretion of insulin from perifused lean mouse islets in the presence of 3mM glucose

(Each profile illustrates the mean of 3 separate profiles ± s.e.m., one profile per mouse)

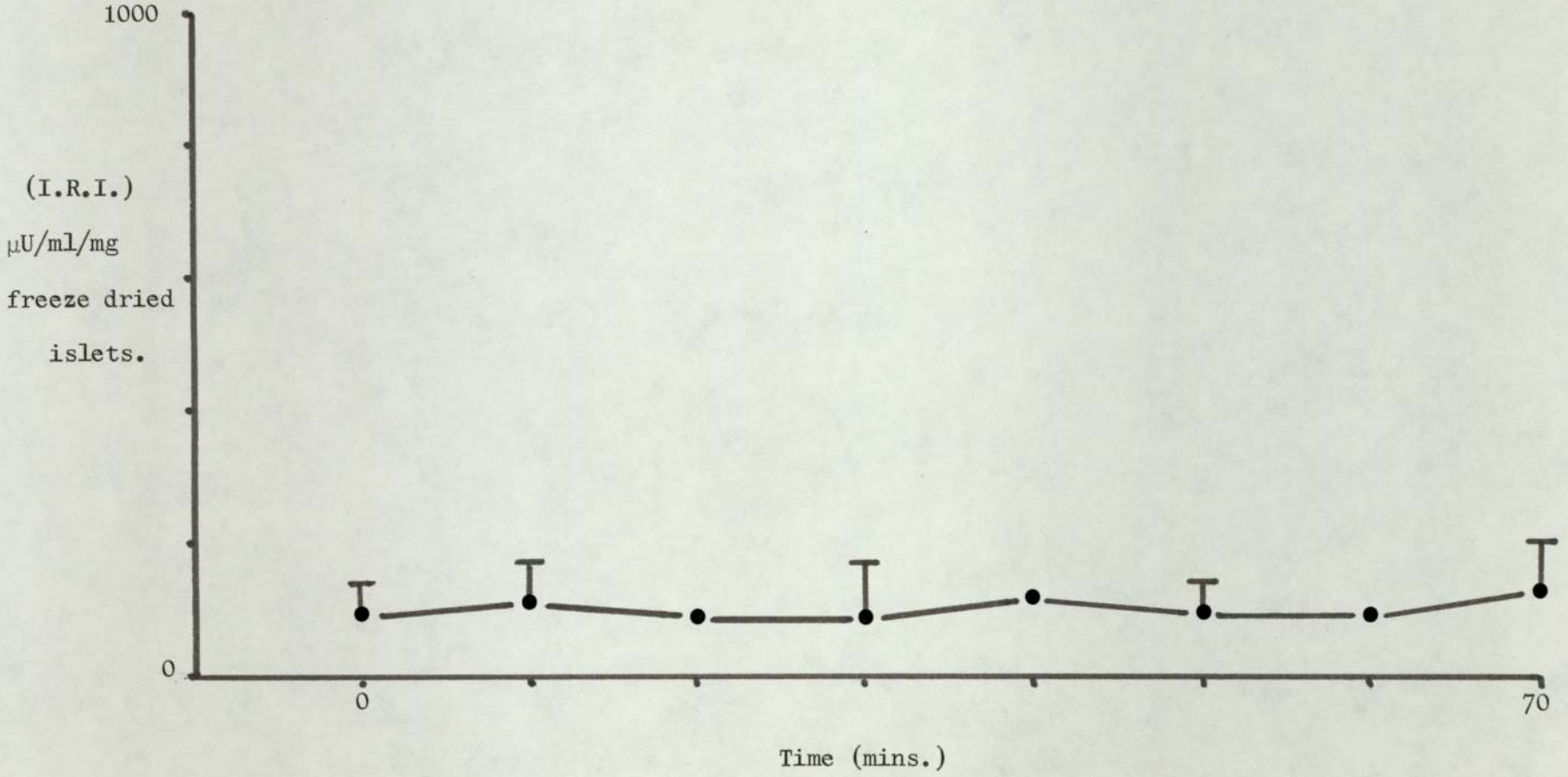


Figure 40 The secretion of insulin from perifused obese mouses islets in the presence of 3mM glucose

(Each profile illustrates the mean of 3 separate profiles ± s.em., one profile per mouse)

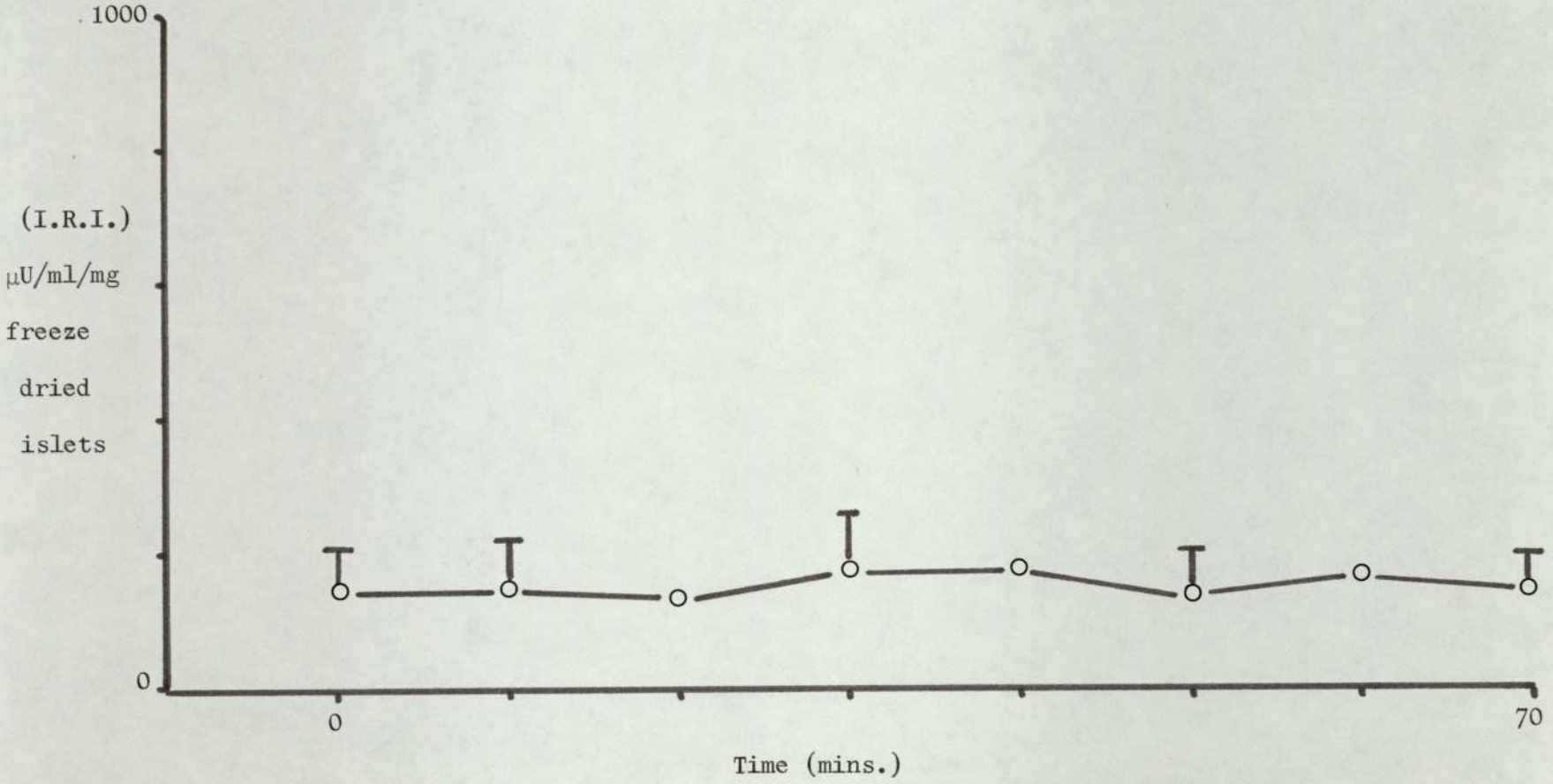


Figure 41 In series perfusion of lean mouse everted jejunal sac (3mM glucose mucosal medium) with perifused islets of lean mice (3mM glucose perfusate).

(Each profile illustrates the mean of 4 separate profiles ± s.e.m., one profile per mouse)

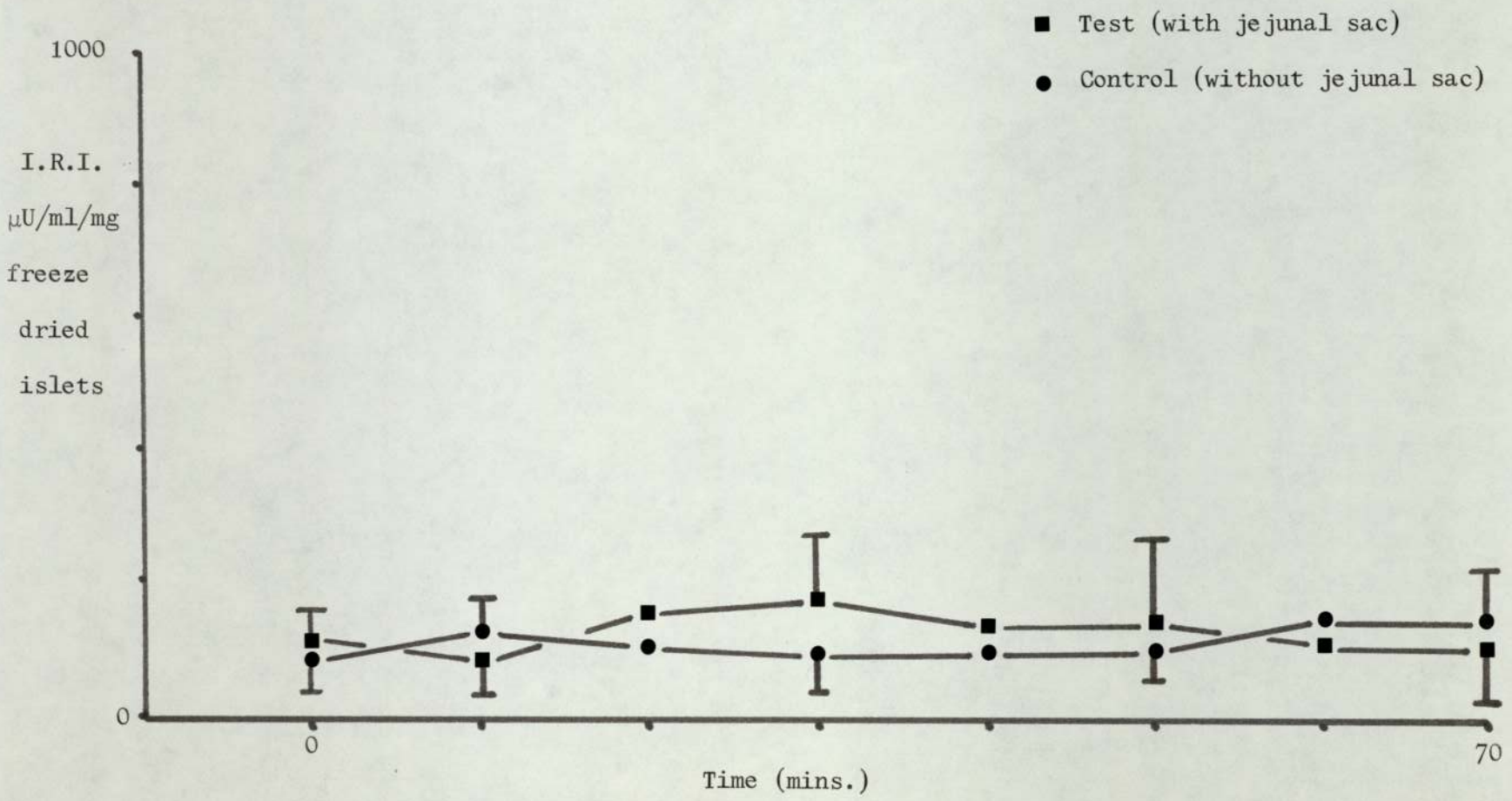


Figure 42 In series perfusion of obese mouse everted jejunal sac (3mM glucose mucosal medium) with perifused islets of obese mice (3mM glucose perfusate).

(Each profile illustrates the mean of 4 separate profiles ± s.e.m., one profile per mouse)

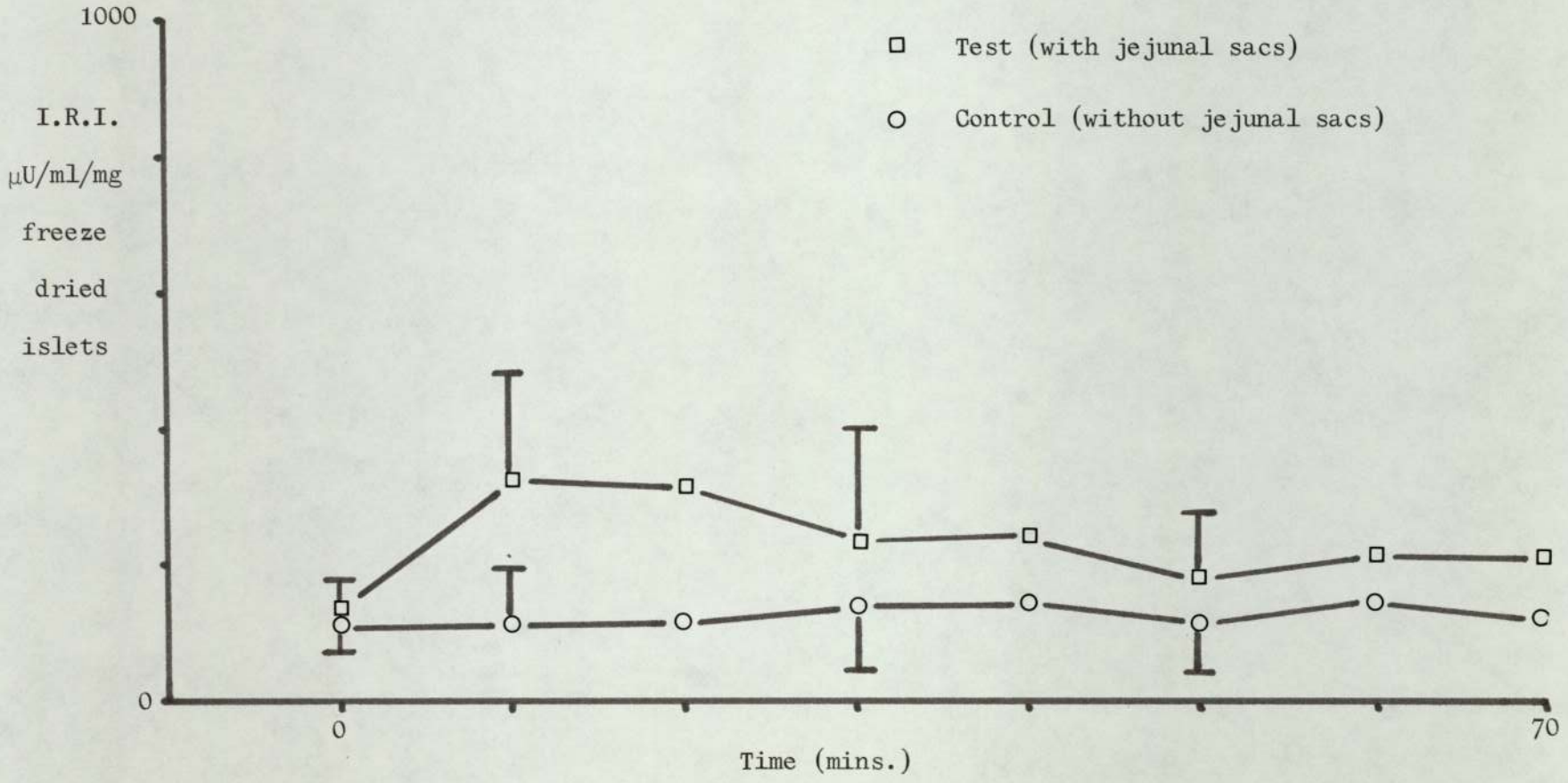


Figure 43 In series perfusion of lean mouse everted jejunal sac (18mM glucose + amino acid supplemented mucosal medium) with perifused islets of lean mice (3mM glucose perfusate).

(Each profile illustrates the mean of 4 separate profiles ± s.e.m., one profile per mouse)

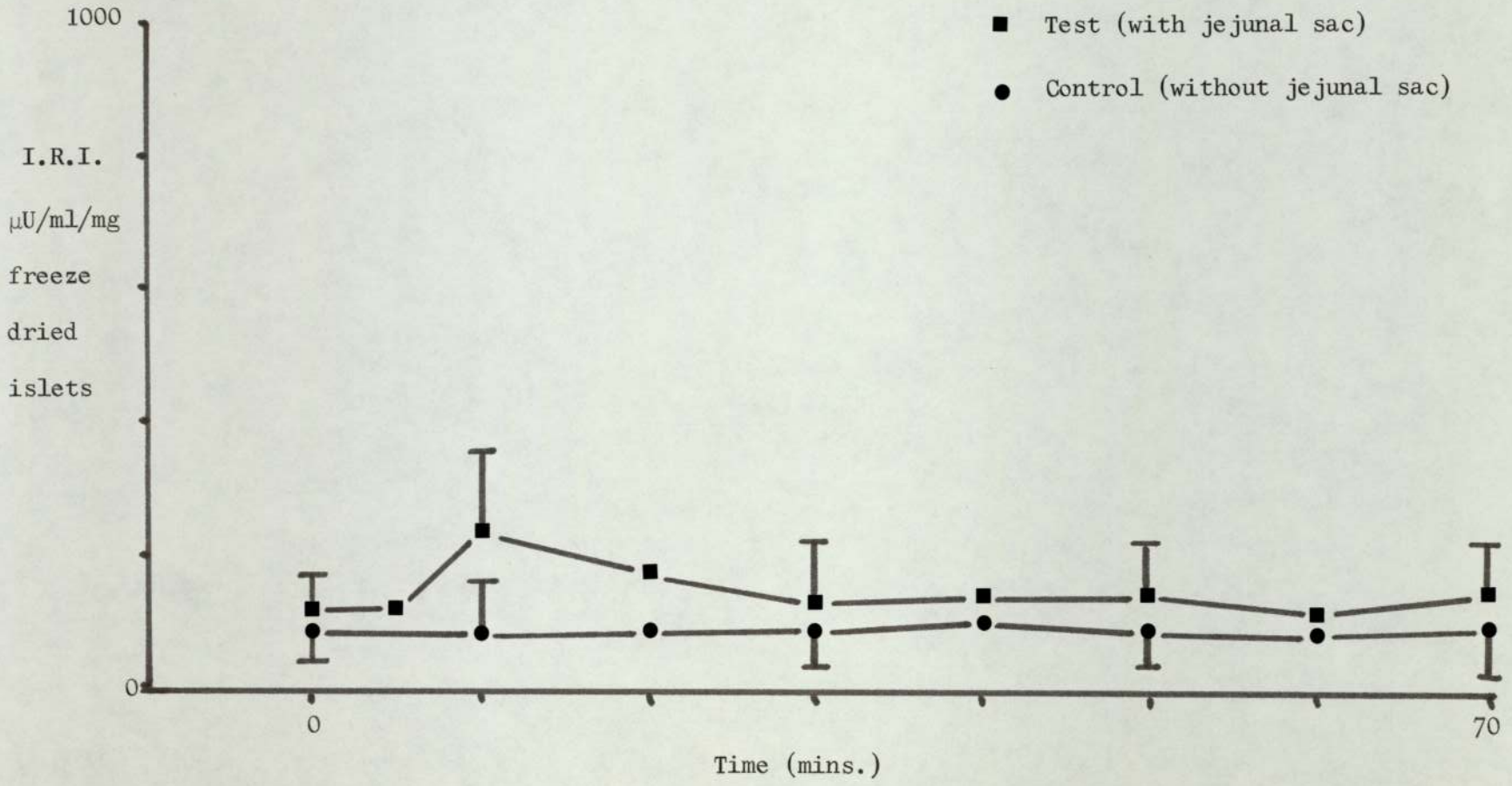


Figure 44 In series perfusion of obese mouse everted jejunal sac (18mM glucose + amino acid supplemented - mucosal medium) with perifused islets of obese mice (3mM glucose perfusate).

(Each profile illustrates the mean of 4 separate profiles ± s.e.m., one profile per mouse)

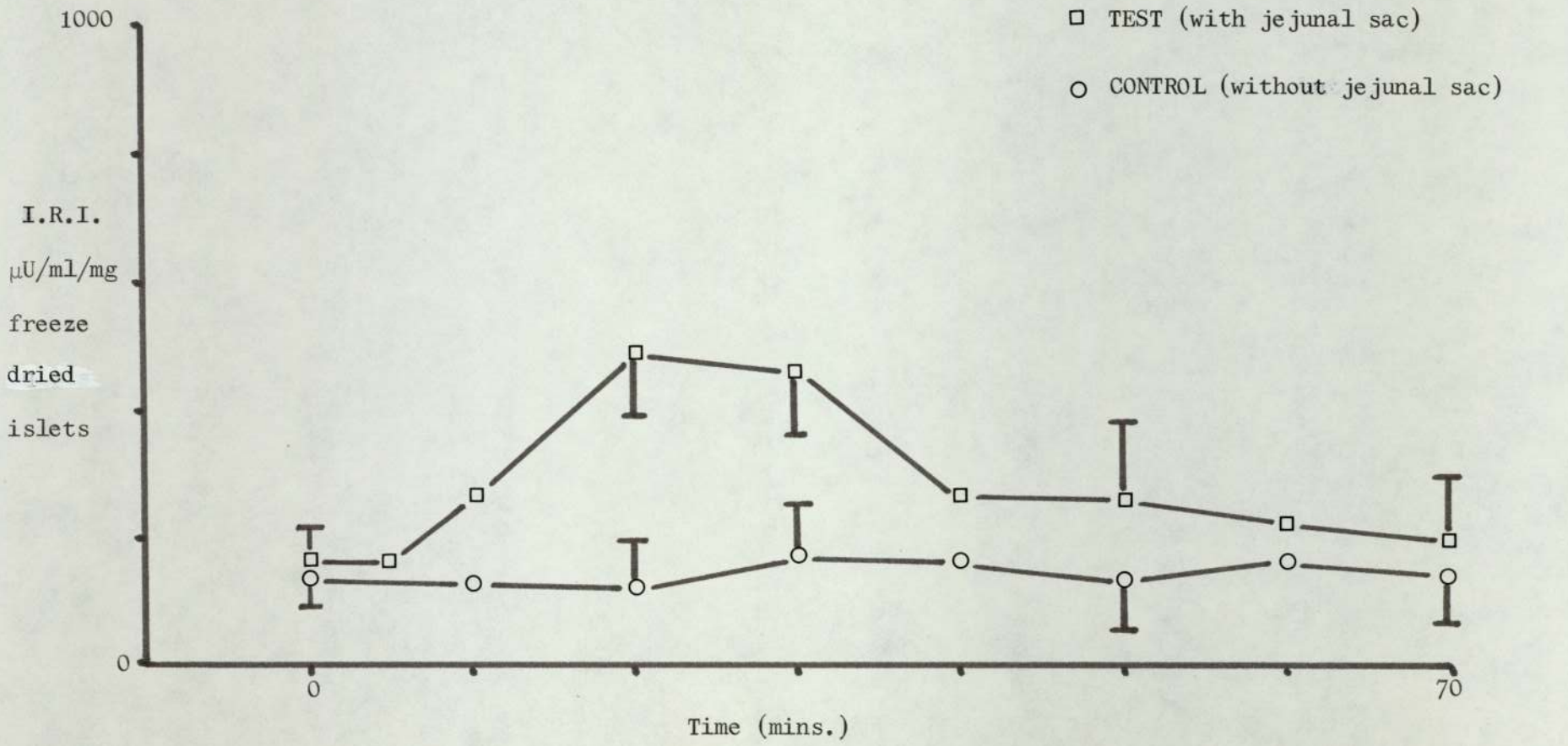


Figure 45 In series perfusion of lean mouse everted jejunal sac (18mM glucose + amino acid supplemented mucosal medium) with perifused islets of obese mice (3mM glucose perfusate).

(Each profile illustrates the mean of 4 separate profiles ± s.e.m., one profile per mouse)

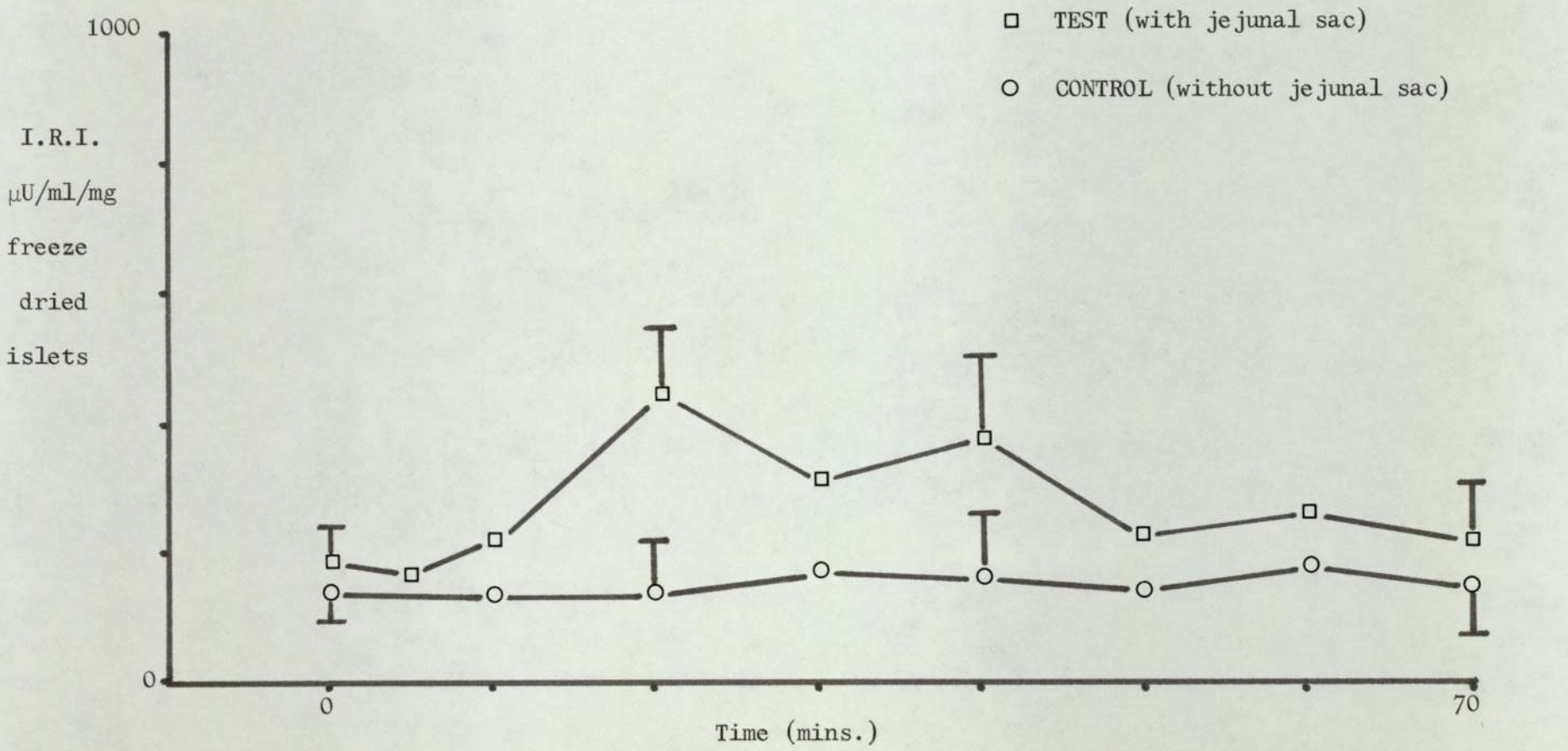
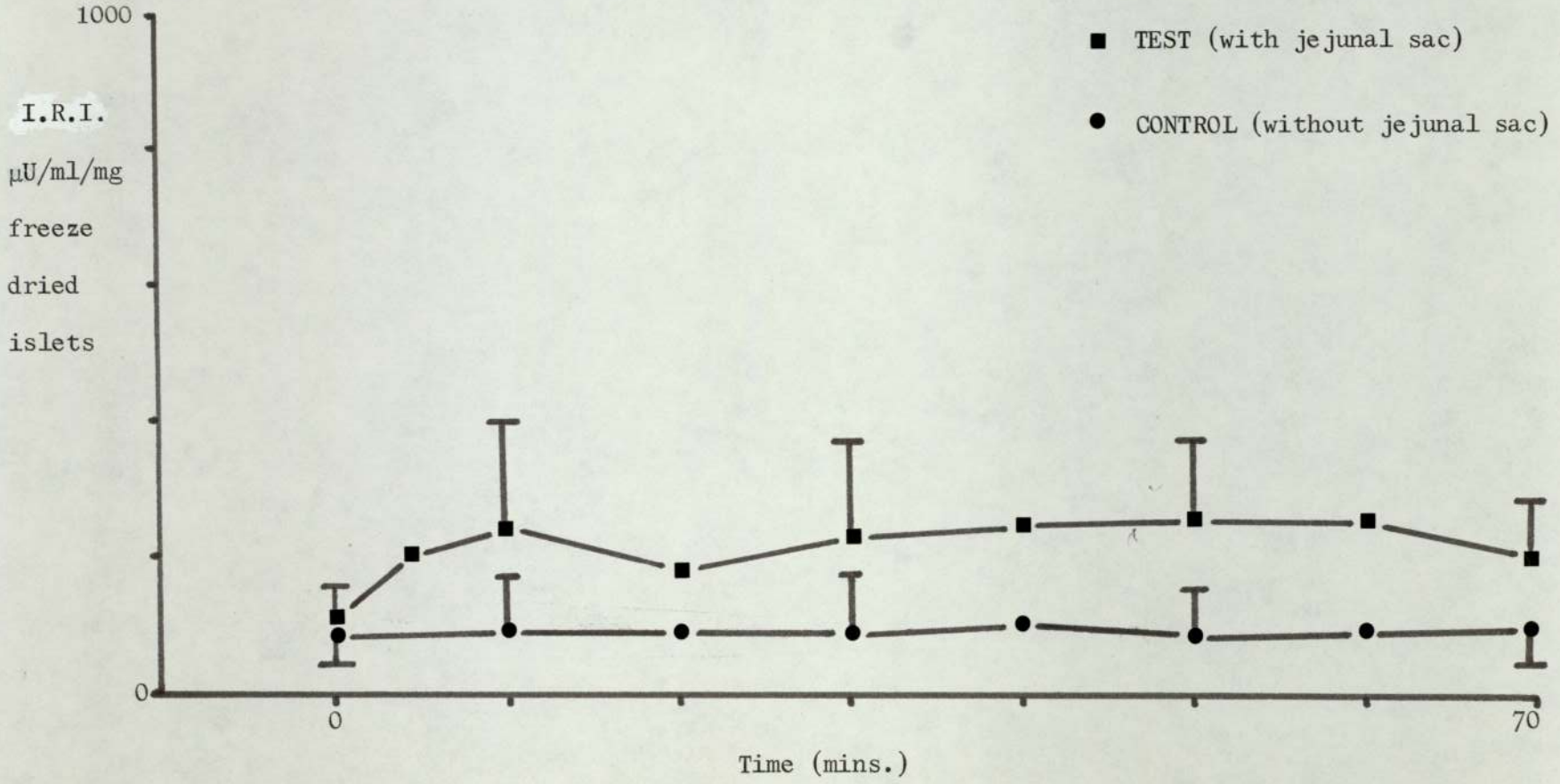


Figure 46 In series perfusion of obese mouse everted jejunal sac (18mM glucose + amino acid supplemented mucosal medium) with perifused islets of lean mice (3mM glucose perfusate).

(Each profile illustrates the mean of 3 separate profiles ± s.e.m., one profile per mouse)



Discussion

In the majority of cases, the incorporation of an everted, perfused jejunal sac from lean or obese-hyperglycaemic mice had little effect on the rate of insulin secretion from either lean or obese mouse islets perfused with buffer containing 3mM glucose. However, with obese mouse islets, it was possible to demonstrate a small significant enhancement of insulin release when placed in series with either lean or obese mouse jejunal sacs, figures 44 and 45. This might well have been due to the hypersensitivity of obese mouse islets to any mucosal substances released from the intestinal preparations. The enhancement of insulin secretion appeared to occur approximately 10 minutes after the incorporation of the everted jejunal sac into the perifusion system.

The experimental design of the in-series system used in the present studies was crude. However, these data suggest that some material might have been released within 5-10 minutes from the jejunal mucosa in response to glucose and amino-acids and that this material was capable of enhancing the release of insulin from islets of obese-hyperglycaemic mice. These findings are consistent with the observations of Felber, Zermatten and Dick (160) who demonstrated that the insulinotropic activity of rat duodenal mucosal extracts was increased five minutes after the ingestion of glucose or amino acids. In addition, these workers were able to show that the release of substances responsible for this insulinotropic activity was biphasic in nature. It was suggested that the biphasic release of gastrointestinal hormones might be analogous to the biphasic release of insulin following glucose stimulation (304). The first phase of gastrointestinal hormone release might represent the movement and secretion of stored hormone by glucose whilst the second phase might correspond to an increase in hormone synthesis.

Which of the known gastrointestinal factors is responsible for the insulinotropic activity of the intestinal mucosa remains to be established. Grenier and coworkers (41), using a preparation of canine jejunum perfused via its vacularisation, demonstrated that the presence of glucose in the intestinal lumen triggered the release of insulin from a perfused canine pancreas, but had no effect on pancreatic exocrine activity. This suggested that the insulinotropic activity released from the jejunum was unlikely to be due to the presence of secretin or pancreozymin, but more likely to be due to the presence of some unknown factor. In the previous section (Chapter IV, Section A, page 62) it was demonstrated that the insulin secretory capacity of obese mouse intestinal extracts was significantly greater than that of lean mouse intestinal extracts. In the present study, however, there appeared to be no significant difference between the insulinotropic capacities of substances released from either lean or obese mouse jejunal sacs.

Critical study of the system

The everted jejunal sac preparation, whilst being a standard technique for the study of intestinal absorption, is essentially unphysiological. The removal and preparation of the everted, perfused jejunal sac involves an inevitable amount of trauma to the tissue. T he blood supply to the tissue is interrupted, resulting in anoxia and a subsequent impairment of absorptive and secretory function. Nevertheless, the technique provides a simplified *in vitro* approximation of the *in vivo* situation.

There existed the possibility that glucose might be absorbed into the perfusate from the mucosal medium, and that this glucose might potentiate any insulinotropic effect of substances released from the jejunal sac. However, it was found that the glucose concentration of

the perifusate never exceeded 4mM and was always too low to stimulate insulin release. Amino acid concentrations in the perifusate were not monitored, but were assumed to be very low and their influence on insulin secretion negligible.

The limited amount of data obtained from the present experiments suggest that humoral factors released from the intestinal mucosa in response to the absorption of carbohydrates and amino acids might be capable of influencing the secretory activity of isolated islets of Langerhans, especially those from obese hyperglycaemic mice.

CHAPTER V

Studies on the mechanism of action of gastrointestinal hormones on glucose induced insulin secretion from islets of lean and obese hyperglycaemic mice.

INTRODUCTION

The secretion of insulin from the pancreatic islets appears to be induced by agents capable of either stimulating adenylate cyclase activity (e.g. glucagon) or inhibiting cAMP phosphodiesterase activity (335, 343, 349). These observations suggest that adenosine 3'5' cyclic monophosphate might be intimately involved in the insulin secretory process. Turtle and Kipnis first measured total islet concentrations of cyclic AMP and suggested the existence in the islet β-cell of an adenylate cyclase cAMP-phosphodiesterase system (335). There is a large body of evidence to suggest that the adenylate cyclase complex is located in the β-cell plasma membrane (344, 345) and that its activity might be modulated via an adrenergic receptor mechanism (335). According to the 2nd messenger hypothesis of Sutherland (346) the activation of the β-cell adenylate cyclase is accomplished by the binding of a hormone (or first messenger) to a receptor (or discriminator) sub unit located on or at the outer surface of the cell membrane. Hence the complement of receptor units will determine the responsiveness of the adenylate cyclase to hormones and pharmacological agents (343). The breakdown of cyclic AMP is controlled by the enzyme cyclic AMP-phosphodiesterase (347, 348, 349) which is located in the cell cytoplasm (350). The activities of these two enzymes are thought to control the level of cyclic AMP in the β-cell and consequently modulate its insulin secretory activity. It has also been suggested that the adenylate cyclase - cAMP-phosphodiesterase system might well be important in the regulation of glucagon secretion from

the islet of α_2 cells (351).

The role cyclic-AMP plays in the mechanism of insulin secretion is still not fully understood. Elevation of the cyclic AMP concentration in the β-cell is not thought to stimulate insulin release in the absence of a substrate (such as glucose), although the nucleotide might modulate or enhance the stimulatory effect of glucose per se (352). It has been suggested that a unifying mode of action cyclic AMP might involve the activation of protein kinases (337, 353, 354) which catalyse the phosphorylation of proteins intimately involved in a variety of cellular processes possibly including secretion. In addition cAMP might also be involved in insulin biosynthesis since Shatz and colleagues (355) have shown the incorporation of ^{3}H-leucine into insulin and pro-insulin fractions of isolated mouse islets to be stimulated by cAMP. The nucleotide might also modulate the process of insulin secretion by an effect on the movement of insulin granules to the periphery of the β-cell (356) possibly via the phosphorylation of proteins in the proposed microtubular-micro-filamentous system (357). In common with many other cyclic AMP-regulated processes, the secretion of insulin is an exocytotic process (emiocytosis) that appears to require calcium (358). It has been suggested that cyclic AMP alters the rate of uptake and/or translocation of ionised calcium from the bound form (associated with intra-cellular organelles) to the free form in the cytoplasm (359, 360, 361).

Many agents which possess insulinotropic activity also increase islet adenylate cyclase activity (362). Exogenous methylxanthines (e.g. theophylline, aminophylline) are believed to prevent the degradation of cyclic AMP by inhibiting cyclic AMP-phosphodiesterase activity within islet tissue (348, 349). In addition these compounds are themselves capable of stimulating the secretion of insulin (363, 375).

There is conflicting evidence in the literature as to whether glucose, physiologically the most important insulinotropic agent, acts directly upon the adenylate cyclase system. The results of several investigations have suggested that the metabolism of glucose within the β-cell, or a metabolite of the sugar itself was responsible for initiating glucose-induced insulin secretion (313, 323). However, the results of early work failed to demonstrate a stimulatory effect for glucose on either islet adenylate cyclase (328, 362, 364), phosphodiesterase (348, 349) or ^{c}AMP-dependent protein kinase activity (337). This was supported by reports that glucose had no signficant effect on the levels of cyclic AMP in isolated islets of either rats (356) or mice (365).

In contrast, Charles and colleagues recently demonstrated that glucose induced insulin secretion occurred with a concomitant increase in islet cyclic AMP concentration (366). Cerasi and Grill have subsequently shown glucose to be capable of stimulating both cyclic AMP accumulation and adenylate cyclase activity in isolated rat islets (367, 368, 369).

The structural similarities of gastrointestinal hormones, particularly secretin, to pancreatic glucagon has raised the possibility that the effects of gastrointestinal hormones upon the pancreatic islet might be mediated via the adenylate cyclase cAMP-phosphodiesterase system. Intestinal hormones have been shown to elevate cyclic AMP levels in isolated human fat cells (370) and in exocrine pancreatic tissue of the cat (371) and rat (372). Both secretin and pancreozymin have been shown to stimulate adenylate cyclase activity in a membrane preparation of mouse islets (328). Raptis and coworkers (373) have suggested that in man the insulinotropic effects of secretin and pancreozymin might be mediated

via $\underline{\beta}$ and $\underline{\alpha}$-adenergic mechanisms respectively.

In a previous section (Chapter IV, Section A, page 61) it was demonstrated that both secretin and pancreozymin produced a rapid and short-lived release of insulin from isolated islets of both lean and obese hyperglycaemic mice. This suggested that insulin was being released from a small, labile pool in response to gastrointestinal hormone stimulation.

In the following studies, the role of cyclic AMP has been investigated in the phasic release of insulin from islets of lean and obese mice in response to glucose and gastrointestinal hormones.

SECTION A The effect of Imidazole on glucose - and gastrointestinal hormone - induced insulin secretion from islets of lean and obese-hyperglycaemic mice.

Imidazole

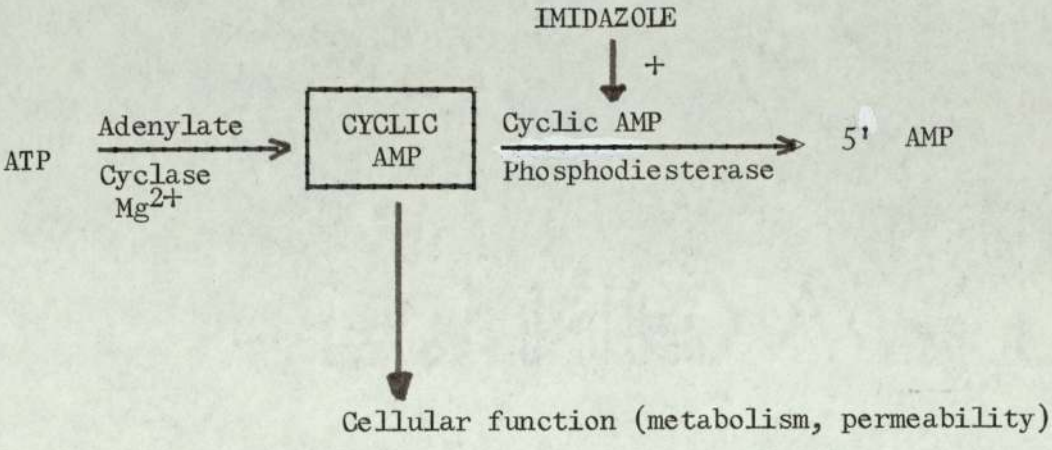
Imidazole is a nitrogenous, pentagonal, heterocyclic molecule with the formula:

NHCH : NCH : CH

The more salient properties of the compound include small size, high solubility in most solvents and an ease of diffusability into cells (374). Most of the metabolic effects of Imidazole are a direct consequence of its ability to lower the intracellular concentration of cyclic AMP by activating the enzyme cyclic AMP-phosphodiesterase (Figure 47). Imidazole has been shown to increase the Vmax. of cyclic AMP phosphodiesterase, and consequently, the compound is only effective at high concentrations of cyclic AMP (374). Imidazole has the capacity to abolish the effect of agents which stimulate adenylate cyclase activity.

The effect of Imidazole on the secretion of insulin has not been extensively investigated. Malaisse, Malaisse-Lagae and King (375) have shown that Imidazole abolished glucagon stimulated insulin secretion from pieces of rat pancreas, whilst that induced by glucose was unaffected. This observation was assumed to be a consequence of β-cell cyclic AMP-phosphodiesterase activation by Imidazole. In contrast, Lefevre & Luyckx (376) reported that insulin secretion in the dog was elevated by the administration of imidazole, although the authors were unable to suggest a possible mechanism for this observation.

Figure 47 The Adenylate Cyclase - cAMP Phosphodiesterase System



In the present study the effect of imidazole was examined on glucose-induced and gastrointestinal hormone induced insulin secretion from islets of lean and obese mice in order to establish a requirement for cAMP in the secretion process.

Materials and Methods

Pancreatic islets from obese-hyperglycaemic mice and their lean littermates fed ad libitum were isolated by freehand microdissection and incubated in a continuous flow perifusion system as described in Chapter IV, Section A, Page 56. The perifusion medium consisted of Gey and Gey bicarbonate buffer, pH 7.4 (277) containing 0.1% w/v Bovine serum albumin fraction V (insulin free, Armour Pharmaceutical Company Ltd., Eastbourne) and glucose (3 or 25 mM). Test hormones were added in the following concentrations: purified secretin (0.2 clin U/ml); purified pancreozymin (1.5 Ivy Dog U/ml) and pancreatic glucagon (5.0 μg/ml; Sigma Chemical Co.Ltd., London). Imidazole was purchased from the Sigma Chemical Co.Ltd., and used at a concentration of 0.5 mg/ml. Two perifusion channels were run simultaneously, one acting as a control (No imidazole).

Islets were pre-perifused 0.5 ml/min for 40 minutes with Gey and Gey bicarbonate buffer, pH 7.4, 37°C containing 3mM glucose in order to establish basal secretory activity. From time zero (fig.48) were then perifused for 70 minutes with fresh medium containing glucose (3 or 25 mM) with or without test hormones. Samples of perifusate were collected at one minute intervals and the insulin content measured by radioimmunoassay. Results were expressed as μU of insulin/ml of perifusate/mg freeze dried islet. Points on the test and control mean profiles were compared statistically $p<0.05$.

Results

The presence of 0.5 mg/ml imidazole in the perifusion medium did not significantly alter the rate of insulin secretion from islets of either lean or obese-hyperglycaemic mice in the presence of 3mM glucose (Figure 48a and b). 25mM glucose-induced insulin secretion from lean mouse islets was slightly, though not significantly depressed in the presence of imidazole compared to the control profile (Figure 49a). However, the secretion of insulin from obese mouse islets in response to 25mM glucose was significantly ($p<0.05$) impaired in the presence of imidazole (figure 49b).

Imidazole produced a complete inhibition of glucagon (5.0μg/ml) stimulated first phase insulin secretion from both lean and obese mouse islets in the presence of 3mM glucose (figure 50a and b).

The immediate stimulation of insulin secretion from both lean and obese mouse islets by pancreozymin (1.5 Ivy Dog U/ml) in the presence of 3mM glucose was significantly depressed by imidazole (figure 51a and b).

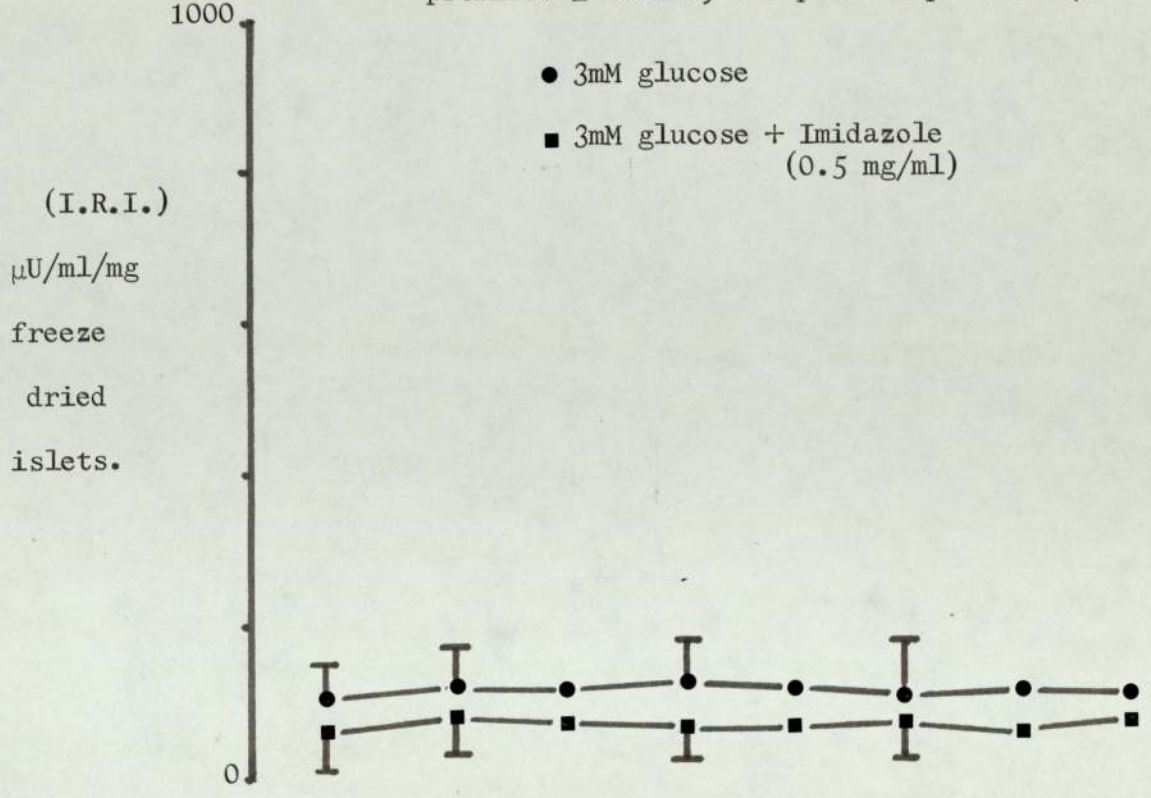
Similarly, secretin (0.2 clin.U/ml) - induced insulin secretion from both lean and obese mouse islets in the presence of 3mM glucose was significantly depressed by imidazole (figure 52a and b).

Imidazole tended to exert its most powerful inhibitory effect on the first phase of hormone induced insulin secretion from both lean and obese mouse islets.

Figure 48 The effect of Imidazole (0.5mg/ml) on 3mM glucose-induced insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(a) Lean

(Each profile represents the mean of 4 separate profiles ± s.e.m., one profile per mouse).



(b) Obese

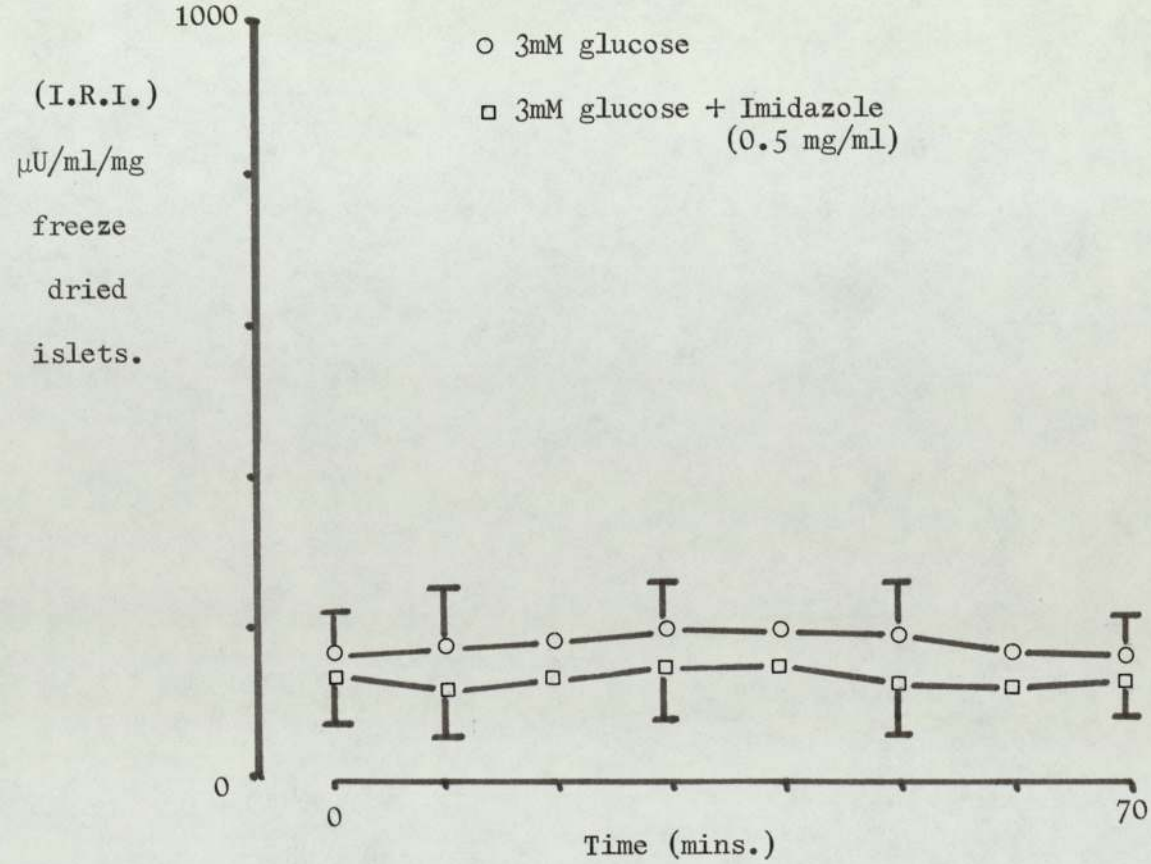


Figure 49 The effect of Imidazole (0.5 mg/ml) on 25mM glucose induced insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile represents the mean of 4 separate profiles ± s.e.m., one profile per mouse).

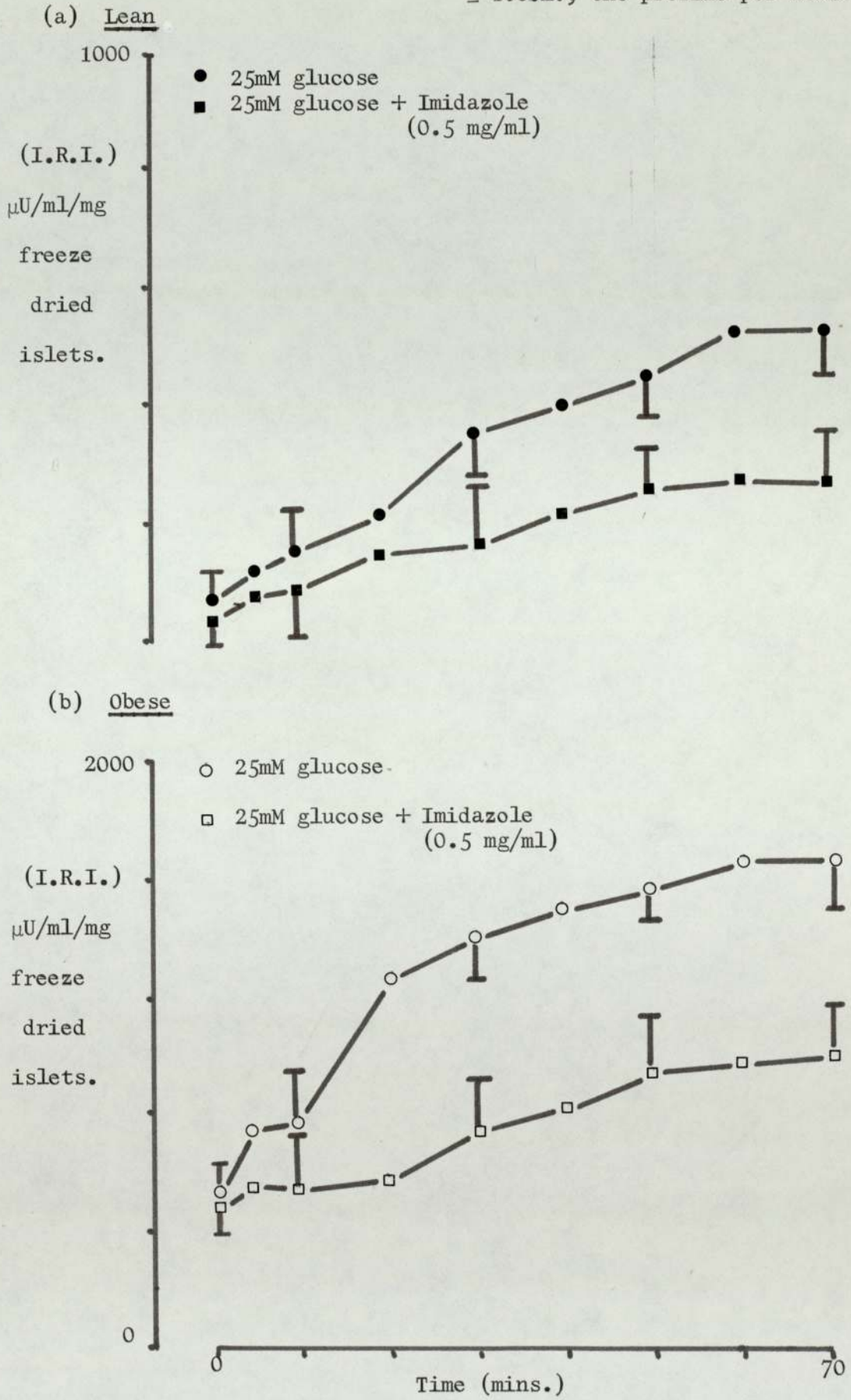


Figure 50 The effect of Imidazole (0.5 mg/ml) on glucagon (5.0μg/ml) ± 3mM glucose induced insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile represents the mean of 4 separate profiles ± s.e.m., one profile per mouse).

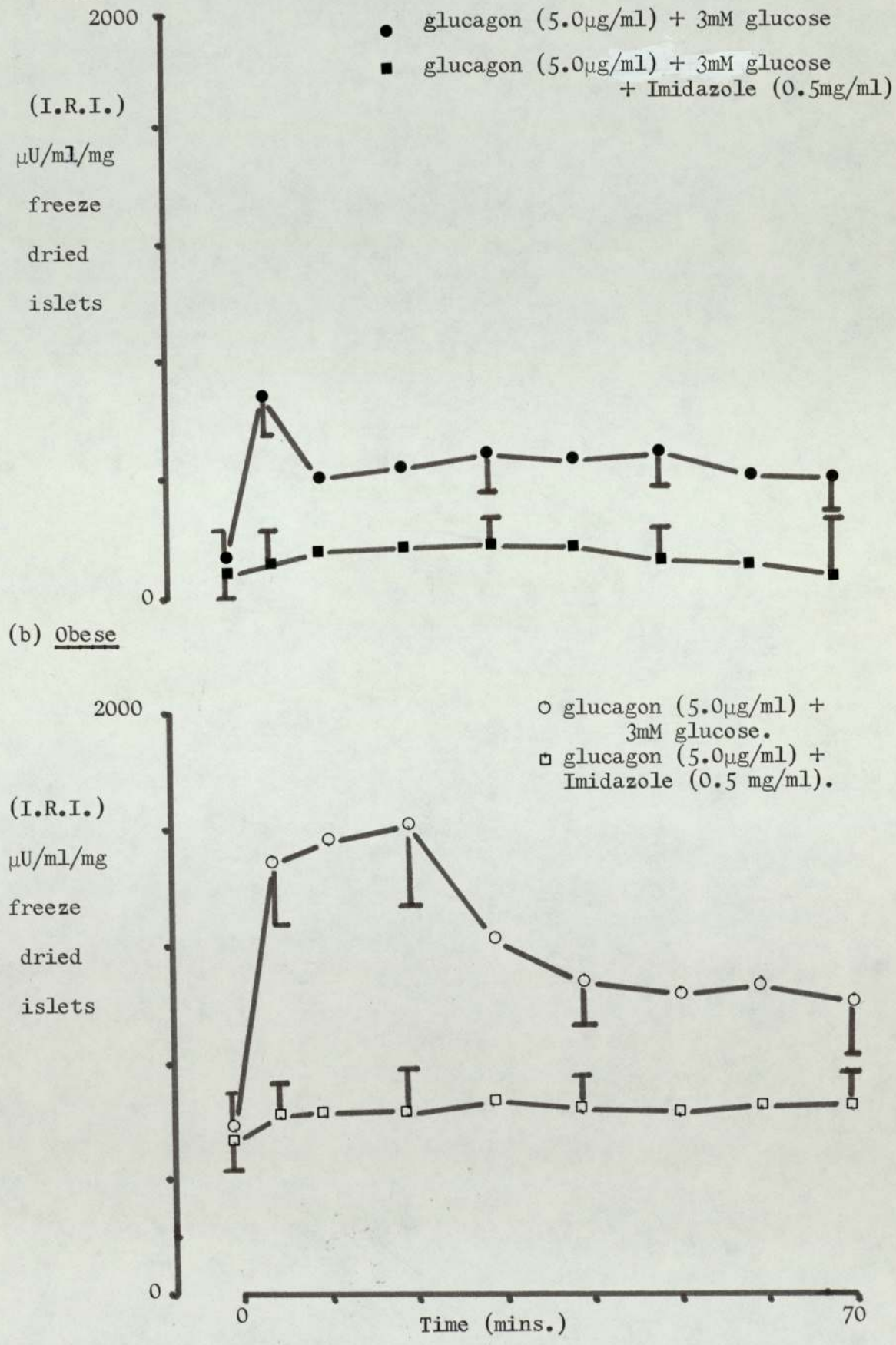
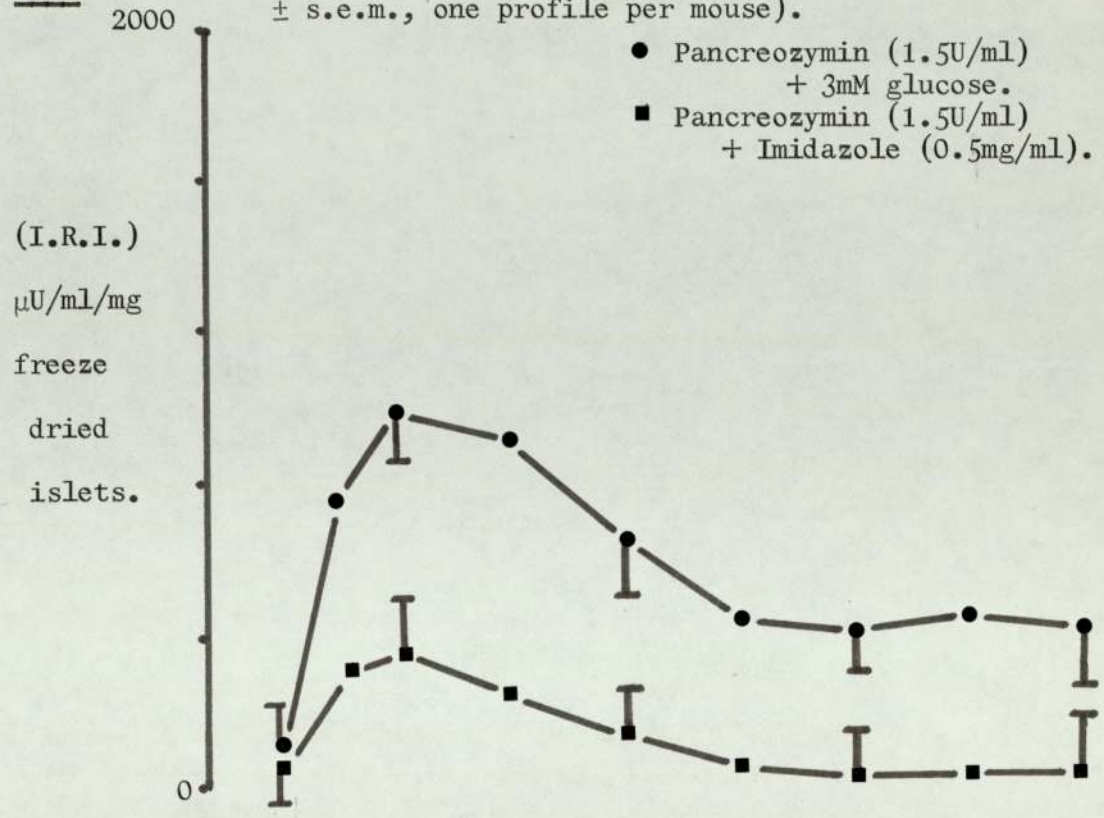


Figure 51 The effect of Imidazole (0.5mg/ml) on pancreozymin (1.5 Ivy Dog U/ml) + 3mM glucose induced insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile represents the mean of 4 separate profiles ± s.e.m., one profile per mouse).

(a) Lean



(b) Obese

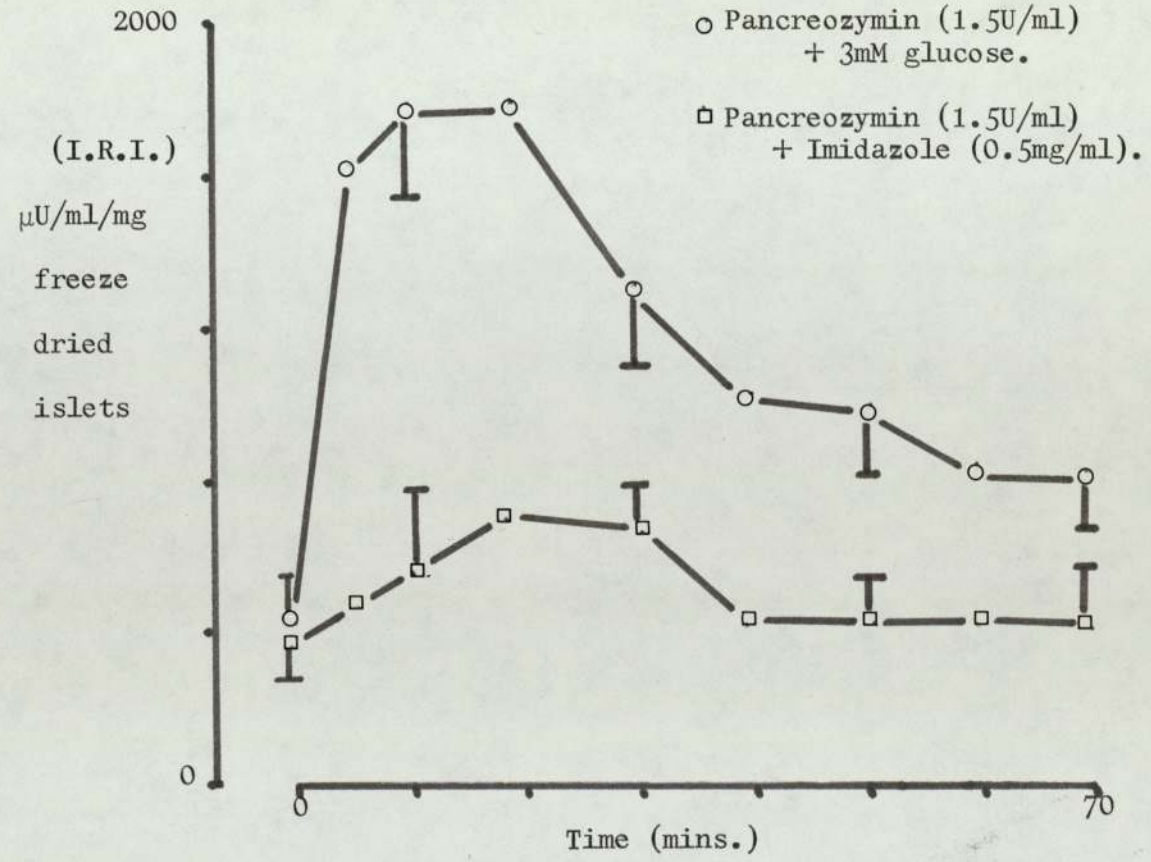
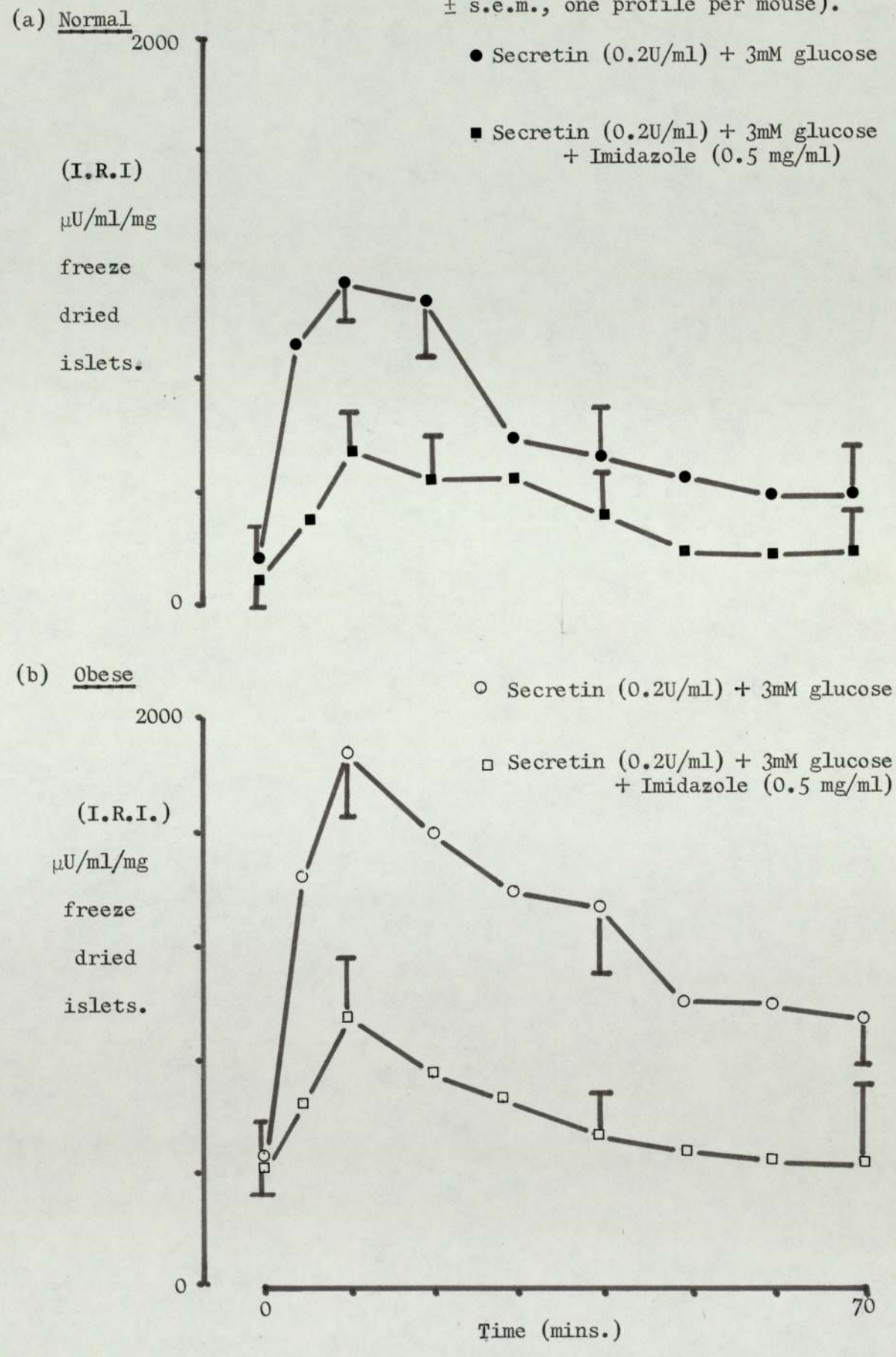


Figure 52 The effect of Imidazole (0.5mg/ml) on Secretin (0.2clin.U/ml) + 3mM glucose-induced insulin secretion from perifused islets of lean and obese-hyperglycaemic mice.

(Each profile represents the mean of 4 separate profiles ± s.e.m., one profile per mouse).



Discussion

The relatively low dose of imidazole used (375) had no significant effect upon insulin secretion from either lean or obese mouse islets in the presence of a nonstimulatory concentration of glucose (3mM).

In addition Imidazole produced a significant inhibition of 25mM glucose-induced insulin secretion from obese mouse islets but had no significant effect on 25mM glucose-induced insulin release from lean mouse islets. Malaisse and colleagues (375) showed that a high concentration of imidazole was capable of inhibiting glucose-induced insulin secretion from pieces of rat pancreas, presumably by reducing intracellular levels of cyclic AMP.

In the present study imidazole produced a complete inhibition of glucagon-induced insulin secretion. This suggested that the activation of phosphodiesterase by imidazole was sufficient to compensate for any elevation in β-cell cyclic AMP concentration induced by glucagon. The inhibition of the first phase of insulin secretion by imidazole might indicate that glucagon exerts its influence on insulin secretion by causing an immediate elevation of β-cell cyclic AMP concentration. Imidazole also produced a significant depression of the early phase of insulin secretion induced by secretin and pancreozymin, suggesting a role for cyclic AMP in the mechanism of gastrointestinal hormone-stimulated insulin secretion. These data also suggest that cyclic AMP might be involved in the first phase of insulin secretion per se following gastrointestinal hormone stimulation. The second phase of glucose-induced insulin secretion appears to be less dependent on intracellular cyclic AMP concentration.

These data are consonant with the hypothesis that glucagon and perhaps gastrointestinal hormones might regulate, via the adenylate

cyclase system, the rate at which glucose metabolism proceeds within the islet β-cell (352) and that at low concentrations, cyclic AMP might well be a rate limiting factor in the secretion of insulin.

SECTION B The effect of glucose and gastrointestinal hormones on lean and obese mouse islet cAMP dynamics.

From the previous studies on the phasic insulin profiles in response to glucose, secretin, pancreozymin and glucagon, it was apparent that glucose and gastrointestinal hormones might stimulate the secretion of insulin via different mechanisms (Chapter IV, Section A, page 83). Investigations involving the effect of imidazole on the phasic release of insulin suggested that cyclic AMP might be involved in the first phase of insulin secretion in response to gastrointestinal hormones (Chapter V, Section A, page 83).

It seemed possible that the usual insulin stimulatory response of the pancreas to glucose or gastrointestinal hormones might be reflected by parallel changes in cAMP content and/or efflux from isolated islets during perifusion. With this in mind the phasic accumulation and efflux of cyclic AMP from islets of lean and obese-hyperglycaemic mice was compared with profiles of insulin secretory activity in response to glucose, glucagon, secretin and pancreozymin.

Materials and Methods

Islets of Langerhans were microdissected from 20-25 week old obese hyperglycaemic mice and their lean littermates and incubated in the perifusion system previously described (Chapter IV, Section A, page 56). The concentrations of glucose and hormones employed were identical to those used in the phasic insulin secretory studies (Chapter IV, page 58). The concentration of cyclic AMP in islets of Langerhans has been reported to be quite low (2.5-25 p.mol/mg. dry weight; 365). Consequently, the number of islets used in the present study was raised to 50 per

perifusion chamber. After the usual 40 minute pre-perifusion period in the presence of 3mM glucose the cyclic AMP content of the tissue was measured at 3, 7, 15, 30 and 50 minutes following the commencement of perifusion with the test substances at time zero. 1 ml aliquots of perifusate collected immediately prior to removal of the tissue were used to estimate cyclic AMP efflux from the islets.

Preparation of samples for cyclic AMP assay

Two separate extraction procedures were investigated and compared for efficiency and reproducibility.

A. Extraction with 5% T.C.A.

Islets were quickly scraped from the millipore filter into 1 ml of 5% (w/v) trichloracetic acid (T.C.A.). The whole operation took approximately 10-15 seconds. The islets were then sonicated for one minute using an ultrasonic disintegrator (Model A350G; Ultrasonics Ltd., Shipley, Yorkshire) fitted with a 3mm diameter titanium probe (power 5, tuning 9-11). The cell debris was removed by centrifugation (4000 r.p.m; 10 minutes) and a 500µl sample removed for processing. The T.C.A. was extracted from the samples by using 5 x 3 volumes of ice-cold water-saturated ether. The upper ether layer was carefully removed after five minutes with a pasteur pipette, and the remaining aqueous extract freeze dried *in vacuo* (0.001 mm Hg., Model 5PS centrifugal freeze drier, Edwards High Vacuum Ltd., Crawley, Sussex). Vials containing lyophilised extract were sealed and stored in a vacuum dessicator for the subsequent estimation of cyclic AMP content.

Samples of perifusate were treated as follows: 1 ml perifusate was added to 100µl 50% (w/v) T.C.A. and shaken using a whirlimixer. The sample was then centrifuged (4000 r.p.m. for 10 minutes) and a 500 µl

aliquot removed and extracted with ether as described above.

2. Extraction with 5% (v/v) HCl - absolute ethanol

Using this procedure, deproteinisation was accomplished with 1 ml of 5% (v/v) hydrochloric acid - absolute ethyl alcohol. This eliminated the need for ether extraction. Islets were similarly homogenised by sonication, centrifuged and a 500µl sample evaporated to dryness under a stream of nitrogen in a water bath at 65-70°C. The dried extracts were stored in a vacuum dessicator for the subsequent assay of cyclic AMP content.

1 ml samples of perifusate were mixed with 100µl 10% (v/v) HCl - ethanol, centrifuged (4000 rpm for 10 minutes) and 500 µl samples evaporated to dryness as described above.

Estimation of cyclic AMP concentration

The assay of cyclic AMP concentration presents many analytical difficulties. In general, these difficulties arise from the low levels of cyclic AMP found in most tissues and from the high relative concentrations of potentially interfering compounds (mainly other nucleotides including cyclic GMP and ATP. Several techniques are available for the measurement of cAMP. These include the phosphorylase activation assay (377), the cyclic AMP-dependent protein Kinase binding assay (378) and radioimmunoassay using an antibody prepared to a succinyl derivative of cyclic AMP (379). In addition, the use of High Pressure Liquid Chromatography (H.P.L.C.) has recently been adapted for the measurement of cyclic nucleotides (380).

In the present work, a modification of the protein-binding assay of Gilman (378) was employed. The component reagents were supplied by the Radiochemical Centre, Amersham, Bucks, in the form of a kit

(Code TRK 342). The method is based on the competition between unlabelled and tritium-labelled cyclic AMP for a binding protein which is highly specific for cyclic AMP. The binding protein is a cyclic AMP-dependent protein kinase isolated from bovine skeletal muscle using the method of Tovey, Oldham and Whelan (381). The cyclic AMP-binding protein forms the basis of the competition between unlabelled (standard or unknown) cyclic AMP and ^{3}H-labelled cyclic AMP of high specific activity for the binding protein. This can be represented as follows:-

C	+	B	$\rightleftharpoons$	CB
unlabelled cyclic AMP		binding protein		
C*	+	B	$\rightleftharpoons$	CB*
labelled cyclic AMP				

At $0^{o}C$, the equilibrium of the reaction shown above is established within sixty minutes. The separation of the bound from the free labelled cyclic AMP is accomplished by the adsorption of the latter onto charcoal, which is rapidly separated by centrifugation. Samples of supernatant (containing bound labelled cyclic AMP) are then removed for β-scinillation counting.

Assay Procedure

The cyclic AMP binding protein was carefully dissolved in 15 ml distilled water by gentle swirling. (8-^{3}H) - cyclic AMP was supplied as a lyophilised powder (activity 5μCi/180 p.mole.) This was reconstituted by the careful addition of 5 ml. distilled water. A standard

aqueous solution of cyclic AMP (80pmol/ml) was prepared and serially diluted to give standards of 16, 8, 4, 2 and 1 pmol/assay tube. Zero tubes containing unlabelled cyclic AMP and blank tubes containing no binding protein were also set up. Lyophilised, unknown cyclic AMP samples were reconstituted with 200µl 0.05 M TRIS-EDTA buffer, pH 7.5. This volume allowed samples to be assayed in duplicate or triplicate. All reagents and samples were kept at 0-4°C in an ice bath during assay. The cAMP assay protocol is shown in table 7.

50µl of labelled cyclic AMP was added to every assay tube. This was followed by the addition of 100µl of binding protein solution to all tubes except the assay blanks. All tubes were then vortex mixed for approximately 5 seconds and incubated in a refrigerator at 0-4°C for 2 hours. This allowed the assay reaction to reach equilibrium. During the two hour incubation period, the charcoal adsorbent was reconstituted by the addition of 20 ml distilled water with constant stirring. After incubation a 100µl aliquot of charcoal suspension was added to each tube. The tube contents were then vortex mixed and centrifuged at 1400 r.p.m. for 15 minutes on a Mistral 4L refrigerated centrifuge at 4°C. This operation was performed as rapidly as possible to avoid the displacement of bound ^{3}H-Cyclic AMP from the binding protein onto the charcoal adsorbent. A 200 µl aliquot of supernatant was carefully added to 10 ml of scintillant (P.C.S. solubliser, Amersham/Searle) in a capped scintillation vial. Each vial was counted for 10 minutes on a Beckman L.S.230 liquid β-scintillation counter.

TABLE 7 Cyclic AMP Assay Protocol

Tube	Buffer	Std/sample	(^{3}H)-cAMP	Binding Protein
Blank	*150	-	50	-
Zero	50	-		100
Stds 1 pmol ^{c}AMP	-	50		
2 pmol "				
4 pmol "				
8 pmol "				
16 pmol "				
Unknown samples				

*All volumes are in microlitres.

All tubes assayed in duplicate.

Construction of standard curves and the calculation of results

A typical cyclic AMP standard curve is shown in figure 47.

The counts for replicate samples were averaged and the blank counts subtracted. The standard curve was constructed by plotting the Co/Cx ratio (i.e. cpm for zero value/cpm for standard) against the respective standard cyclic AMP concentration (pmol./assay tube). The Co/Cx ratio was also calculated for each sample of unknown and the cyclic AMP content in the unknown estimated by reference to the standard curve.

Precision, Range and Specificity

The cyclic AMP assay described has a coefficient of variation of less than 11% over the range 0.2-16 pmol. cyclic AMP/tube and of less than 7% over the range 0.5-14 pmol./tube. In the present studies, the cyclic AMP content of the unknown samples fell consistently within the range 0.5-8 pmol/tube.

Other naturally occurring nucleotides which are likely to cross-react with the cyclic AMP binding protein include ATP and cyclic GMP. However, it has been demonstrated that ATP and cyclic GMP are not likely to interfere with the assay for cyclic AMP at the concentrations found in most tissues (378).

In order to check the possibility that certain components of islet extracts, e.g. endogenous nucleotides, might interfere with the assay for cyclic AMP, several unknown samples were assayed undiluted, and diluted 1 : 2; 1 : 4 and 1 : 8 with 0.05M TRIS-EDTA buffer pH 7.5. The binding curve obtained was found to parallel the cyclic AMP standard curve (figure 48) suggesting that endogenous components of islet extracts had no significant effect upon the assay for cyclic AMP.

Figure 47 A typical cyclic AMP standard curve

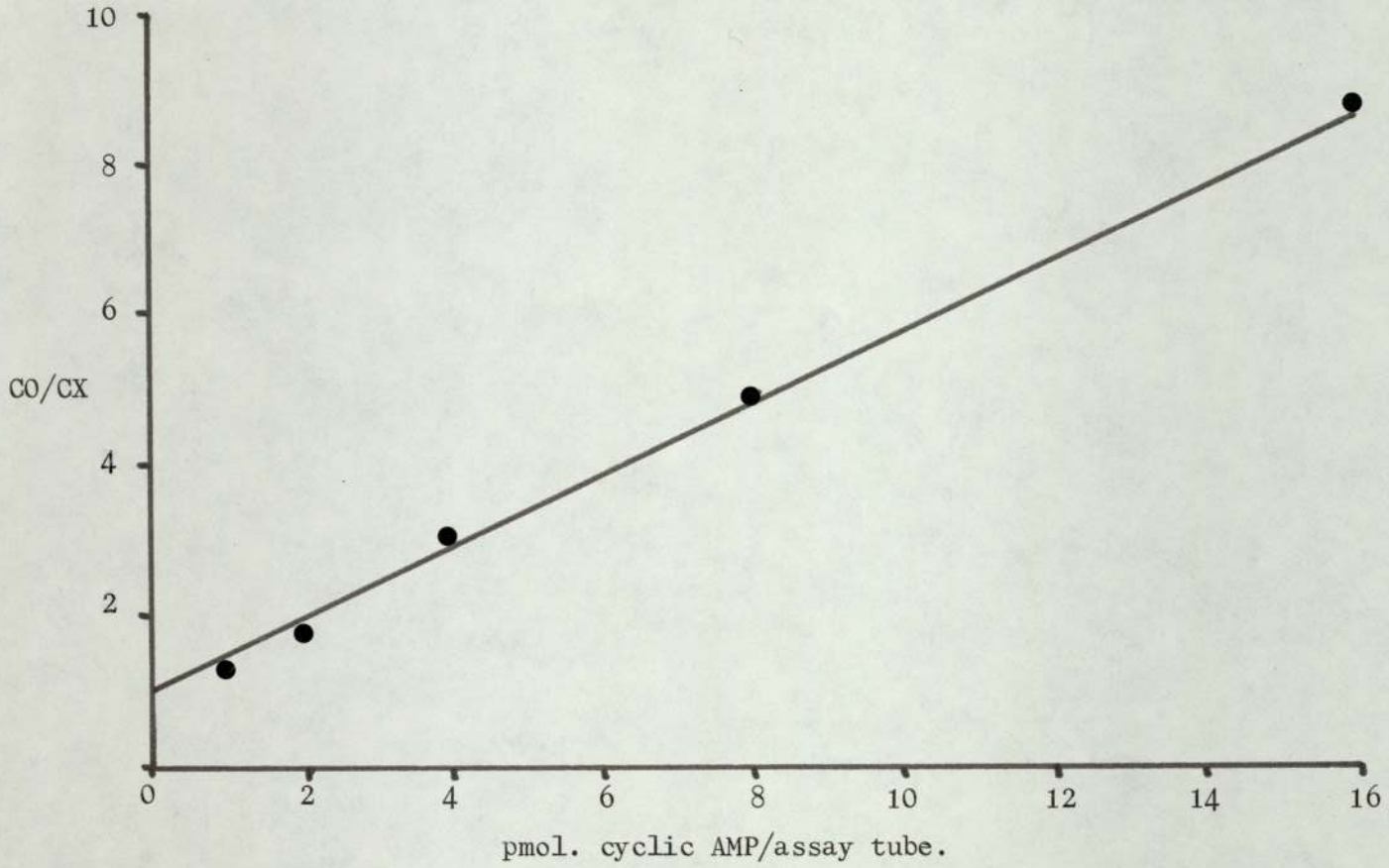
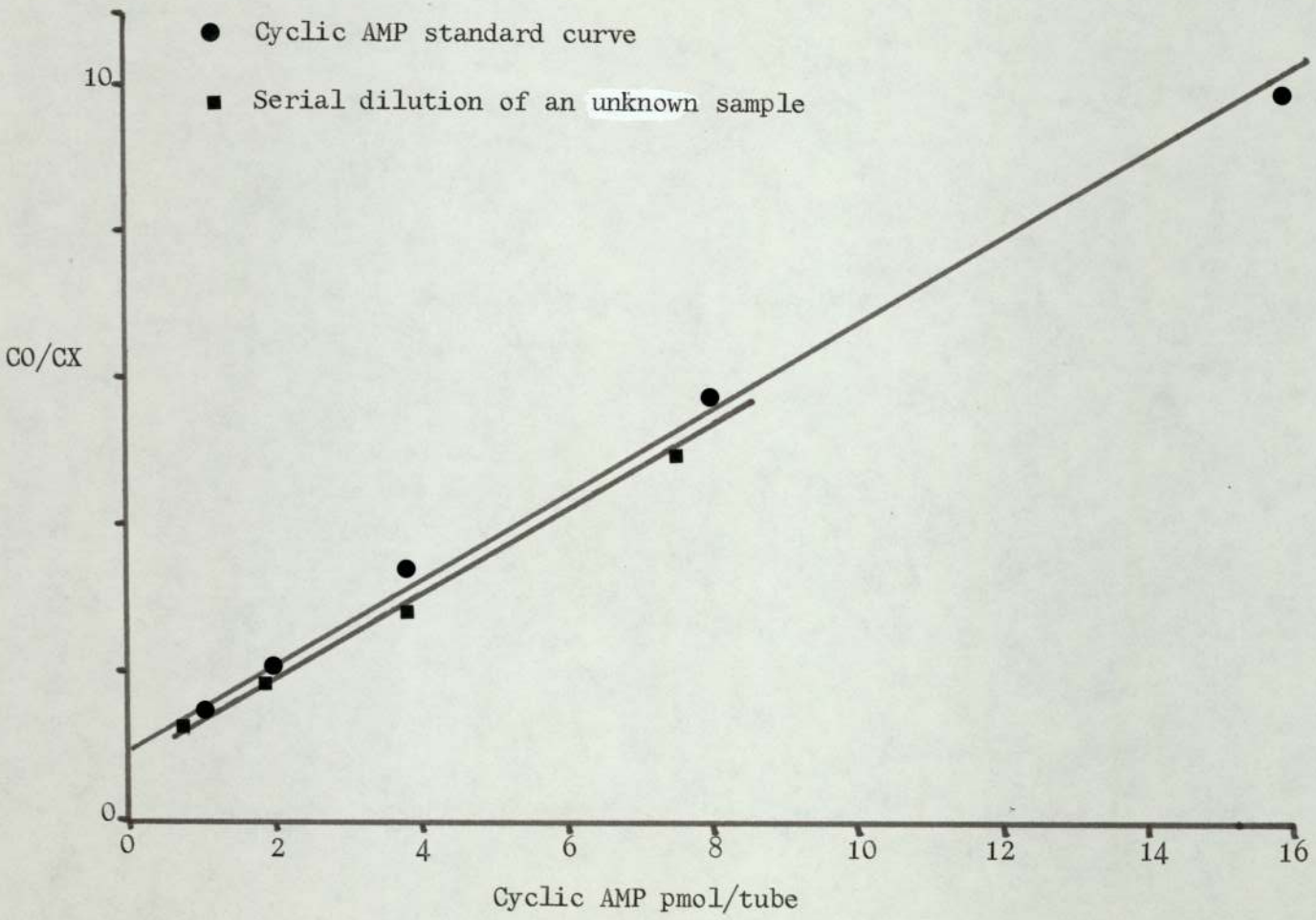


Figure 48 Comparison of a standard curve for cyclic AMP with an unknown sample assayed after serial dilution.



The effect of the extraction procedures on the assay for cyclic AMP

In order to investigate the possibility that one or both of the two extraction procedures (i.e. either T.C.A. or acid-ethanol) might interfere with the binding of cyclic AMP to the binding protein, assays were performed firstly on standards prepared in distilled water, and then secondly on standards prepared in T.C.A. and extracted with ether or prepared in acid-ethanol and evaporated as described previously (page 86).

Profiles of islet cyclic AMP content and efflux were compared with profiles of insulin secretion from islets of lean and obese mice in response to various concentrations - of glucose, glucagon and gastro-intestinal hormones.

Results

The effect of T.C.A.-ether and acid-ethanol extraction procedures on the cyclic AMP standard curve are shown in figure 49. Neither T.C.A.-ether nor HCl-ethanol extraction procedures had any significant effect upon the binding of cyclic AMP to the binding protein. Consequently, T.C.A.-ether extraction was adopted for the preparation of islet cyclic AMP extracts from lean and obese-hyperglycaemic mice. The results were expressed as pmol. cyclic AMP/mg freeze dried islet tissue and pmol.cyclic AMP/ml. of perifusate.

The control secretion of insulin and the content and efflux of cyclic AMP from perifused islets of lean and obese mice in response to 3mM glucose is shown in figure 50. The concentration of the nucleotide in both lean and obese mouse islets in the presence of 3mM glucose fluctuated between 0.5 and 2.0 pmol/mg. The concentration of cyclic AMP lost to the perifusate (efflux) in response to 3mM glucose fluctuated between

zero and 1 pmol./ml for both lean and obese mouse islets (Figure 50).

Increasing the glucose concentration of the perifusion medium from 3mM to 25mM produced a significant ($p<0.05$) stimulation of insulin secretion especially from obese mouse islets (figure 51a). In addition a slight but insignificant early increase in cyclic AMP content was observed for both lean and obese islets which in both cases returned to control levels after approximately seven minutes (figure 51b).

The efflux of cyclic AMP from both lean and obese mouse islets during perifusion with 25mM glucose showed similar slight, but insignificant fluctuations (figure 51c). There was no significant difference between the cyclic AMP content of lean and obese mouse islets in response to either 3 or 25mM glucose.

Pancreatic glucagon (5.0 μg/ml) in the presence of 3mM glucose produced an immediate significant ($p<0.05$) stimulation of insulin secretion from islets of both lean and obese mouse islets compared to controls (figure 52a). The secretion of insulin in response to exogenous glucagon was significantly higher from obese mouse islets than from lean mouse islets. Glucagon also produced a prompt, significant early increase in cyclic AMP content and efflux from both lean and obese mouse islets (figures 52b and c).

Maximum levels of insulin secretion and cyclic AMP content and efflux were observed after 7-10 minutes. After this time the rate of insulin release and the content and efflux of ^{c}AMP from islets of both lean and obese mice started to decline and had returned to approximately control levels by 30 minutes.

Pancreozymin (1.5 Ivy Dog U/ml) in the presence of 3mM glucose elicited a significant stimulatory effect upon the release of insulin from both lean and obese mouse islets (figure 53a). In both cases this

elevated rate of insulin secretion was maintained for some 20 minutes. Pancreozymin also produced a significant, concomitant elevation of cyclic AMP content and efflux from both lean and obese mouse islets (figures 53b and c).

Secretin (0.2 clinical U/ml) in the presence of 3mM glucose also produced an immediate, significant stimulation of the first phase of insulin secretion from islets of both lean and obese mice (figure 54a).

However, this concentration of secretin had no significant effect upon either the content or efflux of cyclic AMP from islets of either lean or obese hyperglycaemic mice (figs. 54b and c).

Figure 49 The effect of different extraction procedures on the cyclic AMP standard curve

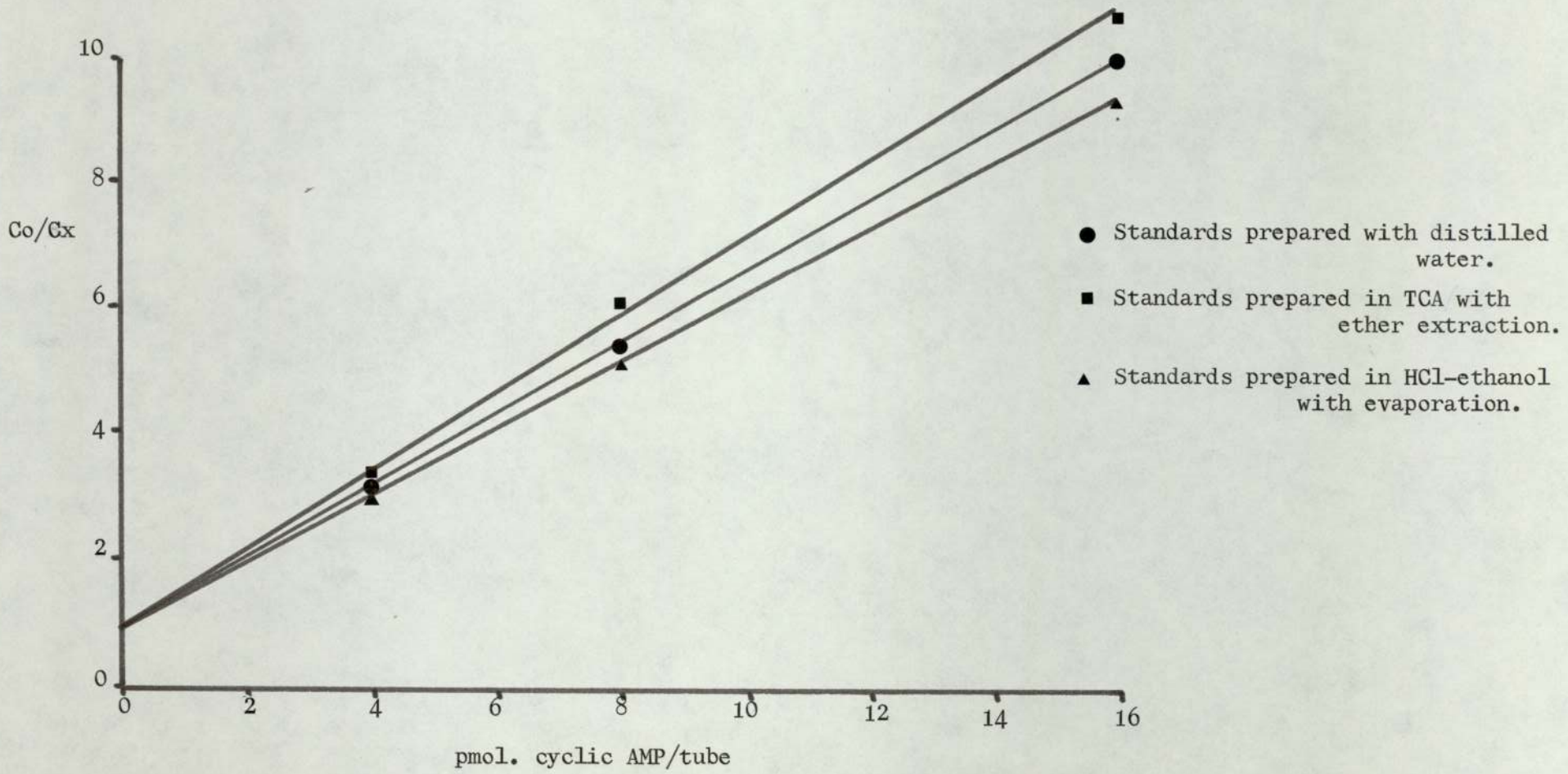


Figure 50 The effect of 3mM glucose on the insulin and cyclic AMP dynamics of lean and obese hyperglycaemic mouse islets.

(Each profile represents the mean of 5 separate profiles ± s.e.m., one profile per mouse)

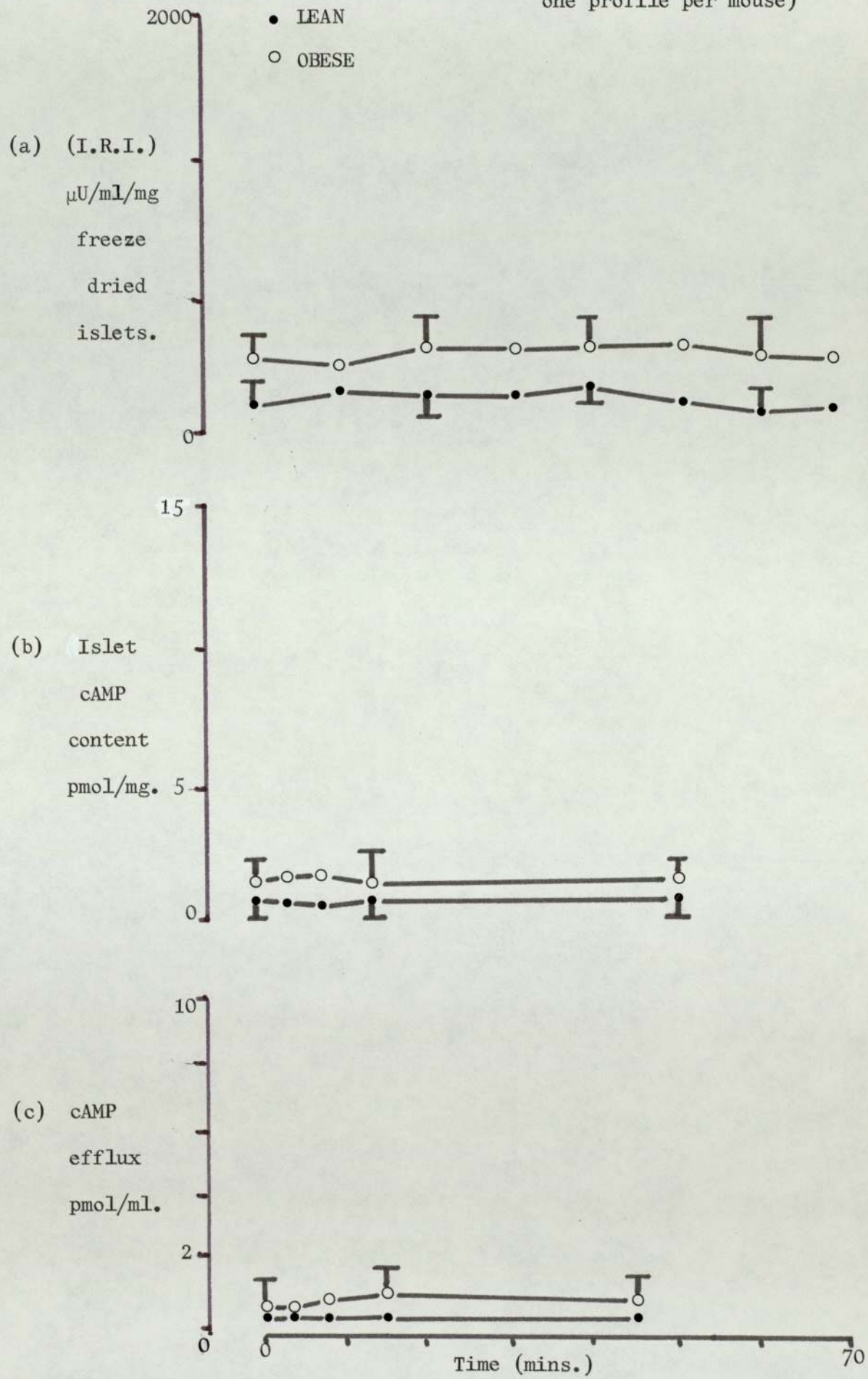


Figure 51 The effect of 25mM glucose on the insulin secretion profile and cyclic AMP dynamics of lean and obese hyperglycaemic mouse islets. (Each profile represents the mean of 5 separate profiles ± s.e.m., one profile per mouse)

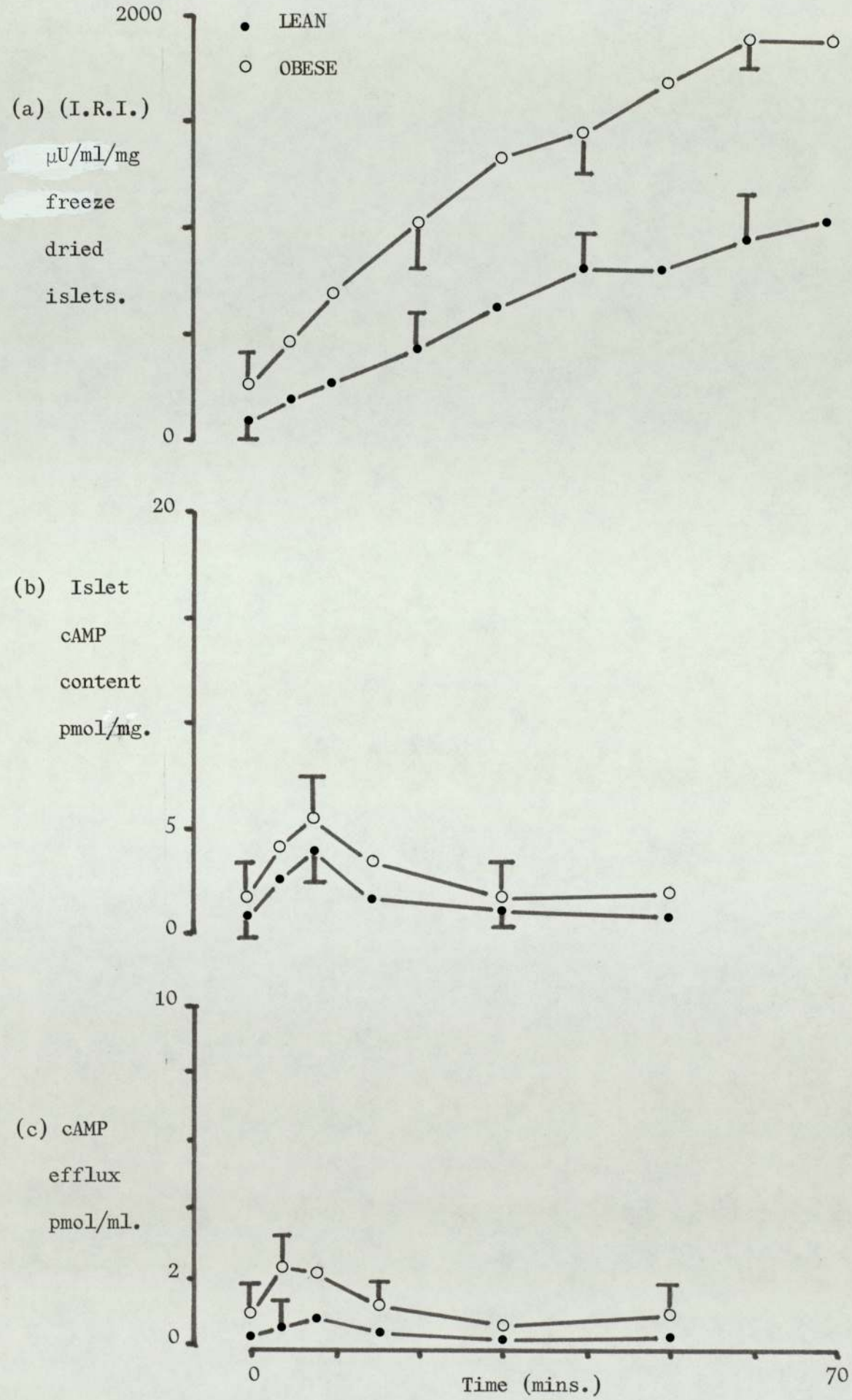


Figure 52 The effect of glucagon (5.0μg/ml) + 3mM glucose on the insulin and cyclic AMP dynamics of lean and obese-hyperglycaemic mouse islets. (Each profile represents the mean of 5 separate profiles ± s.e.m., one profile per mouse).

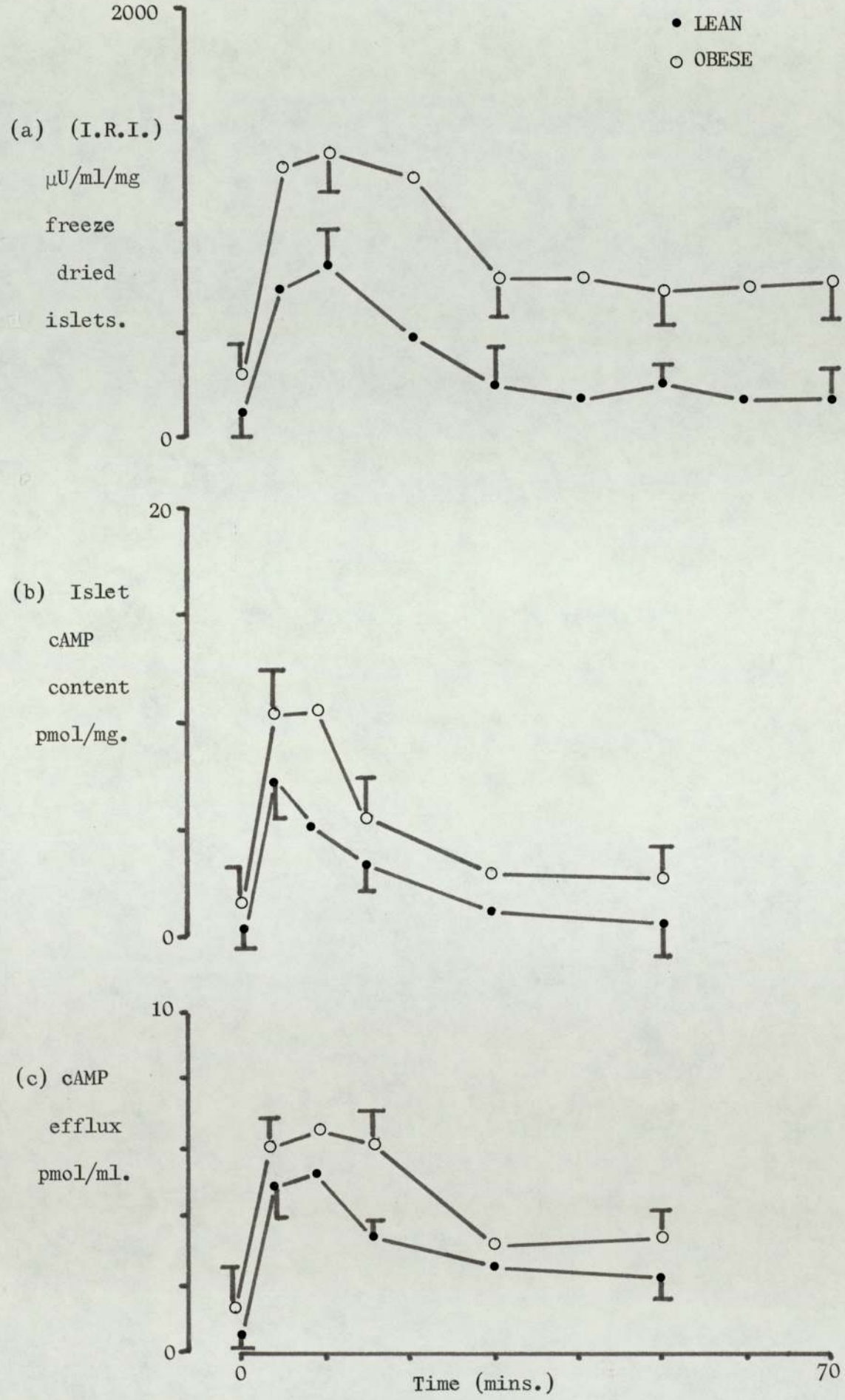


Figure 53 The effect of pancreozymin (1.5 IDU/ml) + 3mM glucose on the insulin and cyclic AMP dynamics of lean and obese-hyperglycaemic mouse islets

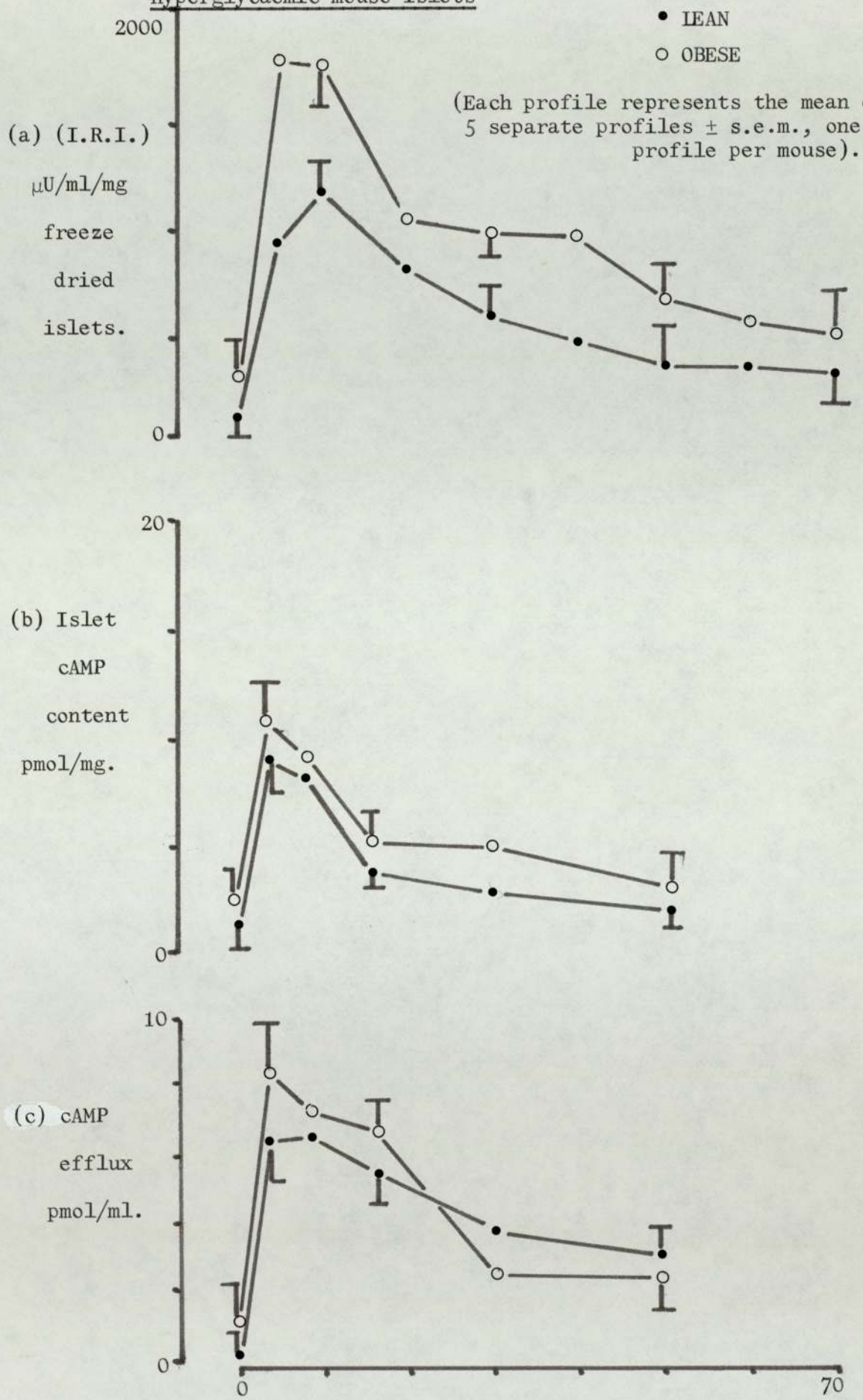
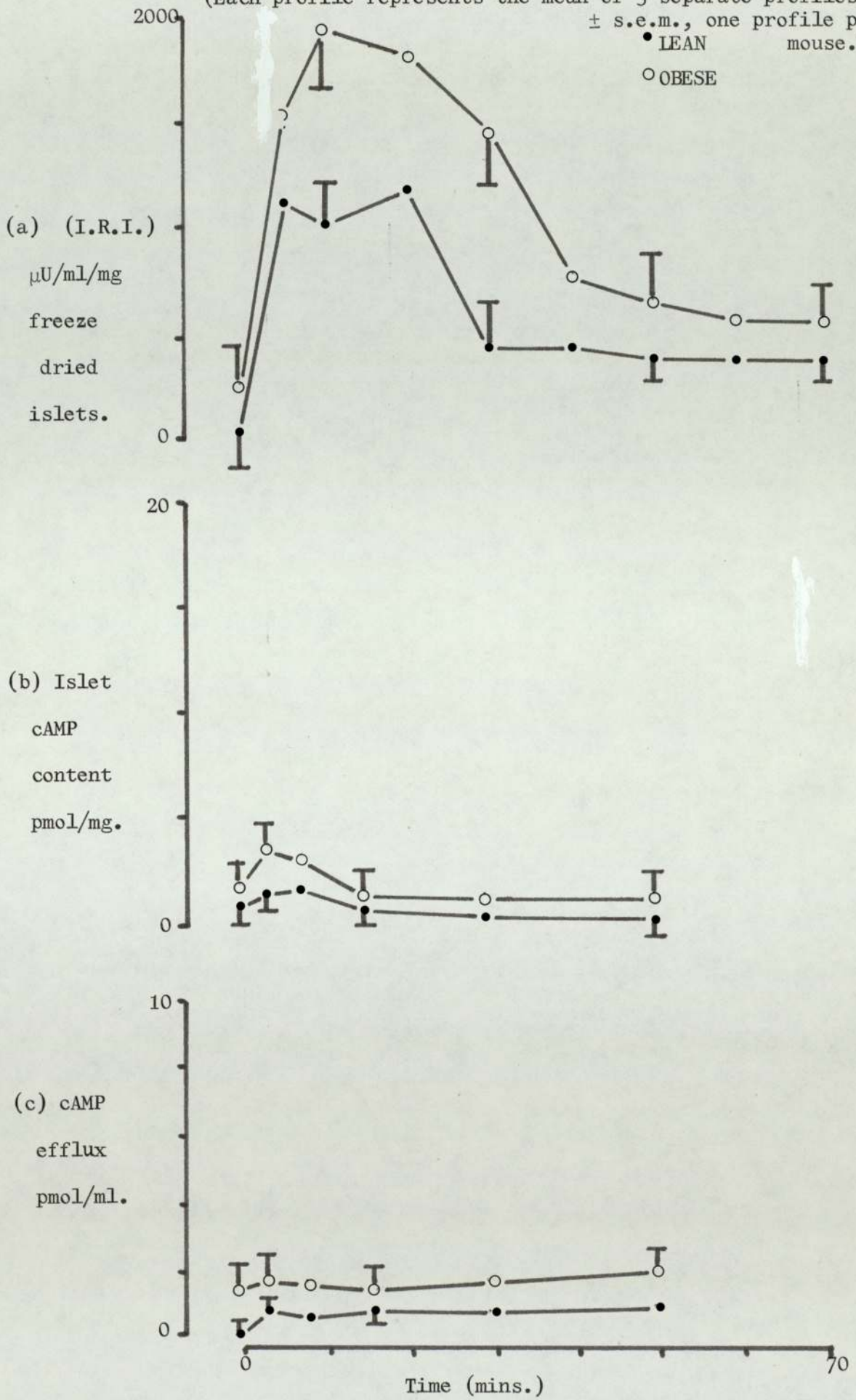


Figure 54 The effect of secretin (0.2 clin U/ml) + 3mM glucose on the insulin and cyclic AMP dynamics of lean and obese-hyperglycaemic mouse islets.

(Each profile represents the mean of 5 separate profiles ± s.e.m., one profile per mouse.)



Discussion

The perifusion of isolated pancreatic islets is a highly suitable model for the study of dynamic changes in islet cyclic AMP content and efflux over short time intervals. The secretion of insulin and the turnover of cyclic AMP within the β-cell are dynamic processes and it is possible that rapid, short lived fluctuations in cyclic AMP concentration might be overlooked when using long term static incubation techniques.

The mean control level of cyclic AMP in both lean and obese mouse pancreatic islets was found to be approximately 1.0 pmol/mg. This figure compares well with the data of Cooper, Ashcroft and Randle (365) and Montague and Cook (356).

25mM glucose initiated a progressive stimulation of the rate of insulin secretion, but had little effect upon either lean or obese mouse islet cyclic AMP levels except for small but insignificant fluctuations after 3 minutes. This finding supports the earlier reports that glucose was without effect upon islet adenylate cyclase activity (328, 338, 345, 362, 364) and islet cyclic AMP content (336, 356, 365, 382).

In a recent publication, Hellman and colleagues (327) using a preparation of perifused obese mouse islets, detected a slight but insignificant increase in islet cyclic AMP content after approximately 3 minutes, in response to 20mM glucose. However, in the presence of a cyclic AMP phosphodiesterase inhibitor, this concentration of glucose produced a significant elevation of islet cyclic AMP levels. In the present studies a phosphodiesterase inhibitor was not added to the perifusion medium. It was decided that when using islets free of acinar tissue, the presence of an exogenous pharmacologically active ^{c}AMP-phosphodiesterase inhibitor, in high concentration, was not consistent with the normal physiological situation.

It has been suggested that glucose, whilst not affecting adenylate cyclase activity directly, might initiate a signal capable of enhancing β-cell membrane ATP formation (327). Howell, Green and Montague (383) and Selawry and colleagues (384) have proposed that glucose, or a glucose metabolite, might exert long term stimulatory effects upon adenylate cyclase activity in the β-cells.

Glucose has been shown to exert a major stimulatory effect upon the second phase of insulin secretion via the metabolism of the sugar within the β-cell (313, 323, 364). However, many investigators claim that glucose induced insulin secretion tends to occur before changes in the levels of glucose metabolites can be detected in the islet (385, 386). This has suggested that the glucose molecule per se might initiate insulin secretion via its interaction with a β-cell plasma membrane-bound glucoreceptor (385, 386, 387).

The effect of glucose on islet cyclic AMP dependent protein kinase has also recently been investigated. Montague and Howell (388) were unable to demonstrate a stimulation of rat islet cyclic AMP dependent protein kinase activity by glucose. However, in a recent study, Davis and Lazarus (389) were able to demonstrate a stimulation of cod islet plasma membrane protein phosphokinase activity by glucose-6-phosphate and phosphoenolpyruvate suggesting that glucose or one of its metabolites might directly stimulate some component of the physical secretion mechanism.

In the present studies pancreatic glucagon, an established activator of adenylate cyclase activity in many tissues including pancreatic islets (338, 345, 362, 390, 391) produced a concomitant stimulation of both insulin secretion and islet cyclic AMP content and efflux. This suggests that glucagon-stimulated insulin secretion might be a direct

consequence of cyclic AMP accumulation within the islet.

Profiles of insulin secretory activity and cyclic AMP accumulation and efflux were also found to be comparable following stimulation with pancreozymin (figure 53). There is some evidence to suggest that pancreozymin might directly stimulate adenylate cyclase activity in both pancreatic exocrine (371, 372) and endocrine tissue (328, 338). However, high concentrations of pancreozymin have also been shown to inhibit cyclic AMP phosphodiesterase activity in exocrine tissue (392). Despite the results of the present studies reports have suggested that pancreozymin was not likely to alter islet cyclic AMP levels sufficiently to influence insulin secretion (393, 394). It has also been suggested that pancreozymin might stimulate the release of insulin either via a cholinergic (395) or an α-adrenergic mechanism (373).

Similar controversy exists as regards the mechanism of secretin-stimulated insulin secretion. The structural similarity between secretin and glucagon suggest that secretin might utilise a second messenger such as cyclic AMP in its mechanism of action and several investigators have shown the hormone to be capable of elevating cyclic AMP levels in exocrine pancreatic tissue (371, 372, 396). It has been suggested that secretin and glucagon binding sites are distinct in liver and fat cell plasma membranes (397), and it has yet to be established conclusively whether secretin stimulates the secretion of insulin via the activation of β-cell adenylate cyclase. In the present studies, secretin did not increase either the content or efflux of cyclic AMP from islets of lean or obese hyperglycaemic mice. This finding is at variance with previous reports suggesting secretin to be capable of stimulating adenylate cyclase activity in isolated mouse islets (338, 345).

Secretin has been shown to elevate guanylate cyclase activity in rat islets suggesting that the hormone might initiate insulin secretion via some cGMP requiring mechanism (398).

In the present investigation, elevated levels of islet cyclic AMP were accompanied by a similar elevation in cyclic AMP efflux. This might suggest that the release of cyclic AMP into the perifusate was merely a passive process whose magnitude was related to the intracellular levels of the nucleotide. Cerasi (367) recently demonstrated that cyclic AMP efflux showed a better correlation with the dynamic secretion of insulin than the accumulation of cyclic AMP within islets. Thus, it was suggested that in the pancreatic islets, as in the perfused liver (399), cyclic AMP efflux could provide a more representative picture of the physiological status of the tissue than its cyclic AMP content. It appears that a large proportion of the cyclic AMP generated within the β-cell might not participate in the events leading to the secretion of insulin but might just leak passively from the cell. Leitner and colleagues (400) proposed that the secretory granule fraction of the β-cell might contain cyclic AMP and other adenine nucleotides which would play an active role in the secretory process and subsequently contribute to c AMP efflux.

The present data suggest that intracellular cyclic AMP levels might play a role in the phasic release of insulin, particularly in response to gastrointestinal hormone stimulation. Burr and coworkers (401) have shown that theophylline, which raises islet cyclic AMP content by an inhibition of cyclic AMP phosphodiesterase activity, also stimulates the first phase of insulin secretion in the absence of glucose. This suggested that cyclic AMP might have a direct effect upon or be implicated in the first phase of insulin secretion, although the

presence of a metabolic substrate, i.e. glucose, is required to provide the major drive for the second phase of insulin secretion.

Cerasi (367) has devised a hypothetical model in which he suggests the insulin secretory process to be controlled by two separate pools of cyclic AMP; one, a specific pool sensitive to glucose, and the other, a non-specific pool under the influence of hormones and other insulinotropic agents.

In the present experimental work, glucose and gastrointestinal hormones appeared to elicit distinct patterns of insulin secretion. Glucagon and gastrointestinal hormones, such as pancreozymin, might initiate an early prompt stimulation of insulin secretion by increasing islet cyclic AMP levels, thus 'priming' the β-cell for a subsequent glucose challenge and also perhaps potentiating the stimulatory effect of glucose itself. The stimulation of the first phase of insulin secretion by secretin might involve cyclic GMP rather than cyclic AMP and the cumulative effect of gastrointestinal hormones might be of particular consequence during the ingestion of a mixed meal, when the pancreas is subjected to sudden increases in glucose amino acid and fatty acid concentrations in the blood. An "early warning" system for the secretion of insulin might counteract the rapid onset of hyperglycaemia seen after the absorption of glucose.

Lean and obese mouse islets appear to contain similar amounts of cyclic AMP per unit weight of tissue. The increased volume of islet tissue in the obese mouse pancreas might be of importance in the development of hyperinsulinism in these animals. Cerasi and Luft (402) proposed that three possible defects in cyclic AMP metabolism could result in an impaired insulin secretion. These were: a decreased adenylate cyclase activity, an increased cyclic AMP phosphodiesterase activity, both leading

to reduced intracellular cyclic AMP levels, and finally a decreased sensitivity of the insulin secretory mechanism to cyclic AMP. Atkins and Matty (364) have shown obese mouse islets to have higher adenylate cyclase and cyclic AMP phosphodiesterase activities than lean mouse islets. It thus seems possible that a higher rate of turnover of cyclic AMP might also be important in the development of hyperinsulinism in the obese hyperglycaemic mouse.

CHAPTER VI

Gastrointestinal hormone like activity in extracts of duodenum-jejunum from lean and obese-hyperglycaemic mice.

INTRODUCTION

In previous chapters obese mouse islets have been shown to be hyper-responsive to glucose, glucagon and certain gastrointestinal hormones, suggesting that an increased secretion of insulin in response to intestinal factors might contribute towards the elevated levels of circulating insulin seen in these animals. The possibility exists that an excessive secretion of gastrointestinal hormones in response to the ingestion of nutrients (e.g. glucose, amino acids) might contribute to the enhanced rate of insulin secretion. Crude acid-ethanol extracts of obese mouse duodenum-jejunum appear to possess a greater insulin-releasing capacity than extracts prepared from the intestinal mucosa of lean mice (see Chapter IV).

The present studies were carried out in order to compare the content of potential insulinotropic gastrointestinal hormones in both lean and obese mouse duodenal-jejunal extracts.

SECTION A Column chromatography of duodenal-jejunal extracts from lean and obese-hyperglycaemic mice

Materials and Methods

The practical and theoretical details of gel filtration techniques have been described extensively (403, 404, 405). The present studies utilised crude acid-ethanol extracts of either lean or obese mouse duodenum-jejunum (designated 'TOT'; 131) which were prepared by the method described previously in Chapter IV, page 58.

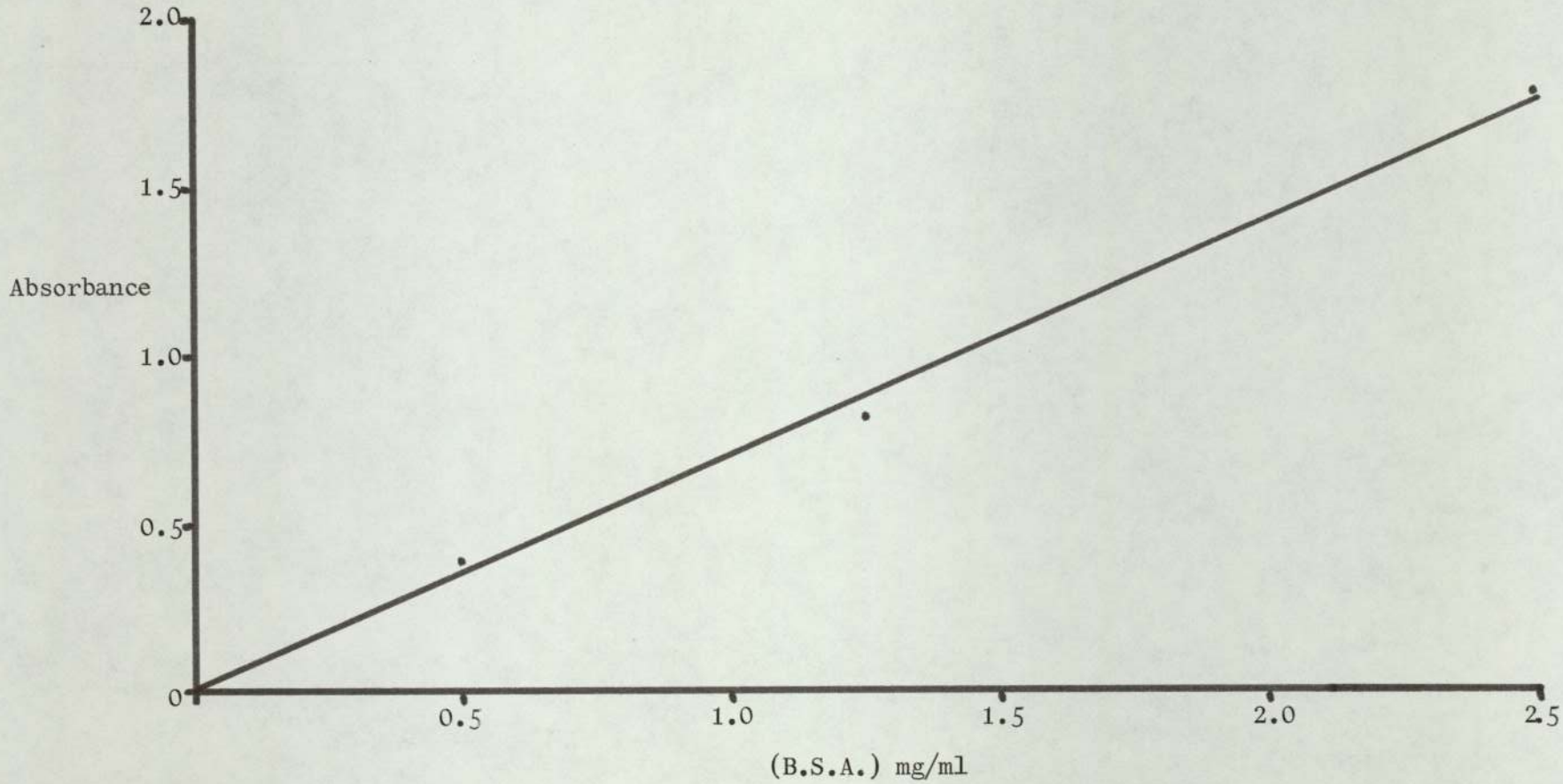
A 1.5 x 30 cm column of Sephadex G50 Fine (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden) was prepared using a glass K15/30 column (Pharmacia) previously rinsed with Repelcote (Hopkin & Williams Ltd, Chadwell Heath, Essex) to prevent the adsorption of protein. The intestinal extracts were carefully applied to the column in 1 ml aliquots at a concentration of 200 mg wet weight intestinal tissue/ml. Acetic acid (IM) was used as the eluent at room temperature (21°C).

Secretin, pancreozymin and glucagon (Sigma Chemical Co. Ltd., London) and Synthetic Human Gastrin I (I.C.I., Macclesfield, Cheshire) were run separately as standards.

1ml samples of effluent were collected in Repelcote ^{R} - coated test tubes, and the absorbance measured at 276 nM on a Beckman DB spectrophotometer.

A bovine serum albumin standard curve (fraction V, insulin free, Armour Pharmaceuticals Ltd., Eastbourne, Sussex) was constructed using the absorbance at 276 nm of a range of standard B.S.Alb. solutions (0-5 mg/ml), figure 55. The protein concentration in samples of column effluent was estimated from this standard curve and expressed in B.S.Alb. equivalents. The protein concentration in each aliquot was then plotted against elution volume and the total protein content of each eluted protein peak estimated by measuring the area under the curve.

Figure 55 Bovine Serum Albumin (fraction V) standard curve, 276 nm



Results

The elution profiles for lean and obese mouse intestinal extracts and for the standard gastrointestinal hormone preparations used are shown in Figures 56 to 61. Both lean and obese mouse intestinal extracts separated into two distinct zones of high absorbance, one peak lying between the volumn void volume and 0.28 of the column volume, the other lying between 0.64 and 0.84 of the column volume. These peaks were arbitrarily designated I and II respectively (Figures 56 and 57). The content of protein in peaks I and II was approximately equal for both lean and obese mouse intestinal extracts, although in each case the second fraction eluted in a somewhat larger volume. Zones I and II of the lean mouse intestinal extract contained some 8.75 mg and 7.0 mg of protein respectively while the obese mouse intestinal extract contained 18.5 mg and 23.75 mg of protein respectively.

The elution profiles for the standard gastrointestinal hormone preparations are shown in Figures 58 to 61. Crude Secretin (Sigma Chemical Co.Ltd.) eluted in a peak between 0.8 and 0.9 of the column volume; crude pancreozymin (Sigma Chemical Co.Ltd.) (Figure 59) between 0.76 and 0.84 of the column volume and synthetic human gastrin (I.C.I.) (Figure 61) between 0.7 and 0.8 of the column volume.

Figure 56 The Elution profile for lean mouse intestinal extract using Sephadex G.50 Fine

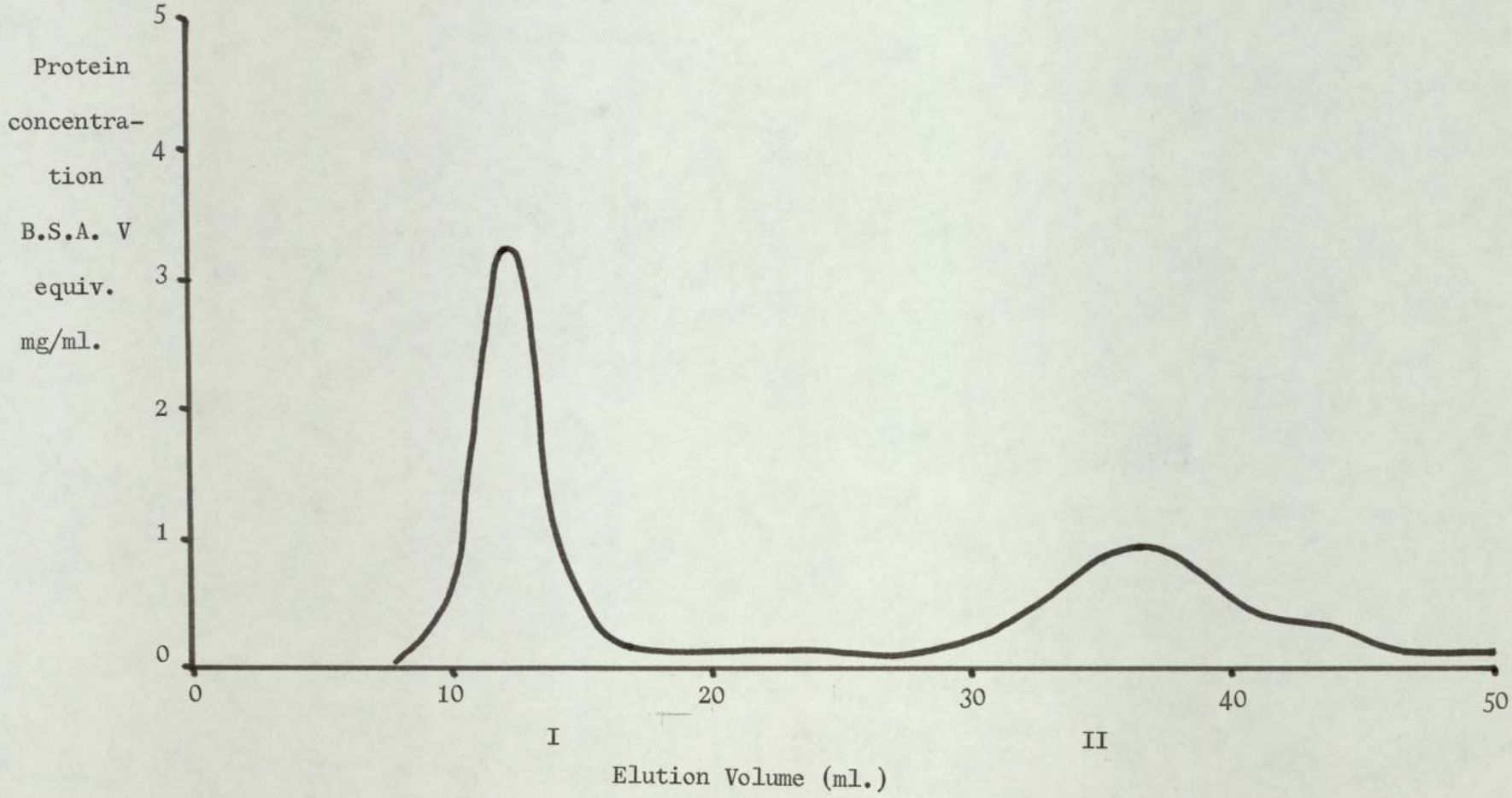


Figure 57 The Elution profile for obese mouse intestinal extract using Sephadex G.50 Fine

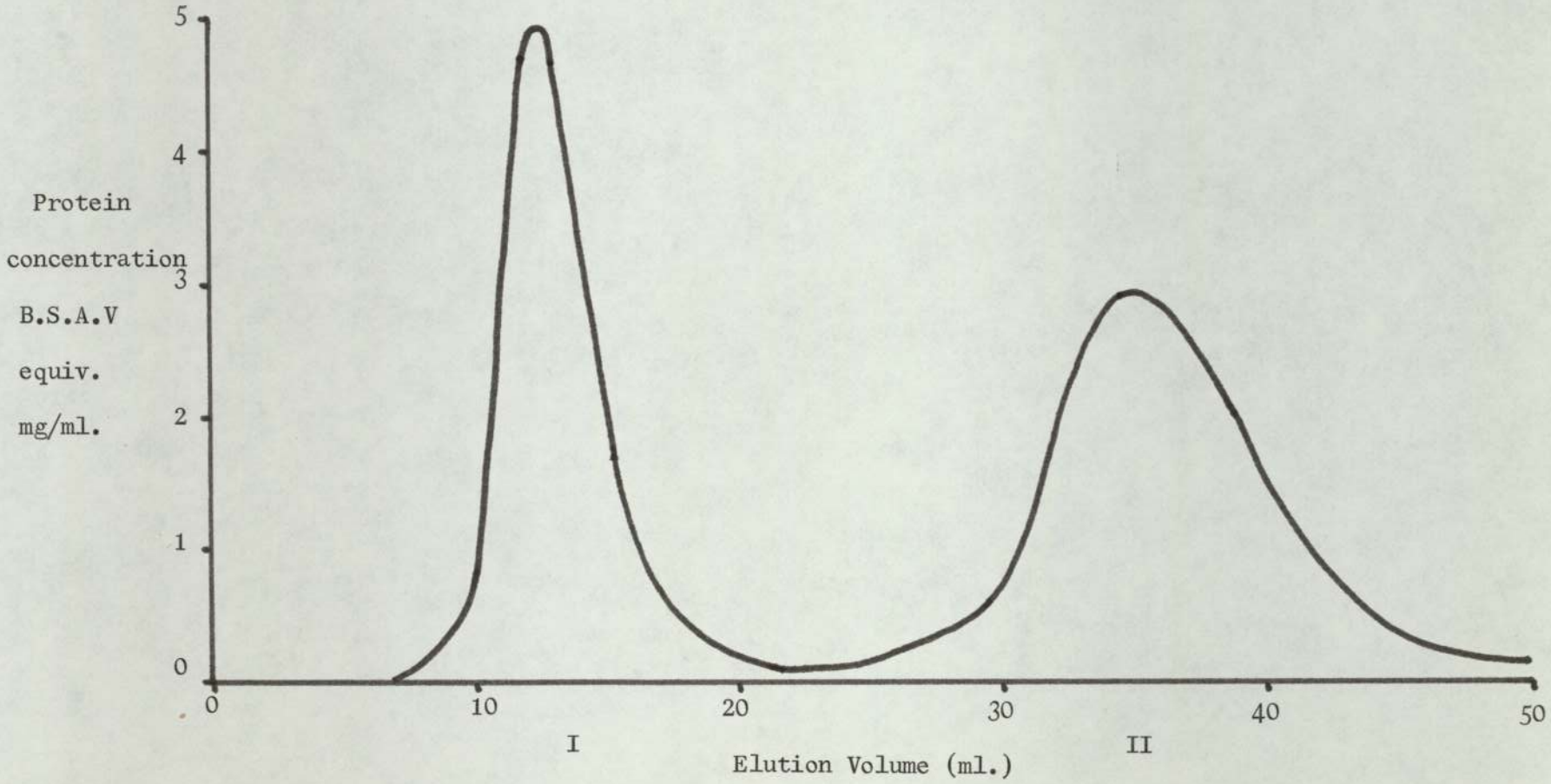


Figure 58 The Elution Profile of crude secretin (2 mg/ml.) using Sephadex G50 Fine

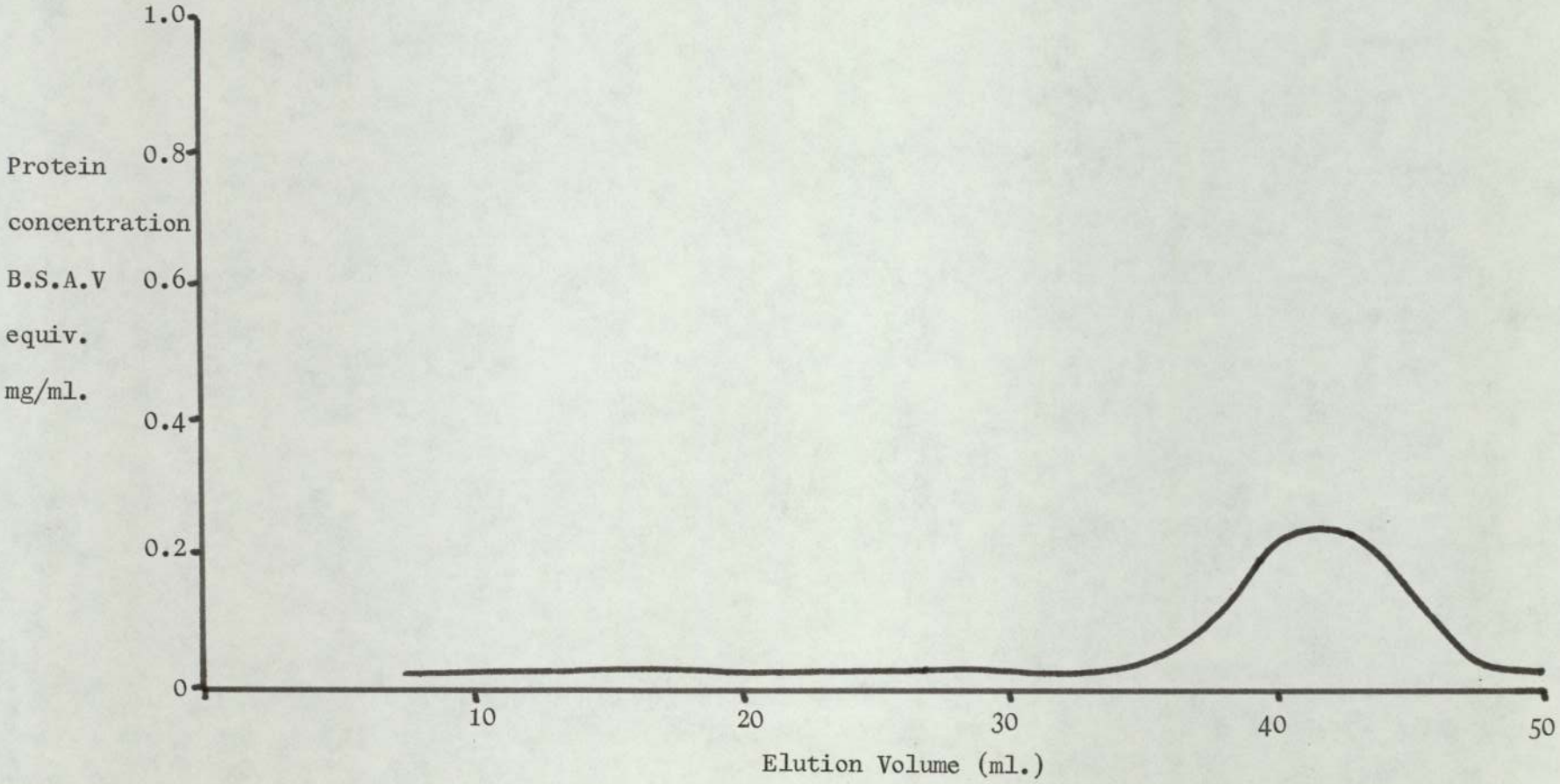


Figure 59 The elution profile of crude pancreozymin (2.0 mg/ml) using Sephadex G.50 Fine

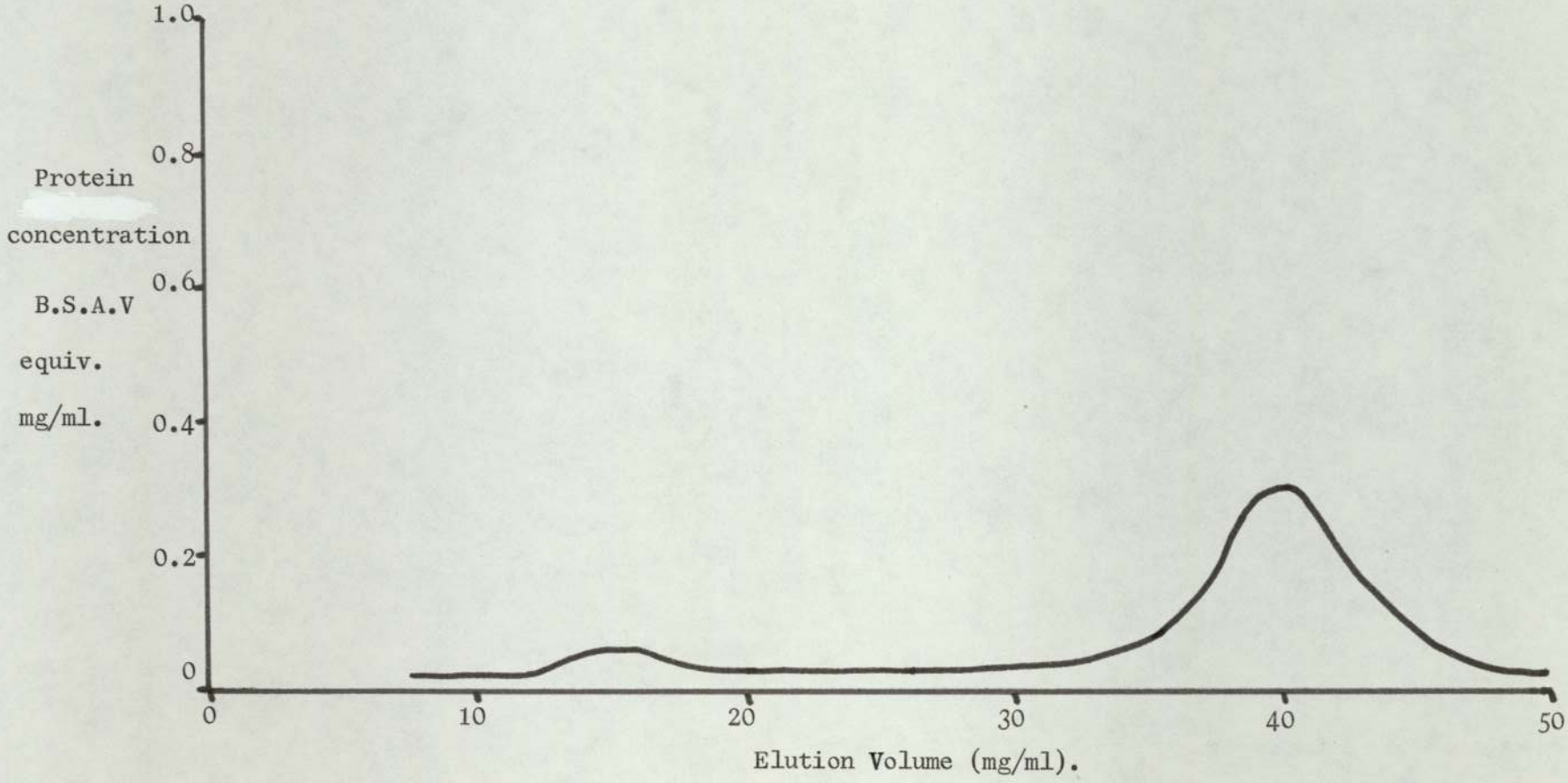


Figure 60 The elution profile of pancreatic glucagon (5.0 mg/ml) using Sephadex G.50 Fine

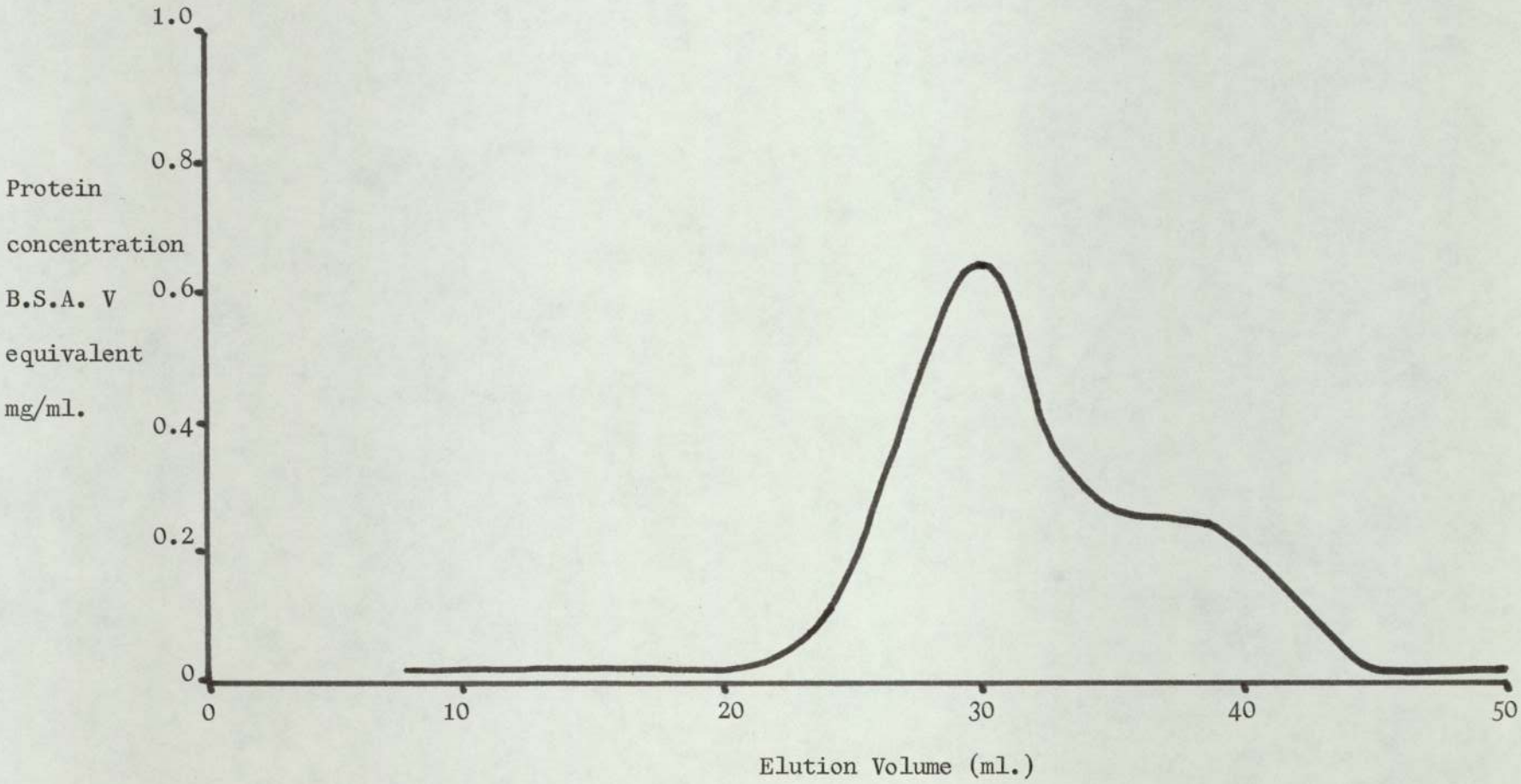
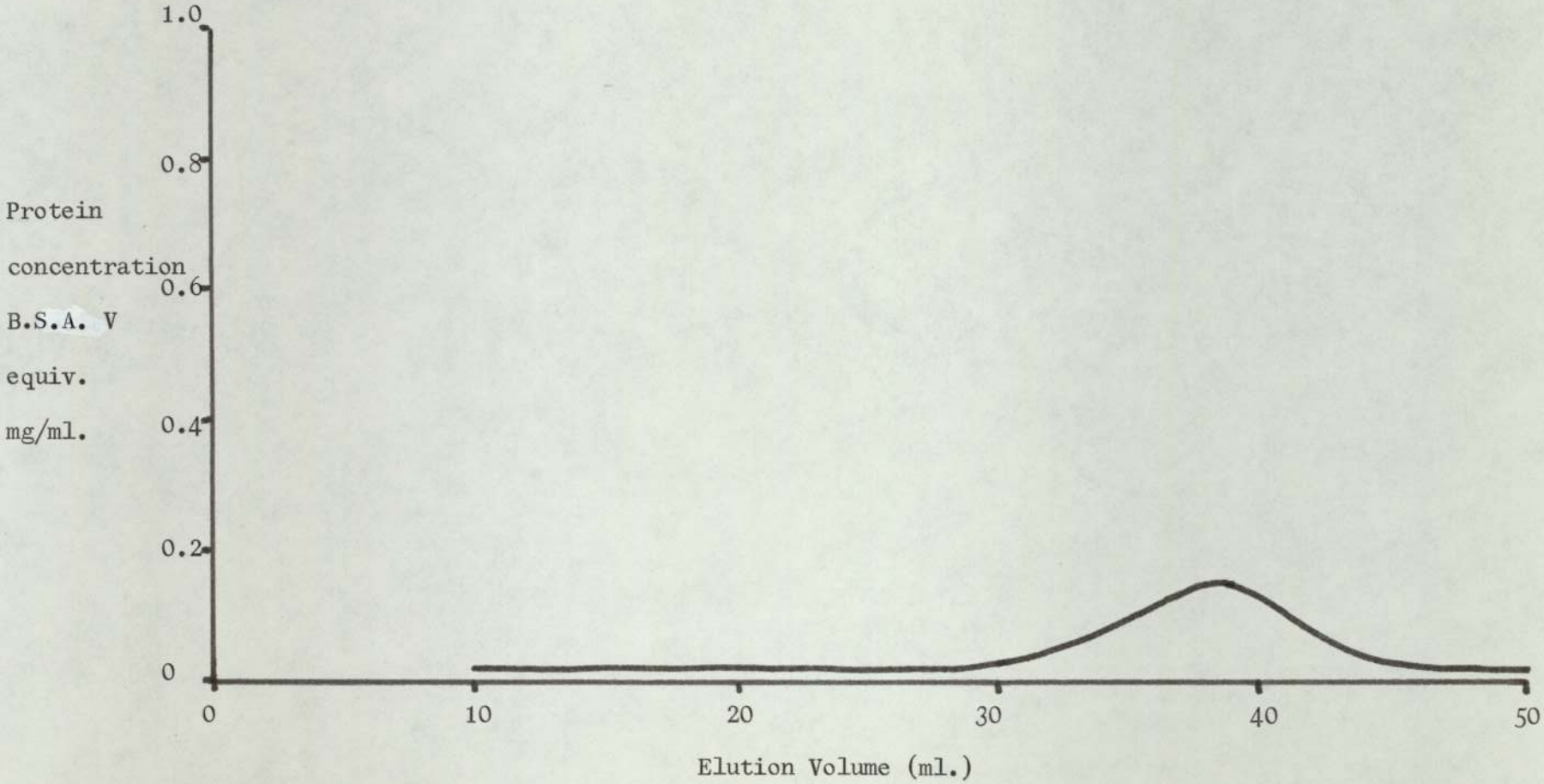


Figure 61 The elution profile of Synthetic Human Gastrin I (1 mg/ml) using Sephalex G.50 fine



Discussion

These profiles illustrated above are consonant with the observations of Moody et al (131) who showed that a crude acid-alcohol extract of porcine intestine could be separated into 2 peaks by Sephadex gel filtration. These workers demonstrated that the components of peak I had no insulin-releasing capacity, whilst the components of peak II were capable of stimulating insulin secretion from rat islets in vitro. The further fractionation of peak II by these workers revealed components containing secretin, pancreozymin and glucagon-like activities. In the present studies, the standard gastrointestinal hormone preparations eluted within the zone represented by peak II of the extracts. Thus, it seems likely that peak II of both lean and obese mouse intestinal extracts contains the intestinal polypeptides secretin; pancreozymin; gastrin and possibly glucagon-like material. It is suggested that the presence of these gastrointestinal hormones in lean and obese mouse intestinal extracts is responsible for the insulinotropic activity previously demonstrated for these extracts in Chapter IVa, page 62. During the fractionation of porcine intestinal extracts, Moody and colleagues demonstrated that fractions containing insulin releasing activity eluted close to fractions containing glucagon-like immunoreactivity although the in vitro system of insulin secretion adopted in their study failed to respond to purified preparations of secretin or pancreozymin.

In the present studies it was not found possible to fully quantitatively resolve each of the gastrointestinal hormones in peak II of the intestinal extracts on Sephadex G50 fine because of their very similar molecular weights and sizes.

The total summed protein content of peaks I and II of obese mouse intestinal extracts was found to be significantly higher than the total summed protein content of peaks I and II of lean mouse intestinal extracts (per mg. original weight of intestinal tissue).

These data suggest the possibility of an increased number and hormone content of endocrine cells in the obese mouse intestine compared to that of the lean littermate. This observation has recently been substantiated by Polak and colleagues (406) who were, however, unable to ascertain whether the differences observed in the gastrointestinal tract of the obese mouse were primary or secondary to the metabolic abnormalities actually seen in these animals. Nevertheless, in the light of this and the present data it seems possible that an increased production of insulinotropic substances from the intestine of the obese mouse in response to ingested food might contribute to the excessive release of insulin from the pancreatic islets of these animals.

SECTION B The estimation of Secretin and Pancreozymin-cholecystokinin like activity in lean and obese mouse intestinal extracts using a rat bioassay technique.

In the previous section (Section A, page 104)it was suggested that obese mouse intestinal extracts might contain a higher proportion of gastrointestinal hormones per unit weight than lean mouse intestinal extracts.

In order to more closely examine the secretin and pancreozymin content of lean and obese mouse intestinal extracts a rat bioassay system has been adopted which is based on the ability of the hormones to stimulate the rate of flow and protein content of pancreatic juice. At the time of these investigations, no convenient radioimmunoassay system was available for the measurement of secretin and pancreozymin.

The bioassay of secretin and pancreozymin

In the rat, pancreozymin stimulates both the flow of juice and the secretion of enzyme protein from the pancreas, whereas secretin stimulates only the flow of juice, having little effect on the secretion of enzyme protein (407). Several investigators have used these facts as the basis of a bioassay for the two hormones (408, 409, 410, 411, 412). Essentially, the secretin bioassay involves the intravenous injection of various doses of secretin via the cannulated jugular vein of an anaesthetised rat and the subsequent measurement of the rate of flow of pancreatic juice from the cannulated pancreatic duct. The technique can be conveniently modified for the simultaneous assay of both secretin and pancreozymin by determining the protein concentration in the pancreatic juice.

Materials and Methods

The Rat Bioassay Preparation

In these investigations, the method of Dockray (407) was used since it allowed the simultaneous bioassay of both secretin and pancreozymin.

Male Wistar rats weighing approximately 250g were fasted overnight before each experiment, but were allowed free access to water. The rats were anaesthetised with sodium pentobarbitone (Nembutal, Abbott Laboratories Ltd., Queenborough, Kent), 45 mg/kg body weight, administered intraperitoneally. The body temperature of the animals were maintained at 38°C during the course of experiments by means of a heated table. The bioassay preparation is illustrated in plate 3.

The right jugular vein was cannulated for the administration of test solutions using polythene tubing (Portex pp25) and a primary dose (0.5 clin.U.) of secretin (G.I.H. Research Unit, Karolinska, Sweden;) was administered in 0.2 ml 0.9% (w/v) saline. The distal end of the common bile duct was then cannulated using a 1" length of polythene tube (Portex pp10) at a point shortly before it entered the duodenum. The proximal end of the bile duct was then ligated above the junction of the bile duct and pancreatic duct. In this way, the flow of bile was abolished whilst the flow of pancreatic juice remained unaffected. A capillary tube calibrated in μl was attached to the free end of the pancreatic duct cannula and the volume of pancreatic juice produced (in μl) noted at intervals of 5 minutes. Following the injection of secretin, pancreozymin or intestinal extract via the jugular vein cannula. The flow rate at each five minute interval was expressed in terms of μl of pancreatic juice produced/5 min.

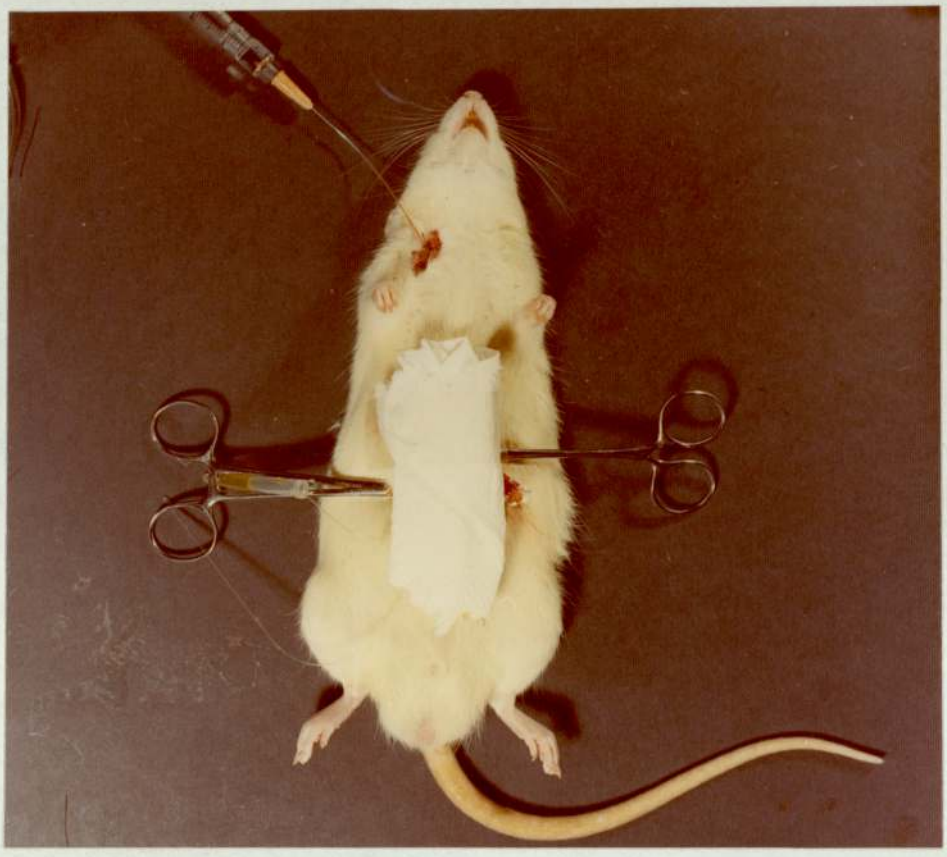


Plate 3. In vivo rat bioassay preparation for the estimation of secretin and pancreozymin-cholecystokinin like activity, in lean and obese mouse intestinal extracts.

The secretion of pancreatic juice in response to the injection of various doses of secretin or pancreozymin (for the production of dose response curves) was calculated by subtracting the basal rate of flow in the absence of any injected material from the peak rate of flow produced after the injection of hormone or extract. These corrected flow rate values were then plotted against the corresponding dose of hormone or extract injected (see figures 64 and 65).

At each five minute interval, the capillary tube on the pancreatic duct cannula were changed, and the 5 minute sample of pancreatic juice diluted with double distilled water to a final volume of 1ml. The absorbance at 276 nm of the sample was determined using a Beckman DB spectrophotometer, and the protein concentration of each sample estimated by reference to a bovine serum albumin (fraction V) standard curve as described in the previous section (Section A).

The rate of protein secretion at each five minute interval was expressed as mg of enzyme protein secreted/5 min. Dose response curves for the secretion of enzyme protein following pancreozymin or secretin injection were determined by subtracting the basal rate of enzyme protein secretion produced in the absence of injected material from the peak rate of enzyme protein secretion following the injection of hormone or extract. These corrected enzyme protein secretion rates were then plotted against the corresponding dose of hormone injected (figures 64 and 65).

A single rat preparation was able to tolerate five injections of either secretin, pancreozymin or intestinal extract. All test materials were administered to the rat in random order to eliminate the effect any reduced sensitivity of the rat pancreas might have on the recorded responses. The doses of secretin and pancreozymin used in these

studies ranged from 0.025-0.8 clinical units and 0.125-2.0 Ivy Dog units respectively.

Crude acid-ethanol extracts were prepared from either lean or obese mouse duodenum-jejunum as described in Chapter IV, (page 58) and administered via the jugular vein cannula in injection volumes of 0.125 and 0.25 ml at a concentration of 200 mg wet weight intestinal tissue/ml. The rate of flow of pancreatic juice (μl/5 min.) and rate of secretion of enzyme protein (mg/5 min) were measured at 5 minute intervals and the secretin-like activity and pancreozymin-like activity of the extracts estimated by reference to the respective hormone dose-response curves.

Results

The effect of secretin (0.025-0.8 Clin U.) on the rate of flow of pancreatic juice and the rate of secretion of enzyme protein are shown in Figure 62. A peak rate of flow of pancreatic juice was observed 10-20 minutes after the injection of secretin (Fig. 62A). There appeared to be a linear relationship between the peak rate of flow of pancreatic juice and the dose of secretin used. Over the range 0.025 clin.U. to 0.8 clin.U. (Figure 64a) secretin had little effect, however, on the concentration of enzyme protein in rat pancreatic juice (Figures 62b and 64b).

Figures 63a and b demonstrate the effect of pancreozymin on the rate of flow and enzyme protein content of rat pancreatic juice. Increasing the injected concentration of pancreozymin from 0.125 to 2.0 Ivy Dog units significantly increased the rate of flow and enzyme protein content of rat pancreatic juice. The peak flow of juice in response to pancreozymin occurred approximately 5 minutes after administration. This was in contrast to secretin (Figure 62a) which produced a peak response after some 10-20 minutes. Furthermore, the juice flow rate response to pancreozymin was shorter in duration than the secretin response. Again, there appeared to be a linear relationship between the dose of pancreozymin administered and both the peak rate of flow of pancreatic juice and the peak rate of secretion of enzyme protein (Figures 65a and b).

The effects of lean and obese mouse intestinal extracts (injection volume 0.25 ml) upon the rate of flow and enzyme protein content of rat pancreatic juice are shown in Figures 66a and b. The lean mouse intestinal extract produced a mean peak flow rate of some 7.5 ± 0.7 µl of pancreatic juice/5 min. By reference to the secretin and pancreozymin

dose response curves (Figures 64 and 65), this response was equivalent to that produced by 0.1 clinical U. of secretin or 0.15 Ivy Dog units of pancreozymin. The same extract produced a peak rate of enzyme protein secretion of some 0.4 ± 0.2 mg/5 min., which was equivalent to 0.07 Ivy Dog units of pancreozymin.

The obese mouse intestinal extract elicited a mean peak flow rate of 13 ± 0.95 µl of juice/5 min., equivalent to 0.5 clinical U. of secretin or 0.25 Ivy Dog units of pancreozymin. The corresponding mean peak rate of pancreatic juice enzyme protein secretion was 1 ± 0.4 mg/ml, which was equivalent to 0.125 Ivy Dog units of pancreozymin.

From the above data it was estimated that lean mouse intestinal extracts contained approximately 0.05 clin.U. Secretin/0.25 ml (≡ 0.20 clin.U/ml) and 0.07 Ivy Dog U. Pancreozymin/0.25 ml(≡ 0.28 I.D. U/ml). The corresponding values for obese mouse intestinal extracts were calculated as:

Secretin; 0.25 clin.U/0.25 ml (≡ 1.0 clin U/ml) and

Pancreozymin; 0.125 I.D.U./0.25 ml (≡ 0.5 I.D.U./ml).

Figure 62 The effect of secretin on the rate of flow of pancreatic juice and the rate of secretion of pancreatic juice enzyme protein in the rat

(Each profile represents the mean of 5 separate profiles; one profile per animal. s.e.m.'s have been omitted for clarity)

○ 0.025 clin.U. secretin
● 0.05 " " "
□ 0.1 " " "
■ 0.4 " " "
▲ 0.8 " " "

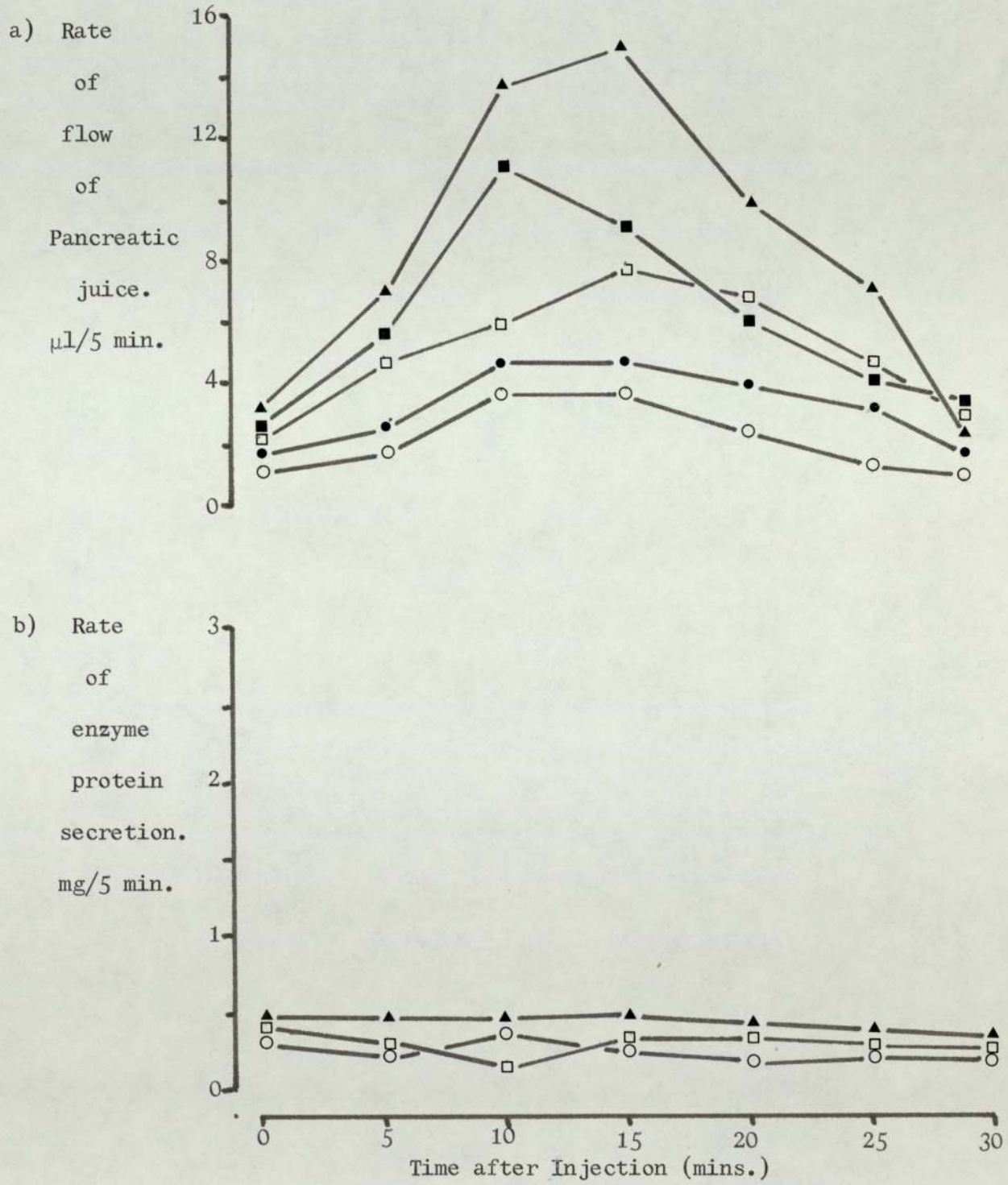


Figure 63 The effect of pancreozymin on the rate of flow of pancreatic juice and the rate of secretion of pancreatic juice enzyme protein in the rat

(Each profile represents the mean of 5 separate profiles, one profile per animal)

○ 0.125 Ivy dog units pancreozymin
● 0.25 " " " "
□ 0.5 " " " "
■ 1.0 " " " "
▲ 2.0 " " " "

s.e.m.'s have been omitted for clarity.

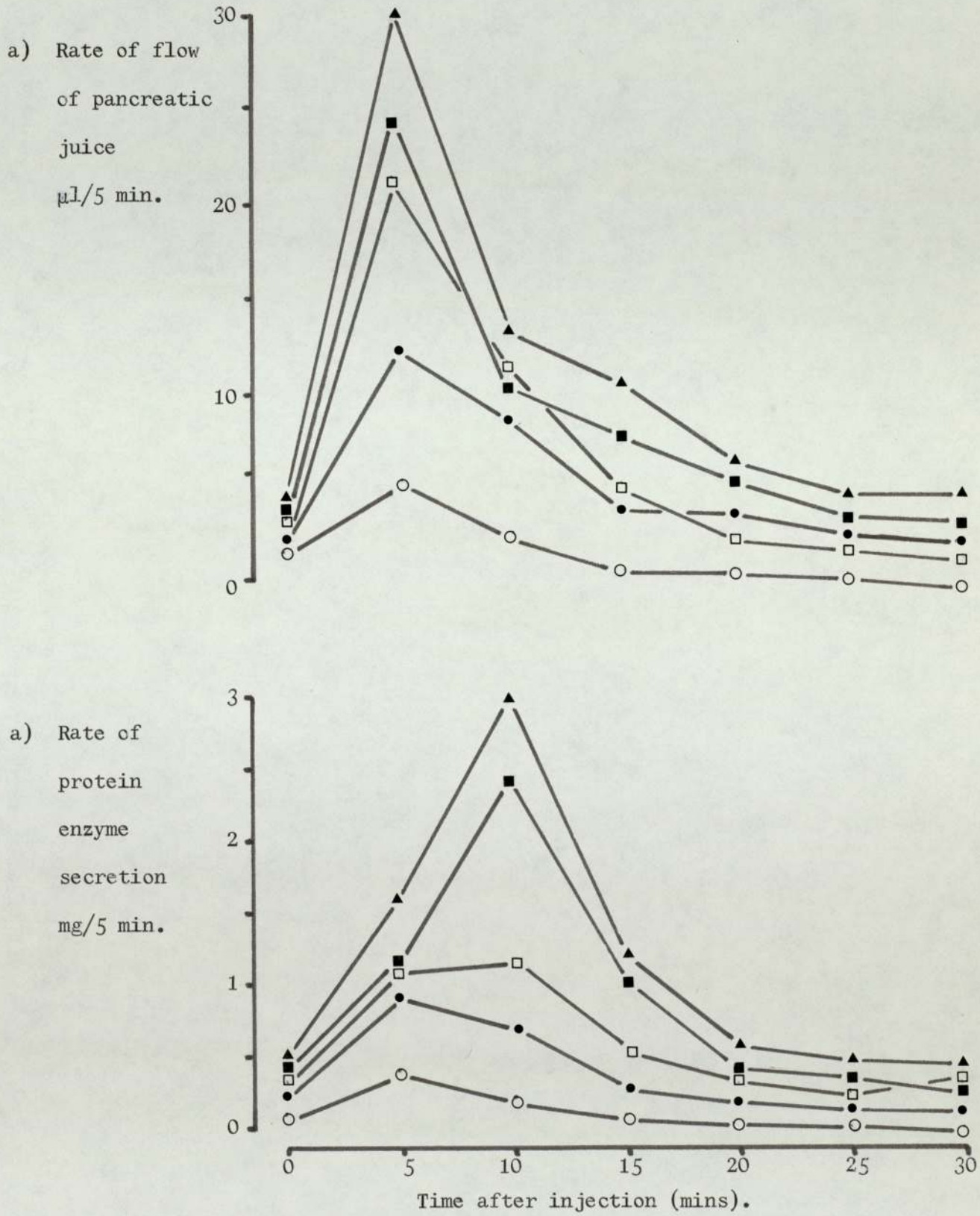


Figure 64 The relationship between the dose of secretin and the peak rate of flow of pancreatic juice and the peak rate of enzyme protein production.

(Each point represents the mean of 5 separate determinations).

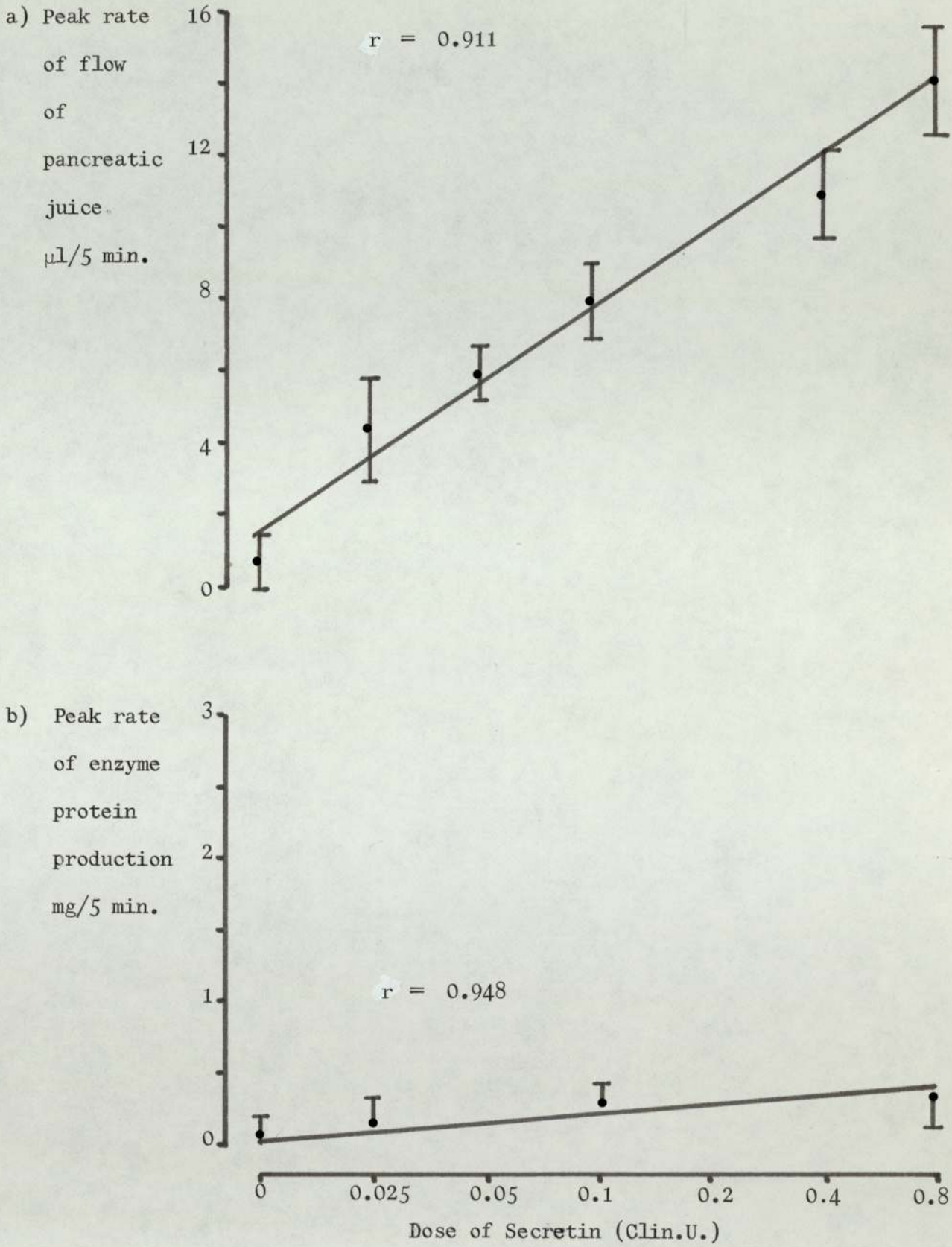


Figure 65 Relationship between the dose of pancreozymin and the peak rate of flow of pancreatic juice and the peak rate of enzyme protein production

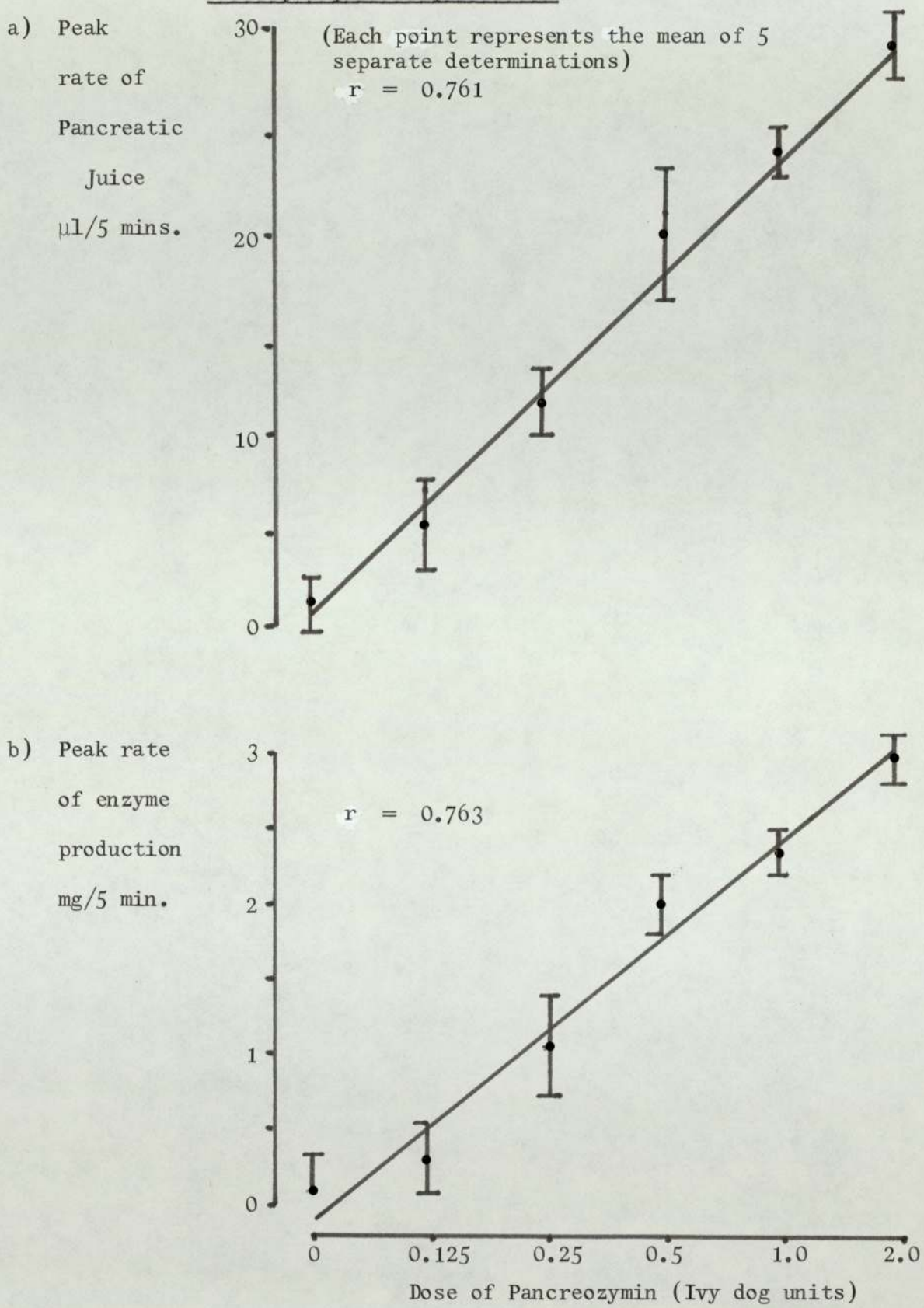
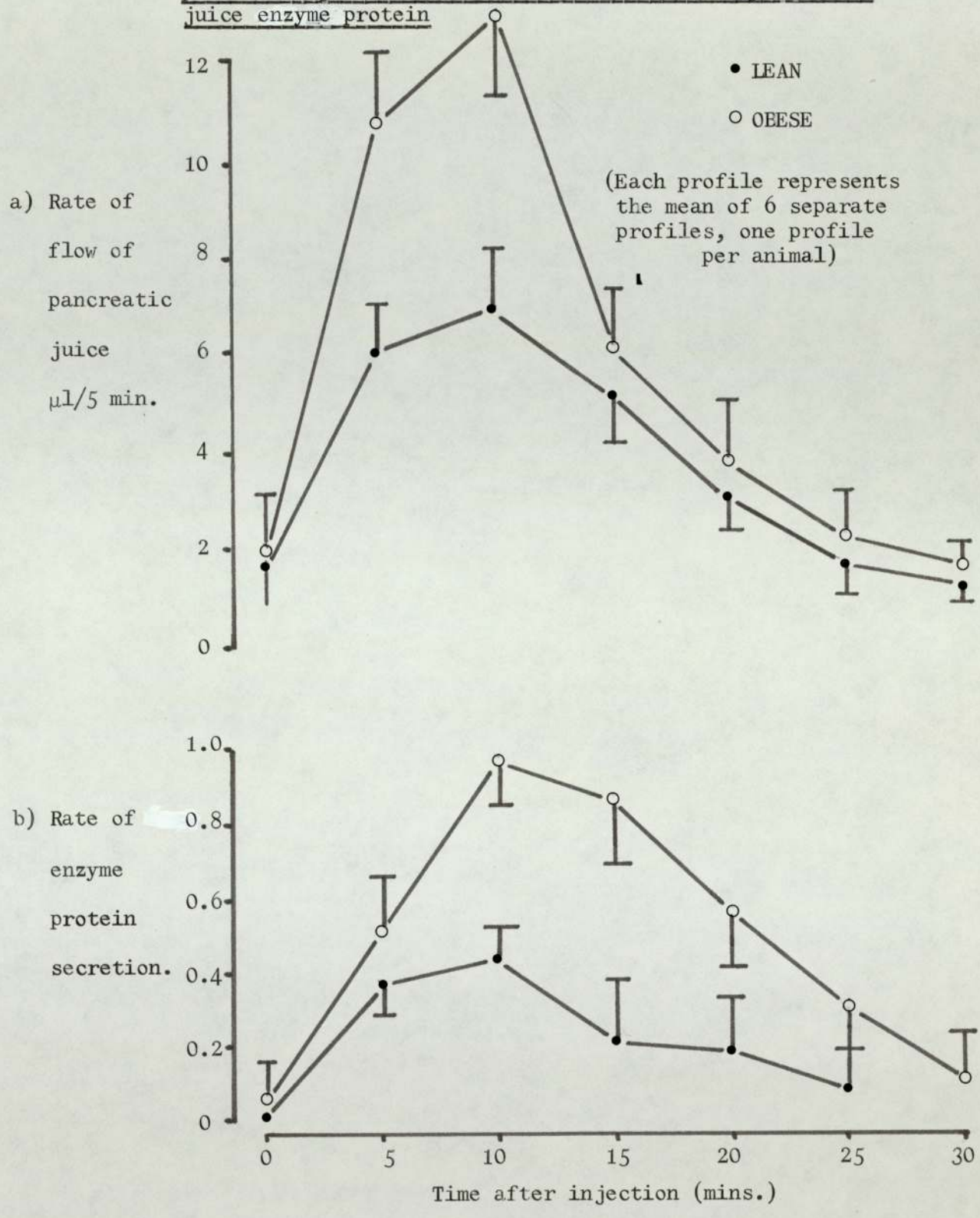


Figure 66 The effect of 0.25 ml injections of lean and obese mouse intestinal extract (200mg/ml) on the rate of flow of pancreatic juice and the rate of secretion of pancreatic juice enzyme protein



Discussion

The present work demonstrates that in the rat, secretin (within the range 0.025 - 0.8 clin.U.) stimulates the rate of flow of pancreatic juice but not the rate of secretion of enzyme protein in the pancreatic juice. Pancreozymin (0.125-2.0 Ivy Dog U/ml) was shown to stimulate both the rate of flow of pancreatic juice and the rate of secretion of enzyme protein. Similar results have been reported by Heatley (411, 412) and by Dockray (407) although in the latter study, a slight stimulation of enzyme protein secretion was observed with secretin. The more rapid stimulatory effect of pancreozymin upon the flow of pancreatic juice in the rat compared to secretin, appears to be less apparent in other mammalian species (407). It has been suggested that the stimulatory effect of pancreozymin upon the flow of pancreatic juice might be a consequence of the presence of certain impurities, particularly secretin, in the preparation used. However, an entirely pure preparation of pancreozymin has yet to be prepared and so the effect of the pure hormone upon the rate of flow of pancreatic juice remains to be determined. It seems unlikely that sufficient quantities of secretin would be present in the pancreozymin preparations used to account solely for the hydrelatic effects observed. The pancreozymin preparation used in these studies is known to contain small quantities of Gastric Inhibitory Polypeptide (GIP) which might conceivably have contributed towards the hydrelatic action of the pancreozymin preparation. However, the effect of GIP on the rate of flow of pancreatic juice has yet to be investigated.

Pancreozymin produced a significantly more rapid stimulation of the rate of flow of pancreatic juice flow than secretin, although the stimulatory effect of the latter was a more protracted one. This

difference in dynamics might reflect a difference in the mechanisms of action of the two hormones within the pancreas, and suggests that secretin and pancreozymin might act upon two distinctly different exocrine cell types.

The effect of secretin and pancreozymin on the rate of flow of pancreatic juice was dose-related, as was the effect of pancreozymin on the rate of secretion of enzyme protein in the pancreatic juice. Since both hormones did significantly affect the rate of flow of pancreatic juice, it was difficult to assay secretin in the presence of unknown amounts of pancreozymin. These difficulties were to some extent overcome in the present study by the simultaneous assay of the two hormones using the same rat preparation as previously described by Heatley (411).

In the present studies, intestinal extracts of obese-hyperglycaemic mice were shown to have a significantly higher bioassayable content of secretin and pancreozymin than extracts prepared from lean mouse intestine. In addition, secretin and pancreozymin have both been shown to stimulate the secretion of insulin from lean and obese mouse islets of Langerhans (Chapter III, page 46). However, it is also probable that the gastrointestinal mucosa of these mice elaborate many other substances with insulinotropic activity besides secretin and pancreozymin (e.g. G.I.P. and I.R.P.).

It is conceivable that the high levels of bioassayable secretin and pancreozymin in the obese mouse intestinal extracts might contribute to an overactive enteroinsular axis in these animals and promote and maintain the hypersecretion of insulin.

CHAPTER VII

The presence and distribution of endocrine like cells in the duodenum and jejunum of lean and obese hyperglycaemic mice

INTRODUCTION

In 1870, Heidenhain (413) first demonstrated the existence of endocrine like groups of cells dispersed throughout the gastric mucosa of both the rabbit and dog. These cells, which did not appear to be immediately involved in the process of digestion, could be selectively stained with bichromate solutions, and were consequently termed "enterochromaffine cells" (E-C cells) (414). Masson (415) demonstrated that certain types of enterochromaffine cells were capable of reducing ammoniac and silver salts and he called these argentaffine cells. The term argyrophil was later introduced to describe a group of cells in the intestine resembling the argentaffine cells, but requiring the presence of a reducing agent to precipitate silver salts (416).

At the Weisbaden Conference of 1969 an attempt was made to resolve the different types of endocrine cells present in the gastrointestinal tract, on the basis of their electron microscopic appearance (417). This led to the identification of three basic types of intestinal endocrine cell; one type was characterised by irregular granules (corresponding to the E-C cell), and the two others were characterised by the presence of spheroidal granules, the "L cell" containing large, and the "S cell" containing small granules (418). Using criteria such as distribution and specific immunofluorescence characteristics, seven types of intestinal endocrine cells have been subsequently identified, although much controversy still exists as to which of these cell types is associated with the secretion of a particular hormone from the

gastrointestinal mucosa. The subject has recently been reviewed extensively by Forssmann and colleagues (419) and others (420, 421, 422). Table 8 lists the enteroendocrine cells currently recognised in the upper gastrointestinal tract of mammals, together with certain of their physical characteristics and purported functions.

The endocrine cells of the upper gastrointestinal tract are most commonly found at the base of the Crypts of Leiberkühn and in the walls of the villi. Under the electron microscope they can be seen as small, round or pyramidal cells containing electron dense granules (421). The pyramidal cell has a narrow apex which often extends to the lumen and bears microvilli. The base of the cell is somewhat wider and faces the submucosa. The nucleus is usually smooth, oval or rounded and occupies a large proportion of the cell. The secretory granules have a diameter of between 200-500 nm and tend to be concentrated in the basal portion of the cell. Some granules appear to have a paler core, which might be artifactual or possibly the result of cutting tangentially through a biconcave granule. It has been suggested that the release of granules from these cells occurs by exocytosis, a process common to several other types of endocrine cell (433).

The cytoplasm of enteroendocrine cells is always less dense than that of surrounding mucosal cells, and often contains micro filaments which may be involved in the secretory activity of the cells.

The average turnover time for gastrointestinal endocrine cells in man has been reported to be approximately four days (424). The cells follow the usual pattern of migration up the side of the crypt and villus, to be subsequently lost at the extrusion zone in the same manner as columnar and mucous-secreting cells. It has been suggested that enteroendocrine cells are embryologically derived from neural crest (423),

TABLE 8 Classification of Enteroendocrine cells (after Lechago & Bencosme, 421)

Cell Type	Distribution	Silver	Shape	Hormone Secreted
EC	Stomach	Fontana +	Elongated	Serotonin
EC	Duodenum	Fontana +	Elongated	Serotonin
ECL	Stomach	Fontana +	Round	Serotonin? Catecholamines?
D	Stomach, Duodenum.	Argyrophil+	Round or irregular	?
A	Stomach, Duodenum.	Grimelius +?	Round	Enteroglucagon
G	Stomach	Grimelius +?	Round or irregular	Gastrin
L	Duodenum	?	Round	Enteroglucagon?
S	Duodenum	?	Round	Secretin
I	Duodenum	?	Round	Pancreozymin?

although it is also suspected that they may be derived directly from crypt-base columnar cells which they closely resemble (424). The ability to carry out cell division is lost upon differentiation into enteroendocrine cells, but rapid cell renewal occurs. The control of crypt-base columnar cell proliferation and differentiation is poorly understood, although it seems possible that food intake might be an influential factor (163, 425).

It would seem possible that the obese hyperglycaemic mouse with its characteristic hyperphagia might possess an increased complement of intestinal endocrine cells compared to the lean mouse. The high rate of food intake would continuously stimulate the secretory activity of these cells.

With this in mind a preliminary histological and electron microscopical examination of lean and obese mouse duodenum and jejunum was carried out.

Materials and Methods

25 week old lean and obese hyperglycaemic mice were used throughout and allowed free access to food and water.

A. Light Microscopy

Animals were killed by cervical dislocation, and portions of duodenum and jejunum were rapidly excised, rinsed in cold water and fixed in 10% (v/v) formal saline for 24 hours at room temperature (21°C). The tissue was then washed in distilled water and dehydrated in graded alcohols (15 mins. each) before being cleared in chloroform and embedded in paraffin wax. Serial sections of 5μ thickness were cut using a rotary microtome (Baird and Tatlock Ltd., London) and subsequently mounted individually on glass slides.

These sections were stained using the silver impregnation technique of Grimelius (426, 427, 428).

B. Electron Microscopy

The techniques used in these studies have been extensively reviewed by Kay (429). The upper portion of the small intestine of lean or obese-hyperglycaemic mice was removed and perfused with ice-cold 6.25% (v/v) glutaraldehyde buffered with sucrose-phosphate (430). The tissue was then cut into small (1 cu.mm) blocks and placed in vials containing 6.25% glutaraldehyde fixative for 4 hours. The tissue was then washed three times in 0.44 M sucrose -0.2 M phosphate buffer, pH 7.2, for 24 hours (431) followed by one hour in sucrose phosphate buffered 2% osmium tetroxide at room temperature (21°C). After fixation, the tissue was washed with distilled water and dehydrated in graded alcohols. The tissue blocks were then mounted in Epon 812 and 0.1 μ gold sections cut using an LKB microtome. The sections were mounted on carbon-coated copper grids and double stained, face upward, with 0.4% lead citrate (20 minutes) and 1% uranyl acetate (15 minutes) (432). The sections were subsequently examined on an AEI EM6B electron microscope.

Results

Light Microscopy

Cells of an argyrophilic nature were characterised by dark, silver-stained granules in the pale coloured cytoplasm. These cells were found sparsely distributed throughout the duodenal and jejunal mucosa in both lean and obese mice (plates 4 to 9). Argyrophil cells were mainly localised at the base of the crypts in both lean and obese mouse duodenum (plates 4 and 5 respectively). A few cells were distributed among columnar cells of jejunal villi (plates 6 and 7), adjacent to capillaries in the lamina propria. In most cases there was a distinct polarisation of granules in the basal regions of the cells (plates 8 and 9). No attempt was made to differentiate the various entero-endocrine cell types and no formal cell counts were performed. However, from a superficial examination, the number of silver staining cells appeared to be greater in obese mouse intestinal mucosa than in lean mouse intestinal mucosa (see plates 4 and 5).

Electron Microscopy

Enteroendocrine cells of both lean and obese mouse duodenal-jejunal mucosa contained characteristic electron dense granules (plates 10 to 13). The cell nucleus occupied a large proportion of each cell, and contained fine, granular chromatin material. In most cases the rod or spheroidal shaped electron dense granules were located in a specific region of each cell and were bounded by well defined granule membranes (plate 11).

The enteroendocrine cells were distributed sparsely throughout the duodenal and jejunal mucosa of both lean and obese mice, but were most commonly identified among the columnar cells at the base of the Crypts of Lieberkühn and in the walls of villi (plate 13). There appeared to be little morphological difference between the enteroendocrine cells of lean or obese mouse duodenal-jejunal mucosa.

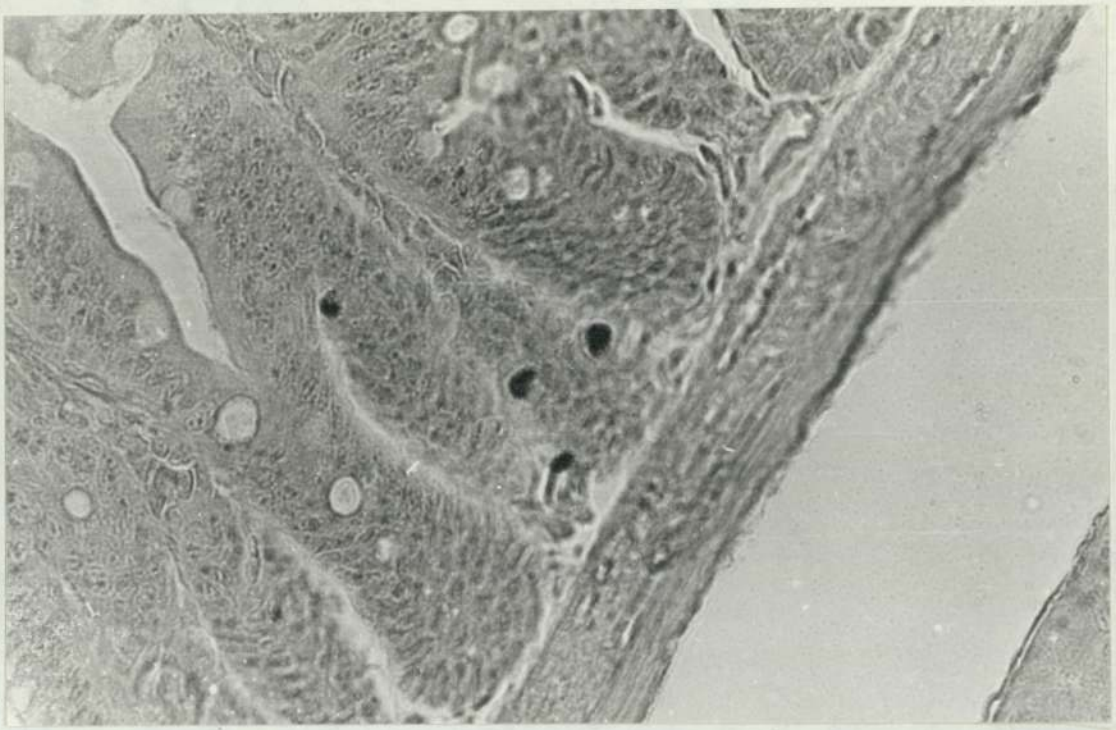


Plate 4. Lean mouse duodenal crypt showing darkly-stained argyrophil cell (x 250).

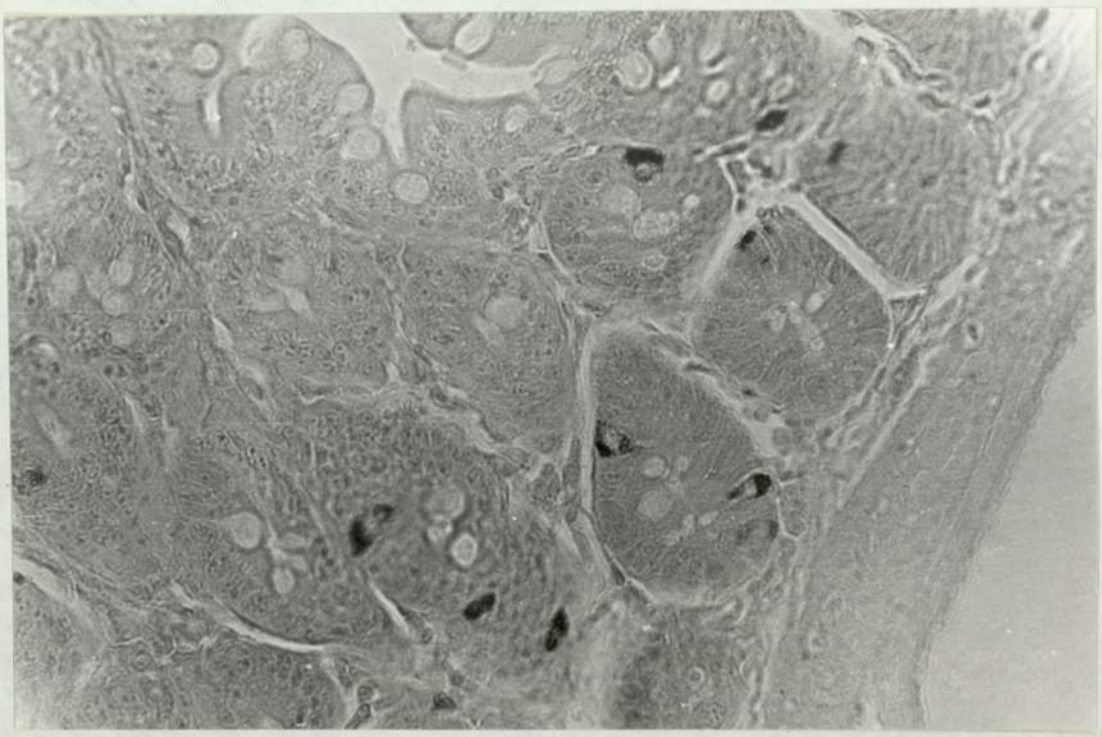


Plate 5. Obese mouse duodenal crypt showing darkly stained argyrophil cells (x 250).

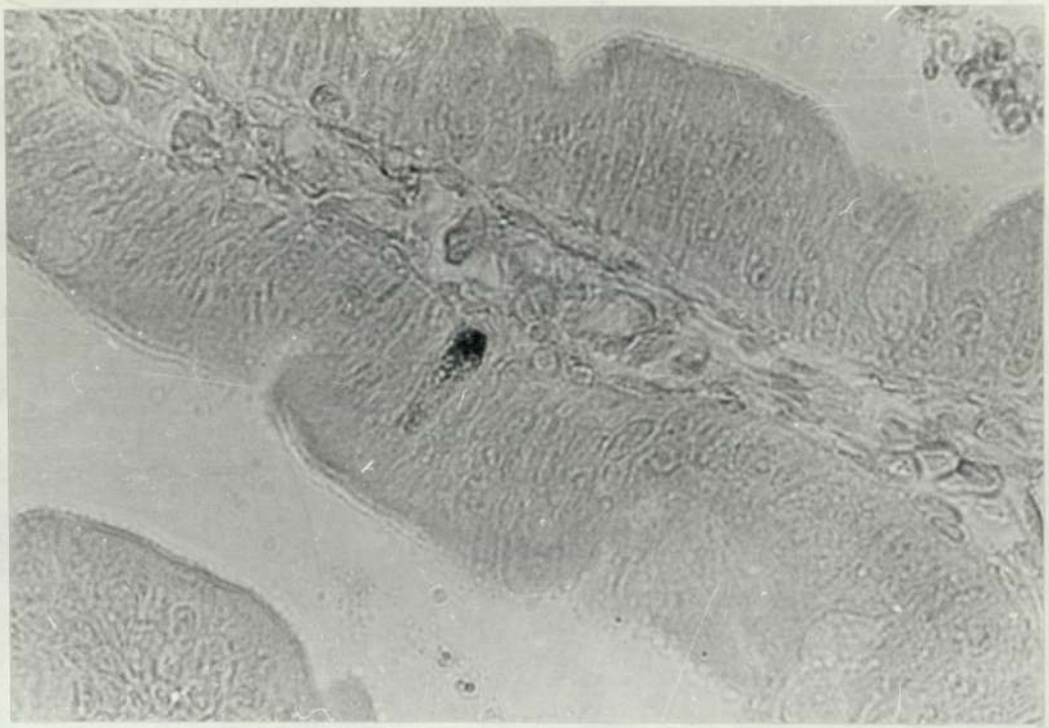


Plate 6. Lean mouse jejunal villus showing a single argyrophil cell (x 250)

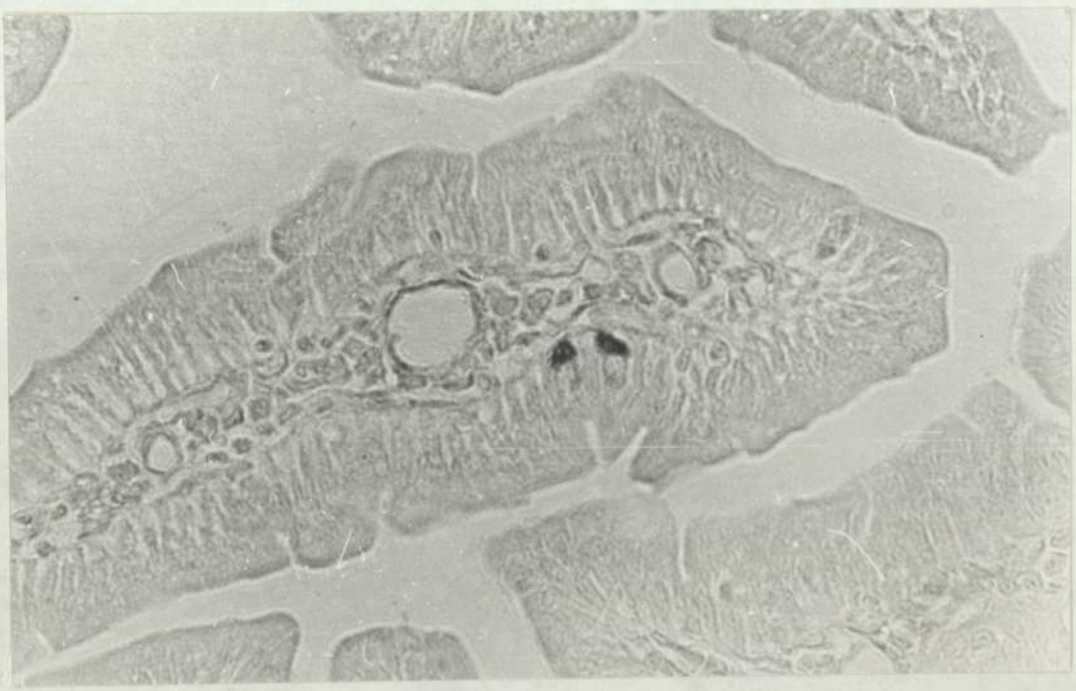


Plate 7. Obese mouse jejunal villus containing two argyrophil cells (x 250).

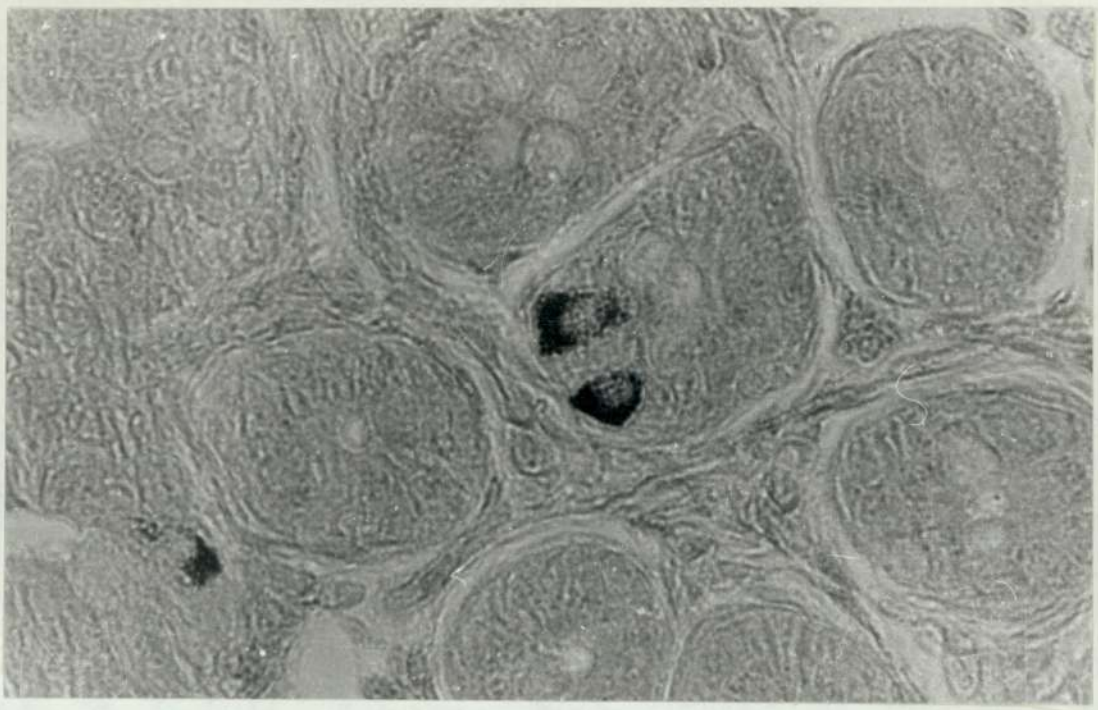


Plate 8. Lean mouse duodenal mucosa showing argyrophil cells. Note the polarisation of silver-stained granules (x 400).

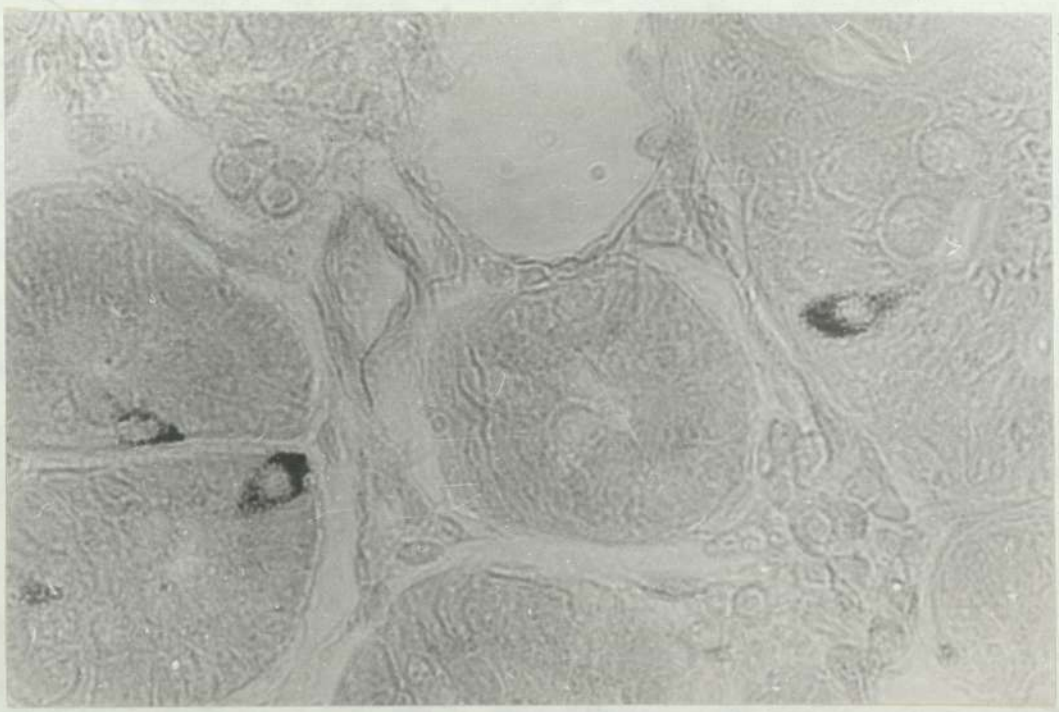


Plate 9. Obese mouse jejunal mucosa showing argyrophil cells adjacent to capillaries in Lamina propria (x 400).

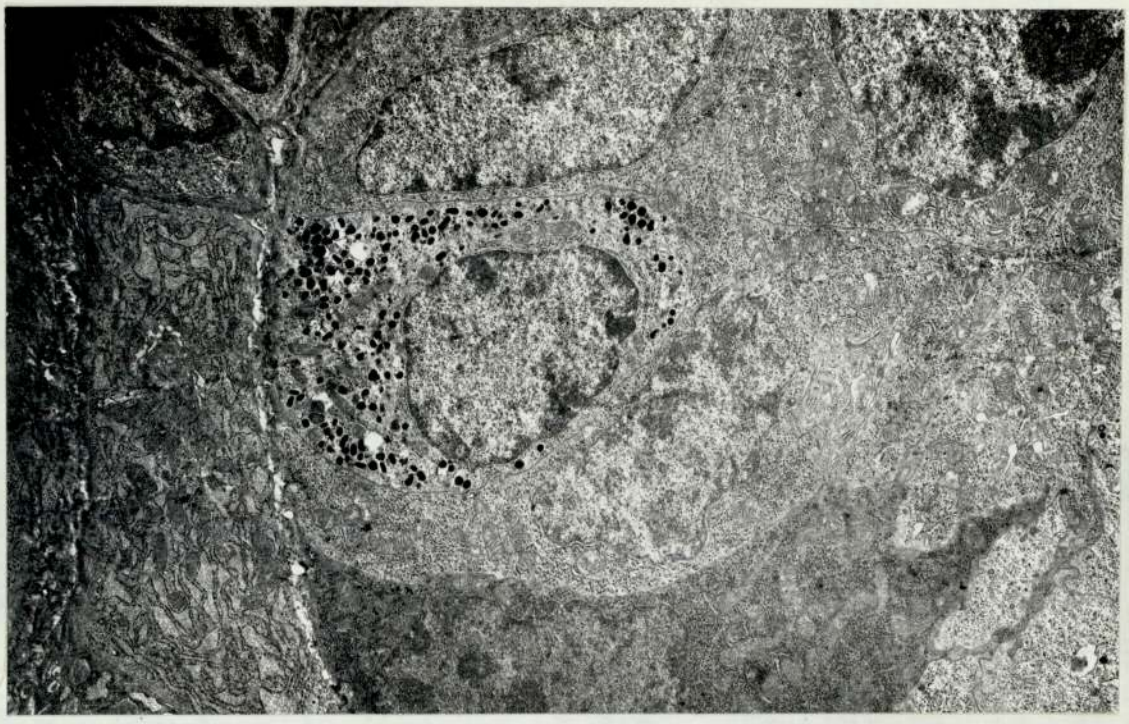


Plate 10. Electron microscope section of enteroendocrine cell of lean mouse duodenal mucosa stained with uranyl-lead (x 3,000)

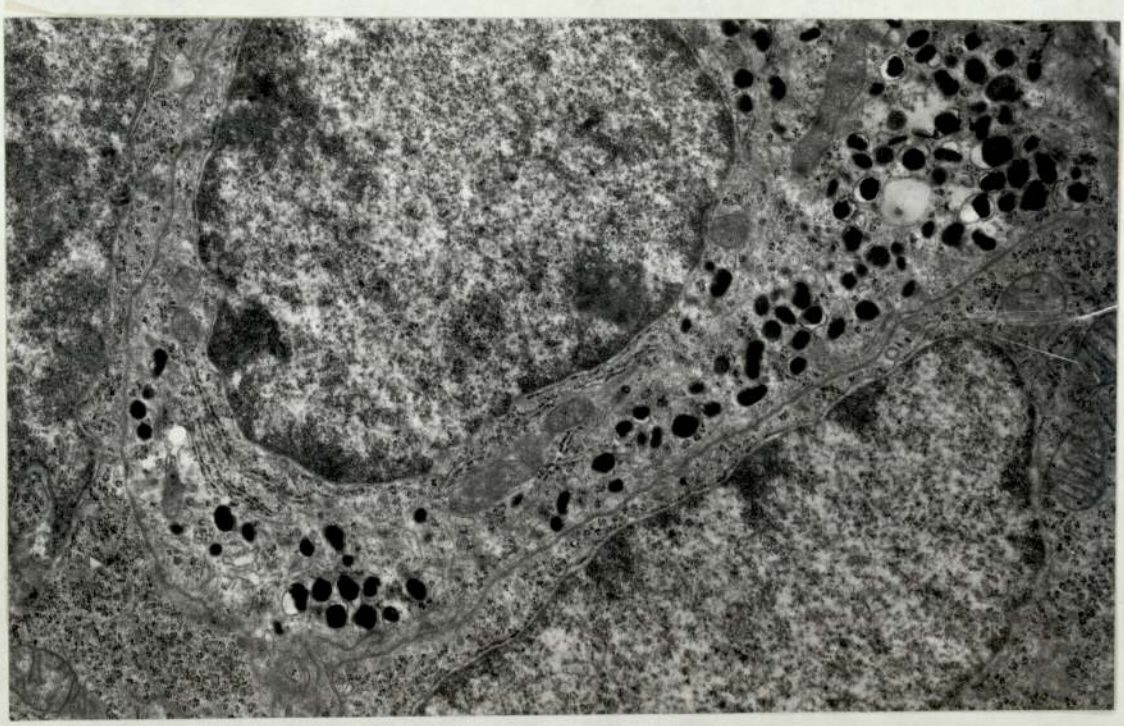


Plate 11. Lean mouse duodenal enteroendocrine cell stained with uranyl-lead (x 10,000)

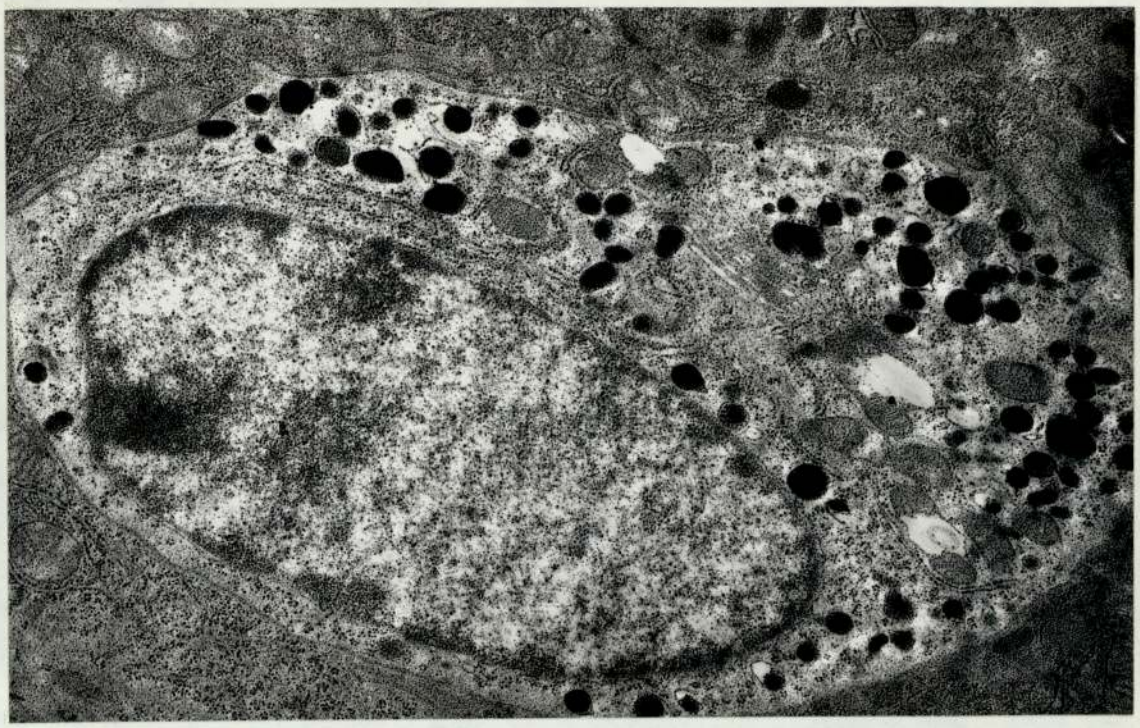


Plate 12. Obese mouse jejunal enteroendocrine cell stained with uranyl-lead (x 10,000).

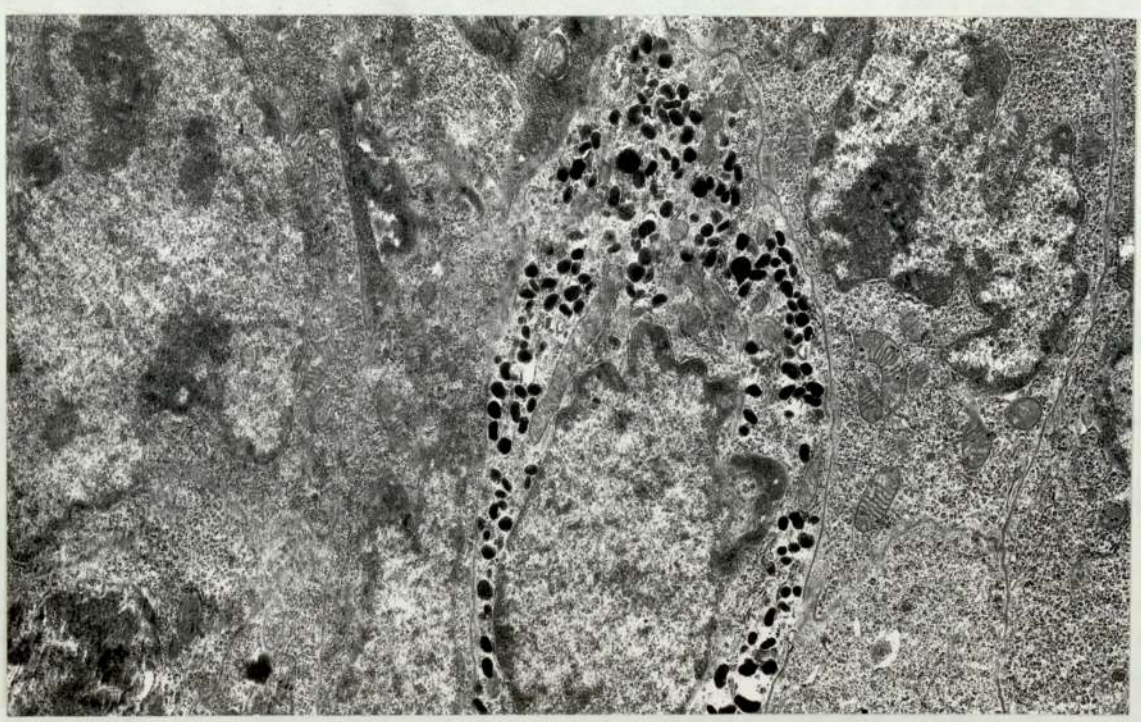


Plate 13. Obese mouse duodenal enteroendocrine cell stained with uranyl-lead (x 7,500).

Discussion

By light microscopy silver staining cells could be identified in the duodenal and jejunal mucosa of both lean and obese mice. Whether the silver deposits in the cells corresponded to secretory granules, or other cytoplasmic organelles could not be determined with certainty by light microscopy. In addition one could not be sure whether these silver staining cells were identical to the cells observed under the electron microscope containing electron dense granules. However, for the present purposes, all these cells were considered to be entero-endocrine cells of the duodenal-jejunal mucosa.

The morphological distribution of enteroendocrine cells observed in the intestinal mucosa of lean and obese mice was consistent with the findings of previous workers in the field (421, 424, 433). With the aid of both light and electron microscopy, enteroendocrine cells were identified in the Crypts of Leiberkuhn and villi of both lean and obese mouse intestinal mucosa. Under the electron microscope, these cells were characterised by the presence of electron dense basally polarised granules with continuous rather than stepwise variation in shape which did not permit a ready classification of the cells with respect to either granule shape or size. These observations are consistent with the work of Nichols (433) who also demonstrated a mixture of spherical and non-spherical cells in mouse enteroendocrine cells. Nichols suggested that the intermediate nature of these cells might represent a multiplicity of function; i.e. several gastrointestinal hormones might be secreted from a single enteroendocrine cell. Cheng and Leblond (424), however, showed that the enteroendocrine cells of mouse duodenum, jejunum and ileum contained exclusively spheroidal granules when stained with iron

haematoxylin. Forssmann and colleagues (419) have been able to categorise rat enteroendocrine cells into at least five distinct types according to their morphological appearance and localisation. No such classification was possible in the present investigation. In addition there appeared to be little morphological difference between the enteroendocrine cells of lean and obese mouse duodenal-jejunal mucosa. This finding contrasts with the recent observations of Polak and colleagues (406) who reported a marked hyperplasia of most of the enteroendocrine cells in the intestinal tract of the obese mouse.

In the present work no attempt was made to quantify the numbers of enteroendocrine cells present in lean and obese mouse intestine. However, superficial observations suggest that duodenal and jejunal enteroendocrine cells are more numerous in obese mice than in lean, a finding that has recently been confirmed on a quantitative basis by Polak and colleagues using immunofluorescent studies (406). These workers noted that GIP containing enteroendocrine cells were present in greater numbers in obese mouse intestine than in lean mouse intestine. This observation is supported by the increased food intake (163) and increased intestinal absorption of glucose (434) in obese animals. In the obese mouse the enteroendocrine cells might respond to the high levels of circulating blood glucose by an elevated synthesis of gastrointestinal hormones. Certainly the concentration of GIP in obese mouse intestinal mucosa is significantly higher than that of its lean littermate (406) and GIP has been reported to be a powerful insulinogenic agent (134, 135). It is conceivable that the intestinal tract of the obese mouse might be the source of an enhanced humoral signal that causes an exaggerated release of insulin from the Islets of Langerhans.

Only further investigation will reveal whether this abnormality is secondary to the numerous other metabolic abnormalities seen in the obese animal, or a primary factor in the development of the obese hyperglycaemic syndrome.

CHAPTER VIII

General Discussion

The gastrointestinal tract has long been known to be physiologically related to the exocrine pancreas. However, a physiological relationship between the intestinal mucosa and the secretory activity of pancreatic endocrine cells has only recently been established.

The gastrointestinal mucosa is a very complex endocrine organ, producing many structurally similar though physiologically distinct hormones (421). The enteroendocrine cells which produce and secrete these hormones appear to be dispersed almost randomly throughout the gastrointestinal mucosa and because of this their morphology and function has proved difficult to study.

Over the last decade a great deal of research has centred on establishing which of the many gastrointestinal hormones are involved in the regulation of insulin secretion from the endocrine pancreas in response to food intake.

The present investigations have confirmed secretin and pancreozymin to be components of an enteroinsular axis. These hormones and pancreatic glucagon were shown to be capable of stimulating the release of insulin from isolated islets of both lean and obese hyperglycaemic mice in the presence and absence of glucose. This suggested that some part of the mechanism of gastrointestinal hormone-induced insulin secretion occurred independently of glucose metabolism within the β-cell. In addition the insulinotropic action of secretin, pancreozymin and glucagon did not appear to require the presence of pancreatic exocrine tissue. This observation is not in accord with the original hypothesis of Hinz and coworkers (68, 72) who suggested that the stimulation of insulin secretion by gastrointestinal hormones might be mediated via the exocrine

pancreas. In their studies, however, islet tissue was obtained by collagenase digestion (271) and it might be that their inability to secure an insulin secretory response to gastrointestinal hormones could have been the result of damaged β-cell membranes incurred during the digestion procedure or residual collagenase activity remaining on the surface of islets due to inadequate washing.

In vitro, experimental work with gastrin-pentapeptide failed to provide conclusive evidence for a stimulatory effect upon insulin secretion. The peptide inhibited the basal and glucose induced release of insulin from obese mouse islets but stimulated the basal secretion from lean mouse islets. Rehfeld (139) has shown the effect of gastrin on insulin secretion to be largely dependent upon the dose of hormone employed. Certainly at first glance one might conclude that the doses of gastrointestinal hormones used in the present study were supra-physiological. However, the circulating levels of gastrointestinal hormones probably bear little relation to their actual concentrations present in the β-cell microclimate and this situation remains to be clarified.

In the present studies, obese mouse islets showed consistently exaggerated insulin secretory responses to glucose; secretin; pancreozymin and glucagon compared to lean mouse islets. Beloff-Chain and colleagues (213) have also reported the hypersecretory activity of obese mouse islets, although it was not clear whether this abnormality manifested itself before or after the appearance of insulin resistance.

Karam suggested that hyperinsulinism in human obesity was merely the result of increased stores of pancreatic insulin and not a consequence of the hypersensitivity of the islet β-cell to stimuli (297). An increased β-cell mass was suggested to be the most likely cause of

the high levels of pancreatic insulin in obesity. Karam proposed that dietary factors, such as an increased food intake and the release of gastrointestinal hormones might contribute towards the enhanced synthesis and secretion of insulin in human obesity. Vinik and co-workers (435, 436) have suggested that overactivity of the entero-insular axis might be the earliest lesion in the development of human maturity-onset diabetes. The subsequent stress on the islet could eventually lead to exhaustion of the β-cells and a relative insulin insufficiency.

Investigation of the phasic release of insulin from perifused islets of lean and obese mouse islets has provided some insight into the mechanism of glucose- and gastrointestinal hormone induced insulin secretion. Perifusion with medium, containing a high concentration of glucose produced a stimulation of insulin secretion from both lean and obese mouse islets. This stimulation was generally maintained for at least one hour and did not always resemble a biphasic response.

In contrast, perifusion with either secretin, pancreozymin or glucagon elicited an immediate stimulation of insulin secretion which lasted only 15-20 minutes in the presence of a low nonstimulatory concentration of glucose (3mM). These observations are consistent with the two pool hypothesis for the secretion of insulin proposed by Porté and Bagdade (332). It would seem likely that gastrointestinal hormones stimulate the release of insulin from a small, labile store of pre-synthesised insulin and have little effect, if any, upon insulin biosynthesis (355). Glucose, on the other hand, appears to exert its major stimulatory influence upon the longterm release pool of insulin which almost certainly involves the initiation of insulin biosynthesis.

Crude preparations of secretin and pancreozymin were observed to be more potent insulin secretagogues than the same concentrations of the purified hormones. It has been shown that crude preparations of gastrointestinal hormones, particularly pancreozymin, contain variable amounts of other gastrointestinal factors, especially GIP and IRP which are extremely powerful insulinotropic agents (134, 135, 330). Somatostatin (Growth Hormone Release-Inhibiting Hormone) a powerful inhibitor of insulin secretion, which was previously thought to be restricted to the hypothalamus and pituitary, has recently been localised in both the intestinal mucosa and pancreatic islets (437). Thus, in the future somatostatin might prove to be a key regulatory factor in the gastrointestinal modulation of islet function.

There is evidence to suggest that the stimulation of insulin secretion, particularly by glucagon and gastrointestinal hormones, might involve an increase in the level of cyclic AMP within the β-cell (338, 345). Controversy exists, however, as to whether glucose-induced insulin secretion involves a direct stimulation of membrane bound adenylate cyclase and the accumulation of cyclic AMP in the β-cell (345, 362, 364, 365, 366, 368). In the present work, glucagon and pancreozymin but not secretin were shown to produce an immediate, pronounced elevation of both lean and obese mouse islet cyclic AMP content. Cyclic AMP profiles obtained with glucagon and pancreozymin closely parallelled their corresponding insulin secretory profiles, suggesting that the secretion of insulin response to these two hormones involved an elevation of the islet intracellular concentration of cyclic AMP (Figure 67). On the other hand it has been suggested that secretin induced insulin secretion involves the elevation of islet cyclic GMP levels (398). In addition stimulatory concentrations of glucose did not significantly

elevate the cyclic AMP concentration of either lean or obese mouse islets. Gastrointestinal hormones elicit a prompt stimulation of insulin secretion presumably via a direct elevation of islet cyclic AMP levels. This may be of physiological importance since it could provide an "early warning" system for the pancreatic islets, stimulating the secretion of insulin in anticipation of the absorption of glucose, amino acids and fatty acids into the blood. Gastrointestinal hormone induced increases in islet cyclic AMP concentration might also provide a "fine-tuning" of glucose induced insulin secretion.

Lean and obese mouse islets were not significantly different as regards their islet cAMP content responses to glucose and gastrointestinal hormones. This would suggest that the exaggerated insulin secretory response of obese mouse islets to glucose and gastrointestinal hormones, compared to lean mouse islets, was not a direct consequence of any altered cyclic AMP metabolism in islets of obese mice. However, Atkins and Matty have demonstrated enhanced adenylate cyclase and cyclic-AMP-phosphodiesterase activities in obese mouse islets (364) suggesting a high rate of turnover of islet cyclic AMP in these animals. Recent work with obese mouse adipocytes has suggested that a perturbation might occur in the coupling step between the adenylate cyclase-hormone receptor sub-unit and the enzyme catalytic site (438). Whether a similar defect exists in the adenylate cyclase system of the obese mouse islet β-cell remains to be established. Certainly the obese mouse islet appears to be hyper-responsive to both cyclic AMP mediated (e.g. glucagon) and non-cyclic AMP mediated (e.g. glucose) insulin secretagogues. It is conceivable that an abnormality might exist in the stimulus secretion coupling process at a point distal to the adenylate cyclase system which might subsequently give rise to the

hypersecretion of insulin.

In the present work gastrointestinal hormone-induced elevations in islet cyclic AMP content were generally accompanied by similar elevations in cyclic AMP efflux. This efflux might well be a passive process whose magnitude is related to the intracellular concentration of the nucleotide. Although Cerasi has shown cyclic AMP efflux to have a better correlation with the dynamic secretion of insulin than the accumulation of cyclic AMP within islets (367) it appears that a large part of the cyclic AMP generated might just passively leak from the β-cell or be released with the contents of β-granules.

In the present work crude extracts of obese mouse duodenum and jejunum were shown to have greater insulinotropic activity than similar extracts of lean mouse intestine. Gel-filtration of these extracts on G50 Sephadex (fine) revealed two peaks of high absorbance at 276 nm. The individual protein content of these two peaks was shown to be greater in extracts of obese mouse intestine than lean mouse intestine. With extracts of both lean and obese mouse intestine the elution profile of the second peak corresponded to that shown by the various standard pure preparations of secretin; pancreozymin; gastrin and glucagon. This suggested that the potential insulin releasing activity of the crude extracts was confined to the second peak.

The bioassayable secretin and pancreozymin content of crude extracts of obese mouse duodenum and jejunum was found to be significantly greater than the bioassayable hormone content of lean mouse intestinal extracts. These studies suggest that the intestinal mucosa of obese mice contain and possibly secrete greater quantities of secretin, pancreozymin and perhaps other potentially insulinotropic

hormones (e.g. GIP, VIP and IRP) than lean mouse intestinal mucosa and would be the consequence of either an increased number of enteroendocrine cells, hyperplasia of individual enteroendocrine cells or both.

Casual examination of histological sections of lean and obese mouse duodenum and jejunum suggested the presence of increased numbers of argyrophilic cells in obese mouse intestinal mucosa. It was not found possible to identify the specific types of enteroendocrine cells in the duodenum-jejunal mucosa of lean and obese mice either by light or electron microscopy. In a recent study using quantitative immunocytochemical techniques, Polak and colleagues were able to demonstrate general endocrine cell hyperplasia in most regions of the obese mouse gastrointestinal tract (406). In particular the number of cells and their content of GIP (a powerful insulin secretagogue) was found to be markedly increased in obese mouse intestine compared to lean.

It has yet to be established whether the initial lesion of the obese mouse syndrome resides in the gastrointestinal tract or the pancreatic islet. Available evidence suggests that an overactive enteroinsular axis could facilitate the development of hyperinsulinism, insulin resistance and obesity in the obese hyperglycaemic mouse. Hyperinsulinism might be maintained by the enhanced intestinal absorption of glucose (434) and the secretion of increased amounts of gastrointestinal hormones in response to food intake. Hyperinsulinism has been reported as the first detectable manifestation of the obese-hyperglycaemic syndrome in mice (187), suggesting that the islet β-cell of the obese mouse is caused initially to oversecrete beyond its productive capacity and that this drive is not explicable in terms of increased adipose tissue stores, hyperglycaemia or insulin resistance

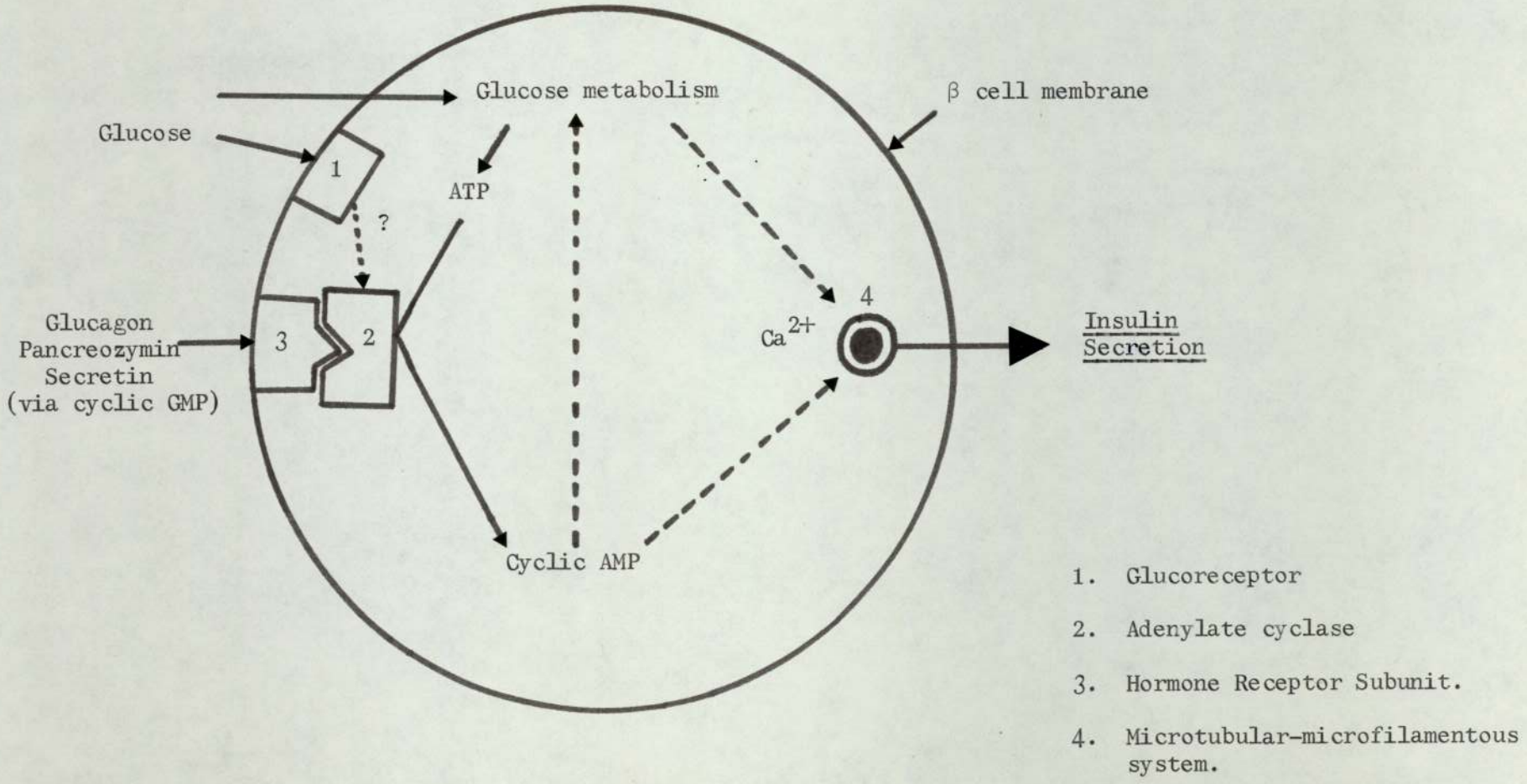
(206). Furthermore, since islet cell hyperplasia appears to be related to increased food intake and can be ameliorated in the adult obese mouse by weight reduction (209), oral feeding would seem to be integral to the development of islet cell hyperplasia in the obese mouse. The fact that carbohydrate-induced obesity results in islet hyperplasia whereas fat-induced obesity does not (439, 440) points to the importance of the quality rather than the quantity of food as the stimulus for the secretion of hormones such as insulin that control body cell composition. Humans and many domesticated animals exist on a largely-carbohydrate diet which probably provides a major stimulus to gastrointestinal-hormone mediated insulin secretion. Consequently, the pancreas is induced to secrete further quantities of insulin which might lead to the development of obesity and maturity onset diabetes. In fact, obesity and maturity-onset diabetes are often characterised by a pronounced early insulin response to oral glucose (230, 435, 436) suggesting that an overactive enteroinsular axis might be one of the earliest lesions common to both abnormalities. It is likely that the gastrointestinal modulation of islet function is a direct action, even though parasympathetic communications may exist between the intestine and pancreas, either direct, or via higher centres such as the hypothalamus (206). Idähl and Martin (220) have demonstrated the existence of a ventro-lateral hypothalamic factor in mouse capable of stimulating the secretion of insulin although the possible role hypothalamic factors and pituitary hormones such as ACTH (352, 441, 442) play in the mechanism of insulin secretion, blood glucose homeostasis and the aetiology and pathogenesis of the obese hyperglycaemic syndrome remains to be elucidated.

In conclusion, it is not yet possible to define the primary lesion leading to the development of the obese-hyperglycaemic syndrome in mice. However, it is proposed that excessive food intake represents a specific stimulus for the induction of hyperinsulinism and pancreatic islet cell hyperplasia, and that this effect might be mediated via the enhanced secretion of gastrointestinal hormones. It is possible that the obese-hyperglycaemic mouse adapts to this over-production of insulin by becoming insulin resistant (especially muscle) and hyperglycaemic.

The genetically obese mouse is an outstanding example of the potential value of animal models in the study of clinical syndromes and their aetiology and pathogenesis in Man.

This work emphasises the importance of the gastrointestinal tract in maintaining nutrient homeostasis and in doing so touches on one of the most important problems in the modern world: the influence of food on body composition and the prevention of disease.

Figure 67 Possible modes of action of glucose and gastrointestinal hormones on the mechanism of insulin secretion.



APPENDIX 1

The effect of pilocarpine on insulin secretion in lean and obese-hyperglycaemic mice.

INTRODUCTION

Pilocarpine is an imidazole alkaloid derived from plants of the genus Pilocarpus. Its pharmacological actions are predominantly those of a peripheral parasympathomimetic agent, and induces profuse secretory activity in many exocrine glands (443). Several authors have described the stimulatory effect of pilocarpine on the secretion of pancreatic juice (444, 445, 446, 447). Administration to dogs and rats as pilocarpine hydrochloride or pilocarpine nitrate at doses of between 0.3 and 20 mg/100 mg body weight i.v. or i.p. produces an almost complete depletion of pancreatic acinar tissue within one hour. This property of pilocarpine has recently been utilised to facilitate the isolation of intact islets of Langerhans from the pancreas of the rat (279). The yield of islets from rat pancreata was shown to be markedly enhanced following the prior administration of pilocarpine hydrochloride three hours earlier.

The present study was undertaken to evaluate whether the prior administration of pilocarpine would facilitate the subsequent freehand microdissection of islets from the pancreata of lean and obese-hyperglycaemic mice. Also, since the islets were required for in vitro studies of insulin secretion, experiments were performed to evaluate the effect of pilocarpine on this process.

Materials and Methods

Lean and obese-hyperglycaemic mice were allowed food and water ad libitum and used at 20-25 weeks of age.

a) The effect of pilocarpine on blood sugar and plasma insulin.

Mice were treated with pilocarpine hydrochloride (13.3 mg/0.2 ml saline per 100g body weight, i.p. : Sigma Chemical Co.Ltd., London) or saline only (0.2 ml/100g body wt. i.p.). After either ten or sixty minutes, the mice were killed by cervical dislocation and blood samples obtained by rapid decapitation. Blood sugar values were estimated by the ferricyanide reduction method (281) and plasma insulin levels were measured by radioimmunoassay (see Chapter II, page 30).

b) The effect of pilocarpine pre-treatment on insulin secretion in vitro

Mice were treated with either pilocarpine hydrochloride (13.3 mg/ 0.2 ml saline per 100g body wt., i.p.) or saline only (0.2 ml/100g body wt., i.p.) and killed sixty minutes later by cervical dislocation. The pancreas was rapidly excised and the islets of Langerhans isolated by freehand microdissection (see Chapter II, page 28). Isolated islets were preincubated in 10ml. 3.33mM glucose bicarbonate buffer (277) for 30 min. at 37°C, with continuous oxygenation and shaking. Groups of 5 islets were then test incubated for a further 30 minutes in 3 ml of buffer containing 0, 8.33 or 16.7 mM glucose. The incubation vials were pre-washed with a solution of bovine serum albumin fraction V (0.4% w/v to reduce insulin loss by adsorption. At the end of each experiment, triplicate 100µl samples of incubation medium were taken and deep frozen (-20°C) for subsequent insulin estimations. The islets were carefully

transferred to perforated aluminium foil weighing boats, freeze dried (-40°C, 0.001 mm.Hg.) overnight and weighed on an ultramicrobalance (Mettler, U.M.6).

c) The effect of pilocarpine addition to the incubation medium on insulin secretion in vitro.

Islets isolated from untreated mice were preincubated in low glucose buffer (3.33mM) as described above and groups of 5 islets were subsequently test incubated for 30 minutes in 3 ml. buffer containing 0, 8.33 or 16.7mM glucose with and without pilocarpine hydrochloride (0.16 and 1.60 g/l). The insulin concentration in the incubation medium, and islet weights were determined as previously.

Mean values of insulin secretion of differently-treated groups of islets, and mean levels of blood sugar and plasma insulin in differently treated mice were compared using Students' t-test. Differences were considered significant for $p<0.05$.

Results

Following pre-treatment with pilocarpine, the pancreas was considerably diminished in volume and characteristically altered in appearance. The normal fleshy appearance of the acinar tissue of control pancreata was replaced by a sparse, translucent tissue exposing the islets of Langerhans as discreet, pale spheroidal bodies clustered in groups adjacent to the larger blood vessels. The freehand microdissection of islets was noticeably easier and more rapid when the mice had been pre-treated with pilocarpine. However, the treated mice also showed several unpleasant characteristic symptoms including increased irritability and stress, diarrhoea, lacrimation and intense salivation.

The isolated islets of lean and obese mice responded to glucose stimulation with a characteristic increase in insulin secretion, and the basal insulin secretion (in the absence of glucose) and glucose-induced insulin secretion were greater from islets of untreated obese mice than from islets of untreated lean mice (Tables 10 and 11).

a) Effect of pilocarpine on blood sugar and plasma insulin levels.

Pilocarpine administration produced a significant rise in blood sugar concentration after 10 minutes in both lean and obese mice (Table 9). After 60 minutes, the blood sugar level had fallen considerably below that of the untreated group in the lean mice, but remained significantly in excess of the untreated group in the obese mice. Plasma insulin levels were significantly increased 10 minutes after pilocarpine treatment in both lean and obese mice. These levels were reduced after 60 minutes, but remained significantly above the untreated group in lean mice.

b) Effect of pilocarpine pre-treatment on insulin secretion in vitro

Pilocarpine pre-treatment produced a slight (though not significant) increase in basal and glucose-induced insulin secretion from islets of lean mice. In contrast islets isolated from pilocarpine pre-treated obese mice, however, exhibited a reduced insulin secretory activity which was significant in the presence of 8.33 and 16.7 mM glucose (Table 10).

c) The effect of pilocarpine addition to the incubation medium on insulin secretion in vitro.

The presence of pilocarpine in the incubation medium enhanced the insulin secretory activity of islets isolated from both lean and obese mice (Table 11). In the case of lean mouse islets, the higher concentration of pilocarpine (1.6 g/l) significantly elevated both basal and glucose-induced insulin secretion while the lower concentration of pilocarpine (0.16 g/l) significantly elevated basal insulin release only. The somewhat elevated insulin secretory activity of obese mouse islets was not statistically significant at either concentration of pilocarpine in the presence and in the absence of glucose.

TABLE 9 Effect of pilocarpine on blood sugar and plasma insulin levels in lean and obese mice.

NORMAL MICE

	Untreated	10 min.	60 min.
Blood Sugar (mg/100ml)	104.0±9.1 (8)	145.6±15.0 (5)	75.2±12.0 (5)
Plasma Insulin μU/ml	31.0±4.1 (8)	268.0±48.5 (5)	87.4±18.5 (5)

OBESE MICE

	Untreated	10 min.	60 min.
Blood Sugar mg/100 ml.	157.7±25.6 (8)	344.0±39.4 (5)	307.0±22.4 (5)
Plasma Insulin μU/ml	239.6±124.8 (8)	915.0±68.9 (5)	413.6±141.4 (6)

Values are means ± s.e.m.

Number of observations in parentheses

$p<0.01$, *$p<0.02$ compared with untreated group.

TABLE 10 Effect of pilocarpine pre-treatment on insulin secretion in vitro from isolated islets of lean and obese mice.

(Values expressed as mU insulin/mg freeze dried islets/ 30 min.)

NORMAL MOUSE ISLETS

Glucose (mM)	0	8.33	16.7
Saline injected	11.9±1.9 (5)	20.9±3.8 (5)	29.7±3.0 (5)
Pilocarpine injected	16.2±2.4 (5)	23.4±2.0 (5)	32.1±4.9 (5)

OBESE MOUSE ISLETS

Glucose (mM)	0	8.33	16.7
Saline injected	21.4±2.4 (5)	31.9±1.8 (5)	42.3±3.6 (5)
Pilocarpine injected	16.7±2.8 (5)	18.4±3.6 (5)	20.8±2.8 (5)

Mean ± s.e.m., Number of observations in parentheses

$p<0.05$ compared to saline injected group.

TABLE 11 Effect of pilocarpine addition to the incubation medium on insulin secretion in vitro from isolated islets of lean and obese mice (Values expressed mU insulin/mg freeze-dried islet tissue/30 min.)

NORMAL MOUSE ISLETS

Glucose	0	8.33	16.7
Control	12.2±1.4 (8)	20.7±2.4 (8)	27.9±1.5 (8)
Low pilocarpine (0.16 g/l)	17.2±1.4 (5)	26.3±5.9 (5)	33.5±3.4 (5)
High pilocarpine (1.60 g/l)	32.0±4.7 (5)	36.5±6.7 (5)	39.2±2.4 (5)

OBESE MOUSE ISLETS

Glucose (mM)	0	8.33	16.7
Control	20.8±1.0 (8)	30.7±1.1 (8)	39.6±1.2 (8)
Low pilocarpine (0.16g/l)	22.6±4.7 (5)	34.9±3.8 (5)	42.6±1.9 (5)
High pilocarpine (0.16g/l)	29.0±5.4 (5)	43.7±9.5 (5)	49.1±6.7 (5)

Mean ± s.e.m. Number of observations in parentheses.
$p<0.05$ compared with control group.

Discussion

Several techniques are now available for the isolation of intact pancreatic islet tissue (see Chapter II, page 27) but many of these procedures have inherent disadvantages when applied to the isolation of mouse islets. The present studies confirm the findings of Kuo et al (279) that pre-treatment of lean and obese mice with pilocarpine one hour prior to the removal of the pancreas produced extensive depletion of the acinar tissue, and thereby facilitated the identification and microdissection of the islets of Langerhans. The pilocarpine pre-treatment regime employed in this investigation produced a rapid hyperglycaemia and hyperinsulinaemia in both lean and obese mice, and constituted a severe insulin challenge in the obese animals (Table 9). This was apparently reflected in the insulin secretory capacity of islets isolated from these mice: pilocarpine pre-treatment was associated with an elevation of basal and glucose-induced insulin secretory activity by the islets of lean mice, but a reduction of this activity by the islets of obese mice (Table 10). Furthermore, the addition of pilocarpine to the incubation medium of islets isolated from untreated mice tended to elevate basal and glucose-induced insulin secretion from the islets of both lean and obese animals (Table 11).

The insulinogenic effect of pilocarpine is consistent with its cholinergic properties, since parasympathetic stimulation and acetylcholine have been shown to promote insulin secretion on several occasions (448, 449, 450, 451).

The rapid onset of hyperglycaemia that accompanies pilocarpine treatment is also likely to provide a stimulus for insulin secretion. The origin of the rapid hyperglycaemia produced by pilocarpine is uncertain, but a sudden increase in hepatic gluconeogenesis (or glycogenolysis) are

likely contributory factors. This effect might involve endocrine modulation since it has been reported that pilocarpine also stimulates the secretion of adrenaline (452, 453, 454) and that acetyl choline stimulates glucagon secretion in the dog (455).

The pilocarpine-induced hyperglycaemia observed after 10 minutes in lean mice appeared to be overcompensated-for after 60 minutes when the raised insulin levels were accompanied by blood sugar levels below those of untreated lean mice. The transient nature of the hyperglycaemia might have been due to the short biological half life of pilocarpine (456). In obese mice, however, the elevated blood sugar levels observed after 10 minutes fell more slowly, in spite of the concomitantly high circulating insulin levels. This observation is consonant with the marked insulin resistance shown by obese-hyperglycaemic mice (179, 183, 186, 187).

In conclusion, the present investigation demonstrates that pilocarpine pretreatment greatly facilitates the microdissection of islets from the pancreas of lean and obese mice. However, the procedure exposes the animals to a great deal of unnecessary stress, making the technique undesirable. Furthermore, since pilocarpine appears to influence the insulin secretory activity of mouse islet tissue, the use of this drug prior to metabolic studies on islets cannot be recommended.

APPENDIX II

The effect of streptozotocin on glucose and hormone induced insulin secretion from isolated islets of lean mice.

INTRODUCTION

Streptozotocin, an N-nitroso-derivative of glucosamine, is an antibiotic derived from streptomyces achromogenes, possessing among its many pharmacological properties the ability to induce experimental diabetes in many mammalian species (457). It is generally accepted that the diabetogenic action of streptozotocin results from its direct effect upon the β-cell and the subsequent impairment of β-cell metabolism and insulin secretion (458). Streptozotocin appears to decrease the rate of biosynthesis and secretion of insulin (458) which results in a depleted pancreatic insulin content, diminished β-cell response to insulinotrophic stimuli and an impaired glucose tolerance (459, 460). Insulin profiles *in vivo*, following the intraperitoneal administration of streptozotocin to mice, have been shown to be multiphasic in nature (461). Following an initial depression, plasma insulin levels were observed to rise after nine hours, subsequently falling off to very low levels. It has been suggested that this secondary transient increase in the concentration of circulating insulin reflected the sudden release of the hormone from the β-cell at necrosis.

Histological studies of the islets of Langerhans of streptozotocin-treated mice and guinea pigs have shown similar degenerative changes to those produced by alloxan, another β-cytoxic agent (460). The β-cells of these animals were characteristically degranulated and contained dilated golgi bodies and endoplasmic reticulum, together with swollen, degenerating mitochondria; all symptoms of cellular necrosis (462, 463).

However, the precise nature of the initial action of streptozotocin on the pancreatic β-cell is not fully understood. Several authors have reported reduced islet levels of nicotinamide adenine dinucleotide (NAD^+) in streptozotocin induced diabetes (461, 464) suggesting that the compound might interfere in some way with the biosynthesis or degradation of the nucleotide in islet tissue (464). The presence of exogenous nicotinamide was observed to reduce the streptozotocin induced depletion of islet NAD^+ and in addition offered some protection for the β-cell against the diabetogenic action of the drug (464, 465, 466). It has been suggested that streptozotocin and nicotinamide might interact in some way at the level of the β-cell plasma membrane (466). Rerup and Tarding (467) proposed that streptozotocin might initially bind to the β-cell membrane for a short period before causing β-cell necrosis.

The present study was undertaken to further investigate the possibility that the primary action of streptozotocin might be at the level of the β-cell membrane, perhaps interacting with glucoreceptor or adenylate cyclase-receptor units. With this in mind, the effect of the drug was examined on glucose and hormone-induced insulin secretion from isolated islets of lean mice.

Materials and Methods

20-25 week old lean animals from the obese mouse colony were split up into groups of five and allowed food and water ad libitum.

a) The effect of pretreating animals with streptozotocin on glucose and hormone induced insulin secretion.

Five lean animals were injected with streptozotocin (200 mg/0.4 ml. saline/Kg. body weight i.p.) and hyperglycaemia (blood glucose levels in excess of 250 mg/100ml, 14 mM) confirmed after 3 days. Five

saline injected animals were used as controls. All mice were killed by cervical dislocation and islets were isolated by microdissection from excised pancreata. The insulin secretory activity of the islet tissue so obtained was investigated using the in vitro perifusion system described previously (Chapter IVA, page 56). Test and control channels (control contained 3mM glucose for both pre-perifusion period and test perifusion period) were run simultaneously. 15 islets were placed in each millipore filter holder and pre-perifused with Gey & Gey bicarbonate buffer, pH 7.4, 37°C for 40 minutes. In the test channel the perifusion medium was changed to one containing either glucose (25mM); secretin (0.2 clin. U./ml., Karolinska Institute, Stockholm) + 3mM glucose; pancreozymin (1.5 clin.U./ml., Karolinska) + 3mM glucose or pancreatic glucagon (5.0 μg/ml., Sigma, London) + 3mM glucose. Samples of perifusate were collected immediately and then subsequently at one minute intervals (flow rate 0.5 ml/Min.). These samples were then quick frozen for the subsequent radioimmunoassay of insulin (μU/ml/mg. freeze dried islet tissue).

b) The effect of streptozotocin (1 mg/ml.) on glucose and glucagon induced insulin secretion from isolated perifused lean mouse islets.

Lean mouse islets were pre-perifused for 40 minutes with 3mM glucose and the subsequent effect of adding streptozotocin (1 mg/ml) to the test perifusion medium investigated on the rate of insulin secretion induced by either glucose (25mM) or glucagon (5 μg/ml) + 3mM glucose. In these experiments streptozotocin (40 mg) was dissolved in 1 ml. of citrate buffer, pH 3.6 and added to the test perifusion medium reservoir just prior to use. Aliquots of perifusate were collected at one minute intervals (flow rate 0.5 ml/Min.) and the insulin content measured by radioimmunoassay.

Results

a) The effect of streptozotocin injection on glucose and hormone-induced insulin secretion.

The mean blood glucose value of 5 mice three days after streptozotocin injection was found to be 302 ± 41 mg/100 ml.

There was no significant difference between the 3mM glucose control insulin secretion profiles of islets incubated in the presence and in the absence of streptozotocin (figure 70 a & b). The presence of streptozotocin in the incubation medium completely abolished the usual insulin secretory response to 25mM glucose (fig. 70a).

In contrast the initial insulin response to glucagon in the presence of 3mM glucose was not abolished by the addition of streptozotocin (1 mg/ml.) to the incubation medium (figure 71). However, after some 40 minutes the glucagon response began to decline in the presence of streptozotocin but was always significantly greater than the 3mM glucose control profile shown in figure 70a.

The effect of 5 minutes pre-perifusion with streptozotocin (1 mg/ml.) on the subsequent insulin secretory response of lean mouse islets to either 25mM glucose or glucagon (5 μg/ml.) + 3mM glucose is shown in figure 72a and b. Pre-perifusion of lean mouse islets for five minutes with medium containing streptozotocin completely abolished the subsequent insulin secretory response to 25mM glucose, but did not significantly impair the subsequent secretion of insulin induced by (5 μg/ml) glucagon + 3mM glucose.

Figure 68 The effect of streptozotocin injection on glucose induced insulin secretion from perifused lean mouse islets.

(Each profile represents the mean of 5 separate profiles, ± s.e.m., one profile per animal)

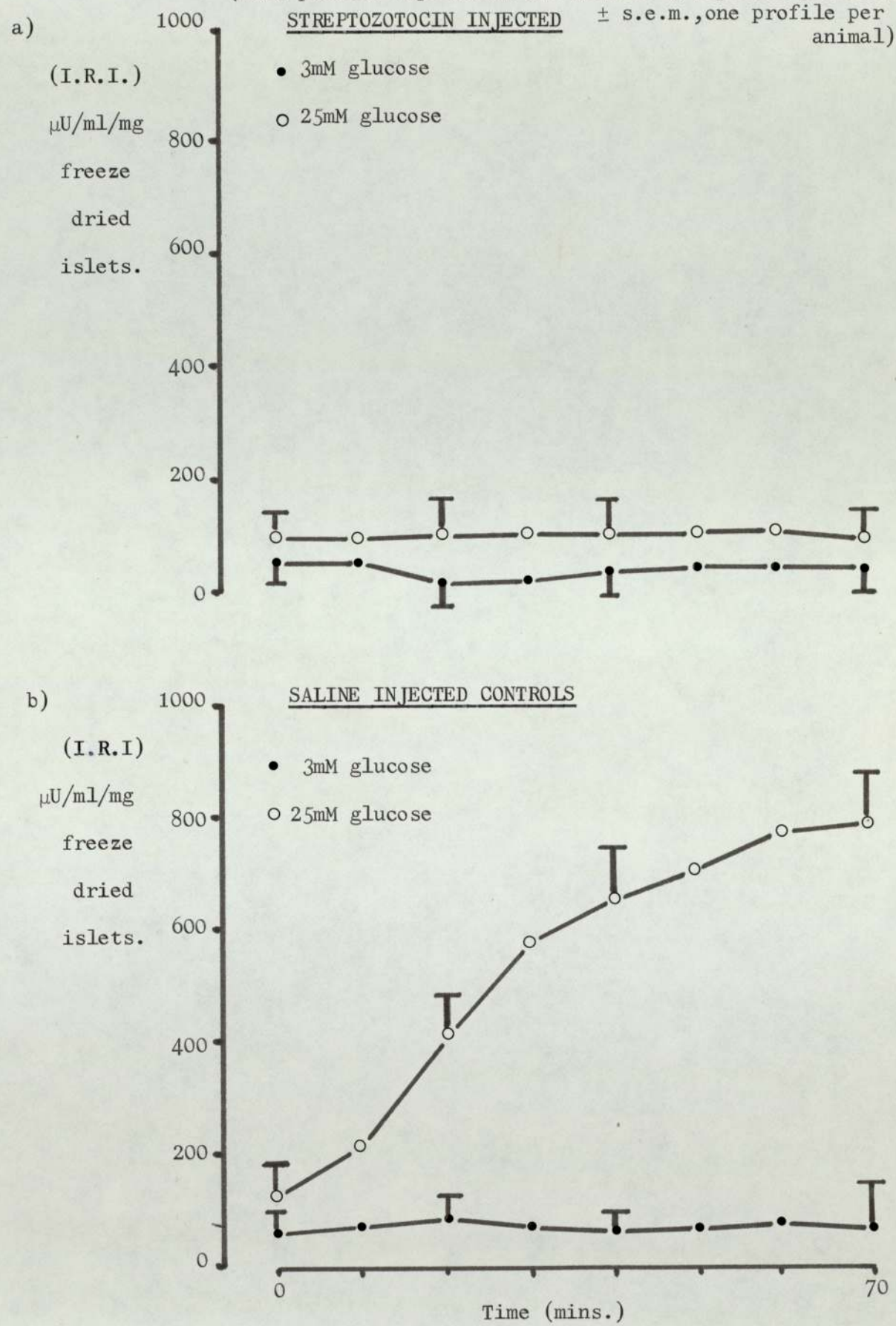


Figure 69 The effect of streptozotocin injection on hormone-induced insulin secretion from perifused lean mouse islets.

(Each profile represents the mean of 5 separate profiles ± s.e.m., one profile per animal).

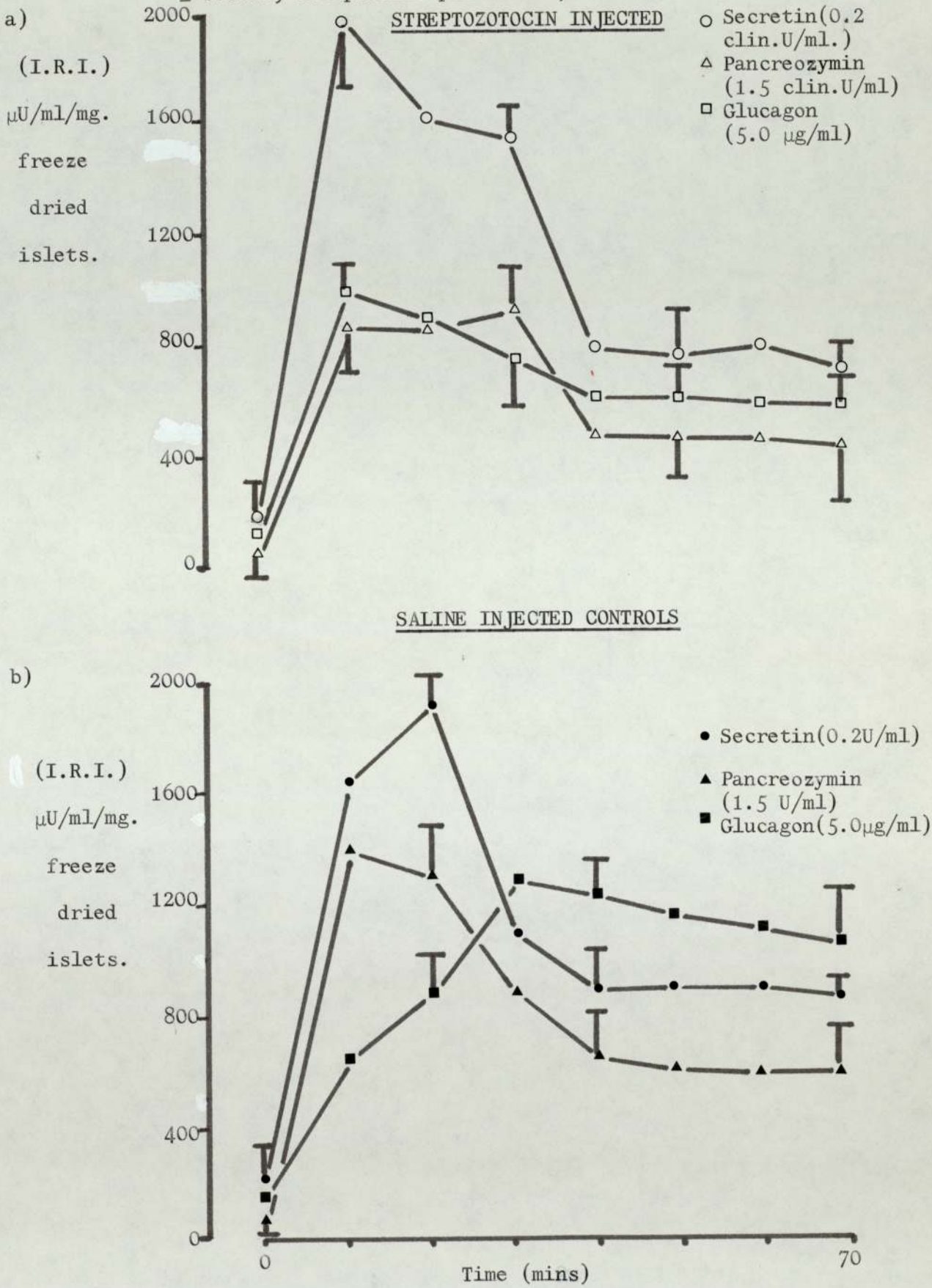


Figure 70 The effect of glucose in the presence of streptozotocin (1mg/ml) on the secretion of insulin from perifused lean mouse islets.
(Each profile represents the mean of 5 separate profiles ± s.e.m., one profile per animal.)

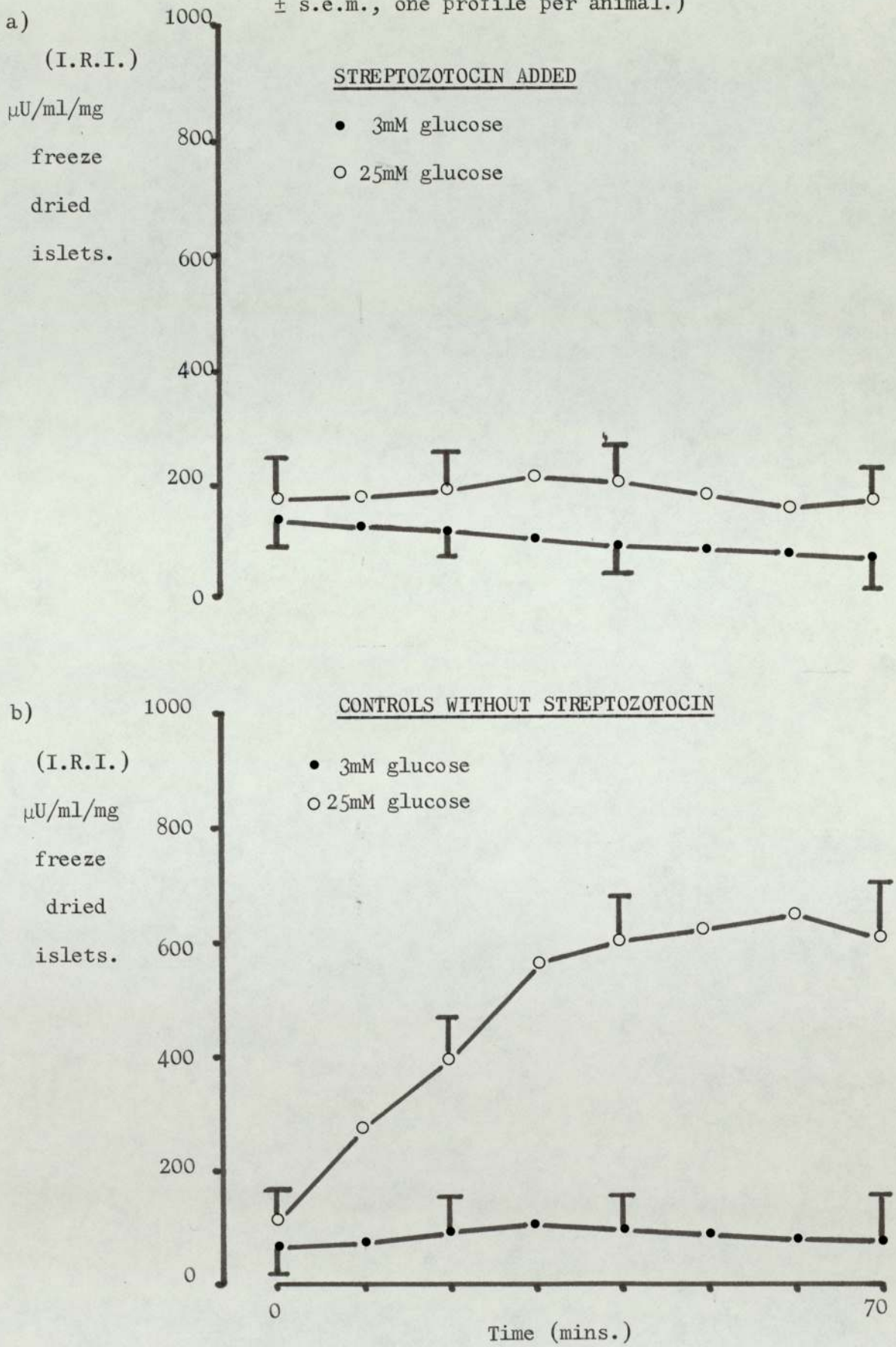


Figure 71 The effect of Streptozotocin (1μg/ml) on glucagon (5μg/ml) + 3mM glucose induced insulin secretion from perifused lean mouse islets

(Each profile represents the mean of 5 separate profiles ± s.e.m., one profile per mouse).

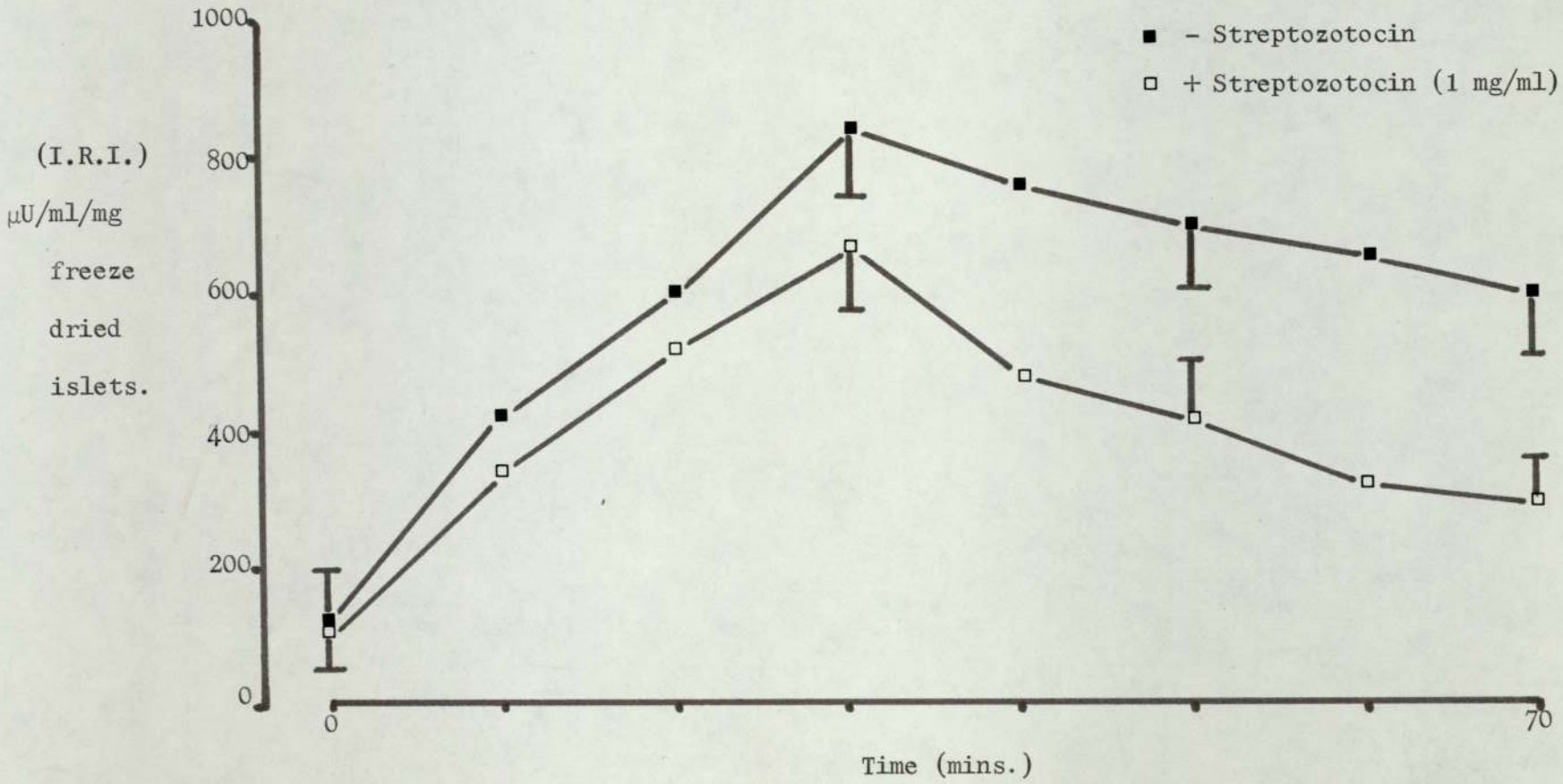
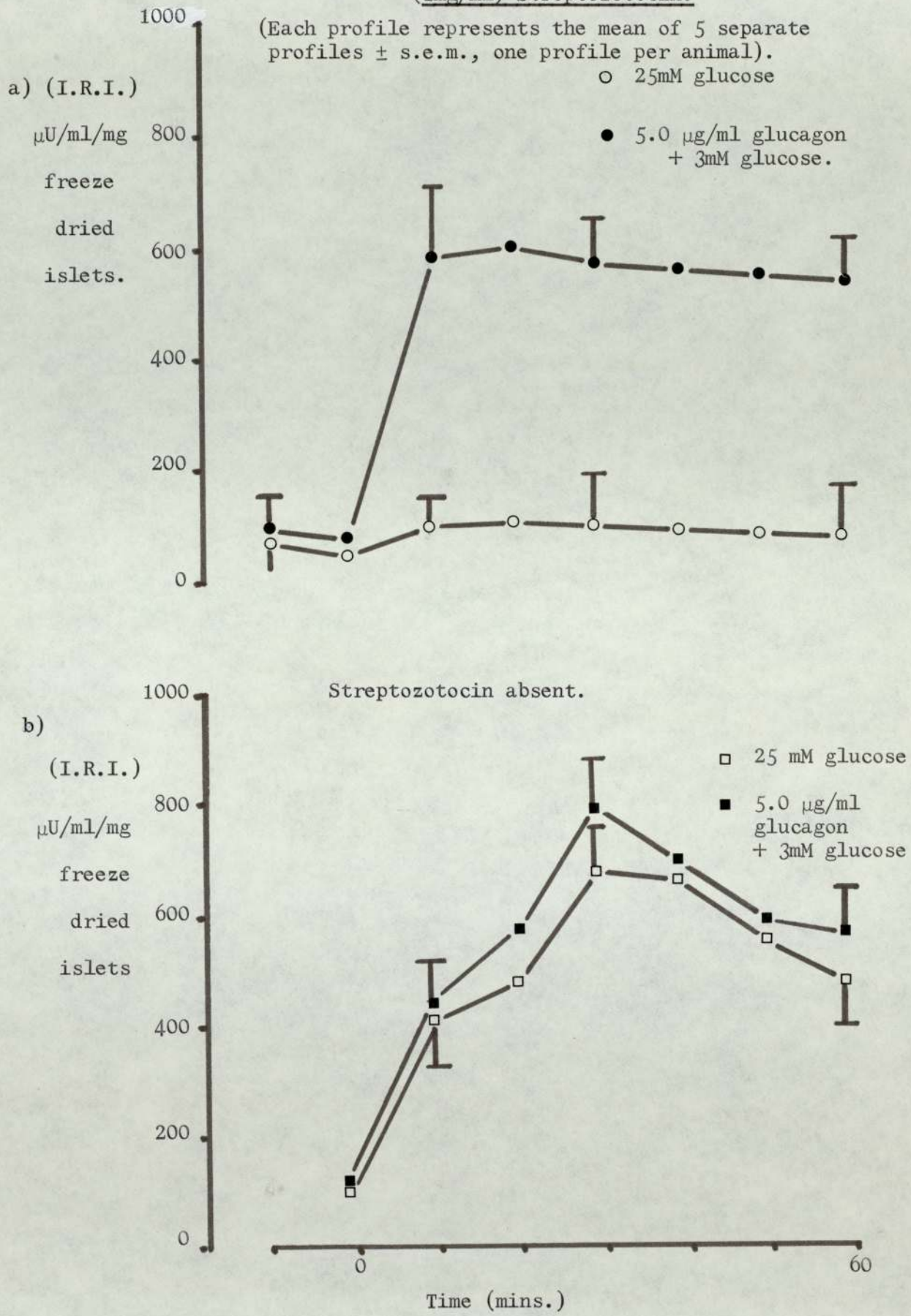


Figure 72 Glucose and glucagon induced insulin secretion from lean mouse islets preperifused with medium containing (1mg/ml) Streptozotocin.



Discussion

Investigations involving the use of streptozotocin (particularly *in vitro*) are complicated by the instability of the compound in solution at physiological temperature and pH (461). At pH 7.0 and 37°C the half life of streptozotocin has been reported to be something less than one hour (471). In the present studies this difficulty was minimised to some extent by the addition of a fairly high concentration of the drug (1 mg/ml.) to the perifusion reservoir immediately prior to use.

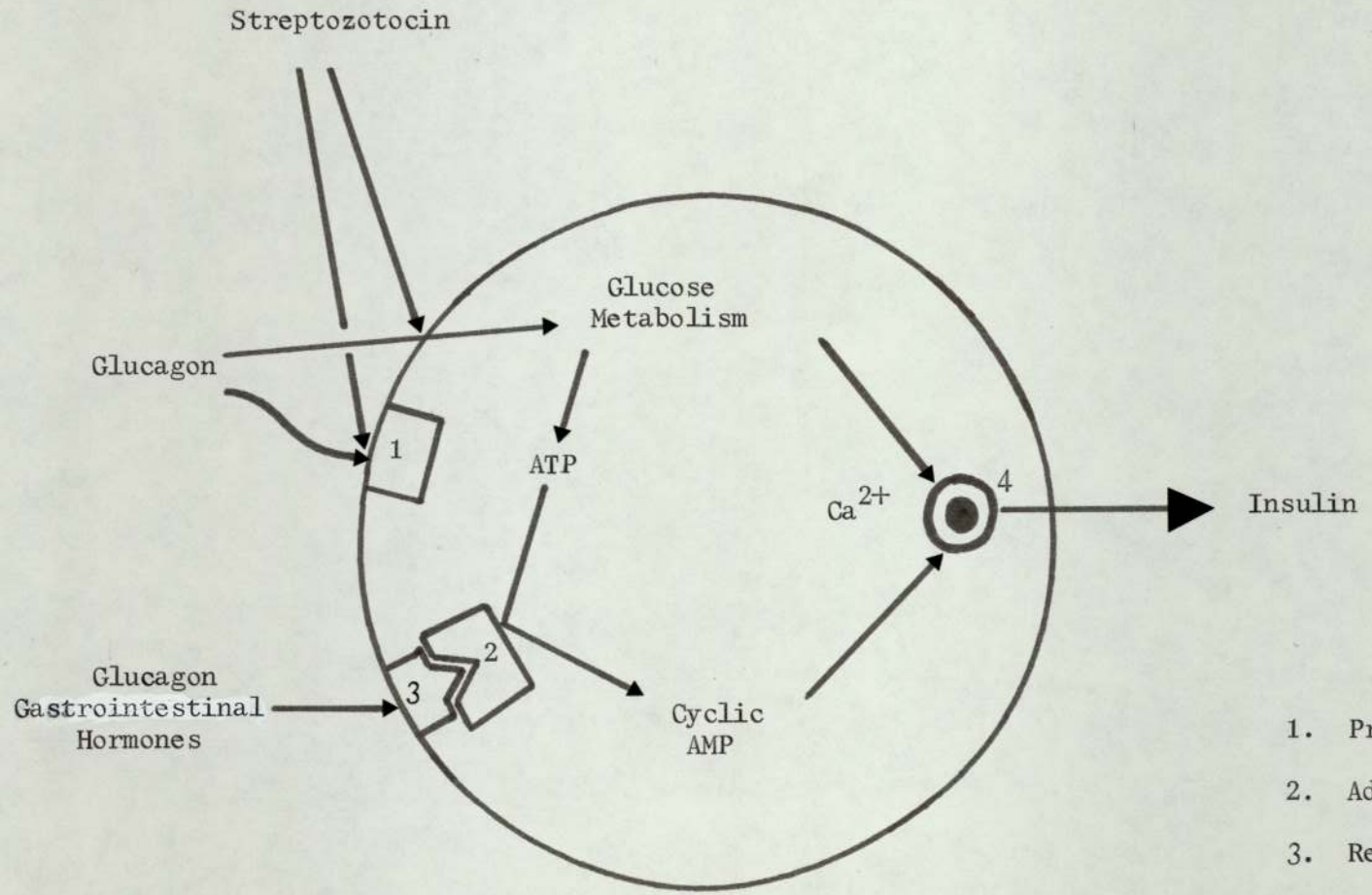
The results of the present work confirm previous observations that the exposure of islet tissue to streptozotocin, both by injection *in vivo* (459) and incubation *in vitro* (465) is associated with a reduced or abolished insulin secretory response to glucose. Perifused islets from streptozotocin injected lean mice and isolated lean mouse islets either pre-perifused or test perifused with medium containing 1 mg/ml streptozotocin were shown to be unresponsive to a stimulatory concentration of glucose (25 mM). Many of these observations have recently been confirmed in the rat by Renold and colleagues (468). However, islets from streptozotocin injected lean mice retained the ability to secrete insulin in response to secretin, pancreozymin and glucagon. This might suggest that streptozotocin interacts with some form of β-cell membrane glucoreceptor and/or glucose transport and metabolism within the β-cell, but does not appear to impair the hormonal stimulation of insulin secretion perhaps via adenylate cyclase. Certainly the presence of the glucose moiety in streptozotocin suggests that the compound is structurally qualified to interact with a glucose transport mechanism at the level of the β-cell membrane (471). Moreover, the presence of 2-deoxy-D-glucose, an inhibitor of glucose transport across the β-cell membrane, has been shown to protect the β-cell from the diabetogenic

action of streptozotocin (466).

Insulin secretion induced by the hormones secretin, pancreozymin and glucagon appears to be largely unaffected by the presence of streptotozotocin suggesting that perhaps hormones and metabolic substrates such as glucose stimulate the secretion of insulin via different molecular mechanisms. It is interesting to note that in human diabetes mellitus, the pancreatic β-cell is relatively unresponsive to glucose, whilst the response to hormones such as glucagon and secretin is often unimpaired(62, 469, 470, 472, 473). In the light of these observations it has been suggested that the early lesion in diabetes mellitus might be an acquired or selective unresponsiveness of the β-cell (and possibly also the α-cell) to glucose in the presence of adequate insulin reserves (474).

It would appear from the present data that streptozotocin might be a useful agent for differentiating between those insulinotrophic agents that are thought to stimulate insulin secretion via some form of glucoreceptor and those which are thought to act via the β-cell membrane adenylate cyclase system (figure 73). This work also suggests that the proposed glucoreceptor and adenylate cyclase systems appear to be functionally separate entities at the level of the β-cell membrane, even though they may be very closely inter-related.

Figure 73 Possible sites of action of Streptozotocin on the islet β-cell



1. Proposed glucoreceptor.
2. Adenylate cyclase.
3. Receptor sub-unit.
4. Microtubular-Microfilamentous system.

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Effect of gastrointestinal hormones on the phasic release of insulin from isolated islets of normal and obese hyperglycaemic mice. By L. Best, T. W. Atkins and A. J. Matty. *Department of Biological Sciences, University of Aston in Birmingham, Gosta Green, Birmingham, B4 7ET*

The present study examines the effects of several gastrointestinal hormone preparations on the insulin secretion dynamics of isolated islets of Langerhans incubated in a continuous flow perifusion system. A modification of the method of Idahl (1972) was used, in which oxygenated buffer containing the appropriate concentration of test hormone and glucose was passed through a perifusion chamber containing 10 islets, microdissected from either normal or obese mice. The effluent was collected at 1 min intervals and the immunoreactive insulin content measured.

Raising the glucose concentration from 60 to 300 mg/100 ml had no significant effect upon the secretion of insulin from either normal or obese mouse islets. A glucose concentration of 500 mg/100 ml produced a small initial 'spike' followed by a sustained rise in insulin secretion for approximately 60 min. This effect was especially marked with obese mouse islets. Crude pancreozymin and secretin, perifused at 1·5 Ivy Dog units/ml and 1·5 Crick units/ml respectively, both produced an immediate rise in insulin secretion, lasting some 20 min. Glucose (300 mg/100 ml) prolonged the effect of pancreozymin on both normal and obese islets. Obese mouse islets were the more sensitive to both hormone preparations. Purified pancreozymin and secretin also had a stimulatory effect on the insulin secretion dynamics of both normal and obese islets.

Obese islets also showed high sensitivity to glucagon. At 5·0 μg/ml, the hormone elicited a marked, sustained rise in insulin secretion from both normal and obese mouse islets. This effect was potentiated by glucose (300 mg/100 ml).

Gastrin pentapeptide (2·0 μg/ml) had no significant effect on insulin secretion from either normal or obese mouse islets and showed no modification of glucose-induced insulin secretion. Similarly, little response was shown with synthetic human gastrin I (1·0 μg/ml). These results substantiate previous reports (Iverson, 1971) of a phasic stimulation of insulin secretion by secretin, pancreozymin and glucagon. The effects elicited by gastrin appear to be largely pharmacological.

The immediate and pronounced effects of gastrointestinal hormones on the phasic secretion of insulin may be consonant with the hypothesis that the enteroinsular axis provides an early warning system and a fine tuning of insulin secretion after food ingestion. The hypersensitivity shown by obese mouse islets to gastrointestinal hormones may contribute to the initial hyperinsulinism characteristic of this syndrome.

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