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Title of Thesis: Gastrointestinal Hormone and Amino Acid Induced Insulin Release from Isolated Pancreatic Islets of Langerhans

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Abstract

Ingestion of glucose and amino acids is associated with a greater rise in serum insulin than is intravenous infusion. Evidence indicates gastrointestinal hormones mediate the response. Mostly on the basis of in vivo studies, it is thought that secretin and enteric glucagon mediate the effect during glucose absorption and pancreozymin and pancreatic glucagon probably are involved during amino acid absorption. Gastrin may produce secretin release and act indirectly.

The present studies were performed using isolated islets of Langerhans of the rat obtained according to the method of Lacy and Kostianovsky. Secretin and glucagon have been demonstrated to produce insulin release at both 50 and 300 mg% glucose. Gastrin was without effect. Pancreozymin produced insulin release at 300 but not 50 mg% glucose. Arginine produced insulin release at both 50 and 300 mg% glucose but leucine was without significant effect. The significance of these findings in relation to in vivo findings is discussed and the need for further work is stressed.

Title for lettering along the back of the bound copies:

GUT HORMONE AND AMINO ACID INDUCED INSULIN RELEASE, in vitro.

GASTROINTESTINAL HORMONE AND AMINO ACID INDUCED INSULIN RELEASE FROM ISOLATED PANCREATIC ISLETS OF LANGERHANS

by

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Introduction

In 1927 Lennox[1] demonstrated that oral glucose was more rapidly assimilated than glucose given intravenously. Scow and Cornfield (1954)[2] felt this was due to rapid removal of glucose from the portal vein by the liver. Although unconfirmed, this was an attractive and lasting explanation for the superiority of oral over intravenous glucose tolerance.

In 1950[3] a previous oral glucose load was found to accelerate the disappearance of a subsequent intravenous load. That insulin might be involved was suggested by Arnould et al.[4] in 1963 who found insulin-like activity (I.L.A.) to be elevated to a higher level for a given rise in blood glucose when glucose was given orally to dogs compared to the intravenous route. The existence of an insulinogenic mechanism in the duodenum or hepatic region was postulated.

Evidence for such a mechanism in the hepatoportal region was suggested by the finding that I.L.A. was strikingly increased by infusion of glucose into the portal vein of dogs when compared with systemic vein infusion.[5] Furthermore, pancreatic venous insulin was found to be increased when glucose was infused into the portal vein even with no appreciable rise in systemic blood glucose.[6] Most other studies present evidence against a portal insulinogenic mechanism although, certainly, the liver plays a significant role in glucose assimilation.

That there is a physiologic secretion of the intestinal wall causing pancreatic insulin release has been the premise of our investigations. The first evidence, albeit indirect, of an

intestinal and not a hepatic insulinogenic mechanism was presented by Dupré in 1964.[7] He confirmed the previous finding that intravenous glucose was assimilated more rapidly when an oral glucose load was given previously. In addition, he found this effect to be unaltered in a patient with liver disease before and after portacaval anastomosis (although in five other cirrhotics without shunts there was no difference in glucose half time after intravenous glucose when preceded by oral glucose).

Elrick et al[8] found similar blood glucose levels and disappearance rates comparing the two routes of administration but found immunoreactive insulin (I.R.I.) levels to be higher after enteric glucose again suggesting the existence of a gastrointestinal hormone although they could not exclude the liver as the source of this stimulus.

McIntyre et al[9] showed higher insulin levels when glucose was infused intraduodenally compared to intravenous infusion even though greater glucose concentrations were obtained with the latter in normals. These workers[10] and Perley and Kipnis[11] reported similar findings in cirrhotics with portacaval shunts. This latter information confirmed Dupré's previous work, providing further evidence for intestinal rather than hepatic involvement in increased insulinogenesis when glucose is given orally compared to intravenously.

Dupré et al[12] demonstrated failure of portal hyperglycemia to account for the enteric glucose induced elevation of blood insulin in experiments in which glucose was infused endoportally in man. Colwell and Colwell[13] also demonstrated the absence of an

hepatic insulinogenic mechanism.

Perley and Kipnis[11] were able to quantitate the relative insulinogenic potencies of the alimentary and glycemic stimuli by reproducing the venous blood sugar profile after 100 gm oral glucose with an intravenous glucose infusion. They found that the alimentary stimulus, in normal subjects, accounted for 30 - 40% of the total insulin released. Furthermore, they calculated that 69 gm of a 100 gm oral glucose load is taken up by the liver and that insulin increases hepatic glucose extraction.

Historically, the existence of an insulinogenic gastrointestinal hormone was suggested in 1906 by Moore et al.[14] Considerable work followed.[15] Initially, attention was focussed on crude secretin preparations which were found to produce hypoglycemia following intravenous injections.[16-18] More recently, glucagon has been extensively investigated. Two other gastrointestinal hormones, pancreozymin and gastrin may also play a role.

Thus, when compared to intravenous infusion glucose ingestion is associated with increased glucose disposal rate and increased insulin levels. The increased disposal rate is a function of portal hyperglycemia and increased hepatic uptake, and increased insulin levels which both increase hepatic uptake and peripheral glucose utilization. The increased insulin response is postulated to be the result of stimulation of an insulinogenic alimentary mechanism. This has been the premise of our investigations.

The existence of such a mechanism would contribute to the maintenance of metabolic homeostasis by insuring the immediate

release of insulin coincident with glucose ingestion thereby accelerating hepatic extraction of absorbed glucose and preventing excessive fluctuations in peripheral blood glucose and insulin levels.

In the following sections the mechanisms of increased insulin release in response to ingested glucose and amino acids compared to intravenous infusion is considered. Then the in vitro techniques are reviewed and the reasons for the selection of the isolated islet technique are considered.

A. Gastrointestinal Secretagogues and Glucose Absorption

That glucose concentration of blood perfusing the pancreatic islets of Langerhans is the primary factor influencing insulin secretion is well accepted.[19-22] The role that the four enteric hormones, secretin, gastrin, glucagon and pancreozymin play in the increased insulinogenesis of oral glucose ingestion will be considered.

I Secretin

Early in this century it was shown that introduction of acid into the upper intestine leads to secretion of pancreatic juice low in enzymes and high in bicarbonate.[23] Bayliss and Starling[24] claimed to have demonstrated the existence of a circulating hormone in this regard which they named secretin. Extraction procedures and bioassays were developed in which the volume of pancreatic juice in dogs (Ivy et al[25]) or the titratable alkali in cats (Hammarsten et al[26]) was determined in response to a secretin injection. In 1951, in their classic paper Wang and Grossman[27] using a unique pancreatic transplant system found hydrochloric acid

(HCl) to be the most powerful stimulus to secretin secretion followed by the products of protein digestion. Isotonic glucose was without effect.

Recent work has produced isolation and purification.[28] Secretin has been found to be a single basic polypeptide chain with 27 amino acid residues. The molecule was synthesized in 1966.[29] There is a striking similarity between the structures of glucagon and secretin with 14 amino acids occupying the same position.

Between 1916 and 1940[15] considerable work was done attempting to elucidate the nature of a possible gastrointestinal humoral substance influencing blood glucose. The leading figures were Zunz and Labarre[16-18, 30] who in 1928 showed intravenous crude secretin, a duodenojejunal extract, caused hypoglycemia in dogs and that the effect was mediated by the pancreas (although they thought there was a direct peripheral effect on metabolism). Laughton and Macallum[31] described a duodenal mucosal extract with negligible secretin activity which had no effect on fasting blood glucose but diminished the hyperglycemic response to a glucose load. These findings were subsequently denied[32] and interest waned.

In 1938 Shay et al[33] found infusion of HCl, among other substances, to diminish the rise in blood glucose after a glucose load. In 1962 Lechin[34] found a secretin lowered serum potassium and provided evidence that insulin was released accounting for the effect. In 1964 after finding oral glucose produced improved assimilation of subsequent intravenous loads in normals and in a patient before and after portacaval anastomosis,[7] Dupré[35] was led to investigate the effect of the gastrointestinal hormone secretin.

He found in 7 patients that secretin given with the second intravenous glucose load reproduced the effect of a previous oral load.

Pfeiffer et al[36] using rabbit and dog pancreas slices, then found secretin to increase insulin release even without glucose in the incubation medium. Similar observations were reported by McIntyre et al.[37] Hadjikhani et al[38] demonstrated secretin-induced insulin release from isolated rat islets and on intravenous injection. However, recently, Lazarus et al[39] failed to demonstrate secretin-induced insulin release from rat pancreas slices. They did, however, produce insulin release by intravenous injection into fed rats. Secretin was also found to be lipolytic but not glycogenolytic.

Dupré and Beck[40] demonstrated insulin (I.L.A.) was elevated when an active extract of gastrointestinal mucosa obtained according to the method of Crick et al[41] was given with intravenous glucose. The insulin level was comparable to that obtained by oral ingestion of glucose and glucose tolerance was improved. Thus, the insulinogenic agent in the extract may be a humoral factor secreted during glucose absorption.

Using a similar extract in dogs, Ketterer et al[42] demonstrated increased insulin and glucagon of pancreatic venous blood without hyperglycemia. In the same paper they produced insulin release with glucagon administered by mesenteric vein and thus postulated the participation of two gastrointestinal insulin releasing factors - glucagon and an unknown factor in the extract which may act indirectly by increasing pancreatic glucagon.

Dupré et al[43] studied patients with liver disease and

umbilical vein catheters. They found crude and purified secretin administered by the physiologic endoportal route to produce a large rise in I.R.I. in portal blood and a smaller rise in peripheral blood without changes in glucose or glucagon suggesting the insulin release produced by secretin was not mediated by glucagon although a local effect within the islets could not be excluded. Similarly Unger et al[44] reported a rapid rise in insulin levels on endoportal injection of secretin in dogs.

In 1966 Boyns et al[45] attempting to produce the release of secretin and/or pancreozymin infused 25 ml of 0.05N citric acid over 10 minutes. There was no effect on serum insulin and no enhancement of the insulin rise when glucose was given simultaneously. These results contrast with those of Shay et al[33], Boyns et al[46,47] found intravenous secretin produced a short-lived elevation of I.R.I. and augmented the I.R.I. response to intravenous glucose. They felt, therefore, that although secretin is insulinotropic, the dose was probably unphysiologic and the failure to influence serum insulin levels by infusion of acid into the duodenum case doubt on the physiological role of secretin in carbohydrate metabolism. Furthermore, Wang and Grossman[27] and Sum and Preshaw[48] showed that isotonic glucose infused into the small bowel had no effect on pancreatic bicarbonate secretin and therefore could not provoke insulin release via secretin.

Unger et al[49] injected secretin by mesenteric vein and produced a brief but striking rise in pancreaticoduodenal vein I.R.I. without a change in glucagon-like immunoreactivity (G.L.I.) or glucose.

Dupré et al[50-52] then presented data demonstrating augmentation of I.R.I. and improved glucose assimilation but no change in G.L.I. when secretin was added to a 40 minute intravenous glucose infusion. Thus the physiologic rise in G.L.I. with glucose ingestion was not reproduced by secretin and further doubt is cast upon its role as the sole physiologic mediator. When secretin was infused with arginine a short initial I.R.I. increment but a diminished increment at 30 - 40 minutes was demonstrated. More important, however, the addition of an infusion of 30 meq HCl into the duodenum, or intravenous gastrin or betazole, during 40 minute glucose infusions produced higher levels of I.R.I. but no change in G.L.I. and surprisingly no significant change in glucose half-time. The dose used produced the approximated normal gastric acid output in response to a meal and is greater than that of Boyns et al,[45] thus possibly explaining their failure to obtain similar results.

Mahler and Weisberg[53] gave 10 achlorhydric patients 300 ml 0.1N HCl by mouth and found no I.R.I. elevation (four patients) or enhancement of I.R.I. elevation when acid was followed by intravenous glucose (three patients). Secretin given intravenously, however, did cause significant transient I.R.I. elevation. Thus the findings of Boyns et al[45] that endogenous stimulation of secretin does not affect glucose tolerance or serum insulin, were confirmed. Evidence was cited that achlorhydric patients should respond normally but the possibility remains that the action of endogenous secretin or insulin secretion may be less readily demonstrated when the blood glucose is falling after rapid intra-

venous infusion of glucose.[52]

In summary, secretin is insulinogenic on intravenous administration especially if the blood glucose is raised. Evidence of its in vitro effect is conflicting, as is the effect of endogenous secretin produced by increasing duodenal acidity. Elucidation of the role of secretin ultimately awaits the development of a secretin radioimmunoassay. There is some evidence radioimmunoassayable secretin is elevated very quickly after glucose ingestion.[54]

II Glucagon

Glucagon is another obvious candidate for the gastrointestinal mediator of insulin secretion and improved oral glucose tolerance.

The existence of a hyperglycemic substance in some early preparations of insulin was recognized[55] and was named glucagon in 1923.[56] The work in the late 40's and early 50's of de Duve[57] and Sutherland[58,59,60] has elucidated more clearly the nature of glucagon. Bioassays were developed and its hyperglycemic - glycogenolytic action was found to be due to increasing the amount of active hepatic phosphorylase, a rate limiting factor.[60] It was also shown to stimulate lipolysis[61] and protein catabolism.[62]

In 1948 it was determined that glucagon originated from the pancreatic alpha cells.[63] Sutherland[58] demonstrated in the same year that glucagon could be extracted from gastric mucosa and to a lesser extent duodenum and ileum.

Glucagon was obtained in 1953 in a highly purified crystalline form and found to be a polypeptide.[64] Bromer[65] determined the amino acid sequence in 1956.

The history of glucagon-induced insulinogenesis began in 1952 when Foa et al,[66] on the basis of cross-circulation studies in dogs, suggested that glucagon was insulinogenic. In 1960, Yalow and Berson,[67] in their classic paper describing the insulin immunoassay found that glucagon produced an increase in plasma insulin but this was felt to be due to hyperglycemia. Unger et al,[68] having developed the first glucagon immunoassay showed that canine pancreatico-duodenal vein G.L.I.* levels were elevated with fasting, phlorizin-induced chronic hypoglycemia and insulin-induced hypoglycemia and depressed by rapid intravenous glucose administration.[69] They showed as well that in total starvation in humans glucagon levels rise[70] and postulated glucagon was a hormone of glucose need and that the pancreatic islets exhibit bihormonal regulation of the disposition of glucose according to the body's needs. More important to the present thesis, however, they found oral glucose produced a fall in G.L.I. at 45 - 240 minutes with a concomitant fall in human growth hormone (H.G.H.), but then a rise in G.L.I. with H.G.H.[69] These points have been disputed and the contrary later was demonstrated by Unger as will be described below.

In 1965 Samols et al[71] presented clear evidence that glucagon given intravenously in large doses (1 mg) increased I.R.I. independently of its effect on blood glucose and improved glucose disappearance. The insulinemic effect was potentiated by the

* (G.L.I. or glucagon-like immunoreactivity denotes the sum of pancreatic and gut glucagon).

simultaneous administration of glucose. It was suggested that oral glucose may stimulate gut glucagon which may be one of the factors responsible for the greater rise in insulin when glucose is taken orally compared to intravenously. They forsaw a dual physiologic role: glucagon would suppress hepatic uptake and stimulate insulin secretion, thereby promoting the peripheral utilization of glucose.

Crockford et al[72] corroborated these findings using smaller but still probably unphysiologic doses.

Using Coore and Randle's rabbit pancreas slices technique[73] Turner and McIntyre[74] and Devrim and Recant[75] using rat pancreas slices demonstrated glucagon in large doses causes insulin release at low glucose concentrations. Vecchio et al[76] and Lambert et al[77] demonstrated insulin release by glucagon in organ cultures of fetal rat pancreas.

Samols et al[78] then administered large 200 gm glucose loads orally and found that plasma immunoreactive glucagon rose to a peak at the 45 minute mark and again at 180 - 240 minutes. This provided further evidence that oral glucose stimulates the release of gut glucagon although it certainly did not exclude the release of pancreatic glucagon by reflex or humoral mechanisms. The explanation for a second late rise in glucagon was felt to involve growth hormone.

Lawrence[79] then found increased immunoreactive glucagon with starvation but no changes with insulin induced hypoglycemia. Small (1 gm/kg) oral glucose loads produced a small diminution in glucagon but larger (1.75 gm/kg) loads produced an elevation.

Intravenous glucose produced no change. She concluded glucagon plays a role in stimulating insulin release with orally administered glucose since glucagon levels are not changed with intravenous glucose despite similar blood glucose levels.

In June 1966 at the American Diabetes Association meeting Ketterer et al[42] presented data showing that in dogs administration of glucagon in physiologic doses via the physiologic portal venous route caused a doubling of insulin secretion with mild hyperglycemia and only small increments in arterial glucagon. In addition, they found similar effects with glucagon extracted from dog stomach and the Crick-Harper-Raper extract of hog duodenal mucosa, (probably secretin) which contained negligible amounts of glucagon, raised pancreatic venous insulin and glucagon. Thus, it was concluded there are at least two gastrointestinal insulin releasing factors: glucagon, and an unknown factor in the Crick-Harper-Raper extract (probably secretin) that may act by causing glucagon release.

The problem at this stage was although immunoreactive[42] and biologically active[80] glucagon had been extracted from gastrointestinal tissues, serum G.L.I. rises with oral glucose, and glucagon stimulates insulin secretion and improves glucose tolerance, it had not been determined that the gut secreted the hormone. Unger et al[81] then claimed that gut and pancreatic glucagon were identical with respect to immunoreactivity and gel filtration characteristics and in producing in vivo hyperglycemia. Samols et al,[83] however, demonstrated immunochemical non-identity. (Unger et al later conceded this point, vide infra). Samols

postulated that alimentary glucose produces an increase in serum G.L.I. due to a huge pancreatic glucagon release overflowing into the portal circulation and peripheral blood. Pancreatic glucagon then acts primarily physiologically as an insulinogenic agent due to the close proximity of the alpha and beta cells. Gut glucagon is elevated in fasting and has a primary physiologic role in glycogenolysis.

In 1966 Dupré et al[43] using patients with umbilical vein catheters demonstrated that the insulinogenic effect of secretin was not mediated by circulating glucagon but an effect on glucagon release within the islets could not be excluded.

A report from Unger's laboratory in 1967[83] demonstrated in dogs that rapid or prolonged endoportal infusion of pancreozymin caused a sharp and striking rise in pancreaticoduodenal vein insulin and a rapid rise in pancreaticoduodenal vein glucagon with a later small rise in glucose. Pancreozymin, thus, was the first hormone known to have glucagon-releasing properties. Secretin did not produce glucagon release. Since intraduodenal instillation of protein hydrolysates was shown to cause a striking rise in both insulin and glucagon secretion[84] and pancreozymin is secreted in response to amino acid ingestion[27] it was postulated that pancreozymin produces pancreatic glucagon release which is then directly or indirectly insulinogenic.

Unger and his co-workers[85,86] then demonstrated in dogs that intravenous glucose suppressed pancreatic venous G.L.I. secretion and that intraduodenal glucose produced a rise in gut glucagon specifically (differentiated from pancreatic glucagon by

removing the pancreas and cannulating mesenteric veins) confirming Samols' and Lawrence's findings that oral glucose produces increased glucagon release. They confirmed Samols' finding of the non-identity of the two glucagons. Gut and pancreatic glucagon of dogs were extracted and data was produced suggesting gut G.L.I. has a molecular weight twice that of pancreatic G.L.I. and that jejunal G.L.I. is devoid of glycogenolytic and hyperglycemic activity but does cause insulin release. Thus, the substance isolated from gut is either quite different from glucagon but cross reacts with glucagon antibodies (thus C.R.M. or cross-reacting material) or it may be an aggregate of glucagon or glucagon complexed to another molecule. They concluded that increased secretion of pancreatic glucagon is not but that gut glucagon (C.R.M.) may be involved in the response to glucose ingestion. Pancreatic glucagon may be involved in the heightened insulin response to ingested amino acids.

Buchanan et al[87] confirmed the above finding that circulating glucagon after oral glucose was of intestinal origin using pancreatectomized animals. Basal G.L.I. was also found to be of intestinal origin.

A recent report from Unger's laboratory[88] describes immunologic descrimination between pancreatic and enteric glucagon.

Dupré et al[50-52] studied the effects of intravenous and intraduodenal infusions of glucose and amino acids with and without the enteric hormones, gastrin, secretin and pancreozymin. When pancreozymin was added to an intravenous glucose infusion insulin release was potentiated but there was no change in G.L.I. which is

in accord with the finding of Ohneda et al[89] that hyperglycemia suppresses the glucagon stimulating effect of pancreozymin. However, when pancreozymin was added to an arginine infusion not only was insulin release potentiated and amino nitrogen levels diminished but there was a significant G.L.I. elevation and a concomitant small rise in blood of glucose. More rapid infusion accentuated the effect. Slower intravenous arginine infusions were performed to duplicate the amino nitrogen levels obtained on intraduodenal arginine infusions. A sustained rise in I.R.I. in the latter instance was noted presumably due to G.L.I. through pancreozymin. Thus it seems that pancreozymin may play a physiologic role in enhancing the insulin response to ingested protein by stimulating the release of pancreatic glucagon.

In summary, the situation at the present with respect to glucagon is presently accepted as follows: Two substances react in the glucagon immunoassay - pancreatic (alpha cell) glucagon and a substance isolated from the gut which may be called gut glucagon although these may be differentiated. Gut glucagon is insulinogenic whereas pancreatic glucagon is both insulinogenic and glycogenolytic. Glucose ingestion is associated with an increase in glucagon of intestinal origin. Glucose ingestion produces neither of the enteric hormonal effects on the exocrine pancreas. Secretin and gastrin administration has not been reported to produce glucagon release. Pancreozymin can produce glucagon release but its effect is suppressed by hyperglycemia. Thus, carbohydrate ingestion produces release of enteric glucagon which is insulinogenic. Pancreatic glucagon is probably the hormone of glucose

need and probably not involved in insulinogenesis.

Amino acid ingestion is associated with a much greater rise in glucagon than is intravenous infusion. Since pancreozymin is alphacytotrophic and released from gut by amino acids, it is presumed to be the physiologic mediater of the increased glucagon and therefore insulin response to ingestion of protein.

III Gastrin

The existence of a gastric secretagogue was first postulated in 1905 by Edkins[90] who found aqueous extracts of gastric antral but not fundic mucosa stimulated acid secretion. Workers[91,92] seeking to confirm this finding found a gastric acid stimulant in virtually all tissues of the body and it was assumed Edkins was demonstrating the activity of histamine. In 1938 Komorov[95] using different extraction techniques was able to separate the activities in antral mucosa from histamine. In 1948 Grossman[94] conclusively established the existence of an antral hormone mechanism for the stimulation of gastric acid secretion. In 1961 Gregory and Tracy[95,96] reported the first reliable method for extraction of gastrin from hog antrum. They then purified two gastrins, characterized them and accomplished total synthesis.[97] The two hormones have identical physiologic properties. The gastrins of other species are very similar and all share an identical N-terminal tetrapeptide which by itself has the same but weaker physiologic properties as the entire molecule.

Gastric distention and certain substances especially partially hydrolyzed protein stimulates gastrin release from the antral mucosa (gastric phase). The cephalic or vagal phase plays

an ill defined role serving as a priming device for regulation of gastric secretion, causing basal secretion and mediating "emotional" secretion. Gastrin is released into the circulation and stimulates the parietal cells (acid) especially, but also the chief cells (pepsin).[98] Grossman[99] has postulated that the two phases share similar dual mechanisms. He suggested that both are mediated by vasovagal cholinergic reflexes that cause direct cholinergic stimulation of the parietal cells or cholinergic release of antral gastrin and that thus "gastrin is a hormonal link in a neural chain".

In addition, the small intestine plays an important role in regulating and especially in inhibiting gastric secretion. A small amount of acid is secreted as long as there is chyme in the intestine. More important, however, are the inhibitory effects. The presence of food, especially acid in the duodenum, protein breakdown products, glucose and especially fat produce inhibition of gastric secretion either by an enterogastric (vagal reflex) or a postulated hormone enterogastrone.[98] Excess gastric acid per se inhibits further secretion.

Insulin hypoglycemia and intravenous injection of certain amino acids (alanine, glycine, glutamic acid) produce gastric secretion of both acid and pepsin by stimulating the vagal release of gastrin. Hyperglycemia inhibits gastric secretion.[100]

The primary action of gastrin is to produce gastric acid secretion. In some animals[101] but not man[102] gastric acid secretion is inhibited by large doses of gastrin but pepsin secretion is increased. Gastric and small bowel motility is increased.

Water and bicarbonate output of hepatic bile is increased,[103] intrinsic factor secretion is produced[104] while the effects on the circulatory system are variable but not significant. There are gross differences in effects among species. Of interest is that endogenous and exogenous gastrin is a weak stimulant of pancreatic water and bicarbonate output like secretin but is a strong stimulant of pancreatic enzyme secretion almost as potent as pancreozymin in dogs.[105] In humans, a background of secretin stimulation is necessary for pancreatic stimulation.[106]

Relation of Gastrin to the Present Study

In 1967 Unger et al[83] found endoportally administered gastrin containing antral extracts and pure gastrin produces a rapid 5 - 10 fold elevation of pancreatico-duodenal venous I.R.I. with very slight rises in G.L.I. and glucose, thus very similar to the effects of secretin. Gastrin thus appears to have a direct insulin-releasing action when given in large doses (0.06 µg).

Dupré et al[50-52] have presented data in which glucose was infused intravenously over 40 minutes without and with 5 minute gastrin infusions in a dose (5 µg/min. over 5 minutes) known to produce a prolonged (greater than one hour) increase in gastric acid and therefore presumably secretin release. Gastrin increased the I.R.I. levels and reduced the glucose half-time. Thus gastrin may act also indirectly by producing acid secretion and consequently secretin release.

Jarrett et al[47] failed to confirm these findings. Gastrin I (2.5 µg/min. over 20 minutes) and pentagastrin (7.5 µg/min. over 20 minutes) failed to change fasting I.R.I. or glucose and produced

no increase in I.R.I. when infused with glucose compared to a control glucose infusion. The reason for this discrepancy is unknown since there was only a minor variation is dosages used.

IV Pancreozymin

In 1928 the presence of a hormone that caused contraction of the gall bladder was demonstrated in the upper small bowel mucosa and called cholecystokinin.[107] Other actions were later demonstrated. In 1943 a hormone causing increased pancreatic enzyme secretion was found in the upper small bowel mucosa. It was called pancreozymin.[108] On the basis of isolation studies, purification and chromatography it is now felt that these hormones are one and the same.[28] The molecule is a polypeptide but is, as yet, incompletely characterized.

Dupré[35] and Dupré and Beck[40] published the reports concerning pancreozymin and insulin release. Crude intravenous pancreozymin in large doses with intravenous glucose was found to produce no effect on glucose tolerance. However, in 1967, Meade et al[109] demonstrated pancreozymin - induced insulin release in dogs. The gastrointestinal hormone increased I.R.I. and I.L.A. (rat diaphragm technique) especially in the presence of hyperglycemia. It was noted the high insulin levels failed to influence blood glucose. Explanations offered for this phenomenon were pancreozymin causes release of both insulin and glucagon; pancreozymin both stimulates insulin release and antagonizes its action; or, the increased insulin secretion was of such short duration that the total amount was insufficient to materially affect peripheral glucose utilization. They postulated that pancreozymin may in part explain the increased

insulin response to glucose but that since glucose utilization was unaffected, an additional factor, probably involving secretin, was involved.

Boyns, Jarrett and Keen[46] then reported that pancreozymin affected I.R.I. levels neither in fasting man or when given along with glucose. However, in this study and in Dupré's previous studies the preparation was of unverified exocrine activity.[52]

Using triply catheterized dogs, Unger et al[49] found rapid endoportal pancreozymin administration produced a sharp peak in insulin secretion in one minute. As well, pancreaticoduodenal vein glucagon rose immediately, and peaked at three minutes followed shortly by hyperglycemia. Glucagon was probably of pancreatic origin, as suggested by later work.[83,87] A constant infusion over twenty minutes produced similar findings. Thus pancreozymin was the first hormone shown to have glucagon-releasing properties. Work from the same laboratory[85,86] demonstrated previously mentioned observations regarding the differences between gut and pancreatic glucagon. Both are insulinogenic but gut glucagon does not produce glycogenolysis. These workers[84] then demonstrated intraduodenal instillation of glucose and especially amino acids produced a glucagon elevation. The glucagon produced by intraduodenal glucose was of enteric origin whereas that released by amino acids was of pancreatic origin. Since amino acids are potent pancreozymin-releasing agents[27] the evidence was strongly suggestive that ingested amino acids or protein breakdown products cause pancreozymin release which in turn produces pancreatic glucagon release.

Either pancreozymin or glucagon, possibly both produce insulin release.

Dupré et al[50-52] presented data in humans showing addition of pancreozymin to an intravenous glucose infusion increased insulin release and enhanced glucose disappearance rates. There was no detectable change in G.L.I. compatible with the finding of Ohneda et al[89] that hyperglycemia suppresses the glucagon stimulating effect of pancreozymin. Infusion of pancreozymin with arginine and duodenal instillation of amino acids produced interesting results discussed in the section dealing with amino acids. In short, there is good evidence in man, as well as dogs that ingested amino acids produce pancreozymin release which is alphacytotrophic and therefore indirectly insulinogenic.

Thus pancreozymin has been found to be insulinogenic in fasting animals and to potentiate the insulinemic response to hyperglycemia. In fasting animals G.L.I. is released. However, hyperglycemia inhibits the pancreozymin-induced release of glucagon. Pancreozymin may therefore be directly insulinogenic without the mediation of glucagon.

Enhancement of glucose disposal by oral ingestion is associated with an elevation of G.L.I. Hyperglycemia inhibits pancreozymin's effect on alpha cells. Infusion of isotonic glucose into the small bowel is without exocrine pancreatic effect in dogs and in man. Therefore, it appears unlikely that pancreozymin is the enteric mediator involved in increased insulin release and glucose disposal rates.

B. Gastro-Intestinal Secretagogues and Amino Acid Absorption

Although it has long been known that glucose is a potent stimulus to insulin secretion, it has only been in the past few years that amino acids have been known to be insulinogenic. In 1932 Schenck[110] found oral glycine to produce moderate hypoglycemia in normal and diabetic adults. Manzini and Arullani[111] discovered that subcutaneous leucine injections produced a 25% fall in blood sugar. An intravenous amino acid mixture was noted to produce a small drop in blood glucose in two of four subjects by Carballeira et al.[112] Intensive investigation of this question in recent years was prompted by Cochrane et al[113] in 1956, who, while investigating patients with idiopathic hypoglycemia, found oral leucine or protein in isocaloric amounts produced a profound fall in blood glucose but had no effect in normals. One of the four patients (who had "islet cell hyperplasia") had a high I.L.A. by the rat diaphragm method. The first evidence I.R.I. was elevated in patients with leucine-induced hypoglycemia was presented by Yalow and Berson[67] in 1960.

The Ann Arbor group has dominated most of the recent advances in knowledge concerning amino acid stimulation of insulin release. In attempting to devise a model for the study of leucine-induced hypoglycemia, Fajans et al[114] produced marked blood sugar diminutions with intravenous leucine in normal subjects pretreated with sulphonylurea compounds. Mabry et al[115] found valine produced slight hypoglycemia in normals then in 1962 Fajans et al[116] presented evidence that a modest but significant hypoglycemic effect and a concomitant rise in serum insulin could be

produced by administering leucine intravenously or orally to healthy adults without sulphonylurea pretreatment. The evidence thus suggested a rising level of blood leucine was a physiologic stimulus to insulin release and a dose-response relationship was evident.

In 1964[117] leucine-rich meals were found to produce a greater insulin output than could be expected from the leucine content alone and it was suspected other amino acids were involved. Consequently, 30 gm mixtures of 10 amino acids were infused and found to produce striking I.R.I. elevations. A dose-response relationship was present and insulin release was not dependent upon leucine or isoleucine. Subsequently, Floyd et al[118] infused amino acids singly and found the insulinogenic potency to be as follows in descending order: arginine, lysine, phenylalanine, leucine, valine, methionine and histidine. No consistent common physico-chemical property or configuration characterizing the more potent or less potent amino acids can be evoked. Blood sugar was elevated moderately (possibly due to gluconeogenesis) but could not account for the striking insulin release.

Of particular interest to gastrointestinal hormone potentiation of insulin release is the data concerning oral ingestion of protein or amino acids. As mentioned previously, Fajans et al[116] in 1962 found oral leucine produced insulin release in some subjects. Similar doses of leucine given intravenously were more potent presumably because of lower plasma amino acid levels. Subsequently, it was found other ingested amino acids

were insulinogenic in varying potencies.[119]

However, increases in plasma amino nitrogen after ingestion of protein meals and increases in plasma insulin were not always concurrent. In some subjects plasma insulin rose before plasma amino nitrogen had increased significantly and there was not a temporal correlation between mean levels of amino nitrogen and insulin. There is, therefore, a suggestion that in addition to the plasma level of amino acids, an additional stimulus to insulin release during protein ingestion exists, possibly gastrointestinal hormones.

Additional evidence for such a mechanism has been obtained by studying diabetics. Berger and Vongaraya[120] showed diabetics demonstrated an increased response of plasma insulin after oral protein. Plasma insulin peaked before plasma amino acids. However, Floyd et al[121] and Merrimee et al[122] found greatly diminished insulin responses to intravenous amino acids in diabetics. Regarding these papers it could be postulated that the systemic and alimentary effects are separated. Thus, whereas intravenous amino acids porduced only a small rise in I.R.I., oral amino acids produce a much greater rise, which effect is quite possibly mediated by the enteric secretagogues. Diabetics may be hyper-responders to these hormones.

As described previously, Wang and Grossman[27] demonstrated that products of protein digestion - peptone and amino acids, were powerful stimulators of pancreatic enzyme secretion similar to that produced by pancreozymin. Dupré[35,40] found a lack of effect of pancreozymin on insulin secretion in man. However, in 1967

Meade et al[109] reported that intravenous pancreozymin in dogs stimulated insulin secretion without the expected fall in blood glucose suggesting a glucagon effect as well. Simultaneous administration of pancreozymin and glucose produced a potentiation of the insulin response. Unger et al,[83] injecting pancreozymin endoportally in dogs, found a sudden large rise in pancreaticoduodenal vein insulin and glucagon followed by an elevation of blood glucose presumably due to the glucagon release.

Ohneda et al[84] and Unger et al[123] found both intravenous and oral amino acids produced elevations of pancreaticoduodenal vein insulin and glucagon that was much higher when given orally despite less aminoacidemia. The glucagon was thought to be of pancreatic origin based on the site of sampling. Thus, pancreozymin released by ingested amino acids appears to be an alpha-cytotrophin.

Dupré et al[52] found that addition of pancreozymin to an intravenous arginine infusion to increase insulin and G.L.I. release and some elevation of blood glucose. The G.L.I. release was more convincingly demonstrated when the infusion was given rapidly. Duodenal infusion of arginine duplicated the amino nitrogen levels produced by intravenous infusion but was associated with a greater and more sustained elevation of I.R.I. G.L.I. was not measured. Secretin was without effect on I.R.I. or amino nitrogen levels but did reduce the glucose elevation.

In summary, pancreozymin is insulinogenic probably via

pancreatic glucagon and is released from the upper small bowel mucosa by amino acids. There is, thus, strong evidence that pancreozymin is physiologically important in stimulating the endocrine pancreas during amino acid absorption.

c. Techniques Used to Study Insulin Release

Various techniques have been devised in an effort to study factors which might influence insulin secretion. The first and most obvious method would be the ingestion or injection of various substances with measurement of subsequent peripheral venous changes in glucose or insulin. It has been assumed that the glucose concentration perfusing the islets of Langerhans is the normal variable influencing insulin secretion. Many more techniques have been devised since ranging from in vivo selective vessel sampling to study of isolated islets in vitro. Some of these techniques will be discussed briefly in an effort to explain why the technique used in the present report was chosen. In addition, any pertinent studies with respect to this thesis will be mentioned.

In Vivo Techniques

In 1927 Zunz and Labarre[124] joined two dogs by pancreatic jugular anastomosis and observed the effect of pancreatic blood of the donor animal upon the blood sugar of the recipient animal. Gayet and Guillaumie[125] and Foglia and Fernandez[126] transplanted the pancreas from one dog to the neck of another dog that had been previously depancreatized and observed the blood sugar changes in the latter animal. In 1937 London and Kotschneff[127] placed an angiostomy cannula in the pancreaticoduodenal vein and determined

the glucose and insulin (bioassay) content of blood from the pancreas after a glucose meal. By these methods each group has shown that hyperglycemia stimulates the release of insulin.

Numerous studies[128] have been performed attempting to correlate changes in beta cell function with alterations in the histologic and electron microscopic appearance of beta cells. Growth hormone, glucagon, and adrenal steroids, among many others, have been studied. This technique has proved useful in partially delineating the process of insulin synthesis and release but would be unsuitable for quantitation of insulin release.

In Vitro Techniques

Although in vivo techniques have contributed considerably to knowledge, in vitro studies have usually been necessary for a fuller understanding. An in vitro pancreatic perfusion preparation offers several advantages in the evaluation of the direct effects of various stimuli. Secondary effects resulting from changes in pituitary, hepatic or adrenal function are eliminated. Substances studied can be conveniently maintained at constant and known levels throughout the experiment. Once secreted, insulin is not subject to extrapancreatic degradation processes but remains in the perfusate where it can be continuously measured. Anderson and Long[129] were the first to devise such a technique perfusing pancreas, stomach and duodenum. They found insulin (bioassayable) to be produced by a high concentration of glucose. Grodsky et al[22] using a similar preparation measured insulin by immunoassay with the advantages of precision, sensitivity and specificity. They demonstrated insulin secretion is directly and quantitatively

controlled by glucose concentration. They also observed degranulation suggesting secretion was occurring at a faster rate than synthesis.

Using this technique Sussman et al[130] and Grodsky et al[131] found glucagon to increase insulin release in the presence and absence of glucose. Penhos et al[132] have recently adapted an in situ pancreas-duodenum preparation for perfusion studies to examine the influence of duodenal hormones on insulin release. Recirculation and glucose in the duodenum both produced increased insulin release.

Tissue culture of the pancreas was first reported in 1960 by Kim et al.[128] Vecchio et al[76] cultured fetal rat pancreas in Eagle's Hela medium and found the tissue to respond to tolbutamide and glucagon, but not glucose. Similar results were reported by Lambert et al[77] and Jeanreaud.[134] Caffeine and theophylline enhanced the effect suggesting that insulin release is at least partially dependent upon intracellular accumulation of cyclic AMP.

The study of insulin release in response to various stimuli using pancreatic slices was initially reported by Bouman in 1960.[135] He found an insulin effect only with duct tied pancreatic tissue of rats. He could demonstrate an insulin effect when normal slices of pancreatic tissue were incubated jointly with rat diaphragm showing that the proteolytic enzymes of acinar tissue quickly degrade released insulin. R.-Candela et al[136] presented a brief note but virtually no data was given except that rabbit pancreas slices produced I.L.A. Coore and

Randle[73] published the definitive work in this regard in 1964 quantitating insulin release by the immunoassay and showing that release of insulin by pieces of rabbit pancreas in vitro provides a simple and reproducible model for the behavior of beta cells in vivo. Among many other observations they found a graded response to glucose and no response to glucagon (5 mg/ml). Creutzfeldt et al[137] found a high glucose concentration to stimulate I.L.A. release and felt this was not just efflux from dying cells but stated emphatically that release cannot be said to be the same process as the mechanism of insulin secretion in vivo in which there is degranulation and in which there is a greater loss of insulin. Recently, however, Gomez-Acebo et al[138] reported physiologic granule release using the same preparation.

Rapid destruction of released insulin has been reported by Mialhe and Meyer,[139] Bakker and Bouman[140] and by Telib et al,[141] none of whom could detect insulin secretion in vitro from normal rat pancreatic tissue. Pancreatic tissue from other species may have a less marked destructive effect. An exception to this was reported by Frerichs et al[142] but they used a bioassay technique. As well chymotrypsin has been reported to falsely elevate immunoassayable insulin.[143] Malaisse et al[144] have circumvented some of these difficulties by using antiinsulin serum (A.I.S.) to bind immediately the insulin released. This has become an accepted technique although A.I.S. itself has actions on the beta cell.

With respect to the present work, Pfeiffer et al[36] incubated sections of rabbit and dog pancreas with glucose,

secretin and glucose and secretin and measured I.L.A. (fat pad) and I.R.I. Optimal insulin secretion was found at a glucose concentration of 200 mg%. Secretin 0.01 U/ml produced the optimal increase in I.L.A. whereas 0.1 U/ml produced the optimal increase in I.R.I. Addition of glucose to the secretin-containing mixture was without effect. They concluded that secretin was directly insulinogenic independent of glucose concentration. Similar results were reported by McIntyre, Turner and Holdsworth.[37] In 1966, Turner and McIntyre,[74] using the same technique found glucagon (0.5 µg/ml) to produce increased insulin (I.R.I.) release at 200 mg%. glucose concentration and a higher concentration (5 µg/ml) to produce increased insulin release at both 60 mg% and 200 mg%. In 1967 Hildebrandt et al[145] reported leucine was insulinogenic using the Malaisse method, although Martin[146] found rat pancreas slices did not respond to leucine. Lazarus et al[39] found secretin and gastrin were without effect whereas glucagon and pancreozymin did increase insulin release.

The admixture and uneven distribution of pancreatic islets throughout the much larger bulk of exocrine tissue of mammals has hindered quantitative biochemical investigations and has accounted for widely varying secretion rates from different pieces. Furthermore, the presence of acinar tissue around the islets may modify the action of substances present in the incubation medium either by destroying them or preventing their gaining close access to the secreting cells. However, various approaches have been used to aleviate these problems.

Certain fish teleosts have their insulin secreting tissue separate from the acinar portion of the pancreas comprising a discrete mass in the mesentery known as the principal islet or Brockmann body. Although a considerable volume of work has been presented using these islets there is little evidence that the fish islet responds to the same stimuli as mammalian islets.[128]

Histochemical techniques have been applied to islet cells under a variety of situations.[147] (Lacy, Hellman and Hellerstrom).[148] In particular, certain enzymes have been studied. The microchemical techniques of Lowry[150] were adapted by Lacy[147] to the islets to study quantitatively the activities of these enzymes. With this procedure it was possible to recognize islets in frozen-dried sections of the pancreas, dissect beta cells from the central portion of the islets in rats and rabbits, weigh them on a quartz-fibre balance, determine enzyme activity quantitatively in the dissected samples and measure the insulin content of the samples. Histochemical studies have also been performed without isolation of the islets. Dixit et al[151] incubated islets obtained in this manner in the presence of radioactive glucose but did not report the results. The undesirability of using freeze-dried tissue for metabolic studies is readily apparent and the indirect nature of enzymatic studies and the lack of availability of equipment militated against use of this technique.

Homotransplantation of the pancreas to various parts of the body of alloxanized or depancreatized animals is usally followed by degeneration of exocrine tissue and proliferation of

endocrine islet tissue and hence, could theoretically be a method of obtaining isolated islet tissue.[152] Browning and Resnik,[153] Coupland[154] and House et al implanted fetal or neonatal pancreas to the anterior eye chamber of mice or rats and to the cheek pouch of hamsters and reported survival and growth of islets and decay of exocrine tissue. Similar results were reported by Gonet's laboratory[155] using the testis as the donor site. The exocrine pancreas and alpha cells disappeared. I.L.A. was present in the islets but no metabolic studies were performed. Roughly similar results were reported by Grimelius et al.[156] Others have transplanted the organ to subcutaneous sites on the anterior abdominal wall, the wall of the intestine or stomach, or the mammary gland. Metabolic studies have not been extensively carried out using these ostensibly isolated islets but the undesirability of using fetal pancreas that has been subjected to non-physiologic processes for a prolonged period and probably without alpha cells is obvious.

More physiologic methods of isolation of pancreatic islets have been described by three investigators.[157] In 1964 Hellerstrom described a method employing microdissection using a dissecting stereomicroscope to tease apart islet and acinar tissue of mice, rats and guinea pigs in a cooled medium without any pretreatment of the animal. Islets of the obese hyperglycemic mouse were obtained in the best yield.

In 1965, Keen, Sells and Jarrett[158] described a method similar to that of Hellerstrom in which the animal was subjected to duct ligation 4 to 6 weeks prior to removal of the pancreas.

The acinar tissue atrophied and the islets removed by microdissection. They performed metabolic studies related to CO_2 production from C^{14} glucose at high and low glucose concentrations. Survival was estimated to be at least four hours, the rate of glucose oxidation continuing linearly during this period and increasing with glucose concentration.

In 1965 Moskalewski[159] used collagenase for the first time to free the islets from acinar tissue. Using guinea pigs he injected Hanks solution subperitoneally to break up the tissue then incubated small pieces of pancreas with collagenase and found the islets to separate fairly readily from the acinar tissue. He then cultured the islets and later performed histologic studies. Degranulation was detected at high glucose concentrations of the culture medium over a period of days. Other histologic changes similar to in vivo changes were described.

The present work was performed according to the technique of Kostianovsky and Lacy[160] which was adapted from Moskalewski's technique for use with rat pancreas. The acinar parenchyma of rat pancreas is disrupted by injection of Hank's solution into the pancreatic duct system. The pancreas is removed, cut into small pieces and incubated with collagenase. After washing the islets could be separated readily with the aid of a dissecting microscope. They reported insulin (I.R.I.) release in response to glucose concentration over time (1 1/2 hours). Light and electron microscopy showed intact islets but studies were not performed after incubation.

Very few reports have followed the initial presentation of this technique. Some, however, have a direct relation to the present work. Vance et al[161] found increased insulin release with increased glucose concentration and reported the curious observation that arginine (200 mg%) produced significantly increased insulin release at 60 mg% glucose but not at 30, 150 or 300 mg% glucose. Lacy[162] later reported glucagon and theophylline produced insulin release. Secretin and intestinal fragments taken during orally provoked hyperglycemia in man produced increased insulin release in isolated islets of the rat.[38]

Vance et al[163] measured both I.R.I. and I.R.G. in the incubation medium using Lacy's technique. They found addition of the kallikrein inhibitor, Trasylol protected insulin and glucagon from proteolytic degradation. Glucagon release is augmented and insulin release is increased by a high glucose concentration.

Howell[164] studied the secretion granules of isolated rat islets before and following incubation with various stimulants. Erlandsen, Vance and Williams[165] found freshly isolated islets and preincubated islets (90 minutes) in glucose 30 mg% to maintain their initial granulation. Higher glucose concentration (300 mg%) produced physiologic degranulation and increased insulin release. Ashcroft and Randle[166] found a direct relationship between the rate of oxidation of radioactive glucose to carbon dioxide and glucose concentration in the medium.

Thus it appears that the behavior of these islets closely approximates that which has been demonstrated in vivo, it is reasonably simple to perform and therefore admirably suited for our purposes.

Materials and Methods

Chemicals

The following chemicals were used in these experiments: l-leucine, Fisher Scientific Co., lot 744174; l-arginine, Eastman Organic Chemicals, lot 2475; crystalline glucagon, Eli Lilly & Co., lot 258-234B-167-1; cholecystokinin - pancreozymin, G.I.H. Research Unit, Karolinska Institutet, lot 26731; secretin, G.I.H. Research Unit, Karolinska Institutet, lot 16761; synthetic human gastrin, Eli Lilly & Co., lot P-88484; collagenase, Worthington Biochemical Corporation, lot CLS7HF and Schwartz Bioresearch, lot 6808P; bovine serum albumin, fraction $\overline{V}$, Armour Pharmaceuticals Incorporated; Trasylol (Preparation A128), F.B.A. Pharmaceuticals Incorporated, lot 71166; $6C^{14}$ glucose, New England Nuclear Corporation, lot 292612; crystalline rat insulin, Novo Terapeutisk Laboratorium; Insulin - Toronto, lot 1055-1 and N.P.H. Insulin, lot 501153, Connought Medical Research Laboratories; I^{131} insulin, Abbott Laboratories.

Isolation of Pancreatic Islets of Langerhans

Islets were isolated according to the technique of Kostianovsky and Lacy[160] with certain modifications. Two male 180 to 200 gm Wistar rats, fasted overnight but allowed water ad libitum, were used. Anesthesia was with intraperitoneal Nembutal. A mid line and two lateral incisions were made and the common bile duct ligated as it entered the duodenum. The common bile duct was then clamped at its beginning and 15 to 20 ml of either Hanks solution[167] or Gey and Gey buffer[168] was injected into the lumen of

the bile duct through a 25 gauge needle. In this way the entire pancreas was distended producing a lacy appearance. The splenic portion of the pancreas was then removed, transferred to buffer solution (Hanks or Gey and Gey) and washed free of blood and the fat trimmed away. The pancreatic tissue of two rats prepared in this manner was then transferred to a fresh volume of buffer and cut with scissors into small pieces in a beaker. The contents were then transferred to a 50 ml plastic centrifuge tube. Pancreatic tissue settled to the bottom and fat rose to the top. The supernatant was aspirated. Buffer was added to the 5 ml mark and the entire contents transferred to a 50 ml plastic conical flask containing 30 - 40 mg of collagenase. The mixture was then incubated at 37°C., and gassed with 95% O_2 and 5% CO_2, and shaken rapidly in a Dubnoff Metabolic Shaking Incubator for 8 to 15 minutes when a relatively homogenous appearance of the small pieces of tissue was observed. The contents of the flask were transferred to a 50 ml plastic centrifuge tube, diluted to about 40 ml with buffer and spun briefly. The supernatant was then aspirated. This washing was repeated 5 to 6 times. The digested pancreatic tissue was then transferred to a shallow Petri dish and examined with a dissecting stereomicroscope.

Although most reports have described perfectly isolated islets in large numbers, I encountered considerable difficulty in obtaining such a preparation. Most islets required a minimum of teasing away from the acinar tissue which was done with 20 guage needles on a 2 ml syringe. Free islets were obtainable, however,

with further digestion but usually these were of diminished durability presumably because of collagenase-induced damage. There is a fine point in time between the stage of perfect acinar tissue-free isolation and disintegration or damage.

The islets were loaded on to the bevel of the disposable needles and gently injected into an incubation "basket" in buffer in a Petri dish. The basket consisted of an Technicon 3 ml polystyrene sample cup with the bottom removed and replaced by a piece of 200 mesh bolting cloth held firmly in place. The islets rested on the bolting cloth. With this device washing is simple since the washing buffer passes easily through the bolting cloth but the islets do not.

When 15 islets were collected in the basket they were washed in fresh buffer and were then ready for incubation. The incubation medium consisted basically of Kreb's Ringer Bicarbonate solution (KRB) with bovine serum albumin 2 mg% and 5 mM each of sodium fumarate, pyruvate and glutamate according to Coore and Randle.[73] The incubation vessel consisted of a 10 ml disposable Worthington chromagen vial.

The islets were preincubated for 15 minutes in incubation medium with glucose 50 mg% and for 5 minutes between each incubation. Before each incubation the islets were washed in a 20 ml volume of fresh buffer. Incubation was 15 minutes in duration and usually 8 incubations were performed during each experiment. The islets were examined at the one and two hour mark with the dissecting microscope and the previous hour's specimens rejected if there was significant fragmentation, loss of opacity or disapp-

earance of the islets.

Insulin release was determined initially by bioassay and more recently by immunoassay.

Insulin Bioassay

Insulin-like activity (I.L.A.) was determined using isolated epididymal fat pad cells according to the methods of Rodbell,[169] Glieman[170] and Dupré[171]. Fed male hooded 140 to 160 gm rats were killed and the epididymal fat pad was removed. The fat tissue was then incubated in 5 ml KRB with 4 gm% bovine serum albumin, glucose 10 mg% and collagenase 10 - 15 mg in a metabolic shaker at 37°C for 15 to 25 minutes with gassing with 95% O_2 and 5% CO_2 for the initial five minutes. When digestion was complete the cells were washed with KRB with albumin 4 gm% and glucose 10 mg%. The cells were then dispensed into incubation flasks with beef-pork insulin standards or test specimens in the presence of $6C^{14}$ glucose (approximately 0.002 mc per flask) in a total volume of 2 ml of KRB with 4 gm% albumin and glucose 10 mg%. The incubation period was one hour. The reaction was stopped with Dole extraction mixture. Fat was extracted with heptane, saponified with alcoholic KOH, and acidified and the resulting fatty acids were extracted with heptane and weighed. Radioactivity was counted in a Packard Tricarb Liquid Scintillation Spectrometer using toluene popop as the scintillator. Values obtained were expressed as counts per minute (cpm) per mg of fatty acid incorporated from glucose. An insulin standard curve was calculated and test specimen values

obtained therefrom and expressed as μU of insulin per ml of islet incubation medium.

Insulin immunoassay

The insulin immunoassay was based on that of Yalow and Berson[67] with many modifications. Insulin antibody was raised in guinea pigs by four weekly subcutaneous injections of N.P.H. (pork) insulin, 1 unit, in Freund's complete adjuvant. An antibody was selected that produced an acceptable asymptotic standard curve and the antibody was diluted to obtain 60 - 70% binding of labeled insulin in the absence of added insulin.

All dilutions of the rat insulin standards, test specimens, antibody and radioactive insulin were in borate buffer in bovine serum albumin (fraction V) 2 gm% at pH 7.4. Radioactive (I^{131}) insulin was purified by passing it through a DEAE Cellulose column washed with 5 ml 0.1 TRIS buffer pH 7.4 in 0.5 gm% bovine serum albumin. Elution with 0.5 gm% bovine serum albumin in 0.1N HCL was followed by dilution to 2 ng/ml. In the assay 0.1 ml of I^{131} insulin was used (0.2 ng/ml or 5 μU/ml). Standards were made with crystalline rat insulin from 0 to 75 μU/ml and test specimens 0.02 to 0.05 ml were measured.

Antisera, radioactive insulin and rat insulin standards or test specimens in a total volume of 1 ml were incubated 48 hours at 4°C. Two ml 0.25 gm% dextran 80 and 2.5 gm% charcoal in borate buffer[172] was then added. The mixture was vortexed, centrifuged and separated. Radioactivity was counted in a Packard Auto Gamma Spectrometer. The fractions were counted usually to 5000 preset counts and the bound (supernatant) over

free (charcoal) ratio was calculated. If the damage (non-specific binding) exceeded 4% of the total counts the damage of each specimen was subtracted from the bound fraction before calculating the bound/free (B/F) ratio. A pooled specimen of rat insulin was measured in each assay to demonstrate any variability from week to week.

Results were expressed as μU of insulin per ml of incubation medium released by islets (usually 15 islets per 3 ml) in 15 minutes.

Types of Incubation

1. Initially, groups of 5 islets were incubated in 1 ml of incubation medium for 15 minutes and ILA determined. An effort was made to determine glucose responsiveness, duration of viability of the islets, reproducibility of insulin response from one incubation to another, similarity of response of one batch of islets to another and a few experiments were done to detect any increased insulin release due to secretin.

2. Once the immunoassay had been developed several additional preliminary experiments were performed.

(a) Dose-response relationship.

Fifteen islets were incubated for 15 minutes in incubation medium containing varying concentration of glucose and the insulin release determined.

(b) Insulin release with time.

Islets were incubated in incubation medium containing 50 and 300 mg% glucose and aliquots taken at intervals over one hour and insulin release measured.

(c) Insulin breakdown.

In order to detect possible insulin breakdown by islet enzymes or possible residual acinar tissue three experiments were done as follows. Islets were obtained in the usual fashion and 15 islets put into a basket, A, and 15 more islets put in another basket labeled B. In baskets C and D there were no islets. After preincubations as above baskets A and B were incubated in 50, 300 mg% glucose in the incubation medium for 15 minutes each. After each of the first two incubations the baskets with the islets were removed and 25000 to 50000 cpm I^{131} insulin was added to the incubation medium and the mixture incubated for a further 15 minutes. Islets were incubated for a third 15 minute period with I^{131} insulin. In baskets C and D labeled insulin was added to incubation medium with 50 and 300 mg% glucose respectively and incubated for 15 minutes.

At the termination of each incubation with labeled insulin the medium was immediately frozen with dry ice. Crystalline zinc insulin 80 U/ml 0.1 ml was added as a carrier. Then 1 ml 10% trichloracetic acid (T.C.A.) was added, the solution melted, was vortexed, centrifuged, and the supernatant transferred to the appropriate counting tube. Radioactivity was counted in a Packard Auto Gamma Spectrometer. Damaged insulin was calculated from the amount of radioactivity in the supernatant of the two control incubations. Damage or non-specific binding was then subtracted from the supernatant counts of the test specimens.

Thus the I^{131} labeled insulin proteolytic action of the

medium in which islets had been incubated at both 50 and 300mg% glucose (first two incubations) and the destructive action of islets per se (third incubation) were calculated by subtracting the non-specific failure of adsorption to charcoal of radioactivity in control vessels C and D incubated under the same conditions but without islets or previous exposure to islets.

(d) Effect of gastrointestinal hormones and amino acids on insulin release.

A series of experiments was carried out in which the effects on insulin release of four hormones and two amino acids were determined at low (50mg%) and high (300mg%) glucose concentrations. In each experiment 15 islets were incubated for 15 minutes in 3 ml of incubation medium. For each substance tested four incubations were carried out, that is, incubations were at both high and low glucose concentration, each with and without added test substance in random order; and then these incubations were repeated, again at random for a total of eight test incubations. Trasylol 1000K.I.U. (kallikrein inhibiting units) was added to each 3 ml incubation medium in experiments in which a polypeptide hormone was used. The substances tested were secretin 0.02µg per ml, glucagon 0.05µg per ml, pancreozymin 0.02µg per ml, leucine 25mg% and arginine 25mg%. In addition to the above, glucagon was tested at an intermediate glucose concentration of 150mg%.

The insulin content of the four hormonal preparations was determined.

Statistical treatment of the observed results was carried out as follows. Student's t test was performed to determine the

level of significance between insulin release at 50 and 300mg% glucose. Then the values obtained during the first and second sets of incubations were examined. Since a significant difference in the values obtained in the two sets of observations was found, the mean of the two incubations carried out under the same conditions in one experiment were considered. Student's t test was carried out on these means to determine the level of significance of the mean values at both glucose concentrations, each with and without added test substance. Student's t test was also performed on paired differences at both glucose concentrations, and on the difference between insulin release obtained with added test substance at both high and low glucose concentrations to determine the presence or absence of synergism. Synergism of the test agent with glucose in this situation may be defined as an effect of the test agent on the rate of insulin release exceeding the effect of the agent at low glucose concentration.

Mean per cent change in insulin release at each glucose concentration was calculated for each substance.

Results are expressed in terms of μU IRI per ml incubation medium in 15 minutes and as μU IRI per islet per minute, the latter as an attempt at some standardization with respect to the data of others.

Results:

1. ILA Results:

The figures used for determination of p values regarding (i) glucose responsiveness, (ii) reproducibility of insulin response from one incubation to another, and (iii) similarity of response of one batch of islets (that is one experiment) to another are

tabulated in Table I.

Table I Response of isolated islets to two glucose concentrations

Experiment	Glucose Concentration of Incubation Medium	
	50mg%	300mg%
1	52,*43	133,96
2	47,46	88,81
3	130,80	160,140
4	90,80	220,205
5	92,76	420,320
6	88,80	440,320
7	125,125	120,120
8	130,110	230,210
9	60,44	340,280
10	68,50	212,192

*values expressed as μU insulin released per ml of incubation medium in 15 minutes.

(i) The islets responded to an increase in glucose concentration by increasing ILA output significantly ($p < 0.01$). There was usually a 2 - 4 fold increase in ILA release when islets were incubated at 300mg% glucose compared to 50mg%.

(ii) Consideration of the two (ILA) values obtained at the same glucose concentration using the same islets by Student's t test on the paired differences revealed no significant difference in ILA release at 50mg % glucose ($p < 0.01$) or at 300mg% glucose ($p < 0.05$). In the latter group one experiment tended to diminish the significance.

(iii) There was considerable discrepancy between the ILA values obtained in one experiment compared to another at both 50 and 300mg% glucose. Presumably this variation from experiment to experiment is due to variation in the size of the islets or more exactly the total number of beta cells in each experiment. The metabolic state of the animal from which they were obtained, the degree of damage due to collagenase and variable other unknown factors may have been involved.

(iv) In assessing the duration of viability, islets were incubated alternatively in 50 and 300mg% glucose (G_{50} and G_{300}, respectively) for 15 minute periods for three hours. The results are tabulated in Table II.

Table II Duration of Islet Viability

Incubation Medium	Time (min.)	Experiment	
		120	122	124
G_{50}	15	76*	250	60
G_{300}	30	320	240	208
G_{50}	45	80	220	68
G_{300}	60	320	460	340
G_{50}	75	92	280	44
G_{300}	90	420	240	340
G_{50}	105	88	280	42
G_{300}	120	440	400	212
G_{50}	135	60	220	42
G_{300}	150	270	240	280
G_{50}	165	76	240	60
G_{300}	180	24	440	92

* values expressed as μU insulin released per ml of incubation medium in 15 minutes.

It can be seen that in 2 of 3 experiments there was a significant fall in insulin release at 3 hrs. Consequently, total incubation periods of two hours were not exceeded.

(v) When secretin (0.2μg/ml) was incubated with islets at two glucose concentrations the results in Table III were obtained.

Table III. Effect of Secretin on ILA Release

Experiment	Incubation Medium			
	Glucose 50mg%	Glucose 50mg% with Secretin	Glucose 300mg%	Glucose 300mg% with Secretin
108	20*	30	96	70
	20	26	60	68
112	130	86	140	170
	84	90	200	224
118	40	40	140	180
	64	40	110	170
mean ± SE	60 ± 17	52 ± 12	124 ± 19	147 ± 26

* values expressed as μU insulin released per ml incubation medium in 15 minutes.

Although there is a tendency for secretin to increase glucose-induced insulin release at 300mg% glucose neither the means nor paired differences are significant. The failure to obtain significance is probably related to the small number of experiments and to the lack of precision of the bioassay. Insulin release in subsequent experiments was assessed by measuring immunoreactive insulin.

2. IRI Results

(a) Dose response relationship

The results obtained in three experiments demonstrating insulin release by islets incubated in various glucose concentrations ranging from 0 to 400mg% are tabulated in Table IV and depicted graphically in Figure 1.

FIGURE 1. DOSE RESPONSE RELATIONSHIP

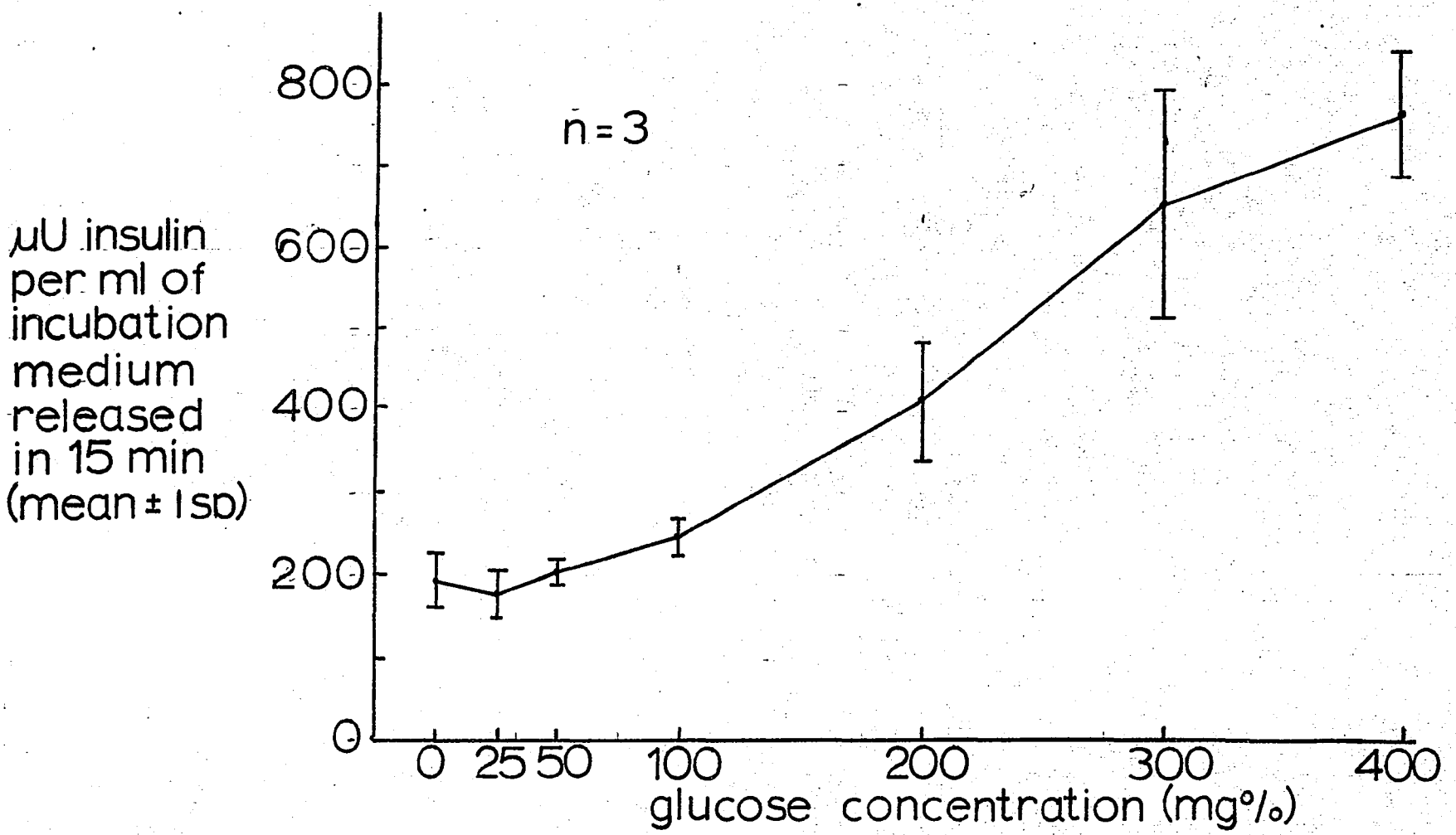


Table IV Dose response relationship

Glucose Concentration in Incubation Medium in mg%	Experiment 109	110	111	Mean ± SE µU/ml in 15 min.	Mean ± SE µUIRI/islet in 1 min.
0	250*	157	180	196 ± 28	2.61 ± 0.37
25	225	180	135	180 ± 26	2.40 ± 0.35
50	220	200	184	201 ± 10	2.68 ± 0.13
100	274	250	200	241 ± 22	3.21 ± 0.29
200	435	475	300	403 ± 53	5.37 ± 0.71
300	800	500	600	633 ± 88	8.44 ± 1.17
400	850	700	700	750 ± 50	10.00 ± 0.67

* values expressed as µU insulin per ml of incubation medium released in 15 minutes.

For future experiments the glucose concentrations used were 50mg% when insulin secretion may be considered at a basal level and 300mg% when insulin release is submaximal.

(b) Insulin release over time

For results obtained in two experiments in which the release of insulin was determined over one hour see Table V and Figure 2.

FIGURE 2. INSULIN RELEASE OVER ONE HOUR

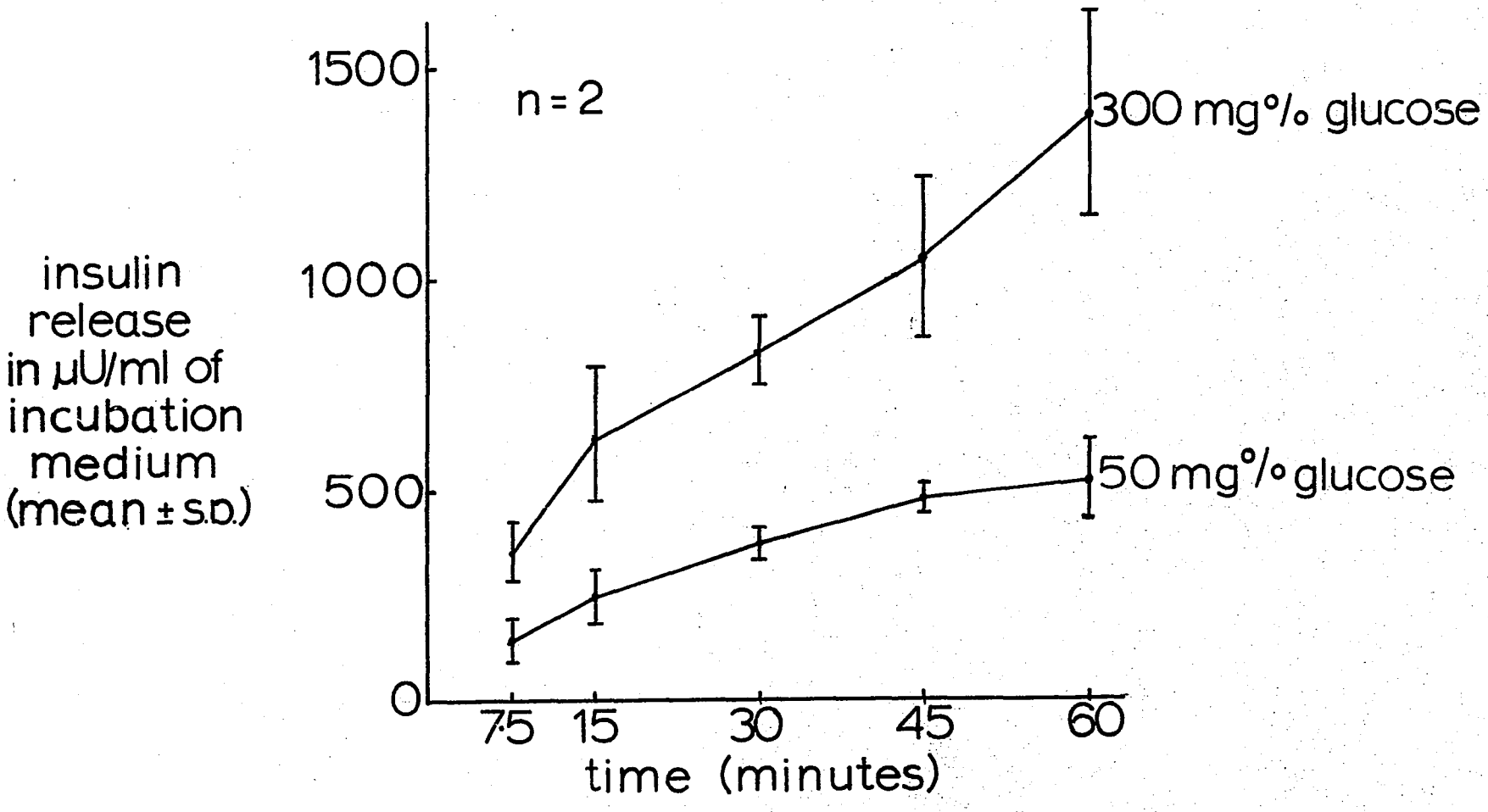


Table V Insulin Release Over One Hour

Time (min.)	Incubation medium glucose concentration 50mg% Exp.214	Exp.258	mean ±SD	300mg% Exp.214	Exp.258	mean ±SD
7.5	97*	193	145±45	285	415	350±62
15	190	310	250±55	470	750	610±131
30	341	419	380±36	726	894	810±80
45	436	504	470±32	855	1235	1045±180
60	470	570	520±47	1135	1635	1385±232

* data expressed as μU IRI/ml/15min.

(c) Insulin Breakdown

See Table VI

In three experiments described in the Method non-specific binding or damage of labeled insulin accounted for 3.7% of total added labeled insulin. The so-called damaged fraction was subtracted from the mean of experimental supernatant values obtained from each incubation (numbered 1,2 and 3). Insulin proteolysis as determined in this system was found to be negligible (mean = 0.23%).

Table VI Insulin Breakdown

Exp.	Basket	Incubation 1	2	3	Estimation of supernatant cpm attributed to insulin destruction as % of total added cpm: 50 mg% glucose medium (inc. 1)	300 mg% glucose medium (inc. 2)	medium with islets (inc. 3)
196	A	1124*	1050	1005	1219-1004=215	1084-1004=80	1090-1004=86
	B	1315	1118	1185			
	C	961			215/26581 x 100	80/26581 x 100	86/26581 x 100
	D	1055			=0.81%	=0.30%	=0.32%
	Total counts per min. added 26581						
	Damage=(961 + 1055)/2=1004						
259	A	2220	1880	1680	1950-2060=-90	2090-2060=30	2030-2060=-10
	B	1680	2300	2420			
	C	2000			-90/56510 x 100	30/56510 x 100	-10/56510 x 100
	D	2120			=0.0%	=0.05%	=0.0%
	Total counts per min. added 56510						
	Damage=(2000 + 2120)/2=2060						
260	A	2410	2391	2210	2413-2250=163	2386-2250=136	2305-2250=55
	B	2416	2381	2401			
	C	2160			163/59327 x 100	136/59327 x 100	55/59327 x 100
	D	2340			=0.27%	=0.23%	=0.09%
	Total counts per min. added 59327						
	Damage=(2160 + 2340)/2=2250						

* figures refer to counts per min. (cpm) of non-TCA-precipitable I^{131}

(d) Effect of gastrointestinal hormones and amino acids on insulin release.

The higher glucose concentration produced significantly greater insulin release than 50mg% glucose ($p<0.01$).

Initial determination of the difference between insulin release in control incubations (50 and 300mg% without added substances), the first set compared to the second set of incubations, revealed no significant differences ($p<0.05$ for both 50 and 300mg% glucose, $n = 10$). However, consideration of all the values after termination of the experimentation ($n=45$) did reveal a significant diminution in the 300mg% glucose control value during the second set of incubations compared to the first ($p>0.05$). Furthermore, paired differences for the 50mg% glucose control incubations were significantly smaller in the first set of incubations ($p>0.05$). Therefore, since two estimates of insulin release under the same conditions could not be treated as replicates, these two values were averaged and the significance of the paired differences of these means determined in each set of experiments. It is recognized that this manipulation limits the inferences that can be drawn.

The results of the experiments in which secretin was tested are represented in Table VII and Figure 3. Synergism was absent.

In Figure 3 and the subsequent three figures the bar graph on the left represents the mean insulin output at the two glucose concentrations indicated with and without added hormone. For no substance tested was mean insulin output increased by the presence of hormone. The figures on the right represent the mean $\pm$SEM of paired differences and the level

of significance, that is, the increment in insulin secretion attributed to added hormone at each glucose concentration.

Figure 3

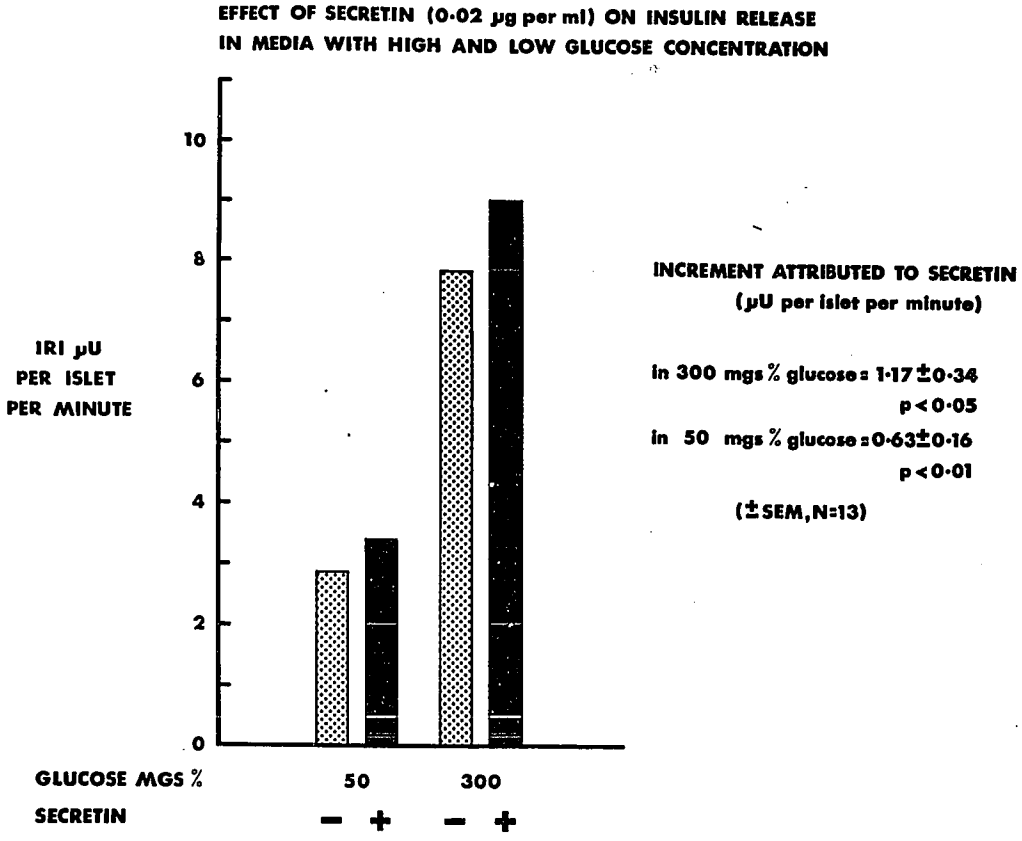


Table VII Effect of Secretin on Insulin Release

Experiment	Incubation Medium			
	Glucose 50mg%	Glucose 50mg% with Secretin	Glucose 300mg%	Glucose 300mg% with Secretin
197	350,* 325	380,400	825,725	875,675
201	295,300	310,340	775,712	1000,825
205	310,330	530,340	900,850	1250,1100
207	274,215	310,256	575,590	600,680
208	40,50	40,40	275,475	575,535
209	194,194	140,150	250,475	485,275
215	430,254	290,540	725,925	575,1100
216	184,220	200,230	425,475	600,475
222	214,200	250,240	900,485	835,535
223	230,224	340,270	1075,750	950,825
225	80,104	80,120	225,185	310,225
226	130,100	194,120	510,425	585,710
227	130,234	250,300	425,400	575,500
	* µU of IRI per ml incubation medium released in 15 min.			
mean$\pm$SEM µU IRI/ml in 15 min.	216$\pm$19	256$\pm$25	591$\pm$47	680$\pm$51
mean$\pm$SEM µU IRI/islet /min.	2.88$\pm$0.35	3.41$\pm$0.45	7.87$\pm$0.84	9.07$\pm$0.89
level of significance of difference at same glucose concentration	n.s		n.s	
	$G_{50}+S - G_{50}$		$G_{300}+S - G_{300}$	
mean$\pm$SEM of paired differences attributed to secretin in µU/islet/min.	0.63$\pm$0.16		1.17$\pm$0.36	
level of significance of paired differences	$p<0.01$		$p<0.01$	

Table VIII and Figure 4 represent the results obtained when the effect of glucagon was examined at 50 and 300mg% glucose. Synergism was absent ($p>0.05$).

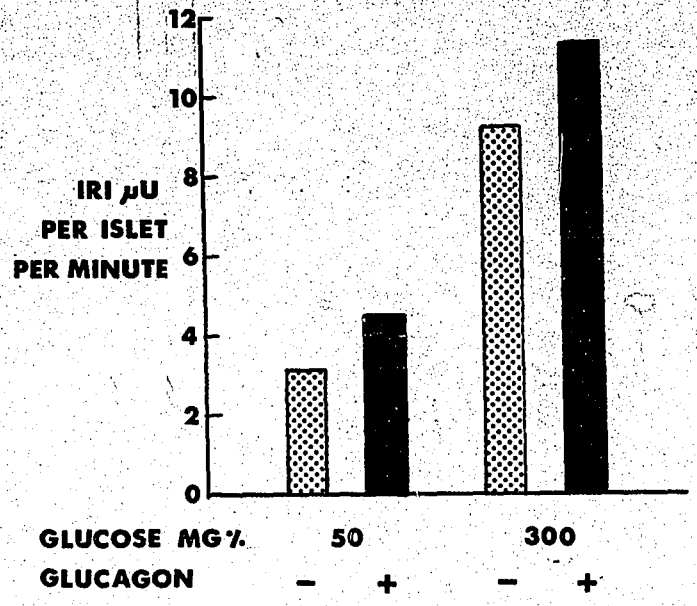
Table VIII. Effect of Glucagon on Insulin Release

Experiment	Glucose 50mg%	Glucose 50mg% with Glucagon	Glucose 300mg%	Glucose 300mg% with Glucagom
203	290*,410	480,450	875,,600	950,,900
204	300,360	450,480	700,,525	750,660
210	184,150	310,190	875,525	975,,600
211	140,220	384,290	800,950	1630,1000
212	240,260	300,320	600,460	725,,525
217	110,170	170,230	485,,960	575,,925
	* µU IRI/ml/15 min.			
mean$\pm$SEM µU/ml/15min.	236$\pm$36	337$\pm$45	696$\pm$48	851$\pm$101
mean$\pm$SEM µU/islet/min.	3.15$\pm$0.48	4.49$\pm$0.60	9.28$\pm$0.64	11.35$\pm$1.35
level of significance of difference at same glucose concentration	n.s		n.s	
	G_{50}+Glu-G_{50}		G_{300}+Glu-G_{300}	
mean$\pm$SEM of paired differences attributed to glucagon in µU/islet/min.	1.37$\pm$0.22		2.07$\pm$0.88	
level of significance of paired differences	$p<0.01$		$p<0.05$	

An intermediate glucose concentration of 150mg% was found to have the effect shown in Table IX on insulin release. Synergism of glucagon and glucose was of borderline significance ($p=0.05$). The failure to

Figure 4

EFFECT OF GLUCAGON (0·05µg per ml) ON INSULIN RELEASE IN MEDIA WITH HIGH AND LOW GLUCOSE CONCENTRATION



INCREMENT ATTRIBUTED TO GLUCAGON (µU per islet per minute)

in 300 mg % glucose 2·07 ± 0·88 $p < 0·05$

in 50 mg % glucose 1·37 ± 0·22 $p < 0·01$

(± SEM, n=6)

detect glucagon stimulated insulin release at 50mg% glucose as demonstrated in Table VIII is probably related to the small number of experiments.

Table IX Effect of Glucagon on Insulin Release at 150mg% glucose

Experiment	Glucose 50mg%	Glucose 50mg% with Glucagon	Glucose 150mg%	Glucose 150mg% with Glucagon
263	177,283*	235,219	1365,525	2000,613
265	513,846	639,968	1345,1370	1635,2270
267	545,670	900,1250	447,650	1170,1690
268	424,414	316,494	1170,1185	1730,2510
269	508,480	450,374	600,690	675,610
	* µU IRI/ml/15min.			
mean$\pm$SEM µU/ml/15min.	485$\pm$78	584$\pm$154	934$\pm$154	1490$\pm$262
mean$\pm$SEM µU/islet/min.	6.47$\pm$1.04	7.79$\pm$2.05	12.45$\pm$2.05	19.87$\pm$3.49
level of significance of difference at same glucose concentration	n.s		n.s	
	G_{50}+Glu-G_{50}		G_{150}+Glu-G_{150}	
mean$\pm$SEM of paired differences attributed to glucagon in µU/islet/min.	1.31$\pm$1.31		7.40$\pm$2.32	
level of significance of paired differences	n.s		p=0.05	

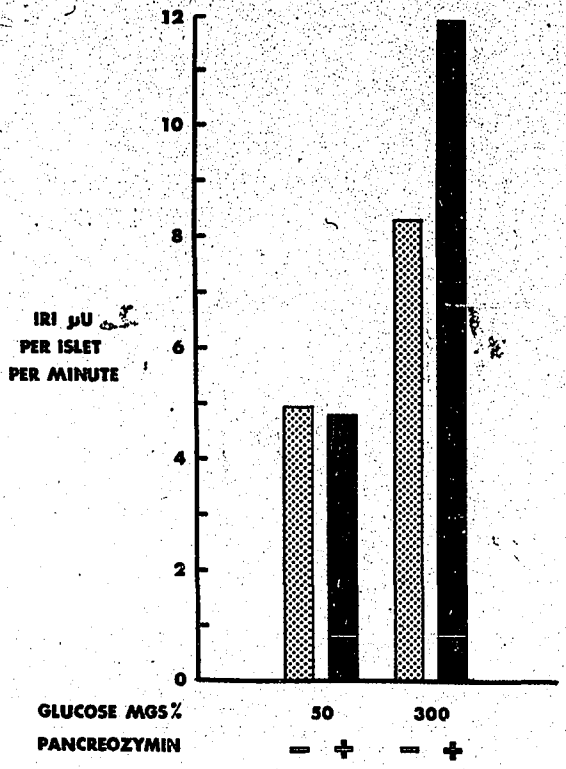
Pancreozymin was found to produce insulin release only at 300mg% glucose concentration as shown in Table X and Figure 5. Synergism was present ($p<0.05$).

Table X Effect of Pancreozymin on Insulin Release

Experiment	Glucose 50mg%	Glucose 50mg% with Pancreozymin	Glucose 300mg%	Glucose 300mg% with Pancreozymin
229	224,270*	250,240	575,560	435,550
232	174,180	170,144	325,450	700,550
246	110,124	110,134	275,275	585,525
247	194,134	164,104	375,300	600,435
248	920,820	940,710	1575,950	1785,1625
249	694,610	720,614	1100,750	1600,1350
	* µU IRI/ml/15min.			
mean±SEM µU/ml/15min.	371±128	358±125	625±159	894±222
mean±SEM µU/islet/min.	4.95±1.71	4.77±1.67	8.33±2.12	11.92±2.96
level of significance of difference at same glucose concentration	n.s		n.s	
	G_{50}+PZ-G_{50}		G_{300}+PZ-G_{300}	
mean±SEM of paired differences attributed to pancreozymin in µU/islet/min.	-0.17±0.12		3.59±1.19	
level of significance of paired differences	n.s		$p<0.05$	

Figure 5

EFFECT OF PANCREOZYMIN (0·2 µg per ml) ON INSULIN RELEASE IN MEDIA WITH HIGH AND LOW GLUCOSE CONCENTRATION



INCREMENT ATTRIBUTED TO PANCREOZYMIN (µU per islet per minute)

In 300 mgs% glucose = 3·59 ± 1·19
$p < 0·05$

In 50 mgs% glucose = −0·15 ± 0·13
ns

(± SEM, N = 6)

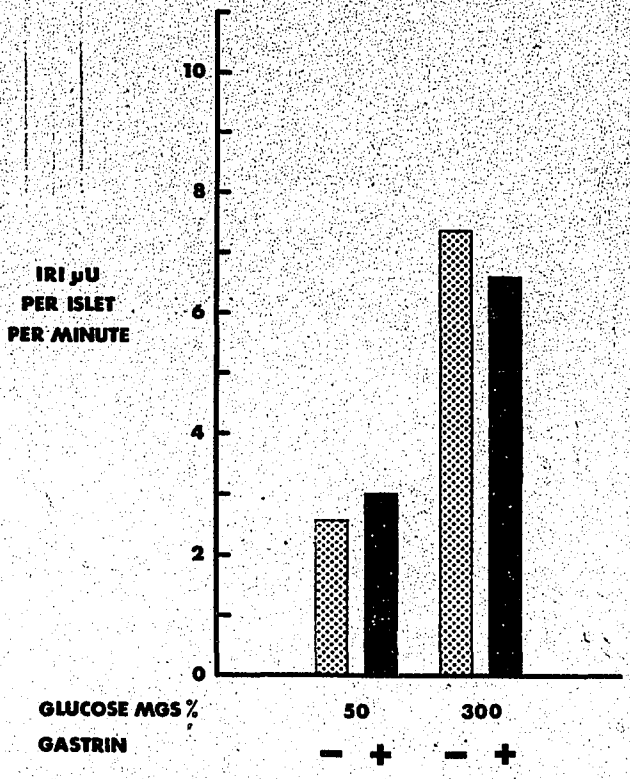
Gastrin was without effect at either glucose concentration. See Table XI and Figure 6.

Table XI Effect of Gastrin on Insulin Release

Experiment	Glucose 50mg%	Glucose 50mg% with Gastrin	Glucose 300mg%	Glucose 300mg% with Gastrin
224	104,160	144,140	460,300	375,325
237	420,290	644,250	900,400	1110,650
236	100,190	120,284	600,450	360,385
238	84,200	230,224	485,385	385,300
244	150,120	194,110	550,500	500,285
245	320,150	204,254	725,850	785,500
	* µU IRI/ml/15 min.			
mean±SEM µU/ml/15min.	190±36	233±45	550±60	496±89
mean±SEM µU/islet/min.	2.53±0.48	3.11±0.60	7.33±0.80	6.61±1.19
level of significance of difference at same glucose concentration	n.s		n.s	
	G_{50}+Ga-G_{50}		G_{300}+Ga-G_{300}	
mean±SEM of paired differences attributed to gastrin in µU/islet/min.	0.56±0.22		-0.72±0.80	
level of significance of paired differences	n.s		n.s	

Figure 6

EFFECT OF GASTRIN (4·0 μg per ml) ON INSULIN RELEASE IN MEDIA WITH HIGH AND LOW GLUCOSE CONCENTRATION



INCREMENT ATTRIBUTED TO GASTRIN
(μU per islet per minute)

In 300 mgs % glucose = −0·75 ± 0·52
ns

In 50 mgs % glucose = 0·36 ± 0·27
ns

(±SEM, N=6)

These experiments have demonstrated arginine induced insulin release at 50 and 300mg% glucose concentration. Synergism, however was absent. See Table XII.

Table XII Effect of Arginine on Insulin Release

Experiment	Glucose 50mg%	Glucose 50mg% with Arginine	Glucose 300mg%	Glucose 300mg% with Arginine
190	420*,320	580,460	850,775	1125,825
191	1020,1000	1200,830	1886,1312	1947,1947
202	170,180	290,205	1425,1900	3150,1925
220	270,270	260,250	700,500	8835,550
239	214,164	314,174	675,460	535,520
240	264,210	240,160	450,500	460,510
241	254,230	410,230	575,335	700,585
266	200,106	232,321	1477,1226	1496,1329
267	260,434	262,468	1725,680	2060,1355
	* μU IRI/ml/15min.			
mean±SEM μU/ml/15min.	332±88	382±85	969±163	1213±242
mean±SEM μU/islet/min.	4.43±1.17	5.09±1.13	12.92±2.17	16.17±3.23
level of significance of difference at same glucose concentration	n.s		n.s	
	$G_{50}+A-G_{50}$		$G_{300}+A-G_{300}$	
mean±SEM of paired differences attributed to arginine in μU/islet/min.	0.65±0.28		3.25±1.29	
level of significance of paired differences	$p<0.05$		$p<0.05$	

Leucine did not produce a significant increment in insulin release at either glucose concentration. Although the mean values were roughly similar to those of arginine the smaller number of experiments diminished the level of significance. See Table XIII.

Table XIII Effect of Leucine on Insulin Release

Experiment	Glucose 50mg%	Glucose 50mg% with Leucine	Glucose 300mg%	Glucose 300mg% with Leucine
183	125,395*	460,400	1100,1350	1087,1125
189	250,380	560,440	500,675	650,725
195	400,420	315,420	700,660	792,1650
230	300,320	270,224	685,600	1150,1135
231	274,214	200,200	610,600	1085,975
	* µU IRI/ml/15min.			
mean±SEM µU/ml/15min.	305±31	349±56	748±120	1037±93
mean±SEM µU/islet/min.	4.07±0.41	4.65±0.75	9.97±1.60	13.83±1.24
level of significance of difference at same glucose concentration	n.s		n.s	
	$G_{50}+L-G_{50}$		$G_{300}+L-G_{300}$	
mean±SEM of paired differences attributed to leucine in µU/islet/min.	0.59±0.73		3.85±1.67	
level of significance of paired differences	n.s		n.s	

Insulin content of various hormonal preparations used was as follows: glucagon 2.3 μU/ml of incubation medium,, secretin 0.54 μU/ml, gastrin 3.7 μU/ml, pancreozymin 0.011 μU/ml. In view of the concentrations of insulin released, none of these values are significant.

Discussion

Using each set of islets as its own control has obvious advantages. However, the reproducibility of responses was at the lower limits of acceptability and sometimes necessitated a large number of experiments. Coore and Randle[73] found this a feasible technique with 30 minute rest periods at low glucose concentrations between incubations. However, Vance et al[163] found a wide variation in glucagon release using isolated islets although the difference in insulin response was not significantly different. They elected not to incubate according to this protocol.

Preincubation at low glucose concentration has been found necessary by virtually all authors.[73,74,163] We demonstrated isolated islets are usually viable for 2 hours. Most authors[73,74,144] are also in agreement with this finding.

Our insulin-release-over-time curves demonstrate approximately linear increases and significant differences in insulin release from 15 to 60 minutes at low and high concentration of glucose. Some authors[74,173] have incubated for 15 minute periods, others[73,144] for 30 minutes.

The necessity of rest periods at low glucose concentrations

between incubations was demonstrated in early experiments when the effect of high medium glucose concentration was found to carry over to subsequent low glucose medium incubations. Five minutes rest at 50mg% glucose was felt to be adequate. Coore and Randle[73], however, rested for 30 minutes. Presumably, this period allows the effect of metabolites to subside and increases the efficiency of the wash.

The initial ILA data revealed the islets did indeed, respond consistently to a change in glucose concentration in a reproducible manner. Although the results obtained in one experiment differed considerably from those of another carried out in the same way, the pattern of response was similar. However, more numerous IRI data revealed a significantly different value of control incubation at 300mg% glucose when the second set was compared with the first. Consequently, the values were meaned, as explained above. The explanation for the fall in insulin output during the second set of four incubations must involve cellular damage either due to collagenase or other unknown in vitro factors or to failure of synthesis or secretion. It is to be noted that the insulin secretion in these experiments is far in excess of that in vivo and may account, in part, for the apparently minor responses of added substances.

We chose the two glucose concentrations of 50 and 300mg% for most of our experiments following the lead of Coore and Randle[73]. These levels are apparently at the lower and near the upper limits of glucose concentration for induction of insulin release according to the dose-response relationships of our preparation. However, it appears that the strong stimulus of 300mg% glucose may have dwarfed the effects of some of the gastrointestinal hormones since incubation

at an intermediate glucose concentration allowed demonstration of synergism of glucose and glucagon. Similarly, the high basal insulin release diminishes the apparent significance of hormone-induced insulin release.

Insulinolysis prompted Malaisse[144] to use anti-insulin serum to immediately bind released insulin in his pancreatic slices technique. Vance et al[163] detected a "small amount" of insulin degradation by isolated islets which was prevented by the addition of Trasylol. However, we have demonstrated negligible destruction without Trasylol. Trasylol was used, however, to prevent degradation of gastrointestinal hormones in the incubation medium.

Secretin

The results depicted in Table VII show that whereas secretin induced insulin release at both 50 and 300mg% glucose there was no synergism involving secretin and glucose at 300mg% glucose.

That secretin is insulinotrophic has been demonstrated in vivo[35,40,43-45,47,49,52] and in vitro[36-38]. Lazarus et al[39] demonstrated secretin-induced insulin release on injection in rats but not in vitro using pancreatic slices. Hadjikani et al[38] demonstrated insulin release both in vivo and with isolated pancreatic islets in rats. The contrast in results points to the possibility of secretin breakdown by pancreatic enzymes. Pfeiffer et al[36] demonstrated maximal insulin release from dog and rabbit pancreatic slices by 0.01 and 0.1 U/ml secretin in the absence of glucose. Addition of glucose had no effect. They concluded secretin per se stimulates insulin secretion independent of blood glucose concentration. Our findings in Table VII are in agreement.

Potentiation of secretin-induced insulin release by a simultaneous glucose infusion has been demonstrated[46,52]. The data are therefore in accord with interpretation of in vivo findings except for the lack of demonstrable synergism.

Secretin release is produced by acid and protein breakdown products in the duodenum. Secretin produces insulin release both in vivo and in vitro. Serum immunoassayable secretin is reportedly elevated after oral and intraduodenal glucose, after intraduodenal acid and administration of intravenous Histalog, and the elevation precedes the rise in blood insulin[179]. If these findings are confirmed secretin can be said to be one of the hormones involved in increased insulin release during glucose absorption.

Glucagon

These experiments have also demonstrated the well known effect of increased insulin release by glucagon at both 50 and 300mg% glucose. Insulin release attributed to glucagon at high glucose concentration exceeded that at low glucose concentration but the difference was not statistically significant. At an intermediate glucose concentration (150mg%) in a small number of experiments synergism was of borderline significance suggesting the possibility that the high glucose concentration (300mg%) dwarfed the glucagon effect. The failure at low glucose levels to produce significantly increased output shown in the second group of glucagon experiments (Table IX) as opposed to the first group (Table VIII) is probably related to the small number of experiments.

Glucagon has been shown to produce insulin release in vivo in fasting man[47,71,72,174] and dogs[42,49] and when given in conjunction

with a glucose infusion,[71,174] in which case synergism has been demonstrated. In perfused isolated rat pancreas[130,131], organ culture,[76] isolated fetal pancreatic tissue[77] and in rat pancreatic slices[74,75] glucagon is insulinogenic. Potentiation was demonstrated by one group[74] but not by another[75] using pancreatic slices. Montague et al[175] have demonstrated glucagon-induced insulin release using isolated islets.

Our findings are thus in accord with previous in vitro and in vivo findings regarding glucagon and we have demonstrated synergism of glucose and glucagon found by some investigators.

The pattern of effect of secretin is similar to that of glucagon though the mean differences are smaller. This finding may be related to the structural similarities between glucagon and secretin. Both hormones exert lipolytic effects on rat adipose tissue in closely similar concentrations. Secretin and gut glucagon-like-immunoreactivity are not glycogenolytic in contrast to pancreatic glucagon. Therefore, if secretin and gut GLI on the one hand, and gut GLI and pancreatic glucagon on the other hand, stimulate insulin release by common mechanisms respectively this must be independent of stimulation of beta cell glycogenolysis.

Use of the isolated islet technique has provided suggestive evidence that pancreatic glucagon may be insulinotrophic by activating adenyl cyclase[180].

The role of gut glucagon is unclear. It appears to be released by ingested glucose and is probably insulinotrophic. It may act in conjunction with secretin. Pancreatic glucagon is an inducer of insulin release and appears to be stimulated by pancreozymin

in the absence of hyperglycemia. What other roles it plays in the gastrointestinal hormone story remain to be determined.

Gastrin

Gastrin failed to produce significant insulin releases at both 50 and 300mg% glucose. Impure gastrin has been shown to cause immediate insulin release in dogs after rapid endoportal injection of high doses.[49] Jarrett and Cohen[47] failed to demonstrate gastrin-induced insulin release in fasting man or during glucose infusion. Dupré et al[50-52] found insignificant changes in blood insulin levels in fasting subjects in response to gastrin, but did demonstrate increments when gastrin was given simultaneously with glucose. Although the aim of the latter experiments was to produce maximal acid output and thereby secretin release, Unger et al[49] presumably demonstrated a direct action of gastrin on the islets and the possibility of a direct action in humans remains.[54] Lazarus et al[54] have demonstrated gastrin induces secretin release directly but the doses used may be pharmacologic. Lazarus et al[39] failed to demonstrate insulin release with intravenous gastrin injections in rats or in vitro using pancreas slices.

Thus, although there is evidence of a direct and/or indirect insulin-releasing action of gastrin in vivo, there was no stimulation in vitro. It appears that in man the effect of gastrin which is manifest during glucose infusions may be mediated by stimulation of release of endogenous secretin. While pancreozymin-like effects of gastrin on pancreatic exocrine function in animals have been related to structural similarities in the N-terminal sequences of these two hormones, the present findings suggest that this resemblance alone does not necessarily lead to stimulation of insulin release.

Pancreozymin

Pancreozymin produced significant insulin release only at 300mg% glucose in these experiments. Insulin release has been induced by pancreozymin in dogs[83,109]. Meade et al[109] demonstrated a synergistic effect of hyperglycemia and pancreozymin and Unger et al[83] demonstrated a prompt glucagon response. In man there are conflicting data[35,40,46, 50-52,176]. At normoglycemic levels pancreozymin-induced insulin release may be via pancreatic glucagon, which effect may be teleologically related to protection against hypoglycemia. Furthermore, it has been shown that hyperglycemia suppresses the glucagon-stimulating effect of pancreozymin[89], although enhancement of insulin release by pancreozymin persists. Thus, pancreozymin may act directly on beta cells at hyperglycemic levels and this is supported by the present experiments.

High oral doses of glucose(200gm) are associated with an elevation of GLI which is followed temporally by insulin and pancreozymin increments[179]. However, more physiologic oral doses of isotonic glucose do not consistently produce GLI increments.

Intraduodenal administration of isotonic glucose has no effect on the exocrine pancreas and it appears that although intravenous pancreozymin is insulinogenic (probably directly, that is, not through glucagon), it does not have a physiologic role during glucose absorption.

Only Lazarus et al[39] have studied pancreozymin in vitro. Using pancreatic slices, pancreozymin increased insulin release.

The results of our experiments, therefore, are in agreement with the in vivo and in vitro work published to date. The pattern of response to pancreozymin in vitro suggests the effect is not mediated

by glucagon within the islet and corresponds with the effect of exogenous pancreozymin observed during glucose infusion. Probably pancreozymin is not involved during glucose absorption. However, pancreozymin is important in insulinogenesis during amino acid absorption. The isolated islet technique should prove to be a useful method in studying insulin release with arginine with and without pancreozymin and in elucidating the role of glucagon in pancreozymin-induced insulin release.

Amino Acids

Leucine and arginine are both clearly insulinogenic in vivo[119,122] and their effect is increased in the presence of hyperglycemia in normals[117]. Rabinowitz et al[177] demonstrated synergism of ingested glucose and protein with respect to insulin levels attained. Colwell and Berger[178] demonstrated a direct action of amino acids on the beta cell using intrapancreatic amino acid infusions. Vance et al[161] produced arginine induced insulin release using isolated islets using 200mg% arginine only at 60mg% glucose. Martin[146] found leucine to be without effect using pancreas slices but Hildebrandt et al[145] did find insulin release using the Malaisse method.

Our work has demonstrated significant insulin release at both glucose concentrations due to arginine but not leucine. The increments were similar but there were fewer leucine experiments, thereby diminishing significance. Arginine is, however, a more potent insulin releasing agent than leucine in vivo. The biochemical mechanism of amino acid induced insulin release is at present unknown.

The isolated islet technique as a method of study offers distinct advantages alluded to above. However, disadvantages lie in

the difficulty in obtaining islets, the possibility of collagenase-induced changes, and the large variation in results in similar incubation media necessitating a large number of experiments to obtain statistical significance.

With respect to future developments in this field, measurement of glucagon levels, as Vance et al[163] have done, particularly in the presence of pancreozymin and amino acids should prove helpful. Ablation of the alpha cells by synthalin or cobaltous chloride might provide additional information. The roles of secretin and pancreozymin in amino-acid-induced insulin release should be investigated. The hormone doses, glucose and amino acid concentrations should be varied to determine minimum and optimum levels. The ultimate answer regarding much of the in vivo work awaits confirmation of the results obtained by Lazarus et al[54], a pancreozymin immunoassay and further definitive data regarding glucagon and gut glucagon-like-immunoreactivity. The isolated islet technique will quite probably be a very useful tool in the elucidation of the biochemical mechanisms of action of the various hormones in the islet.

Conclusion and Summary

Using the isolated islet technique of Kostianovsky and Lacy we have demonstrated the following:

1. The technique is feasible for the study of insulin release.
2. Insulinolysis is negligible.
3. Secretin induces insulin release at 50 and 300mg% glucose.
4. Glucagon produces insulin release at 50,150 and 300mg% glucose. Synergism of glucagon and glucose was demonstrated at 150mg% glucose.
5. Pancreozymin is insulinotrophic at 300mg% glucose but not at 50mg%.

6. Gastrin was without effect at either glucose concentration.
7. Arginine produced statistically significant insulin release at both 50 and 300 mg% glucose concentration.

These data support the hypotheses that secretin and pancreozymin, released in response to physiologic stimuli, contribute to the effects of intestinal hormones on the endocrine pancreas in response to intake of nutrients.

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